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

Análise genética de papilomavírus bovino da região Norte do Brasil

FLAVIO ROBERTO CHAVES DA SILVA

Porto Alegre
2017

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Flavio Roberto Chaves da Silva

Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias, junto à Faculdade de Medicina Veterinária da Universidade Federal do Rio Grande do Sul.
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Análise genética de papilomavírus bovino na região Norte do Brasil

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RESUMO

Os papilomavírus (PV) são vírus epiteliotrópicos e constituem um grande grupo geneticamente diverso da família *Papillomaviridae* que infecta diferentes espécies de mamíferos, répteis e aves. Estes vírus caracterizam-se por sua propriedade oncogênica, sendo responsáveis pela indução de tumores benignos e malignos nos epitélios cutâneo e mucoso de suas espécies hospedeiras. Nos bovinos, a papilomatose causa importantes prejuízos econômicos à pecuária de corte e leiteira, dentre as quais destacam-se as infecções secundárias, a dificuldade de amamentação do bezerro, a depreciação do couro e o descarte precoce de animais e, ainda, no caso de rebanhos de aptidão leiteira, a papilomatose mamária ocasiona dificuldade de ordenha, retenção de leite e predispõe à mastite. Independente do nível de tecnificação da exploração na pecuária, estes agentes podem ser encontrados em quase todos os países. No entanto, poucos tipos de papilomavírus bovinos (BPV) foram caracterizados até o presente momento. Desta forma, este trabalho teve como objetivo realizar um estudo de caracterização genética e patológica das infecções causadas pelo BPV na região norte do Brasil. No Capítulo 1, buscou-se avaliar a diversidade dos BPVs em lesões cutâneas de bovinos na região Amazônica brasileira utilizando o par de *primers* degenerados FAP (FAP59 e FAP64). Foram encontrados os BPVs tipo 1, 2, 11, e 13 além de quatro prováveis novos tipos virais que apresentaram baixa identidade com os BPVs 7, 8, 11 e 12. No Capítulo 2, o genoma de um novo tipo de papilomavírus bovino pertencente ao gênero *Xipapillomavirus,* espécie X*ipapillomavirus 1,* foi caracterizado aplicando a técnica de amplificação por círculo rolante seguida de sequenciamento de alta eficiência utilizando a plataforma Illumina. Este novo tipo apresentou maior similaridade com o BPV3 (79% de identidade a nível genômico) e preenche os requisitos para ser considerado um novo tipo de BPV. Histopatologicamente, a lesão causada por esse novo BPV foi caracterizada como papiloma exofítico, apresentando células bem diferenciadas, marcada acantose e paraqueratose. No Capítulo 3, foi realizado o sequenciamento de um genoma completo de BPV5 utilizando o mesmo protocolo do Capítulo 2. Este foi o primeiro genoma de BPV5 da América do Sul completamente sequenciado e caracterizado. Assim, os resultados obtidos nesta tese contribuem para o melhor conhecimento da variabilidade genética do BPV e mostra a importância da utilização de ferramentas moleculares na sua caracterização, principalmente em regiões carentes de estudos e que podem abrigar tipos desconhecidos.

Palavras-chave: BPV; PCR; identificação; sequenciamento; filogenia.

ABSTRACT

Papillomaviruses (PV) are epitheliotropic viruses that constitute a large genetically diverse group of the *Papillomaviridae* family which can infect different species of mammals, reptiles and birds. These viruses are characterized by their oncogenic properties and are responsible for the induction of benign and malignant tumors in cutaneous and mucosal epithelia. In cattle, papillomatosis causes significant economic losses to beef and dairy cattle characterized by secondary infections, calf breastfeeding difficulties, leather depreciation, early animal discarding, mammary papillomatosis, retention of milk and predisposes to mastitis. Regardless of the level of technification of livestock farming, these agents can be found in almost all countries. However, few types of bovine papillomavirus (BPV) have been characterized to date. Thereby, this work had as objective to carry out a study of genetic and pathological characterization of the infections caused by the BPV from Northern Brazil. In Chapter 1, we evaluated the genetic diversity of BPVs in bovine cutaneous lesions from Brazilian Amazon using the degenerate FAP pairs of primers (FAP59 and FAP64). BPV1, 2, 11, and 13 were found in addition to four probable new viral types that had low identity with BPVs 7, 8, 11 and 12. In Chapter 2, the whole genome of a new BPV type belonging to genus *Xipapillomavirus*, species Xipapillomavirus 1, was characterized applying the technique of rolling circle amplification followed by high-throughput sequencing using the Illumina platform. This new type showed greater similarity with BPV3 (79% identity at the genomic level) and fulfills the requirements to be considered a new BPV type. Histopathology was characterized by exophytic papilloma, presenting well differentiated cells, marked acanthosis and parakeratosis. In Chapter 3, a complete BPV5 genome was sequenced using the same protocol used in Chapter 2. This was the first fully sequenced and characterized BPV5 genome from South America. Thus, the results obtained in this thesis contribute to a better knowledge of the genetic variability of BPV and shows the importance of the use of molecular tools in their characterization, especially in regions that are lacking in studies and which may harbor unknown types.

Keywords*:* BPV; PCR; identification; sequencing; phylogeny.

LISTA DE ILUSTRAÇÕES

Figura 1: O genoma do papilomavírus e seu ciclo de vida.....15

Fifura 2: Reconstrução da árvore filogenética representando a disposição dos grupos de papilomavírus bovino.....18

Figura 3: Papilomas cutâneos típicos de cabeça, pescoço e teto.....20

Figura 4: Lesões histopatológicas da papilomatose bovina.....24

Figura 5: Distribuição geográfica dos BPVs bovinos.....27

LISTA DE TABELAS

Tabela 1: Esquema da classificação das espécies e tipos de papilomavírus bovino dentro dos respectivos gêneros.....17

Tabela 2: Distribuição dos diferentes tipos virais a associação com diferentes tipos de lesões em bovinos.....28-30

LISTA DE ABREVIATURAS E SIGLAS

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	*Equus caballus* papilomavírus
EDTA	Ácido etilenodiaminotetracético sal sódico
ELISA	*Enzyme-Linked Immunosorbent Assay*
EserPV	*Epitesicus serotimus* papilomavírus
HPV	Papillomavirus Humano
kg	Quilograma
L	*Late* (tardio)
LCR	*Long control region* (Região longa de controle)
MaPV	*Mesocricetus auratus papilomavírus*
mg	Miligrama
min	Minutos
mL	Mililitro
mM	Milimolar
MmiPV	*Micromys minutus* papilomavírus
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	Epistemiologia dos papilomavirus

pb	Pares de base
PBS	Solução salina tamponada com fosfato
PCR	*Polymerase Chain Reaction* (reação em cadeia da polimerase)
pmol	Picomol
PmPV	*Peromyscus* papilomavírus 1
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

SUMÁRIO

1 INTRODUÇÃO **12**

2 REVISÃO BIBLIOGRÁFICA **13**
2.1 Papilomavírus bovino 13
2.2 Organização genômica 13
2.3 Ciclo viral dos papilomavírus 14
2.4 Classificação dos papilomavírus bovino 15
2.5 Transmissão 18
2.6 Patogenia e sinais clínicos 19
2.7 Diagnóstico 23
2.8 Tratamento 24
2.9 Prognóstico e profilaxia 25
2.10 Epidemiologia 26

3 OBJETIVOS **31**
3.1 Objetivo geral 31
3.2 Objetivos específicos 31

4 ARTIGOS CIENTÍFICOS **32**
4.1 CAPÍTULO 1: Caracterização genética de papilomavírus bovino revela a existência de quatro prováveis novos tipos na região da Amazônia 33
4.2 CAPÍTULO 2: Descoberta de um novo tipo de BPV amplificado por círculo rolante seguido do sequenciamento de alto desempenho 42
4.3 CAPÍTULO 3: Sequenciamento de um genoma completo de BPV5 54

5 CONCLUSÕES **59**

6 PERSPECTIVAS **60**

7 REFERÊNCIAS **61**

8 ANEXOS **68**

1 INTRODUÇÃO

O agronegócio é um dos principais segmentos da economia nacional, situação que faz com que o País seja o maior exportador de carne bovina e o quarto maior produtor mundial de leite (USDA-FAS, 2017). A papilomatose bovina está amplamente distribuída em rebanhos de todo o mundo e é considerada endêmica no território brasileiro (DA SILVA et al., 2015; LUNARDI et al., 2016). Entre seus efeitos, destacam-se as infecções secundárias, a dificuldade de amamentação do bezerro, a depreciação do couro e o descarte precoce de animais e ainda, no caso de rebanhos de aptidão leiteira, a papilomatose mamária ocasiona dificuldade de ordenha, retenção de leite e predisposição a mastite (LUNARDI et al., 2013a; TOZATO et al., 2013). Nos bovinos de corte, os problemas estão associados a tumores de pele, neoplasias de bexiga urinária e do trato gastrointestinal superior (GIL DA COSTA et al., 2016). Os papilomavírus (PV) constituem um grande grupo de vírus epiteliotrópicos, altamente diversos e que podem induzir proliferações benignas e malignas no epitélio estratificado pavimentoso da pele e em mucosas (CAMPO, 1997a; ZUR HAUSEN, 2002). Uma grande variedade de tipos de PVs têm sido detectados em diferentes espécies de mamíferos e pássaros, incluindo a maioria dos animais domésticos e selvagens e, provavelmente, ocorrem na maioria dos vertebrados superiores (RECTOR et al., 2006).

As técnicas de biologia molecular permitem a identificação, a diferenciação e a quantificação viral, além de identificar variantes virais e novos tipos de papilomavírus (COBO, 2012). Com a difusão e maior facilidade de acesso às tecnologias de sequenciamento convencional e de última geração (HARISMENDY et al., 2009; SCHUSTER, 2008), estudos de epidemiologia molecular e caracterização de novas variantes virais podem atuar como importantes ferramentas no controle de enfermidades causadas por estes agentes infecciosos.

Uma estrutura eficiente para o diagnóstico e informações adequadas sobre a ocorrência da doença e caracterização dos agentes são fundamentais para o desenvolvimento de estratégias de prevenção e controle, beneficiando economicamente este setor produtivo. Visando isso, o presente projeto teve como objetivo realizar um estudo de caracterização genética e patológica das infecções causadas pelo papilomavírus bovino na região norte do Brasil, onde não existem dados disponíveis.

2 REVISÃO BIBLIOGRÁFICA

2.1 Papilomavírus bovino

Os papilomavírus bovinos (BPVs) são vírus pequenos com 55 a 60 nm de diâmetro que pertencem à família *Papillomaviridae* (BERNARD et al., 2010). Esses vírus são desprovidos de envelope lipoprotéico, apresentam simetria icosaédrica e a sua replicação ocorre no núcleo de células epiteliais pavimentosas. Os vírions dos PVs são constituídos por uma única molécula de DNA fita dupla e circular com aproximadamente 8000 pares de bases (pb) (BORZACCHIELLO; ROPERTO, 2008). O genoma está conjugado com histonas, formando um complexo semelhante à cromatina. O DNA está contido em um capsídeo composto de 72 capsômeros, sendo que sessenta 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 (DE VILLIERS et al., 2004).

2.2 Organização genômica

Três diferentes regiões compõem o genoma dos BPVs: a região longa de controle (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 frames* - ORF), correspondendo aos genes precoces (*early* - E) e tardios (*late* - L) (BORZACCHIELLO; ROPERTO, 2008). Todas as ORFs estão localizadas em uma das fitas do DNA viral, indicando que apenas uma fita é utilizada como molde para codificar as proteínas virais (BORZACCHIELLO; ROPERTO, 2008). As três regiões são separadas por dois sítios de poliadenilação (pA), o sítio da região precoce (pA_E) e o sítio da região tardia (pA_L) (ZHENG; BAKER, 2006).

Aproximadamente dez ORFs estão contidas na fita codificante e são classificadas em dois segmentos principais, conforme a fase de transcrição. O segmento E contém oito ORFs, chamadas de precoces ou iniciais (*early* – E), e o segmento L contém duas ORFs tardias (*late* – L). O segmento E contém aproximadamente 45% do genoma viral, codifica proteínas necessárias para as primeiras fases de replicação e transcrição viral. O segmento L representa aproximadamente 40% do genoma viral e codifica as proteínas do capsídeo (L1 e L2), que são produzidas nas fases tardias da replicação viral (HOWLEY; LOWY, 2001). A região longa de controle (LCR) é um segmento que contém aproximadamente 15% do genoma viral e é uma região não

codificante. Esta região contém aproximadamente 500-1000 nucleotídeos (nt) entre o 3' final da ORF tardia e o 5' final da ORF precoce, que contém todos os sinais regulatórios para replicação e transcrição do DNA viral (ROPERTO et al., 2008).

Os genes precoces (E) codificam as proteínas envolvidas tanto na replicação e transcrição do DNA quanto na transformação celular, já os genes tardios (L) codificam as proteínas do capsídeo viral. Os papilomavírus apresentam ORFs sobrepostas e aninhadas, compactando diversos genes em uma pequena extensão do genoma (BERNARD et al., 2010). A Figura 1A mostra um esquema da organização do genoma do BPV.

As principais proteínas para replicação do DNA viral são as proteínas E1 e E2, que são responsáveis pelos primeiros passos do inicio da transcrição e replicação (LAMBERT, 1991; 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). 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). Os PVs induzem tumores benignos que, eventualmente, quando fatores genéticos ou ambientais estão envolvidos, podem resultar em conversão maligna (CAMPO, 2006). Um achado recente sugere que a oncoproteína viral BPV E5 tem efeito similar à proteína humana HPV E5, induzindo neoplasias de bexiga urinária (CORTEGGIO et al., 2011). As oncoproteínas E5, E6 e E7, modulam o processo de transformação (MÜNGER; HOWLEY, 2002).

2.3 Ciclo viral dos papilomavírus

Um aspecto importante que caracteriza os PVs é a dificuldade de propagação *in vitro* em culturas celulares, uma vez que *in vivo* estes vírus se replicam, naturalmente, em epitélio pavimentoso estratificado (HOWLEY; LOWY, 2001).

A infecção pelo papilomavírus inicia-se com a adsorção dos vírions à superfície das células basais do epitélio. Embora a interação com a superfície celular dependa, principalmente da proteína L1, é provável que a L2 também auxilie nesta interação. O vírus provavelmente penetra por endocitose e é transportado pelo citoesqueleto em direção ao núcleo. Durante esta etapa ocorre a desestruturação e a perda do capsídeo viral. Utilizando os poros nucleares, o DNA viral entra no núcleo da célula hospedeira (ZUR HAUSEN, 2002). Os PVs se replicam no epitélio escamoso estratificado da pele

e mucosas e a diferenciação das células do epitélio está intimamente relacionada com o ciclo infeccioso (Figura 1B). A migração das células infectadas para as camadas suprabasais e a sua diferenciação ativam os genes tardios, resultando na formação do capsídeo viral e, consequentemente, do vírion (ZUR HAUSEN, 2002).

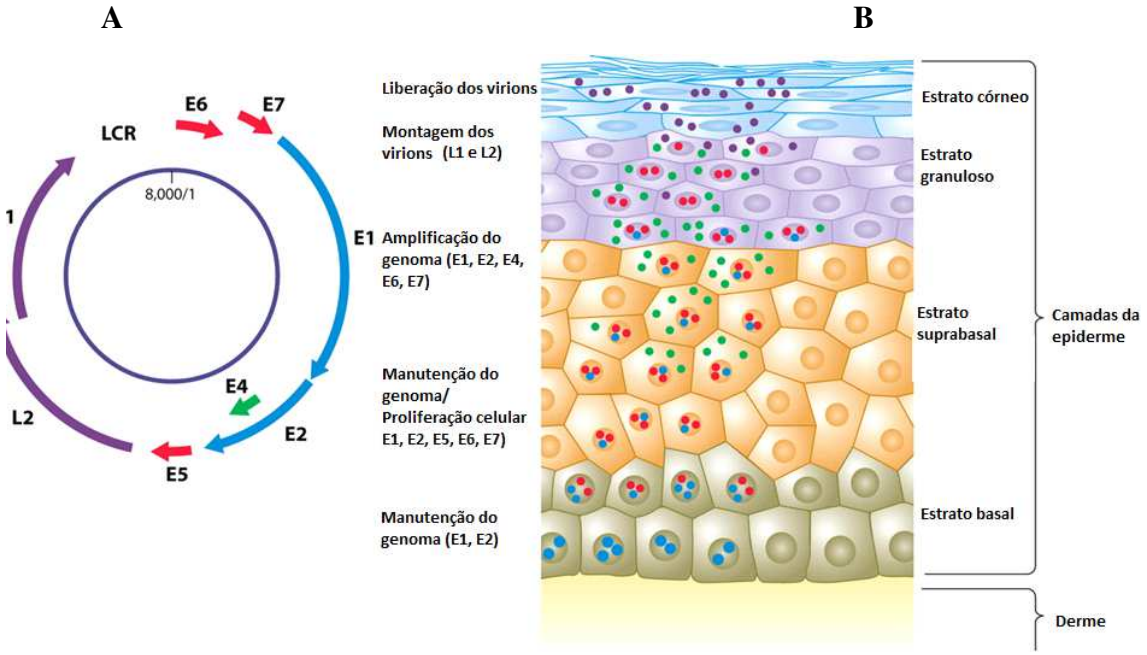

Figura 1. O genoma viral do papilomavírus e seu ciclo de vida. A) Representação do genoma viral circular demonstrando as posições dos genes virais E6, E7, E1, E2, E4, E5, L2, L1 e o LCR entre os genes L1 e E6. B) O ciclo de vida dos papilomavírus, seguindo a diferenciação do epitélio. Adaptado de Lazarczyk e colaboradores (2009).

2.4 Classificação dos papilomavírus bovino

Os critérios de classificação taxonômica dos tipos de PVs mudaram várias vezes em paralelo ao progresso das técnicas de biologia molecular e às análises filogenéticas. As diretrizes do Comitê de Nomenclatura dos Papilomavírus (VAN DOORSLAER et al., 2013) são seguidas na identificação de novos tipos de PVs, onde a sequência de nucleotídeos do gene L1 é utilizada para identificação de novos tipos de PVs por ser o gene mais conservado do genoma. Um papilomavírus é reconhecido como novo tipo se o seu genoma completo for totalmente sequenciado e a região codificante L1 diferir mais de 10% na identidade com os papilomavírus conhecidos. Diferenças de identidade entre 2% e 10% definem um subtipo e diferenças de identidade inferiores a 2% definem uma variante viral. Quando as sequências avaliadas são provenientes de produtos de PCR, representando apenas uma parte do gene L1, a estirpe viral é denominada como um provável novo tipo de PV (DE VILLIERS et al., 2004).

Os métodos recentes para a identificação de novos PVs são baseados na PCR empregando iniciadores (*primers*) genéricos. Estes *primers* são projetados a partir de sequências conservadas do gene que codifica a proteína L1 de tipos de PVs anteriormente caracterizados. Essa estratégia tem sido utilizada com sucesso na detecção de um amplo espectro de tipos virais já conhecidos e de novos tipos de PV, uma vez que a ORF L1 é a mais conservada no genoma do papilomavírus. Uma destas metodologias, envolvendo a utilização do par de *primers* genéricos FAP (FAP59 e FAP64), foi originalmente desenvolvida para a amplificação de HPVs cutâneos (FORSLUND et al., 1999). Porém, essa estratégia tem também possibilitado a detecção de novos tipos virais tanto em bovinos quanto em outras espécies animais (ANTONSSON; HANSSON, 2002; FORSLUND et al., 1999; LITERÁK et al., 2006; OGAWA et al., 2004).

A técnica de amplificação por círculo rolante tem sido utilizada com sucesso para amplificar genomas circulares de vários vírus (RECTOR; TACHEZY; RANST, 2004), utilizando a enzima phi 29. Seguindo de sequenciamento de alta eficiência que tem contribuído para a descoberta e caracterização de novos tipos de BPVs (DAUDT et al., 2016a; MUNDAY et al., 2015).

A família *Papillomaviridae* é composta por 39 gêneros que são subdivididos em espécies, tipos, subtipos e variantes (BERNARD et al., 2010; DE VILLIERS et al., 2004; VAN DOORSLAER et al., 2013).

Atualmente, de acordo com o Base de dados genômicos de Papilomavírus (PaVE), existem 21 tipos de BPV conhecidos, sendo que os BPVs 16 a 21 foram descritos por nosso grupo de pesquisa. Estes estão distribuidos em cinco gêneros: *Xipapillomavirus, Deltapapillomavirus, Epsilonpapillomavirus*, *Dyoxipapillomavirus e Dyokappapapillomavirus*. Além disso, os recentes tipos de BPVs 19 e 21 formam um novo gênero ainda não nomeado. Na classificação dos BPVs, o gênero *Xipapillomavirus* possui duas espécies: *Xipapillomavirus 1* e *Xipapillomavirus 2.* A espécie *Xipapillomavirus 1* é composta por sete tipos de BPVs: BPV3, BPV4, BPV6, BPV9, BP10, BPV11 e BPV15. A espécie *Xipapillomavirus* 2 compreende o BPV12. Ainda, os recentes descritos BPVs 17 e 20 pertencem ao gênero *Xipapillomavirus*. O gênero *Deltapapillomavirus* é composto por uma espécie, *Deltapapillomavirus 4*, compreendendo os tipos BPV1, BPV2, BPV 13 e BPV14.

O gênero *Epsilonpapillomavirus* contém uma espécie (*Epsilonpapillomavirus 1*), compreendendo os tipos BPV5 e BPV8. O gênero *Dyoxipapillomavirus* contém uma espécie (*Dyoxipapillomavirus 1*) e um tipo (BPV7). O BPV 16 e BPV18 pertencem ao gênero *Dyokappapapillomavirus* (Tabela 1). A Figura 2 mostra as relações filogenéticas dos tipos de BPV dentro dos gêneros da família *Papillomaviridae*.

Tabela 1. Esquema da classificação das espécies e tipos de papilomavírus bovino dentro dos respectivos gêneros.

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

*Representa os tipos ainda não classificados dentro de um gênero ou espécie.

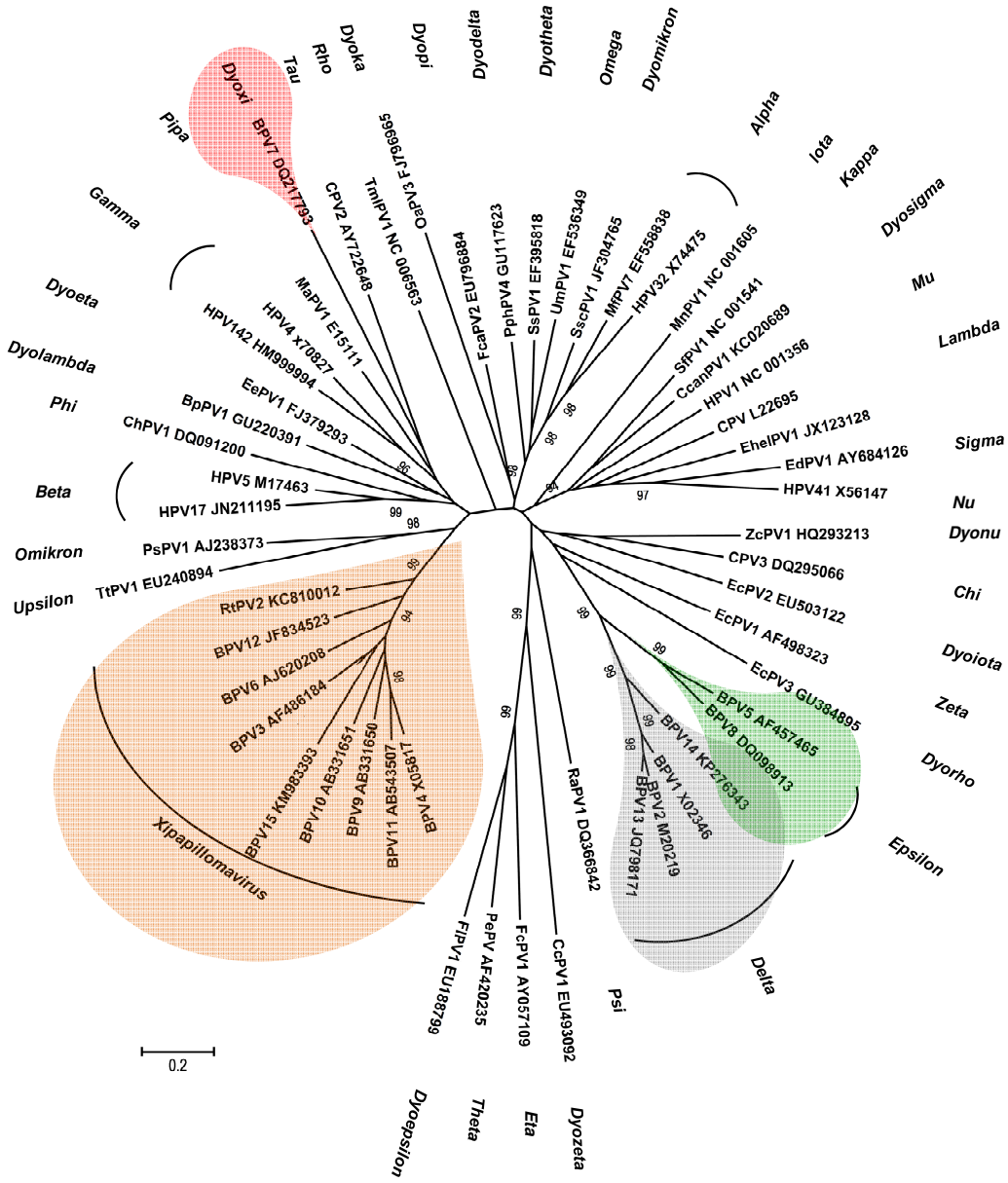

Figura 2. Reconstrução da árvore filogenética representando a disposição dos grupos de papilomavírus bovino. Um PV representativo de cada gênero da família *Papillomaviridae* e todos os tipos de papilomavírus bovinos existentes foram utilizados na construção da árvore filogenética. A árvore foi reconstruída pelo método de máxima verossimilhança no programa MEGA6. Fonte: próprio autor.

2.5 Transmissão

Em geral, os PVs são altamente espécie-específicos e a transmissão interespécie é considerada um evento raro, somente sendo observada entre espécies animais próximas, não havendo exemplos de um PV específico de uma espécie causando uma infecção produtiva em uma segunda espécie (CHAMBERS et al., 2003; MUNDAY et

al., 2015; NASIR; CAMPO, 2008). O caso mais frequentemente relatado de infecção heteróloga é a infecção de equídeos por Delta BPVs tipos 1, 2 e 13. Após uma infecção acidental da derme, os equídeos podem desenvolver tumores dermais benignos conhecidos como sarcóide equino (CAMPO, 2002a; LUNARDI et al., 2013b; NASIR; CAMPO, 2008).

A transmissão do vírus ocorre pelo simples contato direto ou indireto, por fômites e instalações e, possivelmente, insetos como moscas e carrapatos (CAMPO et al., 1994; FINLAY et al., 2009). Também pode ocorrer transmissão por monta natural, quando os animais apresentam papilomas genitais assim como através das técnicas de inseminação artificial (SILVA et al., 2011), transferência de embrião e de fertilização *in vitro* (DE CARVALHO et al., 2003).

A possibilidade de transmissão por técnicas não-naturais é evidenciada através da presença do BPV em amostras uterinas, ovarianas, fluidos e oócitos, oriundos de bovinos abatidos não afetados pela papilomatose cutânea e também a presença do BPV em amostras de sêmen usadas em programa de inseminação artificial, indicando que o vírus não infecta somente tecidos epiteliais (DE CARVALHO et al., 2003). Existem trabalhos que evidenciam que o BPV2 contamina a maioria dos bovinos e os linfócitos possam ser uma fonte de infecção (MUNDAY, 2014). Na forma mamária, a transmissão ocorre pela ordenha mecânica e pelas mãos dos ordenhadores que transportam o vírus e infectam a pele nas áreas de pele com abrasões (CAMPO, 2002).

A presença de tumores no trato digestório é sugerida pela interação entre os BPVs 2 e 4 associado a ingestão da samambaia *Pteridium aquilium*, propiciando o desenvolvimento de carcinomas devido à imunodepressão que limitaria as respostas imunológicas capazes de controlar tumores (CAMPO, 2006). Cogita-se que, no câncer de bexiga em bovinos, o BPV2 possa se disseminar no animal através de via hematogênica, fazendo com que o BPV2 possa atingir a bexiga urinária não somente pela área paragenital (ROPERTO et al., 2008).

2.6 Patogenia e sinais clínicos

A replicação viral ocorre nas camadas basais do epitélio escamoso estratificado da pele ou de membranas mucosas e, provavelmente, os vírus tem acesso às células do nível inferior através de lesões e/ou abrasões. O vírus atinge o núcleo das células basais através de microlacerações, sendo que os primeiros sinais de transcrição do genoma viral aparecem cerca de 4 semanas após a infecção (BUCK et al., 2005).

A infecção pelo PV nos queratinócitos basais e a expressão dos genes precoces provoca a transformação celular, induzindo hiperplasia e hipertrofia. Os genes precoces são expressos nas camadas basal e suprabasal do epitélio e, durante a diferenciação celular da camada espinhosa e granular, o vírus replica seu genoma (DOORBAR, 2005; LAZARCZYK et al., 2009; O'BRIEN; SAVERIA CAMPO, 2002). A diferenciação celular ativa a expressão dos genes tardios, que codificam as proteínas do capsídeo viral L1 e L2, e do gene E4, culminando na formação do vírion, que é liberado ao ambiente com a descamação natural da camada queratinizada (DOORBAR, 2005; LAZARCZYK et al., 2009; O'BRIEN; SAVERIA CAMPO, 2002).

2.6.1 Lesões cutâneas e de mucosas

Nos bovinos as lesões podem apresentar morfologias variadas e são classificados macroscopicamente em filiformes, na forma típica pedunculada e formas não típicas, planas (GRINDATTO et al., 2015a). A forma típica pedunculada apresenta aspecto verrugoso, sugerindo a forma de "couve-flor". Os papilomas são encontrados na cabeça, pescoço, dorso, ventre, abdome, úbere, mucosas dos tratos digestivo, genital, paragenital e também generalizados, sendo que a extensão e duração das lesões dependem do tipo de vírus, áreas afetadas e grau de suscetibilidade (Figura 3) (MERCK, 2001). Normalmente, essas lesões afetam um ou mais animais. Casos de papilomas generalizados indicam baixa resposta imunológica do animal (SMITH, 2006b). Nos bovinos, os papilomas de pênis, pele interdigital ou trato alimentar podem produzir sinais clínicos de dor ou de obstrução (MERCK, 2001).

A

B

Figura 3. Papilomas cutâneos típicos. A) Papiloma de cabeça e pescoço. B) Papiloma de teto. Fonte: próprio autor.

A doença, ainda, pode provocar o surgimento de mastite e dificuldades na ordenha, quando os papilomas encontram-se no úbere ou teto dos bovinos. Essas lesões constantes causam sangramentos e podem servir de porta de entrada para infecções

secundárias e, em casos extremos, esta enfermidade pode levar à morte do animal. As feridas e os sangramentos dos papilomas atraem moscas e, conseqüentemente, há o desenvolvimento de miíases que podem piorar o quadro clínico. Diante de todos esses problemas, se o bovino não responder bem ao tratamento, o produtor vê-se obrigado a descartar animais de alto valor zootécnico prematuramente (RADOSTITS et al., 2002).

Na maioria das vezes, os gêneros de BPV estão associados com diversas patogenias, de forma geral os *Xipapillomavirus* compreendem os PV puramente epiteliotrópricos (BPV3; BPV4 e BPV6); os *Deltapapillomavirus* compreendem vírus associados com fibropapilomas e papilomas cutâneos (BPV1, 2 e 13) e câncer de bexiga urinária (BPV1 e 2) (BORZACCHIELLO; ROPERTO, 2008; LUNARDI et al., 2013a); os *Epsilonpapillomavirus* compreendem o BPV5 e o BPV8, associados a papilomas cutâneos (CLAUS et al., 2009; DA SILVA et al., 2015, 2016; TOMITA et al., 2007) e o gênero *Dyoxipapillomavirus* tem sido encontrado em papilomas cutâneos, de teto e em pele saudável (OGAWA et al., 2007; TOZATO et al., 2013).

2.6.2 Hematúria enzoótica bovina (HEB)

Estudos demostram que algumas lesões benignas podem sofrer transformações malignas em respostas a agentes genéticos ou ambientais. Neste contexto a HEB e o tumor de trato gatrointestinal superior (TGS) em bovinos são doenças relacionadas à associação entre alguns tipos de BPVs e a ingestão da planta conhecida popularmente como samambaia (*Pteridium aquilinum*) e a alterações nutricionais, como a falta ou excesso de molibdênio no solo (WOSIACKI et al., 2002; CAMPO et al., 1992).

A *Pteridium aquilinum* é uma pteridófita pertencente à família *Dennstaedtiaceae*, cuja distribuição biogeográfica é observada em solos pobres, ácidos, com baixos níveis de cálcio e fósforo e umidade relativamente elevada (WOSIACKI et al., 2002). O *Pteridium aquilinum* possui compostos mutagênicos e imunodepressores que podem levar a neoplasia maligna de bexiga. Os principais compostos mutagênicos e imunodepressores são a prunasina, os ptaquilosídeos, o ácido xiquímico e a tiaminase (CAMPO, 1997b). A infecção pelo BPV2 e a ingestão crônica do *Pteridium aquilinum* são associadas com lesões vesiculares e apresentação clínica da HEB. Estudos mostram que a progressão maligna das lesões do aparelho urinário depende do sinergismo do BPV2 com os princípios carcinogênicos e tóxicos da samambaia (CAMPO, 1995; WOSIACKI et al., 2002). De maneira semelhante, a infecção pelo BPV4 pode ser um

fator predisponente para papiloma no TGS que pode evoluir para câncer quando associado aos compostos carcinogênicos e tóxicos da samambaia (CAMPO, 2002a).

Os sinais clínicos decorrentes da intoxicação crônica pela pteridófita são: inapetência, anorexia, emagrecimento progressivo, andar cambaleante, diarreia sanguinolenta, tosse e disfagia (RADOSTITS et al., 2002). 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). Esta doença se caracteriza por lesões hemorrágicas e hiperplásicas da mucosa vesical que, frequentemente, evoluem para neoplasia, afetando bovinos entre três e cinco anos, sem predileção por raça (ROPERTO et al., 2010).

2.6.3 Sarcóide equino

Os papilomavírus são considerados espécie-específicos e, mesmo em condições experimentais, não infectam nenhum outro hospedeiro além daquele que lhe é natural. Os únicos casos conhecidos e bem documentados de infecção cruzada são em equinos infectados pelos BPVs tipo 1 e 2 e 13 (CAMPO, 2002a; ALCÂNTARA et al., 2015; LUNARDI et al., 2013a), também já foi relatado o BPV1 infectando zebra (*Equus burchellii*) (LÖHR et al., 2005). Além disso, também foi encontrado o BPV14 como um possível agente causador do sarcóide felino (MUNDAY et al., 2015).

O sarcóide geralmente se localiza no animal onde a pele é mais fina ou em locais que ocorreu uma lesão traumática anteriormente e o vírus foi introduzido. Os BPVs também podem ser transmitidos para os equinos pelos fômites, mosquitos e moscas. Os BPV1 e 2 que estão envolvidos na patogênese do sarcóide, como na grande maioria dos tumores, tem seu DNA epissomal não integrado no genoma da célula (NASIR; CAMPO, 2008).

O sarcóide equino pode ser definido como tumores fibroblásticos benignos localmente agressivos da pele equina. Raramentes progridem para lesões malignas (BERGVALL, 2013). Podem ocorrer lesões únicas ou múltiplas em diferentes formas, desde uma pequena lesão semelhante a verrugas até grandes lesões ulceradas. São considerados os tumores mais comuns da pele em cavalos e também são encontrados em outros equídeos, como zebras, burros e mulas (LÖHR et al., 2005). Estes tumores raramente regridem e muitas vezes tem recidivas após a terapia. A classificação

morfológica do sarcóide pode variar como: verrucoso, nodular, fibroblástico, misto e maligno (CAMPO, 2002b). Mesmo que o sarcoide equino não seja letal, o tamanho das lesões e a localização dos tumores podem comprometer gravemente a sanidade e a utilidade dos equinos, o que pode levar a decisão de eutanaziar o animal (ANJOS et al., 2010).

2.7 Diagnóstico

O diagnóstico do papilomavírus é basicamente clínico, pois as alterações são bem características, observa-se progessões pendunculadas ou planas com coloração acinzentada (SCHUCH, 2001a). Pode ser diagnósticado também por análise histopatológica (BETIOL et al., 2012) e detecção do DNA viral através de PCR.

A técnica de PCR é uma técnica adequada e utilizada para identificação de PV devido ao alto grau de especificidade e sensibilidade (FORSLUND et al., 1999). No entanto, a detecção de PVs pode ser afetada por fatores primários como a concentração de DNA viral e o grau de purificação da amostra (OGAWA et al., 2004). Entretanto, a identificação e tipificação do BPV através da PCR utilizando *primers* degenerados, seguida de purificação, sequenciamento e análise de similaridade das sequências através de programas e ferramentas como o BLAST (*Basic Local Alignment Search Tool*) mostrou-se o método mais sensível para detecção e identificação viral (BORZACCHIELLO et al., 2003; ZHU et al., 2012).

Outras técnicas como, *southern blot, dot blot* e imunohistoquímica (IHQ) podem ser utilizadas (MUNDAY et al., 2007). A IHQ é uma importante ferramenta que permite avaliar a presença de proteínas expressas pelo vírus, indicando a presença e atividade viral (NAKAMURA et al., 1997). O princípio desta técnica consiste no emprego de anticorpos primários, capazes de se ligarem a epítopos específicos de PVs. A identificação do BPV através de IHQ é geralmente feita através do emprego de anticorpos monoclonais comercialmente disponíveis (anti-L1 e anti-E7, que permitem a imuno-detecção da proteína principal do capsídeo viral –L1 e da oncoproteína E7, respectivamente).

As lesões macroscópicas são classificadas em típicas, pedunculadas e planas (DA SILVA et al., 2015; MONTEIRO et al., 2008). Histologicamente, os papilomas são neoplasias epiteliais com projeções digitiformes a partir da superfície celular. Observa-se também hiperqueratose, acantose da camada espinhal e coilocitose (DAUDT et al., 2016b; LUNARDI et al., 2016; MONTEIRO et al., 2008).

Estudos de papilomas cutâneos em bovinos mostraram proliferação da epiderme, e grânulos querato-hialinos (CAMPO, 2006; GRINDATTO et al., 2015b; LUNARDI et al., 2016). Outras pesquisas em papiloma de teto mostraram a presença de várias ilhas de células epiteliais degeneradas rodeadas por um halo espesso de epiderme hiperplásica (LUNARDI et al., 2016). Algumas dessas características podem ser observadas na Figura 4.

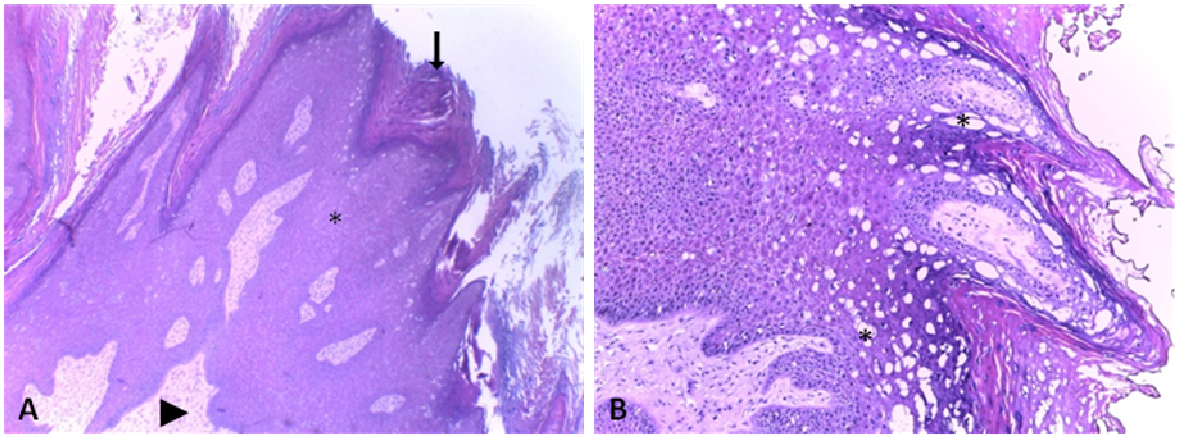

Figura 4. Papilomatose bovina. A) Acantose (*), fibroplasia (cabeça de seta) e hiperqueratose acentuadas (seta). B) Espogiose acentuada (*), displasia da camada espinhosa e hiperplasia. Fonte: próprio autor.

2.8 Tratamento

Existem vários tratamentos descritos para a papilomatose bovina, embora não exista uma concordância com relação à eficácia. Os papilomas pequenos podem ser removidos cirurgicamente, podendo-se usar criocirurgia em papilomas grandes, muitos regridem espontaneamente dentro de alguns meses sem tratamento (SMITH, 2006a).

Um dos tratamentos utilizados para a papilomatose animal é o uso da vacina autógena (MERCK, 2001). Estas são preparadas para um problema específico (individual ou do rebanho), possuindo a vantagem de incluir os tipos de vírus presentes nas lesões. A vacina é produzida a partir de homogeneizados de tecidos de papiloma, com adição de vermelho fenol, sulfato duplo de alumínio e potássio, hidróxido de sódio e solução de formol 1% para inativar o vírus. O protocolo vacinal consiste geralmente de três aplicações subcutâneas (5 mL por animal) com intervalos de dez dias entre as doses. Os resultados dependem do tipo de papiloma, da preparação da vacina e do estágio de evolução dos papilomas do animal afetado (RADOSTITS et al., 2002; SILVA et al., 2004). No Brasil, a vacina autógena tem sido utilizada como tratamento

terapêutico para animais extensamente atingidos por papilomatose e apresenta resultados com aproximadamente 50% de recuperação. No entanto, este tratamento, além de não ser muito eficaz, possui custo relativamente elevado (SILVA et al., 2004). Outra forma de tratamento consiste na utilização de uma ou duas aplicações de clorobutanol, na dose de 50 mg/Kg, em solução alcoólica via subcutânea (RIET-CORRÊA et al., 2001; SCHUCH, 2001b) e diaceturato de diaminazina 3,5 mg/kg (SMITH, 2006a).

2.9 Prognóstico e profilaxia

A papilomatose cutânea geralmente tem prognóstico favorável, com regressão natural dos tumores benignos normalmente após alguns meses da infecção (SCHUCH, 2001b). Porém, a papilomatose extensiva e sem regressão natural e tratamento pode levar à miíases e infecções secundárias, o que comumente ocorre em animais imunodeprimidos (SCHUCH, 2001b). Nos casos de papilomatose das mucosas do trato gastrintestinal e de bexiga, o prognóstico é reservado, já que os papilomas adiantados podem evoluir para carcinomas das células escamosas e é de difícil diagnóstico (RIET-CORREIA et al., 2001; SMITH, 2006a).

Atualmente não existem vacinas comerciais para o tratamento da papilomatose bovina, porém, existem alguns estudos com vacina profilática em bovinos que utilizam partículas semelhantes a vírus (*virus like particles* – VLPs) ou vírus purificados. Contudo, estas vacinas experimentais induzem uma boa proteção somente ao tipo viral homólogo e não confere nenhum efeito terapêutico em tumores estabelecidos (KIRNBAUER, 1996). Pesquisas utilizando a proteína menor do capsídeo viral (L2) produzido 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; RUBIO et al., 2011). Além disso, estudos experimentais *in vitro* e *in vivo*, demostraram que a porção terminal da proteína L2 apresenta uma imunidade heteróloga contra um grande números de PVs, incluindo o BPV1 (RUBIO et al., 2011). O BPV1 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).

O cuidado na aquisição de animais é uma importante medida profilática, assim

como o cumprimento da quarentena. Da mesma forma, o isolamento de animais (que apresentem papilomas) do restante do plantel é bastante relevante no controle da doença. O cuidado no manejo dos animais afetados (separadamente ou por último), a esterilização de materiais cirúrgicos e o controle de invertebrados hematófagos também são importantes medidas profiláticas (SCHUCH, 2001a; SMITH, 2006a). As fêmeas em fase de lactação que possuírem papilomas nos tetos ou úbere, devem ser ordenhadas por último e o ordenhador deve utilizar antissépticos nas mãos como solução de iodo e também fazer assepsia da ordenhadeira após o uso (RIET- CORRÊA et al., 2001).

2.10 Epidemiologia

Os papilomas são tumores benignos presentes em quase todas as espécies de amniotas (RECTOR; VAN RANST, 2013). Em bovinos, são encontrados com maior frequência em animais jovens, com menos de seis meses de idade, especialmente em condições de aglomeração (estabulação). A desnutrição, as más condições de higiene e instalações precárias são fatores de risco para o desenvolvimento de papilomas, uma vez que essas situações provocam estresse e uma consequente imunodepressão, levando ao desenvolvimento da enfermidade. A imunodepressão influi na presença e na gravidade da enfermidade (DA SILVA et al., 2015; SMITH, 2006b).

O BPV é um vírus cosmopolita e diferentes tipos são encontrados em todas as regiões do mundo, embora a distribuição de tipos específicos não apresente a mesma prevalência nas regiões amostradas. A distribuição global dos diferentes tipos de BPV pode ser visualizada na Figura 5. O BPV1 e o BPV2 são os tipos virais com a maior distribuição, embora mais estudos epidemiológicos sejam necessários para determinar a real prevalência dos tipos virais.

No Brasil, independentemente do nível de tecnologia da exploração pecuária, as lesões pelo BPV estão presentes em rebanhos bovinos de corte e, principalmente, nos de aptidão leiteira. Apesar da alta frequência das lesões, a determinação dos tipos virais circulantes ainda é esporádica. Os dados de prevalência tipo-específica de BPVs, em sua maioria, procedem do Brasil e do Japão. No Brasil, um dos maiores produtores mundiais de carne bovina e leite(USDA-FAS, 2017), já foi relatada a presença de praticamente todos tipos de BPVs conhecidos e vários prováveis novos tipos virais. A Tabela 2 mostra a distribuição tipo-específica de BPVs e associação destes com lesão em bovinos.

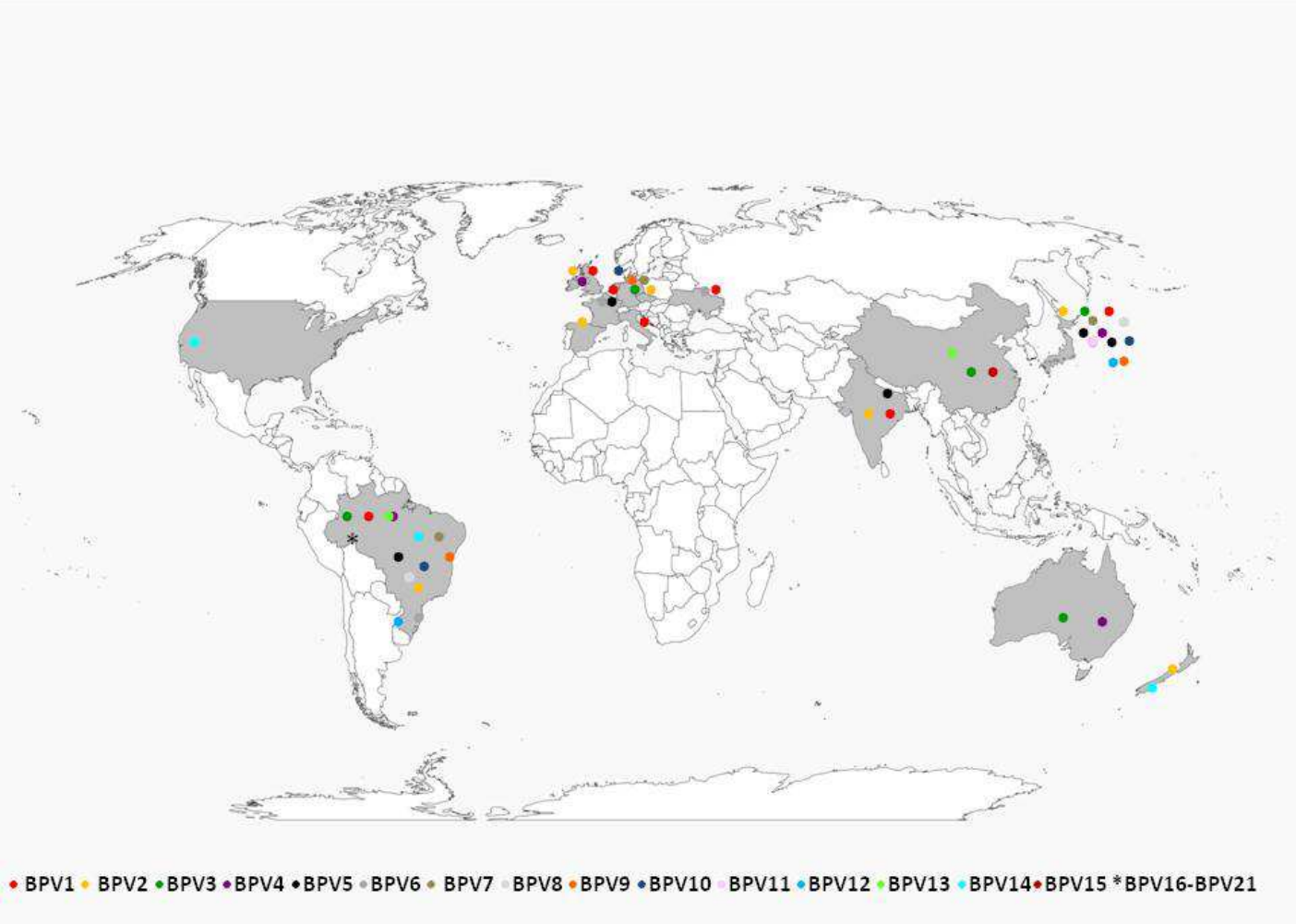

Figura 5. Distribuição geográfica dos BPVs bovinos. Informações provenientes da Tabela 2.

Tabela 2. Distribuição dos diferentes tipos virais e associação com diferentes tipos de lesões em bovinos.

Tipo viral	Associação com lesões	Localização/País	Referências
BPV1	Papiloma e fibropapilomas cutâneos	Japão, Brasil, Itália e Alemanha	(BATISTA et al., 2013; CLAUS et al., 2007, 2008, 2009, DA SILVA et al., 2015, 2012; HATAMA et al., 2011; OGAWA et al., 2004; SCHMITT; FIEDLER; MULLER, 2010; SILVA et al., 2010; SILVESTRE et al., 2009)
	Suabe de pele saudável	Japão	(OGAWA et al., 2004)
	Tumores de bexiga urinária	Itália, Índia e Europa	(BORZACCHIELLO; ROPERTO, 2008; NASIR; CAMPO, 2008)
BPV2	Papiloma cutâneo	Áustria, Brasil, Nova Zelândia, Alemanha e Japão	(ARALDI et al., 2014b; BATISTA et al., 2013; CLAUS et al., 2007, 2008, 2009, DA SILVA et al., 2015, 2012; HATAMA et al., 2011; MELO et al., 2014; MUNDAY et al., 2007; PFISTER et al., 1979; SCHMITT; FIEDLER; MULLER, 2010; SILVA et al., 2010)
	Tumor de bexiga urinária	Escócia, Itália e Europa	(BATISTA et al., 2013; BORZACCHIELLO; ROPERTO, 2008; CAMPO et al., 1992; ROPERTO et al., 2013)
	Tumor de trato gastrointestinal superior	Escócia	(JARRETT et al., 1984)
BPV3	Papiloma e fibropapiloma	Áustria, Autrália, Japão, Brasil, China	(ARALDI et al., 2014b; BATISTA et al., 2013; DA SILVA et al., 2012; HATAMA et al., 2011; HE et al., 2014; OGAWA et al., 2004; PFISTER et al., 1979)
BPV4	Papilomas de trato digestório superior	Escócia e Itália	(BORZACCHIELLO et al., 2003; BORZACCHIELLO; ROPERTO, 2008; CAMPO, 1997b)
	Papiloma e carcinoma de	Reino Unido Itália e Escócia	(BORZACCHIELLO et al., 2003;

	esôfago		BORZACCHIELLO; ROPERTO, 2008; NASIR; CAMPO, 2008)
	Papiloma cutâneo	Alemanha, Japão, Brasil	(ARALDI et al., 2014a; BATISTA et al., 2013; DA SILVA et al., 2012; HATAMA et al., 2011; MELO et al., 2014; SCHMITT; FIEDLER; MULLER, 2010)
BPV5	Papiloma cutâneo	Alemanha, Japão, Brasil e Índia	(ARALDI et al., 2014a; BATISTA et al., 2013; HATAMA et al., 2011; SCHMITT; FIEDLER; MULLER, 2010; SILVA et al., 2013)
BPV6	Papiloma cutâneo	Escócia, Alemanha, Brasil e Japão	(CLAUS et al., 2007, 2008; DA SILVA et al., 2012; HATAMA et al., 2011; JARRETT et al., 1984; SCHMITT; FIEDLER; MULLER, 2010; SILVA et al., 2010)
BPV7	Papiloma cutâneo, de teto e suabe de pele saudável	Japão	(OGAWA et al., 2007)
	Papiloma cutâneo	Alemanha	(SCHMITT; FIEDLER; MULLER, 2010)
	Papiloma de teto	Brasil	(TOZATO et al., 2013)
BPV8	Suabe de pele saudável	Japão	(OGAWA et al., 2004)
	Papiloma cutâneo	Japão e Brasil	(BATISTA et al., 2013; CLAUS et al., 2009; DA SILVA et al., 2012; SILVA et al., 2013; TOMITA et al., 2007)
BPV9	Papiloma de teto	Japão	(HATAMA; NOBUMOTO; KANNO, 2008)
	Papiloma cutâneo	Alemanha, Japão e Brasil	(ARALDI et al., 2014a; DA SILVA et al., 2012; HATAMA et al., 2011; SCHMITT; FIEDLER; MULLER, 2010; SILVA et al., 2013)
	Papiloma de úbere	Brasil	(BATISTA et al., 2013)
BPV10	Papiloma de teto	Japão e Brasil	(HATAMA; NOBUMOTO; KANNO, 2008; TOZATO et al., 2013)

	Papiloma e fibropapiloma cutâneo	Alemanha, Japão e Brasil	(DA SILVA et al., 2012; HATAMA et al., 2011; SCHMITT; FIEDLER; MULLER, 2010; SILVA et al., 2013)
	Papiloma de úbere	Brasil	(BATISTA et al., 2013)
BPV11	Papiloma cutâneo	Japão e Brasil	(DA SILVA et al., 2015, 2012; HATAMA et al., 2011; SILVA et al., 2013)
BPV12	Papiloma teto	Japão	(ZHU et al., 2012)
	Papiloma cutâneo	Brasil	(ARALDI et al., 2014a)
BPV13	Papiloma epitelial	Brasil	(DA SILVA et al., 2015; LUNARDI et al., 2013a)
		China	(PANG et al., 2014)
BPV14	Isolado em sarcóide de felinos	Estados Unidos	(MUNDAY et al., 2015)
	Fibropapiloma cutâneo	Nova Zelândia e Brasil	(DA SILVA et al., 2012; MUNDAY; KNIGHT; HOWE, 2010)
BPV15	Sarcóide bovino	China	(Hu et al., 2015 - não publicado)
BPV16	Papiloma cutâneo	Brasil	(DAUDT et al., 2016a)
BPV17			
BPV18			
BPV19			
BPV20			
BPV21			
BPV04AC14*	Papiloma cutâneo	Brasil	(DA SILVA et al., 2016)

* Provável novo tipo com sequenciamento completo do genoma.

3 OBJETIVOS

3.1 Objetivo geral

Analisar a diversidade genética dos papilomavírus bovinos na região Norte do Brasil.

3.2 Objetivos específicos

- Realizar um estudo das infecções pelo BPV em bovinos e identificar os diferentes tipos de BPV presentes em lesões cutâneas de bovinos nos estados do Acre e Rondônia;
- Detectar o BPV em lesões cutâneas de bovinos dos Estados de Acre, Rondônia;
- Sequenciar os fragmentos do gene L1 obtidos pela amplificação nas amostras de campo e realizar a comparação com as sequencias disponíveis na literatura;
- Realizar a análise filogenética dos BPVs detectados nas amostras estudadas;
- Sequenciar o genoma completo de prováveis novos tipos de BPV através de sequenciamento de alta eficiência.

4 ARTIGOS CIENTÍFICOS

Os resultados, bem como os materiais e métodos utilizados para a realização dos experimentos que fazem parte desta tese serão apresentados na forma de 3 artigos científicos. Adicionalmente, artigos em que o candidato participou como “co-autor” serão apresentados na sessão “Anexos”.

4.1 CAPÍTULO 1: Caracterização genética de papilomavírus bovino revela a existência de quatro prováveis novos tipos na região da Amazônia

O presente experimento já foi concluído e um artigo científico foi publicado no periódico *Virus Genes*. O artigo científico será apresentado a seguir, tal qual foi publicado.

Virus Genes
DOI 10.1007/s11262-015-1220-y

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.

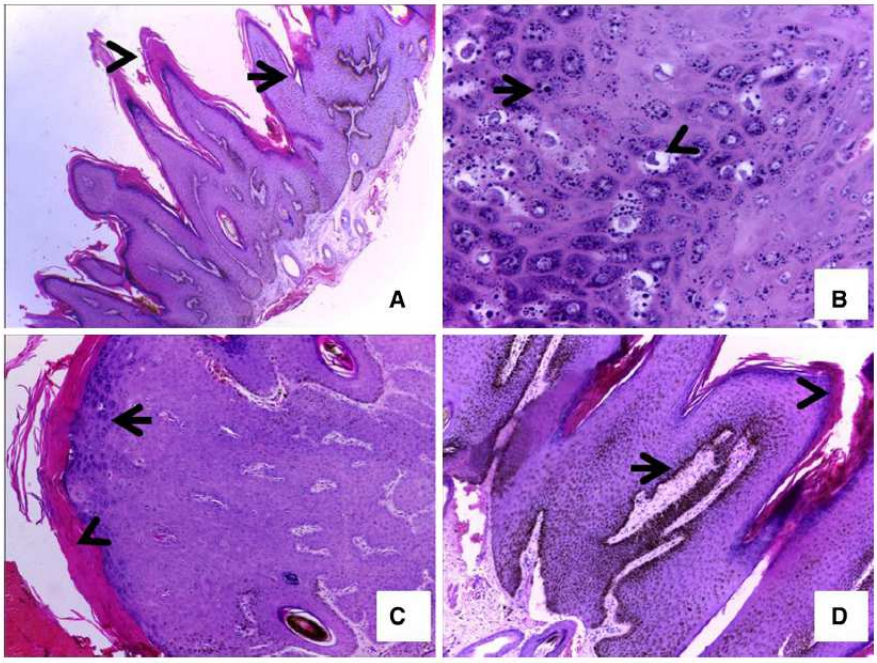


Fig. 1 Exophytic papillomatous proliferation of the epithelium of bovines sampled in this study. (**a**) Acanthosis (*arrow*) and moderate orthokeratotic hyperkeratosis (*head of arrow*). Obj.10x. (**b**) Increased deposition of keratohyalin granules (*arrow*) in the stratum granulosum and occasional 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].

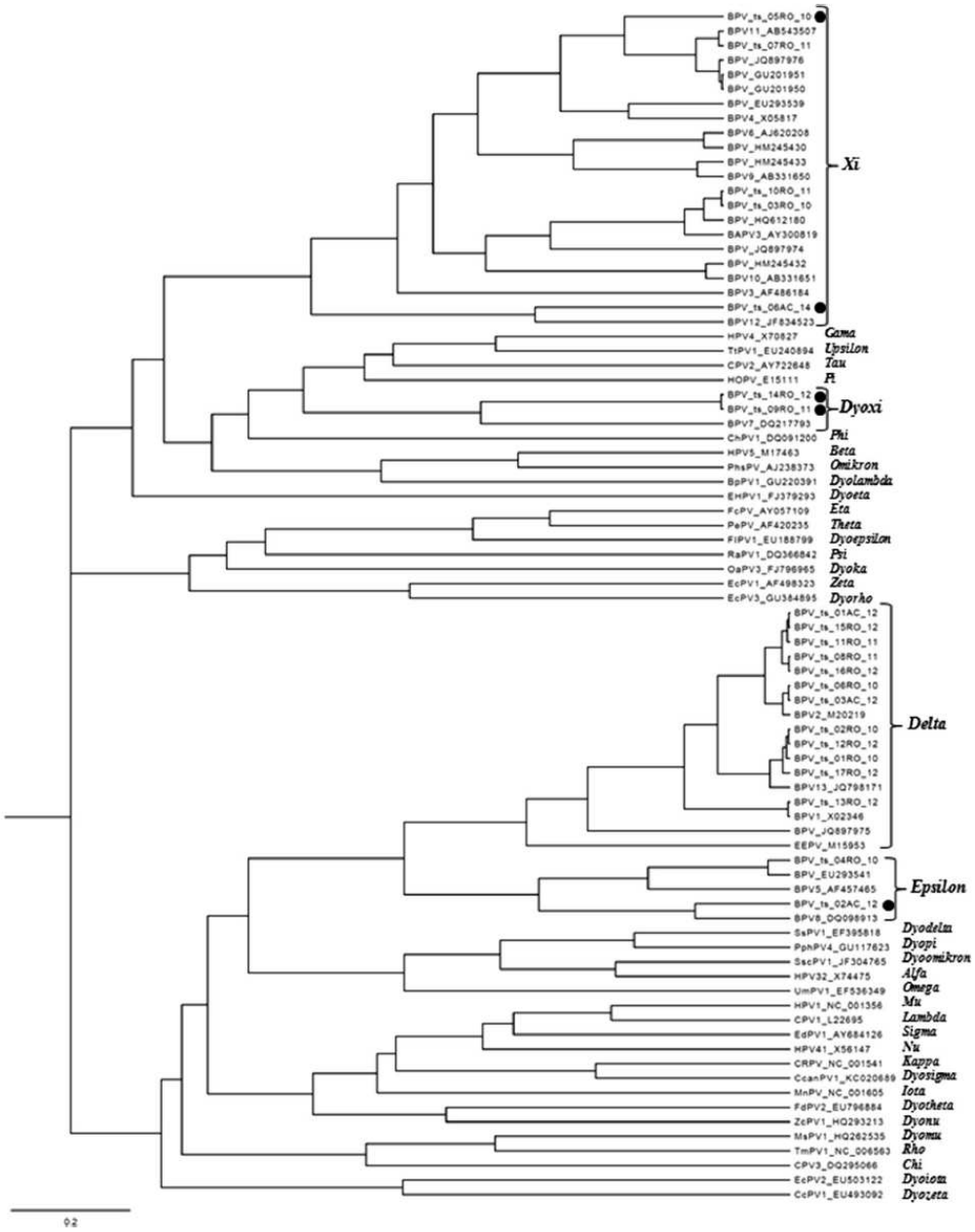
Based on the partial sequence of the L1 gene the strains detected encompassed the BPV1, 2, 11, and 13 types and two putative new types that were previously reported. Additionally, nine putative new subtypes and four putative new types of BPVs were characterized. The histopathological findings of the 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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4.2 CAPÍTULO 2: Descoberta de um novo tipo de BPV amplificado por círculo rolante seguido do sequenciamento de alto desempenho

O presente experimento já foi concluído e um artigo científico foi publicado no periódico *PLoS ONE.* O artigo científico será apresentado a seguir, tal qual foi publicado.

PLOS | ONE

RESEARCH ARTICLE

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

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Data Availability Statement: Sequence of the BPV 04AC14 was deposited in GenBank under accession number KX098515.

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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

Competing Interests: The authors have declared that no competing interests exist.

to the >200 types of viruses identified in humans (HPVs) (http://pave.niaid.nih.gov). The BPVs are assigned into four genera: the genus *Deltapapillomavirus*, with one species; *Deltapapillomavirus 4*, comprising four types (BPV1, 2, 13 and 14); the genus *Epsilonpapillomavirus*, comprising the species *Epsilonapapillomavirus 1*, with two types (BPV5 and BPV8); *Dyoxipapillomavirus*, which comprises *Dyoxipapillomavirus 1* species (BPV7); and the *Xipapillomavirus* genus, composed by the *Xipapillomavirus 1* (BPV3, 4, 6, 9, 10, 11 and 15) and *2* species (BPV12) (http://pave.niaid.nih.gov).

The multiply primed rolling-circle amplification (RCA) strategy has been successfully used for the identification of the circular genomes of a number of viruses [8], e.g., anelloviruses [9], circoviruses [10] and papillomaviruses [11]. The method utilizes bacteriophage ϕ29 DNA polymerase for the selective amplification of circular DNA [8]. Unlike PCR, the primers used in the amplification reaction are exo-resistant random. Therefore, as the technique does not need any specific primer, previous knowledge of the nucleotide sequences is not necessary. Furthermore, ϕ29 DNA polymerase has linear kinetics at 30°C, eliminating the need for thermal cycling. By strand displacement synthesis, repeated copies of the complete genome are synthesized, leading to a high molecular mass double-stranded DNA.

In 2014, a specimen consisting of papillomatous-like lesions was received in the laboratory for the production of a BPV autogenous vaccine. Confirmation of the presumptive diagnosis and typing of the BPV involved was performed by PCR with consensus PV primers and Sanger sequencing [12]. Nucleotide sequencing indicated a BPV type not previously described. The full genome of this novel BPV type was recovered directly from the papillomatous lesions by multiply primed rolling-circle amplification (RCA) followed by NGS. The genome sequence was characterized and the phylogenetic relationship between this novel BPV and the other BPVs was determined.

Materials and Methods

Sample

A specimen consisting of ~20 grams of papillomatous lesions (skin warts; sample 04AC14) was received in the lab. The specimens were derived from one animal in Acre State (within the Brazilian Amazon region), clinically diagnosed as papillomatosis, and collected for the production of a BPV autogenous vaccine. The lesions were removed using scalpels after local anaesthesia (performed with 2% lidocaine). The sample was individually wrapped and stored at −20°C for DNA extraction and in 10% buffered formaldehyde for histopathological analyses. DNA extraction, PV consensus PCR and Sanger sequencing was present in S1 File.

Histopathology

Tissue were fixed in 10% buffered formalin, trimmed, and processed routinely for histopathology. Tissue sections were cut at 3 μm and stained with haematoxylin and eosin (HE).

Rolling-circle amplification (RCA)

Multiply primed rolling-circle amplification (RCA) was performed as previously described [10,13]. Briefly, 100 ng of total DNA (2 μL of 50 ng/μL solution) from papillomatous tissue was denatured at 95°C for 5 minutes and immediately cooled on ice. Twenty-three microlitres of a previously prepared solution containing 1.5 mM of each dNTP (Invitrogen), 6.2 mM random exonuclease-resistant hexanucleotides (Thermo), 2 U of ϕ29 DNA polymerase (Thermo) and 2.5 μL of reaction buffer [50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM dithiothreitol] were added to denatured DNA. The amplification solution was incubated for 18

hours at 30°C, followed by 10 min at 65°C to inactivate the enzyme. The amplicon was electrophoresed in a 0.8% agarose gel and visualized on a UV light source after ethidium bromide staining. The RCA products were purified with a commercial kit (GFX™ Purification Kit; Amersham Biosciences).

High-throughput sequencing and sequence analysis

RCA DNA was tagged and fragmented using the Nextera DNA Library Prep Kit (Illumina) according to the manufacturer's instructions for the preparation of DNA libraries. After amplification 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 (2x150 cycles).

The data were *de novo* assembled using SPAdes genome assembler (version 3.6) [14]. Open reading frame (ORF) predictions and genome annotations of the 04AC14 genome were performed with the aid of Geneious software (version 8.1.4). Gene and protein comparisons were performed in the programmes BLASTn and BLASTp. Sequence of the BPV 04AC14 was deposited in GenBank under accession number KX098515.

Phylogenetic inferences

Local sequence alignments were constructed to determine the sequence identity with BLASTn [15]. Representative PV sequences were retrieved from GenBank. Nucleotide alignments were performed using MUSCLE software [16].

The best selection model to generate the phylogenetic trees was selected with the programme Modeltest 3.7 [17]. A phylogenetic tree with 1000 bootstrap resamples of the alignment data sets was generated using the Maximum Likelihood method in MEGA5 [18]. Bootstrap values (based on 1000 replicates) for each node are given.

Results and Discussion

De novo sequencing and genome assembly

Illumina MiSeq (2×150 cycles run) generated a total of 110,820 high quality paired-end reads. These sequences were *de novo* assembled into 3,885 contigs using SPAdes 3.6 assembler. These contigs were analysed using BLASTn/BLASTx with the National Center for Biotechnology Information (NCBI) databases. One contig (named Node_1) related to *Papillomaviridae* with a circular genome of 7,282 nucleotides (nt) was identified. This contig was composed by 857 reads (mean coverage ~17). The remaining contigs were either related to the bovine genome or to unknown sources.

Genomic characterization of a new BPV type

The PV recovered genome is 7,282 bp in length, arranged in a circular DNA molecule with a GC content of 42.5%. Seven BPV ORFs were identified in the positive strand: five of those corresponded to the genes coding for early proteins (*E8*, *E7*, *E1*, *E2* and *E4*) and two genes (*L1* and *L2*) coded for the late capsid proteins. A long control region (LCR) of 399 bp was recognized between the *L1* and *E8* gene, located at nt positions 6,884–7,282. The major genome features of 04AC14 BPV are shown in Fig 1.

Whole-genome sequence alignments revealed that the closest related PVs were BPV3 (AF486184; 77% of identity to 04AC12), BPV6 (AJ620208; 74%) and BPV4 (X05817; 73%). When each ORF was compared with other PVs, the degree of nucleotide identity varied between 74% and 81% (Table 1).

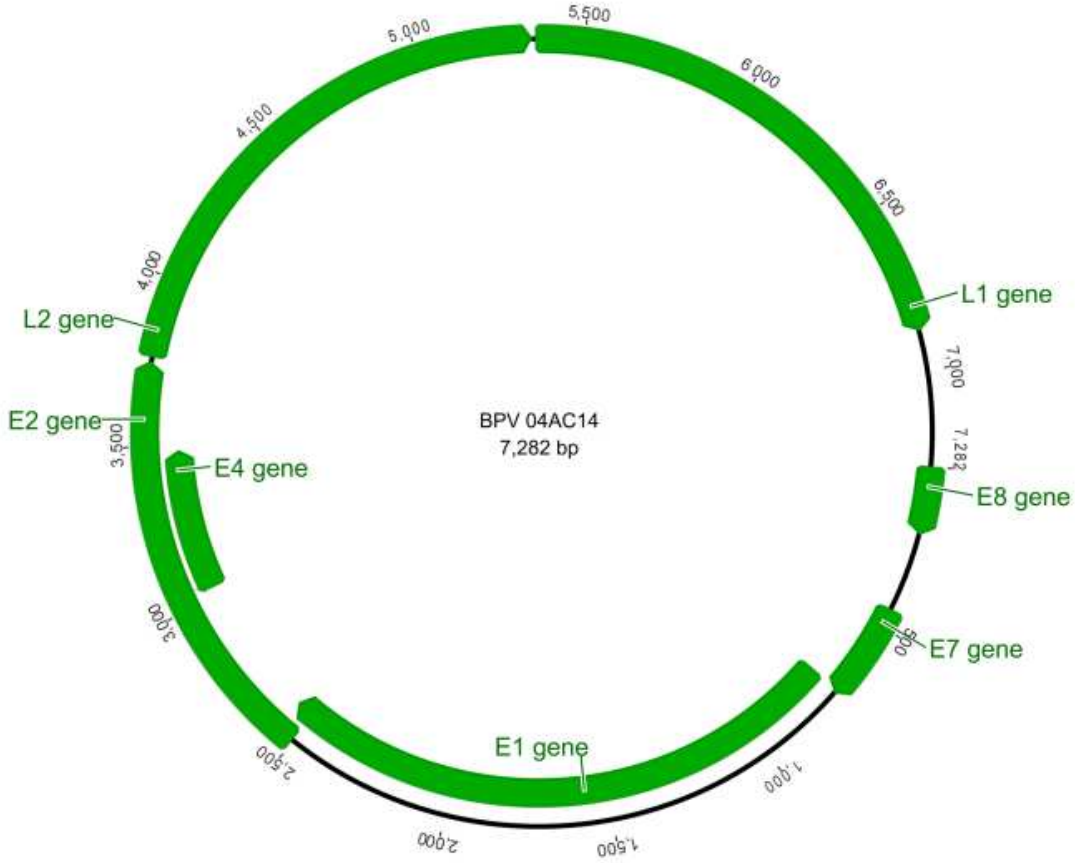

Fig 1. Genomic organization of BPV 04AC14. The first position of the BPV 04AC14 genome was set as the first ATG of the E8 ORF. The ORFs were identified with the aid of the ORF Finder (NCBI). The figure was drawn in Geneious 8.1.4.

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The LCR of BPV 04AC14 contained typical PV features [19] 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 highly conserved distribution of E2 DNA binding sites [20].

Table 1. Main features of the BPV 04AC14 genome.

	Position	Size (nt/aa)	Predicted MW (kDa)	Best blastn hit/Identity
EI	726–2,564	1,839/612	69.6	BPV3 (AF486184)/81%
E2	2,506–3,753	1,248/415	46.8	BPV3 (AF486184)/77%
E4	3,029–3,487	459/152	17.7	BPV3 (AF486184)/76%
E7	440–736	297/98	10.8	BPV3 (AF486184)/80%
E8	1–204	204/67	7.7	BPV11 (AB543507)/77%
L1	5,354–6,883	1,530/509	57.6	BPV3 (AF486184)/79%
L2	3,765–5,342	1,572/525	56.8	BPV3 (AF486184)/75%
LCR	6,884–7,282	399/-	-	BPV3 (AF486184)/74%

doi:10.1371/journal.pone.0162345.t001

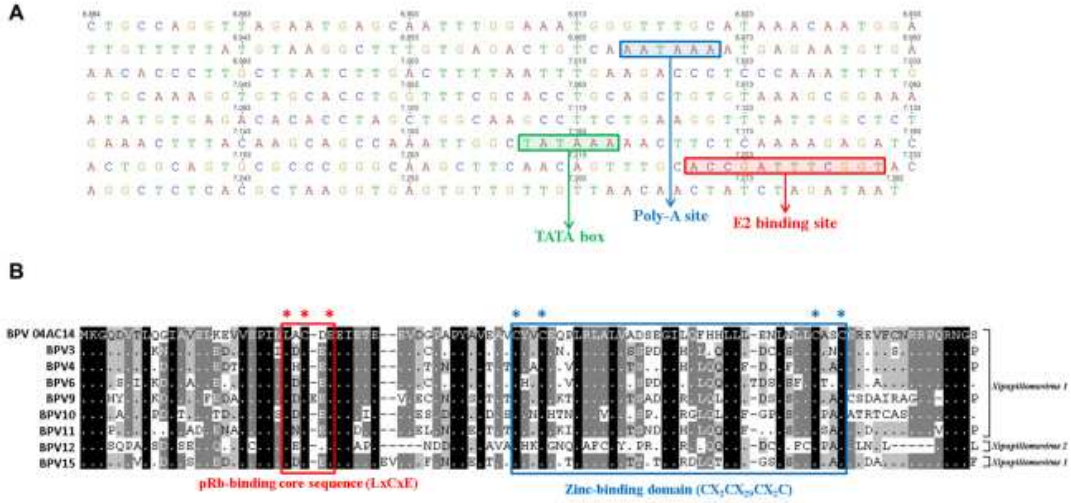

Fig 2. Features of the BPV 04AC14 non-coding region and E7 protein. (A) Non-coding region: coloured boxes display the genomic locations of the E2 binding site (ACCN$_6$GGT), polyadenylation site (AATAAA), and TATA box (TATAAA). (B) Amino acid alignment of E7 proteins with corresponding proteins of closely related types from *Xipapillomavirus 1* and *2*. The location of the pRb-binding core sequence (LXCXE) is marked with a red box, and the location of the zinc-binding domain ($CX_2CX_{29}CX_2C$) is indicated by a blue box. Multiple sequence alignments were performed in MUSCLE, and the figure was drawn in GeneDoc.

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BPV 04AC14 lacks a second LCR, similar to the majority of BPVs. Both E1 and E2 bind to the origin of virus replication, located in the LCR, and activate genomic DNA replication. Most PVs possess one E1-binding site (E1BS) and at least two E2-binding sites (E2BS) [21]. Nevertheless, the BPV 04AC14 LCR shows only one copy of E2BS (ACCN$_6$GGT). In addition, the BPV 04AC14 LCR possesses one poly-A site (AATAAA) and one TATA box (TATAAA), both of which are important transcription and replication regulatory elements. The E2BS, poly-A site, and TATA box are located at positions 7,220–7,231, 6,966–6,971 and 7,160–7,165, respectively (Fig 2A).

The early region of PV genomes encodes the non-structural viral proteins involved in viral DNA replication, transcription and cell transformation [22]. The early region of BPV 04AC14 encodes 5 ORFs: *E8* (204 bp), *E7* (297 bp), *E1* (1,839 bp), *E2* (1,248 bp) and *E4* (459 bp).

The BPV 04AC14 genome encompasses a putative *E8* gene. This gene encodes a protein that is chemically and functionally similar to the protein encoded by the HPV *E5* gene and may also substitute for the function of *E6* as an oncogene, primarily by activating cell growth-promoting signalling [23]. Therefore, E8 may play the role of E5 or E6 because it is located in a similar position in the genome (nt 1–204). Yet, BPV 04AC14 E8 shares a low degree of amino acid identity with BPV3 E8 (34.3% identity and 47.8% similarity).

The BPV 04AC14 E7 protein exhibits one conserved zinc-binding domain (ZnBD: **$CX_2CX_{29}CX_2C$**) (Fig 2B) [24] and possesses a conserved retinoblastoma tumour-suppressor protein-binding domain (pRbBD: **LxCxE**) at amino acid positions 47–83 and 24–28 (Fig 2B) [21,25]. The pRbBD is associated with oncogenesis in human papillomaviruses 16 (HPV16) and 18 (HPV18) [26,27]. The major cellular target for PV E7 and other viral oncoproteins involved in cell transformation is the retinoblastoma tumour suppressor (pRb) [28,29]. The ZnBD in E7, together with pRbBD, are responsible for the immortalization and transformation

of host cells [30]. All *Xipapillomavirus* members contain a pRbBD, which suggests that it could play a significant biological role for epitheliotropic papillomaviruses [31,32].

The putative E1 protein (with helicase function) has an ATP-dependent helicase **GX$_4$GK[T/S]** (**GPPNTGKS** in BPV 04AC14) domain at amino acid positions 441–448 [33]. The novel PV E1 has a cyclin A interaction motif (RXL), which is thought to be important for the initiation of papillomavirus replication [34].

The E1 and E2 proteins are essential for genome transcription and replication [35]. The 04AC14 E2 protein harbours a hinge region with a sequence rich in arginine, serine, and glycine residues. The protein has 73.6% amino acid identity with BPV3 E2. One leucine–zipper domain (**L$_X$6LX$_6$LX$_6$L**) was identified at E2 (aa 331–351) [36]. The BPV 04AC14 E4 ORF is contained within the E2 gene and is 63.9% similar to BPV3 E4. This composition is similar in *Xipapillomavirus* (BPV4, 10, 11, 12), *Epsilonpapillomavirus* (BPV8) and *Dyoxipapillomavirus* (BPV7) [21,32,37–40].

The late regions, *L1* and *L2*, were predicted to encode the major and minor capsid proteins, respectively. Both capsid proteins contain a nuclear localization sequence (NLS), which consists of a high proportion of positively charged residues (K and R) in their C-terminal ends that are likely to play a role in the nuclear translocation of L1 and L2 during the viral life cycle. Furthermore, the L1 gene has been chosen as yardstick for building PV comparisons, and taxonomic categories are based on the percentages of identity at the nucleotide level in this gene [5].

A new PV type can be proposed if the *L1* gene sequence shares less than 90% identity with the closest known PV type [5,7,41]. Here, it can be observed that the BPV 04AC14 *L1* gene diverges 21% in relation to the nucleotide sequence of the closest BPV type (BPV3). Based on this criterion, the PV strain identified in this study should be designated as a novel PV type. Different PV genera share less than 60% nucleotide sequence identity in the L1 ORF. Our results indicate that 04AC14 BPV should be regarded as a new PV type within the genus *Xipapillomavirus*.

Additionally, a phylogenetic tree was reconstructed with optimized alignments based on the nucleotide sequence of the *L1* gene. Using a set of genus-representative sequences of PV, BPV 04AC14 clustered in the *Xipapillomavirus* genus along with most known bovine PVs (Fig 3A).

According to the new ICTV *Papillomavirus* taxonomy proposal, the *Xipapillomavirus* genus comprises three papillomavirus species: *Xipapillomavirus 1* (BPV3, 4, 6, 9, 10, 11 and BPV15), *Xipapillomavirus 2* (BPV12) and *Xipapillomavirus 3* (*Rangifer tarandus* papillomavirus 2). A phylogenetic tree reconstructed with all members of the *Xipapillomavirus* genus and BPV 04AC14 is shown in Fig 3B. BPV 04AC14 grouped in the *Xipapillomavirus 1* branch, which also contains BPV3, 4, 6, 9, 10, 11 and 15 (Fig 3B). This result, combined with the genomic and L1 gene identities of BPV3 (the prototype of *Xipapillomavirus 1* species), clearly demonstrate that BPV 04AC14 is a *Xipapillomavirus 1* species. The phylogenetic evidence from the present study indicates that BPV 04AC14 is closely related to bovine epitheliotropic papillomaviruses.

Histopathological findings

Based on the histopathological findings, the diagnosis of epidermal papillomatosis was confirmed for specimen 04AC14. Neoplastic tissue of sample 04AC14 consisted of exophytic papillomatous lesions, epithelium proliferation, and well-differentiated cells, showing marked acanthosis (Fig 4A and 4B). This pattern has also been observed in other BPV-associated lesions [21,42,43]. *Xipapillomaviruses* infect only epithelial cells to induce true epithelial papillomas [3,32,38]. Some *Xipapillomavirus* types have been reported to cause teat and udder papillomatosis in dairy cattle worldwide [42–44]. Such lesions inflict serious economic losses on the dairy industry. The identification of distinct types/species of BPVs may be highly

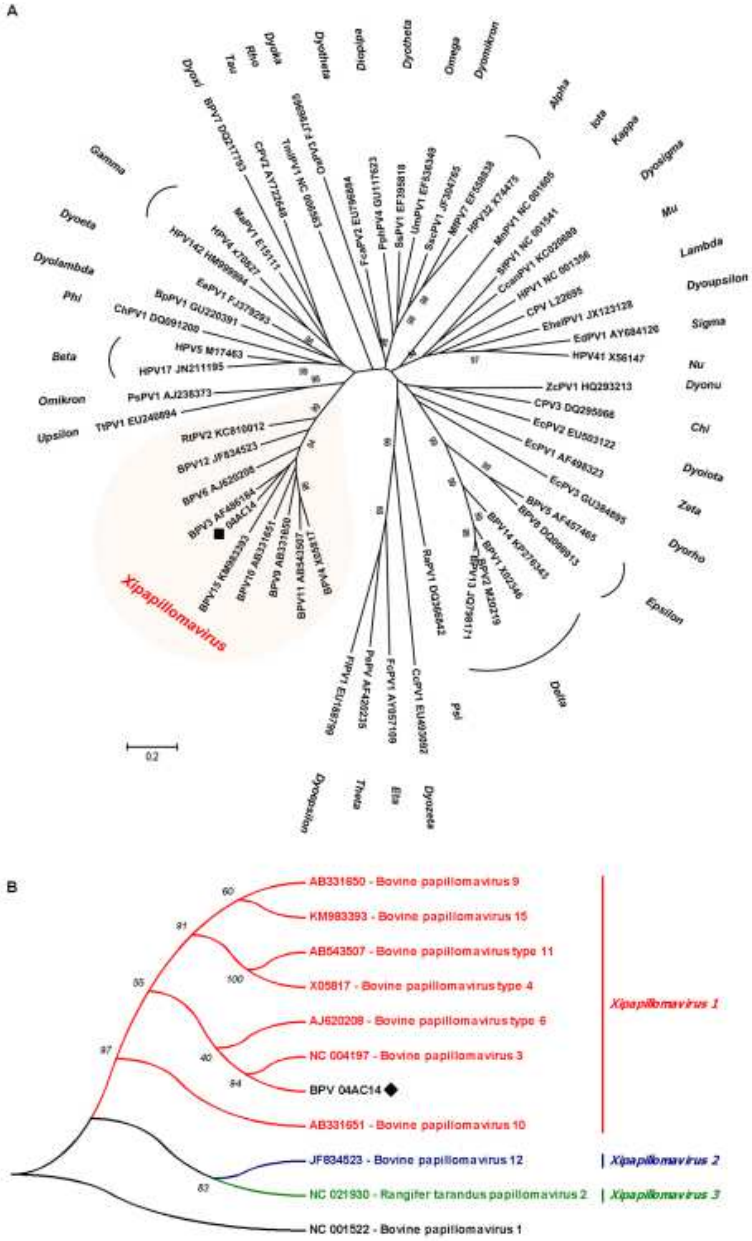

Fig 3. Maximum likelihood phylogenetic trees. (A) Maximum likelihood phylogenetic tree of *L1* gene sequences of genus-representative papillomaviruses. Nucleotide sequences were compared to those of 54 papillomavirus sequences, representing all known papillomavirus genera. The sequences were retrieved from GenBank. Bootstrap values are indicated above the branches. The putative new BPV type introduced here is indicated with a black diamond. (B) Maximum likelihood phylogenetic tree of the *Xipapillomavirus* genus. Nucleotide sequences of the *L1* gene were compared to those of nine bovine *Xipapillomavirus* sequences, representing the three known *Xipapillomavirus* species. The sequences were retrieved from GenBank. Bootstrap values are indicated above the branches. The putative new BPV type is indicated with a black diamond.

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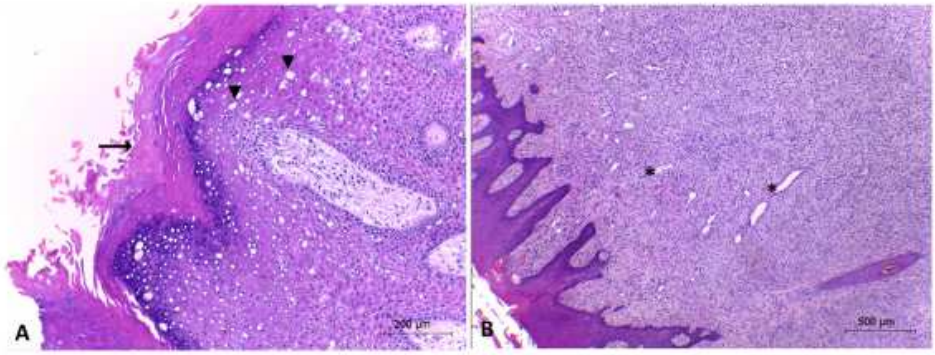

Fig 4. Exophytic papillomatous proliferation of the epithelium. (A) Exophytic papillomatous proliferation, acanthosis and multifocal spongiosis (arrowhead) and multifocal ortokeratosis (arrow) (obj. 10X). (B) The dermis shows fibroplasia and moderate neovascularization (*) (obj. 4X).

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important not only to improve knowledge on BPV biology but also to aid in defining the appropriate antigens for candidate vaccines.

Conclusion

A new putative BPV type—BPV 04AC14 –is introduced. The *L1* gene shares ≤90% identity with previously described BPVs (Table 1). The reconstructed phylogenetic tree with members of the *Xipapillomavirus* genus reveals that BPV 04AC14 is clearly a new member of this genus (Fig 3A). The genome of BPV 04AC14 aligned close to the *Xipapillomavirus 1* branch, which also contains BPV3, the representative species of the genus (Fig 3B). These findings add to the expanding genetic diversity of bovine papillomaviruses. Additionally, because there was no evidence of BPV co-infection in the sample, we can infer that BPV 04AC14 is implicated in the aetiology of bovine papillomatosis.

Supporting Information

S1 File.
(DOCX)

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Author Contributions

Conceptualization: FRCS SPC CD.

Data curation: SPC FQM.

Formal analysis: SPC CD MNW.

Investigation: AS CWC.

Methodology: FRCS SPC LLBG.

Software: SPC CD AS.

Supervision: FQM PMR.

Writing – original draft: FRCS SPC LLBG.

Writing – review & editing: FRCS SPC CD MNW PMR CWC.

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4.3 CAPÍTULO 3: Sequenciamento de um genoma completo de BPV5

O presente experimento já foi concluído e um artigo científico foi publicado no periódico *Virus Genes*. O artigo científico será apresentado a seguir, tal qual foi publicado.

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

Flavio R. C. da Silva[1,2] · Cíntia Daudt[1] · Samuel P. Cibulski[1] · Matheus N. Weber[1] · Ana Paula M. Varela[3] · Fabiana Q. Mayer[3] · Paulo M. Roehe[4] · Cláudio W. Canal[1]

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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).

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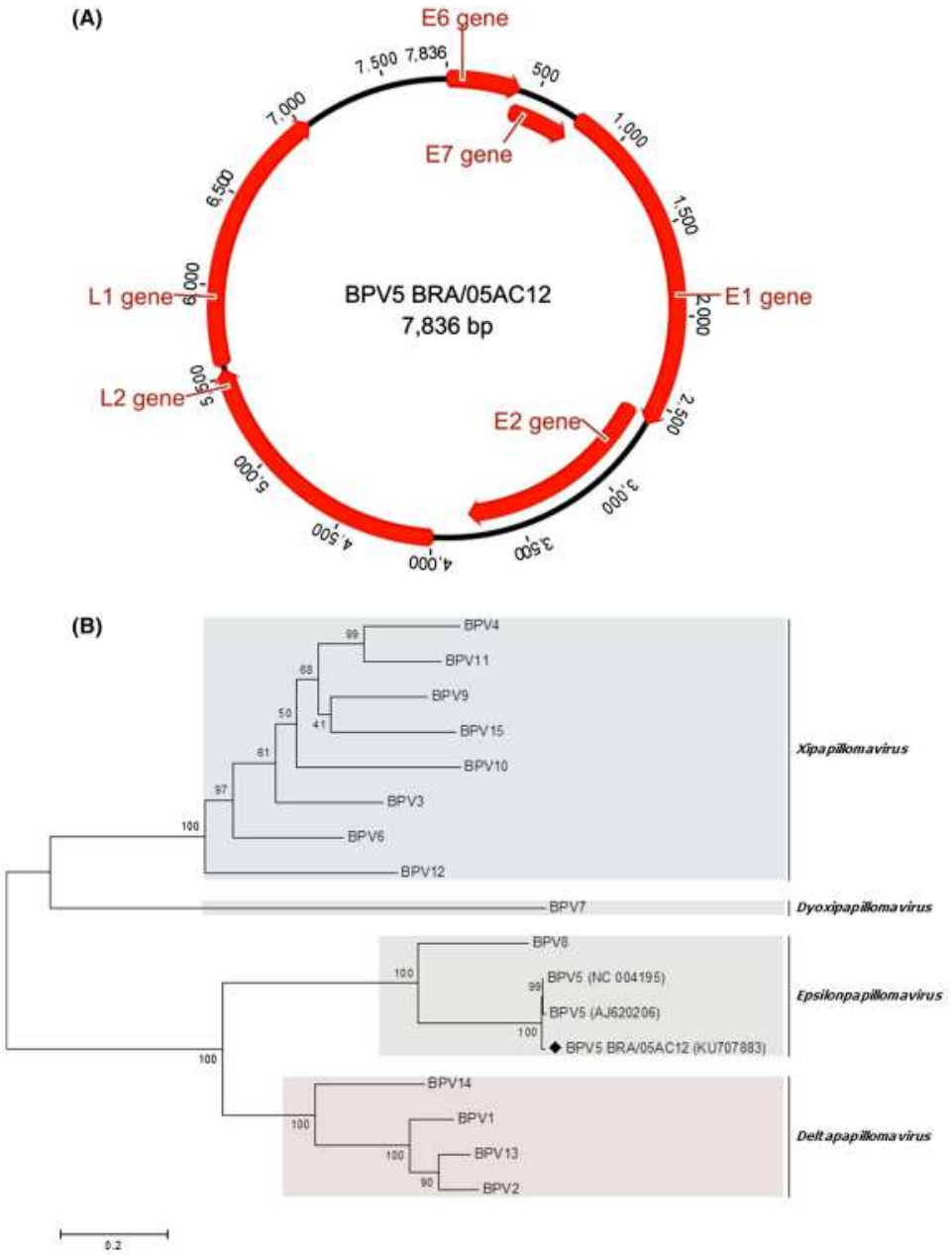
(A)
E6 gene
E7 gene
E1 gene
E2 gene
L2 gene
L1 gene
BPV5 BRA/05AC12
7,836 bp
7,500
7,836
500
1,000
1,500
2,000
2,500
3,000
3,500
4,000
4,500
5,000
5,500
6,000
6,500
7,000
(B)
BPV4
BPV11
BPV9
BPV15
BPV10
BPV3
BPV6
BPV12
BPV7
BPV8
BPV5 (NC 004195)
BPV5 (AJ620206)
BPV5 BRA/05AC12 (KU707883)
BPV14
BPV1
BPV13
BPV2
Xipapillomavirus
Dyoxipapillomavirus
Epsilonpapillomavirus
Deltapapillomavirus
0.2

◀**Fig. 1** **a** Schematic diagram showing the genome organization of BPV type 5 strain BRA/05AC12. Putative coding regions for early (E1, E2, E6, and E7) and late proteins (L1 and L2) are marked by *arrows*. BPV5 strain BRA/05AC12 was deposited in GenBank under the accession no. KU707883. **b** Rectangular phylogenetic tree of bovine papillomavirus whole genomes. Sequences were analyzed by maximum likelihood method with GTR+G+I model. Bootstrap (1000 replicates) values are indicated at the internal nodes. Brazilian isolates detected in the present study are highlighted with a symbol (*Filled diamond*). The length of each pair of branches represents the distance between sequence pairs in the rectangular tree. The *scale bar* represents the percentage of nucleotide differences in the rectangular tree. GenBank accession numbers: BPV1: NC_001522; BPV2: M20219; BPV3: NC_004197; BPV4: X05817; BPV5: NC_004195; BPV6: AJ620208; BPV7: NC_007612; BPV8: DQ098913; BPV9: AB331650; BPV10: AB331651; BPV11: AB543507; BPV12: JF834523; BPV13: JQ798171; BPV14: KP276343 and BPV15: KM983393

BPV type 5 (BPV5; *Epsilonpapillomavirus 1*) has been associated to "rice grain" fibropapillomas, "cauliflower-type" lesions of the udder [4–6], and gastrointestinal wart-like lesions [7]. To date, only two BPV5 genomes have been sequenced: one in Europe and one in North America. This scenario reflects the lack of availability of information for studying the genetics and biology of BPVs. In the present report, the characterization of a complete genome sequence of a BPV5 extracted from papillomatous lesions excised from cattle raised in the Amazon region, Northern Brazil, is provided.

Materials and methods

Sample of skin wart was collected from a bovine with papillomavirus characteristic lesion from Acre state, in Northern Brazil, Amazonian region. The lesions were removed using scalpels after local anesthesia was performed with 2% lidocaine (Bravet, Brazil). The sample was individually wrapped and stored at −20 °C for DNA extraction. Total DNA was isolated from papillomatous lesions using the PureLink® Genomic DNA kit (Invitrogen, USA). Initially, a screening PCR followed by Sanger sequencing was used in order to confirm the presence of BPV genomes in the DNA extracted from lesions [8].

To amplify the complete BPV genome, rolling circle amplification (RCA) was performed as previously described [9, 10]. RCA products were purified; quality and quantity of the DNA were assessed by spectrophotometry and fluorometry. DNA fragment libraries were prepared with 50 ng of purified DNA using a Nextera DNA sample preparation kit (Illumina, USA) and sequenced using an Illumina MiSeq System with MiSeq reagent kit v2 300 cycles. Reads were assembled into contigs using SPAdes 3.5 and compared to sequences in the GenBank nucleotide and protein databases using BLASTn/BLASTx. Geneious software was used for open reading frame (ORF) prediction and genome annotation.

Whole genome sequences of 16 bovine papillomaviruses, including representative within each PV genera and BPV type, were retrieved from GenBank® (http://www.ncbi.nlm.nih.gov/genbank/) and aligned with MAFFT [11]. Molecular Evolutionary Genetics Analysis version 6 (MEGA6) was used for phylogeny inference according to the maximum likelihood method. General time reversible with gamma distribution and invariant sites (GTR+G+I) was selected after using the "Find Best DNA/Protein Model" tool available in MEGA6. The robustness of the hypothesis was tested with 1000 nonparametric bootstrap analyses. The sequence data from the present study were deposited in GenBank® under accession number KU707883.

Results and discussion

A total of 132,900 reads were produced (with average length of 139 nt) and 3276 of these sequences were related to PVs. One full-length circular contig related to BPV5 genome was identified (coverage mean of 67), which was named BPV5 BRA/05AC12. Sequencing analysis of this genome revealed 7836 bp in length, with a GC content of 44.2% (Fig. 1a). The genome potentially encodes four early (E1, E2, E6, and E7) and two late ORFs (L1 and L2). The noncoding region of 791 bp is typically positioned between ORFs L1 and E6.

The noncoding region of BPV5 BRA/05AC12 contains seven consensus palindromic E2-binding sites ($ACCN_6$-GGT), two putative TATA boxes (TATAAA) of the E6 promoter, and the polyadenylation site (AATAAA) for L1 and L2 transcripts [12, 13]. The putative E6 protein exhibits two conserved zinc-binding domains of CX_2CX_{29}-CX_2C, separated by 36 amino acids [14]. E1 encodes for the largest viral protein, which contains 625 amino acids; the ATP-binding site (GPPDTGKS) of the ATP-dependent helicase is present in the carboxy-terminal part of E1 [15].

BPV5/BRA/05AC12 shares a high degree of nucleotide identity with the two other full BPV5 genomes available at GenBank (99.1% with a German BPV5 genome AJ620206 and 99.2% with AF457465, a North American BPV5 genome). As expected, most differences were found in the noncoding region [16].

The phylogenetic tree (Fig. 1b) presented four well-defined branches supported by bootstrap values of 100% corresponding to the *Xipapillomavirus*, *Dyoxipapillomavirus*, *Epsilonpapillomavirus*, and *Deltapapillomavirus* genera. BPV5 strain BRA/05AC12 grouped in the BPV5 node in an independent terminal node separated for the

other two BPV5 included in the analysis detected in the United States and Germany which grouped in the same terminal node. The present findings showed that even PVs are a distinct and variable group of viruses; they are conserved inside the BPV-type troop that is reflected of the similarity of samples detected in different and distant geographic regions.

The present report describes the first South American BPV5 genome fully sequenced that showed high nucleotide identity degree in whole genome when compared with strains detected in North America and Europe. The findings reported in the present study are expected to contribute to studies on BPV biology, since available full BPV genome sequences are still scarce.

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Author Contributions Conceptualization: FRCS, SPC, and CD. Data curation: SPC and FQM. Formal analysis: SPC, CD, and MNW. Investigation: CWC. Methodology: FRCS and SPC. Software: SPC and CD. Supervision: FQM and PMR. Writing—original draft: FRCS and SPC. Writing—review & editing: FRCS, SPC, CD, MNW, PMR, and CWC.

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5 CONCLUSÕES

Capítulo 1

- A caracterização genética de 39 amostras de papilomas bovinos, revelou a presença dos BPVs 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 *Xipapillomavirus, Epsilonpapilomavirus* e *Dyoxipapillomavirus* de BPVs na região da Amazônia;
- A região Amazônica apresenta uma grande diversidade de tipos de BPVs;
- A análise histopatológica mostrou a presença de lesões papilomatosa exofítica, proliferação celular do epitélio, células bem diferenciadas, mostrando acantose acentuada, aumento da quantidade de grânulos na camada granular, e grânulos de querato-hialina, sendo estas características bem relatadas em trabalhos anteriores;

Capítulo 2

- Foi descoberto um novo tipo de BPV (BPV04AC14) no estado do Acre, amplificado por círculo rolante e usando sequenciamento de alto desempenho, pertencente ao gênero *Xipapillomavirus* e espécie *Xipapillomavirus 1*;
- Com base nos achados histopatológicos, o diagnóstico de papilomatose foi confirmada. O tecido neoplásico apresentou papilomatose exofítica, proliferação do epitélio e as células bem diferenciadas com marcante acantose;

Capítulo 3

- Foi relatado o primeiro sequenciamento completo do genoma de um BPV5 na América do Sul;
- Este BPV apresentou um alto grau de identidade de nucleotídeos com os dois outros genomas BPV5 completos disponíveis no GenBank (99,1% com um genoma de origem alemã e 99,2% com um de origem genoma norte-americano).

6 PERSPECTIVAS

A região amazônica é rica em biodiversidade, e sua característica de floresta tropical, possuindo mais de 400 espécies endêmicas de mamíferos, apresenta possibilidades de exploração científica quase inesgotáveis. Por outro lado, a antropização desta região, com a introdução de gado bovino, zebuíno e ovino, possibilitou interações espécies-hospedeiros que parecem deter características próprias.

Desta forma, tendo em vista os resultados obtidos nos trabalhos publicados nesta tese, serão amostradas lesões papilomatosas de diversas espécies de ungulados para a caracterização genética dos papilomavírus, ajudando na reconstrução da história evolutiva destes vírus, na elucidação das interações intra e interespecíficas.

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8 ANEXOS

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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

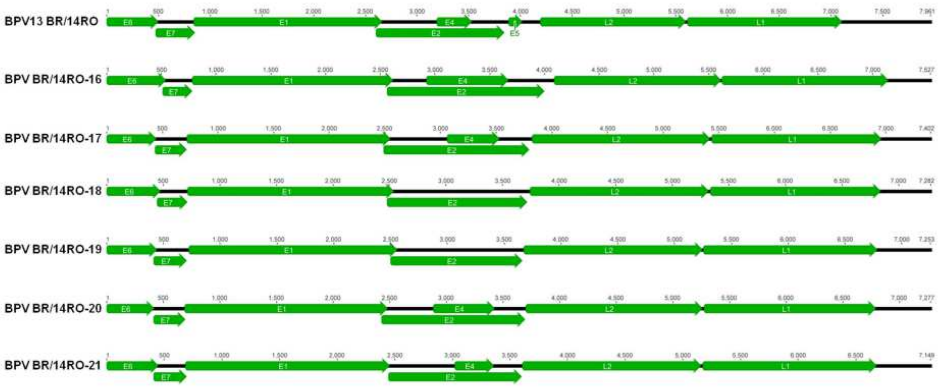

Figure 1. PV genomes found in this study and their archetypical organization. The first nucleotide in the ORF6 was assigned a number 1 in the sequences. All putative new papillomavirus genomes (BPV BR/RO-16 to BPV BR/RO-21) were predicted to contain six to eight ORFs, coding for early (E6, E7, E1, E2,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

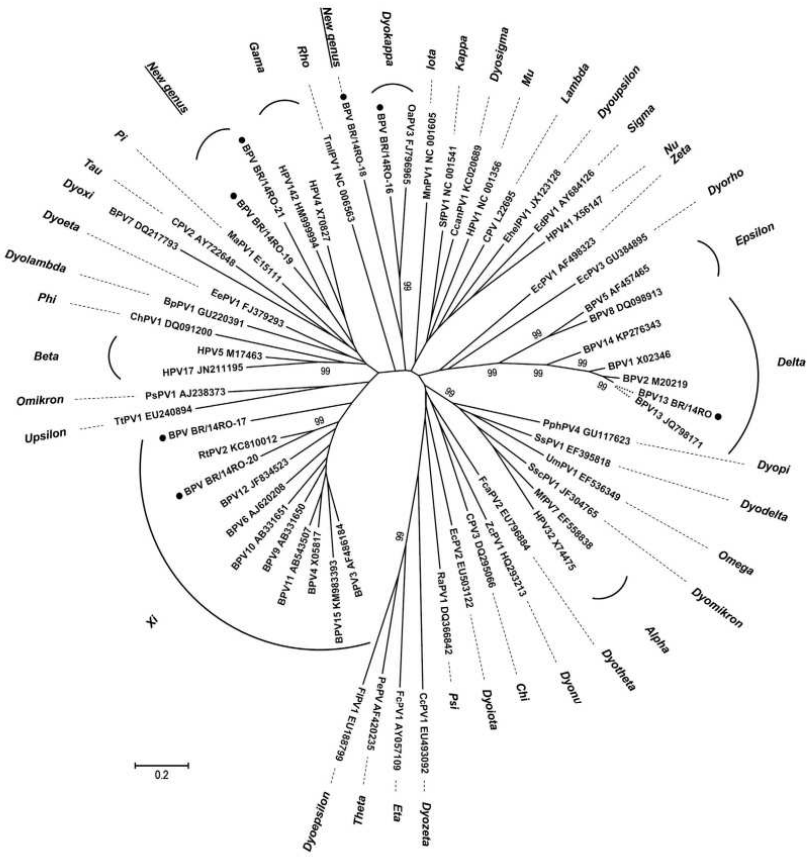

Figure 2. Phylogenetic tree of the papillomaviruses based on complete sequences of the L1 ORF. Bootstrap repetitions (higher than 99%) are indicated above the main branches. Samples belonging of this study with representatives of other PV genera were included on the analysis. A total of 61 PV types of 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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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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