MICHELE LUNARDI

CARACTERIZAÇÃO GENÉTICA DE UM NOVO MEMBRO DE GÊNERO *DELTAPAPILLOMAVIRUS* (PAPILOMAVÍRUS BOVINO TIPO 13) IDENTIFICADO EM LESÕES EPITELIAIS DE BOVINOS E EQUINOS

Londrina
2011

MICHELE LUNARDI

CARACTERIZAÇÃO GENÉTICA DE UM NOVO MEMBRO DE GÊNERO *DELTAPAPILLOMAVIRUS* (PAPILOMAVÍRUS BOVINO TIPO 13) IDENTIFICADO EM LESÕES EPITELIAIS DE BOVINOS E EQUINOS

Tese apresentada para obtenção do título de Doutor em Ciência Animal (Área de Concentração: Sanidade Animal) da Universidade Estadual de Londrina.

Orientador: Prof. Dr. Amauri Alcindo Alfieri

Londrina
2009

MICHELE LUNARDI

CARACTERIZAÇÃO GENÉTICA DE UM NOVO MEMBRO DE GÊNERO DELTAPAPILLOMAVIRUS (PAPILOMAVÍRUS BOVINO TIPO 13) IDENTIFICADO EM LESÕES EPITELIAIS DE BOVINOS E EQUINOS

Tese apresentada para obtenção do título de Doutor em Ciência Animal (Área de Concentração: Sanidade Animal) da Universidade Estadual de Londrina.

BANCA EXAMINADORA

____

Prof° Dr° Amauri Alcindo Alfieri
UEL - Londrina - PR

____

Prof°. Dr°. João Pessoa Araújo Júnior
UNESP - Botucatu - SP

____

Prof°. Dr°. Marcos Bryan Heinemann
UFMG - Belo Horizonte - MG

____

Laurival Antonio Villas-Boas
UEL - Londrina - PR

____

Selwyn Arlington Headley
UNOPAR - Londrina - PR

Londrina, 20 de dezembro de 2011.

O presente trabalho foi realizado no Laboratório de Virologia Animal, Departamento de Medicina Veterinária Preventiva, Centro de Ciências Agrárias, Universidade Estadual de Londrina, como requisito para a obtenção do título de Doutor em Ciência Animal pelo Programa de Pós-Graduação em Ciência Animal (Área de Concentração: Sanidade Animal), sob orientação da Prof. Dr. Amauri Alcindo Alfieri.

Os recursos financeiros para o desenvolvimento do projeto foram obtidos junto às agências e órgãos de fomento à pesquisa abaixo relacionados:

1. CNPq: **Conselho Nacional de Desenvolvimento Científico e Tecnológico / MCT**

2. CAPES: **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior / MEC**

3. FAP/PR: **Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Paraná / SETI**

4. FINEP: **Financiadora de Estudos e Projetos / MCT**

DEDICATÓRIA

Ao Pai Celestial, cuja obra deslumbrante e complexa convida a mente humana a confirmar sua perfeição.

Aos meus queridos pais, Arnaldo e Claudia, e a minha amada irmã, Milena, pelo imensurável amor e carinho, pelas palavras de incentivo e encourajamento e, principalmente, pela transmissão de valores morais valiosos.

A todos os familiares de Osasco, especialmente à minha querida avó e madrinha, Guiomar, e ao meu avô, João, pela compreensão de que a privação do convívio diário seria necessária para minha formação profissional.

Aos meus queridos avós, Francisca e Arnaldo (*in memorian)*, pelo incentivo nos estudos desde a infância.

AGRADECIMENTOS

Ao professor e orientador Dr. Amauri A. Alfieri, e à professora e co-orientadora Dra. Alice F. Alfieri, pelos exemplos de dedicação a área de Virologia Animal, pela oportunidade e confiança em meu trabalho, assim como pela sinceridade em apontar minhas falhas para que eu pudesse me aperfeiçoar. Agradeço também pelo carinho e amizade, e pelas eventuais críticas construtivas e elogios revigorantes.

Aos membros da banca de qualificação: Prof. Dr. Laurival A. Vilas-Boas e Prof. Dr. Selwyn A. Headley, pelas indispensáveis contribuições.

Agradecimentos especiais Profa. Dra. Maria Helena P. Fungaro, coordenadora do Laboratório de Genética Molecular de Microrganismos do Departamento de Biologia Geral, Centro de Ciências Biológicas, por ter disponibilizado seu laboratório para a realização dos procedimentos de clonagem gênica utilizados neste trabalho. Agradeço também ao Prof. Dr. Antônio Basílio de Miranda, coordenador do Laboratório de Genômica Funcional e Bioinformática do Instituto Oswaldo Cruz – Fiocruz, e aos seus alunos Leandro Mattos e Fernando Encinas, pela colaboração na anotação do genoma do BPV13.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da Bolsa de Doutorado.

A todos os professores do Programa de Pós Graduação em Ciência Animal pela formação acadêmica científica.

Às técnicas de laboratório, Kerlei, Dalíria (*in memorian*), Maria e Juliana pela colaboração indireta neste trabalho, e pelos momentos de descontração e amizade.

Aos secretários Valdecir e Helenice, pela paciência e ajuda.

Aos amigos do laboratório de Virologia: Rodrigo, Marlise, Wagner, Elis, Thaís, Brígida, Noemi, Raquel, Juliane e Aline, pela amizade que vai deixar saudades.

Às amigas do laboratório de Genética Molecular de Microrganismos Dani e Lara, pela participação fundamental neste trabalho, pela amizade e excelente convívio.

Às minhas grandes amigas da veterinária, Valeska, Pamela, Vani, Josy, Roberta e Mari, que sempre torceram por mim e cuja amizade espero que perdure para sempre.

Aos meus amigos Brianna, Julius, Rodrigo e Miguel, que mesmo estando longe sempre torceram pela realização deste momento especial.

RESUMO

LUNARDI, M. **Caracterização genética de um novo membro do gênero *Deltapapillomavirus* (Papilomavírus bovino tipo 13) identificado em lesões epiteliais de bovinos e equinos.** 2011. 101f. Tese (Doutorado em Ciência Animal, Área de Concentração: Sanidade Animal) – Universidade Estadual de Londrina, Londrina. 2011.

O papilomavírus (PV) pertence a um grupo muito diversificado de vírus que infecta grande variedade de espécies de vertebrados. O PV está relacionado com proliferações da pele e mucosa de seus hospedeiros. Em bovinos, o papilomavírus bovino (BPV) é o agente etiológico da papilomatose cutânea e dos cânceres da bexiga urinária e do trato gastrointestinal superior. Nos equinos, os sarcóides são neoplasias fibroblásticas locais com característica agressiva, reconhecidas como o tumor de pele mais comum em equinos de todo o mundo. Os sarcóides raramente regridem e, frequentemente, recorrem após a terapia. Pela identificação sucessiva do DNA do BPV em sarcóides, e a demonstração da expressão de diversos genes virais nestas lesões, o envolvimento direto do BPV na patogênese do sarcóide foi corroborado. Atualmente, doze tipos de BPV foram identificados e caracterizados em bovinos. Estes BPVs estão classificados nos gêneros *Deltapapillomavirus* (BPV1 e 2), *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11, e 12) e *Epsilonpapillomavirus* (BPV5 e 8), com a exceção do BPV7 que pertence a um gênero ainda indefinido. Além dos tipos de BPV caracterizados por meio da sequência completa do genoma viral, dezenas de prováveis novos tipos virais, identificados por meio de PCR com primers degenerados que amplificam fragmentos parciais do gene L1, têm sido descritos em rebanhos bovinos provenientes de regiões geográficas diversas. Recentemente, estudos realizados no Brasil revelaram notável diversidade entre os BPVs detectados em papilomas bovinos, sendo descritos quatro prováveis novos tipos virais que foram designados BPV/BR-UEL2 a BPV/BR-UEL5. O presente estudo descreve a sequência genômica completa e o posicionamento filogenético de um destes novos tipos de BPV (BPV/BR-UEL4) identificado em bovinos e tentativamente denominado de papilomavírus bovino tipo 13. Adicionalmente, o envolvimento deste novo tipo de BPV em sarcóides equinos também é relatado. Em cavalos, seis sarcóides que foram coletados cirurgicamente, a partir de várias regiões anatômicas de dois cavalos, foram avaliados com oito pares de primers degenerados. Estes primers foram desenhados para detectar regiões conservadas dos genes L1 e E1 dos PVs cutâneos. Os produtos de PCR obtidos foram sequenciados diretamente. Nos sarcóides avaliados, as quatro sequências geradas dos produtos de PCR apresentaram 100% de identidade com o BPV13. O BPV13 foi identificado em um papilomas localizado na orelha de bovino pertencente a um rebanho da região sul do Brasil. Para amplificar a sequência completa do genoma do BPV13, a amostra de DNA foi submetida à técnica de amplificação por círculo rolante (RCA). Para confirmar que o produto obtido era DNA de papilomavírus, PCR com primers degenerados foi realizada no produto RCA. Estes produtos de PCR foram sequenciados e, baseando-se nestas sequências parciais dos genes E1 e L1, dois pares de primers para PCR de fragmentos longos foram selecionados para a amplificação da maior parte do genoma do BPV13. A sequência completa dos fragmentos longos clonados foi determinada por sequenciamento contíguo, iniciando-se a partir do sítio para os primers universais no vetor de clonagem pCR4-TOPO. As falhas remanescentes nas sequências foram eliminadas por sequenciamento direto do produto RCA. A sequência genômica completa do BPV13 possue 7961 pb, com conteúdo GC de 45,1%, e oito ORFs, codificando para seis proteínas não-estruturais (E1, E2, E4, E5, E6 e E7) e duas proteínas estruturais (L1 e L2). A análise filogenética, realizada a partir do alinhamento de sequência de nucleotídeos concatenada dos genes E7, E1, E2, L2 e L1 do BPV13 e de outros PVs de ungulados, mostrou que o BPV13 é um membro do gênero

Deltapapillomavirus, que já contém os BPVs 1 e 2. A sequência do gene L1 do BPV13 apresentou identidade de 85,5-88,2% com as mesmas sequências dos BPVs 1 e 2. A identificação do BPV13 nos sarcóides examinados sugere a possibilidade de que muitos PVs isolados de sarcóides, e que foram previamente diagnosticados por meio de PCR com primers específicos para o BPV1/2, podem ter sido classificados erroneamente como BPV2, especialmente devido ao fato do sequenciamento direto não ter sido realizado para muitas amostras. Assim como o relatado para os BPVs 1 e 2, é tentador sugerir que o BPV13 também é capaz de induzir fibropapilomas em seus hospedeiros naturais. O agrupamento do BPV13 no gênero *Deltapapillomavirus*, o qual reune PVs que determinam fibropapilomas em artiodactilos ruminantes, assim como os achados da presença do gene E5 e da perda do motivo de ligação do fator pRB na proteína E7, dão suporte a esta hipótese. Os PVs são espécie-específicos e a ocorrência de transmissão entre espécies é relatada como evento muito raro, somente ocorrendo entre espécies hospedeiras próximas, como a infecção de cavalos pelos BPVs 1 e 2. Assim como os outros representantes da espécie Delta4, este novo tipo viral foi identificado em associação com casos de sarcóide equino em rebanhos brasileiros. A caracterização de novos BPVs colabora para a compreensão da filogenia do PV e para a interpretação das enfermidades relacionadas a estes vírus. A obtenção de informações adicionais sobre a base molecular e epidemiologia dos BPVs caracterizados, assim como dos ainda não caracterizados, será necessária para a completa compreensão da patogenia das doenças associadas ao BPV.

Palavras-chave: Bovino, Equino, Papilomatose cutânea, Sarcóide, BPV, Novo tipo de BPV, Genoma completo, Análise filogenética, Histopatologia.

ABSTRACT

LUNARDI, M. **Genetic characterization of a novel member of the *Deltapapillomavirus* genus (Bovine papillomavirus type 13) identified in epithelial lesions from cattle and horses.** 2011. 101f. Thesis (Ph.D. in Animal Science) - Universidade Estadual de Londrina, Londrina. 2011.

Papillomaviruses (PVs) represent a large and highly diverse group of pathogens that infect a wide variety of vertebrate species. PVs are linked to epithelial proliferations of the skin or the mucosa of their hosts. In cattle, the bovine papillomavirus (BPV) is thought to be the casual agent of cutaneous papillomatosis, cancer of the urinary bladder and upper gastrointestinal tract. Equine sarcoids are locally aggressive fibroblastic neoplasms, considered as the most common skin tumor in horses worldwide. These tumors rarely regress and very often recur after therapy. Through consistent identification of BPV DNA in sarcoids as well as the demonstration of expression of diverse viral genes, the direct involvement of BPV in the pathogenesis of sarcoid tumors was corroborated. Until now, twelve BPV types were identified and characterized from cattle. These BPVs are classified in the genera *Deltapapillomavirus* (BPV1 and 2), *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11, and 12) and *Epsilonpapillomavirus* (BPV5 and 8), with the exception of BPV7, which belongs to an undesignated PV genus. In addition to the fully sequenced BPV types, through PCR assay employing consensus or degenerate primers which amplify partial fragments of the L1, the presence of numerous putative new BPV types have been described in cattle herds from diverse geographical regions. Recently, an investigation using the strategy above mentioned revealed notable diversity among BPVs detected in papillomas from Brazilian cattle herds. The study identified four putative new BPV types designated as BPV/BR-UEL2 to BPV/BR-UEL5. This study describes the complete genomic sequence and phylogenetic position of one of these novel BPV types (BPV/BR-UEL4) isolated from cattle: the bovine papillomavirus type 13. Additionally, it reports the involvement of this new BPV type in equine sarcoid lesions. In horses, six sarcoids which were individually and surgically collected from diverse anatomic locations of two horses were tested with eight degenerate primer pairs, designed to detect conserved regions on L1 and E1 genes of a broad-spectrum of diverse cutaneous PV strains. The obtained amplicons were directly sequenced. In the evaluated equine sarcoids, the four different sequences generated from obtained amplicons were shown to present 100% identity with the BPV13, the new BPV type identified from cutaneous warts of cattle. BPV13 was isolated from a cutaneous papilloma of the ear of a cow from a herd located in South region of Brazil. In order to amplify the total genome sequence of the BPV13, the PV genome was submitted to multiply-primed rolling circle amplification (RCA) technique. To confirm that the band was indeed PV DNA, PCR with degenerate primers specific for cutaneous PVs was performed on the RCA product. These PCR products were sequenced and based on the sequences generated, two primer sets for long template PCR were chosen in the partial obtained E1 and L1 sequences in order to amplify the most part of genome of the BPV13. The complete nucleotide sequences of the cloned long PCR products were determined by primer-walking sequencing, starting from the universal primers in the pCR4-TOPO vector. The remaining gaps in the sequences were determined by primer-walking directly on the RCA product. The complete genomic sequence of the BPV13 counts 7961 bp, with a GC content of 45.1%. BPV13 contains eight PV ORFs, coding for six early (E) proteins E1, E2, E4, E5, E6, and E7, and two late (L) proteins L1 and L2. The phylogenetic analysis using a concatenated nucleotide sequence alignment of E7, E1, E2, L2 and L1 ORFs of BPV13 isolated from a cow and other ungulate PVs showed that BPV13 is a member of the genus *Deltapapillomavirus*, closely related to BPV1and BPV2. The entire L1 ORF of BPV13 had 85.5–88.2% sequence

identity, respectively, with Delta-PVs BPV1and 2. The identification of BPV13 in sarcoid lesions herein examined gives rise to the hypothesis that many PV isolates from sarcoid lesions previously diagnosed worldwide through PCR employing BPV1/2 specific primers were misclassified as BPV2, specially due to the direct sequencing of numerous amplicons were not performed. As it has been described for BPVs 1 and 2-induced lesions, it is tempting to suppose that BPV13 is also capable of inducing fibropapillomas in its natural hosts. The grouping of the BPV13 in Deltapapillomavirus genus, which lump fibropapilloma-inducing artiodactyls ruminant PVs together, as well as the finding of the E5 ORF in BPV13 genome and the lack of the pRB-binding motif in E7 encoded protein, support this hypothesis. PVs have a species-specific nature, with interspecies transmission being very rare event and only occurring between closely related species, such as infection of horses with BPV1 and 2. Like other Delta4 representatives, the novel BPV type was detected in association with cases of equine sarcoid in Brazilian herds. The characterization of novel BPVs improves our understanding on PV phylogeny and our interpretation of the described pathologies related to these viruses. Additional information about the molecular basis and epidemiology of characterized and uncharacterized BPVs will be needed to fully elucidate pathogenecity regarding diseases associated with BPV.

Key Words: Bovine, Equine, Cutaneous papillomatosis, Sarcoid, BPV, New BPV type, Complete genome, Phylogenetic analysis, Histopathology.

LISTA DE FIGURAS

Revisão de literatura

Fig.1. Micrografia eletrônica de vírions do papilomavírus bovino 1 (BPV1)........................ 16

Fig.2. Representação esquemática do genoma do papilomavírus bovino 1 (BPV1) 23

Fig.3. Reconstrução filogenética baseada nas sequências nucleotídicas do gene L1 dos 12 tipos de BPV.. 31

Genetic characterization of a novel bovine papillomavirus member of the *Deltapapillomavirus* genus

Fig.1. Diagrammatic genomic organization of BPV13 ……………………………………. 61

Fig.2. Amino acid sequence alignments of artiodactyl PVs' E6, E7, and E5 proteins, respectively …………………………………………...……………………….. 62

Fig.3. Neighbor-joining phylogenetic tree of concatenated nucleotide sequence of E7, E1, E2, L2 and L1 ORFs of ungulate PVs, including that of the BPV13 ……..………… 63

A new bovine delta papillomavirus type associated with equine sarcoid tumors

Fig.1. Histological features characteristic of equine sarcoid ...………………………….. 85

Fig.2. Neighbor-joining phylogenetic tree reconstructed from E1/L1 combined sequences (nt) of BPVs, including that of the BPV/BR-UEL4 isolate obtained from equine sarcoid DNA samples ………..…………………………………….… 86

LISTA DE TABELAS

Revisão de literatura

Tabela 1. Relação entre os diferentes níveis taxonômicos e a identidade observada na sequência de nucleotídeos da ORF L1 18

Tabela 2. Classificação da família *Papillomaviridae*.. 19

Tabela 3. Espécies de papilomavírus que infectam animais domésticos 21

Genetic characterization of a novel bovine papillomavirus member of the *Deltapapillomavirus* genus

Table 1. Sequences and features of degenerate primers used for PCR amplification of partial sequences of the RCA product ………………………………………………… 59

Table 2. Identities shared among Delta-PVs on the L1 nucleotide sequence ……………... 64

A new bovine delta papillomavirus type associated with equine sarcoid tumors

Table 1. Anatomical distribution and pathological classification of evaluated sarcoids ….. 82

Table 2. Sequences and features of degenerate primers used for PCR amplification of partial sequences of BPV/BR-UEL4 strain ……………………………………… 83

SUMÁRIO

1. REVISÃO DE LITERATURA 15

2. OBJETIVOS

2.1. Objetivo Geral 41
2.2. Objetivos Específicos 41

3. ARTIGO PARA PUBLICAÇÃO

Genetic characterization of a novel bovine papillomavirus member of the *Deltapapillomavirus* genus

Introduction 46
Materials and Methods 47
DNA extraction 47
DNA amplification 47
Molecular cloning and DNA sequencing 49
Phylogenetic Analysis 50
Results 50
Discussion 52
References 56

A new bovine delta papillomavirus type associated with equine sarcoids

Introduction 67
Materials and Methods 68
Sarcoidal lesions 68
DNA extraction 69
PCR amplification 69
Sequencing and sequence analyses 70
Phylogenetic analysis 70
Results 71
Discussion 72
References 77

4. CONCLUSÕES 88

APÊNDICES

A. Lista de reagentes 90
B. Soluções e tampões 91
C. Protocolo de técnicas 92

ANEXOS

A. Sequência nucleotídica completa do BPV13.. 95
B. Representação da organização do genoma do BPV13... 99
C. Código das bases degeneradas 100
C. Reconstrução filogenética com os 13 representantes de BPV............................... 101

1. REVISÃO DE LITERATURA

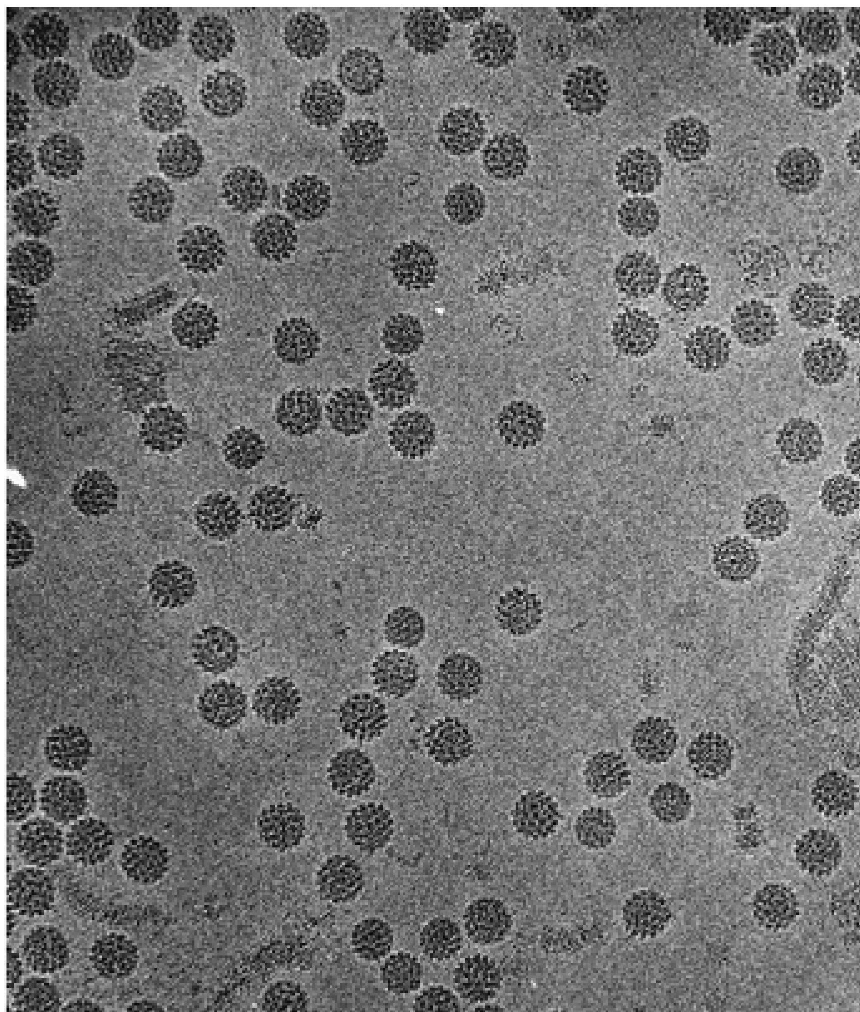
1. REVISÃO DE LITERATURA

a) Introdução

O papilomavírus (PV) é um vírus epiteliotrópico que determina lesões proliferativas benignas tanto na pele (verrugas ou papilomas) quanto em mucosas (condilomas) de seus hospedeiros naturais. Entretanto, também tem sido atribuído a alguns PVs o desenvolvimento de lesões epiteliais malignas, especialmente casos de câncer do cérvix uterino e outros tumores do trato urogenital em humanos (Howley e Lowy, 2007).

O primeiro oncovírus DNA reconhecido corresponde ao primeiro PV animal identificado. Este vírus, denominado CRPV, foi detectado na década de 30 a partir de verrugas cutâneas em coelhos cauda de algodão (do inglês, *cottontail rabbits*) (Shope e Hurst, 1933).

Os PVs são vírus pequenos, não-envelopados e icosaédricos que replicam no núcleo de células epiteliais escamosas. As partículas virais apresentam diâmetro de 52 a 55 nanômetros (figura 1) (Howley e Lowy, 2007).

1 

2 **Figura 1.** Micrografia eletrônica de vírions do papilomavírus bovino 1 (BPV1) (55nm de
3 diâmetro). Fonte: Howley e Lowy, 2007.

4

5 Atualmente, o PV é tido como um grupo de vírus DNA altamente diverso, tendo sido
6 demonstrado em numerosas espécies de mamíferos e em algumas aves e répteis. Além dos
7 seres humanos, os PVs foram identificados na maioria dos animais domésticos, como nos
8 bovinos (BPVs); cães (CPVs); caprinos (ChPV1); equinos (EcPVs); felinos domésticos
9 (FdPVs); ovinos (OaPVs); e suínos (SsPV1) (Bernard et al., 2010).

10

11 **b) Classificação taxonômica dos PVs**

1 Originalmente, os PVs foram agrupados com os poliomavírus na família
2 *Papovaviridae*. Esta classificação inicial foi justificada pela presença de características
3 comuns aos dois vírus, tais como capsídeo não-envelopado semelhante e genoma circular
4 constituído de DNA fita dupla. Atualmente, devido ao reconhecimento de que estes grupos de
5 vírus possuem genomas com extensões e organizações diferentes e similaridades limitadas nas
6 sequências de nucleotídeos e aminoácidos, os PVs estão classificados na família
7 *Papillomaviridae* (van Regenmortel et al., 2002; de Villiers et al., 2004).
8 Os PVs são mencionados, tradicionalmente, como "tipos virais", sendo que o tipo viral
9 representa um genoma completo, cuja sequência nucleotídica do gene L1 apresenta ao menos
10 10% de dissimilaridade com a de qualquer outro tipo de PV anteriormente reconhecido (de
11 Villiers et al., 2004).
12 A classificação dos PVs em gênero unifica espécies relacionadas filogeneticamente
13 que diferem quanto às propriedades biológicas, enquanto a classificação em espécie agrupa
14 tipos virais muito próximos filogeneticamente e que apresentam características biológicas e
15 patológicas comuns. A tradução destas relações taxonômicas para a identidade observada
16 entre sequências de nucleotídeos ocorre da seguinte forma: i) Diferentes gêneros
17 compartilham similaridade inferior a 60% na ORF L1 e entre 23 e 43% quando sequências
18 genômicas completas são comparadas; ii) Espécies diversas em um gênero apresentam
19 similaridades entre 60 e 70% na ORF L1 (tabela 1) (de Villiers et al., 2004).
20
21
22
23
24

Tabela 1. Relação entre os diferentes níveis taxonômicos e a identidade observada na sequência de nucleotídeos da ORF L1.

Nível Taxonômico	Identidade ORF L1
gênero	<60%
espécie	60-70%
tipo viral	71-89%

Fonte: De Villiers *et al.*, 2004.

Atualmente, a família *Papillomaviridade* contém 29 diferentes gêneros nos quais quase 200 tipos de PV estão classificados. O alfabeto grego foi utilizado na criação da nomenclatura dos gêneros que são denominados de *Alphapapillomavirus* a *Dyoiotapapillomavirus*. As espécies são designadas pelo tipo viral que as representam, sendo que os outros tipos de PV classificados nas mesmas são chamados de cepas virais (tabela 2).

Tabela 2. Classificação da família *Papillomaviridae*.

Gênero	Espécies
Alphapapillomavirus	*Human Papillomavirus 2, 6, 7, 10, 16, 18, 26, 32, 34, 53, 54, 61, 90*
	Macaca mulata Papillomavirus 1
Betapapillomavirus	*Human Papillomavirus 5, 9, 49, 92, 96*
	Macaca fascicularis Papillomavirus 2
Gammapapillomavirus	*Human Papillomavirus 4, 48, 50, 60, 88, 101, 109, 112, 116, 121*
Deltapapillomavirus	*Alces alces Papillomavirus 1*
	Bos taurus Papillomavirus 1
	Capreolus capreolus Papillomavirus 1
	Odocoileus virginianus Papillomavirus 1
	Ovis aries Papillomavirus 1
Epsilonpapillomavirus	*Bos taurus Papillomavirus 5*
Zetapapillomavirus	*Equus caballus Papillomavirus 1*
Etapapillomavirus	*Fringilla coelebs Papillomavirus*
Thetapapillomavirus	*Psittacus erithacus Papillomavirus 1*
Iotapapillomavirus	*Mastomys natalensis Papillomavirus 1*
Kappapapillomavirus	*Oryctolagus cuniculus Papillomavirus 1*
	Sylvilagus floridanus Papillomavirus 1
Lambdapapillomavirus	*Canis familiaris Papillomavirus 1* e *6*
	Felis domesticus Papillomavirus 1
	Procyon lotor Papillomavirus 1
Mupapillomavirus	*Human Papillomavirus 1* e *63*
Nupapillomavirus	*Human papillomavirus 41*
Xipapillomavirus	*Bos taurus Papillomavirus 3*
Pipapillomavirus	*Mesocricetus auratus Papillomavirus 1*
	Micromys minutus Papillomavirus 1
Rhopapillomavirus	*Trichechus manatus latirostris Papillomavirus 1*
Sigmapapillomavirus	*Erethizon dorsatum Papillomavirus 1*
Taupapillomavirus	*Canis familiaris Papillomavirus 2*
Upsilonpapillomavirus	*Tursiops truncatus Papillomavirus 1* e *2*
Phipapillomavirus	*Capra hircus Papillomavirus 1*
Chipapillomavirus	*Canis familiaris Papillomavirus 3* e *4*
Psipapillomavirus	*Rousettus aegyptiacus Papillomavirus 1*

Tabela 2. Classificação da família *Papillomaviridae* (continuação).

Gênero	**Espécies**
Omegapapillomavirus	*Ursus maritimus Papillomavirus 1*
Dyodeltapapillomavirus	*Sus scrofa Papillomavirus 1*
Dyoepsilonpapillomavirus	*Francolinus leucoscepus Papillomavirus 1*
Dyozetapapillomavirus	*Caretta caretta Papillomavirus 1*
Dyoetapapillomavirus	*Erinaceus europaeus Papillomavirus 1*
Dyothetapapillomavirus	*Felis domesticus Papillomavirus 2*
Dyoiotapapillomavirus	*Equus caballus Papillomavirus 2*

Fonte: Bernard *et al.* (2010).

Os PVs isolados de animais vertebrados estão classificados em 24 gêneros, enquanto as espécies virais que ocorrem exclusivamente em aves e répteis estão agrupadas em três e um gêneros. A designação taxonômica para os tipos de PV animais baseia-se no nome científico do hospedeiro, utilizando-se a indicação do gênero e espécie do mesmo, tendo-se como exemplo a denominação FdPV1 para o PV tipo 1 do *Felis domesticus* (Bernard et al., 2010). A tabela 3 apresenta os gêneros e espécies nos quais estão classificados os PVs identificados em espécies de animais domésticos.

1 **Tabela 3.** Espécies de papilomavírus que infectam animais domésticos.

Gênero	Espécie	Cepas Virais
Deltapapillomavirus	*Bos taurus Papillomavirus 1*	*Bos taurus Papillomavirus 1*
		Bos taurus Papillomavirus 2
	Ovis aries Papillomavirus 1	*Ovis aries Papillomavirus 1*
		Ovis aries Papillomavirus 2
Epsilonpapillomavirus	*Bos taurus Papillomavirus 5*	*Bos taurus Papillomavirus 5*
		Bos taurus Papillomavirus 8
Zetapapillomavirus	*Equus caballus Papillomavirus 1*	*Equus caballus Papillomavirus 1*
Lambdapapilloamvirus	*Canis familiaris Papillomavirus 1*	*Canis familiaris Papillomavirus 1*
	Canis familiaris Papillomavirus 6	*Canis familiaris Papillomavirus 6*
	Felis domesticus Papillomavirus 1	*Felis domesticus Papillomavirus 1*
Xipapillomavirus	*Bos taurus Papillomavirus 3*	*Bos taurus Papillomavirus 3*
		Bos taurus Papillomavirus 4
		Bos taurus Papillomavirus 6
		Bos taurus Papillomavirus 9
		Bos taurus Papillomavirus 10
		Bos taurus Papillomavirus 11
		Bos taurus Papillomavirus 12
Taupapillomavirus	*Canis familiaris Papillomavirus 2*	*Canis familiaris Papillomavirus 2*
		Canis familiaris Papillomavirus 7
Phipapillomavirus	*Capra hircus Papillomavirus 1*	*Capra hircus Papillomavirus 1*
Chipapillomavirus	*Canis familiaris Papillomavirus 3*	*Canis familiaris Papillomavirus 3*
		Canis familiaris Papillomavirus 5
	Canis familiaris Papillomavirus 4	*Canis familiaris Papillomavirus 4*
Dyodeltapapillomavirus	*Sus scrofa Papillomavirus 1*	*Sus scrofa Papillomavirus 1*
Dyothetapapillomavirus	*Felis domesticus Papillomavirus 2*	*Felis domesticus Papillomavirus 2*
Dyoiotapapillomavirus	*Equus caballus Papillomavirus 2*	*Equus caballus Papillomavirus 2*
-	-	*Bos taurus Papillomavirus 7*

2 Fonte: Adaptado de Bernard *et al.* (2010).

3

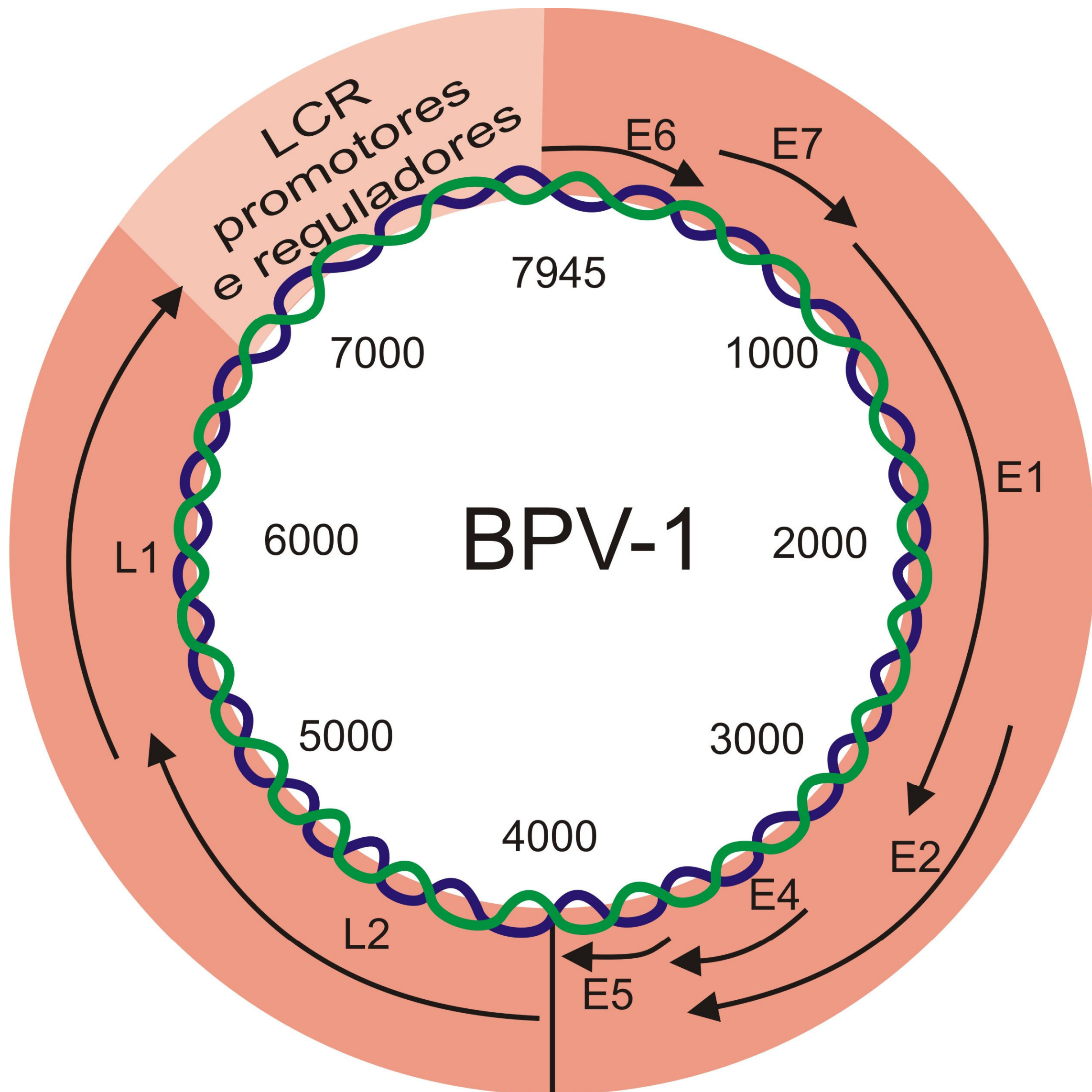
4 Devido ao fato dos PVs não serem passíveis de isolamento por técnicas clássicas de
5 cultivo celular e não gerarem resposta imune humoral intensa no respectivo hospedeiro, a
6 aplicação dos termos taxonômicos “cepa” e “sorotipo” não foi, inicialmente, implementada
7 para esta família viral. Consequentemente, sua classificação baseou-se nas similaridades das

sequências de nucleotídeos e em limitadas propriedades biológicas e médicas (de Villiers et al., 2004; Fauquet et al., 2005).

c) Organização genômica

Nos anos 70, a clonagem de genomas de PVs contribuiu substancialmente no estudo de suas propriedades biológicas e bioquímicas. O sequenciamento dos genomas clonados tornou possível a identificação dos quadros abertos de leitura (ORFs – *open reading frames*) como prováveis genes virais (Danos et al., 1982).

A organização genômica dos diversos PVs é muito similar. Uma característica compartilhada pelos PVs é o fato de todas as ORFs estarem contidas em uma das fitas do DNA viral, demonstrando que somente uma das fitas desempenha a função de molde na transcrição. A fita codificante pode apresentar até 10 ORFs que são classificadas, segundo a fase da diferenciação celular em que são expressas, em iniciais (E – *early*) e tardias (L – *late*). O segmento inicial do genoma (E) é constituído por até oito ORFs que são expressas em células epiteliais em fase inicial de maturação, enquanto o segmento final (L) geralmente contém duas ORFs que são expressas em queratinócitos diferenciados. Uma terceira região, a qual é desprovida de ORFs, tem sido identificada em todos os genomas dos PV. Esta região tem sido denominada de LCR (*long control region*) ou URR (*upstream regulatory region*) e contém tanto a origem de replicação quanto elementos de controle da transcrição (figura 2) (Howley e Lowy, 2007).



1

2 **Figura 2.** Representação esquemática do genoma do papilomavírus bovino 1 (BPV1).

3

4 A expressão das seis proteínas não-estruturais e regulatórias mais comuns (E1, E2, E4,
5 E5, E6 e E7), codificadas pela região inicial do genoma viral, ocorre em células basais ou em
6 etapas intermediárias de maturação. A expressão das duas proteínas estruturais virais (L1 e
7 L2), codificadas pelo segmento final do genoma, ocorre em queratinócitos em fase final de
8 maturação (Zheng e Baker, 2006).

9

d) Proteínas virais

1) Proteínas E1 e E2

A proteína E1 é codificada pela maior ORF encontrada no segmento inicial dos genomas dos PVs. Na comparação das sequências inferidas para as proteínas codificadas pelo gene E1, foi observada homologia significativa entre os diferentes PVs (Wilson et al., 2002).

Conjuntamente com a proteína viral E2, a proteína E1 reconhece a origem de replicação, representando o fator central da replicação dos PVs, sem a qual o mecanismo de replicação do DNA viral não poderia ser iniciado. Além desta função principal, a E1 está envolvida no recrutamento de proteínas da replicação da célula hospedeira, apresenta atividade intrínseca de ATPase/helicase, que proporciona tanto o relaxamento da torção da molécula de DNA na região da origem, quanto a progressão da abertura da forquilha de replicação (Wilson et al., 2002).

A realização de estudos de transformação com o BPV1 demonstrou que a presença da ORF E1, na forma intacta, é fundamental para que ocorra a manutenção estável do genoma viral na célula, por meio de múltiplas cópias do genoma na forma epissomal (Sarver et al., 1984; Lusky et al., 1985; Rabson et al., 1986).

No entanto, a interação da proteína E1 com a origem de replicação se dá com baixa especificidade. O reconhecimento específico e eficiente da origem é obtido somente através da ligação cooperativa das proteínas E1 e E2 aos sítios adjacentes a origem. Portanto, a proteína E2 participa neste mecanismo como um fator de agregação que promove o recrutamento da helicase E1 na origem de replicação (Sanders e Stenlund, 2001).

A ORF E2 do BPV1 codifica uma família de proteínas que compõem o sistema regulatório central do vírus, exercendo controle na expressão gênica e replicação viral. A proteína E2 também modula a transcrição dos promotores virais iniciais através dos seus sítios de ligação (Spalholz et al., 1985). Além disso, a E2 participa da manutenção do genoma

1 viral, na sua forma epissomal, por meio da ligação que promove entre estes genomas e os
2 cromossomos mitóticos durante a divisão celular (Ilves et al., 1999; Lehman e Botchan,
3 1998).
4 *2) Proteína E4*
5 A proteína não-estrutural E4 é encontrada abundantemente no citoplasma de
6 queratinócitos diferenciados dos papilomas. Portanto, embora o gene que codifica para esta
7 proteína esteja localizado na região inicial do genoma viral, a produção da E4 ocorre
8 tardiamente. A proteína E4 do HPV16 tem sido associada ao colapso dos filamentos de
9 citoqueratina, sugerindo a função de auxiliar no processo de saída do vírus da célula (Howley
10 e Lowy, 2007).
11 *3) Proteínas E5, E6 e E7*
12 Em humanos, as oncoproteínas E5, E6 e E7, codificadas pelos genomas de alguns
13 HPVs, representam os fatores virais primários relacionados ao início e progressão do câncer
14 do cérvix uterino. Tais produtos gênicos, têm se mostrado capazes de ultrapassar as barreiras
15 do processo de regulação negativa do crescimento celular, mediados por proteínas das células
16 hospedeiras. Além disso, acredita-se que estas oncoproteínas virais promovam a instabilidade
17 genômica que é observada nos cânceres associados à infecção pelo HPV (Moody e Laimins,
18 2010).
19 A principal maneira pela qual a proteína E7 colabora no escape da célula infectada dos
20 mecanismos de regulação negativa do crescimento celular é através da sua ligação com
21 proteínas membros da família retinoblastoma. No caso do HPV, a E7 interage com estes
22 fatores celulares, direcionando-os à degradação (Dyson et al., 1989). O resultado desta ligação
23 é a liberação e ativação dos fatores da transcrição E2F que comandam a expressão dos genes
24 da fase S do ciclo celular. A interação eficiente da E7 com estes fatores desencadeia a inibição

1 compensatória do crescimento celular e apoptose, mediadas pela via dependente da proteína
2 supressora de tumores p53 (Moody e Laimins, 2010).
3 O encaminhamento da proteína p53 para a degradação, conduzido pela proteína viral
4 E6 de HPVs de alto risco, evita a inibição do crescimento celular tanto em células
5 indiferenciadas quanto nas diferenciadas (Moody e Laimins, 2010). A ação destes dois
6 produtos virais, E6 e E7, na inviabilização destes fatores regulatórios do ciclo celular, permite
7 que células infectadas em processo de diferenciação permaneçam na fase S. Como resultado
8 deste processo, tem-se o cancelamento de muitos pontos de checagem do ciclo celular. Como
9 consequência, o acúmulo de mutações e progressão ao câncer ocorrem nas células
10 persistentemente infectadas por estes vírus (Duensing e Munger, 2004).
11 Em bovinos com hematúria enzoótica, tem-se a expressão da oncoproteína E5 do
12 BPV2 na maioria dos tumores de animais afetados (Campo et al., 1992; Borzacchiello et al.,
13 2003a; Roperto et al., 2008). O início da transformação celular desencadeada pela E5 deve
14 ocorrer, principalmente, por meio de sua interação e ativação do receptor β do fator de
15 crescimento derivado de plaquetas (PDGF). Desta maneira, faz-se o início de uma resposta
16 mitogênica que ocorre mesmo na ausência de PDGF (DiMaio e Mattoon, 2001; Borzacchiello
17 et al., 2006).
18 *4) Proteínas L1 e L2*
19 O capsídio viral é constituído pelas duas proteínas estruturais (L1 e L2). A principal
20 proteína do capsídio (L1) tem aproximadamente 55 kD e representa cerca de 80% da
21 quantidade total de proteína do vírus. Por outro lado, a proteína L2 tem tamanho molecular
22 aproximado de 70 kD (Frave, 1975). Além disso, sabe-se que partículas semelhantes ao vírus
23 (VLP – *virus-like particles*) podem ser produzidas a partir de diferentes PVs somente pela
24 expressão da L1 ou pela combinação da L1 com a L2, utilizando tanto sistemas de expressão
25 protéica de mamíferos quanto outros sistemas eucarióticos e procarióticos (Rose et al., 1993;

1 Zhou et al., 1993). Embora a L2 não seja necessária para o processo de montagem viral, ela é
2 incorporada em VLPs quando co-expressa com a L1. Quando visualizadas por microscopia
3 crioeletrônica, a morfologia das VLPs, contendo somente a L1, parece ser idêntica à das
4 partículas virais intactas (Hagensee et al., 1994). Os epítopos que induzem a produção de
5 anticorpos neutralizantes estão presentes principalmente na L1, sendo também localizados na
6 L2 (Roden et al., 1994).
7
8 **e) BPVs**
9 A infecção por diversos tipos de papilomavírus bovino (BPV – *bovine papillomavirus*)
10 tem sido relacionada a diferentes quadros clínicos em bovinos. A ocorrência de tumores
11 cutâneos benignos, caracterizando a papilomatose cutânea, pode ser verificada em diversas
12 regiões do corpo do animal. Na dependência da extensão das lesões, há o comprometimento
13 do desenvolvimento corporal dos animais, predisposição a infecções e/ou infestações
14 secundárias, depreciação do couro, entre outras consequências que podem acarretar prejuízos
15 econômicos à exploração pecuária de corte e, principalmente, leiteira. Papilomas localizados
16 no úbere e nos tetos de vacas em lactação dificultam a amamentação dos bezerros, a ordenha
17 tanto manual quanto, principalmente, mecânica e, devido às infecções bacterianas
18 secundárias, predispõem a mastites clínicas e/ou subclínicas ascendentes (Campo, 2002).
19 A interação entre tipos específicos do BPV e a ingestão prolongada da planta
20 samambaia (*Pteridium aquilinum*), tem sido apontada como a etiologia tanto da hematúria
21 enzoótica crônica quanto do câncer do trato gastrointestinal superior em bovinos. No primeiro
22 caso, acredita-se que inicialmente ocorra uma infecção latente ou subclínica pelo BPV1 ou 2
23 na mucosa da bexiga. Uma vez que a bexiga urinária representa o maior alvo das toxinas desta
24 planta, após o estabelecimento do vírus, a infecção seria reativada e induzida à neoplasia por

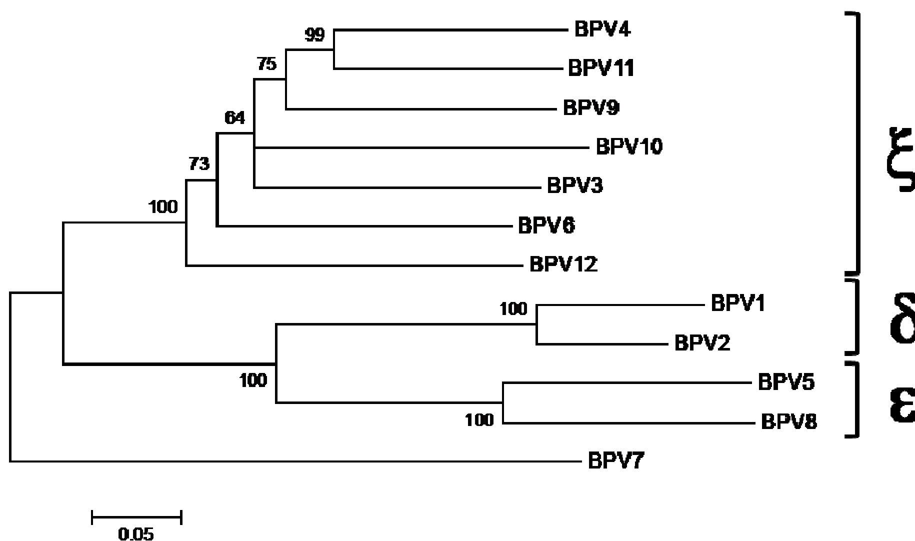
1 meio de compostos químicos imunossupressivos e carcinogênicos presentes na samambaia,
2 culminando na progressão à malignidade (Roperto et al., 2010).
3 A incidência destes tumores é variável entre rebanhos alocados em pastos
4 contaminados pela samambaia, porém este índice pode alcançar percentuais maiores que 90
5 em animais adultos (Pamukcu et al.,1976; Roperto et al., 2010).
6 Quanto aos tumores do canal alimentar, a imunossupressão causada pela ingestão da
7 samambaia define a persistência dos papilomas, induzidos pelo BPV4, que por ação de
8 elementos carcinogênicos da planta podem evoluir para carcinomas malignos (Campo et al.,
9 1994, Campo, 1997; Borzacchiello et al., 2003b).
10 Portanto, embora a infecção por estes BPVs tenha participação central na patogênese
11 destas neoplasias em bovinos, a presença de co-fatores ambientais e biológicos é fundamental
12 no desenvolvimento das lesões (Roperto et al., 2006, 2008).
13 Enquanto cerca de 120 tipos de HPVs já foram definidos, até o início da década de
14 1980 apenas seis tipos de BPV (BPV1 a BPV6) tinham sido identificados a partir de casos de
15 papilomatose cutânea e câncer em bovinos (Pfister et al., 1979; Campo et al., 1980, 1981;
16 Campo e Coggins, 1982; Chen et al., 1982; Jarrett et al., 1984).
17 Entretanto, estudos realizados a partir do início da década passada, com o objetivo de
18 investigar a real diversidade do BPV, têm indicado a existência de numerosos tipos de BPV, a
19 exemplo do que ocorre nos seres humanos. O primeiro trabalho realizado envolveu o emprego
20 dos *primers* genéricos FAP59/FAP64 em amostras (*swabs)* provenientes de pele saudável de
21 animais pertencentes a 19 espécies de vertebrados. Dentre os dez bovinos analisados, e que
22 não apresentavam qualquer sinal clínico compatível com a infecção pelo BPV, em seis foram
23 detectados um ou dois prováveis novos tipos de BPV. Estes prováveis novos tipos virais
24 foram denominados BAA1 a BAA5 (Antonsson e Hansson, 2002).

Posteriormente, visando à determinação da prevalência do BPV em papilomas e pele saudável de tetos, um estudo que envolveu a utilização dos *primers* FAP59/FAP64 e MY09/MY11, analisou 15 papilomas de teto e 122 *swabs* de pele saudável de animais, provenientes de cinco municípios do Japão (Ogawa et al., 2004). Neste estudo, quatro tipos previamente caracterizados de BPV (BPV1, 3, 5 e 6), dois prováveis novos tipos de BPV anteriormente identificados (BAA1 e 5) e 11 prováveis novos tipos (designados BAPV1 a 10 e BAPV11MY) foram encontrados nas 39 amostras positivas para o BPV. Porém, os prováveis novos tipos BAA1 e BAPV7 a 10 foram somente detectados a partir de amostras obtidas de pele saudável. Adicionalmente, em um surto de papilomatose mamária (tetos), ocorrido no Japão em um rebanho constituído por 560 novilhas, foi confirmada a presença do BPV6 na maioria das 14 amostras analisadas (Maeda et al., 2007). Nessa amostragem também foram identificados os prováveis novos tipos BAA5 e BAPV1, anteriormente descritos.

Recentemente, a realização de análises complementares de alguns prováveis novos tipos de BPV, por meio da determinação da sequência completa do genoma viral, permitiu a caracterização de novos tipos virais (Bernard et al., 2010). O primeiro novo tipo caracterizado foi o BPV7, inicialmente denominado BAPV6. Como a sequência de nucleotídeos da ORF L1 do BPV7 foi mais relacionada àquela de PVs membros dos gêneros *Betapapillomavirus*, *Gamapapillomavirus* e *Pipapillomavirus*, os quais contêm representantes responsáveis por lesões cutâneas em humanos e em mucosa de hamsters, este novo tipo de BPV, constituiu um novo gênero ainda não nominado (Ogawa et al., 2007).

O segundo tipo de BPV recentemente descrito foi o BPV8, anteriormente designado como BAPV2 e identificado no Japão. A definição deste novo tipo viral foi realizada juntamente com a descrição da sua variante denominada BPV8-EB, proveniente de um caso de papilomatose cutânea em um bisão europeu nascido na Itália (Tomita et al., 2007). Nesta análise, a elevada similaridade observada entre as sequências da ORF L1 do BPV8 e do BPV5

1 (75%), assim como a análise filogenética, constituíram a base para a classificação deste novo
2 tipo viral no gênero *Epsilonpapillomavirus*. Adicionalmente, a estrutura genômica das regiões
3 inicial e tardia destes dois diferentes membros do gênero se mostrou quase idêntica, diferindo
4 apenas na ORF E4, que está presente no BPV8 e ausente no BPV5.
5 Recentemente, dois tipos de BPV, os BPVs 9 e 10, foram identificados a partir de
6 papilomas de tetos (Hatama et al., 2008). Inicialmente estes tipos virais foram denominados
7 prováveis novos tipos BAPV1 e BAA5 (Antonsson e Hansson, 2002; Ogawa et al., 2004). A
8 análise filogenética e a constatação da maior similaridade na ORF L1 com o BPV3 (74,2 e
9 71,2%, respectivamente) classificaram os dois novos isolados no gênero *Xipapillomavirus*
10 (Hatama et al., 2008).
11 Por meio de PCR, clonagem e sequenciamento, Hatama et al. (2011) avaliaram os
12 genótipos virais presentes em 167 verrugas cutâneas coletadas de rebanhos bovinos japoneses.
13 Entre as lesões avaliadas, 124 apresentaram resultado positivo para a presença do BPV na
14 PCR. Das sequências parciais obtidas no sequenciamento dos produtos de PCR obtidos, três
15 prováveis novos tipos de BPV, assim como oito tipos de BPV anteriormente descritos (BPVs
16 1, 2, 3, 4, 5, 6, 9 e 10), foram identificados. A determinação da sequência completa de um
17 destes prováveis novos tipos de BPV (BPV11), e a comparação da sua sequência nucleotídica
18 no gene L1 com a de outros representantes desta família viral, determinou a sua classificação
19 no gênero *Xipapillomavirus* (Hatama et al., 2011). A sequência completa do genoma de outro
20 isolado, identificado a partir de lesão do epitélio da língua de bovino do Japão, foi
21 recentemente determinada, culminando na designação deste isolado como BPV12 (Zhu et al.,
22 2011). A comparação da sequência de nucleotídeos do gene L1 do BPV12 com outros tipos
23 virais isolados de bovinos sugere sua classificação também no gênero *Xipapillomavirus*
24 (figura 3).
25



1

2 **Figura 3.** Reconstrução filogenética baseada nas sequências nucleotídicas do gene L1 dos 12

3 tipos de BPV. Com exceção do BPV7, os gêneros nos quais os diversos tipos virais estão

4 classificados estão representados pelas letras gregas δ (delta), ε (épsilon) e ξ (xi).

5

6 No Brasil, a associação entre a infecção pelo BPV e a ocorrência de papilomatose

7 cutânea, hematúria enzoótica e neoplasias do trato digestório superior, tem sido confirmada

8 em bovinos (Dos Santos et al., 1998; Claus et al., 2007, 2008; Wosiacki et al., 2005). Estudos

9 anteriormente realizados, empregando *primers* específicos para um único tipo viral,

10 demonstraram a presença do BPV1 em lesões cutâneas, sangue total e plasma sanguíneo de

11 animais adultos com papilomatose cutânea, e em placenta e líquido amniótico provenientes de

12 uma vaca apresentando o mesmo quadro clínico (De Freitas et al., 2003). O BPV2 foi

13 identificado em sangue periférico e bexiga urinária de animais com hematúria enzoótica, e em

14 amostras de papilomas e do trato reprodutivo feminino de bovinos (De Carvalho et al., 2003;

15 Wosiacki et al., 2005, 2006).

16 Apesar da papilomatose cutânea representar um problema sanitário considerável, tanto

17 para rebanhos bovinos de corte quanto, principalmente, leiteiros, a realização de estudos

1 visando à identificação dos tipos de BPV envolvidos na determinação de lesões cutâneas em
2 bovinos no Brasil ainda é esporádica. Recentemente, a detecção do BPV1, 2, 6 e 8 em
3 papilomas provenientes de rebanhos bovinos do Estado do Paraná foi possível por meio da
4 utilização dos *primers* genéricos FAP (Claus et al., 2007, 2009). Em outro estudo, a
5 identificação de quatro prováveis novos tipos de BPV, denominados BPV/BR-UEL2 a 5,
6 ainda não descritos no mundo, apontou para a ocorrência de considerável diversidade viral
7 nos rebanhos bovinos brasileiros (Claus et al., 2008). A caracterização genética de um destes
8 prováveis novos tipos de BPV, o BPV/BR-UEL2, por meio do sequenciamento da sequência
9 completa do gene L1, confirmou a sua classificação no gênero *Xipapillomavirus* (Lunardi et
10 al., 2010).
11
12 **f) EcPVs**
13 Em cavalos, a infecção pelo PV tem sido descrita extensivamente no contexto do
14 sarcóide equino (Otten et al., 1993; Bloch et al., 1994; Yuan et al., 2007; Wobeser et al.,
15 2010). Sarcóides podem ser definidos como tumores de pele fibroblásticos que são localmente
16 invasivos. Estes tumores representam a neoplasia mais comum em eqüinos, com taxas de
17 prevalência variando entre 12,9 e 67% dentre todos os tipos de tumores equinos (Sullins et al.,
18 1986). Após muita especulação quanto ao papel do PV como agente etiológico do sarcóide
19 equino, atualmente, tem-se muitos relatos na literatura demonstrando o envolvimento do BPV
20 tipos 1 e 2 na patogênese deste tumor (Otten et al., 1993; Bloch et al., 1994; Nasir e Reid,
21 1999; Yuan et al., 2007; Wobeser et al., 2010) . Contudo, uma vez que os PVs são tidos como
22 altamente espécie-específicos, devido à associação com o BPV, esta neoplasia cutânea tem
23 sido considerada uma exceção dentre as afecções determinadas pelos PVs (Howley e Lowy,
24 2007).

Além do sarcóide equino, ao menos três outras neoplasias epiteliais potencialmente associadas com o PV, os papilomas equinos clássicos, os papilomas genitais e as placas aurais, são descritas em equinos (Scott e Miller, 2003).

Os papilomas equinos clássicos tem sido associados ao papilomavírus equino tipo 1 (EcPV1). Estas lesões cutâneas são tipicamente observadas em cavalos com menos de 3 anos de idade e ocorrem, principalmente, no focinho e lábios destes animais (Scott e Miller, 2003; Ghim et al., 2004).

A ocorrência de papilomas genitais, tanto em fêmeas quanto em machos, também tem sido relacionada à infecção pelo PV. A regressão espontânea destas lesões parece ser rara, sendo que a progressão para o carcinoma pode ocorrer ocasionalmente (Smith et al., 2009).

Os papilomas aurais ou placas aurais dos equinos têm sido descritos em animais de todas as idades. Embora tal neoplasia apresente um caráter progressivo, a evolução para o câncer não tem sido relatada (Scott e Miller, 2003).

Recentemente, por meio da caracterização genética de DNA viral purificado de um caso de papiloma peniano que evoluiu para carcinoma *in situ*, e de um caso de placa aural, foi possível o reconhecimento do envolvimento dos EcPV2 e 3 em casos de papilomas genitais e placas aurais, respectivamente, em equinos (Lange et al., 2011).

1 **REFERÊNCIAS**

2 ANTONSSON, A.; HANSSON, B.G. Healthy skin of many animal species harbors
3 papillomaviruses which are closely related to their human counterparts. *Journal of Virology*,
4 v.76, p.1253712542, 2002.
5
6 BERNARD, H.U.; BURK, R.D.; CHEN, Z.; van DOORSLAER, K.; HAUSEN, H.; DE
7 VILLIERS, E.M. Classification of papillomaviruses (PVs) based on 189 PV types and
8 proposal of taxonomic amendments. *Virology*, v.401, p.70–9, 2010.
9
10 BLOCH, N.; BREEN, M.; SPRADBROW, P.B. Genomic sequences of bovine
11 papillomaviruses in formalin-fixed sarcoids from Australian horses revealed by polymerase
12 chain reaction. *Veterinary Microbiology*, v.41, p.163–72, 1994.
13
14 BORZACCHIELLO, G.; IOVANE, G.; MARCANTE, M.L.; POGGIALI, F.; ROPERTO, F.;
15 ROPERTO, S.; VENUTI, A. Presence of bovine papillomavirus type 2 DNA and expression
16 of the viral oncoprotein E5 in naturally occurring urinary bladder tumors in cows. *Journal of*
17 *General Virology*, v.84, p.29212926, 2003a.
18
19 BORZACCHIELLO, G.; AMBROSIO, V.; ROPERTO, S.; POGGIALI, F.;
20 TSIRIMONAKIS, E.; VENUTI, A.; CAMPO, M.S.; ROPERTO, F. Bovine papillomavirus
21 type 4 in oesophageal papillomas of cattle from the south of Italy. *Journal of Comparative*
22 *Pathology*, v.128, p.203206, 2003b.
23
24 BORZACCHIELLO, G.; RUSSO, V.; GENTILE, F.; ROPERTO, F.; VENUTI, A.; NITSCH,
25 L.; CAMPO, M.S.; ROPERTO, S. Bovine papillomavirus E5 oncoprotein binds to the
26 activated form of the platelet-derived growth factor ß receptor in naturally occurring bovine
27 urinary bladder tumors. *Oncogene*, v.25, p.12511260, 2006.
28
29 CAMPO, M.S.; MOAR, M.H.; JARRETT, W.F.H.; LAIRD, H.M. A new papillomavirus
30 associated with alimentary cancer in cattle. *Nature*, v.286, p.180–182, 1980.
31
32 CAMPO, M.S.; MOAR, M.H.; LAIRD, H.M.; JARRETT, W.F.H. Molecular heterogeneity
33 and lesion site specificity of cutaneous bovine papillomaviruses. *Virology*, v.113, p.323–335,
34 1981.
35
36 CAMPO, M.S.; COGGINS, L.W. Molecular cloning of bovine papillomavirus genomes and
37 comparison of their sequence homologies by heteroduplex mapping. *Journal of General*
38 *Virology*, v.63, p.255–264, 1982.
39
40 CAMPO, M.S.; JARRETT, W.F.H.; BARRON, R.J.; O'NEIL, B.W.; SMITH, K.T.
41 Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle.
42 *Cancer Research*, v.53, p.17, 1992.
43
44 CAMPO, M.S.; O'NEIL, B.W.; BARRON, R.J.; JARRETT, W.F. Experimental reproduction
45 of the papilloma-carcinoma complex of the alimentary canal in cattle. *Carcinogenesis*, v.15,
46 p.15971601, 1994.
47
48 CAMPO, M.S. Bovine papillomavirus and cancer. *Veterinary Journal*, v.154, p.175188,
49 1997.

CAMPO, M.S. Animal models of papillomavirus pathogenesis. *Virus Research*, v.89, p.249261, 2002.

CHEN, E.Y.; HOWLEY, P.M.; LEVINSON, A.D.; SEEBURG, P.H. The primary structure and genetic organization of the bovine papillomavirus type 1 genome. *Nature*, v.299, p.529534, 1982.

CLAUS, M.P.; VIVIAN, D.; LUNARDI, M.; ALFIERI, A.F.; ALFIERI, A.A. Phylogenetic analysis of bovine papillomavirus associated with skin warts in cattle herds from the state of Paraná. *Pesquisa Veterinária Brasileira*, v.27, p.314318, 2007.

CLAUS, M.P.; LUNARDI, M.; ALFIERI, A.F.; FERRACIN, L.M.; FUNGARO, M.H.P.; ALFIERI, A.A. Identification of unreported putative new bovine papillomavirus types in Brazilian cattle herds. *Veterinary Microbiology*, v.132, p.396401, 2008.

CLAUS, M.P.; LUNARDI, M.; ALFIERI, A.F.; SARTORI, D.; FUNGARO, M.H.P.; ALFIERI, A.A. Identification of the recently described new type of bovine papillomavirus (BPV8) in Brazilian beef cattle herd. *Pesquisa Veterinária Brasileira*, v.29, p.2528, 2009.

DANOS, O.; KATINKA, M.; YANIV, M. Human papillomavirus la complete DNA sequence: A novel type of genome organization among papovaviridae. *The EMBO Journal*, v.1, 231236, 1982.

DE CARVALHO, C.; DE FREITAS, A.C.; BRUNNER, O.; GÓES, L.G.B.; CAVALCANTE, A.Y.; BEÇAK, W.; DOS SANTOS, R.C.S. Bovine papillomavirus type 2 in reproductive tract and gametes of slaughtered bovine females. *Brazilian Journal of Microbiology*, v.34, p.8284, 2003.

DE FREITAS, A.C.; DE CARVALHO, C.; BRUNNER, O.; BIRGEL-JUNIOR, E.H.; DELLALIBERA, A.M.M.P.; BENESI, F.J.; GREGORY, L.; BEÇAK, W.; DOS SANTOS, R.C.S. Viral DNA sequences in peripheral blood and vertical transmission of the virus: a discussion about BPV1. *Brazilian Journal of Microbiology*, v.34, p.7678, 2003.

DE VILLIERS, E.M.; FAUQUET, C.; BROKER, T.R.; BERNARD, H.U.; ZUR HAUSEN, H. Classification of papillomaviruses. *Virology*, v.324, p.1727, 2004.

DIMAIO, D.; MATTOON, D. Mechanisms of cell transformation by papillomavirus E5 proteins. *Oncogene*, v.20, p.78667873, 2001.

DOS SANTOS, R.C.S.; LINDSEY, C.J.; FERRAZ, O.P.; PINTO, J.R.; MIRANDOLA, R.S.; BENESI, F.J.; BIRGEL, E.H.; PEREIRA, C.A.B.; BEÇAK, W. Bovine papillomavirus transmission and chromosomal aberrations: an experimental model. *Journal of General Virology*, v.79, p.21272135, 1998.

DUENSING, S.; MUNGER, K. Mechanisms of genomic instability in human cancer: insights from studies with human papillomavirus oncoproteins. *International Journal of Cancer*, v.109, p.157–162, 2004.

DYSON, N.; HOWLEY, P. M.; MUNGER, K.; HARLOW, E. The human papilloma virus16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, v.243, p.934–937, 1989.

FAUQUET, C.M.; MAYO, M.A.; MANILOFF, J.; DESSELBERGER, U.; BALL, L.A. Virus taxonomy. The Eighth Report of the International Committee on Taxonomy of Viruses. Family Papillomaviridae. Elsevier, p. 239–255, 2005.

FAVRE, M. Structural polypeptides of rabbit, bovine, and human papillomaviruses. *Journal of Virology*, v.15, p.12391247, 1975.

GHIM, S.J.; RECTOR, A.; DELIUS, H.; SUNDBERG, J.P.; JENSON, A.B.; VAN RANST, M. Equine papillomavirus type 1: complete nucleotide sequence and characterization of recombinant virus-like particles composed of the EcPV1 L1 major capsid protein. *Biochemical and Biophysical Research Communications*, v.324, p.11081115, 2004.

HAGENSEE, M.E.; OLSON, N.H.; BAKER, T.S.; GALLOWAY, D.A. Three-dimensional structure of vaccinia virus-produced human papillomavirus type 1 capsids. *Journal of Virology*, v.68, p.45034505, 1994.

HATAMA, S.; NOBUMOTO, K.; KANNO, T. Genomic and phylogenetic analysis of two novel bovine papillomaviruses, BPV9 and BPV10. *Journal of General Virology*, v.89, p.158163, 2008.

HATAMA, S.; ISHIHARA, R.; UEDA, Y.; KANNO, T.; UCHIDA, I. Detection of a novel bovine papillomavirus type 11 (BPV11) using xipapillomavirus consensus polymerase chain reaction primers. *Archives of Virology*, v.156, p.12815, 2011.

HOWLEY, P.M.; LOWY, D.R. Papillomaviruses. In: Knipe, D.M., Howley, P.M. (Eds.), *Fields' Virology*, Lippincott Williams and Wilkins, Philadelphia, cap. 62, p.23002354, 2007.

ILVES, I.; KIVI, S.; USTAV, M. Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which is mediated by the viral E2 protein and its binding sites. *Journal of Virology*, v.73, p.4404–4412, 1999.

JARRETT, W.F.H.; CAMPO, M.S.; O'NEIL, B.W.; LAIRD, H.M.; COGGINS, L.W. A novel bovine papillomavirus (BPV6) causing true epithelial papillomas of the mammary gland skin: a member of a proposed new BPV subgroup. *Virology*, v.136, p.255264, 1984.

LANGE, C.E.; TOBLER, K.; ACKERMANN, M.; FAVROT, C. Identification of two novel equine papillomavirus sequences suggests three genera in one cluster. *Veterinary Microbiology*, v.149, p.8590, 2011.

LEHMAN, C.W.; BOTCHAN, M.R. Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America*, v.95, p.4338–4343, 1998.

LUNARDI, M.; CLAUS, M.P.; ALFIERI, A.A.; FUNGARO, M.H.P.; ALFIERI, A.F. Phylogenetic position of an uncharacterized Brazilian strain of bovine papillomavirus in the

1 genus Xipapillomavirus based on sequencing of the L1 open reading frame. *Genetics and*
2 *Molecular Biology*, v.33, p.745–749, 2010.
3
4 LUSKY, M.; BOTCHAN, M.R. Genetic analysis of bovine papillomavirus type 1 trans-acting
5 replication factors. *Journal of Virology*, v.53, p.95565,1985.
6
7 MAEDA, Y.; SHIBAHARA, T.; WADA, Y.; KADOTA, K.; KANNO, T.; UCHIDA, I.;
8 HATAMA, S. An outbreak of teat papillomatosis in cattle caused by bovine papilloma virus
9 (BPV) type 6 and unclassified BPVs. *Veterinary Microbiology*, v.121, n.34, p.242248, 2007.
10
11 MOODY, C.A.; LAIMINS, L.A. Human papillomavirus oncoproteins: pathways to
12 transformation. *Nature Reviews. Cancer*., v.10, p.55060, 2010.
13
14 NASIR, L.; REID, S.W. Bovine papillomaviral gene expression in equine sarcoid tumors.
15 *Virus Research*, v.61, p.171–5, 1999.
16
17 OGAWA, T.; TOMITA, Y.; OKADA, M.; SHINOZAKI, K.; KUBONOYA, H.; KAIHO, I.;
18 SHIRASAWA, H. Broad-spectrum detection of papillomaviruses in bovine teat papillomas
19 and healthy teat skin. *Journal of General Virology*, v.85, n.8, p.21912197, 2004.
20
21 OGAWA, T.; TOMITA, Y.; OKADA, M.; SHIRASAWA, H. Complete genome and
22 phylogenetic position of bovine papillomavirus type 7. *Journal of General Virology*, v.88,
23 n.7, p.19341938, 2007.
24
25 OTTEN, N.; VON TSCHARNER, C.; LAZARY, S.; ANTCZAK, D.F.; GERBER, H. DNA
26 of bovine papillomavirus type 1 and 2 in equine sarcoids: PCR detection and direct
27 sequencing. *Archives of Virology*, v.132, p.121–31,1993.
28
29 PAMUKCU, A.M.; PRICE, J.M.; BRYAN, G.T. Naturally occurring and bracken fern
30 induced bovine urinary bladder tumors. *Veterinary Pathology*, v13, 110122, 1976.
31
32 PFISTER, H.; LINZ, U.; GISSMANN, L.; HUCHTHAUSEN, B.; HOFFMAN, D.; ZUR
33 HAUSEN, H. Partial characterization of a new type of bovine papillomavirus. *Virology*, v.96,
34 p.1–8, 1979.
35
36 RABSON, M.S.; YEE, C.; YANG, Y.C.; HOWLEY, P.M. Bovine papillomavirus type 1 3'
37 early region transformation and plasmid maintenance functions. *Journal of Virology*, v.60,
38 p.62634, 1986.
39
40 RODEN, R.B.; WEISSINGER, E.M.; HENDERSON, D.W.; BOOY, F.; KIRNBAUER, R.;
41 MUSHINSKI, J.F.; LOWY, D.R.; SCHILLER, JT. Neutralization of bovine papillomavirus
42 by antibodies to L1 and L2 capsid proteins. *Journal of Virology*, v.68, p.75707574, 1994.
43
44 ROPERTO, S.; RUSSO, V.; BORZACCHIELLO, G.; SANDULLI, S.; DE MARTINO, L. *et*
45 *al*. Infezione batteriche della vescica in bovini affetti da tumori uroteliale. *Proceedings of the*
46 *Annual Meeting of Italian Society of Veterinary Sciences*, v.60, p.99e100, 2006.
47
48 ROPERTO, S.; BRUN, R.; PAOLINI, F.; URRARO, C.; RUSSO, V.; BORZACCHIELLO,
49 G.; PAGNINI, U.; RASO, C.; RIZZO, C.; ROPERTO, F.; VENUTI, A. Detection of bovine

1 papillomavirus type 2 (BPV2) in the peripheral blood of cattle with urinary bladder tumors:
2 possible biological role. *Journal of General Virology*, v.89, p.30273033, 2008.
3
4 ROPERTO, S.; BORZACCHIELLO, G.; BRUN, R.; LEONARDI, L.; MAIOLINO, P.;
5 MARTANO, M.; PACIELLO, O.; PAPPARELLA, S.; RESTUCCI, B.; RUSSO, V.;
6 SALVATORE, G.; URRARO, C.; ROPERTO, F. A Review of Bovine Urothelial Tumors and
7 Tumor-Like Lesions of the Urinary Bladder. *Journal of Comparative Pathology*, v.142,
8 p.95108, 2010.
9
10 ROSE, R.C.; BONNEZ, W.; REICHMAN, R.C.; GARCEA, R.L. Expression of human
11 papillomavirus type 11 L1 protein in insect cells: in vivo and in vitro assembly of viruslike
12 particles. *Journal of Virology*, v.67, p.19361944, 1993.
13
14 SANDERS, C.M.; STENLUND, A. Mechanism and requirements for bovine papillomavirus,
15 type 1, E1 initiator complex assembly promoted by the E2 transcription factor bound to distal
16 sites. *The Journal of Biological Chemistry*, v.276, p.23689–23699, 2001.
17
18 SARVER, N.; RABSON, M.S.; YANG, Y.C.; BYRNE, J.C.; HOWLEY, P.M. Localization
19 and analysis of bovine papillomavirus type 1 transforming functions. *Journal of Virology*,
20 v.52, p.37788, 1984.
21
22 SCOTT, D.W.; MILLER JR., W.H. Equine Dermatology. Saunders, St. Louis, p. 700–712,
23 2003.
24
25 SHOPE, R.E.; HURST, E.W. Infectious papillomatosis of rabbits; with a note on the
26 histopathology. *The Journal of Experimental Medicine*, v.58, p. 607624, 1933.
27
28 SMITH, M.A.; LEVINE, D.G.; GETMAN, L.M.; PARENTE, E.J.; ENGILES, J.B. Vulvar
29 squamous cell carcinoma in situ within viral papillomas in an aged Quarter Horse mare.
30 *Equine Veterinary Education*, v.21, p.11–16, 2009.
31
32 SPALHOLZ, B.; YANG, Y.; HOWLEY, P. Transactivation of a bovine papilloma virus
33 transcriptional regulatory element by the E2 gene product. *Cell*, v.42, p.183–191, 1985.
34
35 SULLINS, K.E.; ROBERTS, S.M.; LAVACH, J.D.; SEVERIN, G.A. Equine sarcoid. Equine
36 Practice, v.8, p. 21–7, 1986.
37
38 TOMITA, Y.; LITERAK, I.; OGAWA, T.; JIN, Z.; SHIRASAWA, H. Complete genomes
39 and phylogenetic positions of bovine papillomavirus type 8 and a variant type from a
40 European bison. *Virus Genes*, v.35, p.243249, 2007.
41
42 VAN REGENMORTEL, M.H.V.; FAUQUET, C.M.; BISHOP, D.H.L.; CALISHER, C.H.;
43 CARSTEN, E.B.; ESTES, M.K.; LEMOn, S.M.; MANILOFF, J.; MAYO, M.A.;
44 MCGEOCH, D.J.; PRINGLE, C.R.; WICKNER, R.B. Virus Taxonomy. Seventh Report of
45 the International Committee for the Taxonomy of Viruses. Academic Press, New York, San
46 Diego, 2002.
47
48 WILSON, V.G.; WEST, M.; WOYTEK, K.; RANGASAMY, D. Papillomavirus E1 proteins:
49 form, function, and features. *Virus Genes*, v.24, p.27590, 2002.
50

1 WOBESER, B.K.; DAVIES, J.L.; HILL, J.E.; JACKSON, M.L.; KIDNEY, B.A.; MAYER,
2 M.N.; TOWNSEND, H.G.; ALLEN, A.L. Epidemiology of equine sarcoids in horses in
3 western Canada. *The Canadian Veterinary Journal*, v.51, p.1103–8, 2010.
4
5 WOSIACKI, S.R.; BARREIROS, M.A.B.; ALFIERI, A.F.; ALFIERI, A.A. Semi-Nested-
6 PCR for detection and typing of bovine papillomavirus type 2 in urinary bladder and whole
7 blood from cattle with enzootic haematuria. *Journal of Virological Methods*, v.126, n.12,
8 p.215219, 2005.
9
10 WOSIACKI, S.R.; CLAUS, M.P.; ALFIERI, A.F., ALFIERI, A.A. Bovine papillomavirus
11 type 2 detection in the urinary bladder of cattle with chronic enzootic haematuria. *Memórias*
12 *do Instituto Oswaldo Cruz*, v.101, p.635638, 2006.
13
14 YUAN, Z.; GALLAGHER, A.; GAULT, E.A.; CAMPO, M.S.; NASIR, L. Bovine
15 papillomavirus infection in equine sarcoids and in bovine bladder cancers. *Veterinary*
16 *Journal*, v.174, p.599–604, 2007.
17
18 ZHENG, Z.M.; BAKER, C.C. Papillomavirus genome structure, expression, and post-
19 transcriptional regulation. *Frontiers in Bioscience*, v.11, p.22862302, 2006.
20
21 ZHOU, J.; STENZEL, D.J.; SUN, X.Y.; FRAZER, I.H. Synthesis and assembly of infectious
22 bovine papillomavirus particles in vitro. *Journal of General Virology*, v.74, p.763768, 1993.
23
24 ZHU, W.; DONG, J.; SHIMIZU, E.; HATAMA, S.; KADOTA, K.; GOTO, Y.; HAGA, T.
25 Characterization of novel bovine papillomavirus type 12 (BPV-12) causing epithelial
26 papilloma. *Archives of Virology*, *in press*.
27
28
29
30
31
32
33
34
35

2. OBJETIVOS

2. OBJETIVOS

2.1. Objetivo geral

- Determinar a sequência genômica completa de um provável novo tipo de BPV (BPV/BR-UEL4), confirmar o seu posicionamento taxonômico na família *Papillomaviridae* e investigar sua associação com lesões de sarcóide em equinos.

2.2. Objetivos específicos

- Delinear estratégia para amplificação e seqüenciamento do genoma completo da cepa viral BPV/BR-UEL4;
- Determinar o posicionamento taxonômico deste novo tipo viral por meio da comparação da sequência do gene L1 desta cepa viral com a de outros representantes da família *Papillomaviridae*;
- Identificar os prováveis quadros de leitura na sequência genômica do BPV13 e inferir algumas propriedades biológicas por meio da comparação com as sequências correspondentes de tipos de PV anteriormente caracterizados;
- Amplificar e sequenciar fragmentos parciais dos genes L1 e E1 dos PVs associados a lesões de sarcóides equinos.

3. ARTIGOS PARA PUBLICAÇÃO

GENETIC CHARACTERIZATION OF A NOVEL BOVINE PAPILLOMAVIRUS MEMBER OF THE *DELTAPAPILLOMAVIRUS* GENUS

Formatação segundo normas para publicação no periódico *Veterinary Microbiology*

1 **Genetic characterization of a novel bovine papillomavirus member of the**

2 ***Deltapapillomavirus* genus**

3

4 **Abstract**

5 The family *Papillomaviridae* is composed of 29 genera, grouping about 189 viral types.
6 While more than 100 human papillomavirus (HPV) types were reported and characterized,
7 only twelve BPV types had been described in cattle. Recently, an investigation using PCR
8 assay employing degenerate primers, revealed notable diversity among BPVs detected in
9 Brazilian cattle herds. That study identified four putative new BPV types (BPV/BR-UEL2 to
10 BPV/BR-UEL5). This report describes the complete genomic sequence and taxonomic
11 position of one of these novel PV types (BPV/BR-UEL4), the bovine papillomavirus type 13.
12 BPV13 DNA was isolated from a selected papilloma found in cattle from Southern region of
13 Brazil. In order to amplify the total genome sequence of the BPV13, the PV genome was
14 submitted to multiply-primed rolling circle amplification (RCA) technique. To confirm that
15 the band was indeed PV DNA, PCR with degenerate primers specific for cutaneous PVs was
16 performed on the RCA product. Based on the sequences generated, two primer sets for long
17 template PCR were chosen in order to amplify the most part of genome of the BPV13. The
18 complete nucleotide sequences of the cloned long PCR products were determined by primer-
19 walking sequencing. The complete genomic sequence of the BPV13 has 7961 bp, with a GC
20 content of 45.1%. BPV13 contains eight PV ORFs, coding for E1, E2, E4, E5, E6, E7, L1,
21 and L2 proteins. The phylogenetic analysis showed that the novel BPV13 is sorted into
22 *Deltapapillomavirus* genus which held a group dominated by artiodactyl ruminant PVs. This
23 group is notable in that PV infection largely results in the development of fibropapillomas,
24 pathogenic mechanism considered to be unique among papillomaviruses. The grouping of the
25 BPV13 in *Deltapapillomavirus* genus as well as the finding of the E5 ORF in its genome, and

1 the lack of the pRB-binding motif in E7 encoded protein, support the hypothesis that BPV13
2 is also capable of inducing fibropapillomas in its natural host.
3
4 Keywords: Cattle; Bovine papillomavirus; New BPV type; BPV13; BPV/BR-UEL4 strain;
5 Complete genome sequence; Deltapapillomavirus; Taxonomic classification.
6
7
8
9
10
11
12
13
14
15

1 **1. Introduction**

2

3 Papillomaviruses (PVs) form a large and highly diverse group of pathogens that are
4 widespread among vertebrate species. These viruses have been considered the causative
5 agents of epithelial proliferative lesions of the skin or mucosa of their natural hosts (De
6 Villiers et al., 2004). In cattle, bovine papillomavirus (BPV) induces cutaneous papillomatosis
7 and cancer of the urinary bladder and upper alimentary canal (Campo, 2002). Besides the fact
8 of PVs being known as strictly species specific pathogens, BPVs 1 and 2 represent the only
9 case of interspecies infection of heterologous host species. This is the case of infection of
10 equids by BPVs 1 and 2, giving rise to sarcoids, the most common equine neoplasm (Nasir
11 and Campo, 2008).

12 Currently, the family *Papillomaviridae* is composed of 29 genera
13 (*Alphapapillomavirus* to *Dyoiotapapillomavirus*), grouping about 189 viral types. While most
14 viral strains were characterized from humans (120), 64 viral types were identified from non-
15 human mammals, three from birds, and two from reptiles. In cattle, only twelve BPV types
16 were fully characterized, and are classified in the genera *Deltapapillomavirus* (BPV1 and 2),
17 *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11 and 12) and *Epsilonpapillomavirus* (BPV5 and 8),
18 with the exception of BPV7, which belongs to an as yet undesignated PV genus (Bernard et
19 al., 2010).

20 By PCR assay using consensus or degenerate primers which amplify partial
21 fragments of the L1 gene, the presence of numerous putative new BPV types have been
22 described in cattle herds from diverse geographical regions. Initially, by using the primer pairs
23 FAP59/FAP64 and MY09/MY11, 11 putative new BPV types were detected in skin warts
24 from teat and healthy skin of cattle from Japan and Sweden (Manos et al., 1989; Forslund *et*
25 *al.*, 1999; Antonsson and Hansson, 2002; Ogawa *et al.*, 2004).

1 Recently, investigations using the strategy above mentioned revealed notable
2 diversity among BPVs detected in papillomas from Brazilian cattle herds (Claus et al., 2008;
3 Lunardi et al., 2010). These studies identified four putative new BPV types designated as
4 BPV/BR-UEL2 to BPV/BR-UEL5. This report describes the complete genomic sequence and
5 taxonomic position of the BPV/BR-UEL4 strain, herein designated as bovine papillomavirus
6 type 13.
7
8 **2. Materials and Methods**
9
10 2.1. *DNA extraction*
11
12 BPV13 was isolated from a cutaneous papilloma from the ear of a cow belonging to a
13 herd located in Southern Brazil. Total genomic DNA was extracted from the lesion, which did
14 not harbor any other BPV or putative BPV types (Claus et al., 2008), by using the protocol for
15 purification of total DNA from animal tissues of the DNeasy Blood and Tissue kit (Qiagen,
16 Hilden, Germany).
17
18 *2.2. DNA amplification*
19
20 In order to amplify the BPV13 genome, the purified DNA isolated from the
21 papilloma was submitted to the multiply-primed rolling circle amplification (RCA) technique,
22 by using the illustra TempliPhi 100 amplification kit (GE Healthcare, Little Chalfont, UK),
23 following an optimized protocol for amplification of papillomaviral genomes (Rector et al.,
24 2004).

1 To investigate whether PV DNA had been amplified, 2 µl of the RCA product was
2 run on a 0.8% agarose gel to look for a band consistent with multiple copies *in tandem* of the
3 full-length PV DNA. Additionally, PCR with degenerate primers specific for cutaneous PVs
4 was performed on the RCA product. The following primer pairs were used: AR-E1F1/AR-
5 E1R2, AR-E1F2/AR-E1R4, AR-L1F8/AR-L1R9, AR-E1F2/AR-E1R3, AR-L1F1/AR-L1R3
6 and MY09/MY11 (Table 1). PCR reactions were carried out with 1 µl of 10-fold-diluted RCA
7 product as the template. PCR amplification was performed with the following cycling
8 conditions: an initial denaturation of 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1
9 min at an optimum temperature for primer annealing, and 1 min at 72°C, and a final
10 elongation step of 5 min at 72°C. With all primer pairs used, amplicons suggestive of PV-
11 specific amplification were generated (data not shown). These PCR products were purified
12 through extraction of the PV-specific bands from the agarose gel by using illustra GFX PCR
13 DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont, UK) and sequenced with
14 the same degenerate primers as those used for PCR.

15 Based on the sequences generated, two primer sets for long template PCR were
16 chosen in the partial obtained E1 and L1 sequences in order to amplify the most part of
17 genome of the BPV13 in two long PCR fragments: L1/E1 fragment of approximately 2 kb,
18 amplified with forward and reverse primers 3121 L1 fp 1 fw (5'-
19 CAAAGGAAGACCCGTATG3') and 3121 E1 fa 1 rv (5'- TTCCGCACTCCCCAATAC3'),
20 and E1/L1 fragment of approximately 3 kb, amplified with primers 3121 E1 fp 1 fw (5'-
21 GTCACTTTTGGCTTGCTTC3') and 3121 L1 fa 1 rv (5'-
22 CTTGCTTACTGGGGTTGGAG3').

23 PCR amplification was performed with the following cycling conditions: an initial
24 denaturation of 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 56°C (L1/E1
25 fragment) or 58°C (E1/L1 fragment), and 4 min at 72°C, and a final elongation step of 12 min

1 at 72°C. Amplicons of the appropriate sizes were excised from the agarose gel and purified by
2 using illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont,
3 UK).
4
5 *2.3. Molecular cloning and DNA sequencing*
6
7 The two long fragments obtained were ligated into thepCR4-TOPO vector, followed
8 by transformation into One Shot TOP10 competent cells (TOPO TA Cloning kit for
9 Sequencing, Invitrogen). One clone containing the 2 kb L1/E1 DNA fragment and another
10 containing the 3 kb E1/L1 DNA fragment were selected. The complete nucleotide sequences
11 of the L1/E1 and E1/L1 inserts were determined by primer-walking sequencing, starting from
12 the universal primers sites in the pCR4-TOPO vector. The remaining gaps in the sequences
13 were determined by primer-walking directly on the RCA product, with the use of 23
14 sequencing primers to cover the complete genome.
15 Sequencing was performed by using the BigDye Terminator v.3.1 Cycle Sequencing
16 kit (Applied Biosystems, Carlsbad, USA) on the 3500 Genetic Analyzer (Applied Biosystems,
17 Carlsbad, USA). The sequences obtained were examined with the PHRED software for
18 quality analysis of chromatogram readings. The sequences were accepted if the base quality
19 was equal to or higher than 20. Consensus sequences were assembled by PHRAP software
20 and sequence identities were compared with all sequences deposited in GenBank using the
21 BLAST program.
22 Putative open reading frames (ORFs) were located by using the ORF Finder tool
23 (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and through comparison with BPVs 1 and 2
24 genomes. The molecular weight of the putative proteins was estimated with the ExPASy
25 Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). Peptide motif searches were

performed with ScanProsite tool (http://prosite.expasy.org/scanprosite/). Prediction of transmembrane regions in the amino acid sequences were made through TMHMM server v 2 (http://www.cbs.dtu.dk/services/TMHMM/).

2.4. Phylogenetic analysis

Pairwise sequence alignments and sequence similarities were calculated using ClustalW and identiy matrix option on BioEdit (Hall, 1999). Multiple sequence alignments at nucleotide and amino acid levels were performed on MEGA v 4 software (Tamura et al., 2007). Initially, the sequences were aligned at the amino acid level, after which the nucleotide sequences were aligned according to the aligned amino acid sequences. The unambiguously alignable parts of E7, E1, E2, L2, and L1 ORFs were pasted together to form a concatenated sequence. Phylogenetic trees were reconstructed from the contatenated sequence alignment by the neighbor-joining method with the Kimura two-parameter distance estimate (Kimura, 1980). Bootstrap support values were determined for 1000 replications.

All previously characterized BPV types as well as other ungulate PVs were included in the analysis. The GenBank accession numbers of the above mentioned sequences are: AaPV1 (M15953), CcaPV1 (EF680235), EcPV1 (AF498323), EcPV2 (EU503122), EcPV3 (GU384895), OaPV1 (U83594), OaPV2 (U83595), OvPV1 (M11910), RtPV1 (AF443292), BPV1 (X02346), BPV2 (M20219), BPV3 (AF486184), BPV4 (X05817), BPV5 (AF457465), BPV6 (AJ620208), BPV7 (DQ217793), BPV8 (DQ098913), BPV9 (AB331650), BPV10 (AB331651), BPV11 (AB543507), and BPV12 (JF834523).

3. Results

1 The complete genomic sequence of the BPV13 contains 7961 bp, with a GC content
2 of 45.1%. Eight ORFs were identified in BPV13 genome, encoding for six early (E) proteins,
3 E1, E2, E4, E5, E6, and E7, and two late (L) proteins, L1 and L2. The relative location of the
4 ORFs in BPV13 genome and their predicted molecular masses are represented in Figure 1.
5 The position of the first nucleotide of the BPV13 genome corresponds to the start of the first
6 ORF in the early region, E6.

7 The putative BPV13 E6 protein presents two typical zinc-binding domains,
8 CxxC29/30(x)CxxC, separated by 36 amino acids. Similarly, the putative E7 protein contains
9 one such domain (figure 2). The retinoblastoma tumor suppressor-binding domain (LxCxE) is
10 absent in the BPV13 putative E7 protein. Similarly to BPVs 1 and 2 E5 proteins, the BPV13
11 E5 ORF encodes a leucine-rich small transforming protein which contains a hydrophobic
12 transmembrane domain. The E1 ORF encodes for the largest BPV13 protein (606 amino
13 acids), and hasss the conserved ATP-binding site (GPPNTGKS) in its ATP-dependent
14 helicase carboxy-terminal part. The BPV13 E4 ORF is found completely overlapping E2 ORF
15 but in a different frame. In its predicted amino acid sequence, a start codon was identified,
16 which is not the case in most other PVs. Additionally, a high proline content, which is typical
17 for the E4 ORF, was observed (14 proline residues out of 113 amino acids). The late region
18 contains the major (L1) and minor (L2) capsid protein genes. Both BPV13 putative L1 and L2
19 proteins contain a series of arginine and lysine residues at their carboxy terminus, likely to
20 function as a nuclear localization signal.

21 The non-coding region (NCR) between the stop codon of L1 and the start codon of
22 E6 has 854 bp length in BPV13, from nt 7115 to 7. PVs usually contain an E1 recognition site
23 (E1BS) in the middle of two E2-binding sites, for binding of an E1/E2 complex in order to
24 activate the origin of replication. In the BPV13 NCR, an E1BS could not be identified.

1 Diferently, nine typical E2-binding sites (E2BS) with the consensus sequence ACC-N6-GGT
2 were found in the BPV13 NCR at nt 7214–7225, 7376–7387, 7420–7431, 7471–7482, 7604–
3 7615, 7633–7644, 7772–7783, 7793–7804, and 7912–7922 (Androphy et al., 1987; Li et al.,
4 1989). One additional modified E2-binding site (E2BS*), with the sequence AAC-N4-GGT at
5 nt 7142–7151, was also identified. The NCR 5'end presented two polyadenylation sites
6 (AATAAA) at nt 7131–7136 and 7167–7172; the second site is located upstream of a CA
7 dinucleotide and a G/T cluster, probably being necessary for the processing of late mRNA
8 transcript. In the NCR, the characteristic TATA box of the E6 promoter could not be
9 recognized at the 3' end.

10 The phylogenetic analysis using an E7, E1, E2, L2, and L1 concatenated nucleotide
11 sequence alignment of the BPV13 and other ungulate PVs (Figure 3) demonstrated that
12 BPV13 is a member of the genus *Deltapapillomavirus*, being closely related to other BPV
13 representatives classified as Delta-PVs. The entire L1 ORF of the BPV13 had 85.5 and 88.2%
14 sequence identity, respectively, with Delta-BPVs 1 and 2. The identities obtained through
15 pairwise comparison of the L1 nucleotide sequence with corresponding sequences of PVs also
16 classified in *Deltapapillomavirus* genus is indicated in Table 2.

17

18 **4. Discussion**

19

20 BPV13 presents the core PV E1, E2, L1, and L2 genes, comparable in length and
21 position with other PVs. The proteins encoded by these ORFs are extremely important to the
22 functionality of structural and biochemical properties of every PV sequenced so far
23 (Narechania et al., 2004).

24 The phylogenetic analysis showed that the novel BPV13, isolated from a cutaneous
25 benign lesion of a cow, is sorted into *Deltapapillomavirus* genus which held a group

1 dominated by artiodactyl ruminant PVs. This group is notable in that PV infection largely
2 results in the development of fibropapillomas, indicating that the pathogenic mechanism of
3 Delta-PVs appears to be unique among papillomaviruses (Narechania et al., 2004).
4 Interestingly, BPVs lead to two types of pathology (Jarrett et al., 1984). BPVs 3, 4, 6, 9, 10,
5 11, and 12, which are classified in *Xipapillomavirus* genus induce epithelial papillomas in
6 cattle without fibroblast involvement, whereas BPVs 1 and 2 (Delta-PVs) cause
7 fibropapillomas in their natural hosts. In the case of BPV5, a single PV appears to present a
8 dual pathology (De Villiers et al., 2004).

9 The PV classification criteria defines an isolate as a new PV type if the DNA
10 sequence of the L1 ORF differed by more than 10% from the closest known PV type. PVs
11 that share between 60 and 70% nucleotide similarity in L1 gene are considered distinct
12 species whereas different PV genera share less than 60% nucleotide sequence identity in the
13 L1 ORF (de Villiers et al., 2004). Once that highest identity observed in BPV13 L1 nucleotide
14 sequence was 88.2% with the corresponding sequence of BPV 2, this new isolate meets the
15 main criteria stablished to declare detection of a new PV type. Consequently, BPV13 is herein
16 recognized as a new PV type to be classified in the *Deltapapillomavirus* genus, being the third
17 representative of the Delta4 species.

18 Although not considered ubiquitous among animal PVs, E4 and E5 are largely
19 conserved among the artiodactyl PVs. The close similarity observed in alignments of the
20 artiodactyl PV E5 ORFs, indicates that the protein encoded by this gene is likely to participate
21 on transformation among this group of PVs. Since the pathological and typical feature of
22 artiodactyls PVs is the development of fibropapillomas, the molecular basis of fibroblast
23 transformation is thought to involve the E5 transforming factor (Munger and Howley, 2002).

24 The E6 and E7 oncogenes are thought to interfere with host cell-cycle through
25 ligation with p53 and pRB factors, respectively (Moody and Laimins, 2010). While binding to

p53 prevents the anti-proliferative signaling system of differentiated cells, binding to pRB prevents its interaction with E2F transcription factor, promoting activation of cell replication (Munger et al., 2001). Interaction of E7 protein with pRB factor has been pointed by recognition of the pRB-binding motif (LxCxE), shared by most E7 ORFs (Chan et al., 2001; Dahiya et al., 2000; Dick and Dyson, 2002). Conversely, as is the case of BPV13, there is no evidence for presence of the pRB-binding domain in E7 encoded protein in artiodactyl PV group. In fact, it has been observed that the ability to cause fibropapillomas almost exactly coincide with the pRB-binding motif loss. Consequently, it has been shown that every animal PV known that induce fibropapillomas lacks the pRB-binding motif, including those PVs which seem to determine lesions with dual patterns (Narechania et al., 2004).

The grouping of the BPV13 in *Deltapapillomavirus* genus, which lump fibropapilloma-inducing artiodactyls ruminant PVs together, as well as the finding of the E5 ORF in BPV13 genome and the lack of the pRB-binding motif in E7 encoded protein, support the hypothesis that BPV13 is also capable of inducing fibropapillomas in its natural hosts. The identification of these shared molecular features is thought to be expected since other BPV representatives classified in Delta4 species are known for causing fibropapillomas in their hosts. These observations are in agreement with criteria previously stablished by De Villiers et al. (2004) in which it is stated that different PV types classified in a specific species present close phylogenetic relationship as well as shared biological and pathological properties.

PVs have a species-specific nature, with interspecies transmission being a very rare event and only occurring between closely related species, such as infection of horses with BPVs 1 and 2 (De Villiers et al., 2004). Like other Delta4 representatives, the novel BPV type was detected in association with cases of equine sarcoid in Brazilian herds (Lunardi, unpublished data). Thus, exclusive biological properties shared by Delta4 PVs such as

potential to have fibroblasts as primary target cells, ability to promote fibroblast proliferation, and to cross the host species barrier, are also present in BPV13.

Based on close phylogenetic relationship, and shared molecular features evidenced through the comparison of genomic and amino acid sequences between BPV13 and BPVs 1 and 2, it can be theorized that the BPV13 might also be associated with PV-related malignances that occur in cattle (e.g. urinary bladder tumors). So far this cattle distressing disease have been exclusively associated with representatives of Delta4 species in *Deltapapillomavirus* genus (Nasir and Campo, 2008).

We suggest BPV13 to be classified in *Deltapapillomavirus* genus due to two main facts: i) highest L1 nucleotide identity observed by pairwise comparisons of the corresponding region of Delta-PVs was obtained with BPV2 (88.2%) which was confirmed by its position in obtained phylogenetic trees; ii) likely participation of the BPV13 in etiology of equine sarcoids, as has been classically reported for BPVs 1 and 2, demonstrates common phenotypic features shared by these three BPV types.

In the present study, the taxonomic classification and specific genomic properties of BPV13 isolated from cattle from Brazil was depicted. This BPV type represents the third member to be grouped in Delta4 species from *Deltapapillomavirus* genus. The characterization of novel PVs is useful for both understanding of PV phylogeny and interpretation of the described pathologies related to these viruses. Additional information about the molecular basis and epidemiology of characterized and uncharacterized BPVs will be needed to fully elucidate pathogenecity regarding cattle and horse diseases associated with BPV.

1 **References**

2

3 Antonsson, A., Hansson, B.G., 2002. Healthy skin of many animal species harbors
4 papillomaviruses which are closely related to their human counterparts. J. Virol. 76, 12537–
5 42.

6

7 Bernard, H.U., Burk, R.D., Chen, Z., van Doorslaer, K., Hausen, H., de Villiers, E.M., 2010.
8 Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic
9 amendments. Virology. 401, 70–9.

10

11 Campo, M.S., 2002. Animal models of papillomavirus pathogenesis. Virus. Res. 89, 249–261.

12

13 Chan, H.M., Smith, L., La Thangue, N.B., 2001. Role of LXCXE motif dependent
14 interactions in the activity of the retinoblastoma protein. Oncogene 20, 6152–6163.

15

16 Claus, M.P., Lunardi, M., Alfieri, A.F., Ferracin, L.M., Fungaro, M.H., Alfieri, A.A., 2008.
17 Identification of unreported putative new bovine papillomavirus types in Brazilian cattle
18 herds. Vet. Microbiol. 132, 396–401.

19

20 Dahiya, A., Gavin, M.R., Luo, R.X., Dean, D.C., 2000. Role of the LXCXE binding site in Rb
21 function. Mol. Cell. Biol. 20, 6799–6805.

22

23 De Villiers, E.-M., Fauquet, C., Broker, T.R., Bernard, H.U., Zur Hausen, H., 2004.
24 Classification of papillomaviruses. Virology 324, 17–27.

1 Dick, F.A., Dyson, N.J., 2002. Three regions of the pRB pocket domain affect its inactivation
2 by human papillomavirus E7 proteins. J. Virol. 76, 6224–6234.
3
4 Forslund, O., Antonsson, A., Nordin, P., Hansson, B.G., 1999. A broad range of human
5 papillomavirus types detected with a general PCR method suitable for analysis of cutaneous
6 tumors and normal skin. J. Gen. Virol. 80, 2437–2443.
7
8 Hall TA., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
9 program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95-98.
10
11 Jarrett, W.F., Campo, M.S., O'Neil, B.W., Laird, H.M., Coggins, L.W., 1984. A novel bovine
12 papillomavirus (BPV6) causing true epithelial papillomas of the mammary gland skin: a
13 member of a proposed new BPV subgroup. Virology 136, 255–264.
14
15 Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitution
16 through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
17
18 Lunardi, M., Claus, M.P., Alfieri, A.A., Fungaro, M.H.P., Alfieri, A.F., 2010. Phylogenetic
19 position of an uncharacterized Brazilian strain of bovine papillomavirus in the genus
20 Xipapillomavirus based on sequencing of the L1 open reading frame. Gen. Mol. Bio. 33, 745–
21 749.
22
23 Manos, M.M., Ting, Y., Wright, D.K., Lewis, A.J., Broker, T.R., Wolinsky, S.M., 1989. The
24 use of polymerase chain reaction amplification for the detection of genital human
25 papillomaviruses. Cancer Cell 7, 209–214.

1
2 Moody, C.A., Laimins, L.A., 2010. Human papillomavirus oncoproteins: pathways to
3 transformation. Nat. Rev. Cancer. 10, 550–560.
4
5 Munger, K., Basile, J.R., Duensing, S., Eichten, A., Gonzalez, S.L., Grace, M., Zacny, V.L.,
6 2001. Biological activities and molecular targets of the human papillomavirus E7
7 oncoprotein. Oncogene 20, 7888–7898.
8
9 Munger, K., Howley, P.M., 2002. Human papillomavirus immortalization and transformation
10 functions. Virus. Res. 89, 213–228.
11
12 Narechania, A., Terai, M., Chen, Z., DeSalle, R., Burk, R.D., 2004. Lack of the canonical
13 pRB-binding domain in the E7 ORF of artiodactyl papillomaviruses is associated with the
14 development of fibropapillomas. J. Gen. Virol. 85, 1243–50.
15
16 Nasir, L., Campo, M.S., 2008. Bovine papillomaviruses: their role in the aetiology of
17 cutaneous tumors of bovids and equids. Vet. Dermatol. 19, 243–54.
18
19 Ogawa, T., Tomita, Y., Okada, M., Shinozaki, K., Kubonoya, H., Kaiho, I., Shirasawa, H.,
20 2004. Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy
21 teat skin. J. Gen. Virol. 85, 2191–7.
22
23 Rector, A., Bossart, G.D., Ghim, S.J., Sundberg, J.P., Jenson, A.B., Van Ranst, M., 2004.
24 Characterization of a novel close-to-root papillomavirus from a Florida manatee by using

1 multiply primed rolling-circle amplification: Trichechus manatus latirostris papillomavirus
2 type 1. J. Virol. 78, 12698-12702.
3
4 Rector, A., Van Doorslaer, K., Bertelsen, M., Barker, I.K., Olberg, R-A., Lemey, P.,
5 Sundberg, J.P., Van Ranst, M., 2005. Isolation and cloning of the raccoon (Procyon lotor)
6 papillomavirus type 1 by using degenerate papillomavirus-specific primers. J. Gen. Virol. 86,
7 2029-2033.
8
9 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics
10 Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

1 **Table 1**

2 Sequences and features of degenerate primers used for PCR amplification of partial sequences of the RCA product

Primer	Genomic region target	Polarity	Sequence (5' – 3')[1]	Nucleotide positions[2]	Degree of Degeneracy	Expected amplicon length (bp)
AR-E1F1[3]	E1	forward	CAGGGVMWTTCCCTGBARYTGTTYC	962–986	288	836
AR-E1R2[3]	E1	reverse	TCATANGCCCACTGNACCAT	1797–1778	16	
AR-E1F2[3]	E1	forward	ATGGTNCAGTGGGCNTATGA	1778–1797	16	552
AR-E1R4[3]	E1	reverse	ATTNCCATCHADDGCATTTCT	2329–2309	108	
AR-L1F8[3]	L1	forward	GGDGAYATGDGKGAMATWGG	6016–6035	144	704
AR-L1R9[3]	L1	reverse	GGRCATTTKGTWGCWADGGA	6719–6697	48	
AR-E1F2[3]	E1	forward	ATGGTNCAGTGGGCNTATGA	1778–1797	16	371
AR-E1R3[4]	E1	reverse	TTNCCWSTATTNGGNGGNCC	2148–2129	1024	
AR-L1F1[3]	L1	forward	TTDCAGATGGCNGTNTGGCT	5425–5444	48	600
AR-L1R3[3]	L1	reverse	CATRTCHCCATCYTCWAT	6024–6007	24	

MY09[5]	L1	forward	GCMCAGGGWCATAAYAATGG	6379–6398	8	450
MY11[5]	L1	reverse	CGTCCMARRGGAWACTGATC	6830–6811	16	

1

2 [1]Degenerate nucleotides: B = T, C or G; D = A, T, or G; H = A, T, or C; K = T or G; M = A or C; N = A, G, C, or T; R = A or G;

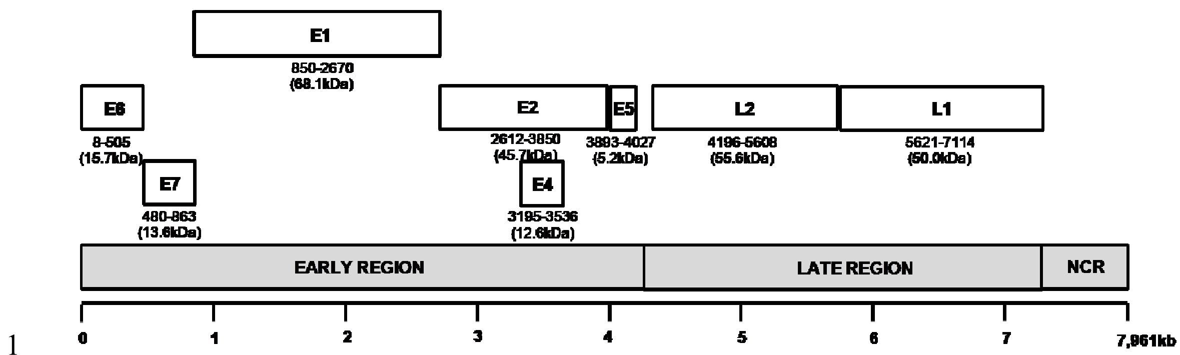
3 S = C or G; V = A, C or G; W = A or T; Y = C or T.

4 [2]Position relative to the sequence of HPV1a.

5 [3]Rector *et al.* (2005).

6 [4]Rector *et al.* (2004).

7 [5]Manos *et al.* (1989).



1

2 **Fig. 1.** Diagrammatic genomic organization of BPV13. The three main regions of the genome

3 are shown as gray rectangles above the ruler. The viral genome is represented as linear, with

4 ORF as white rectangles. Numbers below each ORF indicate nucleotide position from the

5 start to the stop codon and the corresponding molecular mass for the putative proteins.

```
AaPV1 E6     ------MCGECYAYLTCIWCKKGLDKVDAKRCHEKKIRIACRNGKHCAVCTSCLENGLYLERSLFPGRPIYP
CcaPV1 E6    ------MPDNAFTSLSCLWCKNKLKRVDALRCELKDIQPVTRDGFVFAACTGCLELALWMERNVFPGIVVHP
OvPV1 E6     ------MSADYYEHLYCVFCYCVLGKVEARRCYDKKIRTVVRGGLRCAVCTACLEKGLYLERVLNAPQPVYQ
RtPV1 E6     ------MCADYYEPLQCIWCKATLGKVEAKRCLEKRIRPACRDMKSYAACTHCLENALYLERTLFPGKAIHP
BPV1 E6      MDLKPFARTNPFSGLDCLWCREPLTEVDAFRCMVKDFHVVIREGCRYGACTICLENCLATERRLWQGVPVTG
BPV2 E6      MDLQSFSRGNPFSGLACVWCREPLTEVDAFRCMIKDFHVVYRDGVKFGACTTCLENCLDKERRLWKGVPVTG
BPV13 E6     MDLQSFSRGNPFSGLACLWCKEPLTELDAFRCMIKDFHVVYRNGCTFGACTPCLEGCLAKERRLWKGVPVTG
                       : :  * *::*   * .::* **  * :: . *     ..** ***  *  ** :     :

AaPV1 E6     GDLYEPD-PWVMFNDIRCMYCGGCLTRDEKERHRLFCEDFWIFRH-QVRGRCYLCTRHGSR-PPYKETPAAV
CcaPV1 E6    GDCSSNP-PWITSLRIRCMYCGAKLTADEKDRHRYFEEPYVSFRG-RVRGRCYDCCRDGTR-PQYKQGASE-
OvPV1 E6     GSIEEPD-PFIQKACIRCMYCGGILTRDEKDRHRYFEELYVIFRN-QVLGRCYTCTRHGMCSAPYRANATG-
RtPV1 E6     GHLQQPD-PWITQHVIRCMYCGGQLSRDEKERHRLFFENFWLFRS-QVRGRCYSCTRDGAR-PPYEKSAAFR
BPV1 E6      EEAELLHGKTLDRLCIRCCYCGGKLTKNEKHRHVLFNEPFCKTRANIIRGRCYDCCRHGSR-SKYP------
BPV2 E6      EEAQLLHGKSLDRLCIRCCYCGGKLTKNEKQRHVLYNEPFCKTRSNIIRGRCYDCCRHGSR-SNYP------
BPV13 E6     EEAELLHGKSLDKLCIRCCYCGGKLTKNEKHRHVLYNEPFCKTRANIIRGRCYDCCRHGSR-SKYP------
                      :    *** ***. *: :**.**  : * :   *   : **** * *.*   . *

AaPV1 E7     MVHG-PRTKKHLPPYESPPLTLL--------LEPVAPVQQTGIQAPQRKPPSQK---------GHKKG-----------HKKVYSVTVPCNGCDKNLEFC
OvPV1 E7     MACARPLTGRTLPADESPCLTLI--------LEPVSGEAAKNSTPVVVDKPGKP---------PPKR------------HRRQYNVTVSCNDCDKRLNFS
RtPV1 E7     MVHG-PRTKKVLPSDESPCLTLL--------LNPIT-VDPHCTD----DRPPLK---------PPRKP-----------PRRLYYITVACTECDKRLGFC
CcaPV1 E7    MVHG-PNTSKVLPNESLIPLTLH--------LKPLKPEKPAVTVACSLTHEQPR---------PSRPPPKPPR-------MSVYYVGVCCGCCEKKLDFA
BPV1 E7      MVQG-PNTHRNLDDSPAGPLLILSPCAGTPTRSPAAPDAPDFRLPCHFGRPTRKR-------GPTTPPLSSPGKLCATGPRRVYSVTVCCGNCGKELTFA
BPV2 E7      MVQG-PTTHRNLDDSPAGPLLILSPCAGTPTRVPAAPDAPDFRLPCHFGRPTRKR-------GPSTPPLSSPGKVCATGPRRVYSVTVCCGHCGKDLTFA
BPV5 E7      MVAG-PACSKFLPQDPPPPS----VTLVLHDLTQEE-DEQDFVTLHAQYRPTFKDKTPRRPGYNPRAAPCHI--QRLSARCSVFFVQVGCP-CGQPLKIA
BPV8 E7      MVAG-PACTKNLPKEGEPVGGASGVTLLLSRITQPPFDSPDFASLHESFKP--------GDGCFPKPASCQIVKQRVTLPPGIFFVTLQCP-CTFPLRVA
BPV13 E7     MVQG-PNTHRNLDDSPAGPLLILSPCAGTPTRVPAAPDAPDFRLPCHFGRPTRKR-------GPSTPPLSSPGKVCATGPRRVYSVTVCCGDCGKDLTFA
             *. . *   : *                                                                          : : : *  *   * ..

AaPV1 E7     ARTSSATILTLQNLL-LKDLDFLCSTCETN--HG--
OvPV1 E7     VKTTCSTILTLQQLL-TEDLDFLCSFCEAK--NG--
RtPV1 E7     AKTSKDSILSFEKLL-LADLDILCSACENK--HG--
CcaPV1 E7    VRTSQTSITSFHALL-LEDLDILCALCEGKTRHG--
BPV1 E7      VKTSSTSLLGFEHLL-NSDLDLLCPRCESRERHGKR
BPV2 E7      VKTGSTTLLGFEHLL-NSDLDLLCPRCESRERHGKR
BPV5 E7      VQSTPDCISQFEQLL-RGPLDLLCPHCASRCYGR--
BPV8 E7      VRAGNGGIRDFQELLTRNDFDLLCPHCASRHYGK--
BPV13 E7     VKTGSTTLLGFEHLL-NSDLDLLCPRCESRERHGKR
             .::    :  :. **    :*:**. *  .

BPV1 E5      MPNLWFLLFLGLVAAMQLLLLLFLLLFFLVYWDHFECSCTGLPF
BPV2 E5      MPNLWFLLFLGLVAAMQLLLLLFLLLFFLVYWDHFECSCTGLPF
BPV13 E5     MPNLWFLLFLGLVAAMQLLLLLFLLLFFLVYWDHFDCSCTGLPF
             ************************************:********
```

Fig. 2. Amino acid sequence alignments of artiodactyl PVs' E6, E7, and E5 proteins, respectively. Shaded residues indicate amino acids forming zinc-binding motifs while grid highlights transmembrane domain in E5 proteins.

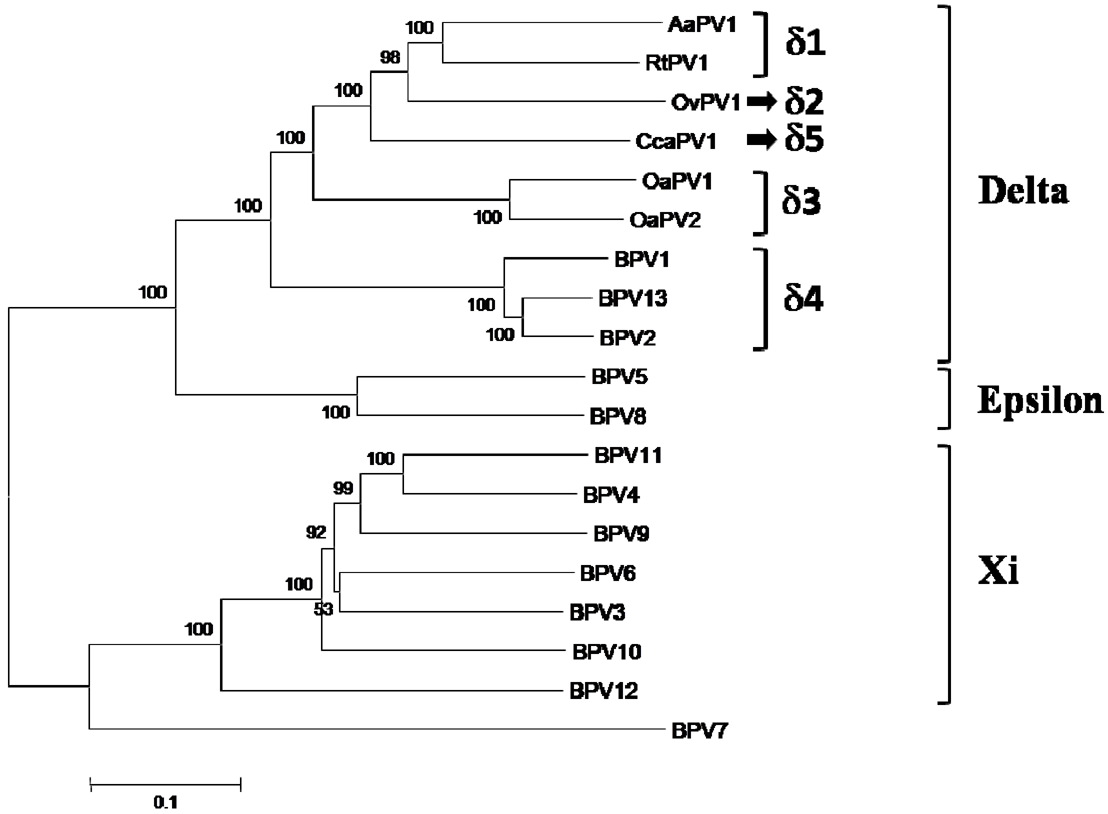


Fig. 3. Neighbor-joining phylogenetic tree of a E7, E1, E2, L2, and L1 concatenated nucleotide sequence of ungulate PVs, including that of the BPV13. Besides the undesignated PV genus represented by BPV7, the genera *Deltapapillomavirus*, *Epsilonpapillomavirus*, and *Xipapillomavirus*, which group the remaining BPVs are indicated in the tree. Additionally, the five species classified within *Deltapapillomavirus* genus are shown. The numbers at the internal nodes represent the bootstrap support values determined for 1000 replications.

1 **Table 2**

2 Identities shared among Delta-PVs on the L1 nucleotide sequence

	BPV13	**BPV1**	**BPV2**	**CcaPV1**	**OvPV1**	**AaPV1**	**RtPV1**	**OaPV1**	**OaPV2**
BPV13	-	0,857	0,882	0,684	0,671	0,679	0,669	0,679	0,681
BPV1		-	0,847	0,687	0,683	0,687	0,688	0,684	0,693
BPV2			-	0,690	0,674	0,675	0,680	0,679	0,691
CcaPV1				-	0,731	0,730	0,716	0,682	0,692
OvPV1					-	0,734	0,746	0,669	0,671
AaPV1						-	0,772	0,681	0,689
RtPV1							-	0,684	0,696
OaPV1								-	0,796
OaPV2									-

3

4

A NEW BOVINE DELTA PAPILLOMAVIRUS TYPE ASSOCIATED WITH EQUINE SARCOIDS

Formatação segundo normas para publicação no periódico *Veterinary Microbiology*

A new bovine delta papillomavirus type associated with equine sarcoids

Abstract

Equine sarcoids are locally aggressive fibroblastic neoplasms, considered as the most common skin tumor of horses worldwide. BPV types 1 and 2 have been classically associated with sarcoids in equids. Investigations aiming to identify papillomavirus (PV) strains other than BPV1 and 2 that might be associated with sarcoidal lesions are still lacking. The aim of this study was to report the involvement of a third BPV type, the BPV13, in equine sarcoids. Six sarcoids were collected from diverse body sites of two horses from Southern Brazil. In order to detect a broad- spectrum of PV strains, eight degenerate primer pairs, designed to detect conserved regions on L1 and E1 genes, were tested on the DNA samples. Then, direct sequencing was performed on the obtained amplicons and sequence identities were compared with sequences from all BPV types. The FAP59/FAP64, MY09/MY11, and AR-E1F2/AR-E1R4 sequences generated from the sarcoids were shown to present 100% identity with the BPV13, a new BPV type previously described in cattle. The results from this study suggest the need for investigations aiming to identify the BPV13 type as well as other PV strains that might be associated with sarcoids collected from herds from diverse geographical areas. In that way, the frequency of occurrence of this viral type could be evaluated in this common tumor of equids.

Keywords: Horses; Equine sarcoid; Bovine papillomavirus; new BPV type; BPV13; BPV/BR-UEL4 strain; phylogenetic analysis; histopathology.

1 **1. Introduction**

2

3 Equine sarcoids are locally aggressive fibroblastic neoplasms, considered the most
4 common skin tumor in horses worldwide. Other equids also affected are zebras, donkeys, and
5 mules. These tumors rarely regress and very often recur after therapy. Clinically, sarcoids can
6 vary in their gross aspect, being classified into four different clinical types: verrucous,
7 fibroblastic, mixed, and occult (Goldschmidt and Hendrick, 2002; Ginn et al., 2007).

8 Once equine sarcoids histologically resemble the fibrotic section of bovine
9 fibropapillomas, the participation of bovine papillomaviruses (BPVs) in their etiology was
10 suspected in the past (Lancaster et al., 1977; Sundberg et al., 1977). However, intradermal
11 inoculation of cell-free extract from cattle warts into healthy horses represented the first
12 successful attempt to demonstrate an association of this clinical entity with BPVs, resulting in
13 the growth of sarcoid-like lesions (Olson and Cook, 1951). More recently, both the consistent
14 identification of BPV DNA in sarcoids as well as the demonstration of expression of diverse
15 viral genes have corroborated the direct involvement of BPV in the pathogenesis of sarcoids
16 (Otten et al., 1993; Nasir and Reid, 1999; Carr et al., 2001a; Chambers et al., 2003; Yuan et
17 al., 2007; Wobeser et al., 2010).

18 BPV types 1 and 2, which are known to cause bovine fibropapillomas, have been
19 classically associated with sarcoids in equids. Previous investigations have established this
20 association either through DNA-DNA hybridization, using BPV1 and 2 labeled genomes as
21 probes, or by polymerase chain reaction (PCR) assays with type-specific primers (Lancaster et
22 al., 1979; Angelos et al., 1991; Otten et al., 1993).

23 Variation in the frequency of recovery of BPV1 and 2 from sarcoids has been
24 demonstrated by studies conducted in diverse geographic areas. While BPV1 has been shown

as more prevalent in affected horses from Europe and Australia (Teifke and Weiss, 1991; Otten et al., 1993; Bloch et al., 1994), BPV2 has been more commonly reported in lesions from horses in Western United States and Canada (Carr et al., 2001b; Wobeser et al., 2010).

Currently, twelve BPV types were identified and characterized from cattle. These BPVs are classified in the genera *Deltapapillomavirus* (BPV1 and 2), *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11, and 12) and *Epsilonpapillomavirus* (BPV5 and 8), with the exception of BPV7, which belongs to an as yet undesignated PV genus (Bernard et al., 2010). In addition to the fully sequenced BPV types, through PCR assay employing consensus or degenerate primers which amplify partial fragments of the L1, the presence of numerous putative new BPV types have been described in cattle herds from diverse geographical regions (Antonsson and Hansson, 2002; Ogawa *et al.*, 2004). Recently, an investigation using PCR with degenerate primers revealed notable diversity among BPVs detected in papillomas from Brazilian cattle herds. The study identified four putative new BPV types designated as BPV/BR-UEL2 to BPV/BR-UEL5 (Claus *et al.*, 2008). Recently, the sequencing of the complete genome of BPV/BR-UEL4 strain allowed its classification as a third member of *Deltapapillomavirus* genus (Lunardi, unpublished data).

Investigations aiming to detect and characterize papillomavirus (PV) strains other than BPV1 and 2 that might be associated with sarcoids are still lacking. The aim of this study was to report the involvement of a third BPV type, the BPV13, in equine sarcoids.

2. Materials and Methods

2.1. *Sarcoidal lesions*

1 Six sarcoids were individually and surgically collected from diverse anatomic
2 locations of two horses from a farm located in the northern region of Parana state, Southern
3 Brazil (Table 1). A portion of each lesion was fixed in 10% buffered formalin, and routinely
4 processed for histopathological evaluation. The remaining fragment of each tumor was kept at
5 -20°C for molecular analyses.
6
7 *2.2. DNA extraction*
8
9 Fragments from each specimen were grounded in liquid nitrogen before DNA
10 extraction procedure. DNA extraction was done by using the protocol for purification of total
11 DNA from animal tissues of the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany)
12
13 *2.3. PCR amplification*
14
15 In order to detect potential additional PV strains that might have been associated with
16 equine sarcoids, eight degenerate primer pairs, designed to detect conserved regions of the L1
17 and E1 genes of a broad-spectrum of diverse cutaneous PV strains, were tested on all of the
18 DNA samples. Table 2 shows the sequences and features of the primers employed.
19 PCR reactions were set using 2 µl of the extracted DNA and 48 µl of PCR-mix
20 consisting of 1 µM from each primer; 200 µM of each dNTP (Invitrogen, Life Technologies,
21 Carlsbad, USA); 2.5 units of Platinum *Taq* DNA polymerase (Invitrogen, Life Technologies,
22 São Paulo, BR); 1× PCR buffer (20 mM Tris– HCl, pH 8.4 and 50 mM KCl); 1.5 mM of

1 $MgCl_2$ and ultrapure sterile water to the final volume. Amplification was performed in a
2 thermocycler (PTC 200, MJ Research Co., USA) with the following cycling profile: an initial
3 step of 5 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at an optimum
4 temperature for primer annealing, 1 min at 72°C, and a final extension step of 5 min at 72°C.
5 Aliquots from the PCR amplified products were analyzed by electrophoresis in 1.5% agarose
6 gel, stained with ethidium bromide (0.5 mg/ml), and examined under UV light.
7
8 *2.4. Sequencing and sequence analyses*
9
10 Initially, all PCR products were purified using illustra GFX PCR DNA and Gel Band
11 Purification kit (GE Healthcare, Little Chalfont, UK). Then, direct sequencing was performed
12 by using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad,
13 USA) with the forward and reverse corresponding primers, in the 3500 Genetic Analyzer
14 (Applied Biosystems, Carlsbad, USA), according to the manufacturer's instructions. The
15 sequences obtained were examined with the PHRED application for quality analysis of
16 chromatogram readings. The sequences were accepted if base quality was equal to or higher
17 than 20. Consensus sequences were determined by CAP3 software and sequence identities
18 were compared with all sequences deposited in GenBank using the BLAST program.
19
20 *2.5. Phylogenetic analysis*
21
22 Alignment and degree of similarity between sequences at nucleotide and amino acid
23 levels were determined by using MEGA v 4 software (Tamura et al., 2007). Phylogenetic
24 trees were reconstructed from multiple nucleotide sequence alignments of concatened E1/L1
25 sequences by the neighbor-joining method with the Kimura two-parameter

distance estimate (Kimura, 1980). Bootstrap support values were determined for 1000 replications.

All previously characterized BPV types as well as the new viral type BPV13 (Lunardi, unpublished data) were included in the analysis. The GenBank accession numbers of the sequences used are: BPV1 (X02346), BPV2 (M20219), BPV3 (AF486184), BPV4 (X05817), BPV5 (AF457465), BPV6 (AJ620208), BPV7 (DQ217793), BPV8 (DQ098913), BPV9 (AB331650), BPV10 (AB331651), BPV11 (AB543507), and BPV12 (JF834523).

3. Results

Following the pathological classification of equine sarcoids proposed by Goldschmidt and Hendrick (2002), in which four different gross morphological types can be recognized, the lesions herein evaluated were categorized as fibroblastic or verrucous (table 1).

The six skin lesions were confirmed as equine sarcoids by the histopathological evaluation. Characteristic features such as epithelial extensions projecting into the underlying dermis (*rete pegs*) and proliferating dermal fibroblasts forming whorls or interlacing bundles were observed in the sarcoid tumor specimens. In addition, epidermal hyperplasia and hyperkeratosis, particularly in verrucous tumors, as well as fibroblasts perpendicularly arranged at the dermo-epidermal junction (*picket fence*) were also identified (Figure 1).

PCR reactions employing the primer pairs FAP59/FAP64, MY09/MY11, and AR-E1F2/AR-E1R4, yielded amplicons of the expected molecular size on DNA samples from all lesions evaluated.

1 By using nBLAST program, all the sequences generated from the sarcoids were
2 shown to demonstrate 100% identity with the viral type BPV13, a new BPV type
3 characterized from a cutaneous wart isolated from a cow (Lunardi, unpublished data).
4 In order to obtain a longer sequence from the L1 gene, the alternative combination
5 FAP59/MY11 of the primers was tested on all DNA samples, generating an amplicon of
6 approximately 1100bp length (data not shown).
7 The partial nucleotide (nt) sequence of the putative E1 gene obtained from all equine
8 lesions presented identities of 90.7% and 91.4% with the Delta-PVs classically associated
9 with sarcoids, BPVs 1 and 2, respectively. Their predicted amino acid (aa) sequences showed
10 similarities of 97.7 and 96.5% with the same viral types. The identities shared with BPVs
11 classified in other genera varied from 44.9 to 61% (nt) and from 39.2 to 57.9% (aa).
12 For the L1 partial DNA fragment obtained from the same equine samples, the
13 identities exhibited at nt sequence with BPVs 1 and 2 were 84.3 and 86.4%, respectively,
14 whereas the predicted aa sequence showed similarities of 94.6 and 94.0 with the same BPV
15 types. Similarities with other BPVs ranged from 51.1 to 61.8% (nt) and from 41.4 to 59.5%
16 (aa).
17 The phylogenetic analysis using an E1/L1 combined nucleotide sequence from the
18 BPV13 obtained from equine sarcoid DNA samples, and the corresponding sequence from all
19 other previously described BPV types, confirmed that this viral strain is grouped together with
20 other *Deltapapillomavirus* representatives (Figure 3).
21
22 **4. Discussion**
23

1 In this study, we describe the presence and participation of the BPV type 13 in equine
2 sarcoids from two horses that were maintained in Southern Brazil. This viral strain was firstly
3 detected by PCR using the degenerate FAP primer pair in three cutaneous papillomas from
4 cattle derived from two different herds within Parana state and designated as the putative new
5 BPV type BPV/BR-UEL4. Through sequence analysis of a partial L1 ORF fragment, 440 bp
6 length, it was shown that this viral type was putatively grouped together with other
7 *Deltapapillomavirus* genus representatives (Claus et al., 2008).

8 The *Deltapapillomavirus* genus comprises papillomaviruses that are known to infect
9 diverse species of wild or domestic ungulates. Currently, five different species are classified
10 in this genus: i) Delta-PV 1 groups viral strains from *Alces alces* (European elk) and *Rangifer*
11 *tarandus* (Reindeer); ii) Delta-PV 2 contains the isolate identified in *Odocoileus virginianus*
12 (American white-tailed deer); iii) Delta-PV 3 clusters PVs obtained from *Ovis aires* (domestic
13 sheep); iv) Delta-PV 4 lumps together viral types that infect *Bos taurus* (domestic cow); and
14 v) Delta-PV 5 includes a representative isolated from *Capreolus capreolus* (Western roe
15 deer). Apart from causing fibropapillomas in their respective host, some representatives of
16 this genus were related to trans-species transmission. BPVs 1 and 2, which were firstly
17 isolated from cattle, have been detected in equine sarcoids worldwide (De Villiers et al., 2004;
18 Bernard et al., 2010).

19 The phylogenetic tree obtained by employing L1/E1 combined sequences (1569 bp
20 length) from BPV type 13 isolated from equine sarcoids and other previously described BPV
21 types showed the grouping of this viral strain with representatives from species delta4. In
22 addition to this grouping pattern, the highest identity presented with BPV2 in the L1 pairwise
23 alignment, the histopathological alterations similar to those verified in BPVs 1 and 2-induced
24 sarcoids, and the ability to infect both cattle and horses confirms the probable classification of
25 this strain into the *Deltapapillomavirus* genus. Since all viral types that were initially isolated

from cutaneous warts from cattle and have been consistently associated with sarcoids are classified in *Deltapapillomavirus* genus, the fact that BPV type 13 is a candidate to be classified in this genus makes its association with sarcoid pathogenesis to be biologically expected.

The presence of PV has been confirmed in sarcoids from many geographical areas by using PCR assay with BPV1/2 specific primers. These studies have focused on the frequency variation of recovery of BPVs 1 and 2 from lesions examined in diverse regions (Teifke and Weiss, 1991; Otten et al., 1993; Bloch et al., 1994; Carr et al., 2001b; Wobeser et al., 2010).

In order to evaluate if the BPV type 13 could be amplified by a PCR assay widely employed in previous epidemiological investigations, DNA samples from the six lesions herein examined were tested with primers designed by Teifke and Weiss et al. (1991) (data not shown). Interestingly, an amplicon of the expected size (approximately 250 bp), representing a partial sequence of E5 ORF, was obtained from each sample evaluated. The nucleotide sequence generated from these amplicons presented a 94% identity with the corresponding E5 fragment of BPV2, confirming the infection with a different BPV type, in this case BPV13 strain. Once the distinction between BPVs 1 and 2 on these PCR products has been achieved through verification of presence (BPV1) or absence (BPV2) of *Bst*XI restriction site, an *in silico* analysis of the sequences obtained indicated that the BPV type 13 could be misclassified as BPV2 by using this diagnostic system.

The detection of the BPV/BR-UEL4 strain, through amplification and sequencing of a partial L1 gene fragment, in a lesion obtained from an affected horse from another geographical area in Brazil, Southeastern region (Rio de Janeiro state) confirms our finding (Silva et al., 2010).

Due to the information that there were around 40 papillomatosis-infected cattle on this farm, which were kept in close contact with the two evaluted horses, the authors believe that those sarcoid-affected horses were likely infected by direct or indirect contact with cattle presenting warts (Bogaert et al., 2005). The previous identification of the BPV/BR-UEL4 strain in cattle warts collected from two other farms located in nearby cities sustains this speculation (Claus et al., 2008).

Although the equine sarcoid occurrence is reported in equids worldwide, investigations with the aim of genotyping PVs associated with these lesions from herds from South America are virtually absent. According to the findings presented in this study, investigations aiming to identify the BPV type 13 as well as other PV strains that might be associated with sarcoids collected from herds from diverse geographical areas are needed to evaluate the frequency of occurrence of this new viral type in this common tumor of equids.

The identification of BPV13 in sarcoids herein examined give rise to the hypothesis that many PV isolates previously diagnosed from sarcoids worldwide by PCR using BPV1/2 E5 specific primers were probably misclassified as BPV2, specially because direct sequencing of numerous amplicons were not performed (Block et al., 1994; Martens et al., 2001a,b; Bogaert et al., 2005; Wobeser et al., 2010). In order to avoid misindentification of PV types the use of general PCR with broad-spectrum primers, and sequencing, seems reasonable in investigations aiming to genotype PV types involved in this disease from now on.

The importance of our findings relies on the possibility of the implementation of adjustments to the diagnostic tools currently employed for the genotyping of PV strains found in association with sarcoid tumors. Furthermore, once that the use of both protective and curative vaccines, based on VLPs produced from BPVs 1 and 2, have been considered promising alternatives in preventing or treating equine sarcoids, the identification of a

1 third BPV type (BPV13 strain) in equine sarcoids becomes relevant for the future design of an

2 immunogen against this common disease of equids.

1 **References**

2

3 Angelos, J.A., Marti, E., Lazary, S., Carmichael, L.E., 1991. Characterization of BPV-like
4 DNA in equine sarcoids. Arch. Virol. 119, 95–109.

5

6 Antonsson, A., Hansson, B.G., 2002. Healthy skin of many animal species harbors
7 papillomaviruses which are closely related to their human counterparts. J. Virol. 76, 12537–
8 42.

9

10 Bernard, H.U., Burk, R.D., Chen, Z., van Doorslaer, K., Hausen, H., de Villiers, E.M., 2010.
11 Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic
12 amendments. Virology. 401, 70–9.

13

14 Bloch, N., Breen, M., Spradbrow, P.B., 1994. Genomic sequences of bovine papillomaviruses
15 in formalin-fixed sarcoids from Australian horses revealed by polymerase chain reaction. Vet.
16 Microbiol. 41, 163–72.

17

18 Bogaert, L., Martens, A., De Baere, C., Gasthuys, F., 2005. Detection of bovine
19 papillomavirus DNA on the normal skin and in the habitual surroundings of horses with and
20 without equine sarcoids. Res. Vet. Sci. 79, 253–8.

21

22 Carr, E.A., Théon, A.P., Madewell, B.R., Hitchcock, M.E., Schlegel, R., Schiller, J.T., 2001a.
23 Expression of a transforming gene (E5) of bovine papillomavirus in sarcoids obtained from
24 horses. Am. J. Vet. Res. 62, 1212–7.

25

1 Carr, E.A., Théon, A.P., Madewell, B.R., Griffey, S.M., Hitchcock, M.E., 2001b. Bovine
2 papillomavirus DNA in neoplastic and nonneoplastic tissues obtained from horses with and
3 without sarcoids in the western United States. Am. J. Vet. Res. 62, 741–4.
4
5 Chambers, G., Ellsmore, V.A., O'Brien, P.M., Reid, S.W., Love, S., Campo, M.S., Nasir, L.,
6 2003. Sequence variants of bovine papillomavirus E5 detected in equine sarcoids. Virus Res.
7 96, 141–5.
8
9 Claus, M.P., Lunardi, M., Alfieri, A.F., Ferracin, L.M., Fungaro, M.H., Alfieri, A.A., 2008.
10 Identification of unreported putative new bovine papillomavirus types in Brazilian cattle
11 herds. Vet. Microbiol. 132, 396–401.
12
13 De Villiers, E.-M., Fauquet, C., Broker, T.R., Bernard, H.U., Zur Hausen, H., 2004.
14 Classification of papillomaviruses. Virology 324, 17–27.
15
16 Forslund, O., Antonsson, A., Nordin, P., Hansson, B.G., 1999. A broad range of human
17 papillomavirus types detected with a general PCR method suitable for analysis of cutaneous
18 tumors and normal skin. J. Gen. Virol. 80,2437–2443.
19
20 Ginn, P.E., Mansell, J.E.K.L., Rakich, P.M., 2007. Skin and appendages. In: Maxie, M. (Ed.),
21 Jubb, Kennedy & Palmer's Pathology of Domestic Animals, Elsevier Saunders, New York,
22 pp. 763-764.
23
24 Goldschmidt, M.H., Hendrick, M.J., 2002. Tumors of the skin and soft tissues. In: Meuten,
25 D.J. (Ed.), Tumors in domestic animals, Iowa State Press, Iowa, pp. 88-89.

1

2 Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitution
3 through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
4

5 Lancaster, W.D., Olson, C., Meinke, W., 1977. Bovine papillomavirus: presence of virus-
6 specific DNA sequences in naturally occurring equine tumors. Proc. Natl. Acad. Sci., USA.
7 74, 524–528.
8

9 Lancaster, W.D., Theilen, G.H., Olson, C., 1979. Hybridization of bovine papilloma virus
10 type 1 and type 2 DNA to DNA from virus-induced hamster tumors and naturally occurring
11 equine tumors. Intervirology. 11, 227–33.
12

13 Manos, M.M., Ting, Y., Wright, D.K., Lewis, A.J., Broker, T.R., Wolinsky, S.M., 1989. The
14 use of polymerase chain reaction amplification for the detection of genital human
15 papillomaviruses. Cancer Cell 7, 209–214.
16

17 Martens, A., De Moor, A., Demeulemeester, J., Peelman, L., 2001a. Polymerase chain
18 reaction analysis of the surgical margins of equine sarcoids for bovine papilloma virus DNA.
19 Vet. Surg. 30, 460–7.
20

21 Martens, A., De Moor, A., Ducatelle, R., 2001b. PCR detection of bovine papilloma virus
22 DNA in superficial swabs and scrapings from equine sarcoids. Vet. J. 161, 280–6.
23

24 Nasir, L., Reid, S.W., 1999. Bovine papillomaviral gene expression in equine sarcoid tumors.
25 Virus. Res. 61, 171–5.

1

2 Ogawa, T., Tomita, Y., Okada, M., Shinozaki, K., Kubonoya, H., Kaiho, I., Shirasawa, H.,
3 2004. Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy
4 teat skin. J. Gen. Virol. 85, 2191–7.

5

6 Olson, C., Cook, H.R., 1951. Cutaneous sarcoma-like lesions of the horse caused by the agent
7 of bovine papilloma. Proc. Soc. Exptl. Biol. Med. 77, 81–284.

8

9 Otten, N., von Tscharner, C., Lazary, S., Antczak, D.F., Gerber, H., 1993. DNA of bovine
10 papillomavirus type 1 and 2 in equine sarcoids: PCR detection and direct sequencing. Arch.
11 Virol. 132, 121–31.

12

13 Rector, A., Bossart, G.D., Ghim, S.J., Sundberg, J.P., Jenson, A.B., Van Ranst, M., 2004.
14 Characterization of a novel close-to-root papillomavirus from a Florida manatee by using
15 multiply primed rolling-circle amplification: Trichechus manatus latirostris papillomavirus
16 type 1. J. Virol. 78, 12698–12702.

17

18 Rector, A., Van Doorslaer, K., Bertelsen, M., Barker, I.K., Olberg, R.-A., Lemey, P.,
19 Sundberg, J.P., Van Ranst, M., 2005. Isolation and cloning of the raccoon (Procyon lotor)
20 papillomavirus type 1 by using degenerate papillomavirus-specific primers. J. Gen. Virol. 86,
21 2029–2033.

22

23 Silva, M.S., Weiss, M., Brum, M.C., Dos Anjos, B.L., Torres, F.D., Weiblen, R., Flores, E.F.,
24 2010. Molecular identification of bovine papillomaviruses associated with cutaneous warts in
25 southern Brazil. J. Vet. Diagn. Invest. 22, 603–6.

1

2 Sundberg, J.P., Burnstein, T., Page, E.H., 1977. Neoplasms of equidae. J. Am. Vet. Med. Ass.
3 170, 150–152.

4

5 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics
6 Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

7

8 Teifke, J.P., Weiss, E., 1991. Detection of bovine papillomavirus DNA in equine sarcoids
9 using the polymerase chain reaction (PCR). Berl. Munch. Tierarztl. Wochenschr. 104, 185–7.

10

11 Wobeser, B.K., Davies, J.L., Hill, J.E., Jackson, M.L., Kidney, B.A., Mayer, M.N.,
12 Townsend, H.G., Allen, A.L., 2010. Epidemiology of equine sarcoids in horses in western
13 Canada. Can. Vet. J. 51, 1103–8.

14

15 Yuan, Z., Gallagher, A., Gault, E.A., Campo, M.S., Nasir, L., 2007. Bovine papillomavirus
16 infection in equine sarcoids and in bovine bladder cancers. Vet. J. 174, 599–604.

1 **Table 1**

2 Anatomical distribution and pathological classification of evaluated sarcoids

Animal	Age (months)	Gender	Breed	Anatomical location	Pathological classification
				right ear	verrucous
A	20	male	American Quarter Horse	muzzle	verrucous
				cheek	fibroblastic
				ear	verrucous
B	17	female	American Quarter Horse	neck	verrucous
				cheek	fibroblastic

1 **Table 2**

2 Sequences and features of degenerate primers used for PCR amplification of partial sequences of BPV/BR-UEL4 strain

Primer	Genomic region target	Polarity	Sequence (5' – 3')[1]	Nucleotide positions[2]	Degree of Degeneracy	Expected amplicon length (bp)
AR-E1F1[3]	E1	forward	CAGGGVMWTTCCCTGBARYTGTTYC	962–986	288	836
AR-E1R2[3]	E1	reverse	TCATANGCCCACTGNACCAT	1797–1778	16	
AR-E1F2[3]	E1	forward	ATGGTNCAGTGGGCNTATGA	1778–1797	16	552
AR-E1R4[3]	E1	reverse	ATTNCCATCHADDGCATTTCT	2329–2309	108	
AR-L1F1[3]	L1	forward	TTDCAGATGGCNGTNTGGCT	5425–5444	48	974
AR-L1R5[4]	L1	reverse	CCATTRTTHWKDCCYTG	6398–6382	144	
AR-L1F8[3]	L1	forward	GGDGAYATGDGKGAMATWGG	6016–6035	144	704
AR-L1R9[3]	L1	reverse	GGRCATTTKGTWGCWADGGA	6719–6697	48	
AR-E1F2[3]	E1	forward	ATGGTNCAGTGGGCNTATGA	1778–1797	16	371
AR-E1R3[4]	E1	reverse	TTNCCWSTATTNGGNGGNCC	2148–2129	1024	

AR-L1F1[3]	L1	forward	TTDCAGATGGCNGTNTGGCT	5425–5444	48	600
AR-L1R3[3]	L1	reverse	CATRTCHCCATCYTCWAT	6024–6007	24	
FAP59[5]	L1	forward	TAACWGTNGGNCAYCCWTATT	5558–5578	128	480
FAP64[5]	L1	reverse	CCWATATCWVHCATNTCNCCATC	6035–6013	576	
MY09[6]	L1	forward	GCMCAGGGWCATAAYAATGG	6379–6398	8	450
MY11[6]	L1	reverse	CGTCCMARRGGAWACTGATC	6830–6811	16	

1

2 [1]Degenerate nucleotides: B = T, C or G; D = A, T, or G; H = A, T, or C; K = T or G; M = A or C; N = A, G, C, or T; R = A or G;

3 S = C or G; V = A, C or G; W = A or T; Y = C or T.

4 [2]Position relative to the sequence of HPV1a.

5 [3]Rector *et al.* (2005).

6 [4]Rector *et al.* (2004).

7 [5]Forslund *et al.* (1999).

8 [6]Manos *et al.* (1989).

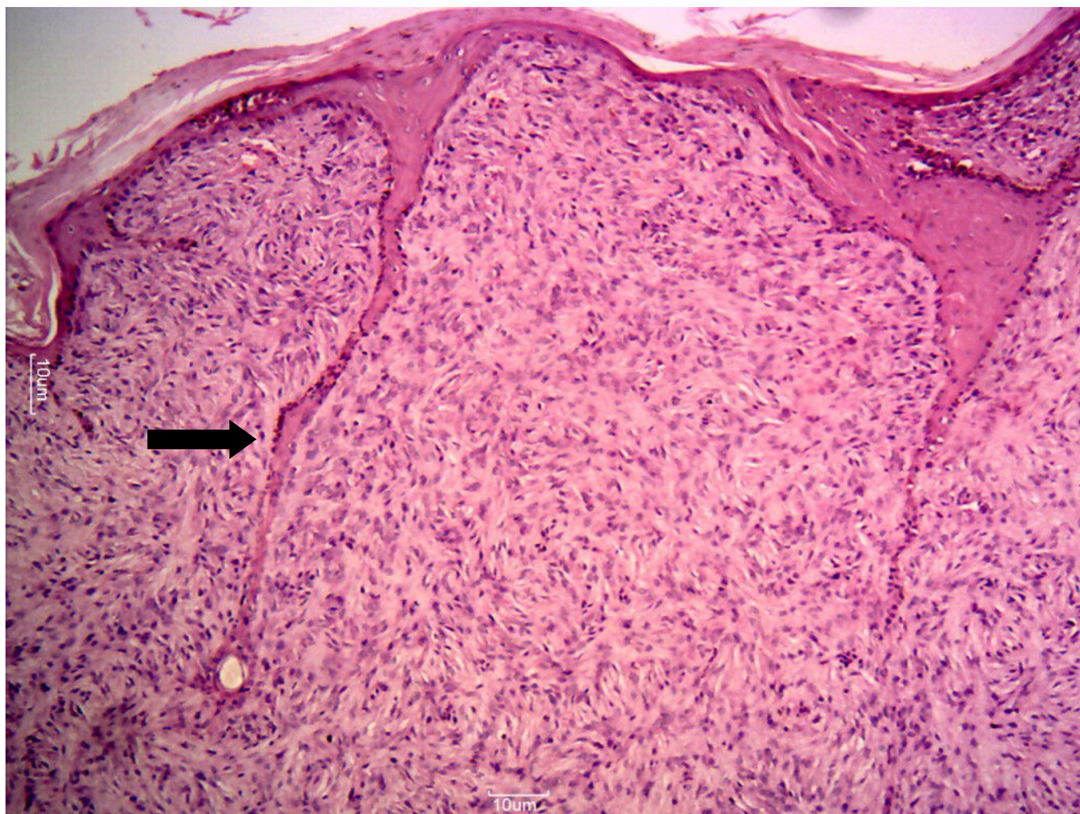
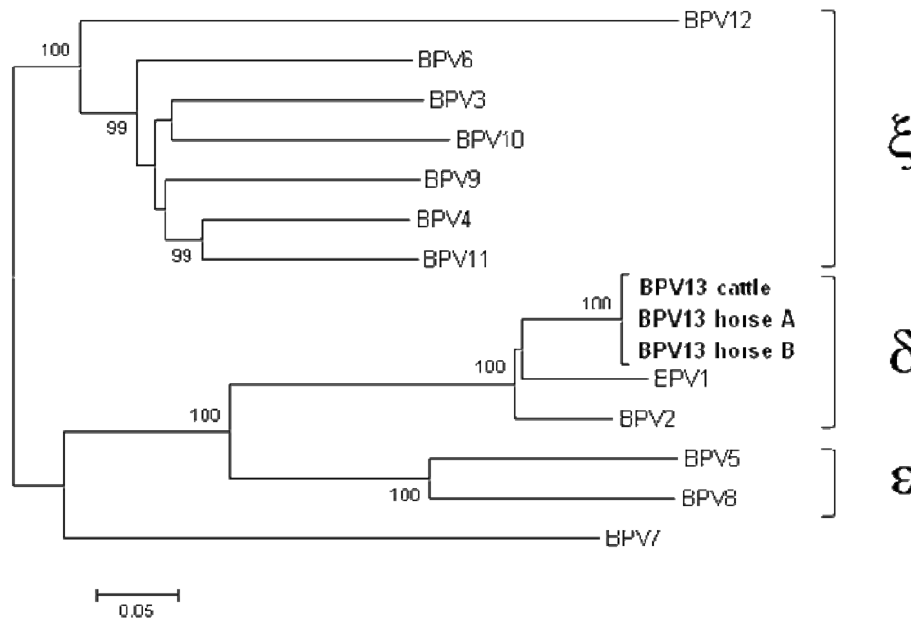


Fig. 1. Histological features characteristic of equine sarcoid observed in lesion collected from right ear of animal A and macroscopically classified as verrucous type. Discrete epidermal hyperplasia and inward projections of the epidermis (*rete pegs* – arrow) into the dermis can be observed as well as proliferation of fibroblasts arranged in short interlacingbundles. Hematoxylin and eosin (HE) stain. Bar=10µm.



1

2 **Fig. 3.** Neighbor-joining phylogenetic tree reconstructed from E1/L1 combined sequences (nt)
3 of BPVs, including that of the BPV type 13 obtained from equine sarcoid DNA samples. The
4 tree is divided into the previously determined genera *Deltapapillomavirus* (BPV1, 2, and 13),
5 *Epsilonpapillomavirus* (BPV5 and 8), *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11, and 12) and
6 an undesignated PV genus (BPV7). *Deltapapillomavirus, Xipapillomavirus* and
7 *Epsilonpapillomavirus* genera are represented by their corresponding greek letter, δ, ξ, and ε,
8 respectively. *The* numbers at the internal nodes represent the bootstrap support values
9 (percentage) determined for 1000 replications.

4. CONCLUSÕES

4. CONCLUSÕES

- A estratégia de amplificação e sequenciamento delineada neste trabalho permitiu a obtenção da sequência genômica completa do BPV13 (BPV/BR-UEL4);

- A comparação da sequência representativa do gene L1 do BPV13 com seqüências do mesmo gene de PVs isolados de outros animais ungulados, permitiu a classificação deste novo tipo de BPV no gênero *Deltapapillomavirus*;

- A comparação das sequências nucleotídicas e/ou de aminoácidos dos diferentes genes identificados no genoma do BPV13, com as de outros PVs, possibilitou a caracterização molecular do genoma deste novo tipo viral, assim como a inferência de propriedades biológicas comumente relatadas para os outros representantes deste gênero;

- A genotipagem dos PVs associados a lesões de sarcóides equinos, utilizando PCR com *primers* degenerados e sequenciamento direto dos amplicons, evidenciou a participação do BPV13, novo representante do gênero *Deltapapillomavirus*, na etiologia do sarcóide equino.

APÊNDICES

APÊNDICE A: Lista de Reagentes

1. 100 mM dNTP Set, 4 x 250 µL; 25 µmol each (100 mM dATP Solution, 100 mM dCTP Solution, 100 mM dGTP Solution, 100 mM dTTP Solution) (Invitrogen Life Technologies®)
2. 10 x PCR-Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen Life Technologies®)
3. 123 bp DNA Ladder (Invitrogen Life Technologies®)
4. Ácido bórico (H_3BO_3) P.M. 61,83
5. Ácido clorídrico (HCl) P.M. 36,46 (Reagen®)
6. Agarose (Gibco BRL®)
7. Ágar (Himedia Laboratories®)
8. Azul de bromofenol (Sigma®)
9. Cloreto de Potássio, P.A. (KCl) P.M. 74,56 (Reagen®)
10. Cloreto de Sódio, P.A. (NaCl) P.M. 58,45 (Reagen®)
11. DNeasy Blood & Tissue Kit (50) (Qiagen®)
12. Ethidium bromide ($C_{21}H_{20}N_3Br$) P.M. 394,3 (Sigma®)
13. Extrato de Levedura (USB®)
14. Glicina, P.A. (Nuclear®)
15. Hidróxido de Sódio, P.A. (NaOH) P.M. 40,00 (Dinâmica®)
16. Hidroximetil amino metano – TRIS 99% P.M. 121,14 (Inlab®)
17. illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare®)
18. illustra plasmidPrep Mini Spin (GE Healthcare®)
19. illustra TempliPhi 100 Amplification Kit (GE Healthcare®)
20. Lambda DNA - (Invitrogen Life Technologies®)
21. *Platinum* Taq DNA Polymerase recombinant 500 units (Invitrogen Life Technologies®)
22. Sacarose, P.A. – sucrose ($C_{12}H_{22}O_{11}$) P.M. 342,31 (Reagen®)
23. TOPO TA Cloning kit for Sequencing (Invitrogen Life Technologies®)
24. Triptona (Acumedia Manufacturers®)

APÊNDICE B: Soluções e Tampões

- **Tampão de Amostra**
  - Azul de bromofenol 0,25%
  - Sacarose – sucrose ($C_{12}H_{22}O_{11}$) 45%

- **Tampão de corrida: TBE (TRIS – Ácido bórico – EDTA) 10 x [ ]**
  - 0,89 M TRIS
  - 0,89 M ácido bórico
  - 0,02 M EDTA dissodium
  - Água bidestilada qsp. 1 litro

  pH = 8,4

- **Meio Luria Bertani (LB) sólido para 500 mL**
  - 5 g de triptona
  - 2,5 g de extrato de levedura
  - 2,5 g de NaCl
  - Dissolver em 400 mL de água destilada
  - Completar para 500 mL (becker).
  - Pesar 3,25 g de ágar em cada erlenmeyer.
  - Adicionar 250 mL da solução a 3,25g de ágar em cada erlenmeyer que será autoclavado.

- **Meio LB líquido**
  - 5 g de triptona
  - 2,5 g de extrato de levedura
  - 2,5 g de NaCl
  - Dissolver em 400 mL de água destilada e completar para 500 mL.
  - Aliquotar a solução em tubos de 3 mL e 5 mL e autoclavar.

APÊNDICE C: Protocolo de Técnicas

- **Gel de agarose a 2%**

  - 1 g de agarose

  - 50 mL TEB 1 x

  - 30 µL de brometo de etídio

- **Ligação do produto da PCR ao vetor**

1. Em um tubo de microcentrífuga (0,6 mL) adicionar:

Solução do DNA a ser clonado	0,5 a 4 µL
Solução salina	1,0 µL
H_2O estéril	6 µL q.s.p
Vetor TOPO	1,0 µL

2. Homogeneizar a reação delicadamente e incubar por 1h.
3. Manter a reação em gelo até proceder a transformação.

- **Pré-inoculação de *E.coli* One Shot®**

1. 3 mL de meio LB líquido autoclavado.
2. Acrescentar 15 µL de células para cada 3 mL de LB.
3. Homogeneizar lentamente o tubo com LB.
4. Incubar em *Shaker* à 37°C e 180 rpm, *overnight*.

- **Preparação de células competentes**

1. Diluir a pré-cultura (150 µL em 5 mL de meio LB líquido) e incubar à 37°C, 180 rpm, até DO_{600nm}=0,4-0,6 (aproximadamente 1h).
2. Logo após, deixar o tubo em banho de gelo por 5 min.
3. Centrifugar 3 mL da cultura em 2 tubos de microcentrífuga (1,5 mL em cada tubo), a 5000 rpm / 5 min.

OBS: Manter as células e soluções em banho de gelo.

4. Eliminar o sobrenadante por inversão do tubo e adicionar ao precipitado 500 µL de 50mM $CaCl_2$.
5. Homogeneizar.
6. Transferir o conteúdo dos 2 tubos para outro tubo.
7. Manter em banho de gelo por 10 min.
8. Centrifugar a 5000 rpm / 5 min e eliminar o sobrenadante posteriormente.
9. Ressuspender as células em 300 µL de 50mM $CaCl_2$.
10. Manter em banho de gelo por 20 min.
11. Aliquotar 50 µL de células competentes em tubos de microcentrífuga e estocar a 20°C.

- **Transformação**

1. Adicionar 3 µL do produto da ligação a 50 µL de células competentes e colocar em banho de gelo por 20 min.
2. Incubar a mistura por 2 min a 42°C em banho-maria.
3. Transferir imediatamente para banho de gelo.
4. Adicionar 1 mL de LB líquido e incubar por 1h a 37°C sob agitação (180 rpm).
5. Centrifugar a 5.000 rpm / 10 min.
6. Descartar o sobrenadante.
7. Ressuspender o sedimento em 100 µL de LB líquido.
8. Semear em duas placas com LB sólido, contendo 75 µg / mL de ampicilina, com o auxílio da alça de Drigalsk.
9. Incubar em estufa a 37°C por 24h.

ANEXOS

ANEXO A: Sequência nucleotídica completa do BPV13

```
1    GTTAACAATG ATCACACCAT CACCGTTTTT TCAAGCGGGA AAAAAAAAGA CAGCTAACTA TAAAAAGCTG CTGACAGACC CTGATTCTCA CATGGACCTG
101  CAAAGTTTTT CCAGAGGCAA TCCTTTCTCA GGATTGGCCT GTCTCTGGTG CAAAGAGCCT CTCACAGAAC TTGATGCTTT TAGGTGCATG ATAAAAGACT
201  TTCATGTTGT ATACCGAAAC GGTTGTACAT TTGGTGCATG CACCCCTTGT CTTGAAGGCT GCCTAGCTAA AGAAAGAAGA CTGTGGAAAG GTGTTCCAGT
301  GACAGGTGAG GAAGCTGAAT TATTGCATGG CAAATCACTT GATAAGCTTT GCATAAGATG CTGCTACTGT GGGGGAAAAC TAACTAAAAA CGAAAAGCAT
401  CGGCATGTGC TTTATAATGA GCCTTTTTGC AAAACCAGAG CTAACATAAT TAGAGGACGC TGCTACGACT GCTGCAGACA TGGTTCAAGG TCCAAATACC
501  CATAGAAACT TGGATGATTC ACCTGCAGGA CCGTTGCTGA TTTTAAGTCC ATGTGCAGGC ACACCTACCA GGGTTCCTGC AGCACCTGAT GCACCCGATT
601  TCAGACTTCC GTGCCATTTC GGCCGTCCTA CTAGGAAGCG AGGTCCTTCA ACGCCTCCGC TTTCCTCTCC CGGAAAAGTG TGTGCAACAG GGCCACGTCG
701  AGTTTATTCT GTTACTGTGT GCTGCGGAGA CTGCGGAAAG GACCTAACAT TTGCTGTCAA GACCGGCTCT ACGACCTTGC TTGGATTCGA ACACCTATTA
801  AACTCAGATT TGGACCTGCT GTGTCCTCGC TGCGAATCTC GCGAGCGTCA TGGCAAACGA TAAAGGTAGC AATTGGGATT CGGGCTTGGG ATGCTCATAT
901  CTGCTGACAG AGGCAGAATG TGAAAGTGAC AAAGAGAATC AGGAACCTGG GGCAGGTGTA GAACTGTCTG TGGAATCCGA TCGGTATGAT AGCCAGGATG
1001 AGGATTTCCT TGACAATGCA TCAGTATTTC AGGGAAATCA CCTGGAGGTC TTCCAGGCTC TAGAGAAAAA GGCGGGTGAG GAGCAGATTT TAAATTTGAA
1101 AAGAAAAGTA TTGGGGAGTG CGGAAAACAG CAGCGGTTCA GAGGCATCTG AAACTCCAGC TAAAAGGCGG AAAGCCGGAG CAAAGAGAAG ATTATTTTCT
1201 GAAAATGAAG CTAACCGTGT TCTTACGCCC TTGCAGGTAC AAGGGGAGGG GGGAGATGGG AGGCAGGGAA TTGATGAGGA ACAGGCCATT AGTCATCTAC
1301 ATCTGCAGCT AGTTAAGTCT AAAAATGCTA CAGCTTTTAA GCTTGGACTG TTTAAGTCTT TGTTTCTTTG TAGCTATCAT GACATAACTA GACTTTTTAA
1401 AAATGATAAG ACTACTAATC AGCAATGGGT TTTAGCTGTA TTTGGACTTG CAGAGGTATT TTTTGAAGCT AGTTTCGAAC TCCTGAAGAA GCAATGTAGC
1501 TTTGTTCAAA TGCAGAAAAG GTCACATGAA GGAGGGACGT GTGCAGTTTA TTTATTATGC TTTAACACAG CAAAAAGCAG AGAAACAGTG CGGAACCTTA
1601 TGGCAAGCAT GCTAAATGTA AGAGAGGAGT GTCTGCTGCT GCAGCCCCCA AAAATACGAG GACTCAGCGC AGCCTTATTT TGGTTTAAAA GTAGTTTGTC
1701 GCCCGCTACT CTTAAACATG GTGCTTTACC TGAGTGGATA CGGGCGCAAA CTACTCTTCA CGACAGCTTG CAGACCGAGA AGTTTGACTT CGGAACTATG
1801 GTGCAATGGG CGTATGATCA TAAATATGCT GAGGAATCAA AAATTGCTTA TGAATATGCT TTGGCAGCAG GTTCTGACAG TAATGCACGA GCTTTCTTAG
1901 CAACTAACAG TCAAGCTAAG CATGTTAAGG ACTGTGCAAC TATGGTCAGG CATTATTTAA GAGCTGAGAC CCAAGCCTTA AGTATGCCTG CATATATTAA
2001 AGCTAGGTGC AAGGTTGCTA CTGGGGAAGG AAGCTGGAAG TCAATTTTAA CTTTTTTTAA CTATCAAAAT ATTGAATTAA TCACCTTTAT TAATGCCTTA
```

```
2101 AAGCTTTGGC TTAAAGGTGT TCCCAAAAAA AACTGCTTAG CATTTATTGG CCCTCCAAAC ACAGGCAAGT CTATGCTATG CAACTCATTA ATTCATTTTC
2201 TAGGGGGAAG TGTTTTATCT TTTGCCAACC ATAAAAGTCA CTTTTGGCTT GCTTCCCTAG CAGATGCTCG AGCAGCTTTA ATAGATGATG CTACACATGC
2301 TTGCTGGAGG TACTTTGACA CATACCTTAG AAATGCATTG GATGGCTATC CCGTCAGTAT TGATAGAAAG CACAAAGCAG CGGTTCAAAT TAAAGCTCCA
2401 CCCCTTTTAG TAACCAGTAA TATTGATGTG CAGGCAGAAG AAAGATACTT GTACTTGCAT AGTCGGGTGC AAACCTTTCG CTTTGAGCAG CCATGCACAG
2501 ATGAATCGGG TGAGCAACCT TTTACTATTA CTGATGCAGA TTGGAAATCT TTTTTTGTAA GGTTATGGGG GCGCTTAGAC CTGGTTGACG AGGAGGAGGA
2601 TAGTGAAGAG GATGGAGACA GCATGCGAAC GTTTACATGT AGCGCAAGAA ACACAAATGC AGTTGATTGA AAAAAGTAGT GATAAGTTGC AAGATCATAT
2701 ACTGTATTGG ACTGCTGTTA GAACTGAGAA CACATTGCTA TATGCTGCAA GGAAAAAAGG GGTGACTGTT CTTGGACACT GCAGAGTCCC ACATTCTGTA
2801 GTGTGTCAAG AAAGAGCAAA GCAGGCAATT GAAATGCAAC TGTCTTTGCA GGAGCTAAGT AAAACTGAGT TTGGGAATGA GCCATGGTCC TTGCTAGATA
2901 CAAGCTGGGA CCGCTATATG TCTGAGCCTA AACGGTGCTT TAAGAAAGGC GCCAGGGTGG TAGAGGTGGA GTTTGATGGA AATGCAAGCA ATACAAACTG
3001 GTACACTGTC TACAGCAAAC TGTACATGCG CACAGAGGAC GGCTGGCAGC TTGCGAAGGC TGGGGCTGAC GGAACTGGGC TCTACTACTG CACCATGGCC
3101 GGTGCTGGAC GCATTTATTA CTCTCGCTTT GGTGAGGAGG CAGCCAGATT TAGCACAACA GGGCATTACT CTGTAAGAGA TCAGGACAGA GTGTATGCTG
3201 GTGTCTCATC CACCTCTTCT GATTTTAGAG ATCGCCCAGA CGGAGTCTGG GTCGCATCAG AAGGACCTGA AGGAGACCCT GCAGGAAAAG AAGCCGAGCC
3301 AGCCCAGCCT GTCTCTTCTT TGCTCGGCTC CCCCGCCTGC GGTCCCATCA GAGCAGGCCT CGGTTGGGTA CGGGACGGTC CTCGCCCGCA CCCCTACCAT
3401 TTTCCTGCAA GCTCGGGGGG CTCTCTTCTC CGCTCTTCCT CCACCCCGGT GCAGGGCTCG GTACCGGTGG ACTTGGCACC AAGGCAGGAA GAAGAAGAAA
3501 ACCAATCGCC CGACTCCACA GAGGAGGAAC CTTTGAGTGT ACCAAGGCGC ACCTCTGATG CTGAAGGATT CCACCTGCTG AAGGCAGGAC AATCATGCTT
3601 TGCTCTAATT TCAGGCACAG CTAACCAGGT AAAGTGCTAC CGCTTTCGTG TGAAAAAAAA CCATAGACAC CGCTACGAGA ACTGCACCAC CACTTGGTTT
3701 ACTGTGGCTG ACAACGGTGC TGAAAGACAA GGACAGGCCC AGATCCTGAT CACCTTTGGA TCTCCAGGTC AAAGGCAAGA CTTTCTGAAA CATGTACCAC
3801 TACCTCCTGG AATGAACATT TCCGGCTTCA CAGCCAGCTT GGACTTTTAA TCACTGCCAT TTGGTGTTCT TCATATCTTG ACTTGGCATA CTATGCCGAA
3901 TCTATGGTTT CTATTGTTCT TGGGACTAGT TGCTGCAATG CAACTGCTGC TGTTACTGTT TCTGCTGCTG TTTTTCTTGG TGTATTGGGA TCATTTTGAC
4001 TGCTCCTGTA CAGGTCTGCC TTTTTAATGC GTCACTGGTT ACTTGCACTA TTGTTACTTC TGTGTGGATT TGATTTGTTT ACTATTCTGT ATGAAGTGTT
4101 TCTGTTTGTG ATTGTATTGT TGTTTGTACA TTTTTTACTA GAATTTGTAT TCCCCCTGCT CAGATTTTTT ATGGTATAAA GCTGCAGCAA TAAACATGAG
4201 TGCACGAAAA AGAGTTAGGC GTGCAAATGT GTATGACCTG TACAGGACTT GCAAGCAAGC GGGCACATGT CCACCAGATG TGATACCCAA AGTAGAAGGG
4301 GACACTATAG CAGATAAAAT TTTAAAATTG GGCGGGCTTG CAATTTATTT AGGGGGACTG GGAATTGGAA CATGGTCTAC AGGAAGAGTG GCTGCAGGTG
4401 GATCACCAAG GTATGTACCA TTAAGGACAT CAGGGTCCAC TAGCAGTCTA GCCTCTGCAG GATCAAGAGC TGGGGCAGCT GCAGGTACTC GAAGCAGCAT
4501 CACCACAGGC ATCCCACTTG ATACTCTGGA AACTATTGGG GCTTTTAGGC CTGGGGCATA TGAGGACACT GTTTTACCAG AAGCTCCTGC TATTGTTACT
```

```
4601 CCTGATGCTG TACCTGCTGA CACAGGAATA GATGGCCTGT CCATAGGTAC TGACTCTTCT ACAGAAACCC TAGTAACACT GTTAGAGCCT GAGGGTCCTG
4701 ATGACATAGC AGTGTTAGAG CTGCAACCCC TAGACCATCC AAATTGGCAA GTTAGCAATG CTGTTCATCA GTCTTCTGCA TACCACGCCC CTCTGCAGCT
4801 GCAGTCCTCC ATAGCAGAAA CATCTGGACT GGAAAATATT TTCGTAGGAG GAGCTGGGCT AGGGGATACA GGGGGAGAAG AAATAGAACT CACATACTTT
4901 GGGTCTCCAC GCACAAGCAC CCCCAGAAGC TTTCCTACAA GTGCACGGGG TATTTTAAAC TGGTTCAGCA AAAGGTACTA CACACAAATA CCCACAGAAG
5001 ACCCTGATAT TTTTACATCA CAGACGTTTG CAAACCCAGT GTATGATCCA GAACCTGTTG TGCTCAAAGG TCCAAGTGGC CGTGTTGGCC TCAGCCAGGT
5101 ATATAGGCCT GACTATATTG AAACACGTGG TGGGGGGCAG GTAGGCCCAC AGTTGCATGT CAGATATTCC TTGAGCACTA TTACAGAAGA TGTTGAAGCC
5201 ATACCGTTAG CAGGTGATGA GGACACACAA GGTTTGGCAT TTATCCCTGT ACATGAAGAA CATGGTGACT TTGAGGAAAT AGAGCTAGAT GATTTTAGTG
5301 AGCAGCAAAG CCTGCTACCA AGAAGAGCAT CAGCCACAAA TCCTGTTGGC AGTGGAGTTC GCAGGTCCAT CATTCCAGGT CAAAGTTTTA GTGCAACACG
5401 TCCCACAGGT GTTGTAACGT ATGGCTCACC TGACATGTAC CCTGCCAGCC CTGTTGGCCC TGACTCTACT TACCCTAGCC TAGTTATTGA TGACAACAGT
5501 ACTACTCCAA TAATTATAAT TGATGGCCAC ACCATTGACC TCTATAGCAA TAACTATAGC TTGCATCCCT CCTTGTTGAG GAAAAGAAAA AAACGCAAAC
5601 ATGCCTAATT TTTTTTGCAG ATGGCGTTGT GGCAACAAGG CCAGAAGCTG TATCTCCCTC CAACCCCAGT AAGCAAGGTG CTATGCAGTG AAACCTATGT
5701 GCAAAGAAAA AGCATATTTT ATCATGCAGA AACGGAACGC CTGTTAACTG TAGGACATCC ATACTACAAA GTCACTGTTG GGGACAAAAC TGTTCCCAAG
5801 GTCTCTGCTA ATCAATTTAG GGTTTTTAAA ATCCAACTAC CTGATCCTAA TCAGTTTGCA TTGCCTGATA AAACAGTTCA CAATCCAAGC AAGGAGCGCC
5901 TTGTCTGGGC AGTTATAGGA GTACAGGTCT CCCGAGGTCA ACCTCTTGGA GGAACAGTTA CTGGGCACCC CACTTTTAAT GCCTTGCTTG ATGCAGAAAA
6001 TGTTAATAGG AAAGTGACCA GCCAAACAAC AGATGACAGG AAGCAAACAG GATTAGATGC TAAGCAACAA CAGATTCTGT TGCTGGGCTG TACCCCTGCA
6101 GAAGGGGAAT ACTGGACAAC AGCCCGGCCA TGTGTAACTG ATCGGGTTGA CAATGGTGCG TGTCCTCCCT TAGAATTAAA GAACAAAAAA ATAGAAGATG
6201 GAGACATGAT GGAAATAGGC TTTGGTGCAG CTGACTTCAA AGCCATTAAT GCCAGTAAAT CAGATCTACC TCTTGACATT CAAAATGAAA TATGCTTATA
6301 TCCTGACTAC CTCAAAATGG CTGAAGATGC AGCTGGAAAT AGCATGTTCT TTTTTGCTAG GAAAGAACAA GTTTATGTTA GGCACATATG GACTAGGGGT
6401 GGCTCAGAAA AAGAAGCACC TAGCACAGAC TTCTACCTCA AGAATAATAA AGGTGATGAA ACCTTAAAGA TACCCAGTGT TCACTTTGGT AGTCCTAGTG
6501 GCTCTCTAGT GTCCACTGAT AATCAAATAT TTAACAGGCC TTATTGGCTT TTTAGAGCCC AAGGCATGAA CAATGGCATT GCATGGAATA ATTTATTGTT
6601 TTTAACTGTA GGGGATAACA CAAGGGGTAC TAACCTTAGT ATTAGTGTAG CTGCAGACGC CCAACCTCTT AAAGAATATG ATACCACTAA GTTTAATCTC
6701 TACCATAGAC ATATGGAAGA ATATAAGCTA GCTTTTATCC TAGAATTGTG CTCTGTTGAA ATCACTGCGC AAACAGTGTC ACATCTGCAG GGACTTATGC
6801 CCACTGTGCT TGAAAACTGG GAGATTGGTG TGCAGCCTCC TACCTCTTCT ATACTTGAAG ATACCTATAG GTACATAGAG TCCCCTGCAA CTAAATGTGC
6901 AGGAAATGTG ATTCCTGCAA AGGAAGACCC GTATGCAGGG CTACGGTTTT GGGATATAGA CTTAAAAGAA AAGCTGTCTT TGGACTTAGA TCAATTTCCC
```

7001 TTGGGAAGAA GATTTCTAGC TCAGCAAGGG GCAGGATGTT CAACTGTGAG AAAGAGGGCT GTTGCAACCA AAAATTCCAG TAAGCCTGCA AAAAGAAAAA

7101 AAATAAAAGC TTAAGCTTCT ATAAATGTAC AATAAATGTG AACCATAAGG TAAGTCAACT GCACCTAATA AAAATCACTT AATAGCAATG TGCTGTGTCA

7201 GTTGTTTATT ACAACCACAC CCGGTACACA TCCTGTCCAG CATTTGCAGT GCATGCATTG AATTATTGTG CTGGCGAGAC TTCATGGCGC CTGGCACTGA

7301 TTCCTGCTTC CTCAGCCAAA ATGAATAATC GCTTTGCTGG CAAGAAACTG AACATCTATG GGACGCGTGC AAAGCACCGG AGGCGGTAGT TGCGGGGTAA

7401 GTACCGAATT TTTATTAGCA CCGATCTCGG TGAAGTGAAA GTTGTCTCTT TTTTTTTCGC GTGGAGCGGT ACCTTAAGCG GTATAAGTAT CGATTAGGTC

7501 TATTTTAGTC TCTGTGTCGG AACCAGAACC GATAAAAGTT TCCATTACGT CTGGGCTTGT CTGCTACTGC CTCTCTATGG TTTGTTGGAG GATTAGACGT

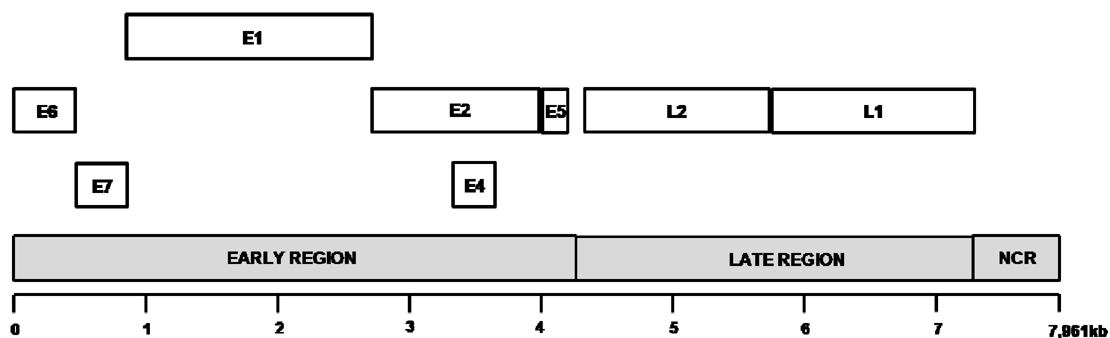
7601 GATACCAATA CTGGTGCATA GCGGATGTCT GTACCGCCAT CGGTGTACCG AAATGGATTG GGGCTCCCCA AGGGATTGAT GGGATGACAG CTTTAGTGTC

7701 AAGTTTATTT AAGCTTGATA GGCTTAATTG GTGAGTACAA GTTGCAAGTT GTAACCTGAT CCCCACAAAG TACCGCTGCC GGTTGGGGTC AAACCGGCAG

7801 CGGTGCAGGG TTACGCCTCA AAGTAGACAG GAATTCATGC CAAGTAGGCG TACCGAAACC TGAAAAAAGC GCGGGAACCA ATCAGAATGC AGAATTTTTT

7901 ATTTCAAGCT ACCGAAACCG GTAAGTAAGT GGTGTATACT TTTTCCCATG GAATAATTGT T

ANEXO B: Representação da organização do genoma do BPV13



ANEXO B: Código das bases degeneradas

R	K	S	W	M	Y	D	V	B	H	N
G	G	G	A	A	T	G	G	G	A	G
A	T	C	T	C	C	A	A	T	T	A
						T	C	C	C	T
										C

ANEXO D: Reconstrução filogenética com os 13 representantes de BPV