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CÍNTIA DAUDT

DIVERSIDADE GENÉTICA DE PAPILOMAVÍRUS BOVINO NO BRASIL

PORTO ALEGRE, DEZEMBRO DE 2017

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DIVERSIDADE GENÉTICA DE PAPILOMAVÍRUS BOVINO NO BRASIL

Tese apresentada como requisito parcial para a obtenção do grau de doutora em Ciências Veterinárias, Especialidade Biologia Celular e Molecular

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Resumo

Os papilomavírus (PV) são vírus epiteliotrópicos que infectam um grande número de vertebrados, incluindo os mamíferos, aves, répteis e peixes. Eles são vírus não envelopados, compostos de um capsídeo estruturado pelas proteínas L1 e L2, que abriga uma molécula de DNA dupla fita e circular. Os PV são vírus oncogênicos que causam lesões benignas e malignas na epiderme e mucosas de seus hospedeiros. Em bovinos, o papilomavírus bovino (BPV) causa papilomas extensivos em animais suscetíveis. Apesar das infecções por BPV apresentarem alta morbidade e baixa mortalidade, elas causam prejuízos econômicos aos produtores, que frequentemente descartam animais precocemente devido à extensão e o local das lesões. Atualmente, 23 tipos de BPV foram classificados e caracterizados, contrastando com o alto número de papilomavírus humanos (HPV) sequenciados e caracterizados (204). Desta forma, acredita-se que a diversidade genética dos BPV seja provavelmente similar à diversidade genética dos HPV existentes. Este trabalho objetivou investigar a diversidade genética dos BPV existentes na região norte e sul do Brasil, assim como realizar o sequenciamento completo de novos tipos virais e tipos já descritos e, desta forma, contribuir para futuros estudos epidemiológicos, filogenéticos e de caracterização genética de BPV. Ainda, esta tese objetivou prover uma atualização da atual epidemiologia, classificação e características genéticas dos PV de ruminantes, com o foco principal nos BPV. Metodologias convencionais (baseadas em amplificação de ácidos nucléicos virais por PCR utilizando oligonucletotídeos específicos para PV) e de última geração (amplificação randômica de genomas circulares e sequenciamento de alta eficiência) foram empregadas, proporcionando a identificação dos vírus circulantes em rebanhos da região Norte e da região Sul do país. Nove novos tipos de BPV foram sequenciados e caracterizados neste estudo (BPV16, 17, 18, 19, 20, 21 e os prováveis BPV24, 25 e 26), evidenciando a grande diversidade genética de BPV presente na região amazônica quando comparada com amostras do sul do país. Além disso, o sequenciamento de genomas completos de outros tipos virais já descritos também foi realizado. Esses resultados proporcionaram uma visão geral dos tipos virais presentes em rebanhos de dois extremos do Brasil, assim como possibilitou a identificação de prováveis novos tipos e a caracterização de novos tipos de BPV. Esses resultados são importantes para entender a evolução e a distribuição dos papilomavírus, bem como para o desenvolvimento de estudos vacinais, que envolvem papilomavírus humano e bovino. Adicionalmente, podemos verificar que pesquisas envolvendo ruminantes selvagens são esporádicas, no entanto seriam importantes para o melhor entendimento da biologia e das relações intra e interespecíficas dos PV.

Palavras-chave: BPV; PCR; amplificação por círculo rolante; sequenciamento de alto desempenho; ruminantes

Abstract

Papillomaviruses (PV) are epitheliotropic viruses that infect a large number of vertebrates, including mammals, birds, reptiles and fish. They are non-enveloped viruses composed of a capsid that is structured by the L1 and L2 proteins, which harbous a circular double-stranded DNA molecule. PVs are oncogenic viruses that cause benign and malignant lesions in the epidermis and mucosa of their hosts. In cattle, bovine papillomavirus (BPV) causes extensive papillomas in susceptible animals. Although BPV infections present high morbidity and low mortality, they cause economic losses to producers, who often discard animals prematurely because of the extent and location of the lesions. Currently, 23 types of BPVs have been classified and characterized, contrasting with the high number of human papillomaviruses (HPVs) sequenced and characterized (204). Therefore, it is believed that the genetic diversity of BPV is probably similar to the genetic diversity of existing HPV. This work aimed to investigate the genetic diversity of BPV in the Northern and Southern regions of Brazil, as well as complete sequencing of new viral types and types already described, and thus contribute to future epidemiological, phylogenetic and genetic characterization studies of BPV. Furthermore, this thesis aimed to provide an update of the current epidemiology, classification and genetic characteristics of ruminant PV, with the main focus on BPV. Conventional methodologies (based on amplification of viral nucleic acids by PCR using specific oligonucleotides for PV) and last generation methodologies (random amplification of circular genomes and high efficiency sequencing) were employed, providing the identification of circular viruses in herds of the North and South regions of the country. Nine new types of BPV were sequenced and characterized in this study (BPV16, 17, 18, 19, 20, 21 and the putatives BPV24, 25 and 26), evidencing the great genetic diversity of BPV present in the Amazon region when compared with samples from the South region. In addition, sequencing of complete genomes from other viral types already described has also been performed. These results provided an overview of the viral types present in Brazilian herds from two distant regions, as well as the identification of potential new types and the characterization of new types of BPV. These results are important to understand the papillomavirus evolution and distribution as well as to development of vaccinal studies, which involve human and bovine papillomaviruses. Additionally, we could verify that researches involving wild ruminants are sporadic, but it would be important for the better understanding of the biology as well as the intra and interspecific of PV relationship.

Keywords: BPV; PCR; rolling circle amplification; high throughput sequencing; ruminant.

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Abreviatura	***Significado***
µg	Micrograma
µL	Microlitro
^{o}C	Graus Celsius
BPV	Papilomavírus bovino
DNA	Ácido desoxirribonucleico
ds	*Double strand* (fita dupla)
dNTP	Desoxinucleosídeo trifosfato
E	*Early* (precoce)
EcPV	Equs caballus papilomavírus
EDTA	Ácido etilenodiaminotetracético sal sódico
ELISA	*Enzyme-Linked Immunosorbent Assay*
EserPV	*Epitesicus serotimus papilomavírus*
HPV	Papillomavirus Humano
kg	Kilograma
L	*Late* (tardio)
LCR	*Long control region* (Região longa de controle)
MaPV	*Mesocricetus auratus papillomavirus*
mg	Miligrama
min	Minutos
mL	Mililitro
mM	Milimolar
MmiPV	*Micromys minutus papillomavirus*

NGS	*Next generation sequence* (Sequenciamento de última geração)
nt	Nucleotídeo
ORF	*Open readin frame* (Fase aberta de leitura)
OvPV	*Ovis aries* papilomavírus
pA	Sítio de poliadenilação
pA_E	Sítio de poliadenilação precoce
pA_L	Sítio de poliadenilação tardio
PaVE	*Papillomavirus episteme*
pb	Pares de base
PBS	Solução salina tamponada com fosfato
PCR	Reação em cadeia da polimerase
PCR	*Polymerase Chain Reaction*
pmol	Picomol
PmPV	*Peromyscus papilomavirus*
PV	Papilomavírus
RCA	*Rolling circle amplification* (Amplificação por círculo rolante)
seg	Segundos
TBE	Tampão Tris, ácido bórico, EDTA
TE	Tris-EDTA
UV	Ultravioleta
V	Volts
VLP	*Virus like particles* (Partículas semelhantes à vírus)

SUMÁRIO

1 INTRODUÇÃO

A papilomatose é uma doença causada por um grande grupo de vírus epiteliotrópicos altamente diversos que ocorre na maioria dos vertebrados, incluindo as aves, mamíferos e peixes (CAMPO, 1997, 2006; DE VILLIERS et al., 2004; LÓPEZ-BUENO et al., 2016; RECTOR; VAN RANST, 2013). Os papilomavírus (PV) podem induzir lesões múltiplas, benignas e proliferativas na epiderme e derme de animais, chamada papilomatose cutânea (SILVESTRE et al., 2009). Entretanto, em alguns casos, as lesões podem progredir para neoplasias malignas (ARAIBI et al., 2004; MUNDAY; KIUPEL, 2010; WOODMAN; COLLINS; YOUNG, 2007).

Em humanos, os papilomavírus estão associados a lesões no trato genital, respiratório superior, digestivo e pele. Infecções não tratadas podem evoluir para neoplasias malignas e metastáticas (MUNDAY; KIUPEL, 2010; WOODMAN; COLLINS; YOUNG, 2007). A associação de câncer do trato genital masculino e feminino com o papilomavírus humano (HPV) já é bem estudada. Os principais HPV envolvidos com a gênese de câncer cervical são os HPV 16 e 18 (SANDERS; STENLUND, 2001; WOODMAN; COLLINS; YOUNG, 2007), assim como os HPV 31 e 33 são comumente relacionados com câncer cervical, vulvar, vaginal, anal e peniano (ZUR HAUSEN, 2002). Ainda, estudos demonstram a relação dos HPV 16 e 18 com câncer de mucosa oral (GONZÁLEZ-MOLES et al., 1994; PIENNA SOARES et al., 2002; WESTRA, 2009).

O papilomavírus bovino (BPV) causa uma enfermidade infecto-contagiosa, crônica, de caráter tumoral que afeta rebanhos de bovinos de leite e de corte em todo o mundo, sendo associado a diversas formas de tumores benignos e malignos (ARAIBI et al., 2004; CAMPO, 1997; CAMPO et al., 1992; DA SILVA et al., 2015; JARRETT et al., 1994; LUNARDI et al., 2016; MARTANO et al., 2013; OGAWA et al., 2004). As lesões associadas à infecção pelo BPV determinam prejuízos econômicos consideráveis à bovinocultura tanto por perdas diretas, causadas por mortes dos animais, quanto indiretas, representadas por reduções na produtividade e no valor comercial dos animais e subprodutos, como o couro (ALFIERI; LUNARDI; ALFIERI, 2012).

Atualmente, mais de 200 tipos de HPV foram totalmente sequenciados, caracterizados e catalogados, contrastando com o baixo número de papilomavírus descritos em animais (MUNDAY, 2014a; MUNDAY et al., 2016; NG et al., 2015; ORBELL; YOUNG; MUNDAY, 2011; RECTOR; VAN RANST, 2013; TSE et al., 2012). Os bovinos são os animais em que os PV são mais frequentemente detectados. Atualmente, os bovinos compreendem 23 tipos (http://pave.niaid.nih.gov) além de vários potenciais novos tipos descritos (BATISTA et al., 2013; DA SILVA et al., 2015; HE et al., 2013; LUNARDI et al., 2016; OGAWA et al., 2004). Apesar de ser um agente etiológico importante, a detecção e a caracterização de PV em animais é ainda deficiente (MUNDAY et al., 2007; RECTOR; VAN RANST, 2013). Esse fato se deve, em parte, as técnicas de detecção moleculares (que utilizam-se de testes baseados no conhecimento prévio de sequências genômicas) (FREITAS et al., 2013) e ao baixo número de estudos focados em papilomavírus não-humanos.

Desta forma, no intuito de colaborar com o estudo de diversidade genética dos BPV, este trabalho objetiva investigar a diversidade genética dos BPV do Brasil, assim como promover uma atualização epidemiológica e filogenética dos PV que infectam ruminantes. Metodologias convencionais (baseadas em amplificação de ácidos nucléicos virais por PCR utilizando oligonucletotídeos específicos para PV) e de última geração (amplificação randômica de genomas circulares e sequenciamento de alta eficiência) foram empregadas, permitindo a identificação dos vírus circulantes em rebanhos da região Norte e da região Sul do país. Esses resultados propiciaram uma visão geral dos tipos virais presentes nos rebanhos brasileiros, assim como a identificação de prováveis novos tipos e a caracterização de nove novos tipos de BPV. O presente trabalho ampliou significativamente o conhecimento acerca da diversidade genética e filogenética dos BPV e servirá de base substancial para futuros estudos epidemiológicos e filogenéticos destes vírus.

2 REVISÃO BIBLIOGRÁFICA

2.1 Estrutura viral dos papilomavírus

Os papilomavírus (PV) pertencem à família *Papillomaviridae* (BERNARD et al., 2010; DE VILLIERS et al., 2004). Seus vírions são desprovidos de envelope lipoprotéico e apresentam simetria icosaédrica, seu genoma é constituído por uma única molécula de DNA circular dupla fita, com aproximadamente 8000 pares de bases (pb) (BERNARD et al., 2010; RECTOR; VAN RANST, 2013; SCHILLER; MÜLLER, 2015). Um esquema da organização do capsídeo viral e suas proteínas é mostrado na Figura 1. Os PV podem infectar um grande número de amniotas (BERNARD et al., 2010; DE VILLIERS et al., 2004; RECTOR; VAN RANST, 2013) e, provavelemente, não amniotas (LÓPEZ-BUENO et al., 2016).

O vírion possui 55 a 60 nm de diâmetro e formam partículas paracristalinas no núcleo das células infectadas (BORZACCHIELLO; ROPERTO, 2008). No vírion maduro, o DNA viral se encontra associado com histonas celulares do hospedeiro, em um complexo semelhante à cromatina (KING et al., 2012). Adicionalmente, genoma de dsDNA está contido em um capsídeo composto de 72 capsômeros (SCHILLER; MÜLLER, 2015). Sessenta destes capsômeros se ligam de forma hexavalente e doze de forma pentavalente. Cada capsômero é composto por duas proteínas codificadas pelo vírus, a proteína principal, L1, e a proteína secundária, L2 (PFISTER; ZUR HAUSEN, 1978).

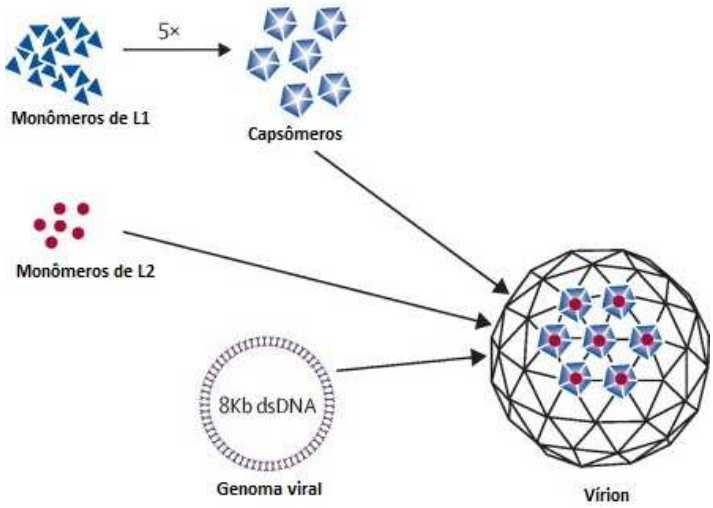

Figura 1. Esquema da organização do capsídeo viral e suas proteínas. Adaptado de (SCHILLER; MÜLLER, 2015).

2.2 Organização genômica dos papilomavírus

O genoma dos PV é dividido em três diferentes regiões: a região longa de controle (*long control region* - LCR), que contém os elementos necessários para replicação e transcrição do DNA viral, e duas regiões que contém as fases abertas de leitura (*open reading frame* - ORF), que correspondem aos genes precoces (*early* - *E*) e tardios (*late* - *L*) (BORZACCHIELLO; ROPERTO, 2008). As três regiões são separadas por dois sítios de poliadenilação (pA), o sítio da região precoce (pA_E) e o sítio na região tardia (pA_L) (ZENG e BAKER, 2006). Todos os tipos de PV seguem um mesmo padrão de organização genômica, com somente uma fita codificante e apresentam ORFs sobrepostas e aninhadas (LAZARCZYK et al., 2009).A Figura 2 mostra um esquema da organização do genoma do BPV1.

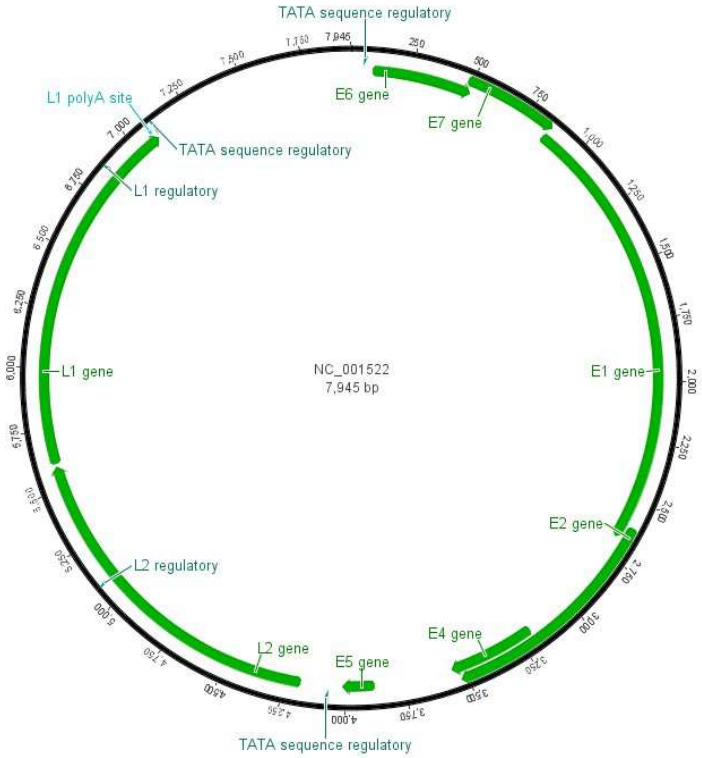

Figura 2. Representação esquemática da organização do genoma do BPV1 A figura da organização genômica foi montada com o programa *Geneious* (versão 8.1). **(acesso NC_001522 do GenBank).**

Aproximadamente dez diferentes ORFs já foram descritas na fita codificante e são classificadas em dois segmentos principais, conforme a fase de transcrição. O segmento E contém até seis ORFs, e o segmento L contém até três ORFs. Os genes precoces (*E1, E2, E4, E5, E6, E7 e E8*) codificam as proteínas (que recebem os mesmos nomes dos genes) envolvidas tanto na replicação e transcrição do DNA viral quanto na transformação celular (CORTEGGIO et al., 2013; LAMBERT, 1991; VENUTI et al., 2011). Por outro lado, os genes tardios (*L1* e *L2*) codificam as proteínas do capsídeo viral L1 e L2. A região longa de controle é uma região não codificante do genoma, com aproximadamente 500-1000 nucleotídeos (nt), localizada a montante da região de transcrição precoce e compreende aproximadamente 10 a 15% do genoma viral (LAZARCZYK et al., 2009).

Esta região contém elementos típicos de regulacão e transcrição viral. Ela possui elementos regulatórios para a replicação viral e controla os genes de transcrição e

transformação celular nos PV. Genericamente as LCR possuem organizações similares: uma região promotora, uma região potencializadora e um ou mais sítios de ligação altamente conservados para a proteína E2 (E2 *binding-sites* - E2BS) (DESAINTES; DEMERET, 1996; ZHENG; BAKER, 2006). As posições dos E2BS nos diferentes genomas pode influenciar nas características da regulação da expressão do genoma viral (LAZARCZYK et al., 2009). Embora alguns autores afirmem que os genomas dos papilomavírus são altamente estáveis e as mutações ou recombinações das sequências são raras (DE VILLIERS et al., 2004), existem evidências de que coinfecções múltiplas com HPV podem levar à emergência de vírus recombinantes e com propriedades patogênicas novas (VARSANI et al., 2006).

2.3 Proteínas virais

As principais proteínas para replicação do DNA viral são as proteínas E1 e E2, que são responsáveis pelos primeiros passos na iniciação da transcrição e replicação (LAMBERT, 1991; MÜNGER; HOWLEY, 2002; SANDERS; STENLUND, 2001). Enquanto a proteína E1 atua na replicação do DNA viral, a proteína E2 ancora na LCR ativando ou reprimindo a transcrição dos genes virais e, desta forma, controlando a atividade transcricional (BAXTER et al., 2005; HOWLEY; LOWY, 2001). As proteínas E2 são estruturalmente bem conservadas entre os diferentes tipos de PV, sendo bem semelhantes em tamanho com aproximadamente 350 a 400 aminoácidos (HARRIS; BOTCHAN, 1999). Essas proteínas (E1 e E2) também tem papel importante na segregação do genoma viral para as células filhas, assim como na manutenção da sua integridade (BRAVO; FÉLEZ-SÁNCHEZ, 2015; DOORBAR et al., 2012).

As proteínas estruturais L1 e L2, compõem o capsídeo viral e as oncoproteínas E5, E6 e E7, modulam o processo de transformação celular (MÜNGER; HOWLEY, 2002). Somente as principais ORFs *E1*, *E2*, *L1* e *L2* estão presentes em todos os papilomavírus já descritos (RECTOR; VAN RANST, 2013) e, teoricamente, essas proteínas poderiam realizar sozinhas as tarefas básicas de replicação, regulação, estabilização e empacotamento do DNA viral, levando à liberação dos vírions (BRAVO; FÉLEZ-SÁNCHEZ, 2015).

2.4 Classificação dos papilomavírus

Esta grande família viral é formada por vírus filogeneticamente classificados em 49 gêneros e composta de várias espécies, tipos, subtipos e variantes (BERNARD et al., 2010; DE VILLIERS et al., 2004; "ICTV", 2017). Atualmente a sequência de nucleotídeos do gene L1 é utilizada para a classificação dos PV por ser o gene mais conservado do genoma (BERNARD et al., 2010; DE VILLIERS et al., 2004). Um novo tipo de PV é considerado quando o seu genoma completo é totalmente sequenciado e a ORF L1 difere mais de 10% dos tipos de PV descritos. As espécies de PV compartilham entre 71% e 89% de identidade de nucleotídeos nesta mesma ORF. As similaridades menores do que 60% definem um novo gênero. Um subtipo é definido quando a diferença entre a ORF L1 é entre 2% e 10%, e uma variante viral ocorre quando esta diferença é menor do que 2% (BERNARD et al., 2010; DE VILLIERS et al., 2004).

Até o momento do início desta tese, os BPV eram classificados em quatro gêneros, cinco espécies e 15 tipos (Tabela 1). O gênero *Deltapapillomavirus*, é constituído pela espécie *Deltapapillomavirus 4* (compreendendo o BPV1, 2, 13 e 14); *Epsilonpapillomavirus,* pela espécie *Epsilonapapillomavirus 1* (BPV5 e BPV8); *Dyoxipapillomavirus*, pela espécie *Dyoxipapillomavirus 1* (BPV7). O gênero *Xipapillomavirus*, era constituído pelas espécies *Xipapillomavirus 1* (BPV3, 4, 6, 9, 10, 11 e 15) e *Xipapillomavirus 2* (BPV12) (http://pave.niaid.nih.gov) (Tabela 1). Durante o desenvolvimento desta tese, nove novos tipos de BPV foram sequenciados e caracterizados (BPV16, 17, 18, 19, 20, 21 e os prováveis BPV24, 25 e 26). A Figura 3 mostra a atual relação filogenética dos atuais grupos de BPV descritos, de acordo com a similaridade do gene L1 de cada um dos representantes.

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

Gênero	Espécie	Tipos de BPV
Deltapapillomavirus	*Deltapapillomavirus 4*	BPV1, 2, 13 e 14
Xipapillomavirus	*Xipapillomavirus 1*	BPV3, 4, 6, 9, 10, 11, 15
	Xipapillomavirus 2	BPV12
Epsilonpapillomavirus	*Epsilonpapillomavirus 1*	BPV5 e 8
Dyoxipapillomavirus	*Dyoxipapillomavirus 1*	BPV 7

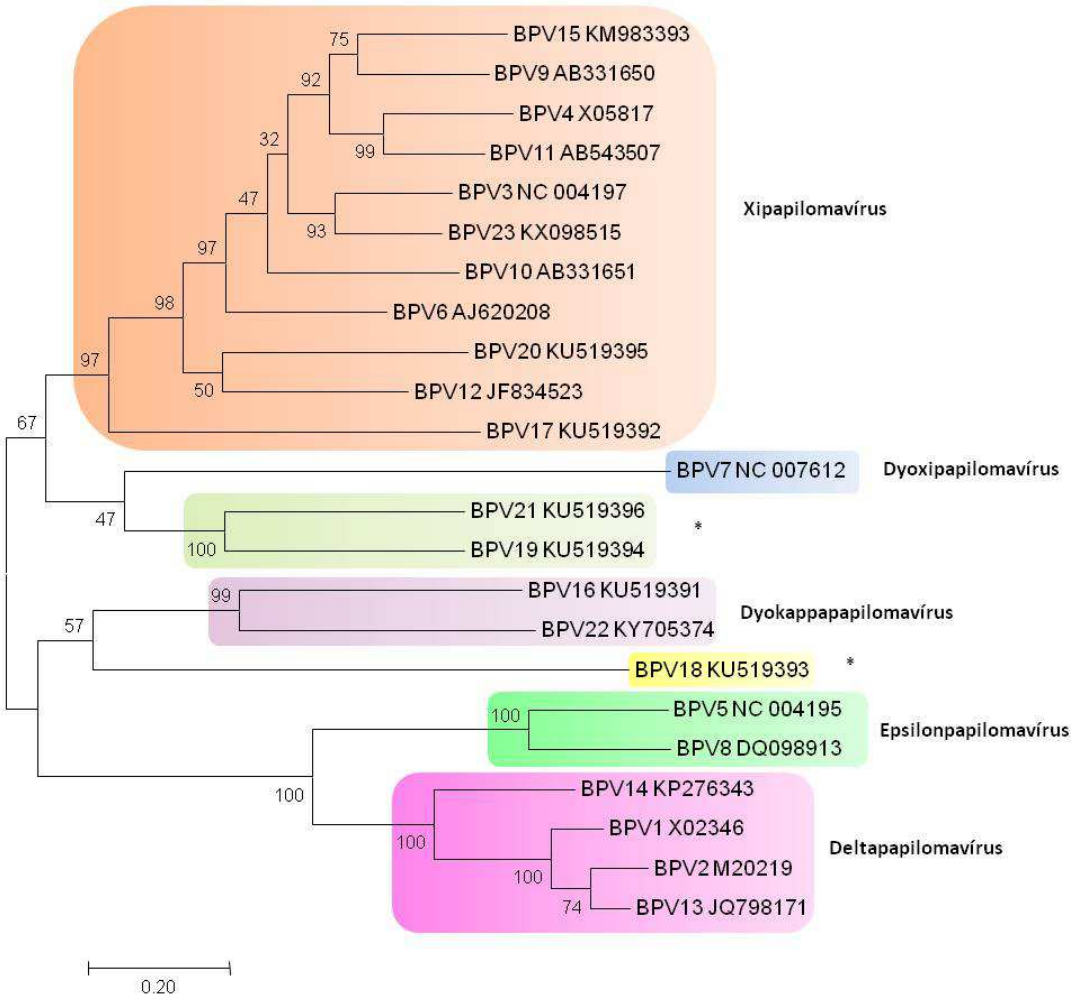

Figura 3: Disposição filogenética dos grupos de papilomavírus bovino utilizando a ORF L1 completa de cada representante de BPV. Gêneros ainda não classificados estão representados por *.

2.5 Epidemiologia

A papilomatose bovina tem distribuição mundial e, embora a mortalidade seja baixa, ela pode causar surtos com alta morbidade. A doença não é letal, mas pode levar à morte do animal pela grande presença de papilomas, causando o enfraquecimento do animal e também míiases devido a traumatismos causados nos papilomas (SCHUCH, 2001). Por outro lado, como muitos vírus, o BPV pode estabelecer uma infecção latente (CAMPO et al., 1994; DOORBAR, 2013; ROPERTO et al., 2010) e ser encontrado em

pele saudável (OGAWA et al., 2004; RECTOR; VAN RANST, 2013), assim como em sangue, esperma e outras secreções em bovinos (LINDSEY et al., 2009).

O vírus se dissemina por contato direto ou indireto, sexual, fômites (agulhas, brincadores e outros aparelhos contaminados), instalações e possivelmente insetos (CAMPO et al., 1994; FINLAY et al., 2009; REID et al., 1994). Parte do genoma do BPV1 já foi detectado em várias espécies diferentes de moscas, incluindo espécies sugadoras e não sugadoras, sugerindo que elas possam ser vetores dos papilomavírus (FINLAY, 2011; FINLAY et al., 2009). Também existem evidências que sugerem que a maioria dos bovinos seja contaminada com BPV2 e os linfócitos possam ser uma fonte de infecção (MUNDAY, 2014b), disseminando o vírus através da via hematogênica (ROPERTO et al., 2008). Outros estudos demonstraram a presença de BPV nos ovários, útero, oócitos, sangue, leite, urina, líquido seminal e espermatozóide de bovinos infectados (DE CARVALHO et al., 2003; LINDSEY et al., 2009; SILVA et al., 2011).

A manifestação do BPV no trato digestório é sugerida pela interação entre o vírus da papilomatose e a ingestão de samambaia *Pteridium spp.*, proporcionando o desenvolvimento de carcinomas devido à imunodepressão que limitaria as respostas imunológicas capazes de controlar tumores (BORZACCHIELLO et al., 2003; BORZACCHIELLO; ROPERTO, 2008; CAMPO, 1997). Por definição, os PV são, em geral, espécie específicos (CAMPO, 2006). No entanto, o BPV1, 2 e 13 consolidadamente infectam equinos (BOGAERT et al., 2008; LUNARDI et al., 2013c; MUNDAY; KIUPEL, 2010; NASIR; CAMPO, 2008) e bubalinos (LUNARDI et al., 2013a; ROPERTO et al., 2010, 2013; SILVESTRE et al., 2009), pulando a barreira das espécies. Além disso, estes vírus, juntamente com o BPV5 também foram descritos em iaques, antas, girafas, burros, bisons e zebras (BOCANETI et al., 2016). Ainda, o HPV9 e o BPV14 foram descritos infectando felinos domésticos (MUNDAY et al., 2007, 2015).

2.6 Patogenia e sinais clínicos

2.6.1 Papilomatose epitelial e de mucosas em bovinos

A propagação dos PV acontece em células epiteliais em diferenciação, podendo infectar fibroblastos, queratinócitos e células epiteliais (CAMPO, 2002) assim como junções entre diferentes tipos de epitélios, que são locais vulneráveis (DOORBAR et al., 2012), formando projeções digitiformes microscópicas ou macroscópicas (SILVA et al., 2004). O PV infecta o epitélio escamoso estratificado da pele e mucosas causando lesões benignas as quais podem progredir para neoplasias malignas (HOWLEY; LOWY, 2001; LOWY; SCHILLER, 2006). Acredita-se que, para a infecção ser estabelecida, é necessário a ocorrência de um microtrauma ou erosão da epiderme para que o vírus possa infectar o epitélio basal (DOORBAR et al., 2012).

Uma vez que o PV infecta a célula basal, o genoma viral é incorporado dentro do núcleo depois de a célula completar um ciclo mitótico, e permanece lá como um cromossomo episomal (BRAVO; FÉLEZ-SÁNCHEZ, 2015; DOORBAR et al., 2012). Os genes virais são expressos na fase proliferativa, e a expressão completa do genoma viral resulta na produção de capsídeos virais, somente após o início da diferenciação celular nas camadas superiores do epitélio. As proteínas E6 e E7 parecem mediar a proliferação das células das camadas basal e parabasal e a liberação do vírus ocorre durante a descamação das células infectadas da superfície das lesões. As células infectadas proliferam e a diferenciação é demorada (DOORBAR et al., 2012). Um esquema da infecção por HPV é ilustrado na Figura 4.

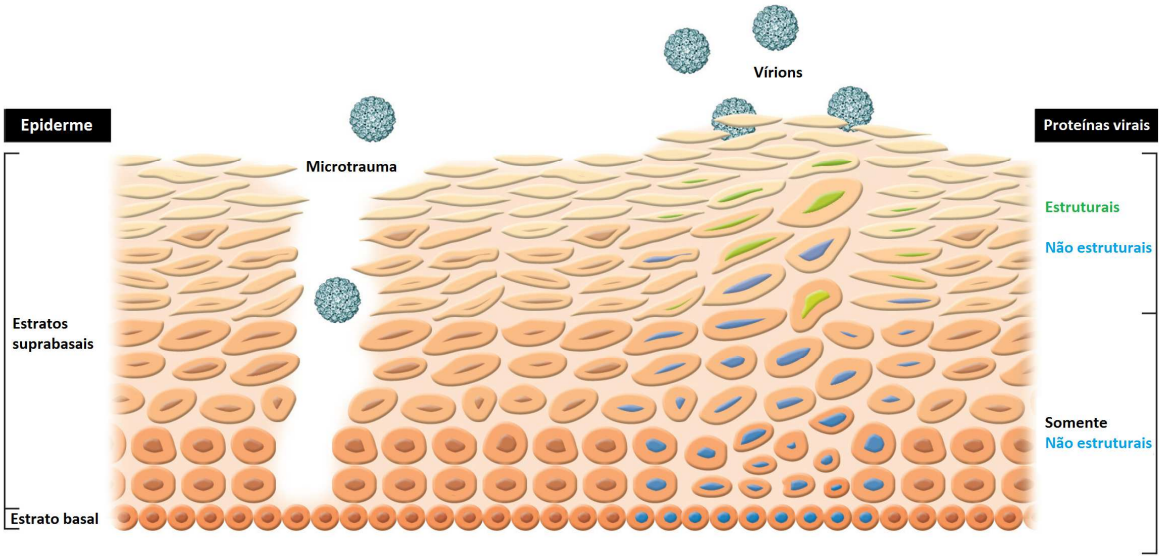

Figura 4: Representação esquemática do ciclo de replicação do papilomavírus. Adatptado de Lowy and Schiller (2006).

As infecções crônicas assintomáticas e os papilomas causados pelos PV parecem estar ligados à estratégia de transformação celular e propagação dos vírions no epitélio e, provavelmente, também às diferentes relações entre eles e o sistema imune do hospedeiro (DOORBAR et al., 2012). Os fatores genéticos do hospedeiro, que controlam o sistema imune inato e as respostas das células T efetoras, podem determinar o aumento do risco de infecção persistente (SHAFTI-KERAMAT et al., 2009), e a fraca resposta imune dos bovinos ao BPV pode estar relacionada ao fato de o ciclo de vida do vírus ser restrito ao epitélio (CAMPO, 2006). Geralmente as infecções causadas por PV regridem naturalmente e o exame histológico das lesões revela uma intensa infiltração de linfócitos na derme e no epitélio (CAMPO, 2003), reforçando o papel da resposta imune celular no combate ao agente infeccioso (O'BRIEN; SAVERIA CAMPO, 2002).

Os PV já foram descritos em várias ordens de mamíferos, répteis, aves e peixes, oriundos tanto de lesões papilomatosas quanto de pele saudável (ANTONSSON; HANSSON, 2002; LÓPEZ-BUENO et al., 2016; RECTOR; VAN RANST, 2013). Nos bovinos, os papilomas são encontrados em diversas áreas do corpo (cabeça, pescoço,

dorso, ventre, abdome, úbere, mucosas do trato digestivo e de bexiga urinária) ou podem se apresentar de maneira generalizada (CAMPO, 1997; CARVALHO et al., 2012; OGAWA et al., 2004; TOZATO et al., 2013).

As proliferações papilomatosas geralmente ocorrem em animais mais jovens, abaixo dos dois anos de idade, porém a ocorrência em animais adultos não é incomum (DA SILVA et al., 2015; SMITH, 2006). Tais proliferações podem ser de coloração branca, castanha ou cinza, são massas firmes que fazem protrusão, com superfície córnea ressecada e podem variar de tamanho, podendo ser solitárias ou múltiplas.

Embora alguns autores hipotetizem que diferentes tipos de BPV tenham predileção por diferentes localidades anatômicas dos bovinos, os BPV tem sido associados com as vários tipos de lesões e localidades corporais. O BPV1 é associado com fibropapilomas de teto e pênis; o BPV2 é associado com papilomas cutâneos, papilomas do trato digestivo e tumores de bexiga (associado com a ingestão de samambaia *Pteridium* spp.) (BORZACCHIELLO; ROPERTO, 2008; CAMPO et al., 1992; MUNDAY, 2014b); o BPV3 é associado com papilomas cutâneos; BPV4 com papilomas do trato gastrintestinal superior (relacionado com a ingestão de samambaia *Pteridium aquilinum*) e também com papilomas cutâneos (ARALDI et al., 2014; MUNDAY, 2014b; NASIR; CAMPO, 2008); o BPV5 com fibropapilomas de úbere; BPV6 é relacionado com papilomas de teto (BORZACCHIELLO; ROPERTO, 2008; LUNARDI et al., 2016; TOZATO et al., 2013); BPV7 é associado com papiloma cutâneo e de teto e foi encontrado em pele saudável de tetos de bovinos (DA SILVA et al., 2015; OGAWA et al., 2004; TOZATO et al., 2013); BPV8 e 11 foram descritos em papilomas cutâneos; BPV9 e 10 são associados com papiloma escamosos do úbere e teto (BORZACCHIELLO; ROPERTO, 2008; LUNARDI et al., 2016; TOZATO et al., 2013); o BPV 12 foi descrito primeiramente em um papiloma epitelial de língua e também é associado com papilomas cutâneos (ARALDI et al., 2014; ZHU et al., 2012); BPV13 é associado com papiloma cutâneo (DA SILVA et al., 2015; LUNARDI et al., 2013b). O BPV14 e 15, foram detectados em pele saudável e fibropapilomas de gado (embora tenha sido primeiramente descrito em uma lesão tipo sarcóide em gato) (MUNDAY et al., 2015), e em sarcóide bovino, respectivamente (“ICTV”, 2017).

2.6.2 Hematúria enzoótica em bovinos

A hematúria enzoótica crônica é uma síndrome caracterizada pela presença de sangue na urina, comumente provocada por neoplasia de bexiga urinária em animais adultos que tem acesso prolongado a pastagens ricas em samambaia (*Pteridium* ssp.), (CAMPO, 2006; ROPERTO et al., 2010). A incidência de tumores varia entre o gado que está no pasto com acesso à samambaia, mas pode ser igual ou maior do que 90% (ROPERTO et al., 2010). A samambaia tem sido identificada como um cofator ambiental importante na indução da carcinogênese no gado, pois naturalmente contém um grande número de toxinas imunossupressoras e mutagênicas (CAMPO, 2006). Estas toxinas induzem a transformação maligna, que promovem instabilidade genômica, resistência à apoptose, desregulando o ciclo celular (GIL DA COSTA; MEDEIROS, 2014).

O BPV2 é uma importante causa da carcinogênese de bexiga urinária em bovinos (MARTANO et al., 2013; ROPERTO et al., 2010; WOSIACKI et al., 2002) em sinergismo com os princípios carcinogênicos e tóxicos da samambaia (CAMPO, 1997; WOSIACKI et al., 2002). O genoma do BPV2 já foi encontrado em epitélio de bexiga saudável (BORZACCHIELLO; ROPERTO, 2008) e outras secreções e órgãos de bovinos clinicamente assintomáticos (LINDSEY et al., 2009). O vírus em estado latente pode ser "ativado" pela ingestão de samambaia, pois substâncias químicas da samambaia podem induzir imunodepressão, o que faz com que inicie a progressão das neoplasias (CAMPO, 1997; ROPERTO et al., 2010).

A expressão da oncoproteína E5, que induz transformações morfológicas e tumorigênicas em fibroblastos humanos e de roedores (DOORBAR et al., 2012), também é comumente detectada em tecidos neoplásicos de bexiga e em células de sangue periférico em bovinos infectados com BPV (CAMPO, 2006; ROPERTO et al., 2010). Em tecidos naturalmente infectados pelo BPV1, a oncoproteína E5 é expressa no citoplasma de células epiteliais basais e suprabasais, principalmente no complexo de Golgi (BORZACCHIELLO; ROPERTO, 2008). Esta oncoproteína interage fisicamente com o fator de crescimento plaquetário derivado β (PDGF β) e forma um complexo estável nas células neoplásicas de bexiga urinária, sugerindo um papel importante para a oncoproteína E5 no câncer de bexiga urinária (BORZACCHIELLO et al., 2006).

A oncoproteína E7 é expressa no citoplasma de células de tecidos tumorais de bexiga urinária em bovinos e pode ser observada na área apical da membrana das células tumorais (CAMPO, 2006; ROPERTO et al., 2010). As duas oncoproteínas virais, E5 e E7 não são expressas em células endoteliais normais, e a expressão de tais proteínas em tecidos neoplásicos demonstra que o BPV pode ter um papel importante no processo neoplásico (BORZACCHIELLO et al., 2007). Em humanos, a elevação dos níveis de E6 e E7 está diretamente relacionada com o aumento da gravidade das neoplasias, e a expressão desregulada desses genes (*E6* e *E7*) é fundamentalmente responsável pela acumulação de erros genéticos nas células infectadas e com a eventual integração dos episssomas virais nos cromossomos da célula hospedeira (DOORBAR et al., 2012).

2.7 Diagnóstico e tratamento

Como as alterações são bem características, o diagnóstico pode ser feito clinicamente. Uma biópsia também pode ser realizada para se observar microscopicamente em cortes histológicos as alterações histológicas provocadas pelo vírus. Também podem ser observadas as partículas víricas por microscopia eletrônica. Contudo, essas técnicas não são utilizadas como rotina (SCHUCH, 2001). O diagnóstico definitivo é baseado na identificação do vírus ou de seus efeitos citopáticos (AIELLO; ASA, 2001).

Em bovinos o diagnóstico viral também é realizado através da Reação em Cadeia da Polimerase (*polymerase chain reaction* - PCR). Esta é uma técnica adequada e utilizada para identificação de PV devido ao alto grau de especificidade e sensibilidade (FORSLUND et al., 1999). Porém, esses parâmetros podem ser afetados por fatores primários como a concentração e a pureza da amostra de DNA (OGAWA et al., 2004). Portanto, a coleta e armazenamento adequados da amostra, assim como o cuidado na extração do DNA, são passos importantes e que devem ser levados em consideração para termos resultados fidedignos na biologia molecular.

A vacina autógena é o tratamento mais comumente utilizado para a papilomatose bovina (SCHUCH, 2001). É feito um macerado com os papilomas do animal afetado e o vírus é inativado (AIELLO; ASA, 2001; SCHUCH, 2001), porém os resultados

dependem do tipo de papiloma, da preparação da vacina e do estágio de evolução das lesões (SCHUCH, 2001). No Brasil, a vacina autógena tem sido utilizada como tratamento terapêutico para animais extensamente atingidos por papilomatose e apresenta resultados de, aproximadamente, 50% de recuperação. No entanto este tratamento, além de não ser muito eficaz, possui custo elevado (SILVA et al., 2004).

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

2.8 Prevenção e controle

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

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

(BORZACCHIELLO et al., 2009). Deste modo, uma vacina contra o BPV foi adaptada para testes em bovinos (MÜLLER, 2014). Vacinas que utilizam proteínas recombinantes induzem proteção homóloga e heteróloga contra os tipos virais de maior importância clínica em bovinos (NICHOLLS; STANLEY, 2000), embora elas não induzam uma resposta imune esterelizante. Desta forma, tais vacinas instigam os pesquisadores a encontrarem uma forma satisfatória de ativar a resposta imunológica, tanto nos tratamentos profiláticos quanto terapêuticos, nas infecções por papilomavírus (NICHOLLS; STANLEY, 2000).

Embora alguns estudos acerca da vacinação profilática e terapêutica contra BPV em bovinos tenham sido feitos, ainda existe um grande desafio em relação à esta questão, uma vez que estudos vacinais profiláticos envolvem alto custo financeiro e de mão-de-obra. Portanto, a adoção de medidas de prevenção são importantes, as quais consistem principalmente em evitar contato de animais com papilomas com animais sem papilomas. Ainda, cuidados básicos de manejo, como não compartilhar cabrestos e ordenhadeiras, de animais com papilomas, isolar o animal afetado, realizar a desinfecção de instrumentos de descorna, marcação e tatuagem (SMITH, 2006) são de grande importância. Pode-se também desinfetar baias, bretes e outros materiais através da fumigação com formaldeído para evitar a contaminação (AIELLO; ASA, 2001).

3 HIPÓTESE

A diversidade genética dos BPV é provavelmente equiparada à diversidade genética dos HPV existentes, e novos tipos virais podem ser identificados e caracterizados, principalmente no Brasil, onde existe uma grande densidade de bovinos de corte e de leite.

4 OBJETIVOS

4.1 Geral

Estudar a diversidade genética do papilomavírus bovino.

4.2 Específicos

- Investigar os tipos de BPV presentes em lesões papilomatosas de bovinos do norte e sul do Brasil, nos estados de Rondônia, Acre e Rio Grande do Sul, através da amplificação parcial do gene L1.
- Sequenciar o genoma completo de um *Deltapapilomavírus 4* (BPV2).
- Investigar diversidade genética de BPV presente em um lesões papilomatosas de bovinos no norte e sul do Brasil através de sequenciamento de alta eficiência.
- Sequenciar os genomas completos de prováveis novos tipos virais encontrados neste estudo.

5 CAPÍTULO 1: CARACTERIZAÇÃO GENÉTICA DE PAPILOMAVÍRUS BOVINOS DA AMAZÔNIA REVELA A EXISTÊNCIA DE QUATRO PROVÁVEIS NOVOS TIPOS

O presente experimento já foi concluído e um artigo científico intitulado "*Genetic characterization of Amazonian bovine papillomavirus reveals the existence of four new putative types*" foi redigido, submetido e aceito para publicação em 18/06/2015 no periódico *Virus Genes*, Qualis B1 e fator de impacto 1,431. O manuscrito será apresentado a seguir, tal qual foi publicado.

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Genetic characterization of Amazonian bovine papillomavirus reveals the existence of four new putative types

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Abstract Papillomaviruses are small and complex viruses that belong to the *Papillomaviridae* family, which comprises 39 genera. The bovine papillomavirus (BPV) causes an infectious disease that is characterized by chronic and proliferative benign tumors that affect cattle worldwide. Different genotypes of BPVs can cause distinct skin and mucosal lesions and the immunity they raise has low cross-protection. This report aimed to genotype BPVs in cattle from Northern Brazil based on nucleotide partial sequences of the L1 ORF. Skin wart samples from 39 bovines clinically and histopathologically diagnosed as cutaneous papillomatosis from Acre and Rondônia States were analyzed. The results revealed four already reported BPV types (BPVs 1, 2, 11, and 13), nine putative new BPV subtypes and four putative new BPV types as well as two putative new BPV types that were already reported. To our knowledge, this is the first record of BPVs from the Brazilian Amazon region that identified new possible BPV types and subtypes circulating in this population. These findings point to the great genetic diversity of BPVs that are present in this region and highlight the importance of this knowledge before further studies about vaccination are attempted.

Keywords Bovine · Papillomavirus · Genotype · Amazon · Phylogeny · Cattle

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Introduction

Papillomaviruses (PVs) are small and complex viruses that belong to the family *Papillomaviridae*, which is composed of 39 genera [1]. They have circular double-stranded DNA containing approximately 8000 base pairs (bp). In cattle, bovine papillomaviruses (BPVs) infection may be asymptomatic, although it can induce skin warts or neoplasia in the mucosa of the urinary bladder and upper digestive tract [2, 3]. Generally, PVs are strictly species and tissue-specific, although equine sarcoids can be caused by BPVs 1, 2 and 13 [4, 5].

The BPVs were previously classified into three genera that used to be divided in thirteen types (BPVs1–13) [6]. However, according to the new suggested nomenclature, they are now classified into four genera, five species and eight types [1]. The *Xipapillomavirus* genus comprises two species, *Xipapillomavirus* 1 (BPV3) and 2 (BPV12), and five types, BPVs 4, 6, 9, 10, and 11. The *Deltapapillomavirus* genus comprises a single species, *Deltapapillomavirus* 4 (BPV1), and two types, BPVs 2 and 13. The *Epsilonpapillomavirus* genus also comprises a single species, *Epsilonpapillomavirus* 1 (BPV5), and one type (BPV8). The BPV7 was generally assigned as an unclassified genus [7] and according to the new classification; this

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is the only BPV species that represents the *Dyoxipapillomavirus* genus [8].

The new PV types were considered when the entire genome was sequenced and the L1 ORF was found to differ by more than 10 % in comparison to any other PV type. Similarities greater than 60 % define the PV genus. A subtype is defined when the difference of the L1 ORF is between 2 and 10 % and a variant is when the difference is less than 2 % [6, 9]. The genetic characterization is usually performed by PCR using the degenerated primer pair (FAP59/FAP64) that amplifies a highly conserved region of the L1 gene from all known PV types, followed by sequencing of the amplification product [10]. This technique has enabled the identification of several putative new BPV types in both dairy and beef cattle from distinct geographical regions worldwide [11–13]. Despite the thirteen BPV types recognized, there are at least 170 human papillomavirus types (HPV) related to skin and mucosal diseases, which reflects the fact that studies in HPV have been more extensive when compared to animal PVs, supporting the importance of more studies in cattle because it appears that many animal PV types have not been detected [8, 14].

Although numerous BPV types have been described and putative and new BPV types have identified in Brazilian cattle from distinct regions [13, 15–18], Brazil is an extensive territory encompassing unstudied regions, which comprise a low number of habitants and one of the largest cattle herd worldwide. In this context, the Amazon region is propitious for the discovery of novel strains because this region has one of the largest beef cattle herds in Brazil and, in addition, the Amazon biome has one of the largest biodiversities worldwide. Therefore, the genetic characterization of this virus is an important basic knowledge to preview the effectiveness of future vaccines, which is what has been done with HPV. In this report, cutaneous papillomatosis from cattle herds in the Brazilian Amazon region were investigated, and novel putative BPV types and subtypes were genetically characterized.

Materials and methods

Samples

Samples of skin warts from 39 bovines of distinct dairy cattle herds from different municipalities of Acre (four samples) and Rondônia (17 samples) States in the Northern Brazil, Amazon region, which were clinically diagnosed as papillomatosis were obtained from diverse bovine body parts. The lesions were removed using scalpels after local anesthesia was performed with 2 % lidocaine (Bravet, Brazil). Half of the sample was individually wrapped and stored at −20 °C for DNA extraction and the other half was stored in 10 % buffered formaldehyde for histopathological analyses.

Histopathology

Tissue sections were fixed in 10 % buffered formalin, trimmed, and processed routinely for histopathology. One section per tissue was cut at 3 µm and stained with hematoxylin and eosin (HE) and Masson's trichrome.

DNA extraction and PCR

Papilloma samples were ground with sterile sand in 10 mL of phosphate buffered saline (PBS) (pH 7.4), centrifuged at 720×*g* for 10 min and 100 µL of the supernatant was stored at −20 °C for DNA extraction. DNA was extracted using a silica based-protocol [19]. The purified DNA was eluted in 10 mM Tris, 1 mM EDTA and stored at −20 °C.

Partial amplification of the L1 gene was performed with the forward primer FAP59 (5′-TAA CWG TIG GIC AYC CWT ATT-3′) (Position in BPV strain X02346: 5712-5752) and the reverse primer FAP64 (5′-CCW ATA TCW VHC ATI TCI CCA TC-3′) (Position in BPV strain X02346: 6206-6185) [10]. The nucleotide numbers in the primers alignments were based on the *Deltapapillomavirus* 4 (BPV1) sequence (GenBank BPV strain: X02346). Aliquots from the reactions were analyzed by electrophoresis in 2 % agarose gels stained with Blue Green Loading DYE I (LGC, Brazil), and examined under UV light with Molecular Imaging Software Gel Logic (Kodak, USA).

Sequencing

The PCR amplification products were purified using the NucleoSpin Extract II Kit (Macherey–Nagel, Düren, Germany), and both strands were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems). The sequences were assembled using the SeqMan software of the Lasergene package (DNASTAR, USA).

Phylogenetic analysis

Local sequence alignments were accomplished to determine the sequence identity with BLAST [20]. Representative sequences and sequences with the highest homology to the sequences from this study that are available in GenBank were retrieved from the NCBI homepage (http://www.ncbi.nlm.nih.gov/) for phylogenetic analysis with the sequences described here. Altogether, the dataset consisted of 82 sequences of the L1 gene. The nucleotide alignment was performed using the back translated amino acid sequenced with the MUSCLE software [21].

The phylogeny was estimated with a Bayesian Markov Chain Monte Carlo (MCMC) method, using BEAST version 1.7.2 [22]. These analyses were run using an estimated GTR + I + G substitution model with partitions into codon positions and six categories of gamma, performing 10 million generations through the MCMC and sub-sampling each 1000 generations. The tree prior used was the Logistic grown model with an uncorrelated exponential relaxed clock and normal distribution of rates. The resulting data were analyzed using the software Tracer (http://tree.bio.ed.ac.uk/software/tracer/) after removing a 10 % "burn in" for each data and compared with different priors. Several runs were performed to optimize the parameters used in the Bayesian Inference. A maximum clade credibility tree for each dataset was generated by the software TreeAnnotator v.1.7.1 (http://beast.bio.ed.ac.uk/treeannotator). The phylogenetic trees were visualized with the software FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/).

Ethics statement

Lesions were collected by veterinarians to prepare autogenous vaccines. All efforts were made to minimize animal suffering.

Results

Histopathology

The neoplastic tissue was comprised by exophytic papillomatous, epithelium proliferation, and well-differentiated cells, showing marked acanthosis, increased amounts of granules in the granular layer, and keratohyalin granules. Koilocytes (enlarged keratinocytes with eccentric pyknotic nuclei surrounded by a clear halo) and marked orthokeratotic hyperkeratosis were occasionally observed. The basal cells were observed in the periphery of the growths, and their surface was moderately hyperkeratotic. In addition, a moderate multifocal inflammatory infiltrate composed mainly of lymphocytes and plasma cells, with occasional eosinophils was observed in the dermis (Fig. 1 a–d). Based on the histopathological findings, the diagnosis of epidermal papillomatosis was confirmed in the 39 samples.

Phylogenetic analysis

In total, 39 samples from Amazonian Region were analyzed, four displayed negative results and 35 were positive.

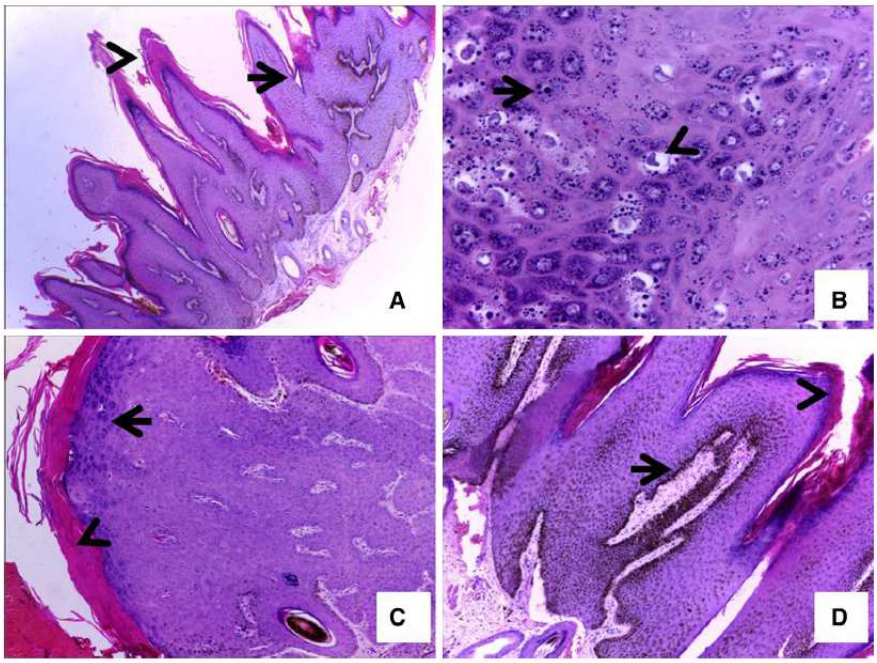


Fig. 1 Exophytic papillomatous proliferation of the epithelium of bovines sampled in this study. (**a**) Acanthosis (*arrow*) and moderate orthokeratotic hyperkeratosis (*head of arrow*). Obj.10x. (**b**) Increased deposition of keratohyalin granules (*arrow*) in the stratum granulosum and occasional koilocytes (*head of arrow*). Obj.40x. (**c**) Keratohyalin granules (*arrow*). Orthokeratotic hyperkeratosis (*head of arrow*). HE, Obj.10x. (**d**) Marked retention of melanin in the stratum basale of the epidermis (*arrow*) and discrete hyperkeratosis orthokeratotic (*head of arrow*). Obj.10x

Nine of those positive samples had not enough genetic material for sequencing. The other 26 samples were sequenced; however, five of those samples that showed double peaks and noises were excluded.

Data about the cattle sampled, lesion morphology, BPV types, and putative new BPV types found in this study are summarized on Table 1. The great majority of papillomavirus strains detected in this study belonged to the genus *Deltapapillomavirus* (12) and *Xipapillomavirus* (5). Two strains were related to the genus *Epsilonpapillomavirus* and two to the genus *Dyoxipapillomavirus*, according to the phylogenetic analysis (Fig. 2).

Those belonging to the *Deltapapillomavirus* genus were strains 01AC12, 03AC12, 06RO10, 08RO11, 11RO11, 15RO12, 16RO12, which was similar to BPV2 (97.5, 99.3, 99.3, 97.5, 97.5, 97.5, 97.5 %, respectively). Among these strains, five were classified as putative subtypes and two as variants, according to their identity with BPV2. Strain 13RO12 was closer to BPV1 (100 % of identity) and four other strains (01RO10, 02RO10, 12RO12, 17RO12) were classified as putative new subtypes of BPV13 (96.7 %). The strain 07RO11 grouped at the genus *Xipapillomavirus* and was classified as variant of BPV11 (99.6 %).

Three strains were closer to two putative new BPV types that were previously described. Two homologous strains from Rondônia State (03RO10 and 10RO11) were more closely related (96.4 %) to two strains described previously as putative new types (GenBank accession numbers HQ612180 and AY300819, respectively). The third strain (04RO10) was closely related to a putative new type reported in Southern Brazil (GenBank accession number EU293541) (97.1 %).

Higher phylogenetic distances were observed in the two strains from Rondônia State that were encompassed into a separated cluster with a same ancestor as BPV7 in the terminal node. Two homologous strains, identified as 09RO11 and 14RO12, had low identity (64.5 %) with BPV7, and therefore represent a putative new type. Another strain (02AC12) was also identified as a putative new type sharing low identity to BPV8 (77.9 %) despite the fact that they grouped in the same terminal node in the genus *Epsilonpapillomavirus*.

Additionally, according to the phylogenetic analysis, the strains 05RO10 and 06AC14 from Rondônia and Acre States, respectively, were classified as putative new types belonging to the genus *Xipapillomavirus*. The strain 05RO10 has the same ancestor that the BPV11 strains but is located on an independent branch (81.9 %). The strain 06AC14 grouped in the same terminal node as BPV12 nevertheless they have low identity (76.5 %).

Discussion

In the present study, papillomavirus DNA was amplified and sequenced in 21 of 39 cutaneous wart samples. Although some BPVs have higher affinities to specific primers [23], in the present study the primer pair FAP59/64 was able to amplify bovine papillomavirus DNA from all known BPVs genera. Despite the lower level of sensitivity of these primers when compared to specific primers [23] they have been used in many studies, being an important tool to enlarge the knowledge of BPVs diversity and enabling the discovery of a broad range of novel BPVs [10–13, 16].

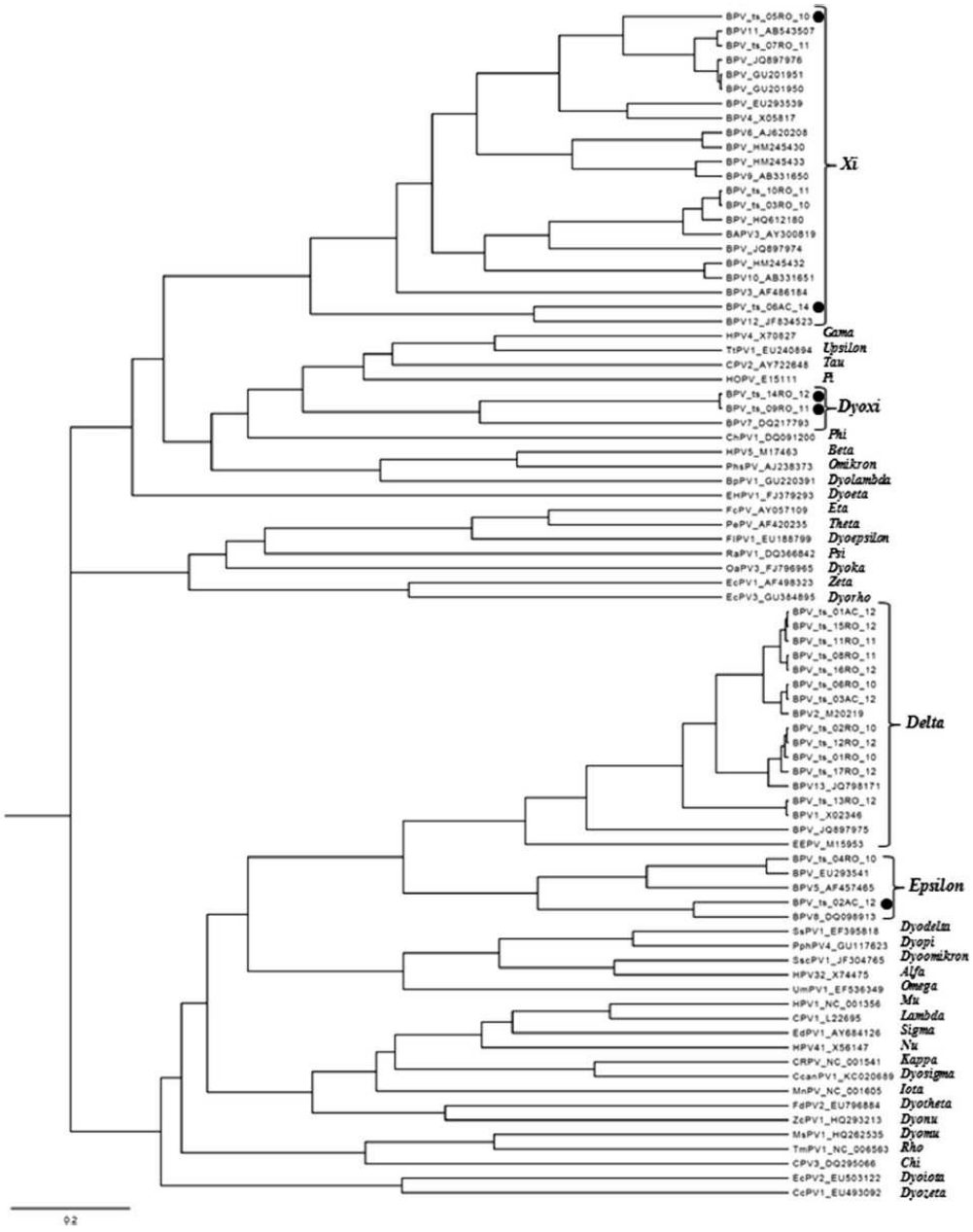
Based on the partial sequence of the L1 gene the strains detected encompassed the BPV1, 2, 11, and 13 types and two putative new types that were previously reported. Additionally, nine putative new subtypes and four putative new types of BPVs were characterized. The histopathological findings of the samples are consistent with those lesions typically observed in papilloma lesions [17, 24, 25] but no fibroblast proliferation was observed, ergo clinical and histopathological findings confirmed the diagnosis of papillomatosis. The majority of the strains clustered within the *Deltapapillomavirus* genus that encompasses the BPV types considered to be etiological agents of papillomatosis in cattle [12, 13, 17, 18], sarcoid disease in horses [4, 5, 26], as well as urinary bladder in cow [27]. BPVs 1 and 2 are the most common BPV types distributed worldwide and unusually, they have the ability to infect not only bovines but also other species such as equines [4, 5, 26], yaks [28], and buffalos [27, 29]. In Brazil, these BPV types were already described in studies carried out in cattle herds from the Southern, South-East and North-East regions [13, 18, 30, 31], and the present findings reveals that they are also present in the Amazon region of Brazil.

Seven BPVs had lower identity compared with the reference BPV strains. Three of these strains had high identity with the strains HQ612180 and EU293541, which are novel putative types recently reported in different regions from Brazil [13, 31]. They have a close relationship with isolates previously described in Japan and recently in the North-East [12, 32] and Southern regions of Brazil [13] that belong to two different PV genera (*Xi* and *Epsilonpapillomavirus*). Furthermore, the histopathological findings are similar to those previously described [32] suggesting that this putative new type is a potential causative of cutaneous papillomatosis. Additionally, the fact that these strains are from distinct years and localities indicates that these viruses are clearly widespread and the detection of those novel putative BPV types also in Northern Brazil suggests that these genotypes are possibly frequent in the virus population rather than rare events.

Table 1 Data about the cattle sampled, lesion morphology, BPV types, and putative new BPV types found in this study

BPV type or PNT	State	Geographical location (lat and long)	Municipality	Age category	Gender	Lesion	Identity %	Strain	GeneBank acession number
BPV1	Rondônia	−10°44′53″ −62°12′57″	Ouro Preto do Oeste	Adult	Male	MI	100	13RO12	KP701423
BPV2	Rondônia	−10°53′07″ −61°57′06″	Ji-Paraná	Young	Female	MI	97.5–99.3	06RO10	KP701434
	Rondônia	−11°10′31″ −61°54′05″	Presidente Médici	Young	Male	Peduncle		08RO11	KP701421
	Rondônia	−61°41′35″ −10°44′53″	Nova Colina	Young	Female	Flat		11RO11	KP701430
	Rondônia	−10°44′53″ −62°12′57″	Ouro Preto do Oeste	Adult	Male	MI		15RO12	KP701426
	Rondônia	−10°44′53″ −62°12′57″	Ouro Preto do Oeste	Adult	Female	MI		16RO12	KP701427
	Acre	−09°58′29″ −67°48′36″	Rio Branco	Adult	Female	Peduncle		01AC12	KP701414
	Acre	−09°49′40″ −66°53′00″	Acrelândia	Adult	Female	Flat		03AC12	KP701416
BPV7	Rondônia	MI	MI	Adult	Female	Peduncle	64.5	09RO11[a]	KP701425[a]
	Rondônia	−10°44′53″ −62°12′57″	Ouro Preto do Oeste	Adult	Female	MI		14RO12[a]	KP701424[a]
BPV8	Acre	−09°49′40″ −66°53′00″	Acrelândia	Adult	Female	Flat	77.9	02AC12[a]	KP701415[a]
BPV11	Rondônia	−09°54′48″ −63°02′27″	Ariquemes	Adult	MI	Peduncle	99.6	07RO11	KP701418
	Rondônia	−11°01′47″ −62°40′30″	Mirante da Serra	Adult	Female	MI	81.9	05RO10[a]	KP701432[a]
BPV12	Acre	−09°58′29″ −67°48′36″	Rio Branco	Adult	Female	Peduncle	76.5	06AC14[a]	KP701433[a]
BPV13	Rondônia	−10°53′07″ −61°57′06″	Ji-Paraná	Adult	Female	MI	96.7	01RO10	KP701417
	Rondônia	−10°53′07″ −61°57′06″	Ji-Paraná	Adult	Female	MI		02RO10	KP701420
	Rondônia	−10°44′53″ −62°12′57″	Ouro Preto do Oeste	Young	Female	MI		12RO12	KP701419
	Rondônia	−10°44′53″ −62°12′57″	Ouro Preto do Oeste	Adult	MI	MI		17RO12	KP701429
HQ612180	Rondônia	−10°44′53″ −62°12′57″	Ouro Preto do Oeste	Adult	MI	MI	96.4	03RO10	KP701422
	Rondônia	−61°41′35″ −10°44′53″	Nova Colina	Young	Female	Flat		10RO11	KP701428
EU293541	Rondônia	−11°01′47″ −62°40′30″	Mirante da Serra	Adult	MI	MI	97.1	04RO10	KP701431

Data about the cattle sampled and their similarities with representative strains and putative new types already described. Gender and lesion morphology are missing (MI) for some samples. Known putative new types are indicated as "PNT". [a]Putative new types found in the present study. The upper coordinate represents Latitude (lat) and the lower coordinate represents longitude (long)



In this report, we detected four novel putative BPV types that belonged to the three distinct genera *Xi*, *Epsilon*, and *Dyoxipapillomavirus*. These putative new types have low identity with BPVs 7, 8, 11, and 12 and the histopathological results indicate their potential for causing papillomatosis since the closest related types were previously linked to cutaneous papillomas [3, 7, 13, 16, 17, 24, 33, 34]. Moreover, to our knowledge, this is the first description of a putative new type related to the new *Dyoxipapillomavirus* genus.

Additionally, the great number of putative new subtypes and the detection of putative new types recently reported also suggest the existence of several prevalent BPV types that have been undetected in unstudied regions, such as the

◀**Fig. 2** Phylogenetic tree of the bovine papillomavirus dataset based on partial sequences of L1 ORF. Posterior probabilities are indicated above the main branches. Putative new types from the present work belonging to the genera *Xi*, *Dyoxi*, and *Epsilonpapillomavirus* are indicated with black dots. Samples belonging of this study with representatives of other PVs genera were included on the analysis and a total of 82 PV types of different species and genera were analyzed. Accession numbers for the sequences used are included. *BPV*, bovine papillomavirus; *HPV*, human papillomavirus; *TtPV*, *Tursiops truncatus* papillomavirus; *CPV*, canine papillomavirus; *HOPV*, hamster oral papillomavirus; *ChPV*, *Capra hircus* papillomavirus; *PhPV*, *Phocoena spinipinnis* papillomavirus; *BpPV*, *Betonggia penicillata* papillomavirus; *EHPV*, European hedgehog papillomavirus; *FcPV*, *Fringilla coelebs* papillomavirus; *PePV*, *Psittacus erithacus timneh* papillomavirus; *FlPV*, *Francolinus leucoscepus* papillomavirus; *RaPV*, *Rousettus aegyptiacus* papillomavirus; *OaPV*, *Ovis aries* papillomavirus; *EcPV*, *Equus caballus* papillomavirus; *EEPV*, European elk papillomavirus; *SsPV*, *Sus scrofa* papillomavirus; *PhPV*, *Phocoena phocoena* papillomavirus; *SscPV*, *Saimiri sciureus* papillomavirus; *UmPV*, *Ursus maritimus* papillomavirus; *EdPV*, *Erethizon dorsatum* papillomavirus; *CRPV*, Cottontail rabbit papillomavirus; *CcanPV*, *Castor canadensis* papillomavirus; *MnPV*, *Mastomys natalensis* papillomavirus; *FdPV*, Feline papillomavirus; *ZcPV*, *Zalophus californianus* papillomavirus; *MsPV*, *Morelia spilota* papillomavirus; *TmPV*, *Trichechus manatus latirostris* papillomavirus; *CcPV*, *Caretta caretta* papillomavirus

Amazon region that is virtually propitious for the discovery of novel strains since this ecosystem has one of the largest biodiversities worldwide. Moreover, in comparison to HPV, which has approximately 170 PV types identified, it appears that many animal PV types have not yet been detected [8, 14] and this is a reflection of the fact that human PVs have been extensively studied [6].

Despite the few number of papilloma specimens analyzed, it was possible to identify several putative new BPV types and subtypes from cattle herds of distinct farms from the Acre and Rondônia States. Additionally, the identification of these Brazilian putative new BPV types related with distinct genera, including the recently defined *Dyoxipapillomavirus* genus, indicates a high diversity of BPVs in this region. The marked viral diversity described here reflects the characteristics of the Amazonian region, which includes a mixture of several breeds, lower sanitary care, and the climate of the tropical forest. Although previous studies have shown that the BPVs distribution is most likely geographically unrelated [17] our findings points to a great viral diversity in this region. This high BPVs diversity on Amazon region could reinforce that the majority of animal PVs are undetected [14], mainly in the unstudied regions.

The paper aimed to genotype BPVs in cattle from the Brazilian Amazon region based on nucleotide partial sequences of the L1 ORF amplified from skin wart samples that were clinically and histopathologically diagnosed as papillomatosis. We identified nine putative new BPV subtypes and four putative new BPV types as well as two putative new BPV types that were already reported. To our knowledge, this is the first record of BPVs from the Brazilian Amazon region that showed the great genetic diversity of BPVs that is present in this region and highlights the importance of this knowledge before further studies about vaccination are attempted.

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6 CAPÍTULO 2: EXPANSÃO DA FAMÍLIA PAPILOMAVIRIDAE EM HOSPEDEIROS BOVINOS: CARACTERIZAÇÃO DE DOIS NOVOS TIPOS VIRAIS PERTENCENTES AOS GÊNEROS XI E EPSILONPAPILMAVÍRUS

O presente trabalho já foi concluído e um artigo científico intitulado "*Expansion of papillomaviridae in bovine host: characterization of two new virus types assigned to Xi and Epsilonpapillomavirus genus*" está em fase de redação e será enviado para publicação no periódico *Transboundary and Emmerging Diseases*. O manuscrito será apresentado a seguir como se encontrava no momento da finalização desta tese.

11 **Expansion of *Papillomaviridae* in bovine host: characterization of two new virus**
12 **types assigned to *Xi* and *Epsilonpapillomavirus* genus**

13

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Abstract

The importance of knowing the diversity, taxonomy and evolution of PVs for diagnostic and therapeutic approaches has been increasingly acknowledged over the past few years. BPV are viruses that affect cattle herds around the world and cause economic relevant economic losses. Brazil is one of the largests beef producers and exporters worldwide. In this way we aim to analyze the BPV types that are circulating in the Southern region of Brazil, in the State of Rio Grande do Sul. Through the PCR technique and using the generic FAP primer pair, we observed mainly the presence of *Delta*-PVs. Additionally,we could verify the presence of two putative new BPV types, which were characterized through the RCA technique and high performance sequencing. A new type characterized in this study belongs to the genus Epsilonpapillomavirus and it is the first *Epsillon*-PV characterized in South America.

Introduction

Since the first description of the human papova group in 60's [1], a numerous other viruses have been identified with genomes composed of a circular double-stranded DNA (dsDNA) [2-4]. Today, the *Papovaviridae* family was extinct and his members were (re)classified into *Polyomaviridae* and *Papillomaviridae* families. Papillomaviruses (PV) are an ancient group of viruses with small, double-stranded DNA circular genomes that are mostly 7–8 kb. They are species-specific and have a strict tropism for mucosal and cutaneous stratified squamous epithelial surfaces of the host. PVs have been isolated from a wide range of host species, including mammals, birds, reptiles and fish [5,6].

The study group of PVs, within the International Committee for the Taxonomy of Viruses, provides guidelines for PV classification and nomenclature (http://ictvonline.org/index.asp). Classically, viral taxonomy standards for PVs do not implement taxonomic categories below viral species. However, for the PV community, the relevant taxonomic level is the 'type': two PV genomes sharing more than 90% nucleotide identity in the L1 gene (that's encodes the major capsid protein) belong into the same PV type. Around a half of the full-length genome PV types entries in the GenBank and PaVE (Papillomavirus Episteme) [7] correspond to HPVs (>300 types). However, in recent years there has been an increase in the discovery of new genomes of animal-PVs.

The BPVs are ubiquitous and appear to be distributed worldwide, especially BPV1 and BPV2, which are the most commonly detected. Notwithstanding, a several of new Bovine papillomaviruses (BPV) were discovered and characterized, thanks to the

new molecular techniques that have facilitated the amplification and sequencing of highly divergent virus types [8-11]. Thus, in order to increase the data on detection of BPVs, in this study, we report the detection and genome characterization of two novel bovine papillomaviruses from Southern Brazilian cows using high-throughput sequencing approaches.

2. Material and Methods

2.1. Samples and detection of PV genomes

Forty-six papillomatous lesions samples (skin warts) derived from twenty municipalities of Rio Grande do Sul State were processed. These samples were received in the laboratory for the production of a BPV autogenous vaccine (from 2010 to 2015) (Table 1 and Figure 1).

Total DNA was extracted with the universal phenol-chloroform protocol [12]. DNA quality and quantity was evaluated spectrophotometrically and fluorometrically, respectively. PV diagnostic PCR were performed with primer pairs FAP59/FAP64 [13]. PCR products were sequenced using a Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) in an ABI Prism 3130 Genetic Analyzer (Applied Biosystem,). Similarity analysis was performed using BLAST tool [14] (http://blast.ncbi.nlm.nih.gov).

2.2. Multiply-primed rolling-circle amplification and high throughput sequencing

Multiply-primed rolling-circle amplification (RCA) was performed according to previously established protocols [15-17]. Briefly, 100 ng of total DNA (in two µL) from infected tissues was denatured at 95 °C during 5 minutes and cooled on ice. Twenty-

three microlitres of a previously prepared solution containing 1.5 mM of each dNTPs (Invitrogen), 6.2 mM of random exonuclease-resistant hexanucleotides (Thermo Fisher Scientific), 2U of ϕ29 DNA polymerase (Thermo Fisher Scientific) and 2.5 µL reaction buffer were added. The amplification solution was incubated for 18 hours at 30 °C, followed by 10 min at 65 °C to inactivate the enzyme. The amplified DNA was electrophoresed in 0.8% agarose gel and visualized on a UV light source after ethidium bromide staining. RCA products were purified with a silica-based protocol (PureLink, Invitrogen).

For preparation of DNA libraries, RCA DNA was tagged and fragmented using the Nextera XT DNA Library Prep Kit (Illumina) according to manufacturer instructions. After the amplification step via a limited-cycle PCR program, PCR cleanup was performed with Agencourt AMPure XP beads (Beckman Coulter). The library was sequenced in a MiSeq System (Illumina) using a MiSeq Reagent Kit V2 (2 x 150 nt; Illumina).

2.3. Sequence analysis and phylogenetic inferences

The data were *de novo* assembled using the CLC Genomics Workbench (version 8). Open reading frames (ORFs) predictions and genome annotations were performed in Geneious software (version 8.1.4) and using the ORFfinder from NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Genes and proteins comparisons were performed in BLASTn and BLASTp programs [14].

Representative sequences within genera and sequences with the highest identities to the sequences from the present study that are available in GenBank were retrieved from the NCBI homepage (http://www.ncbi.nlm.nih.gov/) for phylogenetic

113 inferences. Altogether, the dataset consisted of 46 sequences of the L1 gene. The
114 multiple sequence alignments were performed through the MUSCLE software [18].

115 The phylogeny was reconstructed with a maximum likelihood method using the
116 MEGA6 software [19]. These analyses were performed using the GTR substitution
117 model, and the algorithm was modeled with a gamma distribution (shape
118 parameter = 5). The nucleotide substitution model was defined by the tool "find best
119 DNA/Protein model (ML)" of MEGA6 software [19]. Statistical support was provided
120 by 1,000 non-parametric bootstrap analyses.

121

122 **3. Results and Discussion**

123 **3.1. High frequency of deltapapillomaviruses in papillomatous lesions**

124 BPV1, 2 and 13 were detected in 76% of the samples (Figure 1 and
125 Supplemental Table 1). These viruses belong to *Deltapapillomavirus 4* species and have
126 been related to malignant cell transformation (mainly urinary bladder tumours),
127 fibropapillomas and cutaneous papillomas [1–5]. A high proportion of papillomatous
128 lesions harboring BPV1 and 2 (37% and 48%, respectively) were found, while BPV13
129 was detected only in 2% of the samples. Deltapapillomaviruses are the most detected (or
130 detectable) BPVs so far, mainly BPV1 and 2 [2, 6], and they are well known by
131 infecting other hosts species besides *Bos taurus*, as equids [3, 7] and buffaloes [8].

132 Herein, a high frequency of *Deltapapillomavirus* genus linked to cutaneous
133 papillomas in diverse body parts of cattle from distinct municipalities of Rio Grande do
134 Sul State, in Southern Brazil, was detected. Similarly, other studies have detected
135 mostly *Delta* and *Xi* genus in papilloma lesions from diverse body sites in cattle. In

Northeastern region of Brazil 6 distinct BPV types from *Delta* and *Xi* genus and one putative new type related to *Delta* genus were detected [6]. *Delta* and *Xi*-PVs also were the prevalent BPVs present in studies carried out in Europe [11] and Asia [12].

Mostly *Delta* types were also detected from diverse body sites (excluding teats and udder) of cattle in Amazon Region of Brazil (BPV1, 2 and 13) [2]. However, besides the majority of BPVs belonged to *Delta* genus, a high level of BPV diversity was described in these samples [2, 13]. Additionally, teat papilloma samples analyzed from Southern and Southeastern Brazil have reported mostly *Xi*-PVs as well putative reported and unreported PV types [14, 15].

The diversity of PV types detected in this study in papillomas from cattle body is consistent to other similar studies [6, 14, 15]. Herein were detected five distinct BPV types into 46 samples, mostly belonging to *Delta* genus (BPV1, 2 and 13). Moreover, 2 of these sequences (4%) were putative new types (PNT) related to *Xi* and *Epsilonpapillomavirus* genus. The PNT 14RS13 showed best BLAST hits with the strains BAPV4 (AY426550, e-value 4^{-169} and 93% of identity) and the SW05 (KF751806, e-value 3^{-120} and 82% of identity). The other PNT 29RS14 showed best BLAST hits with the strains GQ471901 and BPV/BR-UEL-2 (EU293538), both with e-value 3^{-150} and 91% of identity.

3.2. Two novel BPV types assigned to *Xi* and *Epsilonpapillomavirus* genus

Illumina MiSeq (2×150 cycles run) generated a total of 2,260,316 and 1,442,984 high quality paired-end reads to 14RS13 and 29RS14 samples, respectively. These sequences were *de novo* assembled into contigs using CLC Genomics Workbench (v.8) and these contigs were analysed using BLASTn/BLASTx with the National Center for Biotechnology Information (NCBI) databases.

From sample 14RS13, two circular contigs related to *Papillomaviridae* were identified, showing a PV co-infection case. The first contig correspond to the PNT founded by FAP-PCR approach (best complete L1 BLASTn hit: Bovine papillomavirus 8, e-value=0.0). These contig was 7,770 nt and was assembled by a huge quantity of reads (898,053), covering a coverage mean of 14,263X. The second PV contig identified in this sample (7,946 nt, coverage mean of 605X) correspond to a well-known BPV1. This BPV1 genome was deposited in GenBank database under accession number XXYYYYY.

In the sample 29RS14, we found only one circular contig related to *Papillomaviridae* family. BLASTn results showed identities with *Xipapillomavirus* members (BPV11, e-value=0.0). Maping reads back to contig (7,261 nt), the coverage mean was 296X.

i: The new Epsilonpapillomavirus genome (putative BPV25)

The PV recovered genome is 7,770 bp in length, arranged in a circular DNA molecule with a GC content of 44.9% (Figure 2). Seven ORFs were identified in the positive strand: five of those corresponded to the genes coding for early proteins (E6, E7, E1, E2 and E4) and two genes (L1 and L2) coded for the late capsid proteins. A long control region (LCR) of 792 bp was recognized between the L1 and E6 gene, located at nt positions 6,979–7,770. The major genome features of 14RS13 BPV are shown in Figure 2.

The LCR of BPV 14RS13 contained typical PV features [20] that hold regulatory elements for virus replication and control the transcription of transforming genes. The LCRs of mucosal epitheliotropic PVs possess a similar genome organization, which typically includes a promoter region, an enhancer region and a

184 highly conserved distribution of E2 DNA binding sites [21]. Both E1 and E2 bind to the
185 origin of virus replication, located in the LCR, and activate genomic DNA replication.
186 Most PVs possess one E1-binding site (E1BS) and at least two E2-binding sites (E2BS)
187 [22]. Nevertheless, the BPV 14RS13 LCR shows eleven copies of E2BS
188 (ACCN6GGT). In addition, the BPV 14RS13 LCR possesses one poly-A site
189 (AATAAA) and three TATA box (TATAAA), both of which are important
190 transcription and replication regulatory elements.

191 The early region of PV genomes encodes the non-structural viral proteins
192 involved in viral DNA replication, transcription and cell transformation [23]. The early
193 region of BPV 14RS13 encodes 5 ORFs: E6 (405 bp), E7 (378 bp), E1 (1,797 bp), E2
194 (1,236 bp) and E4 (423 bp). The BPV 14RS13 E6 protein exhibits two conserved zinc-
195 binding domain (ZnBD: CX2CX29CX2C) [24]. The E7 protein lacks the
196 retinoblastoma tumour-suppressor protein-binding domain (pRbBD: LxCxE) [25]. The
197 pRbBD is associated with oncogenesis in human papillomaviruses 16 (HPV16) and 18
198 (HPV18) [26,27].

199 The putative E1 protein (with helicase function) has an ATP-dependent helicase
200 GX4GK[T/S] (GQPNTGKS in BPV 14RS13) domain at amino acid positions 427–434
201 [28]. The novel PV E1 has two cyclin A interaction motif (RXL), which is thought to be
202 important for the initiation of papillomavirus replication [29]. The E1 and E2 proteins
203 are essential for genome transcription and replication [30].

204 The late regions, L1 and L2, were predicted to encode the major and minor
205 capsid proteins, respectively. Both capsid proteins contain a nuclear localization
206 sequence (NLS), which consists of a high proportion of positively charged residues (K
207 and R) in their C-terminal ends that are likely to play a role in the nuclear translocation

208 of L1 and L2 during the viral life cycle. Furthermore, the L1 gene has been chosen as
209 yardstick for building PV comparisons, and taxonomic categories are based on the
210 percentages of identity at the nucleotide level in this gene [31].

211 A new PV type can be proposed if the L1 gene sequence shares less than 90%
212 identity with the closest known PV type [3,4,31]. Here, it can be observed that the BPV
213 14RS13 L1 gene diverges 18% in relation to the nucleotide sequence of the closest BPV
214 type (BPV8). Based on this criterion, the PV strain identified in this study should be
215 designated as a novel PV type. Our results indicate that 14RS13 BPV should be
216 regarded as a new PV type within the genus *Epsilonpillomavirus* (putative BPV25).

217 *ii. The new Xipapillomavirus genome (putative BPV26)*

218 The complete genome of BPV 29RS14 is a 7,261 bp long (with a GC content of
219 42.7%) and includes the canonical non-coding region (NCR), five early ORFs (E1, E2,
220 E4, E5 and E7), and two late ORFs (L1 and L2). The major genome features of BPV
221 29RS14 are shown in Figure 2.

222 BPV 29RS14 long control region (LCR, 483 bp) was predicted to contain two
223 polyadenylation sites (AATAA) located at nt 6,859-6,864 and 6,878-6,872. Two TATA
224 box were identified at the nucleotide positions: 6,073-6,078 and 7,211-7,216
225 (TATAAA). Three E2 binding sites (E2BS) motifs were found in the LCR region,
226 which with a stringent consensus sequence [ACCN6GGT].

227 The 29RS14 E5 gene encodes a putative high hydrophobic E5 protein. This
228 putative protein is a lysine-rich and responsible by aids the E7 protein in oncogenesis
229 processes [23,32]. The second oncoprotein of BPV 29RS14 is the E7. The E7 protein
230 exhibits one conserved zinc-binding domain (ZnBD: CX2CX29CX2C) [24]. The E6

231 protein presents the retinoblastoma tumour-suppressor protein-binding domain (pRbBD:
232 LxCxE) [25], associated with oncogenesis in high-risk human papillomaviruses [26,27].

233 The predicted E1 gene contains a helicase domain with the conserved ATP-
234 binding site [GPPNTGKS] (aa 438–445). The late region, as expected, was predicted to
235 encode two viral capsid proteins L1 and L2.

236 Local and global alignments with the complete sequence of the 29RS14 L1 gene
237 showed that this BPV has 93% of identity with a PNT detected in Brazil (GQ471901)
238 [33]. However, the complete genome of this sample was not fully sequenced. The
239 second best Blast hit was the well characterized BPV11. The 29RS14 L1 gene diverges
240 18% of L1 from BPV11, thus characterized as a new viral type within the
241 *Xipapillomavirus* genus.

242 **3.3. BPV phylogeny and evolutionary history of new bovine papillomaviruses**

243 A phylogenetic tree was reconstructed with optimized alignments based on the
244 nucleotide sequence of the L1 gene. Using a set of Ruminantia genus-representative
245 sequences of PV, BPV 14RS13 clustered in the *Epsilonpapillomavirus* genus, the third
246 *Epsilon* PV detected in cattle (Figure 3). The 29RS14 clustered in the *Xipapillomavirus*
247 genus, along with most known bovine PVs (Figure 3).

248

249 **4. Conclusions**

250 This study evidenced a high proportion of bovine papillomas lesions harbouring
251 *Deltapapillomaviruses* (85%). Additionally, we identified two novel papillomaviruses
252 belonging to *Epsilonpapillomavirus* (putative BPV25) and *Xipapillomavirus* (putative
253 BPV26) genera. The new type (putative BPV26) characterized in this study is the first

254 *Epsillon*-PV characterized in South America. However, further studies are needed to
255 evaluate the impact of such new BPV types on the outcome of papillomavirus-
256 associated diseases.

257

258 **Competing interests**

259 None of the authors has any potential financial conflict of interest related to this
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261

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267

268

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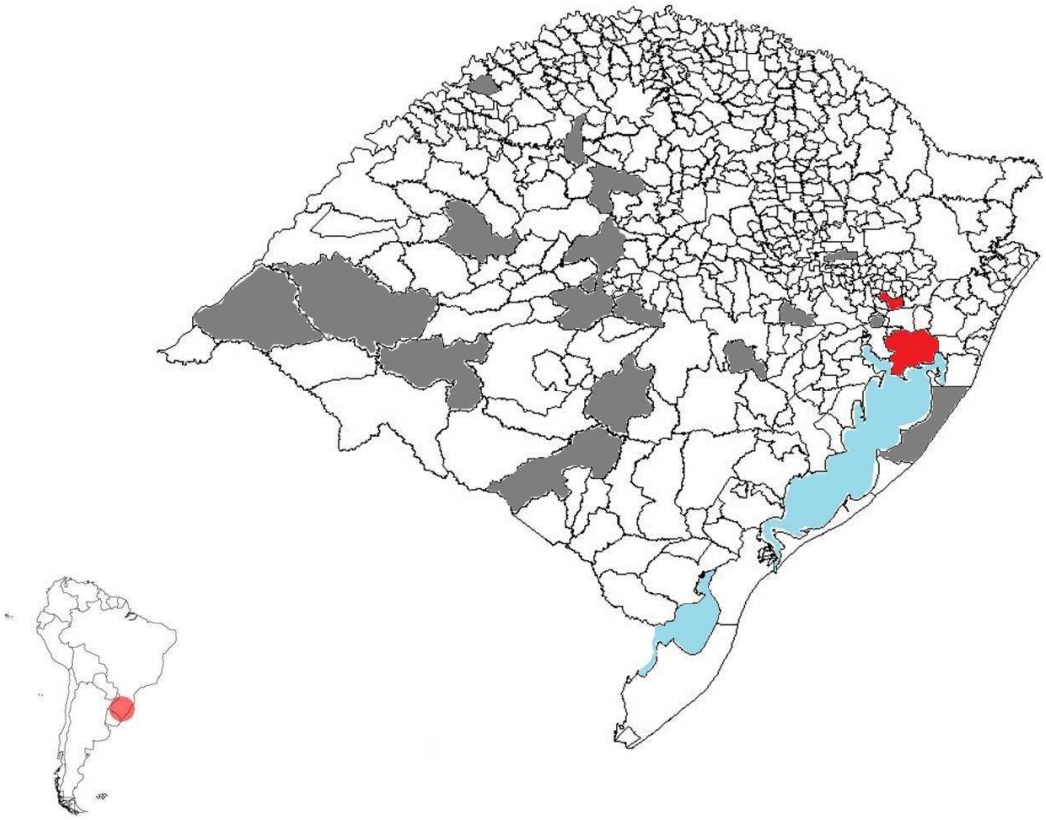


361

362 **Figure 1. Map of the region of Rio Grande do Sul, South Brazil, where the samples**
363 **were collected.** Grey and red colored municipalities show where the viral types were
364 detected. Red colored municipalities show were the new BPV types were detected.

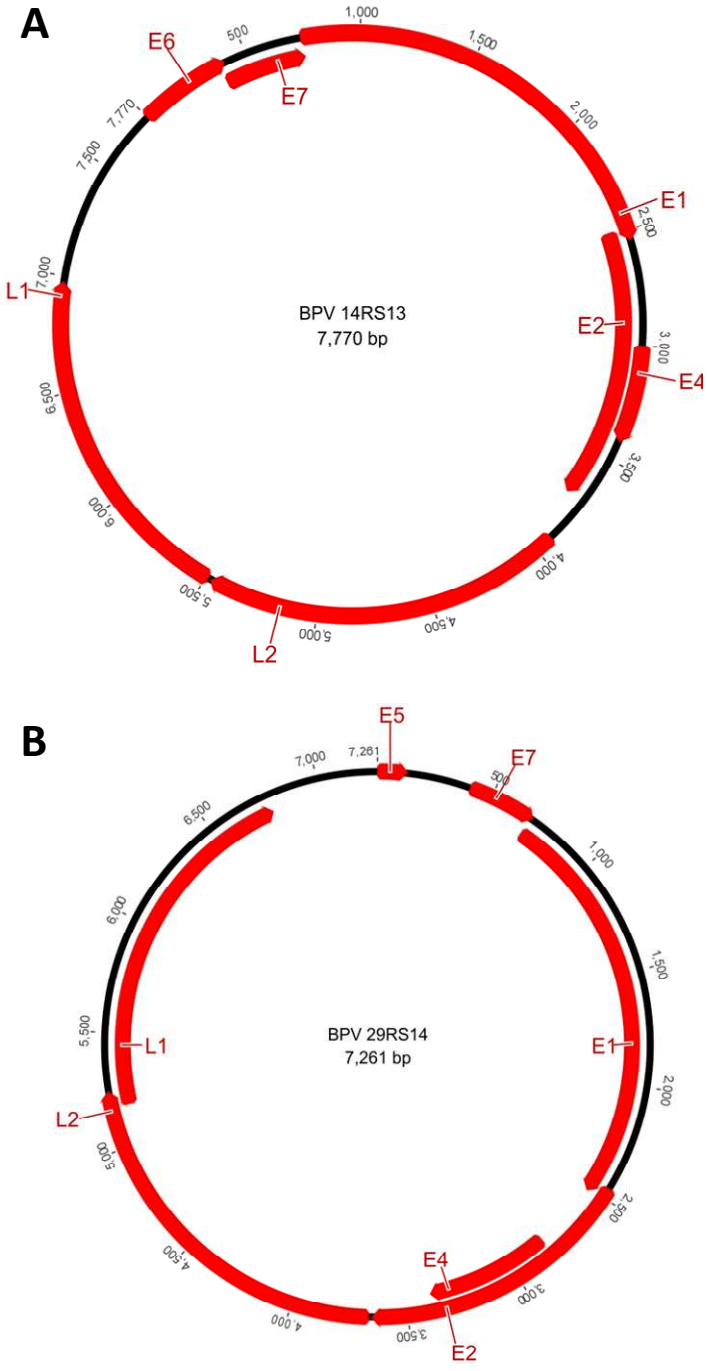
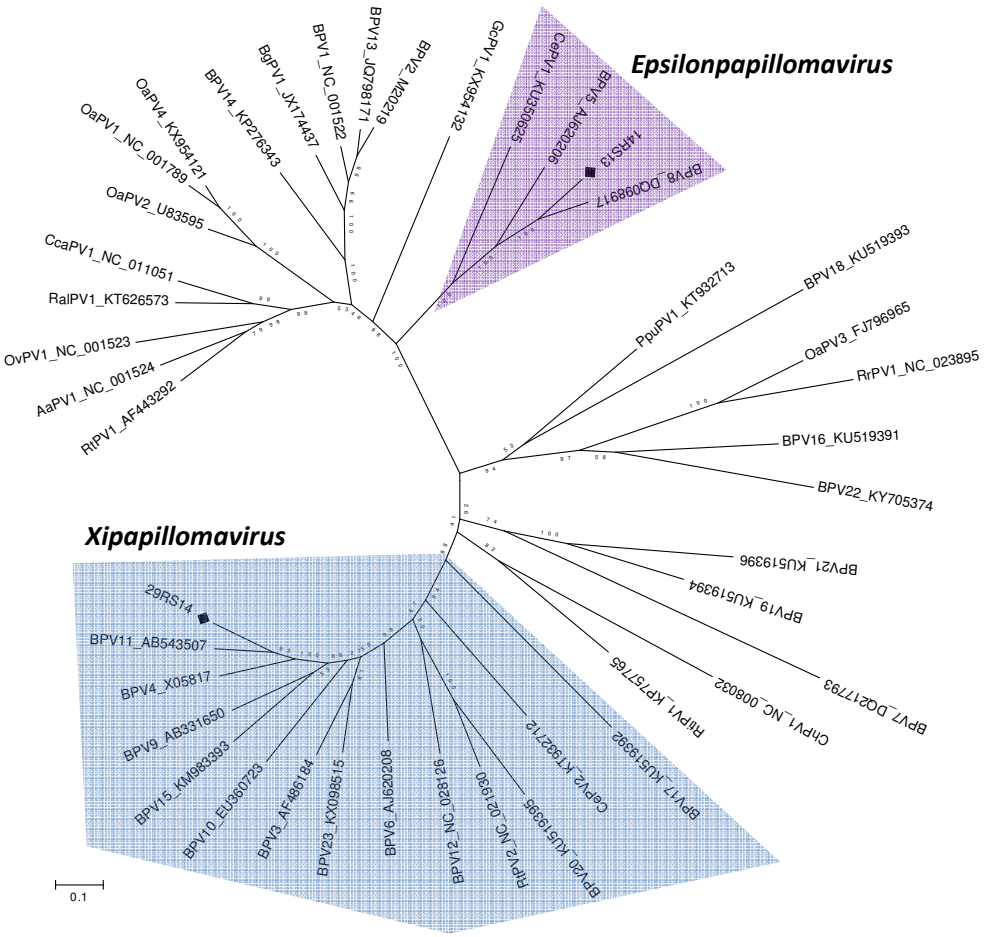


Figure 2. Genomic architecture of news BPVs. (A) The new *Epsilonpapillomavirus* (putative BPV25) genome. (B) The new *Xipapillomavirus* (putative BPV26) genome. The first position of the BPVs genomes was set as the first ATG of the E6 and E5 ORF, respectively. The ORFs were identified with the aid of the ORF Finder (NCBI) and the figure was drawn in Geneious 8.1.4.



372

373 **Figure 3. Maximum likelihood phylogenetic tree.** Maximum likelihood phylogenetic
374 tree of L1 gene sequences of Ruminantia genus-representative papillomaviruses. The
375 sequences were retrieved from GenBank. Bootstrap values are indicated above the
376 branches. The putative new BPV type introduced here is indicated with a black
377 diamond.

Supplemental Table 1. Sample description.

Sample	Year	City	Lesion	PV type	GenBank accession no.
01RS10	2010	Mostardas	Skin wart	BPV2	-
07RS10	2010	Mostardas	Skin wart	BPV2	-
08RS10	2010	NI	Skin wart	BPV2	-
09RS10	2010	NI	Skin wart	BPV13	-
12RS12	2012	Mostardas	Skin wart	BPV2	-
14RS13	2013	Viamão	Skin wart	PNT*	-
26RS14	2014	Uruguaiana	Skin wart	BPV2	-
27RS14	2014	Uruguaiana	Skin wart	BPV1	-
29RS14	2014	Novo Hamburgo	Skin wart	PNT*	-
34RS14	2014	Viamão	Skin wart	BPV1	-
43RS14	2014	Pantano Grande	Skin wart	BPV1	-
45RS14	2014	Restinga Seca	Skin wart	BPV2	-
48RS14	2014	Viamão	Skin wart	BPV1	-
50RS14	2014	Bagé	Skin wart	BPV1	-
51RS14	2014	Canoas	Skin wart	BPV1	-
54RS14	2014	Viamão	Skin wart	BPV6	-
65RS15	2015	Caçapava do Sul	Skin wart	BPV1	-
66RS14	2014	Bagé	Skin wart	BPV2	-

75RS14	2014	Rosário do Sul	Skin wart	BPV1	-
77RS14	2014	Júlio de Castilhos	Skin wart	BPV1	-
78RS14	2014	Santa Maria	Skin wart	BPV1	-
79RS14	2014	Alegrete	Skin wart	BPV2	-
80RS14	2014	Santo Cristo	Skin wart	BPV6	-
84RS14	2014	Alegrete	Skin wart	BPV1	-
85RS14	2014	Júlio de Castilhos	Skin wart	BPV2	-
86RS14	2014	Silveira Martins	Skin wart	BPV2	-
95RS14	2014	Ijuí	Skin wart	BPV1	-
98RS14	2014	Santa Maria	Skin wart	BPV1	-
99RS14	2014	São Gabriel	Skin wart	BPV1	-
118RS15	2015	Cruz Alta	Skin wart	BPV2	-
119RS15	2015	Viamão	Skin wart	BPV1	-
121RS15	2015	NI	Skin wart	BPV2	-
123RS15	2015	Uruguaiana	Skin wart	BPV2	-
124RS15	2015	Uruguaiana	Skin wart	BPV1	-
128RS15	2015	General Câmara	Skin wart	BPV1	-
129RS15	2015	General Câmara	Skin wart	BPV2	-
130RS15	2015	Viamão	Skin wart	BPV2	-
131RS15	2015	Viamão	Skin wart	BPV2	-

132RS15	2015	Viamão	Skin wart	BPV2	-
137RS15	2015	Carlos Barbosa	Skin wart	BPV2	-
138RS15	2015	NI	Skin wart	BPV2	-
139RS15	2015	NI	Skin wart	BPV2	-
141RS15	2015	Santa Maria	Skin wart	BPV2	-
142RS15	2015	Santa Maria	Skin wart	BPV6	-
143RS15	2015	NI	Skin wart	BPV2	-
145RS15	2015	NI	Skin wart	BPV6	-

7 CAPÍTULO 3: SEQUÊNCIA GENÔMICA COMPLETA DE UM DELTAPAPILOMAVÍRUS 4 (*BOVINE PAPILLOMAVIRUS 2*) ORIUNDO DE LESÃO PAPILOMATOSA DE BOVINO DA REGIÃO AMAZÔNICA, BRASIL

O presente experimento já foi concluído e um artigo científico intitulado *"Complete genome sequence of Deltapapillomavirus 4 (Bovine papillomavirus 2) from a bovine papillomavirus lesion in Amazon region, Brazil"* foi submetido para publicação e aceito no dia 07/03/2016 ao periódico Memórias do Instituto Oswaldo Cruz, Qualis B1 e fator de impacto 2,605. O manuscrito será apresentado a seguir, tal qual foi publicado.

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Complete genome sequence of Deltapapillomavirus 4 (bovine papillomavirus 2) from a bovine papillomavirus lesion in Amazon Region, Brazil

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*The complete genome sequence of bovine papillomavirus 2 (BPV2) from Brazilian Amazon Region was determined using multiple-primed rolling circle amplification followed by Illumina sequencing. The genome is 7,947 bp long, with 45.9% GC content. It encodes seven early (*E1, E2, E4, E5, E6, E7, *and* E8*) and two late (*L1 *and* L2*) genes. The complete genome of a BPV2 can help in future studies since this BPV type is highly reported worldwide although the lack of complete genome sequences available.*

Key words: papillomavirus - bovine - BPV2 - complete genome

Papillomaviruses (PVs) are small, oncogenic, highly epitheliotropic viruses with marked tropism for squamous epithelia (Bravo & Felez-Sanchez 2015). The genome of PVs is a circular molecule of double stranded DNA of about 8 kb, which bears one of the slowest evolutionary rates among viruses (Rector et al. 2007).

Fifteen bovine papillomavirus (BPV) types have been recognised to date (BPV1-BPV15) and are classified into four genera and five species. BPV infections have been reported worldwide; among these, BPV2 has been reported as one of the most prevalent types (Hatama et al. 2011, Roperto et al. 2013, Araldi et al. 2014). The BPV2 is assigned to the Deltapapillomavirus genus species 4. Apart from causing infections in the original host (cattle), this virus type has been recovered from lesions in other species, such as the equines and in buffaloes (Corteggio et al. 2013, Kumar et al. 2015).

There are few studies on the genetic diversity and distribution of BPV in Brazil. Despite this paucity of data, it is known that the BPV2 is the most detected virus in Brazilian cattle (Batista et al. 2013, Araldi et al. 2014, da Silva et al. 2015). In order to expand the knowledge on the genetic diversity of the BPV2, the complete genome sequencing of an autochthonous BPV2 from the Brazilian Amazon Region is described.

A rolling circle amplification (RCA) was applied to 100 ng of total DNA isolated from a papilloma lesion as previously described (Dezen et al. 2010, Rijsewijk et al. 2011). Neoplastic tissue was comprised by exophytic papillomatous, epithelium proliferation, and well-differentiated cells, marked acanthosis, koilocytes, increased amounts of granules in the granular layer, and keratohyalin granules. Libraries were prepared with Nextera DNA sample preparation kit (Illumina) using the RCA products and sequenced in an Illumina MiSeq System with MiSeq reagent kit v2 300 cycle. Reads were assembled into contigs using SPAdes 3.6 and compared to sequences in the GenBank nucleotide and protein databases using BLASTn/BLASTx. The Geneious software was used for open reading frame (ORF) predictions and genome annotations.

A total of 27,764 reads were produced, of which 8,116 were related to BPV2 (average reads length 111 nt). One full-length circular contig related to BPV2 was identified and annotated (mean coverage 92). The circular genome was named BPV2 BRA/09RO12. It spans 7,947 bp, with a 45.9% GC content (Figure). The genome potentially encodes seven early (*E1*, *E2*, *E4*, *E5*, *E6*, *E7*, and *E8*) and two late ORFs (*L1* and *L2*). A 934 bp noncoding region (NCR) is located between the *L1* and *E6* ORFs (Figure).

The gene *E1* encodes the largest viral protein (with helicase function), which contains 606 amino acids; the adenosine 5'-triphosphate (ATP)-binding site (GPPNTGKS) of the ATP-dependent helicase is present in the carboxy-terminal part of *E1* (Titolo et al. 1999). The putative E6 protein exhibits two conserved zinc-binding domains of $CX_2CX_{29}CX_2C$ (Lehoux et al. 2009). The E5 protein shows a leucine-rich profile, while E7 exhibits a proline-rich profile. The NCR contains nine consensus palindromic E2-binding sites ($ACCN_6GGT$), three putative TATA boxes (TATAAA) of E6 promoter, and the polyadenylation site (AATAAA) for *L1* and *L2* transcripts (Zheng & Baker 2006, de Villiers & Gunst 2009).

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Nucleotide alignment of bovine papillomavirus 2 (BPV2) BRA/09RO12 with the complete genomes of BPV2 available in GenBank. Putative coding regions of BPV2 BRA/09RO12 for early (*E1*, *E2*, *E4*, *E5*, *E6*, *E7*, and E8) and late proteins (L1 and L2) are marked by arrows.

The sequence reported here (BRA/09RO12) shares a high degree of nucleotide identity among BPV2 genomes available at GenBank (97.7% with a North American BPV2 reference genome M20219 and ~98.5% with recently sequenced Chinese strains KC878306 and KM455051) (Figure). As expected, most differences in the nucleotide sequences were concentrated in the NCR and in the *E8* gene (Garcia-Vallve et al. 2006). Double stranded viruses show the slowest evolutionary rates among viruses (Sanjuan et al. 2010). As example, two BPV1 sequences reported in Sweden and in United States of America more than 30 years apart displayed 99.89% nucleotide identity, not different from the standing genetic variation of this virus (Ahola et al. 1983).

The complete genome of BPV2 BRA/09RO12 is the first complete BPV2 recovered from Brazilian cattle reared in the Amazon Region. It reveals a high degree of identity (> 97%) with previously published BPV2 reported elsewhere, thus confirming the worldwide prevalence of such virus type. This sequence is expected to assist future studies on genetic comparisons and characterisation of PV genomes.

Nucleotide sequence accession - The complete genome sequence of BPV2 strain BRA/09RO12 is available in GenBank under the accession KU674833.

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8 CAPÍTULO 4: PAPILOMAVÍRUS BOVINO 24: UM NOVO MEMBRO DO GÊNERO XIPAPILOMAVÍRUS DETECTADO NA REGIÃO AMAZÔNICA

O presente experimento já foi concluído e um artigo científico intitulado "*Bovine papillomavirus 24: A novel member of Xipapillomavirus genus detected in Amazon Region*" será submetido para publicação no periódico *Archives of Virology*, como *Annotated Sequence Record*. O Manuscrito será apresentado a seguir, tal qual se encontrava no momento de finalização desta tese.

Bovine papillomavirus 24: A novel member of *Xipapillomavirus* genus detected in Amazon Region

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Abstract

Bovine papillomaviruses (BPV) have been described as etiologic agents of cutaneous and mucosal papillomas in cattle. Currently 23 bovine papillomavirus (BPV) types have been fully sequenced and characterized. In the present study, we describe a new BPV that was detected in a cutaneous papilloma from a cow. This papillomavirus is proposed as BPV type 24 and phylogeneic analysis suggests the BPV should be classified within the *Xipapillomavirus* genus, herein named BPV24. Moreover, a co-infection with *Epsilon* and *Delta* BPVs in a cutaneous papilloma from another animal was also detected and the full genomes were sequenced. Both papillomas were from cattle within the Acre State, Amazon Region of Brazil. The data presented herein shows the significance of using high throughput methods to indentify co-infections and allow the characterization of new genomes.

Keywords: papillomavirus; new type; co-infection; next generation sequencing.

Annotated sequence record

Papillomaviruses are non-enveloped viruses that harbour a circular double-strand DNA molecule of about 8 Kbp [1–3]. Currently 23 *Bos taurus* papillomavirus (BPV) types have been fully sequenced and characterized [4–6]. In cattle BPVs are well-recognized to cause hyperplastic cutaneous and mucosal papillomas and have been suggested as a potential cofactors in the development of upper alimentary tract and urinary bladder cancer [7, 8]. In humans, human papillomaviruses (HPV) are also involved in skin and mucosal benign lesions (warts) as well as cervical, penile, anal, vaginal and oropharynx cancers [9]. More than 200 HPV types have been characterized so far and co-infections are frequently reported [10–12].

Currently 49 genera of Papillomaviruses have been proposed. Of these five contain BPVs [4] including the *Delta* (BPV1, 2, 13 and 14), *Xi* (BPV3, 4, 6, 9, 10, 11, 12, 15, 17 and the recently described putative BPV23), *Epsilon* (BPV5 and 8), *Dyoxi* (BPV7) and *Dyokappapapillomavirus* (BPV16 and BPV22). In addition, three new BPVs (18, 19 and 21) remain unclassified, but appear most likely to belong to two unasigned genera [4–6, 13].

Herein is described the use of rolling circle amplification (RCA) followed by high throughput sequencing (HTS) to detect PV types in two papillomas lesions. The method enabled amplification of DNA from three well-characterized PV types as well as the complete DNA sequence of a novel BPV type. It is proposed that the novel BPV is classified within the *Xipapillomavirus* genus.

DNA was isolated from two papilomatous lesions (02AC12 and 06AC14) previously characterized as epidermal papillomatosis, in which BPVs more related to BPV8 (77.9%) and BPV12 (76.5%) respectively [14] had been detected. These were submitted to randomly-primed rolling circle amplification (RCA) in order to enrich

circular DNA [6, 13, 15, 16]. The samples where selected due to their low similarities to other related types [14]. The RCA products were submitted for Rubicon Thruplex DNA-Seq library preparation (as per manufacturers' instructions) and sequencing on the Otago Genomics & Bioinformatics Facility Illumina MiSeq™ platform producing >250,000 paired end reads.

Sequencing reads were quality assessed using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). High quality reads exceeding a quality score of Q20 and greater than 50 bases in length were assembled using CLC genomics workbench 8. All assemblies were confirmed by mapping reads to the contigs. Open reading frames (ORF) predictions and genome annotations of the complete genomes were performed with the aid of Geneious software (version 9.0.5) and ORF finder tool (https://www.ncbi.nlm.nih.gov/gorf/gorf.html). Gene and protein comparisons were performed with programs BLASTn and BLASTp. The phylogenetic analysis was performed in MEGA7 software and the tree was generated using the best selection model with Maximum-likelihood method. Alignments were performed using MUSCLE software [18] using default settings.

A summary of the Illumina MiSeq run is presented in Supplementary Table 1. Sequence reads were *de novo* assembled and the generated contigs were compared to the GenBank database using BLASTn and BLASTx softwares. In the sample 02AC12, we found three different contigs showing high identity with PV sequences. In the sample 06AC14 only one contig with similarity to PV was found. The remaining contigs were either related to the bovine genome or to unknown sources.

One contig with similarity to BPV was idenfied in sample 06AC14 (peduculated skin lesion, see [14]). Assigned as BPV24, this sequence showed most similarity using

BLAST to BPV4 (79%) . The BPV24 genome was predicted to be 7,256 bp in length arranged in a circular DNA molecule with a GC content of 41.5% (Figure 1). The genome shared characteristics with other *Xipapillomavirus* members and it was predicted to contain seven ORFs (five early – E5, E7, E1, E2 and E4, and two late – L1 and L2) and a long control region (LCR). The BPV24 predicted ORFs and their features are summarized in the Table 1.

The PV LCR tipically contains regulatory elements for virus replication and gene transcription. The BPV24 LCR is 480 bp in length and it is located between the L1 stop codon and the start codon of E5 (Figure 1). Two E2 binding sites (E2BS, $ACCN_6GGT$) at nt positions 7,100 and 7,189 were indentified however, no E1 binding site (E1BS) ($ATTGTTN_3AACAAT$) was found. A polyadenylation site (AATAAA) was predicted at nt position 6,854.

The E5 ORF was located btween the LCR and the E7 ORF, as previously found in other *Xipapillomavirus 1* types (BPV9, 10, 11 and 15). The E5 gene encodes a small transforming protein (42 aa) with a highly hydrophobic amino acid composition (33% of lysine residues). The BPV24 E7 predicted protein shows one zinc-binding domain ($CXXC\text{-}X_{29}\text{-}CXXC$) as well as the retinoblastoma protein binding site (LXCXE).

The E1 predicted ORF encodes the largest BPV24 protein containing a putative ATP-binding site (GPPNTGKS) within the ATP-dependent helicase carboxy-terminus (439-446 aa position). Additionaly, four cyclin interaction motifs (RXL) were predicted to be present in E1. Conserved protein domains identified within E2 included a C-terminal DNA-binding domain (aa 307-354) and the N-terminal transactivation domain (N-TAD) (aa 13-205). BPV24 E2 protein lacks the leucine-zipper domain ($LX_6LX_6LX_6L$) that is present in some E2 PVs. The BPV24 E4 ORF overlaps E2 is a

105 predicted to encode a proline-rich (18 proline residues out of 175 amino acids) peptide
106 containing two cyclin A interactions motifs (RXL), via an alternative reading frame.
107 The late region is composed by the two late genes that encodes for major (L1)
108 and minor (L2) capsid proteins. Both L1 and L2 BPV24 proteins contain a series of
109 lysine and arginine residues, mainly at their carboxy termini. The L1 gene sequence is
110 typically used to classify all PV types [2]. Phylogenetic analysis of L1 BPV types and
111 all *Xi* PVs showed BPV24 to be closely related to BPV4 (77%) and was consequently
112 classified as a new member of *Xipapillomavirus 1* species. Since one papilloma
113 contained multiple PV types, the role of each BPV detected could not be determined.
114 However, sample 06AC14 contained only BPV24 suggesting a possible role for this
115 BPV in the development of the papilloma. Nevertheless, due to the frequent
116 asymptomatic infection of skin by BPV types, additional cases have to be examined
117 before it can be confirmed that BPV24 is a cause of papillomas in cattle.
118 Three distinct BPV types, belonging to *Delta* and *Epsilonpapillomavirus* genera
119 were identified co-infecting the 02AC12 sample (flat skin lesion, see [14]). We
120 recovered the genome of BPV2, BPV5 and BPV8, which were named BPV2
121 BR/02AC12, BPV5 BR/02AC12 and BPV8 BR/02AC12 (GenBank accession numbers
122 xxx, xxx and xxx, respectively). BPV2 have been widely descrided in cutaneous
123 papillomas as well as in urinary bladder carcinomas in cattle worldwide [19, 20]
124 however, entire genomes descriptions for this BPV are rare (one North American, two
125 Chinese [21] strains and one Brazilian strain KU674833 until now [22]). The BPV2
126 BR/02AC12 was 7,947 bp and showed 100% nt identity to BPV2 KU674833 (Brazilian
127 strain), 99% to BPV2 KM455051 (Chinese strain), and 98% nt identity to BPV2
128 M20219 (North American) and KC878306 (Chinese strain) strains. The complete

129 genome of BPV2 BR 02/AC12 is the second complete BPV2 recovered from Brazilian
130 cattle reared in the Amazon Region [22].

131 The epsilonpapillomaviruses detected in the 02AC12 sample share high genomic
132 identity with their congeners. BPV5 BR/02AC12 was 7,839 bp in length and showed
133 98% nt identity to all other BPV5 complete genomes available in genome database,
134 even the one from Amazon region recently described [16]. BPV8 BR/02AC12 was
135 7,699 bp in length and represents the first genome sequence of this BPV type in
136 America. It was 98% nt identical to the other two BPV8 decribed ten years ago in Japan
137 [21]. The sequences reported herein are expected to assist future studies on genetic
138 comparisons and characterization as well as evolution and synergism/competition
139 studies of PV genomes. All genomes and their ORFs are represented in Figure 1.

140 In this study, we reported four complete BPV genomes detected in two skin
141 warts samples from cattle located in Northern Brazil, including one putative new BPV
142 type named BPV24. The identification and characterization of animal PVs as well as the
143 detection of mixed and co-infections are important to better understand the virus
144 biology and their intra-specific interactions. This study indicates the Amazon region to
145 be a useful resource for the identification and classification of BPVs, and it is perhaps
146 not surprising given the Amazon forest encodes one of the largest biodiversities
147 worldwide. Therefore, apparently this region could harbours a great diversity of viruses
148 as it happens to animal and vegetal species.

149

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222

223 **Table 1. BPV24 predicted proteins and their features.**

ORF	ORF location	Length (nn)	Length (aa)	Molecular mass (kDa)	pI
E1	680 - 2,509	1830	609	69.147	6.51
E2	2,532 - 3,659	1128	375	41.934	10.50
E4	2,866 - 3,393	28	175	2.072	109.80
E5	1 - 129	129	42	4.874	5.29
E7	394 - 690	297	98	10.736	3.94
L1	5,262 - 6,776	1515	504	57.596	7.69
L2	3,674 - 5,251	1578	525	57.232	4.63

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225

226 **Supplementary Table 1. Summary of the Illumina sequencing data and CLC**
227 **assembly.**

Sample	02AC12	06AC14
Reads	182,776	85,674
Total contigs >5,000 bp in length	6	2
Total PV-related contigs	3	1
BLASTn results*	Contig 1 - BPV2 KM455051 (98.6% ID) Contig 2 - BPV5 NC_004195 (99.6% ID) Contig 3 - BPV8 DQ098913 (98.2% ID)	Contig 1 – BPV4 X05817 (79% ID)
Coverage	Contig 1: 560X (32,340 reads) Contig 2: 200X (10,971 reads) Contig 3: 750X (42,872 reads)	Contig 1 650X (38,765 reads)

228 *Best BLASTn results for PV-related contigs.

229

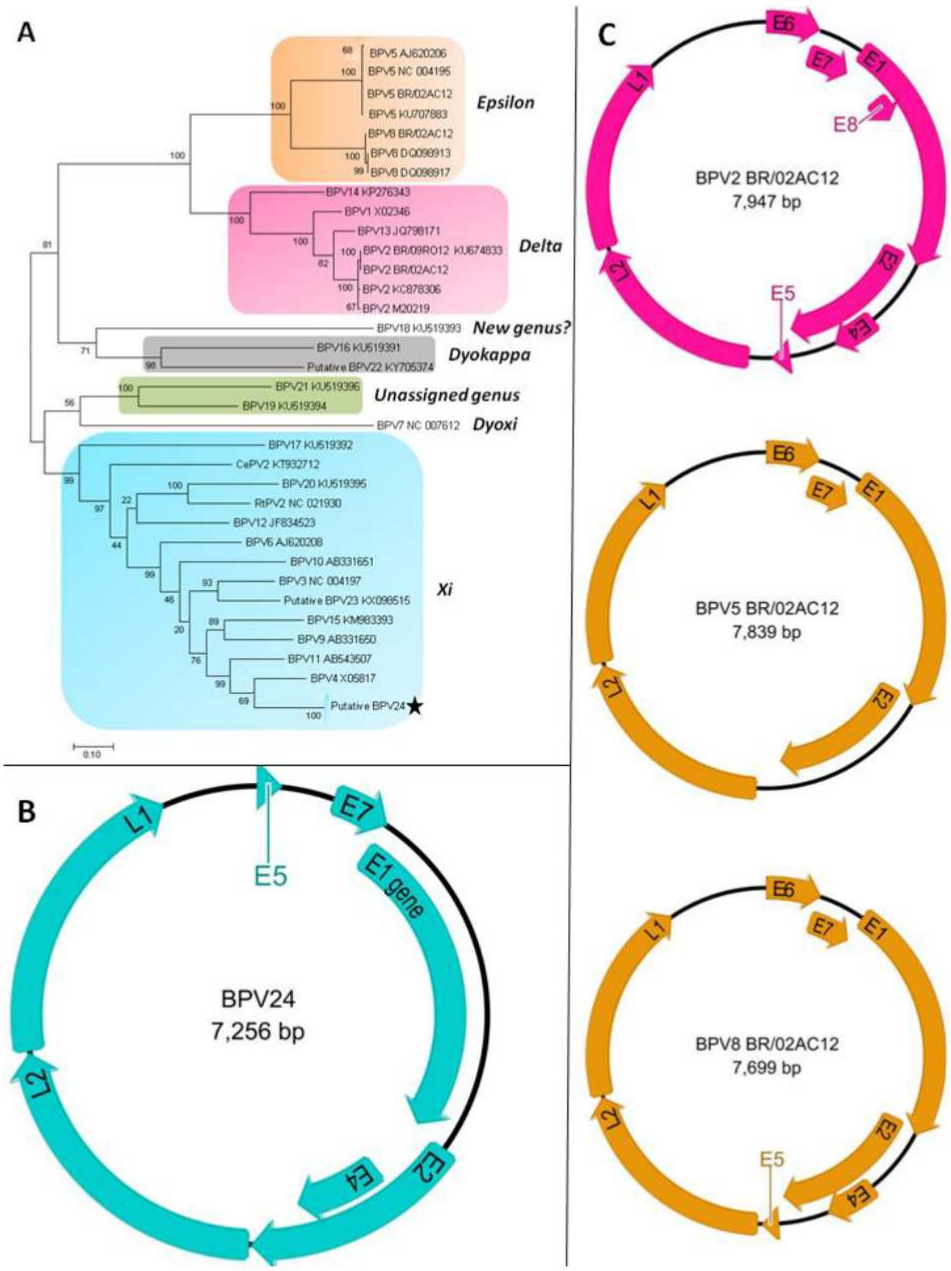

Figure 1. Schematic diagrams showing the genomic organization of BPVs and phylogenetic tree. (A) Maximum-likelihood phylogenetic tree of *Bos taurus* papillomavirus. L1 entire sequences were retrieved from GenBank, aligned with the aid of MUSCLE and phylogenetic analysis were performed in MEGA6 software. (B) Genomic organization of BPV24. The first position of the BPV24 genome was set as

the first ATG of the E5 gene. (C) Genomic organization of BPV2, BPV5 and BPV8 detected in 02AC11 sample. The ORFs were identified with the aid of the ORF Finder (NCBI) and Geneious software version 9.0.5. The figure was drawn in Geneious software 9.0.5.

9 CAPÍTULO 5: QUANTAS ESPÉCIES DE PAPILOMAVÍRUS PODEM NÃO SER DETECTADAS EM LESÕES PAPILOMATOSAS?

O presente experimento já foi concluído e um artigo científico intitulado "*How many papillomavirus species can go undetected in papilloma lesions?*" foi submetido para publicação e aceito no dia 03/11/2016 no periódico *Scientific Reports*, Qualis A1 e fator de impacto 4,259. O Manuscrito será apresentado a seguir, tal qual foi publicado.

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How many papillomavirus species can go undetected in papilloma lesions?

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A co-infection comprising to at least seven papillomavirus (PV) types was detected by next generation sequencing (NGS) of randomly primed rolling circle amplification (RCA) products of a bovine (*Bos taurus*) papilloma lesion from the Brazilian Amazon region. Six putative new PV types that could not be detected by commonly used PCR protocols were identified. Their overall L1 nucleotide identities were less than 90% compared to described PV species and types. L1 nucleotide BLAST sequence hits showed that each new type was related to *Beta*, *Gamma*, *Dyokappa*, *Dyoeta*, and *Xipapillomavirus*, as well as two likely new unclassified genera. Our results show that the employment of NGS is relevant to the detection and characterization of distantly related PV and is of major importance in co-infection studies. This knowledge will help us understand the biology and pathogenesis of PV, as well as contribute to disease control. Moreover, we can also conclude that there are many unknown circulating PVs.

Papillomaviruses (PVs) are circular double-stranded DNA viruses containing approximately 8,000 base pairs (bp)[1,2]. They belong to the *Papillomaviridae* family and these complex viruses can infect a wide range of amniotes[1–3]. This large family is composed of viruses phylogenetically assigned to 39 genera with several species, types, subtypes and variants[1,2]. The entire genome must be sequenced for considering new PV types and the L1 open reading frame (ORF) have to differ by more than 10% in comparison to the closest PV types. PV species share between 71% and 89% nucleotide identity within the complete L1 ORF. A PV genus is defined when the similarities are larger than 60% in the L1 ORF. When this difference is between 2% and 10% or less than 2% a subtype or variant occurs, respectively[1,2].

Human PV (HPV) encompasses more than 200 types that are fully sequenced, characterized and cataloged, in contrast to the low number of *Bos taurus papillomavirus* (BPV), which comprises only 15 types (http://pave.niaid.nih.gov). PVs are usually characterized by PCR amplicon sequencing, which is performed using degenerate primer pairs that amplify a relatively conserved L1 gene region from all known PV types and species[4]. This technique has allowed the identification of divers putative new PVs types in humans and other animals, including BPV types in cattle herds from distinct continents worldwide[5–8].

Although PV detection and characterization in animal species are still poorly studied[3,9], primers directed to the PV genera have been commonly used and have shown an increase in the specificity of detection and characterization of PV DNA compared to general primers[10–12]. Nearly two hundred human PVs (HPVs) have been recognized and PV co-infections have been reported using numerous molecular techniques that detect many distinct HPVs genotypes[13–16]. Several generic PV primer systems have been developed for the detection of HPV types rather than BPV types[17]. Additionally, there is much less research of cattle in this field, reflected by the vague BPV types reported and the few cases of BPV co-infection reported[18–20].

Multiply primed rolling circle amplification (RCA) of circular genomes followed by classic sequencing enabled the discovery of novel animal PVs[21]. Although this study did not show evidence of co-infections, the RCA

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	GenBank acession no.	Read count*	Mean genome coverage#	GC-content (%)
BPV13 BR/14RO	KU519390	1645	31	45.1
BPV BR/14RO-16	KU519391	650	12	44.5
BPV BR/14RO-17	KU519392	1366	27	42.4
BPV BR/14RO-18	KU519393	578	12	41.0
BPV BR/14RO-19	KU519394	774	16	42.7
BPV BR/14RO-20	KU519395	378	8	44.6
BPV BR/14RO-21	KU519396	630	13	47.7

Table 1. Overall genome coverage and GC-content of the seven contig papillomavirus sequences recovered from a papillomatous lesion. *Number of reads mapped to the reference. #Average number of times each base was sequenced.

followed by NGS has enabled the detection of HPV co-infections[22]. Moreover, the extraordinary diversity of PV types that infect the animal skin combined with the low numbers of PV types detected by degenerate primers[23] indicate that these techniques allow the discovery of only closely related PVs from known genera. Furthermore, PVs could be cultivated only in a sophisticated and arduous raft cell culture system, thereby hampering whole genome analysis due to the lack of necessary adequate amount of purified genomic viral DNA[24].

Therefore, an efficient method for amplification and sequencing is essential for improving the identification of PV species that are not detected by current methods mainly in animal researches. The knowledge of PV types is important for epidemiological studies of viral variants in different PV-affected species and to determine the full picture of genetic diversity. Such information will help clarify the biological relationship between distinct viruses with respect to both pathogenesis and treatment. Along these lines, randomly primed RCA followed by NGS was performed to investigate the complete genetic diversity of PVs present in a papilloma lesion.

Results

NGS from RCA products reveals distinct BPV genomes. NGS from RCA products of one papilloma lesion enabled the amplification of seven full-length PV genomes. The contigs associated with PV were assembled from 113,616 high-quality reads (Table 1). The seven contigs were named BPV13 BR/14RO and putative BPV BR/14RO-16 to BPV BR/14RO-21. Chimeric forms were not detected using the RDP4 software. Primer alignment with the Geneious software (version 9) (http://www.geneious.com)[25] found mismatches in all sequences at the 3' end of the FAP59 and MY09 primers (Supplementary Figure 1). A low number of reads probably corresponding to the sequence 14RO_12 (GenBank accession number KP701419) was also identified in the sample. Since the genome was not complete it was not further analyzed.

The genomes are 7,149 to 7,961 Kb in length and display the archetypal organization of PVs (Fig. 1). The first nucleotide in E6 was assigned the number 1 in the sequences. All putative new viruses (BPV BR/14RO-16 to BPV BR/14RO-21) were predicted to contain six (BPV BR/14RO-18 and 19) to seven ORFs (BPV BR/14RO-16, BPV BR/14RO-17, BPV BR/14RO-20 and BPV BR/14RO-21), coding for early (E6, E7, E1, E2, and E4) and late (L1 and L2) proteins (Fig. 1). The BPV13 variant sequence showed the same characteristics as the reference genome[17]. The overall L1 nucleotide identities of the putative new types were less than 90% in comparison to other PV species and types[1].

Phylogenetic inferences. Phylogenetic inferences showed that the six new PV genomes clustered with three known and two unknown PV genera (Fig. 2). Their nucleotide and amino acid identities to the closest related PVs are summarized in Table 2. L1 identities to the most closely related PVs with corresponding GenBank accession numbers following phylogenetic analysis are summarized in Table 2. Putative BPV BR/14RO-16 clustered with members of *Dyokappapapillomavirus* and was most closely related to *Ovis aries* PV3 (OvPV3) (62% sequence identity). Putative BPV BR/14RO-17 was most closely related to BPV3 (62%), and putative BPV BR/14RO-20 was most closely related to RtPV2 (*Rangifer tarandus* PV2) (74% sequence identity), both members of the *Xipapillomavirus* genus. Putative BPV BR/14RO-18was most closely related to members of the *Xi* and *Dyokappapapillomavirus* genera and likely represents a new genus. Putative BPV BR/14RO-19 and BPV BR/14RO-21 constituted a distinct cluster and were most closely related to PVs belonging to *Gamma, Xi* and *Dyoetapapillomavirus*. Both are probable representatives of a new genus in the *Papillomaviridae* family. The BPV13 BR/14RO and the putative new BPV BR/14RO-16 to BPVBR/14RO-21 sequences were deposited in GenBank under accession numbers KU519390 to KU519396.

Discussion

Remarkable efforts have been made to identify human PVs using numerous clinical arrays that can detect dozens of distinct HPV genotypes in the same sample[13,14] using several generic PV primer systems[17]. These efforts reflect over than 200 HPV genomes that are fully sequenced, characterized and cataloged (PaVE). In comparison to HPV, only 15 BPV types have been detected and fully sequenced thus far (PaVE). This scenario is probably pictured because of the lower efforts in BPV studies when compared to HPV due clinical relevance and funding applied. Co-infections comprising HPV are commonly reported in young or immunodepressed women[26–28]. On the other hand, BPV co-infections comprising up to six known PV types based on multiplex BPV genotyping assay[20] or specific primers[18,29] are rarely reported. Although the majority of BPV types and putative new types have been characterized using generic or genus-specific primers[8,12,17,30], such protocols have important limitations

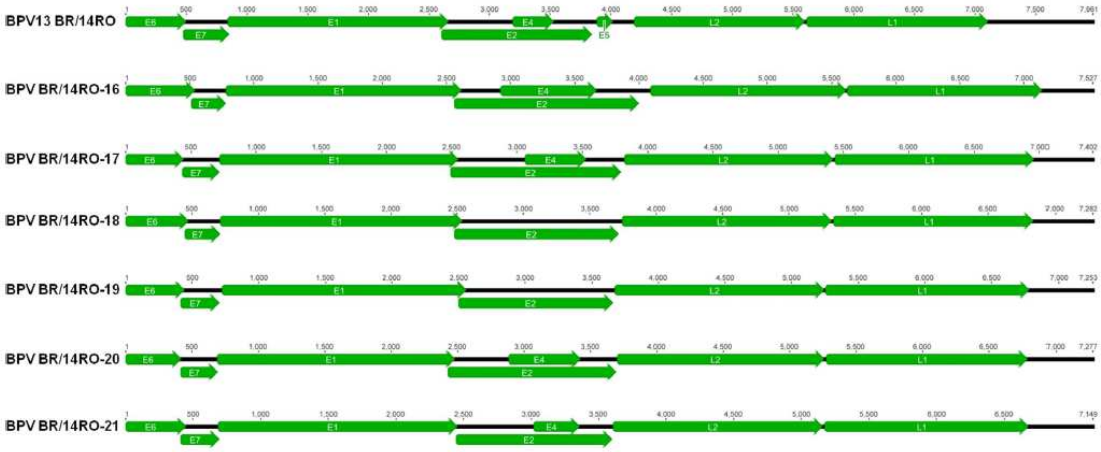

Figure 1. PV genomes found in this study and their archetypical organization. The first nucleotide in the ORF6 was assigned a number 1 in the sequences. All putative new papillomavirus genomes (BPV BR/RO-16 to BPV BR/RO-21) were predicted to contain six to eight ORFs, coding for early (E6, E7, E1, E2, E4 and E5) and late (L1 and L2) proteins.

because they allow the discovery of only closely related PVs but rarely detect mixed infections. In the present study, the combination of RCA and NGS allowed the detection of at least seven BPVs co-infecting the same lesion, including six putative new BPV types.

An isothermal protocol that uses ϕ29 DNA polymerase to amplify complete PV genomes was previously developed[31]. Following the amplification of the genome, there is the need to analyze the DNA using restriction enzymes, cloning, and sequencing which is labor-intensive and time-consuming. Additionally, multiply primed RCA and primer-walking for entire genome sequencing already enabled the discovery of novel PVs[21]. Moreover, there is no evidence of co-infection. One possibility is that the specific degenerate primer pairs used in these studies selected a virus population with higher affinity to primer binding than unknown viruses that may be present in the same sample. This could lead to an underestimation of the detection of other viruses in mixed infections and even a failure to detect distant phylogenetic PVs.

A RCA followed by NGS approach was applied to analyze a sample from which have been previously found a putative new BPV type[8]. The method enabled the detection of seven PVs with six being uncharacterized so far. The randomly primed RCA followed by NGS offers the possibility to amplify and detect any circular DNA that is present in a sample without specificity, thus allowing a great overview of unknown PVs. This technique could magnify the sensitivity of all PVs present in one sample and allow the understanding of the natural history of infection by different PV types. This approach is meaningful once there is more evidence suggesting that cervical infections caused by some HPV types may also depend on the existence of other HPV types[13], thereby suggesting a synergistic pattern. NGS present some restrictions as limiting capability to find mutations like single nucleotide polymorphisms (SNP), insertions and deletions in regions of lower coverage[32,33]. To minimize the possible base calling errors, in the present study, only high quality reads ($Q \geq 30$) were used for *de novo* assembly. Also, although this Illumina platform displays some underestimation in AT-rich and CG- rich regions[33] all putative new types and the BPV13 described in this study presented a GC content considered normal when compared to other PV family members.

The present method enables the detection of a large number of putative new types suggesting the existence of many other BPV types that may have been underestimated thus far. Such a massive PV co-infection indicates that these mammals can harbor genetically diverse PVs similar to humans. Additionally, the Amazon region ecosystem harbors one of the largest global biodiversities and it is quite propitious for the emergence of novel strains. However, there is a need for deeper investigations on this issue by applying this method in all PV affected animals, including other cattle herds worldwide and humans.

We have shown that the enrichment method together with the Illumina NGS platform works for a range of PV genera detection such as *Dyokappa*, *Xi* and *Gammapapillomavirus*. Moreover, the identity of three new types showed inter-genera localization, and these types probably compose two new genera in the *Papillomaviridae* family. These findings indicate a high number of undetected PVs ignored in the usual assays. Therefore, it is essential to use unbiased methods for the discovery of highly divergent novel viruses as has been done with numerous human and animal agents[34].

Most of the xipapillomaviruses present E5 or E8 gene in the E6 genomic position, including BPV4, BPV9, BPV10, BPV11 and BPV15 (GenBank accession no. X05817, AB331650, AB331651, AB543507, and KM983393, respectively) that encode E5, and BPV3, BPV6 and BPV12 (GenBank accession no. AF486184, AJ620208, and JF834523, respectively) that encode E8. The novel xipapillomaviruses detected in the present study present E6 in the E6 genomic position as well as RtPV2 that clustered in the same terminal node that BR/14RO-20. BR/14RO-17 formed a separated branch within *Xipapillomavirus* and probably is a novel species.

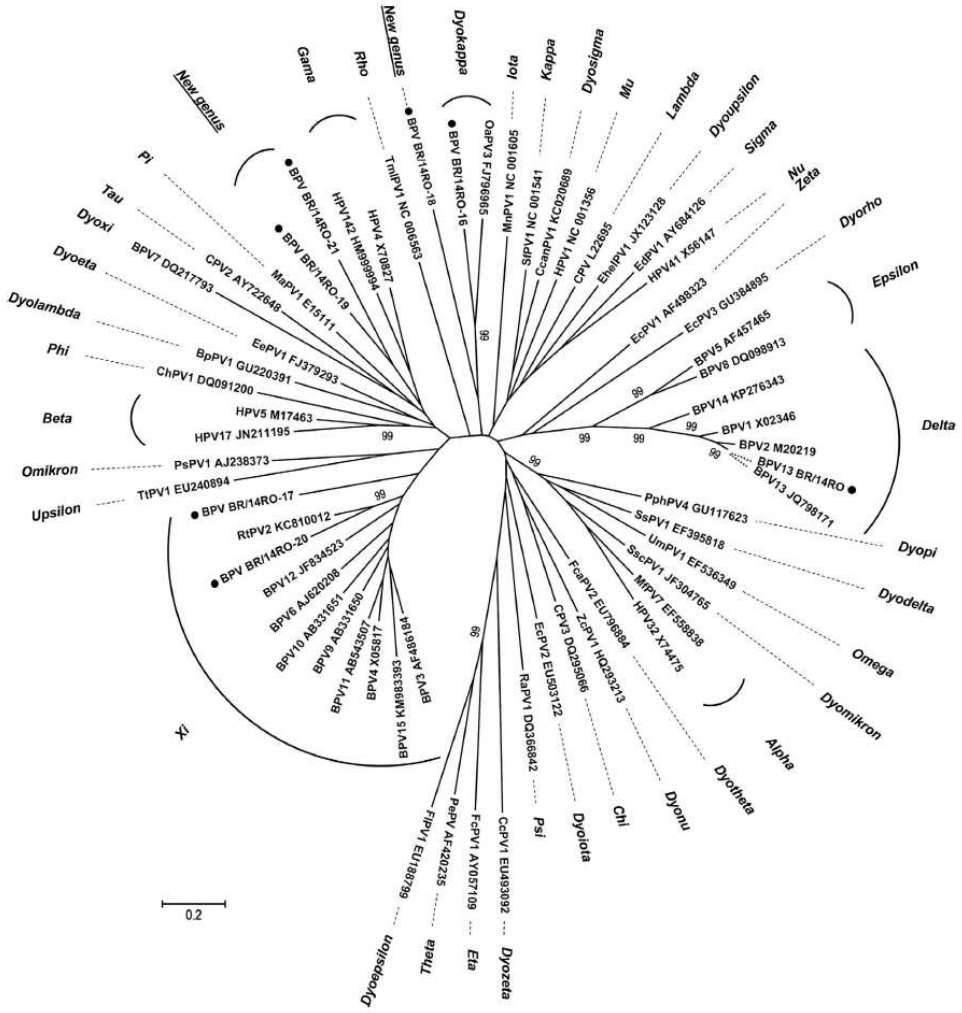

Figure 2. Phylogenetic tree of the papillomaviruses based on complete sequences of the L1 ORF. Bootstrap repetitions (higher than 99%) are indicated above the main branches. Samples belonging of this study with representatives of other PV genera were included on the analysis. A total of 61 PV types of different species and genera were analyzed. Accession numbers for the sequences are included and abbreviations are used according to PaVE. Putative new types and genera are indicated with black dots.

In conclusion, the combination of two relatively simple and fast methods already developed to amplify and genotype PV genomes proved to be very effective in the detection of known and unknown PV viruses using small amounts of DNA derived from one PV lesion. Furthermore, viral genomes can be largely reconstituted by currently available *de novo* assembly algorithms. The presence of multiple PV types and variants in the same lesion will allow the development of new studies regarding the roles of these different viruses in the biology and pathogenesis of the diseases in which they are involved.

Material and Methods

Ethics Statement. Lesions were collected by veterinarians to prepare autogenous vaccines and all efforts were made to minimize animal suffering. All procedures were performed in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series—No. 170 revised 2005) and the procedures of the Brazilian College of Animal Experimentation (COBEA). It must be highlighted that this project was approved by Universidade Federal do Rio Grande do Sul Animal Ethics Committee (number 28460) and we had the owner's permission to access the animals for the purposes of this study.

Sample processing, rolling circle amplification, and Illumina sequencing. Biopsy material was obtained from a bovine of the Brazilian Amazonian region, diagnosed with epidermal papillomatosis. The lesion

PNT#	Identity (%)	PV type	PV species	PV genera	GenBank acession
BPV BR/14RO-16	62	OaPV3	*Dyokappapapillomavirus 1*	*Dyokappa*	FJ796965
	55	BPV3	*Xipapillomavirus 1*	*Xi*	AF486184
	55	BPV6	*Xipapillomavirus 1*	*Xi*	AJ620208
	55	BPV9	*Xipapillomavirus 1*	*Xi*	AB331650
	55	BPV11	*Xipapillomavirus 1*	*Xi*	AB543507
BPV BR/14RO-17	62	BPV3	*Xipapillomavirus 1*	*Xi*	AF486184
	61	BPV4	*Xipapillomavirus 1*	*Xi*	X05817
	61	BPV6	*Xipapillomavirus 1*	*Xi*	AJ620208
	61	RtPV2	*Xipapillomavirus 3*	*Xi*	KC810012
BPV BR/14RO-18	54	RtPV2	*Xipapillomavirus 3*	*Xi*	KC810012
	53	OaPV3	*Dyokappapapillomavirus 1*	*Dyokappa*	FJ796965
	51	BPV BR/14RO-16	*	*Dyokappa*	KU519391
BPV BR/14RO-19	66	BPV BR/14RO-21	*	*	KU519396
	60	HPV4	*Gammapapillomavirus 10*	*Gamma*	X70827
	60	EePV1	*Dyoetapapillomavirus 1*	*Dyoeta*	FJ379293
	59	MaPV1	*Pipapillomavirus 1*	*Pi*	E15111
	59	HPV142	*Gammapapillomavirus 10*	*Gamma*	HM999994
BPV BR/14RO-20	74	RtPV2	*Xipapillomavirus 3*	*Xi*	KC810012
	68	BPV12	*Xipapillomavirus 2*	*Xi*	JF834523
	67	BPV9	*Xipapillomavirus 1*	*Xi*	AB331650
BPV BR/14RO-21	66	BPV BR/14RO-19	*	*	KU519394
	58	HPV142	*Gammapapillomavirus 10*	*Gamma*	HM999994
	58	BPV6	*Xipapillomavirus 1*	*Xi*	AJ620208

Table 2. L1 identities to most closely related PVs with corresponding GenBank accession numbers following phylogenetic analysis. *Genomes not assigned to PV species or genera. #Putative new types (PNT) are designated as BPV BR/14RO-16 to BPV BR/14RO-21.

was removed using scalpels after local anesthesia (performed with 2% lidocaine, Bravet, Brazil). One putative new PV type was previously detected when a L1 fragment was sequenced using FAP primers[8]. To obtain the complete genome sequence of this putative new type, the tissue was processed and genomic DNA was extracted as described previously[8]. To amplify the full PV genomes, randomly-primed rolling circle amplification (RCA) was performed essentially as described by Rector *et al.*[31] using 100 ng of purified DNA from the biopsy specimen. The amplicons were analyzed by agarose gel electrophoresis stained with Blue Green Loading Dye I (LGC, Brazil) and examined under UV light with the Molecular Imaging Software Gel Logic (Kodak, USA).

Following RCA, DNA was purified using a Genomic DNA Clean & Concentrator (Zymo Research). The quality and quantity of the DNA were assessed using a Nanodrop spectrophotometer (Thermo Scientific) and a Qubit fluorometer (Invitrogen). DNA fragment libraries were further prepared with one ng of purified RCA DNA using a Nextera XT DNA sample preparation kit and sequenced using an Illumina MiSeq instrument (2×150 paired-end reads with the Illumina v2 reagent kit).

Genome assemblies and sequence analyses. The paired-end sequence reads were assembled into contigs using SPAdes 3.5[35] and compared to sequences in the GenBank nucleotide and protein databases using BLASTn/BLASTx. Geneious software version 9[25] was used for open reading frame (ORF) and conserved domain predictions as well as genome annotation. Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motifscan) was used to confirm the conserved domain prediction pointed by Geneious software version 9[25].

Similarities searches were performed using local sequence alignments BLAST[36]. Global sequence alignments were accomplished to determine sequence identities with MUSCLE software[37]. Representative sequences within genera and sequences with the highest identities to the sequences from the present study that are available in GenBank were retrieved from the NCBI homepage (http://www.ncbi.nlm.nih.gov/) for phylogenetic analysis. Altogether, the dataset consisted of 45 sequences of the L1 gene. The multiple sequence alignments was performed through the MUSCLE software[37].

The phylogeny was reconstructed with a maximum likelihood method using the MEGA6 software[38]. These analyses were performed using the GTR substitution model, and the algorithm was modeled with a gamma distribution (shape parameter = 5). The nucleotide substitution model was defined by the tool "find best DNA/Protein model (ML)" of MEGA6 software[38]. Statistical support was provided by 1,000 non-parametric bootstrap analyses.

RDP4 software[39], using the RDP[40], GENECONV[41], BOOTSCAN[42], MAXCHI[43], CHIMAERA[44], SISCAN[45] and 3 SEQ[46] methods using default settings were used to look for the presence of chimeric genomes that can arise during the building of contigs. Recombination was considered credible in sequences only if they were detected by more than three methods having significant *P* values coupled with strong phylogenetic support for recombination. To verify any mismatches that could make difficult the annealing of the viruses detected with the degenerate primer regions, all generated sequences were aligned with primers FAP59/64[4] and MY09/11[47] using ClustalX software[38].

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Author Contributions

C.D. conceived the study, performed the rolling circle amplification, analyzed the data and co-wrote the paper. F.R.C.S analyzed the data and co-wrote the paper. A.F.S. performed the phylogenetic analysis and contributed to the discussion of the paper. M.N.W. performed the RDP4 software analysis and contributed to discussion and paper writing. F.Q.M. contributed to discussion, interpretation of the data, and writing of the paper. S.P.C. conceived the data analysis, discussion, interpretation of the data and paper writing. C.W.C. contributed to the data analysis, discussion, interpretation of the data and paper writing. All authors approved the final version of the manuscript.

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10 CAPÍTULO 6: PAPILOMAVÍRUS EM RUMINANTES: UMA ATUALIZAÇÃO

O presente trabalho já foi concluído e um artigo de revisão de literatura intitulado "*Papillomaviruses in ruminants: an update*" foi submetido para publicação no dia 30/10/2017 no periódico *Transboundary and Emmerging Diseases* e se encontra em revisão. O Manuscrito será apresentado a seguir, tal qual foi enviado para publicação.

Papillomaviruses in ruminants: an update

Running head: Ruminant Papillomaviruses

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Abstract

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

Keywords: BPV, ruminantia, papillomavirus, phylogeny, molecular epidemiology.

1. Introduction

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

Within the large group of ungulates (diverse group of mammals that includes odd-toed and even-toed ungulates), bovine papillomavirus (BPV) plays a role in a variety of diseases in domestic and wild ruminants (Borzacchiello et al., 2003; Tomita et al., 2007; Van Dyk et al., 2012; Bam et al., 2013; Kumar et al., 2013; Lunardi et al., 2013a), causing considerable morbidity to the affected animals. The suborder Ruminantia comprises of a large and relevant group of terrestrial herbivorous mammals which embrace the Tragulidae, Giraffidae, Antilo-capridae, Moschidae, Cervidae, and Bovidae families that contain at least 200 extant species (Fernández and Vrba, 2005). Within these families, PVs have been detected and characterized into Bovidae, Cervidae and Giraffidae families until the present moment.

This scenario can lead to economic losses mainly in countries that have vast amount of cattle herds, as BPV induces chronic, tumoral and contagious diseases that affects dairy and beef cattle herds worldwide and is associated with a variety of benign and malignant tumors (Campo et al., 1992; Jarrett et al., 1994; Campo, 1997; Araibi et al., 2004; Ogawa et al., 2004; Martano et al., 2013; Da Silva et al., 2015). BPV is also

64 highly important as an *in vivo* model for Human papillomavirus (HPV) studies beyond
65 veterinary interest (Campo, 1997; Gil da Costa and Medeiros, 2014).
66 Due to the increscent detection number of PV types that mainly affect large
67 ruminants, this review aims to provide an update of the current epidemiology,
68 classification and genomic features of ruminant PVs (infecting Giraffidae, Bovidae, and
69 Cervidae hosts), focusing on BPVs. The review also aims to clarify the key differences
70 between the apparently high-risk *Delta* papillomaviruses and the seemingly low-risk *Xi*,
71 *Epsilon*, *Dyoxi* and *Dyokappa-PVs* as well as the recently described BPVs 19 and 21
72 that belong to an unclassified genera.
73
74 **2. Classification of papillomaviruses: old and new considerations**
75 After being classified into the *Papovaviridae* family, PVs were discriminated as
76 a separate family, the *Papillomaviridae* (King et al., 2012). Traditionally, PVs are
77 classified into Genera, Species, Types, Subtypes and Variants. The type classification is
78 substantial due to its great clinical relevance.
79 Currently, PV classification is based on nucleotide sequence identity of L1 Open
80 Reading Frame (ORF), a relatively conserved region that can be aligned for all known
81 PVs for the construction of phylogenetic trees (de Villiers et al., 2004; Bernard et al.,
82 2010). The type of PV is determined when the entire genome sequenced and the L1
83 ORF differs by more than 10% in comparison to any other PV type. A new PV genera is
84 proposed when the identities are lower than 60%. ORF L1 differences between 2% and
85 10% determine a new subtype, and a variant is defined when the difference is less than
86 2% (de Villiers et al., 2004; Bernard et al., 2010) (Figure 1). Nevertheless, not just the
87 percentage of identity should be taken into account to define the PV's classification.

The interpretation of the phylogenetic position, genome organization, biology, and pathogenicity should also be considered (Bernard et al., 2010).

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

BPVs are a crescent group of epitheliotropic viruses that recognize *Bos taurus* as its classical host, although there are some well-established cross-infections involving BPV1 and 2. Currently, bovine papillomaviruses (BPVs) consist of 23 types (https://pave.niaid.nih.gov/), two of them (BPV22 and 23) recently described (Da Silva et al., 2016; Bauermann et al., 2017). BPVs are distributed into five known genera and one unclassified genera (https://pave.niaid.nih.gov/). The *Xipapillomavirus* genus comprises of two species, *Xipapillomavirus 1* (BPV3, 4, 6, 9, 10, 11, 15) and *2* (BPV12), and two recently described types that are still not classified as species (BPV17 and 20). The *Deltapapillomavirus* and *Epsilonpapillomavirus* genera comprise of one single species each, *Deltapapillomavirus 4* (BPV1, 2, 13 and 14) and *Epsilonpapillomavirus 1* (BPV5 and 8), respectively. The BPV7 also comprises of a single species, the *Dyoxypapillomavirus 1* species.

Lately, two new BPV types named BPV16 and 18 were classified into the *Dyokappapapillomavirus* genus. Other two new BPV types (BPV19 and 21) recently described are representatives of an unclassified genus. Further, two more of the recently described new types (BPV22 and 23) cluster in the *Dyokappa* (Bauermann et al., 2017)

112 and *Xipapillomavirus* genus (Da Silva et al., 2016), respectively. The current
113 classification ("PaVE," 2017) of Ruminantia PVs and some classification suggestions
114 are summarized in Table 2 (see also Figure 6 and Figure 7).

115 The genetic characterization by PCR using the degenerated primer pairs
116 originally designed for HPV detection (FAP59/FAP64 or MY09/MY11), which
117 amplifies a highly conserved region of PV L1 gene (Snijders et al., 1990; Forslund et
118 al., 1999), has enabled the identification and characterization of several PV types in
119 almost all affected PV species (Antonsson and Hansson, 2002; Rector et al., 2004;
120 Gottschling et al., 2008; Munday et al., 2013), as well as in new BPV types in both
121 dairy and beef cattle from distinct geographical regions worldwide (Ogawa et al., 2004;
122 Lunardi et al., 2013b). Also, putative and new BPV types, partially or totally sequenced
123 by Sanger or NGS, have been published recently (Da Silva et al., 2015; Daudt et al.,
124 2016a; Lunardi et al., 2016). However, the development of NGS has enabled the
125 discovery of new BPV types that were not amplified using FAP or MY primer pairs (Da
126 Silva et al., 2016; Daudt et al., 2016a).
127
128 **3. Virus structure and genome architecture: a comparison of large ruminant PV**
129 **genomes**
130 The PV virion shell is composed of the major L1 and minor L2 capsid proteins,
131 which constitute an icosahedral structure devoid of lipoprotein envelope. The capsid
132 shell contains 72 capsomers (pentamers) of L1 protein and possesses approximately 600
133 Å in diameter (Modis et al., 2002).
134 The genome of these viruses is double-stranded and circular DNA of
135 approximately 8,000 base pairs (Modis et al., 2002; de Villiers et al., 2004; Bernard et
136 al., 2010; Rector and Van Ranst, 2013), and in the mature virion, the viral DNA is

associated with host cell histone proteins H2a, H2b, H3 and H4 in a chromatin-like complex (King et al., 2012). Additionally, it is organized within the virion (and infected cells) like cellular DNA in a minichromosomal form (episomal) (King et al., 2012). Genomes of oncogenic PVs are usually found integrated into the host cell chromosome and it is commonly linked with the high level expression of oncogenes and the severity of neoplasia (Doorbar et al., 2012).

Their genome organization comprises of 5 or 6 early (E) ORFs and 2 late (L) ORFs (Bernard et al., 2010). The early ORFs coding for two proteins (E1 and E2) which are involved in replication and transcription, three oncoproteins (E5, E6 and E7), which are involved in cell transformation, and the E4 protein, which contributes to virion production and actually exhibits an expression pattern closer to the late proteins. The late proteins L1(major) and L2 (minor) are the structural components of the viral capsid (Doorbar et al., 2012).

4. *Deltapapillomavirus* and oncogenicity: High-risk PVs?

In humans, most mucosal HPV types cause benign lesions, but there are some types which are considered oncogenic or high-risk PVs that lead to the development of malignant lesions (Martel et al., 2012). The high-risk HPV types (mainly *Alpha* genus) are considered a necessary cause of cervical cancer, and its prevalence in penile, vulvar, vaginal and anal cancers are of major relevance (Martel et al., 2012). Similarly, in cattle, the *Delta-PVs* comprise of the high-risk mucosal types that can cause urinary bladder cancer in their natural hosts. BPV1 and 2 infect both epithelial and mesenchymal cells and the cross-infections are also common between its members (Bergvall, 2013; Munday et al., 2015; Bocaneti et al., 2016).

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

4.1. Ruminantia PV oncoproteins: a comparison

4.1.1. E5 protein

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

187 Studies in cattle have detected the E5 expression only in cancer cells but not in
188 normal ones (Borzacchiello and Roperto, 2008). The E5 protein interacts with the
189 platelet-derived growth factor receptors (PDGF-R) (involved in cancer development) in
190 both epithelial and vascular tumors of the urinary bladder in cattle (Borzacchiello and
191 Roperto, 2008). The comparison between all Ruminantia PV genomes shows that E5
192 oncogene is present in all *Delta-PVs* (high-risk PVs), one *Epsilon-PV* (which has not
193 been related to cancer up till now) and in a variety of *Xi-PVs* (BPV9, 10 and 15), not
194 including BPV4, which is the only *Xi-PV* related to malignancy so far (Table 1, Figure 2
195 and 3). Usually, *Delta* genomes encode the E5 gene at the 3' end of the early genome
196 region and is expressed from a spliced mRNA that initiates upstream of the E2 gene
197 (DiMaio and Petti, 2013). Also, cofactors such as bracken fern ingestion, which
198 contains mutagenic chemicals and immunosuppressants are substantial for the
199 occurrence of cancer (International Agency for Research on Cancer, 2007).
200 Interestingly, when *Xi* genomes contain E5 genes, they are substituting E6/E8
201 genes (first early region ORF). Despite its distinct location, the protein size is almost the
202 same in length (42 aa) when compared to *Delta* E5 (41 to 53 aa).
203 The analysis of E5 predicted aa sequences from the Ruminantia PVs showed that
204 the *Deltapapillomavirus 4* species has great similarity among the PVs analyzed (Figure
205 3). In the *Delta 4* species, the most dissonant E5 aa sequence is from BPV14, which was
206 recently detected in urothelial bladder tumors in cattle from southern Italy (Munday et
207 al., 2015; Roperto et al., 2016b). Also, the *Xi-PVs*, which are not involved with cancer
208 so far, showed the most variable E5 (Figure 3).
209 Interestingly, the high-risk BPVs (BPV1, 2 and 13) and the BgPV1 are almost
210 identical in its E5 aa sequence. Most differences observed between BPV13 and BgPV1
211 E5 are located at the C-terminal protein. BPV13 presents a conservative aa change (E to

212 D) at 46th position (Lunardi et al., 2013b), and the BgPV1 contains a non-conservative
213 change (G to S) at 51st aa position. Additionally, BPV14, as GcPV1, contains non-
214 conservative changes at these positions. Moreover, the BPV1, 2, 13 and BgPV1 consist
215 of a peculiar conserved region from 11th to 15th aa position, prior to the leucine rich
216 region.

217 This conserved region among the *Delta 4 PVs* comprises of a sequence of non-
218 polar and polar aa followed by three non-polar aa. However, BPV14 comprises of a
219 polar and basic aa followed by three non-polar aa, which also occurs in the *Delta 3 PVs*
220 (OaPV1, 2 and 4) and *Delta 5* (CcaPV1). The *Epsilon* 1 representative consists of a
221 basic, polar, neutral and polar aa sequence in this region. Therefore, this conservative
222 region among the high-risk *Delta-PVs* could play an important role in malignancy cell
223 transformation (Figure 3).

224

225 **4.1.2. E6 protein**

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

235 Ruminantia PV E6 oncoprotein contains 123 to 180 aa in length. The aa
236 alignment shows two well-conserved, hypothetical zinc finger structures (Figure
237 4).These zinc finger structures are also similarly conserved among HPVs (Zimmermann

et al., 2017). The most oncogenic BPVs reported (BPV1, 2, 13) and BgPV1 contains an acid aa residue at 70th position (E), a non-conservative change, while the other Ruminantia contains a majority of nonpolar, polar or basic aa. All *Delta 4* also contain an acid aa residue at 80th position (D), as *Delta 5* (CcaPV1 and RalPV1) and the *Dyokappa* member BPV22 and the unclassified BPV18. *Dyoxi 1* (BPV7) contains four more aa (CXX-X33-CXX) that are polar, nonpolar followed by two basic aa in the first zinc finger prediction.

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

Moreover, albeit it is not commonly reported, five E6 predicted proteins have one putative Retinoblastoma protein-binding site (pRB-binding domain LXCXE) overlapping one of the two zinc-binding domains. Also, although the zinc finger do not fold properly, it, somehow, could also play a role in targeting the retinoblastoma protein (Figure 4). Interestingly, these sequences belong to *Delta* genus (*Delta 4*, BPV1 and 13 - aa position 62nd to 66th; *Delta 3*, OaPV1, 2 and the Delta member OaPV4- aa positions 99th to 103rd), which are the most common genera linked to malignant cell transformation (Borzacchiello and Roperto, 2008). The Ruminantia E6 sequences lack a PDZ-binding motif (ETQL) in its C-terminus. Although there are divergences amidst the predicted E6 protein in the Ruminantia PVs, there are some global conservative

263 regions beyond the zinc finger domains that could be essential to its transforming
264 functions (see positions 72nd, 109th, 139th, 151st and 152nd in Figure 4).

265
266 **4.1.3 E7 protein**
267 E7 oncoproteins are thought to interfere with the host cell cycle by targeting the
268 cell cycle regulator pRB, leading to proliferation of the basal and parabasal cells
269 (Doorbar et al., 2012). High expressions of E6 and E7 in cells at the lower layers
270 interferes directly with the cell cycle and stimulate uncontrolled division and is directly
271 related to the increasing severity of neoplasia in humans (Doorbar et al., 2012).
272 The pRB-binding domain is present in 23 of 41 E7 Ruminantia encoded
273 proteins. While all Ruminantia *Xi*, *Dyokappa* and *Phi* genus lodges pBR, *Delta*, *Epsilon*
274 and *Dyoxi* members lack the canonical pRb-binding domain LXCXE. The absence of
275 pRB-binding domain in its E7 proteins with the E5 presence in some Ruminantia PVs
276 has been linked to fibropapilloma-associated viruses (Narechania et al., 2017).
277 However, there are some *Delta* and *Epsilon* PVs related to fribropapilloma lesions
278 (Table 1).
279 The majority of Ruminantia E7 oncoproteins consist of a zinc finger domain
280 (CXXC-29X-CXXC). The exceptions are BPV6, CePV2, BPV8 and PpuPV1, which
281 belong to *Xi*, *Epsilon,* and unclassified genera, wherein the CXXC motifs are separated
282 by 30 aa residues (Figure 5). The set of BPV oncoproteins, E5, E6 and E7, are known to
283 cooperate in cell transformation (Nasir and Campo, 2008). Both the careful analysis of
284 its predicted proteins and the biological sample histological classification are important
285 to elucidate the real role of each oncoprotein in cell transformation amidst Ruminantia
286 hosts.

287

288 **5. Papillomaviruses species in ruminants**

289 Nowadays, there are at least 200 HPV types fully sequenced and characterized.
290 In contrast, only 39 ruminant PV types are recognized up to now
291 (https://pave.niaid.nih.gov/), which leads us to support the importance of improving the
292 efforts to characterize animal PVs. Recent studies, some using high-throughput
293 sequencing platforms, have shown that animal PVs were underestimated and new types
294 have been characterized (Da Silva et al., 2016; Daudt et al., 2016a; López-Bueno et al.,
295 2016; Bauermann et al., 2017; Tore et al., 2017).

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

304 Ruminantia PVs comprise of 6 known genera: *Delta, Epsilon*, *Xi*, *Phi*,
305 *Dyokappa,* and *Dioxypapillomavirus* and one putative unclassified genus ("PaVE,"
306 2017). Here, we suggest one new unclassified genus related to the *Dyokappa* genus
307 (Figure 6, 7 and 8 and Supplementary Figure 1C). This clade is supported by BPV18
308 and PpuPV1, which diverge from *Dyokappa* by more than 55% and represents a diverse
309 branch from *Dyokappa* members. Also, on exploring the identity and the PVs
310 phylogenetic position, the same analysis comprising of the *Delta-PVs* shows that
311 GcPV1 is the most unlike *Delta* genome and, probably, is a representative of a new
312 species in this genus (Supplementary Figure 1A). In the same way, the CePV1 could
313 comprise the *Epsilonpapillomavirus* 2 in the *Epsilon-PVs* (Supplementary Figure 1D),

314 and BPV20 (more related to RtPV2) could be considered as a *Xipapillomavirus* 3
315 species (Supplementary Figure 1B).
316
317 **6. Epidemiology**
318 In *Bos taurus*, BPVs are widespread, besides, the type distribution is not the
319 same in the studied regions. They are present more frequently in young animals,
320 especially in housing conditions. Also, bad nutrition, lack of proper cleaning and
321 inadequate installations are risk factors for the development of papillomatosis, since
322 they can cause stress, and consequently, immunodepression, which influences the
323 presence and the severity of this disease (SMITH, 2006; Da Silva et al., 2015).
324 Independent of technology level of livestock exploration, the papilloma lesions are
325 particularly relevant in dairy cattle herds.
326 Despite the lack of tissue culture systems for the *in vitro* propagation of the PV,
327 detailed genetic and molecular information has been accumulated, especially in human
328 and bovine papillomavirus, using cloning and Sanger sequencing. Lately, the new
329 technology of high efficiency sequencing has enabled the characterization of new and
330 putative new BPVs (Munday et al., 2015; Da Silva et al., 2016; Daudt et al., 2016a). As
331 PVs are cosmopolitan viruses, they have been detected in many regions of almost all
332 continents infecting a large range of amniote species (Rector and Van Ranst, 2013).
333 Besides the high lesions frequency level, the PV genotyping is still sporadic. The
334 majority of BPV type-specific prevalence data are from Japan and Brazil. Brazil is one
335 of the largest beef and milk producer in the world, and 21 out of 23 BPV types were
336 detected and described here, as well as a great number of putative new types (Carvalho
337 et al., 2012; Batista et al., 2013; Lunardi et al., 2013a; Da Silva et al., 2016, 2017;

338 Daudt et al., 2016a , 2016b; Lunardi et al., 2016). The BPV type-specific distribution
339 and their association with specific lesions in cattle are summarized in Table 1.
340 The Bovidae family also consists of PVs that were characterized in three other
341 host species: *Capra hircus* (ChPV1); *Ovis aries* (OaPV1, 2, 3 and the OaPV4 recently
342 described (Tore et al., 2017)); and *Bos grunniens* (BgPV1). The other Ruminantia PVs
343 are sporadically diagnosed. Beyond the Bovidae family, the Cervidae family is the
344 second most studied, comprising of 11 PV types, characterized into 9 animal species
345 nowadays ("PaVE," 2017). The roe deer papillomavirus (CcPV1) infection has been
346 identified as an endemic disease in roe deer population of the Carpathian Basin in
347 Central Europe (Hungary, Austria and Croatia) (Erdélyi et al., 2009). The PV type-
348 specific distribution and their lesion association in Ruminantia are summarized in Table
349 1. The distribution of different BPV types is shown in Figure 8.
350

351 **7. Papillomavirus diseases**

352 **7.1. Cutaneous papillomas**

353 The cutaneous lesions in cattle can display distinct morphologies and have been
354 grossly classified into filiform, pedunculate and atypical forms, as planar shape or
355 squamous papilloma (Da Silva et al., 2015; Grindatto et al., 2015; Lunardi et al., 2016).
356 The typical pedunculate form presents verrucous aspect, known as cauliflower form.
357 Papillomatous lesions are found in head, neck, dorse, abdomen, udder, teat and mucosa
358 of digestive and genital tracts (Claus et al., 2007; Borzacchiello and Roperto, 2008;
359 Batista et al., 2013; Lunardi et al., 2016) (See also Table 1). Virtually all BPVs have
360 been detected in cutaneous lesions, even the ones which are found in malignant lesions,
361 as the *Delta-PVs* represented by BPVs 1, 2, 13 and 14 (Table 1).
362 Cutaneous HPV types cause an asymptomatic or self-limited benign tumors and
363 are usually classified as "low-risk" PV types (mainly from the *Gamma* and *Beta* genera)

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

Commonly, diverse BPV genera are associated with distinct diseases. In general, *Xipapillomavirus* are classified as epitheliotropic PVs (BPV3, BPV4 and BPV6); *Deltapapillomavirus* are associated with fibropapillomas and cutaneous papillomas (BPV1, 2, 13 and 14) as well as the urinary bladder (BPVs 1, 2, 13 and 14) (Borzacchiello and Roperto, 2008; Lunardi et al., 2013a; Roperto et al., 2016b). The *Epsilonpapillomavirus* genus (BPV5 and 8) is linked to cutaneous papillomas (Tomita et al., 2007; Claus et al., 2009; Da Silva et al., 2015; Da Silva et al., 2016) and the *Dyoxipapillomavirus* genus was described in healthy skin swabs and has been linked to cutaneous papillomas in teat and body (Ogawa et al., 2007; Tozato et al., 2013; Savini et al., 2016). BPV6, 7, 8, 9 and 10 are usually linked to benign squamous papilloma (showing varying degrees of hyperkeratosis or parakeratosis, with elongated digital-like proliferation of the squamous epithelium) and fibropapillomas (besides BPV7) (Tozato et al., 2013; Lunardi et al., 2016).

Although considerable efforts have been made to map the preferred body site for each BPV type and it is thought to be important to better understand its biology, it appears that the BPV infection is guided mainly by its tropism for mucosal or cutaneous epitheliums (Table 1). Also, as the animals harbor PVs in healthy skin (Campo et al., 1994; Ogawa et al., 2004) and tumors appear at sites of damaged skin (Campo et al., 1994; Doorbar et al., 2012), there is a probability to find BPV types in sites where there

389 is more abrasion, according to the farm management. In addition, there are evidences
390 that they are not restricted to any anatomical site in cattle (Batista et al., 2013). PV
391 infection regarding Cervidae, Giraffidae, and other members of Bovidae family are
392 poorly detected and studied and it is difficult to speculate the frequent body site where it
393 causes lesion. Therefore, more studies involving this issue are necessary to clarify the
394 relevance of this aspect in PV ruminant infections.
395
396 **7.2. Teat papillomas**
397 BPV6, 7, 8, 9 and 10 were identified in teat papillomas in dairy cows of Japan
398 and southern and southeastern Brazil through sequencing of FAP PCR products
399 (Hatama et al., 2008; Tozato et al., 2013; Lunardi et al., 2016). Recently, the putative
400 new types BPV/BR-UEL6 and BPV/BR-UEL7, BAPV9 and a subtype of putative new
401 type BAPV4 were also reported in teat papillomas in these Brazilian regions (Lunardi et
402 al., 2016). Although the presence of teat and udder lesions could be associated with
403 mastitis and the decrease in milk production (Campo, 2006), which leads to the cow's
404 early disposal, there are few studies regarding teat papillomatous lesions till now.
405
406 **7.3. Bladder carcinomas**
407 Bladder carcinomas are relatively common in cattle grazing on bracken fern in
408 synergy with BPV1 and 2 (Wosiacki et al., 2002; Roperto et al., 2010, 2013; Roperto et
409 al., 2013a). They were also detected in buffaloes and yaks, although sporadically
410 (Roperto et al., 2013a, Roperto et al., 2013b).
411 The clinical signs of chronic intoxication caused by the bracken fern are
412 anorexia, inappetence, progressive weight loss, bloody diarrhea, cough and dysphagia.
413 The immunosuppression caused by bracken fern could promote hematogenous spread of
414 BPV2 to the bladder and allow prolonged infection of the bladder by the PV, leading to

415 hematuria and cystitis progression (Munday, 2014). The urinary bladder tumor
416 incidence among cattle grazing on this pteridophyte is higher than 90% and it is
417 characterized by hemorrhagic and hyperplasic lesions from bladder mucosa, which
418 frequently progress to neoplasm (Roperto et al., 2010).

419
420 **7.4. Upper GI carcinomas**

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

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

434
435 **7.5. Papillomavirus cross infections**

436 Generally, PVs are strictly species- and tissue-specific, although equine sarcoids
437 can be caused by BPV1, 2 and 13 (Nasir and Campo, 2008; Lunardi et al., 2013b).
438 Other examples of cross-species infections determined by *Delta-PVs* are the detection
439 of BPV1 in cutaneous fibropapillomas of a giraffe and a sable antelope (Van Dyk et al.,
440 2012), cutaneous warts from buffalos infected with BPVs 1 and 2 (Pangty et al., 2010),
441 and the involvement of BPV2 in carcinogenesis of urinary bladder of buffalos grazing

442 on pasture containing bracken fern (Roperto et al., 2013). Also, BPV1 and 2 were
443 reported in yaks associated with cutaneous papillomatosis in the North-East region of
444 India (Bam et al., 2013).

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

451

452 **7.6. Mixed and co-infections**

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

461 More recently, the Rolling Circle Amplification followed by next generation
462 sequencing strategy has demonstrated the high level of BPV co-infection, as well as the
463 characterization of several new BPVs (Daudt et al., 2016a). The implications of the high
464 diversity of different BPV genotypes in a single lesion in cattle suffering from
465 cutaneous papillomatosis are still unclear. Nevertheless, the frequent observation of co-
466 infection in skin warts of cattle resembles the scenario shown for cutaneous HPVs
467 where infections by more than 10 HPV types can be identified (Antonsson et al., 2000).

Curiously, examples of BPV co-infections have recently extended beyond skin lesions, and are characterized by frequent presence of several *Delta-PVs* in individual urothelial tumors of cattle grazing on pasture containing bracken fern as well as identification of up to five viral types circulating in the blood from animals with and without cutaneous warts (Santos et al., 2014; Bocaneti et al., 2016; Roperto et al., 2016). Some studies have shown co-infection of BPV13 and BPV2 in cattle urothelial bladder tumor cells, as well as BPV13 itself (Roperto et al., 2016a). Additionally, BPV1 and 2 were detected by qPCR in a wart collected from the reticulum of a buffalo from India (Kumar et al., 2013). In the case of urinary bladder and gastrointestinal tract cancer, prolonged ingestion of immunosuppressant compounds of bracken fern could explain the ease of multiple BPV types infecting their hosts (Roperto et al., 2016a; Roperto et al., 2016b).

7.7. BPV infection in non-epithelial cells

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

In cattle, the presence of BPV DNA was demonstrated through partial amplification of viral segments in blood, urine, milk, semen, placenta, lymph nodes, and cells and tissues of reproductive female tract. Expression of main structural protein and/or oncoproteins was evidenced by RT-PCR, western blotting, immunoprecipitation,

494 immunohistochemistry, and electron microscopy examination of placenta, sperm cells
495 and PBMCs of healthy cattle or animals suffering from urinary bladder tumors
496 (Carvalho et al., 2003; Roperto et al., 2008, 2011, 2012; Lindsey et al., 2009; Santos et
497 al., 2014; Cota et al., 2015), these consistent remarks have confirmed that BPV
498 replication is not restricted to epithelia. Besides, it has been suggested that active
499 infection of $CD4^+$ and $CD8^+$ lymphocytes may represent the main circulating targets for
500 BPV in blood, allowing for the spread of this pathogen to numerous organs at initial
501 stages of infection (Corteggio et al., 2011; Roperto et al., 2011).
502
503 **8. Diagnosis**
504 Diagnosis of papillomatosis can be performed clinically once the alterations are
505 well characterized as peduncle, or planar progressions of the epidermis can be observed
506 (Schuch, 2001). Also, the histopathological findings and the DNA detection are
507 indispensable for confirming the diagnosis (Betiol et al., 2012; Munday, 2014; Da Silva
508 et al., 2015; Lunardi et al., 2016).
509 The PCR technique is an important diagnostic tool and is largely used for PV
510 identification due to its high specificity and sensitivity (Forslund et al., 1999; Ogawa et
511 al., 2004). However, PCR detection can be affected by primary factors such as DNA
512 concentration and its purification (Ogawa et al., 2004). Type identification through PCR
513 and sequencing, using tools such as BLAST (Basic Local Alignment Search Tool) is
514 shown to be the most sensitive method for PV identification and characterization
515 (Borzacchiello et al., 2003; Zhu et al., 2012).
516 Some techniques, such as *southern blot, dot blot* and immunohistochemistry
517 (IHQ), can be used for PV diagnosis (Munday et al., 2007). The IHQ is a substantial
518 tool that allows the identification of the virus proteins, which is important in viral

519 activity observations (Nakamura et al., 1997). The BPV identification through IHQ is
520 generally carried out using commercial monoclonal antibodies (anti-L1 and anti-E7,
521 which allows the immune detection of the major capsid protein and the E7 oncoprotein,
522 respectively).

523 Histologically, papillomas are epithelial neoplasms that present digitiform
524 progression through the cellular surface. It is common to observe hyperkeratosis,
525 acanthosis of the spinal layer and koilocytosis (Monteiro et al., 2008; Da Silva et al.,
526 2015; Lunardi et al., 2016). Also, cutaneous papillomas in cattle show epidermis
527 proliferation and kerato-hyaline granules (Campo, 2006; Grindatto et al., 2015; Lunardi
528 et al., 2016). The presence of several islands of degenerated epithelial cells surrounded
529 by a thick halo of hyperplastic epidermis can also be observed (Lunardi et al., 2016).
530 Some of these characteristics are shown in Figure 9.

9. Conclusion and perspectives

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

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

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553 **Conflict of Interest**

554 None of the authors of this paper has any conflict of interest. This manuscript has not been
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557

558

10. References

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908 **Figure 1. Classification scheme of PVs based on L1 nucleotide sequence.** The green
909 color represents the identity. The red color represents the differences and the yellow color
910 represents the variation percentage in the classification.
911
912 **Figure 2. Ruminant genomes and their putative ORFs.** All *Delta* members contain the
913 E6, E7 and E5 oncogenic ORFs.
914
915 **Figure 3. Alignment of E5 proteins from Ruminantia PV genomes.** The red squares
916 represent the conserved region and the blue squares represent some differences pointed out
917 among the E5 Ruminantia aa sequences.
918
919 **Figure 4. Alignment of E6 proteins from Ruminantia PV genomes.**
920
921 **Figure 5. Alignment of E7 proteins from Ruminantia PVs.**
922
923 **Figure 6. Evolutionary relationship between Ruminantia PV L1 complete gene.** The
924 evolutionary history was inferred by using the Maximum Likelihood method based on the
925 General Time Reversible model. The tree with the highest log likelihood (-38092.55) is
926 shown. Initial tree(s) for the heuristic search were obtained automatically by applying
927 Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the
928 Maximum Composite Likelihood (MCL) approach, and then selecting the topology with
929 superior log likelihood value. A discrete Gamma distribution was used to model
930 evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.8349)). The rate
931 variation model allowed for some sites to be evolutionarily invariable ([+*I*], 9.10% sites).

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 41 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1307 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2013).

Figure 7. Evolutionary relationship between Ruminantia papillomaviruses L1 gene and representatives of each PV genera available in PaVE. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-80284.03) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.9786)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 4.28% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 92 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1156 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2013).

Figure 8. Qualitative world map showing the distinct BPV types detected in each continent.

955 **Figure 9. Bovine papillomatous lesion stained with H & E (200 X).** A) acanthosis (*),
956 fibrosis (head arrow) and hyperkeratosis (arrow). B) Spongiosis (*). Dysplasia and
957 hyperplasia can also be observed.
958
959 **Supplementary Figure 1. Color-coded pairwise identity matrix comparing *Delta*, *Xi*,**
960 ***Dyokappa* and *Epsilonpapillomavirus* members.** The identity matrices were generated
961 using the full length L1 nucleotide sequence from Ruminantia PV representatives of each
962 type. Each colored cell represents a percentage identity score between two sequences (one
963 indicated horizontally to the left, and the other, vertically at the bottom). A colored key
964 indicates the correspondence between pairwise identities and the colors displayed in the
965 matrix. The Software Sequence Demarcation Tool Version 1.2 (SDTv1.2) (Muhire et al.,
966 2014) was used to provide reproducible means of using the pairwise genetic identity
967 calculations to classify the nucleotide sequence set of the Ruminantia PVs.

968 **Table 1. Distinct BPV types and their country distribution and association with different skin papilloma lesions in large ruminants'**
969 **species. Countries: Australia (AU); Brazil (BR); China, (CN); Denmark (DK); Germany (DE); Italy (IT); India (IN); New Zealand**
970 **(NZ); Norway (NO); Sweden (SE); United States of America (US); Belgium (BE); Hungary (HU); Turkey (TR); Great Britain &**
971 **Northern Ireland (GB); South Korea (KOR). Biological Samples: CP (cutaneous papillomas), MI (missing information), UNC**
972 **(unclassified), pulmonary fibromatosis (PF); Epithelial papilloma (EP); fibropapilloma (FP); urinary bladder (UB), Health skin**
973 **(HS); Upper digestive tract (UDT); Sarcoid (SAR); Squamous cell carcinoma (SCC); Squamous papilloma (SP); Urinary bladder**
974 **tumors (UBT).**

PV type	Country	Biological Sample	Location	Animal family	Genera	Species
AaPV1	SE	CP, PF	Lung	Cervidae	*Delta*	*Delta 1*
BgPV1	CN	FP		Bovidae	*Delta*	*Delta 4*
BPV 1	BR, JP, GB, IT, IN, MA	CP, FP, Flies, UB, Semen, Blood, HS	Body, UBT, Teat	Bovidae	*Delta*	*Delta 4*
BPV 2	DE, BR, NZ, JP, IN, IT, TR, KOR	CP, FP, UB,Semen, Blood, Milk, Urine	Body, UBT, Udder	Bovidae	*Delta*	*Delta 4*
BPV 3	DE, JP, BR, CN	CP, FP	Body, Teat, Udder	Bovidae	*Xi*	*Xi 1*
BPV 4	UK, GB, BR	CP, UDT, Milk, Urine, Blood	Body, UDT	Bovidae	*Xi*	*Xi 1*
BPV 5	JP, BR, IN	CP, HS, UDT	Teat	Bovidae	*Epsilon*	*Epsilon 1*
BPV 6	GB, JP, BR	CP, HS, SP, HS	Body, Teat	Bovidae	*Xi*	*Xi 1*
BPV 7	JP, BR, IT	CP, SP	Body, Teat	Bovidae	*Dyoxi*	*Dyoxi 1*
BPV 8	JP, BR	CP, FP, Blood	Body, Teat	Bovidae	*Epsilon*	*Epsilon 1*
BPV 9	JP, BR	CP, FP, SP	Body, Teat, Udder	Bovidae	*Xi*	*Xi 1*
BPV 10	JP, BR	CP, FP, SP, Blood	Body, Teat, Udder	Bovidae	*Xi*	*Xi 1*
BPV 11	JP, BR	CP, Blood, HS		Bovidae	*Xi*	*Xi 1*
BPV 12	SE, JP, BR	EP, HS	Tongue	Bovidae	*Xi*	*Xi 2*

BPV 13	BR, CN, IT	CP, UB		Bovidae	*Delta*	*Delta 4*
BPV 14	USA, NZ, BR	CP, FP, Feline sarcoids		Bovidae	*Delta*	*Delta 4*
BPV15	CN	Bovine "sarcoid"		Bovidae	*Xi*	*Xi 1*
BPV16	BR	CP	Body	Bovidae	*Dyokappa*	UNC
BPV17	BR	CP	Body	Bovidae	*Xi*	UNC
BPV18	BR	CP	Body	Bovidae	*Dyokappa*	UNC
BPV19	BR	CP	Body	Bovidae	UNC	UNC
BPV20	BR	CP	Body	Bovidae	*Xi*	UNC
BPV21	BR	CP	Body	Bovidae	UNC	UNC
BPV22	USA	Vulvovaginitis	Vagina	Bovidae	*Dyokappa*	UNC
BPV23	BR	CP	Body	Bovidae	*Xi*	*Xi* 1
CcaPV1	HU	FP		Cervidae	*Delta*	*Delta 5*
CePV1	IT	FP		Cervidae	*Epsilon*	UNC
CePV2	NZ	CP		Cervidae	*Xi*	UNC
ChPV1	BE	HS		Bovidae	*Phippa*	*Phippa 1*
GcPV1	DK	Giraffe lesions		Giraffidae	*Delta*	UNC
OvPV1	USA	FP		Cervidae	*Delta*	*Delta 2*
OaPV1	AU	MI		Bovidae	*Delta*	*Delta 3*
OaPV2	AU	MI		Bovidae	*Delta*	*Delta 3*
OaPV3	IT	SCC		Bovidae	*Dyokappa*	*Dyokappa 1*
OaPV4	IT	FP	Scrotum	Bovidae	*Delta*	*Delta 3*
PpuPV1	DE	Hair follicles		Cervidae	UNC	UNC
RalPV1	UK	CP		Cervidae	*Delta*	UNC
RrPV1	IT	Nasolabial neoplasia		Cervidae	*Dyokappa*	*Dyokappa 2*
RtPV1	USA	MI		Cervidae	*Delta*	*Delta 1*
RtPV2	NO	Eye swab		Cervidae	*Xi*	*Xi 3*
RtiPV1	DE	Hair follicles		Cervidae	UNC	UNC

975 **Table 2. Classification scheme of Ruminantia PV genus, species and types.**

976

Genus	Species	Ruminantia PV types
Deltapapillomavirus	*Deltapapillomavirus 1*	AaPV1, RtPV1
	Deltapapillomavirus 2	OvPV1
	Deltapapillomavirus 3	OaPV1, 2 and 4
	Deltapapillomavirus 4	BPV1, 2, 13 and 14
		BgPV1
	Deltapapillomavirus 5	CcaPV1, RalPV1
	*	GcPV1
Xipapillomavirus	*Xipapillomavirus 1*	BPV3, 4, 6, 9, 10, 11, 15 and 23
	Xipapillomavirus 2	BPV12
	Xipapillomavirus 3	RtPV2
	*	BPV20 and 17 and CePV2
Epsilonpapillomavirus	*Epsilonpapillomavirus 1*	BPV5 and 8
	*	CePV1
Dyoxipapillomavirus	*Dyoxipapillomavirus 1*	BPV7
Dyokapppapapillomavirus	*Dyokappapapillomavirus 1*	OaPV3
	Dyokappapapillomavirus 2	RrPV1
	*	BPV16 and 22
Phipapillomavirus	*	ChPV1, RtiPV1
*	*	BPV18, PpuPV1
*	*	BPV19 and 21

977 * Indicate types not yet assigned into a species or genera.

978

Figure 1

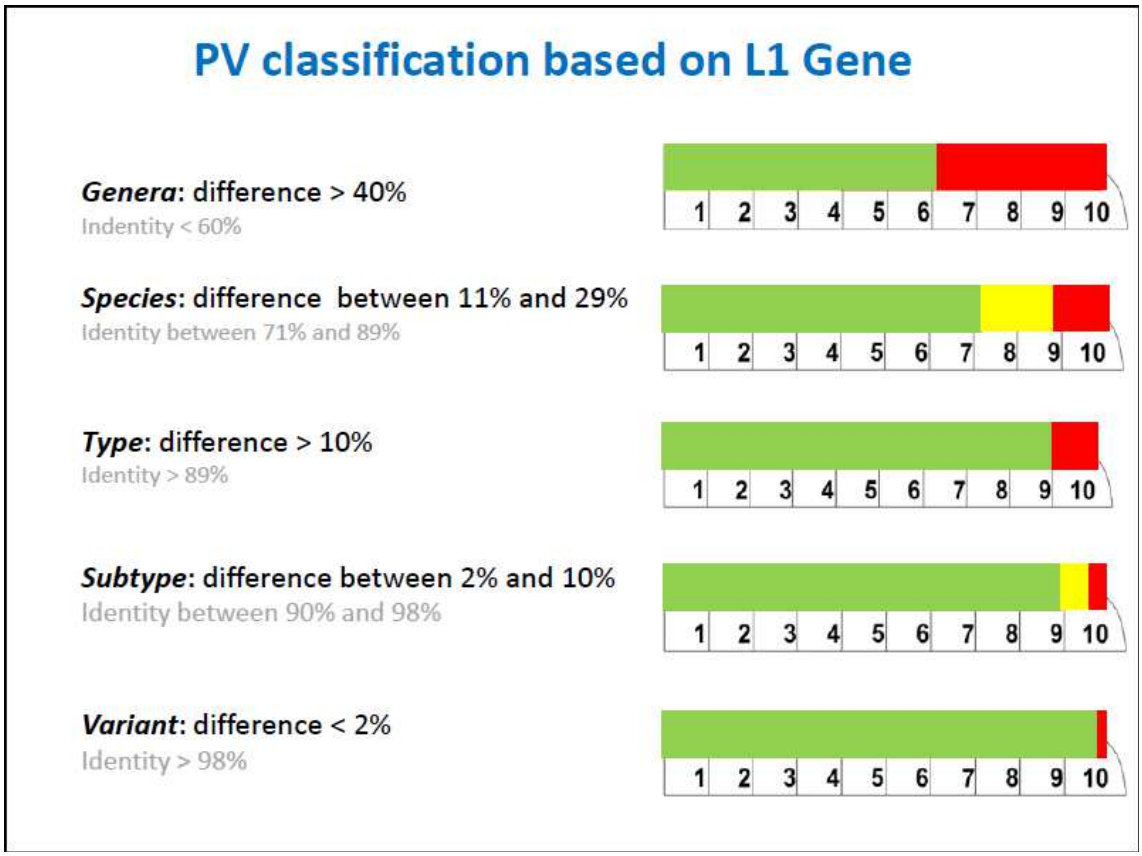

PV classification based on L1 Gene
Genera: difference > 40%
Indentity < 60%
Species: difference between 11% and 29%
Identity between 71% and 89%
Type: difference > 10%
Identity > 89%
Subtype: difference between 2% and 10%
Identity between 90% and 98%
Variant: difference < 2%
Identity > 98%
1 2 3 4 5 6 7 8 9 10

Figure 2

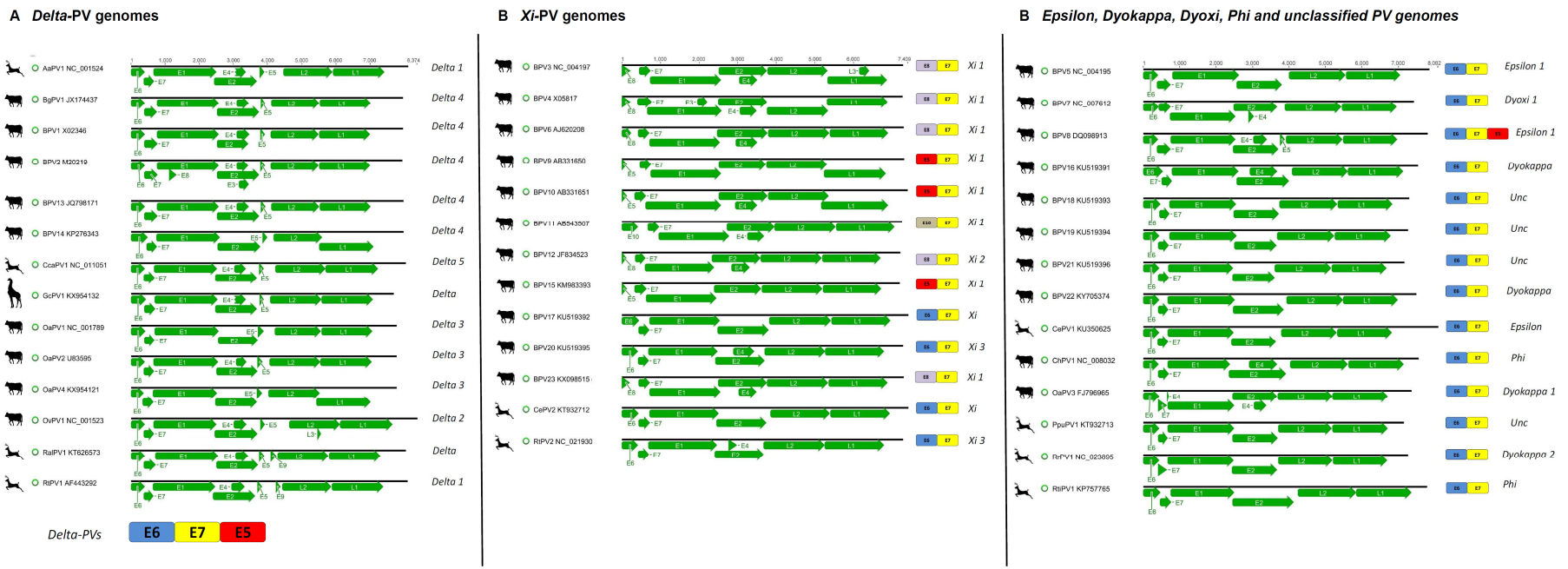
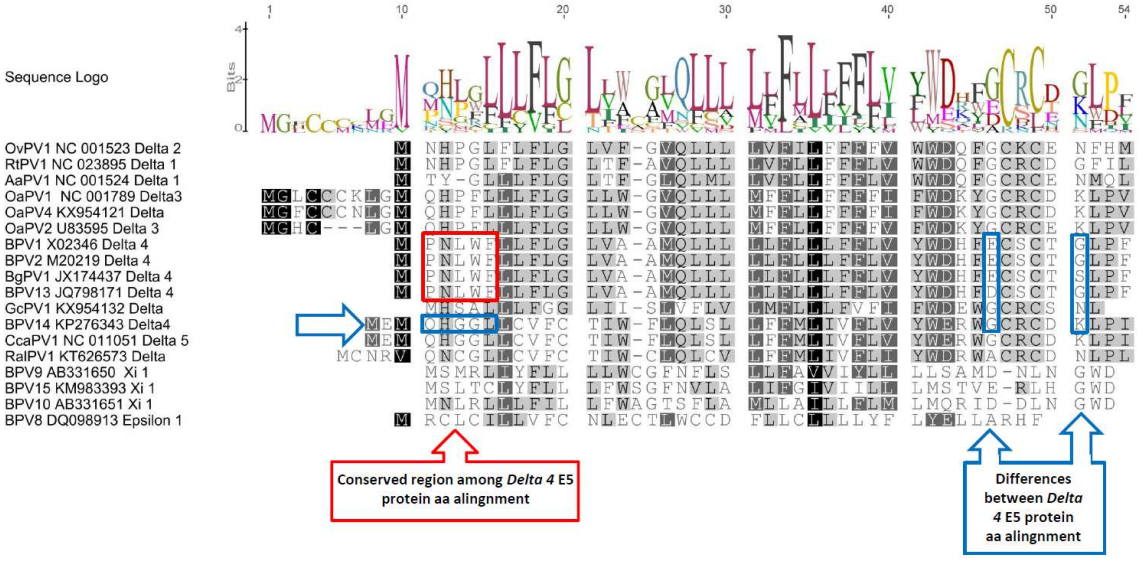

A Delta-PV genomes
AaPV1 NC_001524
BgPV1 JX174437
BPV1 X02346
BPV2 M20219
BPV13 JQ798171
BPV14 KP276343
CcaPV1 NC_011051
GcPV1 KX954132
OaPV1 NC_001789
OaPV2 U83595
OaPV4 KX954121
OvPV1 NC_001523
RalPV1 KT626573
RtPV1 AF443292
Delta 1
Delta 4
Delta 4
Delta 4
Delta 4
Delta 4
Delta 5
Delta
Delta 3
Delta 3
Delta 3
Delta 2
Delta
Delta 1
Delta-PVs
E6
E7
E5
B Xi-PV genomes
BPV3 NC_004197
BPV4 X05817
BPV6 AJ620208
BPV9 AB331650
BPV10 AB331651
BPV11 AB543507
BPV12 JF834523
BPV15 KM983393
BPV17 KU519392
BPV20 KU519395
BPV23 KX098515
CePV2 KT932712
RtPV2 NC_021930
Xi 1
Xi 1
Xi 1
Xi 1
Xi 1
Xi 1
Xi 2
Xi 1
Xi
Xi 3
Xi 1
Xi
Xi 3
B Epsilon, Dyokappa, Dyoxi, Phi and unclassified PV genomes
BPV5 NC_004195
BPV7 NC_007612
BPV8 DQ098913
BPV16 KU519391
BPV18 KU519393
BPV19 KU519394
BPV21 KU519396
BPV22 KY705374
CePV1 KU350625
ChPV1 NC_008032
OaPV3 FJ796965
PpuPV1 KT932713
RtPV1 NC_023095
RtPV1 KP757765
Epsilon 1
Dyoxi 1
Epsilon 1
Dyokappa
Unc
Unc
Unc
Dyokappa
Epsilon
Phi
Dyokappa 1
Unc
Dyokappa 2
Phi

Figure 3


Sequence Logo
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RtPV1 NC 023895 Delta 1
AaPV1 NC 001524 Delta 1
OaPV1 NC 001789 Delta3
OaPV4 KX954121 Delta
OaPV2 U83595 Delta 3
BPV1 X02346 Delta 4
BPV2 M20219 Delta 4
BgPV1 JX174437 Delta 4
BPV13 JQ798171 Delta 4
GcPV1 KX954132 Delta
BPV14 KP276343 Delta4
CcaPV1 NC 011051 Delta 5
RalPV1 KT626573 Delta
BPV9 AB331650 Xi 1
BPV15 KM983393 Xi 1
BPV10 AB331651 Xi 1
BPV8 DQ098913 Epsilon 1
Conserved region among Delta 4 E5 protein aa alignment
Differences between Delta 4 E5 protein aa alignment

Figure 4

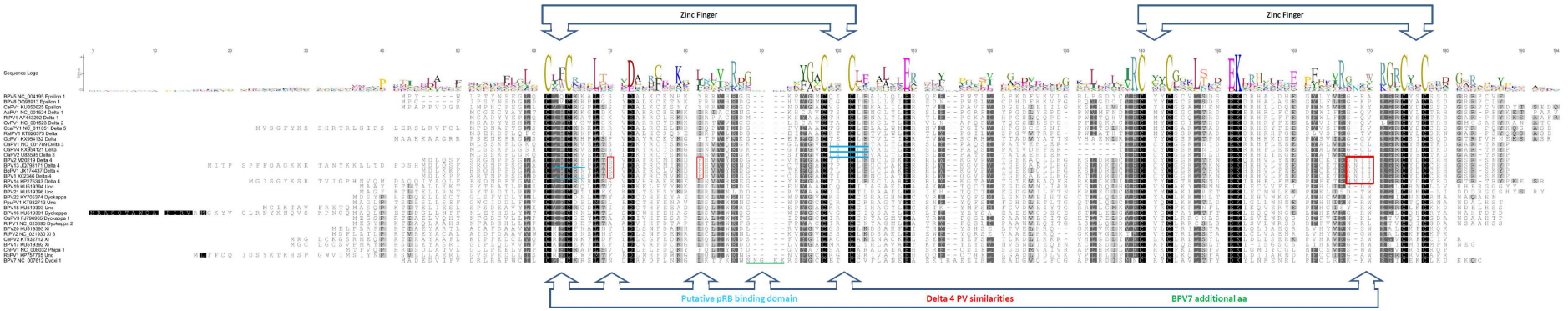
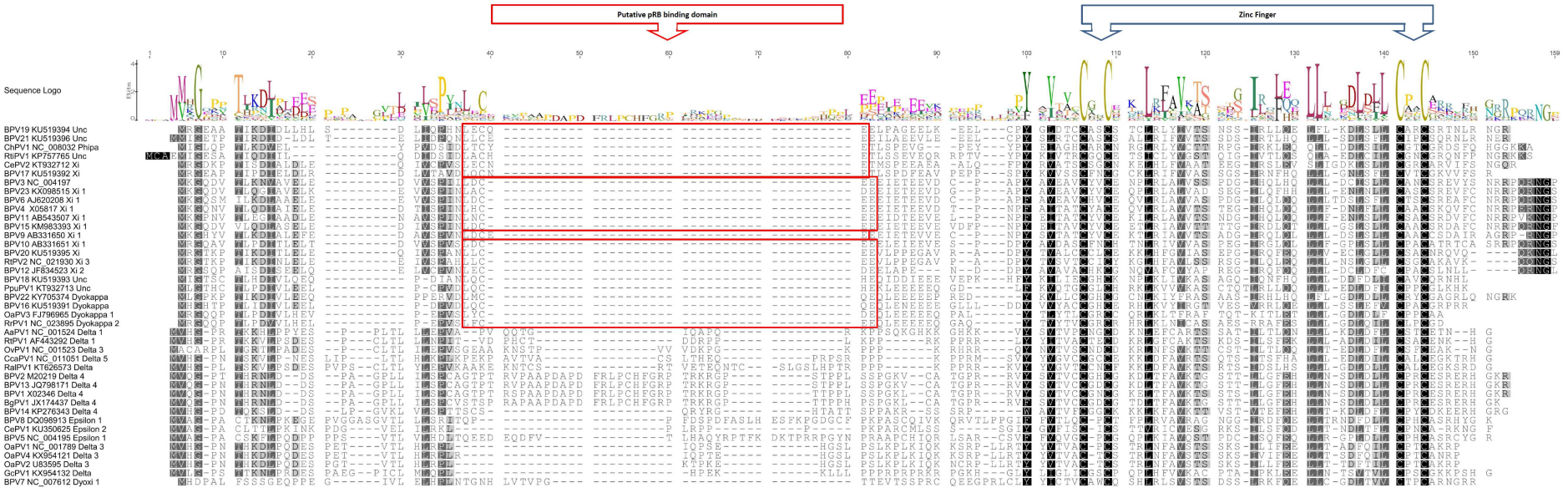

Zinc Finger
Zinc Finger
Sequence Logo
Putative pRB binding domain
Delta 4 PV similarities
BPV7 additional aa

Figure 5


Putative pRB binding domain
Zinc Finger
Sequence Logo
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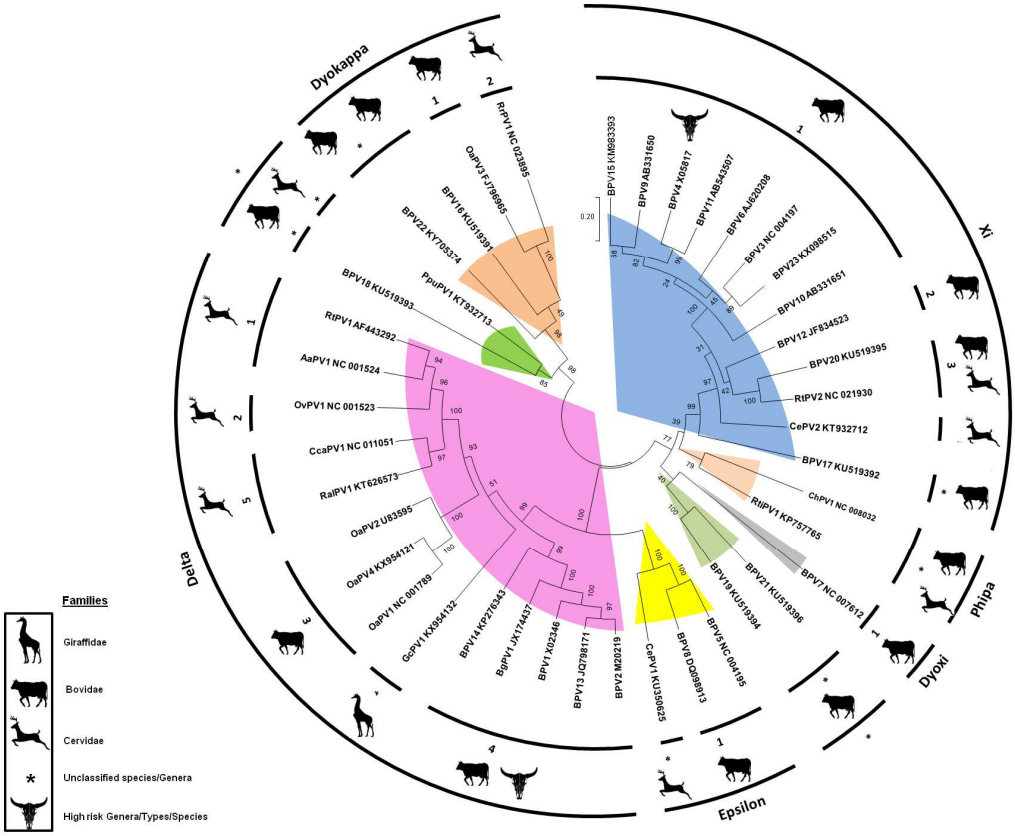
BPV21 KU519396 Unc
ChPV1 NC_008032 Phipa
RtPV1 KP757765 Unc
CePV2 KT932712 Xi
BPV17 KU519392 Xi
BPV3 NC_004197
BPV23 KX098515 Xi 1
BPV6 AJ620208 Xi 1
BPV4 X05817 Xi 1
BPV11 AB543507 Xi 1
BPV15 KM983393 Xi 1
BPV9 AB331650 Xi 1
BPV10 AB331651 Xi 1
BPV20 KU519395 Xi
RtPV2 NC_021930 Xi 3
BPV12 JF834523 Xi 2
BPV18 KU519393 Unc
PpuPV1 KT932713 Unc
BPV22 KY705374 Dyokappa
BPV16 KU519391 Dyokappa
OaPV3 FJ796965 Dyokappa 1
RrPV1 NC_023895 Dyokappa 2
AaPV1 NC_001524 Delta 1
RtPV1 AF443292 Delta 1
OvPV1 NC_001523 Delta 3
CcaPV1 NC_011051 Delta 5
RaPV1 KT626573 Delta
BPV2 M20219 Delta 4
BPV13 JQ798171 Delta 4
BPV1 X02346 Delta 4
BgPV1 JX174437 Delta 4
BPV14 KP276343 Delta 4
BPV8 DQ088913 Epsilon 1
CePV1 KU350625 Epsilon 2
BPV5 NC_004195 Epsilon 1
OaPV1 NC_001789 Delta 3
OaPV4 KX954121 Delta 3
OaPV2 U83595 Delta 3
GcPV1 KX954132 Delta
BPV7 NC_007612 Dyoxi 1

Figure 6


Families
Giraffidae
Bovidae
Cervidae
Unclassified species/Genera
High risk Genera/Types/Species
Dyokappa
Xi
Phipa
Dyoxi
Epsilon
Delta
0.20
BPV15 KM983393
BPV9 AB331650
BPV4 X05817
BPV11 AB543507
BPV6 AJ620308
BPV3 NC 004197
BPV23 KX098515
BPV10 AB331651
BPV12 JF834523
BPV20 KU519395
RtPV2 NC 021930
CePV2 KT932712
BPV17 KU519392
ChPV1 NC 008032
RtPV1 KP757765
BPV7 NC 007612
BPV21 KU519396
BPV19 KU519394
BPV5 NC 004195
BPV8 DQ089913
CePV1 KU350625
BPV2 M20219
BPV13 JQ738171
BPV1 X02346
BgPV1 JX174437
BPV14 KP276343
GcPV1 KX954132
OaPV1 NC 001789
OaPV4 KX954121
OaPV2 U83595
RalPV1 KT626573
CcaPV1 NC 011051
OvPV1 NC 001523
AaPV1 NC 001524
RtPV1 AF443292
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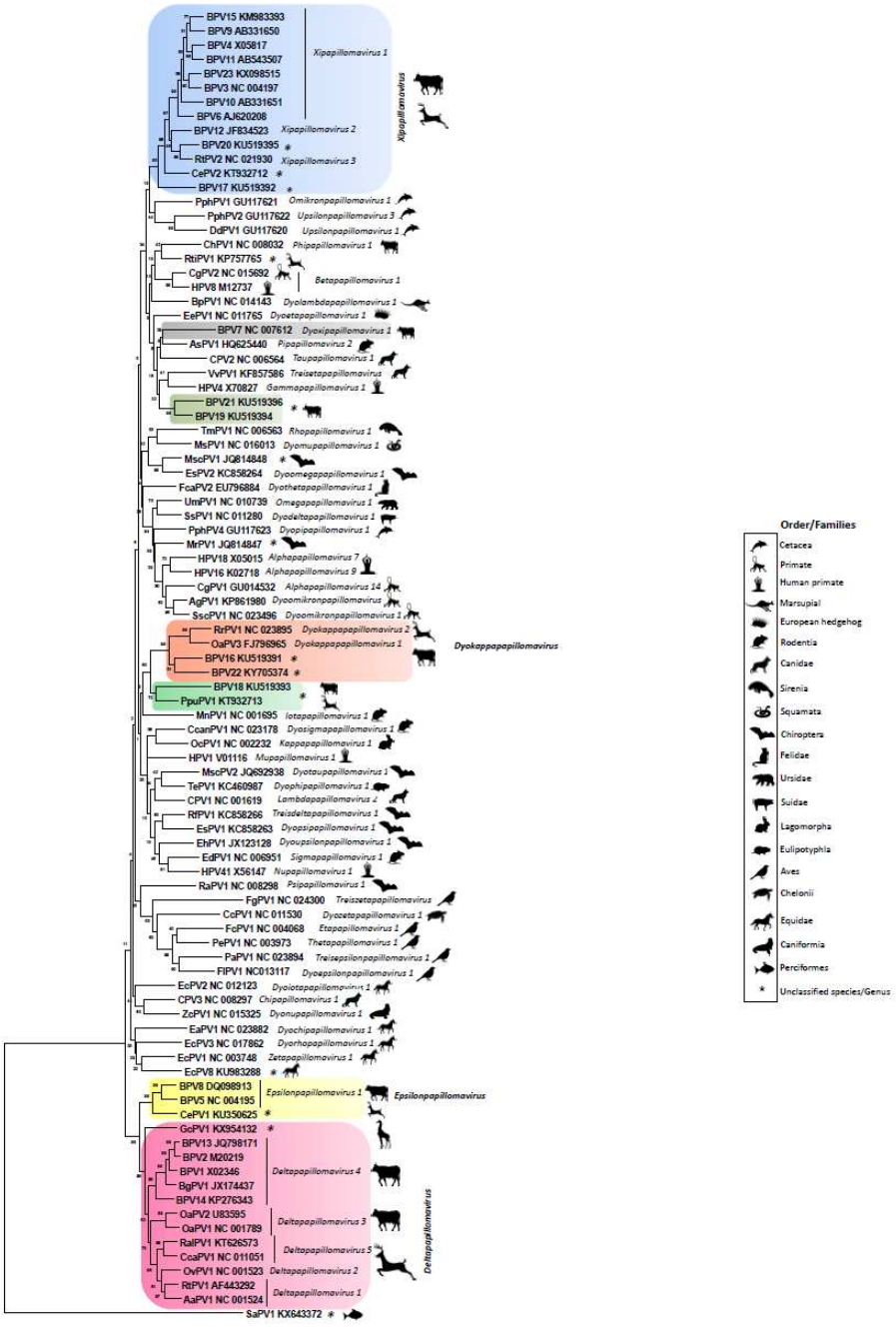
BPV16 KU519391
OaPV3 FJ798965
RrPV1 NC 023895

Figure 7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BPV9 AB331650
BPV4 X05817
BPV11 AB543507
BPV23 KX098515
BPV3 NC 004197
BPV10 AB331651
BPV6 AJ620208
BPV12 JF834523
BPV20 KU519395
RtPV2 NC 021930
CePV2 KT932712
BPV17 KU519392
Xipapillomavirus 1
Xipapillomavirus 2
Xipapillomavirus 3
Xipapillomavirus
PphPV1 GU117621
PphPV2 GU117622
DdPV1 GU117620
ChPV1 NC 008032
RtPV1 KP757765
CgPV2 NC 015692
HPV8 M12737
BpPV1 NC 014143
EePV1 NC 011765
BPV7 NC 007612
AsPV1 HQ625440
CPV2 NC 006564
VvPV1 KF857586
HPV4 X70827
BPV21 KU519396
BPV19 KU519394
TmPV1 NC 006563
MsPV1 NC 016013
MscPV1 JQ814848
EsPV2 KC858264
FcaPV2 EU796884
UmPV1 NC 010739
SsPV1 NC 011280
PphPV4 GU117623
MrPV1 JQ814847
HPV18 X05015
HPV16 K02718
CgPV1 GU014532
AgPV1 KP861980
SscPV1 NC 023496
RrPV1 NC 023895
OaPV3 FJ796965
BPV16 KU519391
BPV22 KY705374
Dyokappapapillomavirus
BPV18 KU519393
PpuPV1 KT932713
MnPV1 NC 001695
CcanPV1 NC 023178
OcPV1 NC 002232
HPV1 V01116
MscPV2 JQ692938
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CPV1 NC 001619
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EsPV1 KC858263
EhPV1 JX123128
EdPV1 NC 006951
HPV41 X56147
RaPV1 NC 008298
FgPV1 NC 024300
CoPV1 NC 011530
FcPV1 NC 004068
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PaPV1 NC 023894
FlPV1 NC013117
EcPV2 NC 012123
CPV3 NC 008297
ZcPV1 NC 015325
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EcPV1 NC 003748
EcPV8 KU983288
BPV8 DQ098913
BPV5 NC 004195
CePV1 KU350625
Epsilonpapillomavirus 1
Epsilonpapillomavirus
GcPV1 KX954132
BPV13 JQ798171
BPV2 M20219
BPV1 X02346
BgPV1 JX174437
BPV14 KP276343
Deltapapillomavirus 4
OaPV2 U83595
OaPV1 NC 001789
Deltapapillomavirus 3
RalPV1 KT626573
CcaPV1 NC 011051
Deltapapillomavirus 5
OvPV1 NC 001523
Deltapapillomavirus 2
RtPV1 AF443292
AaPV1 NC 001524
Deltapapillomavirus 1
Deltapapillomavirus
SaPV1 KX643372
Order/Families
Cetacea
Primate
Human primate
Marsupial
European hedgehog
Rodentia
Canidae
Sirenia
Squamata
Chiroptera
Felidae
Ursidae
Suidae
Lagomorpha
Eulipotyphla
Aves
Chelonii
Equidae
Caniformia
Perciformes
Unclassified species/Genus

Figure 8

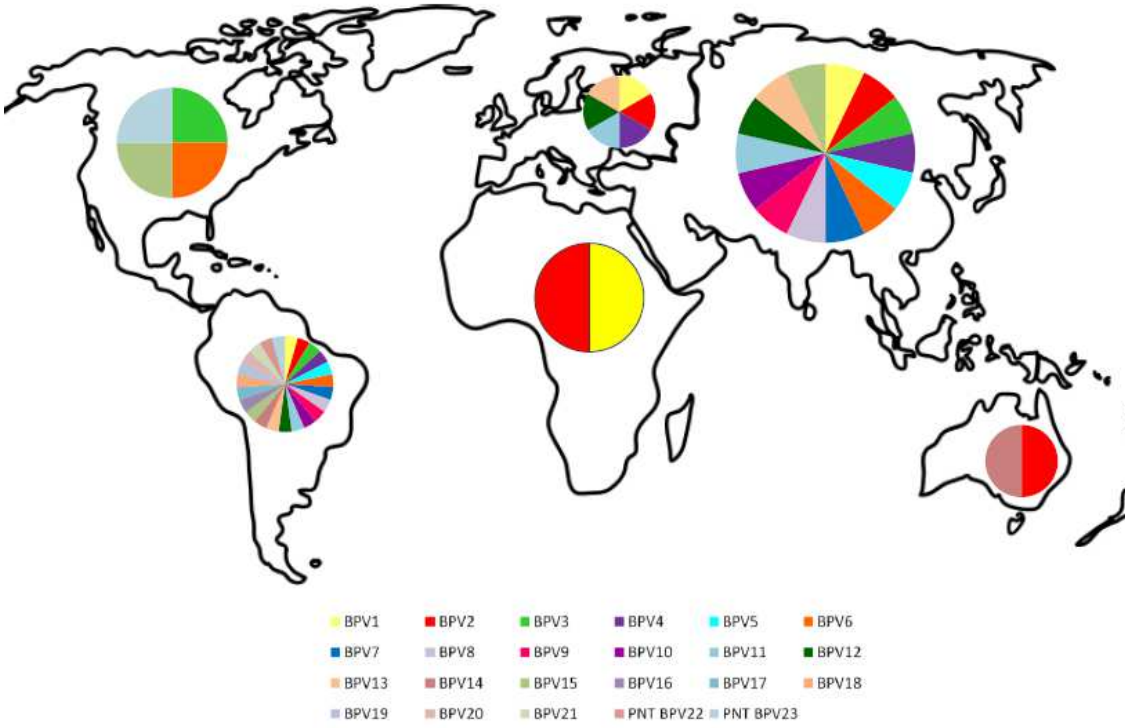
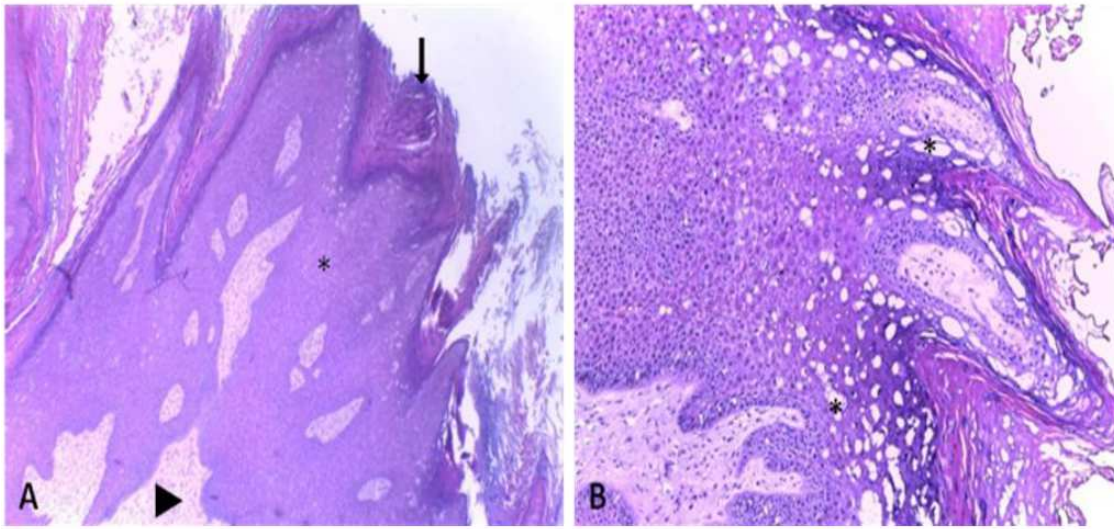
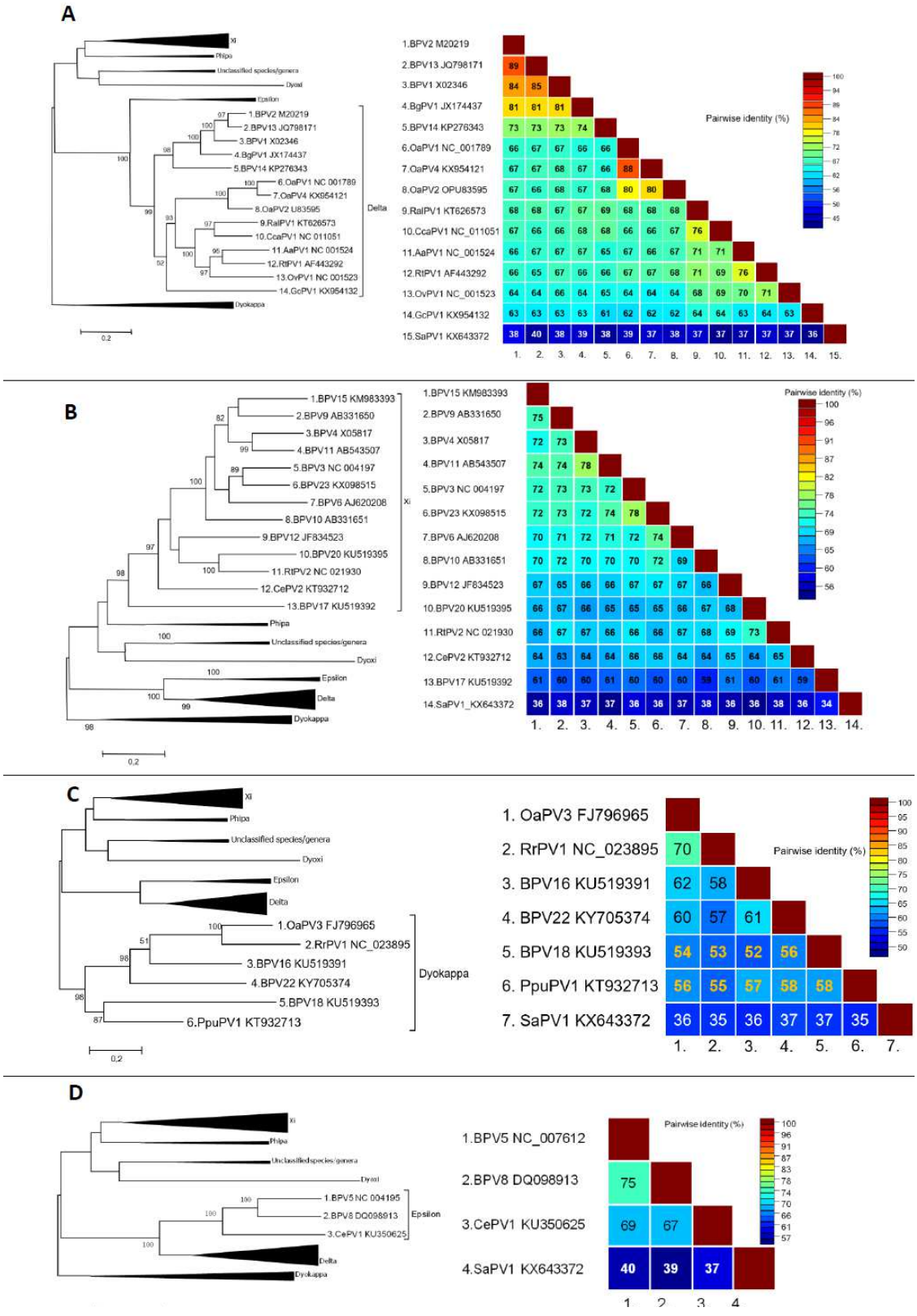

BPV1
BPV2
BPV3
BPV4
BPV5
BPV6
BPV7
BPV8
BPV9
BPV10
BPV11
BPV12
BPV13
BPV14
BPV15
BPV16
BPV17
BPV18
BPV19
BPV20
BPV21
PNT BPV22
PNT BPV23

Figure 9



Supplementary Figure 1



CONCLUSÕES

Capítulo 1

- O sequenciamento parcial do gene L1 de 39 amostras de lesões papilomatosas provenientes de bovinos da região Amazônica Brasileira, revelou a presença dos BPV 1, 2, 11, e 13, sendo o tipo 1 e o 2 os mais frequentes, além de 4 prováveis novos tipos virais, pertencentes aos gêneros *Xipapillomavírus, Epsilonpapilomavírus* e *Dyoxipapillomavírus*;
- A região Amazônica parece apresentar uma grande diversidade de tipos de BPV.

Capítulo 2

- O primeiro genoma completo de um BPV2 oriundo da região Amazônica foi sequenciado revelando um alto grau de identidade com outros genomas completos do mesmo tipo de BPV previamente reportados.

Capítulo 3

- A combinação da amplificação por círculo rolante seguida do sequenciamento de alto desempenho possibilitaram o sequenciamento de sete genomas completos de BPV, oriundos de uma amostra de papiloma de gado proveniente da região Amazônica.
- Estudos de coinfecção são fundamentais para a elucidação do papel dos diferentes tipos virais na biologia e patogênese das doenças em que eles estão envolvidos.
- O grande número de novos BPV encontrados coinfectando uma mesma lesão papilomatosa, juntamente ocom o BPV13, sugere a existência de muito outros tipos de BPV que podem ter sido subestimados até o momento.
- Seis novos tipos de BPV (BPV16 ao BPV21) foram descritos coinfectando uma mesma lesão papilomatosa juntamente com o BPV13.

Esta coinfecção massiva detectada indica que os bovinos podem abrigar uma diversidade genética de PV similar à descrita em humanos.

Capítulo 4

- O sequenciamento de alto desempenho de duas amostras papilomatosas de bovinos revelou um novo tipo de BPV, denominado BPV24 como possível agente etiológico de papilomas em bovinos. Ainda, a segunda amostra apresentou coinfecção relacionado com BPV dos gêneros Epsilon e Deltapapilomavírus.
- A identificação e caracterização de papilomavírus animais, assim como a detecção de coinfecção e infecções mistas são importantes para o entendimento das relações intra e interespecíficas desses virus.
- Este estudo indica que a região Amazônica provavelmente abriga uma diversidade única de BPV, assim como acontece com outras species, por ser uma floresta com uma das maiores biodiversidades no planeta.
- Aparentemente esta região pode abrigar uma grande diversidade de vírus, como acontece com as espécies animais e vegetais.

Capítulo 5

- O desenvolvimento do sequenciamento de alto desempenho possibilitou a descoberta de novos tipos de BPV que não foram amplificados utilizando a técnica de PCR com os pares de iniciadores degenerados FAP e MY.
- A análise dos PV de ruminantes mostrou similaridades interessantes entre os membros da espécie *Delta 4*, que podem estar relacionadas com o seu grande potencial oncogênico.
- Desta forma, pode pode-se dizer que os *Delta 4* podem ser classificados como vírus de alto risco ongogênico para *Bos taurus*.
- A predileção de BPV por partes anatômicas específicas ainda não está clara e, aparentemente, a maioria dos BPV já foi detectado em lesões cutâneas até o presente momento.

- Pesquisas envolvendo ruminantes selvagens ainda são esporádicas, porém seriam importantes para o melhor entendimento da biologia e das relações intra e interespecíficas dos PV, assim como para o melhor entendimento da evolução destes vírus.

Capítulo 6

- De 46 amostras analisadas, 76% delas pertenceram ao gênero Deltapapilomavírus (BPV1, 2 e 13), 9% das amostras mostraram a presença do BPV6 e 4% mostraram a presença de dois prováveis novos tipos que foram totalmente sequenciados e denominados XXXXX.
- Este estudo sugere que a diversidade de BPV no RS parece ser menor quando comparada com regiões de maior diversidade de outras espécies, como acontece na Região Amazônica Brasileira.

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ANEXOS

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

A seguir, serão apresentados os resumos dos artigos científicos publicados e/ou em vias para publicação.

Artigo 1

Experimento concluído e artigo publicado na revista *Plos One*.

Título: *Novel bovine papillomavirus type discovered by rolling-circle amplification coupled with next-generation sequencing*

PLOS | ONE

RESEARCH ARTICLE

Novel Bovine Papillomavirus Type Discovered by Rolling-Circle Amplification Coupled with Next-Generation Sequencing

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Abstract

Currently, fifteen bovine papillomavirus (BPV) types have been identified and classified into four genera: *Deltapapillomavirus*, *Epsilonpapillomavirus*, *Dyoxipapillomavirus*, and *Xipapillomavirus*. Here, the complete genome sequence of a new BPV type (BPV 04AC14) recovered from a papillomatous lesion is reported. The genome is 7,282 bp in length and exhibits the classic genetic organization and motifs of the members of *Papillomaviridae*. Maximum likelihood phylogenetic analyses revealed that BPV 04AC14 clusters with members of the *Xipapillomavirus* genus. The nucleotide sequence of the L1 capsid protein of the novel BPV is closely related to its counterpart, BPV3, with which it shares 79% similarity. These findings suggest that this virus is a new BPV type of the *Xipapillomavirus* genus.

Introduction

Papillomaviruses (PVs) are small viruses whose genomes consist of double-stranded DNA molecules of approximately 8 kb; PVs are widely distributed and probably infect all amniotes [1]. Most PVs are part of the skin microbiota; however, in some cases, infections by certain types manifest in distinct clinical presentations, from highly productive, self-limited warts to invasive cancers [2]. In cattle, bovine papillomavirus (BPV) infections are probably primarily asymptomatic, although on occasion, certain BPV types can induce skin warts or neoplasias in the mucosa of the urinary bladder and upper digestive tract [3,4].

PVs are classified in the *Papillomaviridae* family and subdivided into 39 genera and several species, types, subtypes and variants. This discrimination is based on the degree of nucleotide sequence diversity of the L1 gene [5–7]. Currently, fifteen BPVs have been reported, in contrast

Artigo 2

Experimento concluído e artigo publicado na revista *Virus Genes*.

Título: *Genome characterization of a bovine papillomavirus type 5 from cattle in the Amazon region, Brazil*

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Genome characterization of a bovine papillomavirus type 5 from cattle in the Amazon region, Brazil

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Abstract Papillomaviruses are small and complex viruses with circular DNA genome that belongs to the *Papillomavirus* family, which comprises at least 39 genera. The bovine papillomavirus (BPV) causes an infectious disease that is characterized by chronic and proliferative benign tumors that affect cattle worldwide. In the present work, the full genome sequence of BPV type 5, an *Epsilonpapillomavirus,* is reported. The genome was recovered from papillomatous lesions excised from cattle raised in the Amazon region, Northern Brazil. The genome comprises 7836 base pairs and exhibits the archetypal organization of the *Papillomaviridae*. This is of significance for the study of BPV biology, since currently available full BPV genome sequences are scarce. The availability of genomic information of BPVs can provide better understanding of the differences in genetics and biology of papillomaviruses.

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Keywords *Papillomaviridae* · *Epsilonpapillomavirus* · BPV5 · Complete genome · Phylogeny

Introduction

Viruses from the *Papillomaviridae* family infect epithelia in amniotes and are associated with asymptomatic infections, proliferative benign lesions, and different cancers in humans and other animals [1]. Papillomaviruses (PVs) have circular, double-stranded DNA genomes of ~8 kb in length. The organization of PV genomes consists of the early and the late regions and the noncoding region between them. Viral regulatory proteins are found in the early region, which are necessary for the initiation of virus replication. Moreover, the early region contains E6 and E7 viral proteins that present oncogenic properties [2]. The late region contains the capsid protein genes (L1 and L2 genes) [3].

Bovine papillomaviruses (BPVs) are now classified into four genera (*Xi*, *Delta*, *Epsilon,* and *Dyoxipapillomavirus*), five species, and 15 types (http://pave.niaid.nih.gov), based on the degree of nucleotide similarity of the major capsid gene (L1). The *Xipapillomavirus* genus comprises two species, *Xipapillomavirus 1* (BPV3, 4, 6, 9, 10, and 11) and *2* (BPV12). The *Deltapapillomavirus* genus comprises a single species, *Deltapapillomavirus 4* (BPV1, 2, and 13) as well the *Epsilonpapillomavirus* genus which is composed of *Epsilonpapillomavirus 1* (BPV5 and 8). The *Dyoxipapillomavirus* genus comprises single representative species, *Dyoxipapillomavirus* 1 (BPV7). BPV types 14 and 15 are new types assigned into *Delta* and *Xipapillomavirus* genera, respectively; the classification is pending approval by the International Committee on Taxonomy of Viruses (ICTV).

Experimento concluído e artigo a ser enviado para publicação na revista *Virus Genes*.

Título*: Bovine papillomavirus co-infection: Delta, Xi, Dyokappapapillomavirus and an unasigned genus described in a bovine papillomatous lesion*

Bovine papillomavirus co-infection: Delta, Xi, Dyokappapapillomavirus and an unasigned genus described in a bovine papillomatous lesion

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Abstract

Papillomaviruses (PVs) are small circular double-stranded DNA viruses that belong to the *Papillomaviridae* family. Currently, 22 bovine papillomavirus (BPV) types have been fully characterized. BPV co-infections are rarely reported, contrasting to human papillomaviruses (HPV), which are commonly identified in young or immunodepressed women. In the present study, we described four distinct bovine papillomavirus (BPV) types in one papillomatous lesion. The genomes were detected by rolling circle amplification coupled to next generation sequencing (NGS) in a bovine (*Bos taurus*) lesion from the Brazilian Amazon region. It was identified the well characterized BPV13, together with the recently described BPV17, 18 and 20. The data presented herein reinforces that some new BPV types cannot be accessed by commonly applied PCR protocols and suggest that the knowledge of BPV co-infections can be underestimated due to the limitation of detection techniques.

Keywords: papillomavirus; bovine; co-infection; next generation sequencing.

Artigo 4

Experimento concluído e artigo a ser enviado para publicação na revista *Veterinary Pathology*.

Título: *Canine papillomavirus type 16 associated to squamous cell carcinoma in situ: pathological and virological findings*

Canine papillomavirus type 16 associated to squamous cell carcinoma *in situ*: pathological and virological findings

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Abstract

Papillomaviruses (PV) are circular double stranded DNA viruses belonging to *Papillomaviridae* family. In the infection cycle, PVs produce proteins that can influence cell growth and differentiation, leading to hyperplastic papilloma (warts) or neoplasia. *Canis familiaris* papillomavirus (CPVs) have been associated to different malignancy lesions such as oral and cutaneous papillomatosis, pigmented plates and squamous cell carcinomas *in situ* (SCC *in situ*). Here, we characterize a case of a bitch with uncommon papillomatous-like lesions induced by the CPV16 that progress to SCC *in situ*. In addition, we characterize pathologically lesions caused by this virus, using immunohistochemistry and *in situ* hybridization. *In situ* hybridization and immunohistochemistry for CPV revealed strong signals within the neoplastic tissue. The full genome of the CPV16 recovered directly from the papillomatous lesions was characterized and the phylogenetic relationship of this CPV was determined. These findings add to the expanding the knowledge about the association between CPV16 infection and oncogenesis in dogs.

Keywords: canine papillomavirus; CPV16; carcinoma; oncogenesis.

Anexo B: Projeto Bilateral Brasil-Alemanha

Estudo de eficácia pré-clínica no tratamento profilático contra o papilomavírus bovino utilizando vacinas baseadas na proteína L2

O projeto apresentado a seguir foi realizado em parceria com o Instituto de Pesquisas em Câncer da Alemanha (*German Cancer Research Center* – DKFZ) no período de maio a outubro de 2016 e financiado pelo Serviço Alemão de Intercâmbio Acadêmico (DAAD). As análises ainda estão em andamento.

Resumo

Os papilomavírus (PV) são vírus pequenos, pertencentes à família *Papillomaviridae* que podem causar tumores benignos ou malignos em seus hospedeiros naturais. O papilomavírus humano (HPV) é responsável por um grande número de mortes em mulheres acometidas pelo câncer cervical. Similarmente, o papilomavírus bovino (BPV) causa infecções em bovinos trazendo prejuízos à bovinocultura. O objetivo deste estudo foi analisar a resposta imune homóloga e heteróloga de bovinos conferida por uma vacina desenvolvida com a porção terminal da proteína L2 do capsídeo viral do BPV1, enxertada na tioredoxina com epítopos distintos de células T-*helper* e formulada com diferentes adjuvantes. Três grupos de quatro bezerros de diferentes idades (1,5; 4 e 8 meses) foram imunizados intramuscularmente e a resposta imune humoral dos bovinos foi analisada através do teste ELISA e do teste de neutralização baseado em pseudo-vírions. A imunização com as vacinas contendo epítopos de BPV1L2 foi realizada no Brasil e a detecção de anticorpos contra L2 e os testes de neutralização baseados em pseudo-vírions específicos foram realizadas no Laboratório de Estratégias Específicas de Vacinação contra Vírus Tumorigêncios, no Instituto de Pesquisas em Câncer da Alemanha (DKFZ). O DKFZ possui uma linha de pesquisa consolidada, com métodos, equipamentos, reagentes e conhecimentos necessários a realização de todas as análises e é um Instituto de Pesquisa distinto, o qual foi agraciado duas vezes com os Prêmios Nobel de Química e Medicina. Em 2008, o Prêmio Nobel de Medicina foi recebido pelo trabalho desenvolvido com o HPV, o qual elucidou que o HPV é um dos principais agentes etiológicos do câncer cervical em mulheres. Em 2014 o DKFZ foi honrado com o Nobel de Química, pelo trabalho pioneiro em microscopia fluorescente de alta resolução. Desta maneira, as estratégias que vem sendo pesquisadas no DKFZ, com intuito de aperfeiçoar a vacina contra o HPV e BPV, são de extrema relevância. Os resultados parciais deste estudo demonstram que os bovinos de quatro meses de idade foram os que melhor responderam ao tratamento profilático utilizando epítopos de BPV1L2. Este trabalho visa gerar conhecimento sobre a imunização contra o BPV em tratamento profilático, que servirá de base para estudos posteriores na formulação de uma vacina eficaz composta por HPV.

1 Introdução

Os papilomavírus (PVs) são vírus da família *Papillomaviridae* que infectam diferentes espécies de animais, como aves, répteis e mamíferos, incluindo o homem (BRAVO et al., 2010). Comumente, os PVs causam lesões hiper-proliferativas no epitélio e mucosas dos seus hospedeiros naturais, o que pode induzir tumores benignos ou malignos (JEMAL et al., 2011; FERLAY et al., 2013; BRAY et al., 2012).

O papilomavírus humano (HPV) pode induzir ao câncer cervical, que é o quarto câncer mais prevalente entre as mulheres, sendo a segunda maior causa de mortalidade feminina mundial atualmente (JEMAL et al., 2011; FERLAY et al., 2013; BRAY et al., 2012). Em bovinos, a infecção pelo vírus do papiloma bovino (BPV) pode também induzir lesões cutâneas ou neoplásicas de mucosa causando grandes perdas econômicas na bovinocultura (BORZACCHIELLO & ROPERTO, 2008).

Existem duas vacinas profiláticas comercialmente disponíveis para HPV atualmente, a Gardasil® (Merck Sharpe & Dohme) e a Cervarix® (GlaxoSmithKline, GSK). Tais vacinas são compostas pela proteína L1 agrupada em partículas semelhantes ao vírus (v*irus-like particles* - VLPs) e são altamente imunogênicas (KEAM et al., 2008; SIDDIQUI et al., 2006) para os tipos virais homólogos. Apesar das vacinas profiláticas contra o HPV serem capazes de induzir uma resposta humoral específica para a proteína L1 homóloga (HARPER et al., 2009), elas apresentam uma baixa proteção cruzada contra tipos virais heterólogos (BROWN et al., 2009; WHEELER et al., 2012). Resultados similares também são observados na vacinação profilática de bovinos com VLPs dos seus hospedeiros naturais (KIRNBAUER et al., 1996).

No entanto, vacinas que utilizam proteínas recombinantes induzem uma proteção homóloga e heteróloga contra os tipos virais de maior importância clínica em bovinos (LOWY et al., 2012). A proteína menor do capsídeo viral (L2) apresentou elevado grau de similaridade entre distintos tipos de HPV e, apesar de sua baixa imunogenicidade, a imunização de animais com este antígeno induziu uma elevada proteção heteróloga contra outros tipos virais oncogênicos (MÜLLER et al., 1997). Em estudo realizado em bovinos, uma vacina com formulação mista (BPV1L2, BPV6L2 e BPV8L2 com o adjuvante baseado em hidróxi-saponinas) não induziu uma resposta imune heteróloga eficiente contra os BPVs 1 e 8 (MÜLLER, 2014). Deste modo,

destaca-se a necessidade de investigação mais detalhada de alguns pontos como a idade mínima para indução de uma resposta imune protetora, adjuvante mais apropriado e epítopo que estimule células T *helper*.

Outros estudos vacinais, com intuito de tratamento terapêutico, que utilizaram partículas quiméricas semelhantes a vírus (CVLPs) têm demonstrado seu grande potencial para a imunoterapia de tumores associado aos PVs (MÜLLER et al.; 1997) na prevenção e tratamento experimental de tumores induzidos (GIROGLOU et al., 2001). As CVLPs compreendem nas proteínas L1 e E7 do BPV1 (MÜLLER et al., 1997) e estudos prévios demonstraram uma resposta parcial benéfica em equídeos, assim como não houve evidências de toxicidade relacionada com as doses (MATTIL-FRITZ et al., 2008).

Como ainda não existe uma vacina comercial eficaz destinada à profilaxia contra uma ampla gama de HPV e BPV, é de interesse de saúde humana e veterinária a utilização de bovinos como modelo de infecção natural para o teste de uma vacina contra BPV. Desta forma, o presente projeto visa gerar conhecimento sobre a imunização contra o BPV que deverá servir de base para estudos posteriores na formulação de uma vacina eficaz composta por HPV.

2 Revisão bibliográfica

Os papilomavírus (PVs) são um grande grupo de vírus pequenos e não envelopados, que pertencem à família *Papillomaviridae* (BERNARD et al., 2010). Eles infectam células escamosas da mucosa e epitélio de seus hospedeiros naturais e já foram descritos em várias espécies animais, inclusive no homem (BORZACCHIELLO & ROPERTO, 2008; BRAVO et al., 2010). PVs são considerados espécie-específicos, contudo, há evidências de contaminação entre diferentes espécies que normalmente resulta em uma infecção sem a produção de vírions (CAMPO, 2006; NASIR AND CAMPO, 2008).

Esses vírus são constituídos de DNA fita dupla contendo aproximadamente 8.000 pares de base (pb). Seu genoma é organizado em diferentes regiões conforme o padrão de expressão durante o ciclo infeccioso: região precoce (E), com cinco ou seis fases abertas de leitura (ORFs), região tardia, com duas ORFs (L1 e L2) e a região de

controle (LCR), onde se encontram sítios de ligação a fatores transcricionais virais e do hospedeiro, além da origem de replicação (HOWLEY & LOWY, 2001; BERNARD et al., 2010).

A infecção pelos PVs pode ser assintomática ou induzir lesões hiper-proliferativas na pele ou mucosa de seus hospedeiros naturais, que podem naturalmente regredir ou progredir para neoplasias (HOWLEY & LOWY, 2001). Em humanos os cânceres associados à infecção pelo HPV incluem desde lesões neoplásicas anogenitais até orofaríngeas, contribuindo com uma parcela significativa dos cânceres na população mundial (JEMAL et al., 2011; FERLAY et al., 2013; BRAY et al., 2012). Os BPVs podem persistir como infecção latente e serem reativados nos casos de traumas físicos ou de imunossupressão em bovinos, levando à necessidade de abate desses animais nos casos de persistência dos papilomas, de infecção amplamente espalhada pelo corpo do animal ou ainda a inabilidade de combate ao agente infeccioso (CAMPO et al., 1994), havendo casos em que esses vírus podem atingir até 70% dos animais do rebanho (ROSENBERGER, 1989; RADOSTIS et al., 2007).

De acordo com seu potencial oncogênico, os tipos virais são comumente subdivididos em vírus de "baixo risco" (*low-risk* - LR) ou de "alto risco" (*hight-risk* - HR), sendo 15 tipos de HPVs considerados responsáveis por 99% dos cânceres cervicais em humanos (MUÑOZ et al., 2003) e três tipos de BPVs responsáveis pela maioria dos cânceres de bexiga urinária e trato digestório superior frequentemente relatados em bovinos (CAMPO et al., 2002; BORZACCHIELLO et al., 2001).

O câncer cervical é o quarto câncer mais prevalente entre as mulheres e, de acordo com a Organização Mundial da Saúde, é atualmente a segunda maior causa de mortalidade feminina mundial (JEMAL et al., 2011; FERLAY et al., 2013; BRAY et al., 2012). Já em bovinos, os BPVs 1 e 2 são vírus de grande relevância pois têm a capacidade de infectar células epiteliais e fibroblastos, causando fibropapilomas na pele, mamilos e úbere assim como câncer de bexiga urinária (NASIR & CAMPO, 2008; HOPKINS, 1986). Adicionalmente, eles representam a maioria dos casos de infecção entre espécies, infectando búfalos, equinos e bisões (NASIR & CAMPO, 2008; CAMPO, 2006) e a imunoprofilaxia destes vírus, que estão envolvidos na etiologia de tumores e constituem um problema sanitário de grande importância econômica para

regiões de pecuária bovina no mundo inteiro (HOPKINS, 1986), é de grande importância.

Como tratamento contra o HPV, existem duas vacinas profiláticas comercialmente disponíveis, a Gardasil® (Merck Sharpe & Dohme) e a Cervarix® (GlaxoSmithKline, GSK), que são compostas pela proteína L1, a maior proteína do capsídeo viral, que se agrupa em partículas semelhantes ao vírus (v*irus-like particles* - VLPs) e são altamente imunogênicas (KEAM et al., 2008; SIDDIQUI et al., 2006). Essas vacinas para humanos são baseadas nos dois tipos de HPV mais oncogênicos (HPV16 e HPV18), embora a vacina Gardasil® também contenha as VLPs dos HPVs 6 e 11, que são vírus responsáveis por cerca de 90% das verrugas genitais. Além disso, elas também diferem na composição de seus adjuvantes [aluminum hydroxiphophate sulfate para Gardasil® e AS04 (3-O-deacylated-44-monophosphoryl lipid-A Alum formulation) para Cervarix®] e nos seus sistemas de produção (células de fungo para Gardasil® e células de inseto para Cervarix®) (KEAM et al., 2008; SIDDIQUI et al., 2006). Apesar das vacinas profiláticas contra o HPV serem capazes de induzir uma resposta humoral específica para a proteína L1 homóloga (HARPER et al., 2009) elas apresentam uma baixa proteção cruzada contra outros tipos virais oncogênicos heterólogos (BROWN et al., 2009; WHEELER et al., 2012).

De maneira similar, a vacinação profilática de bovinos com VLPs ou com vírus purificados induz uma alta proteção somente ao tipo viral homólogo e não confere nenhum efeito terapêutico em tumores estabelecidos (JARRETT et al., 1990; KIRNBAUER et al., 1996). Além disso, as vacinas compostas de VLPs apresentam limitações devido ao custo e restrições em relação à replicação viral em sistemas de cultivo celular eucariótico (MÜLLER, 2014). Por outro lado, a proteína menor do capsídeo viral (L2) produzida em sistema procarioto se mostrou eficaz em tratamentos profiláticos e terapêuticos em bovinos, e a massiva infiltração de linfócitos sugere que este peptídeo contenha epítopos específicos que estimulem células T (JARRETT et al., 1991). Adicionalmente, vacinas que utilizam proteínas recombinantes induzem proteção homóloga e heteróloga contra os tipos virais de maior importância clínica em bovinos (LOWY et al., 2012) e, como no tratamento das infecções por papilomavírus muitas terapias são ineficazes, é preciso melhorar o potencial da intervenção imunológica nas infecções por papilomavírus, tanto profilática quanto terapêutica, em humanos e animais

(NICHOLLS AND STANLEY, 2000). No Brasil, a vacina autógena tem sido utilizada como tratamento terapêutico para animais extensamente atingidos por papilomatose e apresenta resultados de, aproximadamente, 50% de recuperação. No entanto este tratamento, além de não ser muito eficaz, possui custo elevado (SILVA et al., 2004).

Dentre as estratégias testadas para o HPV, a utilização da proteína L2 como antígeno vacinal apresentou-se promissora devido ao elevado grau de similaridade entre esta proteína em distintos tipos de HPVs (MÜLLER et al., 1997) e, apesar da baixa imunogenicidade da proteína L2, a imunização de animais com este antígeno induziu uma elevada proteção heteróloga (JARRETT et al., 1991; LOWY et al., 2012).

Estudos posteriores *in vitro* e *in vivo*, demonstraram que a porção terminal da proteína L2 induz uma resposta imune heteróloga contra um grande número de PVs, incluindo o BPV1 (RUBIO et al., 2011). A maioria dos estudos sobre PV, a indução de tumores malignos e o papel dos oncogenes foram primeiramente reconhecidos utilizando o BPV e o bovino como modelos de estudo para depois serem avaliados e validados para o HPV (CORTEGGIO et al., 2013). Consequentemente, o BPV é o modelo animal mais indicado para o teste vacinal, pois ele vem contribuindo de maneira significativa para a elucidação da transformação celular em tumores de ocorrência natural em bovinos assim como para o estudo do papel dos genes precoces na transformação celular (BORZACCHIELLO et al., 2009). Deste modo, uma vacina contra o BPV foi adaptada para teste em camundongos e, posteriormente, em bovinos (MÜLLER, 2014).

Vacinas baseadas em peptídeos sintéticos, embora mais seguras, são menos imunogênicas quando comparadas às vacinas tradicionais (AUCOUTURIER et al., 2001). Desta forma é fundamental o uso de adjuvantes mais potentes, que tenham efeito significativo na resposta imune, modulando-a de acordo com o tipo de resposta necessária, para assim produzirem uma proteção adequada contra diferentes antígenos (MCNEELA & MILLS, 2001), e que ao mesmo tempo tenham baixa toxicidade e possuam baixos efeitos colaterais (AGUILAR & RODRIGUEZ, 2007). Os sais de alumínio são estudados há muito tempo e suscitam uma resposta inflamatória do tipo II que é caracterizada pelo acúmulo de eosinófilos no tecido adjacente à aplicação da vacina (WALLS, 1997), possuem segurança comprovada, inclusive para uso humano (SUN et al., 2009). No entanto, não induzem uma resposta satisfatória de anticorpos

contra subunidades proteicas e é um adjuvante considerado fraco no que diz respeito à indução de resposta imune mediada por células (RELYVELD et al., 1998). Por outro lado, as saponinas estimulam a imunidade celular e humoral assim como a geração de diferentes isotipos de anticorpos (CAMPBELL & PEERBAYE, 1992; KENSIL, 1996) e são utilizadas em vacinas contendo peptídeos sintéticos contra doenças virais em animais domésticos (MARCIANI et al., 1991). As atividades da saponina extraída da *Quillaja saponaria* Molina (Quil A) como adjuvante têm sido comprovadas em pesquisas vacinais veterinárias e humanas (XIAO et al., 2007; Sun et al., 2009) e são candidatas para comporem as vacinas de nova geração para imunoterapia de tumores em combinação com outros adjuvantes (RAGUPATHI et al., 2005). Adicionalmente, a combinação do Quil A com o hidróxido de alumínio como adjuvante é utilizada em uma série de vacinas contra infecção viral na imunização de animais domésticos em diversas vacinas (DALSGAARD et al., 1990; BAR et al., 1998; AUCOUTURIER, 2001; SUN et al., 2009).

Desta forma, como ainda não existe uma vacina comercial eficaz destinada à profilaxia e também terapêutica contra uma ampla gama de HPV e BPV, é de interesse veterinário e de saúde humana a utilização de bovinos como modelo de infecção natural para o teste de uma vacina contra BPV. O presente projeto visa gerar conhecimento sobre a imunidade contra o epítopos do BPV e, assim, servir de base para estudos posteriores na formulação de uma vacina eficaz contra o HPV.

3 Objetivos

3.1 Objetivo geral

Os objetivos deste estudo são analisar as respostas imunes das vacinas contra o BPV utilizando a proteína L2 do capsídeo viral de diferentes epítopos sintéticos estimulantes de células T *helper* e formulada com diferentes adjuvantes.

3.2 Objetivos específicos

- Determinar a melhor idade mínima dos bezerros na indução de uma resposta imune desejável ao antígeno BPV1L2;

- Comparar os diferentes adjuvantes em relação è resposta imune induzida pela vacina BPV1 L2;
- Determinar se os epítopos estimulantes de células T *helper* podem alterar a resposta imune;

4 Material e métodos

4.1 Experimento 1

4.1.1 Imunização com vacinas contendo epítopos de BPVL2

Para determinar a melhor idade de vacinação contra o BPV, em um experimento inicial, três grupos de quatro bezerros de diferentes idades (1,5; 4 e 8 meses) foram imunizados intramuscularmente (Figura 1).

Cada grupo foi imunizado com quatro doses de 800 µg de epítopos de L2 de BPV1 (BPV1L2), utilizando como adjuvante a combinação de hidróxido de alumínio e saponina (QuilA) (4 mg/0.4 mg, respectivamente), conforme Tabela 1. O protocolo vacinal foi de duas semanas de intervalo entre cada dose (Figura 2). As coletas de sangue foram realizadas sempre antes da aplicação de cada dose das vacinas e no septuagésimo dia após a primeira dose. As imunizações com vacinas contendo epítopos de BPVL2 e as coletas sanguíneas foram realizadas em um estabelecimento de gado leiteiro no município de Viamão, Rio Grande do Sul, Brasil.

Tabela 1. Composição das vacinas utilizadas para cada grupo do experimento 1.

Grupo/ idade em meses	**Antígeno**	**Quantidade de antígeno/dose**	**Quantidade de adjuvantes (hidróxido de alumínio combinado + Quil A)/dose**	**Número de Animais por Grupo**
1,5	BPV1L2	800 µg	4 mg/0.4 mg	5
4	BPV1L2	800 µg	4 mg/0.4 mg	4
8	BPV1L2	800 µg	4 mg/0.4 mg	4

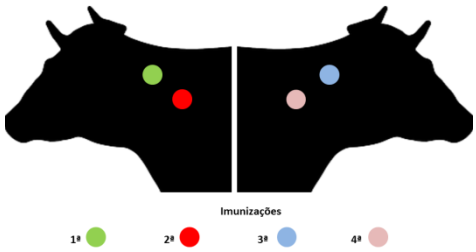

Figura 1: Representação esquemática dos diferentes locais de imunização intramuscular.

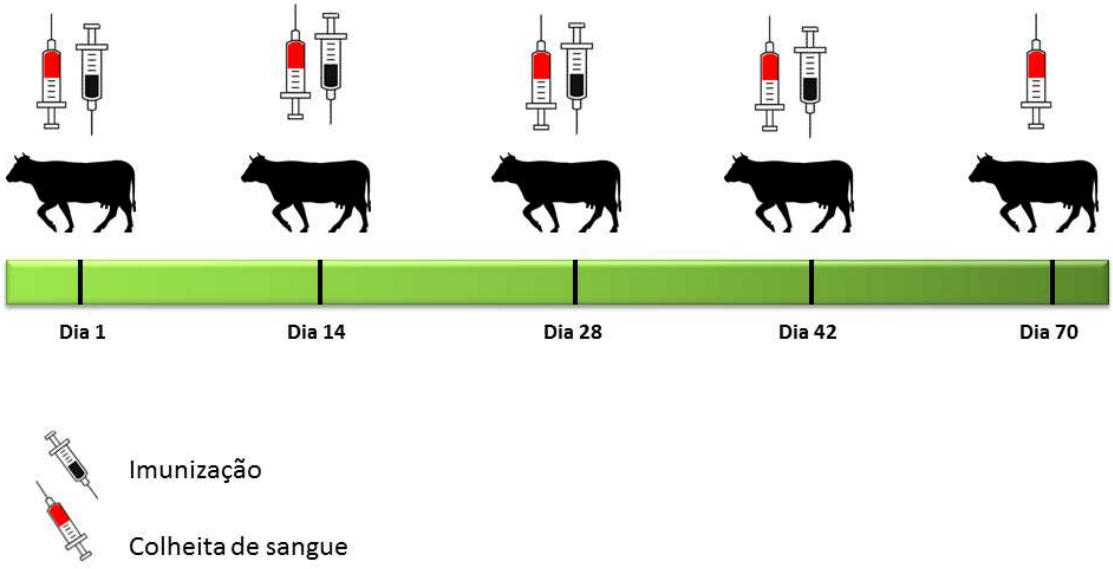

Figura 2: Representação esquemática do programa de imunização e colheita de sangue.

4.1.2 Amostragem sanguínea

Amostras de sangue de cada animal foram coletadas por punção na veia jugular nos dias 1, 14, 28, 42 e 70, armazenadas em tubos de coleta contendo EDTA para análise hematológica. Para obtenção do soro, amostras de sangue foram armazenadas em tubos de coleta simples, à temperatura ambiente por uma hora e em seguida centrifugados a 4000 x g por 10 min a 4 °C. Após o soro foi acondicionado em novos tubos a uma temperatura de -20 °C para a realização da análise imunológica.

4.1.3 Detecção de anticorpos anti-L2

O método de ELISA foi utilizado para testar o soro quanto à presença de anticorpos específicos anti-L2 de acordo com (MÜLLER, 2014). As placas de microtitulação revestidas com 50 µL de tampão de revestimento foram incubadas *overnight* a 37 ˚C. Após, foi adicionado tampão de bloqueio e as placas foram incubadas durante 1 hora à temperatura ambiente. As placas foram lavadas por três vezes com PBS-T. Após, 50 µL de peptídeo específico L2, diluído em tampão de bloqueio a uma concentração final de 1 mg/mL, foi adicionado a cada poço da placa, as quais foram incubadas durante 1 hora à temperatura ambiente. As placas foram lavadas e 50 µL de soro diluído a 1:200 em tampão de bloqueio foi adicionado às placas e incubado durante 1 hora à temperatura ambiente. As placas foram lavadas e 50µL de um conjugado com peroxidase e IgG de rato (Gampo-Dianova) diluído em PBS-T a 1:5000 foi adicionado. Após 1 hora a 37 °C a etapa de lavagem foi repetida e 100 µL de solução de substrato foram adicionados a cada poço. A reação foi realizada durante 10-20 minutos e a absorbância medida em um leitor de ELISA.

4.1.4 Teste de neutralização baseada em pseudovírions

Pseudo-vírions contendo um plasmídeo com marcador genético (*Gaussia luciferase*), capazes de infectar células HeLaT K4 e expressar o gene marcador, foram incubados com soro contendo anticorpos antes de serem inoculados nas células. Os soros e os pseudovírions foram diluídos em 50 µL de DMEM, e adicionados a cada poço da placa. A placa foi incubada à temperatura ambiente durante 20 min antes da adição de 50 µL de suspensão de células HeLaT K4 em cada poço (2,5 x 10^5 células/mL). Em seguida as placas foram incubadas à 37 ˚C por 48 horas (MÜLLER, 2014).

4.2 Experimento 2

Determinar entre os diferentes epítopos estimulantes de células T *helper* e adjuvantes testados, o que melhor induz resposta imune ao BPV1L2. De acordo com os

resultados do primeiro estudo, serão selecionados animais com idade condizente à melhor resposta imune à vacina profilática. Serão utilizados dez grupos de três a cinco animais cada. Cada antígeno (BPV1L2, P25/BPV1L2, PADRE/BPV1L2, CS.T3/BPV1L2 e DEQTGLPIKS/BPV1L2) será testado com dois adjuvantes diferentes (Alum + QuilA e Óleo de parafina) (Tabela 2). O protocolo vacinal, as colheitas sanguíneas e os testes sorológicos serão realizados conforme métodos utilizados no experimento 1.

Tabela 2. Composição das vacinas utilizadas para cada grupo do experimento 2.

Grupo/ Antígenos	Dose da vacina	Dose do ajuvante	Número de animais por grupo
BPV1L2	800 µg	Alum + Quil A (4 mg/ 0.4 mg)	4
P25-BPV1L2	800 µg	Alum + Quil A (4 mg/ 0.4 mg)	4
PADRE-BPV1L2	800 µg	Alum + Quil A (4 mg/ 0.4 mg)	4
CS.T3-BPV1L2	800 µg	Alum + Quil A (4 mg/ 0.4 mg)	4
DEQTGLPIKS –BPV1L2	800 µg	Alum + Quil A (4 mg/ 0.4 mg)	4
BPV1L2	800 µg	Paraffin oil (18.2 mg)	4
P25-BPV1L2	800 µg	Paraffin oil (18.2 mg)	4
PADRE-BPV1L2	800 µg	Paraffin oil (18.2 mg)	4
CS.T3-BPV1L2	800 µg	Paraffin oil (18.2 mg)	4
DEQTGLPIKS –BPV1L2	800 µg	Paraffin oil (18.2 mg)	4

5 Resultados Parciais

Quatro bezerros de diferentes idades (1,5; 4 e 8 meses) foram imunizados intramuscularmente com quatro doses contendo 800 µg de antígenos de BPV1L2,

utilizando como adjuvante a combinação de hidróxido de alumínio e saponina (QuilA) (4 mg/0.4 mg a acada dose) (Tabela 1), obedecendo o protocolo vacinal (Figura 2). As coletas de sangue foram realizadas sempre antes da aplicação de cada dose das vacinas e no septuagésimo dia e enviados para análise hematológica. O soro foi armazenado a uma temperatura de -20 ^{o}C para a realização da análise imunológica.

Os resultados obtidos neste estudo ainda estão sendo analisados e alguns testes ainda estão em andamento. No entanto, a análise dos resultados parciais do experimento 1mostra que os animais com idade de 4 meses foram os que melhor responderam ao tratamento profilático com os epítopos do BPV1L2.

6 Perspectivas

O presente projeto pretende gerar conhecimento sobre a imunidade contra os epítopos do BPV, e, futuramente, associados a diferentes epítopos de células T e a distintos adjuvantes. Desta forma, os resultados gerados neste doutorado sanduíche poderão servir de base para estudos posteriores na formulação de uma vacina eficaz, de ampla proteção e com custos reduzidos contra o HPV, contribuindo também para o possível desenvolvimento de uma vacina eficaz contra o BPV. Desta forma, existem boas perspectivas quanto a publicação dos resultados desses estudos em revistas especializadas em vacinologia internacionalmente renomadas.

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