Universidade Estadual de Londrina

BRUNA FONSECA MATIAS

IDENTIFICAÇÃO MOLECULAR DOS TIPOS DE PAPILOMAVÍRUS BOVINO PRESENTES EM LESÕES HIPERPROLIFERATIVAS DO TRATO GASTROINTESTINAL SUPERIOR DE BOVINOS LEITEIROS

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Tese apresentada ao Programa de Pós-graduação em Ciência Animal – área de concentração Sanidade Animal – da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutor em Ciência Animal.

Orientador: Prof. Dr. Amauri Alcindo Alfieri
Coorientadora: Profa. Dra. Michele Lunardi

Londrina
2025

Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

Matias, Bruna.
IDENTIFICAÇÃO MOLECULAR DOS TIPOS DE PAPILOMAVÍRUS BOVINO PRESENTES EM LESÕES HIPERPROLIFERATIVAS DO TRATO GASTROINTESTINAL SUPERIOR DE BOVINOS LEITEIROS / Bruna Matias. - Londrina, 2025.
66 f. : il.

Orientador: Amauri Alfieri.
Coorientador: Michele Lunardi.
Tese (Doutorado em Ciência Animal) - Universidade Estadual de Londrina, Centro de Ciências Agrárias, Programa de Pós-Graduação em Ciência Animal, 2025.
Inclui bibliografia.

1. Lesões hiperproliferativas em trato gastrointestinal superior de bovinos; - Tese. 2. Tipos de papilomavírus bovino presentes em lesões de trato gastrointestinal de bovinos. - Tese. I. Alfieri, Amauri. II. Lunardi, Michele. III. Universidade Estadual de Londrina. Centro de Ciências Agrárias. Programa de Pós-Graduação em Ciência Animal. IV. Título.

CDU 619

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Londrina, 10 de março de 2025

AGRADECIMENTOS

Primeiramente a Deus, pelo dom da vida e por me permitir chegar até aqui, com sabedoria, empatia e coragem para enfrentar todos os obstáculos no decorrer do processo.

Aos meus pais, por todo o apoio em cada etapa da minha formação, mesmo à distância sempre estiverem presentes desde a graduação até o mestrado e doutorado. Tudo que sou é por vocês e não tenho palavras para agradecer.

Ao meu irmão Lucas, que me transmitia força e coragem para conquistar meus objetivos.

Ao meu esposo Diego, que esteve ao meu lado desde o começo do doutorado, sempre me apoiando e encorajando, te amo e sou grata por tudo.

Aos meus sogros que me acolheram e estavam presentes sempre que precisei, muito obrigada.

Ao meu amigo José Victor, que esteve comigo na maioria das etapas do mestrado e do doutorado, sempre me apoiando e me auxiliando. Obrigada pelas conversas, desabafos e conselhos.

Á minha querida Profa. e coorientadora Michele Lunardi, não tenho palavras para expressar minha gratidão, por todo aprendizado, conselhos e ajuda em cada processo.

Ao Prof. Amauri Alfieri, meu orientador, obrigada por me aceitar como sua orientada e acreditar no meu projeto.

Á Profa. Elis Lorenzetti, que esteve sempre presente e me ajudou em tudo que precisei. Além de aceitar fazer parte da minha banca de qualificação, muito obrigada.

Por aceitar compor a banca de avaliação da minha tese e contribuir com as melhorias do meu trabalho, muito obrigada Profa. Alaís Agnol, Profa. Tatiane Faccin, Profa. Marlise Claus, Profa. Elisabete Takiuchi e Profa. Cíntia Daudt.

A todos que de forma direta ou indireta contribuíram com meu projeto.

Matias, Bruna Fonseca. **Identificação molecular dos tipos de Papilomavírus Bovino presentes em lesões hiperproliferativas do trato gastrointestinal superior de bovinos leiteiros**. 2025. 66f. Tese (Doutorado Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2025.

RESUMO

Papilomavírus (PVs) são considerados oncogênicos, capazes de produzir lesões hiperproliferativas de origem benigna ou maligna em tecido cutâneo ou em mucosa. Estes patógenos são responsáveis por causar perdas econômicas consideráveis na cadeia produtiva bovina, além disso, são negligenciados e pouco estudados, necessitando de estudos mais aprofundados para elucidar a real diversidade de tipos virais existentes. O objetivo deste trabalho foi avaliar a presença do vírus em lesões no trato gastrointestinal superior (TGIS) de bovinos leiteiros criados em pastagens contaminadas com samambaia (*Pteridium spp.*), a fim de demonstrar a diversidade de tipos virais de *Bos taurus papillomavirus* (BPV), já caracterizados e ainda não descritos, associados com essas lesões. As lesões foram coletadas em bovinos leiteiros de descarte abatidos em abatedouro frigorífico localizado na Mesorregião Norte Central do Paraná. No primeiro artigo, PCR (*Polimerase cadeia reaction* – Reação em cadeia da polimerase*)* convencional com primers degenerados foi utilizada, seguida pelo sequenciamento Sanger. Vinte e três lesões hiperproliferativas de esôfago extraídas de desesseis bovinos foram avaliadas. Entre elas foi possível amplificar os genes L1 e E1 em apenas cinco papilomas. Na análise histopatológica, foi possível classificar todas as lesões como papiloma escamoso. Além disso, os BPVs 1 e 4, já caracterizados nestas lesões em estudos anteriores, foram detectados. Pela primeira vez, os BPVs 9 e 39 foram identificados em lesões presentes na mucosa deste sítio anatômico, além de um possível novo tipo relacionado ao BPV12. Já no segundo artigo, duas lesões hiperproliferativas em esôfago de um bovino foram investigadas. Após análise histopatológica, as lesões foram classificadas como papiloma escamoso e carcinoma *in situ*. Para análise molecular, foram utilizadas a PCR convencional com o uso de primers degenerados, seguida de sequenciamento Sanger, além da técnica de RCA (*Rolling circle amplification* - Amplificação por círculo rolante) seguida pelo HTS (*High-throughput sequencing* – Sequenciamento de alto rendimento). Com a utilização da PCR convencional com primers degenerados, foi possível amplificar fragmento genômico parcial do BPV4 no papiloma escamoso e pela primeira vez fragmento genômico parcial do BPV2 em carcinoma *in situ*. Já com o emprego das técnicas RCA e HTS, foi possível obter o genoma completo do BPV4 a partir do papiloma escamoso e demonstrar a coinfecção com os BPVs 2 e 4 no carcinoma *in situ*. Conclui-se, a partir dos achados do presente estudo, que a diversidade viral de BPVs associados a mucosa do TGIS de bovinos é superior a estimada nos estudos iniciais.

Palavras-chave: BPV. Papilomatose. Papiloma escamoso. Lesões esofágicas hiperplásicas. Carcinoma *in situ*.

MATIAS, Bruna Fonseca. **Identification molecular of Bovine Papillomavirus types present in hyperproliferative lesions of the upper gastrointestinal tract of dairy cattle**. 2025. 66p. Thesis (Doctorate in Animal Science) – Universidade Estadual de Londrina, Londrina, 2025.

ABSTRACT

Papillomaviruses (PVs) are considered oncogenic, capable of producing hyperproliferative lesions of benign or malignant origin in skin tissue or mucosa. These pathogens are responsible for causing considerable economic losses in the bovine production chain. Furthermore, they are neglected and little studied, requiring more in-depth studies to elucidate the real diversity of existing viral types. The objective of this study was to evaluate the presence of the virus in lesions in the upper gastrointestinal tract (GIT) of dairy cattle raised on pastures contaminated with bracken (*Pteridium* spp.), in order to demonstrate the diversity of viral types of *Bos taurus papillomavirus* (BPV), already characterized and not yet described, associated with these lesions. The lesions were collected from dairy cattle slaughtered at a slaughterhouse located in the North Central Mesoregion of Paraná. In the first article, conventional PCR (Polymerase chain reaction) with degenerate primers was used, followed by Sanger sequencing. Twenty-three hyperproliferative lesions of the esophagus extracted from sixteen cattle were evaluated. Among them, it was possible to amplify the L1 and E1 genes in only five papillomas. In the histopathological analysis, it was possible to classify all lesions as squamous papilloma. In addition, BPVs 1 and 4, already characterized in these lesions in previous studies, were detected. For the first time, BPVs 9 and 39 were identified in lesions present in the mucosa of this anatomical site, in addition to a possible new type related to BPV12. In the second article, two hyperproliferative lesions in the esophagus of a bull were investigated. After histopathological analysis, the lesions were classified as squamous papilloma and carcinoma *in situ*. For molecular analysis, conventional PCR with degenerate primers followed by Sanger sequencing, in addition to the RCA (Rolling circle amplification) technique followed by HTS (High-throughput sequencing) were used. Using conventional PCR with degenerate primers, it was possible to amplify a partial genomic fragment of BPV4 in squamous papilloma and, for the first time, a partial genomic fragment of BPV2 in carcinoma *in situ*. Using RCA and HTS techniques, it was possible to obtain the complete genome of BPV4 from squamous papilloma and demonstrate co-infection with BPVs 2 and 4 in carcinoma *in situ*. It is concluded from the findings of the present study that the viral diversity of BPVs associated with the mucosa of the GIT of cattle is higher than that estimated in the initial studies.

Key-words: BPV. Papillomatosis. Squamous papilloma. Hyperplastic esophageal lesions. Carcinoma *in situ*.

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LISTA DE ABREVIATURAS E SIGLAS

ATP	Adenosina trifosfato
BPV	Papilomavírus bovino (*Bos taurus papillomavirus*)
CCE	Carcinoma de células escamosas
CIS	Carcinoma *in situ*
COX	Cicloxigenase
DNA	Ácido desoxirribonucleico
dNTP	Desoxinucleosídeo trifosfato (*Deoxynucleoside triphosphate*)
DSB	Rupturas de fita dupla (*Double strand breaks*)
HEB	Hematúria Enzoótica Bovina
HPV	Papilomavírus humano (*Human papillomavirus*)
HTS	Sequenciamento de alto rendimento (*High-throughput sequencing*)
ICTV	Comitê Internacional de Taxonomia de Vírus (*International Committee on Taxonomy of Viruses*)
LCR	Região longa de controle (*Long Control Region*)
MHC-I	Complexo principal de histocompatibilidade classe I (*Major histocompatibility complex class I*)
MHC-II	Complexo principal de histocompatibilidade classe II (*Major histocompatibility complex class II*)
ORF	Fase de leitura aberta (*Open Reading Frame*)
PaVE	*The Papillomavirus Episteme*
PCR	Reação em cadeia da polimerase (*Polimerase cadeia reaction*)
PDGF R	Receptores de fator de crescimento derivado de plaquetas (*Platelet-derived growth factor receptors*)
PULNMP	Neoplasia urotelial papilar de baixo potencial maligno (*Papillary urothelial neoplasm of low malignant potential*)
PV	Papilomavírus
RCA	Amplificação por círculo rolante *(Rolling Circle Amplification)*
TGIS	Trato gastrointestinal superior
UEL	Universidade Estadual de Londrina

SUMÁRIO

1 INTRODUÇÃO

Papilomavírus (PVs) estão classificados na família *Papillomaviridae* e são amplamente disseminados entre inúmeras espécies de animais vertebrados, incluindo os seres humanos. PVs apresentam distribuição mundial e são caracterizados por seu potencial oncogênico, podendo originar lesões hiperproliferativas benignas ou malignas em tecido cutâneo e em mucosas. A formação tumoral maligna é mais incidente em mucosas (Campo, 2002).

A família *Papillomaviridae* possui uma gama de espécies e tipos virais, representadas em sua maioria por papilomavírus humanos (HPVs) que infectam exclusivamente o ser humano. No entanto, a real diversidade de PVs que infectam os animais ainda é desconhecida. Até o momento, mais de 200 tipos de HPVs já foram completamente caracterizados, frente a apenas 44 tipos de *Bos taurus papillomavirus* (BPVs) (PaVE, 2024). Portanto, apesar dos bovinos serem a segunda espécie animal mais estudada em relação aos PVs, ainda é necessária a realização de estudos mais detalhados e abrangentes com o objetivo de elucidar a real diversidade de BPVs associados a diversas entidades clínicas em bovinos. Adicionalmente, investigações completas deverão ser conduzidas com os objetivos de identificar e caracterizar PVs provenientes de outras espécies animais (Claus et al., 2007).

A investigação de BPVs tem sido negligenciada, mesmo estando entre as enfermidades que causam perdas econômicas consideráveis na cadeia produtiva de bovinos de leite. As perdas econômicas causadas pelas infecções pelos BPVs podem ocorrer de forma direta, quando há morte de animais, e de forma indireta, no caso da papilomatose cutânea quando localizada no úbere e tetos das vacas, impossibilitando a ordenha, e depreciando o valor dos animais, devido à perda de peso causada por tumores no trato gastrointestinal superior de bovinos (TGIS) (Alfieri et al., 2017).

Estudos prévios, realizados por meio de técnicas moleculares clássicas como hibridização *in vitro* e PCR com primers específicos, identificaram a participação do BPV4 no desenvolvimento da maioria dos tumores do TGIS de bovinos. Até o momento, apenas seis tipos de BPVs (1, 2, 4, 5, 13 e 44) foram descritos em tipos histológicos distintos deste sítio anatômico em bovinos e bubalinos. Nas últimas décadas, o emprego da técnica de PCR com primers degenerados, capazes de anelar nos genes mais conservados de PVs de uma gama de tipos virais,

demonstrou a ocorrência de alta diversidade genética de tipos de BPV relacionados à papilomatose cutânea.

2 REVISÃO DE LITERATURA

2.1 FAMÍLIA PAPILLOMAVIRIDAE

A família *Papillomaviridae* é atualmente uma família viral distinta segundo o 7º relatório do *International Committee on Taxonomy of Viruses* (ICTV) (de Villiers et al., 2004) e é subdividida em duas subfamílias, a subfamília *Firstpapillomavirinae*, e a subfamília *Secondpapillomavirinae* (ICTV, 2024).

Além de estarem disseminados entre espécies de animais vertebrados, incluindo os seres humanos, estes agentes virais são considerados espécie específicos, uma vez que a replicação viral produtiva só é observada quando a infecção ocorre em determinada espécie (Roperto et al., 2008). Além disso, PVs podem ocasionar a formação tumoral em seus hospedeiros, sendo por este motivo considerados vírus oncogênicos. As lesões hiperproliferativas decorrentes da infecção por PVs podem ocorrer em tecido cutâneo e mucoso e apresentar origem benigna ou maligna. Entretanto, a formação tumoral de origem maligna é observada principalmente em mucosas (Campo, 2002). Apesar de serem considerados espécie específicos, alguns tipos virais possuem capacidade de infectar outras espécies animais, onde não ocorre a replicação viral infecciosa, como por exemplo o sarcóide equino que é causado pelos BPV tipos 1, 2 e 13 (Lunardi et al., 2013).

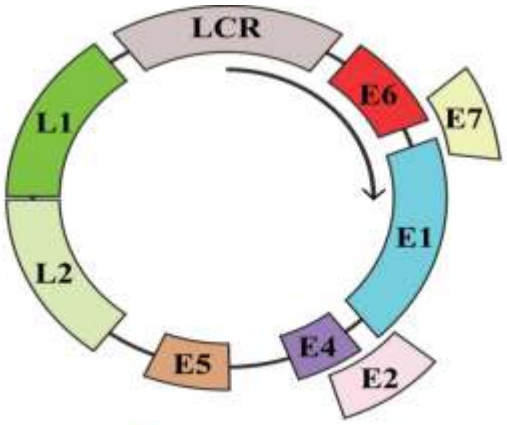
2.2 ESTRUTURA, GENOMA E REPLICAÇÃO

PVs são vírus com potencial oncogênico com 52 a 55 nm de diâmetro, capsídeo icosaédrico desprovido de envelope lipoproteico (Roperto et al., 2008). O genoma viral é constituído por DNA de fita dupla circular, com extensão de aproximadamente 8.000 pares de bases (Munday 2014). O genoma é dividido em três regiões que são classificadas em precoce (*early* - E), tardia (*late* - L) e região longa de controle (*long codon region* - LCR) (Figura 1) (van Doorslaer, 2013).

A região E é responsável por codificar as proteínas não estruturais denominadas de E1 a E7. As proteínas E1, E2 e E4 atuam na replicação e transcrição do genoma, enquanto as oncoproteínas E5, E6 e E7 são responsáveis pela transformação tumoral (Borzacchiello, 2006). Adicionalmente, a proteína E4 está relacionada à remodelação da matriz extracelular e maturação viral (Ferraro et al.,

2011). A região L codifica as proteínas estruturais L1 e L2 que atuam na formação do capsídeo viral (Roperto et al., 2008). A região LCR, apesar de não codificar proteínas virais, possui elementos importantes para a transcrição e replicação viral, além de possuir a origem de replicação (Van Doorslaer, 2013).

Figura 1 – Organização genômica do DNA circular do papilomavírus, demonstrando as subdivisões: Precoce (*Early*), Tardia (*Late*) e Região longa de controle (*Long control region)*.



Fonte: Araldi et al 2017.

A proteína E1 possui três domínios funcionais sendo que o primeiro, a porção N-terminal, induz a fosforilação de Cdk2. O segundo domínio, C-terminal, atua como helicase dependente de ATP. Por fim, o domínio central se liga à proteína E2, formando o complexo E1-E2. As proteínas E1 e E2 são cruciais para que ocorra a replicação viral (Forslund et al., 1999; García-Vallvé et al., 2005; Araldi et al., 2017). O complexo E1-E2 irá se ligar à origem de replicação localizada na LCR do genoma viral (Araldi et al., 2017).

A proteína E4 pode ser facilmente detectada nas camadas suprabasais e granulosas da epiderme, sendo a proteína reconhecida como o principal sinal de patogenicidade dos PVs. A E4 também contribui com a replicação viral por se conectar com a citoqueratina filamentosa (Campo, 1997), além de estar relacionada à remodelação da matriz extracelular e maturação viral (Ferraro et al., 2011).

A oncoproteína E5 é a proteína mais conhecida por fazer a transformação celular, tanto *in vivo* quanto *in vitro* (Campo, 2006). A E5 é responsável

pela indução de alterações na membrana celular, prejudicando o complexo de Golgi, se ligando a subunidade vacuolar H+ -ATPase, fazendo com que haja a alcalinização da membrana interna dessa organela (García-Vallvé et al., 2005). Com isso, se inicia o sequestro da cadeia pesada do MHC-II (*Major histocompatibility complex class* II - complexo de histocompatibilidade principal classe II), promovendo um mecanismo de evasão do sistema imune (Venuti et al., 2011). Outro mecanismo da E5 é a inibição da expressão de MHC-I (*major histocompatibility complex class I* – complexo de histocompatibilidade principal classe I), e cicloxigenase (COX), fazendo com que ocorra a persistência da infecção viral (Borzacchiello, 2007; Araldi et al., 2017). Adicionalmente, a oncoproteína E5 inibe a comunicação celular e compromete a diferenciação celular (Rampias et al., 2014). Outra alteração causada pela oncoproteína E5 é sua ligação ao recepctor beta do fator de crescimento (PDGF R (*Platelet derived growth factor receptors* – receptores de fator de crescimento derivado de plaquetas)), ativando a via Akt mediada por 3-quinase que, consequentemente, irá ativar a ciclina D3 promovendo então a desregulação do ciclo celular (Araldi et al., 2017).

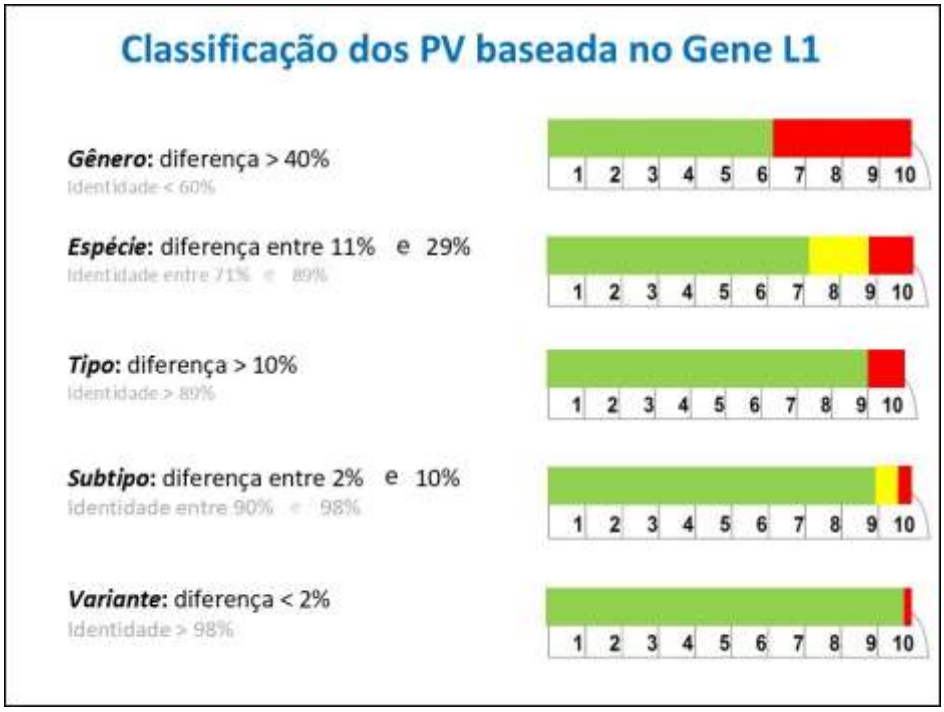
A ativação do PDGF R também é capaz de estimular a angiogênese (Pietras e Ostman, 2010). A oncoproteína E6 atua na transformação e na replicação celular, sendo responsável por causar a degradação proteolítica da p53 que impede a replicação viral (Araldi et al., 2017). A oncoproteína E7 contribui para a desregulação do ciclo celular, por meio do rompimento de DNA causando instabilidade genômica por inibir o reparo de DSB (*double strand breaks* – quebras bifilamentares) (Araldi et al., 2017).

A proteína L1 está diretamente ligada ao fator de virulência, uma vez que está associada ao mecanismo de infecção viral, se ligando aos receptores de sulfato de heparina presentes na membrana celular. A proteína L1 tem sido considerada como um dos fatores de infecção produtiva. Durante a morfogênese, a proteína L2 se liga ao DNA viral, contribuindo com a encapsidação e liberação da progênie viral, assim como está diretamente associada a montagem do papilomavírus e também atua no processo infeccioso (García-Vallvé et al., 2005; Wang e Roden, 2013).

2.3 CLASSIFICAÇÃO DOS PVS E NOMENCLATURA

A identificação dos tipos de PV é realizada por meio do percentual de similaridade de nucleotídeos observado no gene L1. Quando a sequência nucleotídica do gene L1 difere em mais de 10% da mesma sequência de tipos virais já caracterizados, considera-se um novo tipo viral. Já um novo gênero de PV é considerado quando a identidade difere em mais de 40%. Considera-se um novo subtipo quando se observa diferenças nesta sequência entre 2% a 10% e uma nova variante é considerada quando a diferença é <2% (Figura 2). Esta definição foi estabelecida no Workshop Internacional de Papilomavírus realizado em Quebec em 1995 (de Villiers 2004; Daudt et al., 2018). Além disso, foi descrito recentemente que além do percentual de similaridade do gene L1, também se utiliza a posição do novo vírus na árvore filogenética com o gene L1 completo para sua classificação (Van Doorslaer, 2022).

Figura 2 - Esquema de classificação de Papilomavírus (PVs) com base na sequência de nucleotídeos do gene L1. A cor verde representa a identidade. A cor vermelha representa as diferenças, e a cor amarela representa a porcentagem de variação na classificação.



Fonte: Daudt et al., 2018.

Quanto à sua nomenclatura, há uma variação conforme a espécie

animal que o vírus irá infectar, por exemplo *Bos taurus papilllomavirus*, infectando principalmente a espécie bovina. Em relação à classificação taxonômica atual, PV pertence à família *Papillomaviridae*, que é subdividida em duas subfamílias, *Firstpapillomavirinae* e *Secondpapillomavirinae*. A subfamília *Firstpapillomavirinae* possui 52 gêneros e 133 espécies, enquanto a subfamília *Secondpapillomavirinae* contém apenas um gênero, denominado *Alefpapillomavirus*, que é constituído por uma única espécie viral (ICTV, 2024).

2.4 Papilomavírus Bovino

Na espécie bovina, BPVs podem induzir a papilomatose cutânea, caracterizada pela formação de tumores benignos na pele, ou seja, fibropapilomas e papilomas verdadeiros. A papilomatose cutânea pode ocorrer em diversos sítios anatômicos, incluindo tetos e úbere de vacas, comprometendo principalmente animais jovens e/ou imunossuprimidos. Apesar de ser caracterizada por lesões benignas, a papilomatose cutânea pode acarretar diversos prejuízos à cadeia produtiva bovina, seja por redução na ingestão de alimentos, limitação no processo de ordenha e amamentação de bezerros. As principais consequências para a pecuária bovina são queda na produção de leite e descarte de vacas, maior predisposição às infecções secundárias e depreciação do valor do couro (Campo, 2003; Alfieri et al., 2017).

Em bovinos, os tipos virais detectados pertencem aos gêneros *Deltapapillomavirus* (BPV1, 2, 13 e 14), *Dyoxipapillomavirus* (BPV7 e BPV-mTAPV-Isx), *Epsilonpapillomavirus* (BPV5, 8, 25 e 44), *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11, 12, 15, 17, 20, 23, 24, 26, 28, 29, 30, 31, 35, 36, 37, 38, 39, 40, 41, 42 e 43), *Dyokappapapillomavirus* (BPV16, 18 e 22), e sem classificação definida (BPV19, 21, 27, 32, 33 e 34) (Tabela 1) (PaVE 2025).

Tabela 1. Tipos de BPV presentes em lesões hiperproliferativas e sua associação com diferentes sítios anatômicos de bovinos.

Tipo viral	Gênero	Sítio anatômico
BPV1	*Deltapapilomavírus*	Tecido cutâneo, tetos, vesícula urinária e TGIS
BPV2	*Deltapapilomavirus*	Tecido cutâneo, úbere, vesícula urinária e TGIS
BPV3	*Xipapilomavírus*	Tecido cutâneo, teto e úbere
BPV4	*Xipapilomavírus*	Tecido cutâneo e TGIS
BPV5	*Epsilonpapillomavirus*	Tetos
BPV6	*Xipapillomavirus*	Tecido cutâneo e tetos
BPV7	*Dyoxipapillomavirus*	Tecido cutâneo e tetos
BPV8	*Epsilonpapillomavirus*	Tecido cutâneo e tetos
BPV9	*Xipapillomavirus*	Tecido cutâneo, tetos e úbere
BPV10	*Xipapillomavirus*	Tecido cutâneo, tetos e úbere
BPV11	*Xipapillomavirus*	Tecido cutâneo
BPV12	*Xipapillomavirus*	Língua
BPV13	*Deltapapillomavirus*	Tetos e vesícula urinária
BPV14	*Deltapapillomavirus*	Tecido cutâneo
BPV15	*Xipapillomavirus*	
BPV16	*Dyokappapapillomavirus*	Tecido cutâneo
BPV17	*Xipapillomavirus*	Tecido cutâneo
BPV18	*Dyokappapapillomavirus*	Tecido cutâneo
BPV19	*Não classificado*	Tecido cutâneo
BPV20	*Xipapillomavirus*	Tecido cutâneo
BPV21	*Não classificado*	Tecido cutâneo
BPV22	*Dyokappapapillomavirus*	Vulva
BPV23	*Xipapillomavirus*	Tecido cutâneo
BPV24	*Xipapillomavirus*	Tecido cutâneo
BPV25	*Epsilonpapillomavirus*	
BPV26	*Xipapillomavirus*	
BPV27	*Não classificado*	
BPV28	*Xipapillomavirus*	Tecido cutâneo
BPV29	*Xipapillomavirus*	Tecido cutâneo
BPV30	*Xipapillomavirus*	Úbere
BPV31	*Xipapillomavirus*	Tetos
BPV32	*Não classificado*	Tetos
BPV33	*Não classificado*	Tetos
BPV34	*Não classificado*	Tetos
BPV35	*Xipapillomavirus*	Tetos
BPV36	*Xipapillomavirus*	Tetos
BPV37	*Xipapillomavirus*	Tetos
BPV38	*Xipapillomavirus*	Tetos
BPV39	*Xipapillomavirus*	Tetos
BPV40	*Xipapillomavirus*	Tetos
BPV41	*Xipapillomavirus*	Tetos
BPV42	*Xipapillomavirus*	Tetos
BPV43	*Xipapillomavirus*	Tetos
BPV44	*Epsilonpapillomavirus*	Tetos
BPV-mTAPV-lsx	*Dyoxipapillomavirus*	

Fonte: PaVE, 2025.

2.5 HEMATURIA ENZOÓTICA BOVINA

Outro quadro clínico associado à infecção por BPV é a Hematúria Enzoótica Bovina (HEB), caracterizada como uma doença inflamatória crônica, que pode estar associada à presença de lesões neoplásicas na vesícula urinária, relacionada à interação entre a ingestão crônica da samambaia (*Pteridium* spp.) e a presença da infecção por BPV (Falco et al., 2023). A sugestão da participação de BPV na etiologia da HEB foi devido à identificação da presença de transcritos do genoma viral em células tumorais da vesícula urinária de bovinos e búfalos (Campo et al., 2002; Roperto, 2022). A samambaia possui compostos tóxicos, carcinogênicos, mutagênicos e capazes de causar imunossupressão. O consumo de samambaia por animais infectados por BPV aumenta a probabilidade de ativação da replicação do BPV latente, determinando o desenvolvimento de lesões pré-malignas (Campo et al., 1992). A presença de BPV e de fatores carcinogênicos presentes na samambaia agem em sinergismo, desencadeando a progressão tumoral (Jarret et al., 1984; Campo et al., 1992).

Estudos promovendo a indução de carcinogenicidade foram realizados com diversos compostos isolados da samambaia. No entanto, o principal composto capaz de gerar o efeito carcinogênico foi um glicosídeo norsesquiterpeno, denominado ptaquilosídeo (Hojo-Souza et al., 2010). Apesar da samambaia não ser palatável para os animais, a ingestão desta planta ocorre quando há menor oferta de pastagem, superlotação de pasto, deficiência de fibra na dieta ou após o inverno rigoroso caracterizado por longo período seco (Cruz e Bracarense, 2004).

Os achados histopatológicos em vesícula urinária de bovinos comprometidos pela HEB podem ser classificados em alterações não neoplásicas que podem conter hiperplasia, displasia, ninhos de Brunn, vacúolos intraepiteliais no urotélio e papilomas escamosos (Gabriel et al., 2009), e alterações neoplásicas caracterizadas em carcinoma *in situ* (CIS), neoplasia urotelial papilar de baixo potencial maligno (PULNMP), carcinoma papilar de baixo grau, carcinoma papilar de alto grau, carcinoma invasivo de baixo grau, carcinoma invasivo de alto grau e carcinoma urotelial (Roperto et al., 2010). Adicionalmente, hemangiomas, hemangiosarcomas, fibromas, carcinomas em células transicionais e adenocarcinomas também podem estar presentes (Jarret et al., 1978; Souto et al., 2006).

2.6 TUMORES DO TRATO GASTROINTESTINAL SUPERIOR DE BOVINOS

Os tumores do TGIS em bovinos são causados por BPVs e pelo consumo crônico da samambaia. O BPV4 é detectado com maior frequência em lesões da mucosa deste sítio anatômico, tanto em lesões benignas como em lesões malignas (Borzacchiello et al., 2003). Outro tipo viral associado a lesões do TGIS é o BPV2, encontrado em menor frequência e já identificado em fibropapilomas, papilomas verdadeiros e também em carcinoma *in situ* (Gasparotto et al., 2024; Matias et al., 2024), podendo ocorrer a coinfecção entre os BPVs 2 e 4, conforme reportado em investigações recentes (Fernandes et al., 2022; Matias et al., 2024).

Estudo realizado envolvendo 27 lesões do TGIS de bovinos e bubalinos criados na India, identificou a presença dos BPVs 1, 2 e 5 em lesões classificadas como fibropapilomas e papilomas em rúmen, retículo e boca de bubalinos, e BPV5 em rúmen e esôfago de bovinos (Kumar et al., 2013). Além dos tipos virais mencionados nestas lesões, recentemente, os BPVs 13 e 44 foram detectados a partir de lesões ruminais classificadas histopatologicamente como papilomas escamosos e fibropapilomas, em bovinos abatidos e criados na região Amazônica do Brasil (Gasparotto et al., 2024). As principais lesões caracterizadas no TGIS são papilomas, fibropapilomas e carcinomas de células escamosas (Jarrett et al., 1978; Jarrett et al., 1984).

A interação da ingestão crônica de samambaia com a infecção por BPVs pode resultar no desenvolvimento de lesões papilomatosas, ou até mesmo neoplásicas, na mucosa do TGIS de bovinos (Campo et al., 1985). A presença de tumores nestas estruturas do TGIS pode trazer como consequências clínicas a obstrução da passagem de alimentos, disfagia, regurgitação, timpanismo e, consequentemente, perda de peso (Alfieri et al., 2017). Essas ocorrências podem variar conforme a localização do tumor, por exemplo, a regurgitação pode indicar que o tumor está localizado na região esofágica, assim como a distensão abdominal pode demonstrar que o tumor está localizado na região ruminal (Faccin et al., 2018).

Em relação aos achados histopatológicos em TGIS, papilomas escamosos ou fibropapilomas, histologicamente semelhantes aos papilomas cutâneos, são classificados como lesões benignas (Campo, 1985; Gasparotto et al., 2024). Já os carcinomas *in situ* (Matias et al., 2024) e de células escamosas (CCE), são classificados como lesões malignas, com extensões e dimensões variáveis, sendo

comumente encontrados na entrada do rúmen e esôfago, seguidos por epiglote, base da língua e faringe (Jarret et al., 1980; Faccin et al., 2018).

2.7 Reação em Cadeia da Polimerase e Utilização de Primers Degenerados

A utilização da PCR convencional com primers degenerados capazes de amplificar regiões mais conservadas do genoma de PVs, como os genes L1 e E1, têm demonstrado resultados significativos na identificação de tipos virais já caracterizados e ainda não descritos, proporcionando a elucidação da diversidade viral de PVs presentes em lesões hiperproliferativas detectadas em diversas localizações anatômicas de bovinos (Crespo et al., 2019).

Na papilomatose cutânea de bovinos, a utilização desta estratégia molecular, associada ao sequenciamento Sanger de fragmentos provenientes destes genes conservados, tem possibilitado a identificação de prováveis novos tipos virais em lesões hiperproliferativas de bovinos. Em investigações conduzidas em rebanhos bovinos do Brasil, a identificação de prováveis novos tipos virais na papilomatose mamária de rebanhos leiteiros permitiu a detecção dos prováveis novos tipos de BPV denominados BPV/BR-UEL6 e BPV/BR-UEL7, além da identificação dos BPVs 6, 7, 8, 9 e 10, anteriormente caracterizados (Lunardi et al., 2016).

Com a utilização dos primers degenerados FAP59/FAP64 e AR-E1F2/AR-E1R4, foi possível a identificação de infecção mista contendo os BPV2 e 4 em papiloma esofágico de uma vaca leiteira de oito anos de idade, situada na região Centro-Oeste do Brasil (Fernandes et al., 2022). Além disso, utilizando o par de primers FAP59/FAP64, também foi possivel identificar BPVs em lesões no TGIS de bovinos abatidos, detectando o BPV2 e pela primeira vez os BPVs 13 e 44 nesta localidade anatômica (Gasparotto et al., 2024).

2.8 Amplificação por Círculo Rolante e Sequenciamento de Alto Rendimento

A utilização das técnicas de amplificação por círculo rolante (*rolling circle amplification* - RCA) associada ao sequenciamento de alto rendimento (*high-throughput sequencing* - HTS) apresentou grande eficiência na identificação e caracterização de novos tipos de PVs (Silva et al., 2016; Daudt et al., 2016; Sauthier et al., 2021). A estratégia de detecção de PVs por RCA utiliza a DNA polimerase do

bacteriófago *phi*29 para a mplificaçãoa inespecífica de genomas de PVs presentes nas lesões, uma vez que o método envolve o emprego de *primers* randômicos. O produto da amplificação por RCA consiste em múltiplas cópias concatenadas do genoma viral, gerando DNA de dupla fita com elevado tamanho molecular (Rector et al., 2004).

A aplicação da estratégia envolvendo a combinação de RCA e HTS vêm proporcionando a identificação e caracterização de novos tipos de PV, por meio da obtenção da sequências genômicas completas, permitindo a detecção de novos tipos virais distantemente relacionados aos anteriormente caracterizados (Sauthier et al., 2021). Adicionalmente, estas técnicas têm ampliado o conhecimento sobre a frequência de ocorrência e a importância de coinfecções em lesões benignas determinadas por BPV, com a observação da presença de até nove diferentes tipos de BPV em uma única lesão papilomatosa cutânea (Daudt et al., 2016; Sauthier et al., 2021).

Com o uso das técnicas de RCA e HTS, foi possível ampliar a gama de tipos virais associados a papilomatose cutânea em bovinos. Desta maneira, foi possível identificar um provável novo tipo viral de BPV em amostra de lesão hiperproliferativa coletada de um bovino localizado na região amazônica do Brasil. Este novo tipo foi identificado como BPV23, o qual apresentou 90% de similaridade no gene L1 em relação aos tipos virais anteriormente descritos e pertencentes ao gênero *Xipapillomavirus* (da Silva et al., 2016). Ainda na região amazônica do Brasil, foi possível identificar coinfecção de sete tipos virais de BPV em uma única lesão papilomatosa de um bovino, sendo um deles já caracterizado e os outros seis novos tipos virais, os quais foram designados como BPV16, 17, 18, 19, 20 e 21, todos com menos de 90% de similaridade em relação ao gene L1 dos tipos virais anteriormente caracterizados (Daudt et al., 2016).

Em estudos realizados no sul do Brasil, que também utilizaram esta estratégia molecular, foi possível identificar 11 tipos virais já caracterizados e 14 novos tipos de BPV em lesões papilomatosas da glândula mamária de 23 vacas, além de detectar coinfecção em mais de 50% das amostras estudadas. Atualmente estes novos tipos virais são reconhecidos como BPVs 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 e 43 (Sauthier et al., 2021; PaVE 2025).

3 REFERÊNCIAS

ALFIERI, A. A., Lunardi, M., Alfieri, A. F. *Papillomaviridae*. In: FLORES, Eduardo. Virologia Veterinária: Virologia Geral e Doenças Víricas. 3ª Edição. Santa Maria: UFSM, 2017. p. 477-492.

ARALDI, R. P., ASSAF, S. M. R., CARVALHO, R. F. D., CARVALHO, M. A. C. R. D., SOUZA, J. M. D., MAGNELLI, R. F., MÓDOLO, D. G., ROPERTO, F. P., STOCCO, R. C., BEÇAK, W. Papillomaviruses: a systematic review. **Genetics and Molecular Biology**, v. 40, n. 1, p. 1-21, 2017.

BORZACCHIELLO, G., AMBROSIO, V., ROPERTO, S., POGGIALI, F., TSIRIMONAKIS, E., VENUTI, A., CAMPO, M. S., ROPERTO, F. Bovine papillomavirus type 4 in oesophageal papillomas of cattle from the south of Italy. **Journal of Comparative Pathology**, v. 128, n. 2-3, p. 203-206, 2003.

BORZACCHIELLO, G., RUSSO, V., GENTILE, F., ROPERTO, F., VENUTI, A., NITSCH, L., CAMPO, M. S., ROPERTO, S. Bovine papillomavirus E5 oncoprotein binds to the activated form of the platelet-derived growth factor B receptor in naturally occurring bovine urinary bladder tumours. **Oncogene**, v. 25, n. 8, p. 1251-1260, 2006

BORZACCHIELLO, G. Bovine papillomavirus infections in animals. **Communicating Current Research and Educational Topics and Trends in Applied Microbiology**, v.2, p. 673-679, 2007.

CAMPO, M. S. Bovine papillomavirus and cancer. **The Veterinary Journal**, v. 154, n. 3, p. 175-188, 1997.

CAMPO, M. S. Bovine papillomavirus: old system, new lessons? In: CAMPO, M. S. Papillomavirus research: from natural history to vaccine and beyond. **Caister Academic Press**, 2006. p. 1-33.

CAMPO, M. S. Papillomavirus and disease in humans and animals. **Veterinary and Comparative Oncology**, v. 1, n. 1, p. 3-14, 2003.

CAMPO, M. S., JARRET, W. F., BARRON, R., O'NEIL, B. W., SMITH, K. T. Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. **Cancer Research**, v. 52, n. 24, p. 6898-6904, 1992.

CAMPO, M. S., MOAR, M. H., SARTIRANA, M. L., KENNEDY, I. M., JARRET, W. F.

The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. **The EMBO Journal**, v. 4, n. 7, p. 1819-1825, 1985.

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

CLAUS, M.P., LUNARDI, M., ALFIERI, A.F., ALFIERI, A.A. Análise filogenética de papilomavírus bovino associado com lesões cutâneas em rebanhos do estado do Paraná. **Pesquisa Veterinária Brasileira**, v. 27, p. 314-318, 2007.

CRESPO, S.E.I., LUNARDI, M., OTONEL, R.A.A., HEADLEY, S.A., ALFIERI, A.F., & ALFIERI, A. A. Genetic characterization of a putative new type of bovine papillomavirus in the Xipapillomavirus 1 species in a Brazilian dairy herd. **Virus Gene**, v. 55, p. 682-687, 2019.

CRUZ, G. D., BRACARENSE, A. P. F. Toxicidade da samambaia (Pteridium aquilinum (L.) Kuhn) para a saúde animal e humana. **Semina: Ciências Agrárias**, v. 25, n. 3, p. 249-258, 2004.

Da SILVA, F. R., CIBULSKI, S. P., DAUDT, C., WEBER, M. N., GUIMARÃES, L. L., STRECK, A. F., MAYER, F. Q., ROEHE, P. M., CANAL, C. W. Novel bovine papillomavirus type discovered by rolling-circle amplification coupled with next-generation sequencing. **PLoS One**, v. 11, n. 9, p. e0162345, 2016.

DAUDT, C., da SILVA, F. R., STRECK, A. F., WEBER, M. N., MAYER, F. Q., CIBULSKI, S. P., CANAL, C. W. How many papillomavirus species can go undetected in papilloma lesions?. **Scientific Reports**, v. 6, n. 1, p. 36480, 2016.

De VILLIERS, E.M., FAUQUET, C., BROKER, T.R., BERNARD, H.U., ZUR HAUSEN, H. Classification of Papillomaviruses. **Virology**, v.324, n. 1, p. 17-27, 2004

FACCIN, T. C., CARGNELUTTI, J. F., RODRIGUES, F. D. S., MENEZES, F. R. D., PIAZER, J. V. M., MELO, S. M. P. D., LAUTER, B. F., FLORES, E. F., KOMMERS, G. D. Bovine upper alimentary squamous cell carcinoma associated with bracken fern poisoning: clinical-pathological aspects and etiopathogenesis of 100 cases. **PloS one**, v. 13, n. 9, p. e0204656, 2018.

FALCO, F., CUCCARO, B., TULLIO, R., ALBERTI, A., CUTARELLI, A., CARLO, E., ROPERTO, S. Possible etiological association of ovine papillomaviruses with bladder

tumors in cattle. **Virus Research**, v. 328, p. 199084, 2023.

FERNANDES, K.P., ALFIERI, A.A., DAROLD, G.M., BOABAID, F.M., AGNOL, A.M.D., LUNARDI, M. Case report: Mixed infection of bovine papillomaviruses associated with squamous papiloma of the upper alimentary tract in a dairy cow. **Frontiers in Veterinary Science**, v. 9, n. 1, p. 1020166, 2022.

FERRARO, C. T. L., CANEDO, N. H. S., OLIVEIRA, S. P. D., CARVALHO, M. D. G. D. C., DIAS, E. P. Infecção oral pelo HPV e lesões epiteliais proliferativas associadas. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 47, p. 451-459, 2011.

FORSLUND, O., ANTONSSON, A., NORDIN, P., STENQUIST, B. O., HANSSON, B. G. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. **Journal of General Virology**, v. 80, n. 9, p. 2437-2443, 1999.

GABRIEL, A. L, KOMMERS, G. D., MASUDA, E. K., FIGHERA, R. A., PIAZER, J. V. M., BARROS, C. S. L., MARTINS, T. B., ROSA, F. B. Aspectos clínico-hematológicos e lesões vesicais na intoxicação crônica espontânea por Pteridium aquilinum em bovinos. **Pesquisa Veterinária Brasileira**, v.29, n. 7, p. 515-525, 2009.

GARCIA-VALLVÉ, S., ALONSO, Á., BRAVO, I. G. Papillomaviruses: diferente genes have diferente histories. **Trends in Microbiology**, v. 13, n. 11, p. 514-521, 2005.

HOJO-SOUZA, N. S., CARNEIRO, C. M., SANTOS, R. C. D. Pteridium aquilinum: o que sabemos e o que ainda falta saber. **Bioscience Journal**, v. 26, n. 5, p. 798-808, 2010.

GASPAROTTO, P. H. G., SANTOS, I. R., DANTAS FILHO, J. V., SILVA, M. S., SOUZA, F. A., SOUSA, J. C. M., DRIEMEIER, D., CANAL, C. W., SILVA, F. R. C., DAUDT, C. Characterization of Bovine Papillomavirus types detected in cattle rumen tissue from Amazon region, Brazil. **Animals**, v. 14, n. 15, p.2262, 2024.

ICTV **International Committee on Taxonomy of Viruses**. Disponível em: https://ictv.global/. Acesso em: 15 mar. 2024.

JARRETT, W. F. Bracken fern and papiloma vírus in bovine alimentary cancer. **British Medical Bulletin**, v.36, n. 1, p. 79-81, 1980.

JARRETT, W. F. H., MCNEIL, P. E., GRIMSHAW, W. T. R., SELMAN, I, E., MCINTYRE, W. I. M. High incidence area of cattle cancer with a possible interaction

between an environmental carcinogen and a papilomavirus. **Nature**, v. 274, n. 5668, p. 215-217, 1978.

JARRETT, W. F., CAMPO, M. S., BLAXTER, M. L., O'NEIL, B. W., LAIRD, H. M., MOAR, M. H., SARTIRANA, M. L. Alimentary fibropapilloma in cattle: a spontaneous tumor, nonpermissive for papillomavirus replication. **JNCI: Journal of the National Cancer Institute**, v. 73, n. 2, p. 499-504, 1984.

KUMAR, P., NAGARAJAN, N., SAIKUMAR, G.; ARYA, R.S., SOMVANSHI, R. Detection of bovine papilloma viruses in wart-like lesions of upper gastrointestinal tract of cattle and buffaloes. **Transbound and Emerging Diseases**, v. 62, n. 3, p. 264-271, 2015.

LUNARDI, M., ALFIERI, A.A., OTONEL, R. A. A., de ALCÂNTARA, B. K., RODRIGUES, W. B., de MIRANDA, A. B., ALFIERI, A. F. Genetic characterization of a novel bovine papillomavirus member of the Deltapapillomavirus genus. **Veterinary Microbiology**, v. 162, n. 1, p. 207-213, 2013.

MATIAS, B. F., LUNARDI, M., GONÇALVES, K. C. B., VILAS-BOAS, L. A., GUSTANI-BUSS, E., BRACARENSE, A. P. F. L., CUNHA FILHO, L. F. C., ALFIERI, A. F., ALFIERI, A. A. Molecular detection by rolling circle amplification combined with deep sequencing of mixed infection by Bovine Papillomaviruses 2 and 4 in carcinoma in situ of the bovine esophageal mucosa. **Viruses**, v. 16, n. 10, p. 1558, 2024.

MUNDAY, J. S. Bovine and human Papillomaviruses: a comparative review. **Veterinary Pathology**, v. 51, n. 6, p. 1063-1075, 2014.

PATEL, K. R., SMITH, K. T., CAMPO, M. S. The nucleotide sequence and genome organization of bovine papillomavirus type 4. **Journal of General Virology**, v. 68, n. 8, p. 2117-2128, 1987.

PaVE **Papillomavirus Episteme**. Disponível em: https://pave.niaid.nih.gov. Acesso em: 17 fev 2025.

PIETRAS, K., OSTMAN, A. Hallmarks of cancer: interactions with the tumor stroma. **Experimental Cell Research**, v. 316, n. 8, p. 1324-1331, 2010.

RAMPIAS, T., SASAKI, C., PSYRRI, A. Molecular mechanisms of HPV induced carcinogenesis in head and neck. **Oral Oncology**, v.50, n. 5, p. 356-363, 2014.

RECTOR, A., TACHEZY, R., VAN RANST, M. A sequence-independent strategy for

detection and cloning of circular DNA vírus genomes by using multiply primed rolling-circle amplification. **Journal of Virology**, v. 78, n. 10, p. 4993-4998, 2004.

ROPERTO, S. Role of BAG3 in bovine Deltapapillomavirus-mediated autophagy. **Journal of Cellular Biochemistry**, v. 123, n. 1, p. 59-64, 2022.

ROPERTO, S., BORZACCHIELLO, G., BRUN, R., LEONARDI, L., MAIOLINO, P., MARTANO, M., PACIELLO, O., PAPPARELLA, S., RESTUCCI, B., RUSSO, V., SALVATORE, G., URRARO, C., ROPERTO, F. A review of bovine urothelial tumours and tumour-like lesions of the urinary bladder. **Journal of Comparative Pathology**, v. 142, n. 2-3, p. 95-108, 2010.

ROPERTO, S., BRUN, R., PAOLINI, F., URRARO, C., RUSSO, V., BORZACCHIELLO, G., PAGNINI, U., RASO, C., RIZZO, C., ROPERTO, F., VENUTI, A. Detection of bovine papillomavirus type 2 in the peripheral blood of cattle with urinary tumours: possible biological role. **Journal of General Virology**, v. 89, n. 12, p. 3027-3033, 2008.

SAUTHIER, J. T., DAUDT, C., da SILVA, F. R. C., ALVES, C. D. B. T., MAYER, F. Q., BIANCHI, R. M., DRIEMEIER, D., STREIT, R. S. A., STAATS, C. C., CANAL, C. W., WEBER, M. N. The genetic diversity of “papillomavirome” in bovine teat papilloma lesions. **Animal Microbiome**, v. 3, p. 1-13, 2021.

VAN DOORSLAER, K. Evolution of the papillomaviridae. **Virology**, v. 445, n. 1-2, p. 11-20, 2013.

VAN DOORSLAER, K. Revisiting Papillomavirus Taxonomy: A Proposal for Updating the Current Classification in Line with Evolutionary Evidence. **Virology**, v.14, n. 10, p. 2308, 2022.

VENUTI, A., PAOLINI, F., NASIR, L., CORTEGGIO, A., ROPERTO, S., CAMPO, M. S., BORZACCHIELLO, G. Papillomavirus E5: the smallest oncoprotein with many functions. **Molecular Cancer**, v. 10, p. 1-18, 2011.

WANG, J. W., RODEN, R. B. S. L2, the minor capsid protein of papillomavirus. **Virology**, v.445, p. 175-186, 2013.

4 HIPÓTESE

Este trabalho foi delineado com a hipótese de que tipos de BPVs adicionais, tanto aqueles já conhecidos quanto os ainda não descritos, podem ser identificados em associação com tumores do TGIS por meio de PCR com primers degenerados, seguida de sequenciamento Sanger. Adicionalmente, técnicas moleculares inovadoras, como RCA e HTS, podem proporcionar a caracterização de genomas completos dos tipos virais presentes nestas lesões, inclusive a identificação de coinfecções.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Investigar e caracterizar a diversidade de tipos de BPVs presentes em lesões hiperproliferativas do TGIS de bovinos leiteiros.

5.2 OBJETIVOS ESPECÍFICOS

- Detectar a presença de genomas de BPV em lesões hiperproliferativas do TGIS de bovinos por meio da PCR convencional e da amplificação por círculo rolante (RCA);
- Identificar e caracterizar, por meio de sequenciamento de Sanger e HTS (*high-throughput sequencing*), os tipos de BPV já caracterizados e ainda não caracterizados presentes em lesões hiperproliferativas no TGIS de bovinos;
- Correlacionar os tipos de BPV identificados com a classificação histopatológica das lesões hiperproliferativas;
- Identificar os tipos virais já caracterizados e ainda não caracterizados responsáveis pelas lesões hiperproliferativas no TGIS.

6 ARTIGO A

Artigo formatado de acordo com as Normas da ABNT.

MULTIPLE TYPES OF BOVINE PAPILLOMAVIRUS ARE IDENTIFIED IN SQUAMOUS PAPILLOMAS IN THE UPPER GASTROINTESTINAL TRACT OF BRAZILIAN CATTLE

ABSTRACT

Papillomaviruses (PVs) are oncogenic and infect the skin and mucosa of several host species. This study aimed to investigate the diversity of BPV types in hyperplastic lesions identified in the upper alimentary canal of cattle from Parana state, southern Brazil. Twenty-three hyperproliferative lesions were collected from the gastrointestinal tract of 16 dairy cattle, and the lesions were histologically classified as squamous papillomas. The degenerate primer pairs MY11/MY09, FAP59/FAP64, and E1F2/E1R4 were used to partially amplify the BPV L1 and E1 genes. The obtained amplicons were sequenced by the Sanger method. Five (21.7%) lesions were BPV-PCR positives. BPV1 and BPV4, previously found in these lesion types, were identified in two samples. BPV9 and BPV39 were identified for the first time in other two samples. DNA from a putative new BPV type, phylogenetically related to BPV12, was sequenced from one lesion. This study identified five different types of BPV in five lesions of the upper alimentary tract of cattle. The results suggest that similar to cutaneous papillomatosis, which is known to be infected by a wide variety of BPV types, it is likely that multiple BPV types contribute to the development of papillomas in the esophageal mucosa of dairy cattle raised in pastures contaminated by bracken fern. In conclusion, it is important to emphasize the need to use multiple degenerate primers capable of amplifying the partial L1 and E1 conserved genes to elucidate the diversity of BPV types present in lesions in the upper alimentary tract of cattle since the present study detected viral types not yet identified in mucosal lesions in this anatomical site.

Keywords: Upper alimentary tract; Esophagus; Hiperplastic lesion; Sequencing; Polymerase chain reaction.

1. INTRODUCTION

Papillomaviruses (PVs) are oncogenic viruses responsible for causing benign and malignant lesions in the skin and mucosa of vertebrates, including humans. They

are non-enveloped viruses with an icosahedral capsid and a genome composed of double-stranded circular DNA with approximately 8 kbp length (Bernard et al., 2010). The papillomavirus genome contains ORFs that encode early (E) and late (L) proteins. The E1 and E2 proteins promote viral replication, the E4 protein participates in the amplification and production of virions, while the E5, E6, and E7 oncoproteins may promote cellular transformation. The late-phase proteins L1 and L2 compose the viral capsid and are necessary to complete the viral cycle (Daudt et al., 2018).

The L1 gene is considered the most conserved ORF within the viral genome. Therefore, to describe a new viral type, the sequencing of the complete new genome is necessary, with the demonstration that the nucleotide (nt) sequence of the L1 gene presents at least 10% divergence when compared to previous sequences corresponding to any other viral type already recognized. If the new viral strain differs between 2 and 10%, a new subtype is considered, while a new variant is detected when the identity observed is <2% (de Villiers et al., 2004).

The viral types detected in cattle belong to the genera *Deltapapillomavirus* (BPV1, 2, 13 and 14), *Dyoxipapillomavirus* (BPV7 and BPV-mTAPV-Isx), *Epsilonpapillomavirus* (BPV5, 8, 25 and 44), *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11, 12, 15, 17, 20, 23, 24, 26, 28, 29, 30, 31, 35, 36, 37, 38, 39, 40, 41, 42 and 43), *Dyokappapapillomavirus* (BPV16, 18 and 22), and some types remain unclassified (BPV19, 21, 27, 32, 33 and 34) (PaVE 2025).

Bos taurus papillomavirus (BPV) infection may result in cutaneous papillomatosis, forming hyperplastic lesions on the skin of various anatomical sites, such as teats and udder. These lesions are characterized as squamous papillomas or fibropapillomas, being considered benign (Munday 2014). However, BPV infection may also result in malignancy, being the precursor of neoplastic lesions in the urinary bladder mucosa, generally associated with bracken fern (*Pteridium spp.*) consumption, known as bovine enzootic hematuria (BEH) (Jarrett et al., 1980; Campo et al., 1992; Borzacchiello et al., 2003). The bracken fern is known for its carcinogenic and immunosuppressive properties and may act synergistically with BPV, promoting malignancy (Jarrett et al., 1980).

Another clinical condition that can affect cattle and is associated with BPV infection is the development of tumors in the mucosa of the upper alimentary tract of cattle, which is also associated with bracken fern compounds. The viral types already detected in such lesions were BPV1, BPV2, BPV4, BPV5, BPV13, and BPV44

(Borzacchiello et al., 2003; Kumar et al., 2013; Gasparotto et al., 2024), with BPV4 being detected more frequently in both benign and malignant lesions (Borzacchiello et al., 2003). The cytopathic effects caused by BPV infection and observed in such lesions are koilocytosis, ortho and parakeratotic hyperkeratosis, hypergranulosis in the granular layer, and acanthosis (Araldi et al., 2015). The main lesions observed in the alimentary tract are classified as papillomas, fibropapillomas, and squamous cell carcinomas (Jarrett et al., 1978; Jarrett et al., 1984). This study aimed to histologically classify the hyperplastic lesions and investigate the diversity of BPV types in lesions from the alimentary tract mucosa of cattle from Brazil.

2. MATERIALS AND METHODS

2.1 Biological Samples and Histopathological Analysis

Through the post-slaughter inspection of the mucosa lining the whole upper alimentary tract, 23 proliferative lesions were identified and collected from the esophagus of 16 Girolando breed mature cattle from farms located in the North Central mesoregion of the Parana state, southern Brazil, a region known to contain acid soil and bracken fern infestation. A portion of each esophageal lesion was fixed by immersing in 10% neutral-buffered formalin solution, and processed routinerally for histopathological evaluation and stained with hematoxylin and eosin. The proliferative mucosal lesions were evaluated by histopathology as previously described (Araldi et al., 2015). The remaining fragments of each tumor were stored at −80 °C for further molecular evaluations.

This study was approved by the Ethics Committee for Animal Use at the Universidade Estadual de Londrina (Protocol nº 050/2021). All the applicable international, national, and institutional guidelines for animal care and use were followed.

2.2 PCR Amplification of Partial Fragments of the BPV L1 and E1 Genes

All frozen 23 fragments from each esophageal lesion were disrupted and homogenized using TissueLyser LT (Qiagen, Hilden, Germany). Subsequently, total DNA was purified from these tissue homogenates using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Purified DNA was stored at −80 °C for further molecular analyses. Aliquots of ultrapure sterile water

were included as negative controls in all DNA extraction procedures.

To investigate the presence of BPV strains potentially associated with the cattle esophageal mucosa hyperplastic lesions, three degenerate primer pairs (FAP59/FAP64, MY09/MY11, and AR-E1F2/AR-E1R4), designed to amplify regions of the L1 and E1 conserved genes of a broad spectrum of cutaneous and mucosal PV strains, were tested using DNA purified from the lesions. The assays employed for the amplification of fragments from the L1 and E1 genes, as well as the sequence and characteristics of the primers used were described previously (Matias et al., 2024).

2.3 Direct Sequencing by Sanger Method and Sequence Analyses

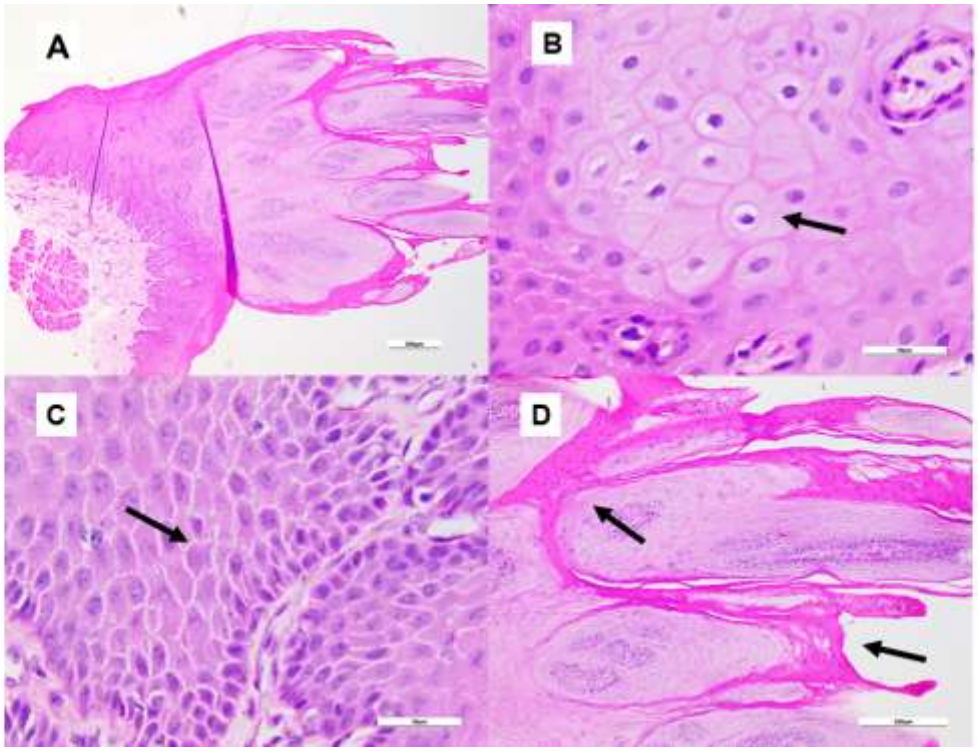
Amplicons representing partial fragments of the BPV L1 and E1 genes were purified using Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA), following the protocol provided by the manufacturer. Next, direct nt sequencing was performed on a 3500 Genetic Analyzer Sequencer (Applied Biosystems, Carlsbad, CA, USA), using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA), and the corresponding forward and reverse primers employed in the PCR assays, according to the instructions provided by the manufacturer. The generated sequences were examined using the Phred application for quality analysis of chromatogram readings (http://lbi.cenargen.embrapa.br/phph/). The sequences with a base quality ≥20 were considered acceptable. The consensus sequences were assembled using CAP3 software Version 3, and their identities were obtained through comparison with nt sequences deposited on the public database GenBank, using the BLASTn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. RESULTS

All 23 examined lesions exhibited similar histological features. The histopathological analyses of the neoplastic lesions located in the esophagus revealed a well-organized proliferation of stratified squamous epithelial cells. These cells were arranged in multiple layers and formed papillary projections (Figure 1A). Within the stratum spinosum of the proliferating epithelium, small clusters of koilocytes were occasionally observed (Figure 1B). Additionally, prominent desmosomal junctions were evident between epithelial cells (Figure 1C) and marked parakeratotic hyperkeratosis was noted (Figure 1D). These histological characteristics are consistent

with a diagnosis of squamous papillomas. Furthermore, lymphocytic infiltrates were present in three of the tumors. Such findings are cytopathic effects that suggest BPV infection. However, only five of the hyperplastic lesions had partial fragments of the BPV L1 and E1 genes amplified (Table 1).

Figure 1 – Photomicrographs of esophageal papillomas from Girolando cattle. A) Well-organized proliferation of epithelial cells forming papillary projections. B) Small clusters of koilocytes (epithelial cells displaying clear cytoplasm and a pyknotic nucleus) indicated by arrows. C) Epithelial cells exhibiting prominent desmosomal junctions (arrows). D) Parakeratotic hyperkeratosis highlighted by arrows. Hematoxylin-eosin staining. Scale bar A 300 µm, B and C 30 µm, D 20 µm.



Fonte: o próprio autor.

Five different types of BPV were amplified from hyperplastic lesions of the TGIS of cattle. Direct sequencing of the partial fragments amplified from L1 and E1 BPV genes revealed the presence of the viral types BPV1, BPV4, BPV9, BPV39, and a putative new BPV type, presenting 77% nt similarity with the previously characterized BPV type 12 (Table 1).

Table 1. PCR detection of different BPV types in esophageal lesions collected from the alimentary tract mucosa of cattle using different primer pairs.

Sample	PCR assays (primer pairs)			BPV
N°	FAP59/FAP64	MY11/MY09	E1F2/E1R4	Type
A1	+	–	+	BPV9
A6	+	–	+	BPV39
A7	+	–	–	Putative new BPV type (~BPV12)
A14	+	–	+	BPV4
A16	–	+	+	BPV1

Fonte: o próprio autor.

The direct sequencing of the partial BPV L1 gene amplified with the primer pair FAP59/ FAP64 in sample A1 revealed 100% similarity with the BPV9 corresponding sequence (GenBank accession number HM245433). E1 gene partial sequence was also amplified in the same lesion. The direct sequencing of the E1 gene showed 99% nt similarity with another strain of the same viral type (GenBank accession number MW436427). In sample A6, Blastn analysis of the FAP sequence demonstrated 96.24% similarity with BPV39 strain BPV/UFPE03BR (GenBank accession number JQ897974) and 93.08% similarity with BPV39 complete genome (GenBank accession number MW727481). The sequence from the partial E1 gene showed 94.41% similarity with the BPV39 partial sequence (GenBank accession number MW428426). As for lesion A14, obtained nt sequences amplified from L1 gene with primer pair FAP59/FAP64 and from E1 gene demonstrated similarities of 98.48% and 99.59% with BPV4 (GenBank accession numbers MW436424 and MW436424, respectively). In lesion A16, obtained nt sequences amplified from the L1 gene with primer pair MY09/MY11 and from the E1 gene demonstrated similarities near 100% with BPV1 (GenBank accession numbers MG263871 and MG977494, respectively). Nonetheless, amplification of the partial fragment of the L1 gene (FAP59/FAP64) in tumor A7 showed only 77.75% nt similarity with BPV12 (GenBank accession number OP682876), which indicates the presence of a putative new BPV type.

4. DISCUSSION

In this study, the degenerate primer pairs that amplify partial fragments of the

conserved PV L1 and E1 genes allowed the detection of BPV genomes in 5 out of 23 esophageal tumors of cattle. The direct sequencing of the obtained amplicons revealed the infection by representatives classified in the *Deltapapillomavirus* genus (BPV1) and the *Xipapillomavirus* genus (BPV4, BPV9, and BPV39), as well as a putative new BPV type most closely related to BPV12 (77% similarity), also to be classified in the *Xipapillomavirus* genus.

BPV type 4, isolated in 1978, is considered the main viral type associated with tumors that develop in the upper alimentary tract of cattle (Jarret et al., 1978; Campo et al., 1980). Studies conducted in diverse geographic areas have confirmed this finding. The analysis of lesions collected in slaughterhouses from Glasgow, representing 70 different cases, evaluated the presence of BPV4 DNA by in situ hybridization technique, and confirmed the infection by this viral type in 61 lesions (Campo et al., 1985). In another investigation involving papillomatosis extending from the mouth and tongue to the reticulum in two different animals from Scotland and England, the molecular analysis demonstrated the identification of the BPV4 genome in those lesions (Tsirimonaki et al., 2003). In Italy, exophytic and inverted papillomas were detected by histopathological evaluation of the esophagus of 147 out of 1,133 cattle slaughtered between 2000 and 2001. Through molecular analysis, the presence of BPV4 was also confirmed in more than 60% of the tumors (Borzacchiello et al., 2003).

Through a combination of different degenerate primer pairs (FAP59/FAP64, MY09/MY11 and AR-E1F2/AR-E1R4), able to amplify conserved genomic segments of the BPV genome, our investigation revealed a broader diversity in BPV types than classically related to hyperplastic lesions that occur in this specific anatomical site. Despite having been used by other authors, the MY09/MY11 primer pair did not demonstrate efficacy in the present study since this PCR assay tested positive in only one esophageal lesion. To the authors' knowledge, this study represents the first report that showed the identification of BPV DNA from BPV9 and 39 in tumors located in the mucosa of the upper alimentary tract of cattle. Recently, a study aiming to detect BPV DNA in 100 papillomatous lesions from ruminal mucosa of cattle raised in Northern Brazil, through PCR assay using FAP primer pair, demonstrated the presence of the BPV13 and 44 in such tumors, besides the BPV2, which is commonly associated with the bovine upper alimentary tract (Gasparotto et al., 2024).

Our findings, in association with the unusual BPV types previously detected in the

rumen from cattle (BPVs 13 and 44), suggest that there is a greater diversity of viral types related to tumors of the upper digestive tract of cattle than previously suggested by initial studies (Jarrett et al., 1984; Borzacchiello et al., 2003). Interestingly, despite having detected the participation of new viral types in the esophageal mucosa of cattle, the BPV types detected herein differed from the viral types previously found in the ruminal mucosa of cattle from the Northern region, highlighting the need for in-depth investigations aiming to elucidate the papillomaviral diversity in the bovine digestive mucosa of cattle raised in different geographic areas.

Another viral type commonly found in lesions located at the upper alimentary tract of cattle is the *Deltapapillomavirus* BPV2, which is usually associated with esophageal fibropapillomas (Jarret et al., 1984; Kumar et al., 2013). The molecular analyses of hyperplastic lesions collected from this anatomical site from an affected bull raised in the Midwestern region of Brazil, demonstrated the occurrence of a mixed infection caused by BPV2 and 4 through sequencing of the L1 and E1 gene fragments amplified by FAP59/FAP64 and E1F2/E1R4 primer pairs (Fernandes et al., 2022). More recently, a mixed infection characterized by the presence of BPV2 and 4 was also detected in a carcinoma *in situ* in the esophagus of a bull from Southern Brazil through RCA followed by HTS (Matias et al., 2024). However, in the present study, BPV2 was not amplified from any evaluated lesion from the esophagus of the affected cattle from North Central mesoregion of the Parana state, southern Brazil.

The present study also detected the BPV1 in a lesion from the esophagus, which was also identified in the upper alimentary tract of buffaloes from India. In addition to this finding in buffaloes, this previous investigation detected the BPV5 in the same anatomical location of cattle from the same geographic area (Kumar et al., 2013).

In addition to the detection of genomic sequences from BPV types previously characterized, the direct sequencing of the partial L1 gene fragment amplified from a tumor in this study showed the presence of a putative new BPV type closely related to the *Xipapillomavirus* BPV12 (77% identity). Regarding the current classification for new PVs, the sequencing and analysis of the identity observed in the nt sequence of the L1 ORFs, the most conserved gene in the PV genome, define the classification of a new PV type, subtype or variant.

The use of degenerate primers able to amplify partial sequences from conserved genes of the BPV genome has represented a useful molecular tool to elucidate the genetic diversity of BPV types involved mainly in cutaneous hyperplastic lesions from

cattle. Previously, the analyses of cutaneous papillomas through FAP59/FAP64 allowed the characterization of a putative new BPV type from a cutaneous papilloma (Crespo et al., 2019). The molecular investigation by FAP PCR assay of cutaneous warts from cattle raised in Northern Brazil, enabled the identification of nine possible new BPV subtypes and four probable new BPV types in cattle affected by papillomatosis (Silva et al., 2015). These studies demonstrate that degenerate primers are an effective tool for the identification of putative new viral types of BPV. The present study highlights the effectiveness of the application of this strategy since the use of a combination of different consensual PCR assays allowed the identification of additional viral types that were not previously detected in the upper alimentary tract of cattle.

5. REFERENCES

ARALDI, R.P., MELO, T.C., NEVES, A.C., SPADACCI-MORENA, D.D., MAGNELLI, D.G., MODOLO, D.G., DE-SÁ-JÚNIOR, P.L., MAZUCCHELLI-DE- SOUZA, J., CARVALHO, R.F., BEÇAK, W., STOCCO, R. C. Hyperproliferative action of bovine papillomavirus: Genetic and histopathological aspects. **Genetics and Molecular Research**, v. 14, n. 4, p. 12942-12954, 2015.

BERNARD, H. U., BURK, R., CHEN, Z., VAN DOORSLAER, K., HAUSEN, H. Z., de VILLIERS, E. Classification of Papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. **Virology**, v. 401, n. 1, p.70-79, 2010.

BORZACCHIELLO, G., AMBROSIO, V., ROPERTO, S., POGGIALI, F., TSIRIMONAKIS, E., VENUTI, A., CAMPO, M. S., ROPERTO, F. Bovine papillomavirus type 4 in oesophageal papillomas of cattle from the south of Italy. **Journal of Comparative Pathology**, v. 128, n. 2-3, p. 203-206, 2003.

CAMPO, M. S., MOAR, M. H., JARRETT, W. F. H., LAIRD, H. M. A new papillomavirus associated with alimentary câncer in cattle. **Nature**, v. 286, n. 1, p. 180-182, 1980.

CAMPO, M. S., JARRET, W. F., BARRON, R., O'NEIL, B. W., SMITH, K. T. Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. **Cancer Research**, v. 52, n. 24, p. 6898-6904, 1992.

CAMPO, M.S., MOAR, M.H., SARTIRANA, M.L., KENNEDY, I.M., JARRET, W.F.H.

The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. **The EMBO Journal**, v. 4, n. 7, p. 1819–1825, 1985.

CRESPO, S. E. I., LUNARDI, M., OTONEL, R. A. A., HEADLEY, S. A., ALFIERI, A. F., & ALFIERI, A. A. Genetic characterization of a putative new Type of bovine papillomavirus in the Xipapillomavirus 1 species in a Brazilian daity herd. **Virus Genes**, v.55, n.1, p. 682-687, 2019.

DAUDT, C., DA-SILVA, F.R.C., LUNARDI, M., ALVES, C.B.D.T., WEBER, M.N., CIBULSKI, S.P., ALFIERI, A.F., ALFIERI, A.A.; CANAL, C.W. Papillomaviruses in ruminants: An update. **Transboundary and emerging diseases**, v. 65, n. 5, p.*1*381–1395, 2018.

De VILLIERS, E.M., FAUQUET, C., BROKER, T.R., BERNARD, H.U., ZUR HAUSEN, H. Classification of Papillomaviruses. **Virology**, v.324, n. 1, p. 17-27, 2004.

FERNANDES, K.P., ALFIERI, A.A., DAROLD, G.M., BOABAID, F.M., AGNOL, A.M.D., LUNARDI, M. Case report: Mixed infection of bovine papillomaviruses associated with squamous papiloma of the upper alimentary tract in a dairy cow. **Frontiers in Veterinary Science**, v. 9, n. 1, p. 1020166, 2022.

FORSLUND, O., ANTONSSON, A., NORDIN, P., STENQUIST, B., HANSSON, B.G. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. **Journal of General Virology**, v. 80, n. 9, p. 2437–2443, 1999.

GASPAROTTO, P. H. G., SANTOS, I. R., DANTAS FILHO, J. V., SILVA, M. S., SOUZA, F. A., SOUSA, J. C. M., DRIEMEIER, D., CANAL, C. W., SILVA, F. R. C., DAUDT, C. Characterization of Bovine Papillomavirus types detected in cattle rumen tissue from Amazon region, Brazil. **Animals**, v. 14, n. 15, p.2262, 2024.

JARRETT, W. F. Bracken fern and papiloma vírus in bovine alimentary cancer. **British Medical Bulletin**, v.36, n. 1, p. 79-81, 1980.

JARRETT, W. F. H., MCNEIL, P. E., GRIMSHAW, W. T. R., SELMAN, I, E., MCINTYRE, W. I. M. High incidence area of cattle cancer with a possible interaction

between an environmental carcinogen and a papilomavirus. **Nature**, v. 274, n. 5668, p. 215-217, 1978.

JARRETT, W. F., CAMPO, M. S., BLAXTER, M. L., O'NEIL, B. W., LAIRD, H. M., MOAR, M. H., SARTIRANA, M. L. Alimentary fibropapilloma in cattle: a spontaneous tumor, nonpermissive for papillomavirus replication. **JNCI: Journal of the National Cancer Institute**, v. 73, n. 2, p. 499-504, 1984.

KUMAR, P., NAGARAJAN, N., SAIKUMAR, G.; ARYA, R.S., SOMVANSHI, R. Detection of bovine papilloma viruses in wart-like lesions of upper gastrointestinal tract of cattle and buffaloes. **Transbound and Emerging Diseases**, v. 62, n. 3, p. 264-271, 2015.

MANOS, M.M., TING, Y., WRIGHT, D.K., LEWIS, A.J., BROKER, T.R., WOLINSKY, S. M. The use of polymerase chain reaction amplifica- tion for the detection of genital human papillomaviruses. **Cancer Cell**, v. 7, n. 1, p.209-214, 1989.

MATIAS, B. F., LUNARDI, M., GONÇALVES, K. C. B., VILAS-BOAS, L. A., GUSTANI-BUSS, E., BRACARENSE, A. P. F. L., CUNHA FILHO, L. F. C., ALFIERI, A. F., ALFIERI, A. A. Molecular detection by rolling circle amplification combined with deep sequencing of mixed infection by Bovine Papillomaviruses 2 and 4 in carcinoma in situ of the bovine esophageal mucosa. **Viruses**, v. 16, n. 10, p. 1558, 2024.

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

PaVE **Papillomavirus Episteme**. Disponível em: https://pave.niaid.nih.gov. Acesso em: 13 mar. 2024.

RECTOR, A., VAN-DOORSLAER, K., BERTELSEN, M., BARKER, I.K., OLBERG-LEMEY, R.A.P., SUNDBERG, J.P., VAN-RANST, M. Isolation and cloning of the raccoon (*Procyon lotor*) papillomavirus type 1 by using degenerate papillomavirus-specific primers. **Journal of General Virology**, v. 86, n. 7, p. 2029-2033, 2005.

Da SILVA, F. R. C., DAUDT, C., STRECK, A. F., WEBER, M. N. FILHO, R. V. L., DRIEMEIER, D., CANAL, C. W. Genetic characterization of Amazonian bovine Papillomaviruses reveals the existence of four new putative types. **Virus Genes**, v.

51, n.1, p. 77-84, 2015.

TSIRIMONAKI, E., O'NEIL, B.W., WILLIAMS, R., CAMPO, M.S. Extensive papillomatosis of the bovine upper gastrointestinal tract. **Journal of Comparative Pathology**, v. 129, n. 2-3, p. 93-99, 2003.

7 ARTIGO B – MOLECULAR DETECTION BY ROLLING CIRCLE AMPLIFICATION COMBINED WITH DEEP SEQUENCING OF MIXED INFECTION BY BOVINE PAPILLOMAVIRUSES 2 AND 4 IN CARCINOMA IN SITU OF THE BOVINE ESOPHAGEAL MUCOSA

Artigo publicado na revista ***Viruses.***

Article

Molecular detection by rolling circle amplification combined with deep sequencing of mixed infection by bovine papillomaviruses 2 and 4 in carcinoma *in situ* of the bovine esophageal mucosa

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Abstract: Papillomaviruses (PVs) are oncogenic and infect the skin and mucosa of various host species. Considering the recent advances in research on PVs using rolling circle amplification (RCA) followed by high-throughput sequencing (HTS), in this study, we aimed to investigate the bovine papillomavirus (BPV) types associated with proliferative lesions in the upper alimentary tract of an affected bull and characterize the viral strains through complete genome sequencing using this strategy. We analyzed the PV strains associated with two hyperplastic esophageal lesions through PCR using degenerate primer pairs and RCA, followed by HTS. HTS of the libraries generated using RCA products provided the whole genome sequence of BPV4 present in squamous papilloma, whereas, the complete genome sequence of BPV2 and subgenomic fragments of BPV4 were identified in carcinoma *in situ* (CIS). For the first time, we have sequenced BPV2 identified from the CIS of the bovine upper alimentary canal. Additionally, RCA followed by HTS allowed characterization of the mixed infection by BPV2 and BPV4 in this lesion. These data reveal that BPV4 is not the only BPV type present in CIS of the esophageal mucous membrane; moreover, a mixed infection caused by BPV2 and BPV4 at the tested anatomical site was demonstrated.

Keywords: Cattle; upper alimentary tract; esophagus; papilloma; histopathology; coinfection; polymerase chain reaction; next generation sequencing

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date

1. Introduction

Papillomaviruses (PVs) are small non-enveloped oncogenic viruses with an icosahedral capsid and a genome composed of double-stranded circular DNA that is approximately 8,000 bp in length [1-2]. PVs infect the skin and mucous membranes of various hosts including humans. These infections usually cause benign lesions that spontaneously regress [3]. However, in some cases showing no occasional regression malignant progression may occur, in which benign papilloma transforms into squamous cell carcinoma [4].

The papillomavirus genome contains open reading frames (ORFs) that encode the early (E) and late (L) proteins; E1 and E2 proteins promote viral replication, E4 participates in the production of virions, and the oncoproteins E5, E6, and E7 are linked to cellular transformation. The late proteins L1 and L2 are structural proteins involved in the formation of the capsid and the completion of the viral cycle [1-2].

Among the *Papillomaviridae* family, 44 types of bovine papillomavirus (BPV) have been reported to infect cattle. These diverse viruses belong to the genera *Deltapapillomavirus* (BPV1, 2, 13, and 14), *Dyoxipapillomavirus* (BPV7, 19, and 21), *Epsilonpapillomavirus* (BPV5 and 8), *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11, 12, 15, 17, and 23), unclassified *Dyokappapapillomavirus* (BPV16, 18, and 22), unclassified *Xipapillomavirus* (BPV20, 24, 26, 28, and 29), unclassified *Epsilonpapillomavirus* (BPV25), and unclassified types awaiting official classification (BPV 27, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, and 44) [5-6].

In cattle, BPVs can cause several clinical entities, such as cutaneous papillomatosis characterized by benign tumors at different anatomical sites on the skin, which can be histologically classified as fibropapillomas and true papillomas [7]. Other BPV infection-associated clinical conditions in cattle include bovine enzootic hematuria (BEH), a chronic inflammatory and neoplastic disease of the urinary bladder [4], and the development of tumors in the upper alimentary tract; both clinical alterations may be related to the chronic ingestion of bracken fern (*Pteridium aquilinum*). In addition to its toxic, carcinogenic, and mutagenic effects, *P. aquilinum* has an immunosuppressive effect, which contributes to papilloma persistence [8-9]. In tumors developed in the upper digestive tract, BPV4 has mainly been detected in squamous papillomas [10], whereas, BPV2 is associated with fibropapillomas [11].

The rolling circle amplification (RCA) followed by high-throughput sequencing (HTS) supported significant advances in recent research on papillomaviruses. This untargeted amplification method has helped to sequence the genomes of existing and potential new viral types of PVs with greater sensitivity [12-14]. In this study, we aimed to investigate the BPV types associated with hyperplastic lesions located in the upper alimentary tract of an affected bull and to characterize the involved viral strains through complete genome sequencing using this molecular methodology.

2. Materials and Methods

2.1. Biological Samples and Histopathological Analysis

Through the post-slaughter inspection of the mucosa lining the whole upper alimentary tract, two proliferative lesions were identified and collected from the esophagus of a Girolando breed mature dairy bull provenient from a farm located in the North Central mesoregion of the Parana state, Southern Brazil. A portion of each esophageal lesion was fixed by immersing in 10% neutral-buffered formalin and stained using hematoxylin and eosin for routine histopathological evaluation. The proliferative mucosal lesions were histopathologically evaluated as previously described [15]. The remaining fragment of each proliferative lesion was stored at −80 °C for further molecular evaluations.

The sample collection and all procedures performed in this study were based on ethical and animal welfare considerations. All the applicable international, national, and institutional guidelines for animal care and use were followed. This study was approved

by the Ethics Committee for Animal Use at the Universidade Estadual de Londrina (Protocol No. 050.2021).

2.2. PCR Amplification of Partial BPV L1 and E1 Genes

Frozen fragments acquired from each esophageal lesion were disrupted and homogenized using TissueLyser LT (Qiagen, Hilden, Germany). Subsequently, total DNA was purified from these tissue homogenates using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the instructions provided by the manufacturer. Purified DNA was stored at −80 °C for further molecular analyses. Aliquots of ultrapure sterile water were used as negative controls in all DNA extraction procedures.

To investigate the presence of PV strains potentially associated with the hyperplastic lesions collected from the esophageal mucosa of the dairy bull, three degenerate primer pairs designed to detect the conserved regions of the L1 and E1 genes of a broad spectrum of cutaneous and mucosal PV strains were tested using DNA purified from both lesions. The sequences and features of the primers used are listed in Table 1.

Table 1. Sequences and features of degenerate primers used for the PCR amplification of partial papillomavirus genes.

Primer	Genomic region target	Polarity	Sequence (5'-3')[a]	Expected amplicon length (bp)	Annealing temperature
MY 11[b]	L1	Forward	GCMCAGGGWCATAAYAATGG	450	55 °C
MY 09 [b]	L1	Reverse	CGTCCMARRGGAWACTGATC		
FAP59[c]	L1	Forward	TAACWGTNGGNCAYCCWTATT	480	50 °C
FAP64 [c]	L1	Reverse	CCWATATCWVHCATNTCNCCATC		
AR-E1F2 [d]	E1	Forward	ATGGTNCAGTGGGCNTATGA	552	50 °C
AR-E1R4 [d]	E1	Reverse	ATTNCCATCHADDGCATTTCT		

[a] Degenerate nucleotides: M= A or C; R= A or G; W= A or T; Y= C or T; V= A, C or G; H= A, T or C; N= A, G, C or T; D= A, T or G. [c] [16]; [d] [17]; [e] [18].

PCR mixtures (50 µl) contained 5 µl of the purified DNA, 0.4 to 1 µM of each primer, 200 µM of each deoxynucleoside triphosphate (dNTP) (Invitrogen, Carlsbad, CA, USA), 2.5 U of HotStar Taq DNA polymerase (Qiagen, Hilden, Germany), 1× PCR buffer, 1.5 to 2.5 mM $MgCl_2$, and ultrapure sterile water used to make up the final volume. Amplification was performed with the following cycling profile: an initial step of 15 min at 95 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at an optimal temperature for primer annealing (Table 1), and 1 min at 72 °C, with a final extension for 10 min at 72 °C. Aliquots of the PCR-amplified products were analyzed via electrophoresis using 2% agarose gels, stained with ethidium bromide (0.5 mg/ml), and examined under UV light.

2.3. Sequencing of Subgenomic Fragments

Amplified partial fragments of the BPV L1 and E1 genes were purified using a Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) following the protocol provided by the manufacturer. Next, direct sequencing was performed on a 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) and the corresponding forward and reverse primers, according to the instructions provided by the manufacturer. The generated sequences were examined using the Phred application for quality analysis of chromatogram readings. The sequences with a base quality ≥20 were considered acceptable. The consensus sequences were determined using CAP3

software, and their identities were analyzed based on the public database GenBank using the BLASTn program.

2.4. Rolling-circle Amplification

The BPV genomes were amplified using DNA purified from two esophageal lesions through the multiple-primed rolling-circle amplification (RCA) technique using the TempliPhi 100 Amplification kit (Cytiva, Marlborough, MA, USA), following a previously reported protocol optimized for the amplification of papillomaviral genomes [19]. We verified the amplification of PV DNA through electrophoretic analysis of the RCA product (2 µl) using a 0.8% agarose gel generating a band corresponding to multiple tandem copies of the full-length PV DNA.

The RCA products were purified using a Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and quantified through fluorometry using a Qubit 4.0 (Invitrogen).

2.5. High-throughput Sequencing (HTS)

Libraries were prepared using 1 ng of purified DNA using an Illumina DNA Library Prep kit (Illumina, San Diego, CA, USA). The Agilent D1000 ScreenTape kit (Agilent Technologies, Santa Clara, CA, USA) was used for the quality control of the library on the Agilent TapeStation 4150 System (Agilent Technologies, Santa Clara, CA, USA) and determination of the library size. The library was quantified on a Qubit 4.0 Fluorometer using the Qubit dsDNA Quantification High Sensitivity Assay kit (Invitrogen, Waltham, MA, USA). The library was sequenced via the paired-end sequence strategy using the MiniSeq High Output kit (2 × 150 cycles) and MiniSeq system platform (Illumina, San Diego, CA, USA). Initially, the quality of the sequencing reads was evaluated using FastQC software version 0.11.3 (www.bioinformatics.babraham.ac.uk/projects/fastqc). To minimize possible base-calling errors, reads were trimmed based on a Phred quality score cutoff of 30; sequence reads shorter than 150 nucleotides (nt) were discarded. Complete BPV genomes were assembled using a *de novo* strategy using SPAdes software version 3.9.0 [20].

The reference guided assembly of the sequencing reads obtained for the CIS was performed using the following steps. Firstly, MultiQC was used for quality control reporting [21], followed by Trimmomatic for trimming and filtering paired-end reads [22]. The BWA (Burrows-Wheeler Aligner) was employed for aligning reads to the BPV4 reference genome (accession number: X05817) [23], and SAMtools was used to process, sort, index, and calculate alignment statistics from BAM files [24]. The BCFtools was employed to generate consensus sequences based on reference guided assembly [25].

To annotate the genomes obtained from HTS, putative ORFs were identified using a combination of the ORF Finder program and BLASTp developed by the National Center for Biotechnology Information (NCBI) and compared with previously characterized BPV strains, while the theoretical isoelectric points and the molecular weigths of the predicted viral proteins were estimated using the Expasy server (https://web.expasy.org/compute_pi/).

2.6. Phylogenetic Analysis

Pairwise and multiple sequence alignments at the nucleotide (nt) and amino acid (aa) levels and sequence similarities were estimated using ClustalW in the MEGA v11 software [26]. Complete nt sequences of the L1 gene derived from PV types, previously characterized through complete genome sequencing and representing the *Deltapapillomavirus* and *Xipapillomavirus* genera, were included in the analyses. Two phylogenetic trees were reconstructed based on the alignment of complete L1 nt sequences using the Maximum Likelihood method with the general time-reversible model in MEGA v11 software.

3. Results

Histopathological analysis revealed marked epithelial hyperplasia, parakeratotic hyperkeratosis, and koilocytosis, resulting in exophytic folding in the first lesion, which is characteristic of a squamous papilloma (Figure 1a and 1b). These cytopathic effects indicate BPV infection (Figure 1a and 1b). In the second lesion, the proliferation of disorganized and anaplastic epithelial cells without invasion through the basement membrane into the submucosa was detected, which characterizes a carcinoma *in situ* (CIS) and preneoplastic lesion (Figure 1c and 1d).

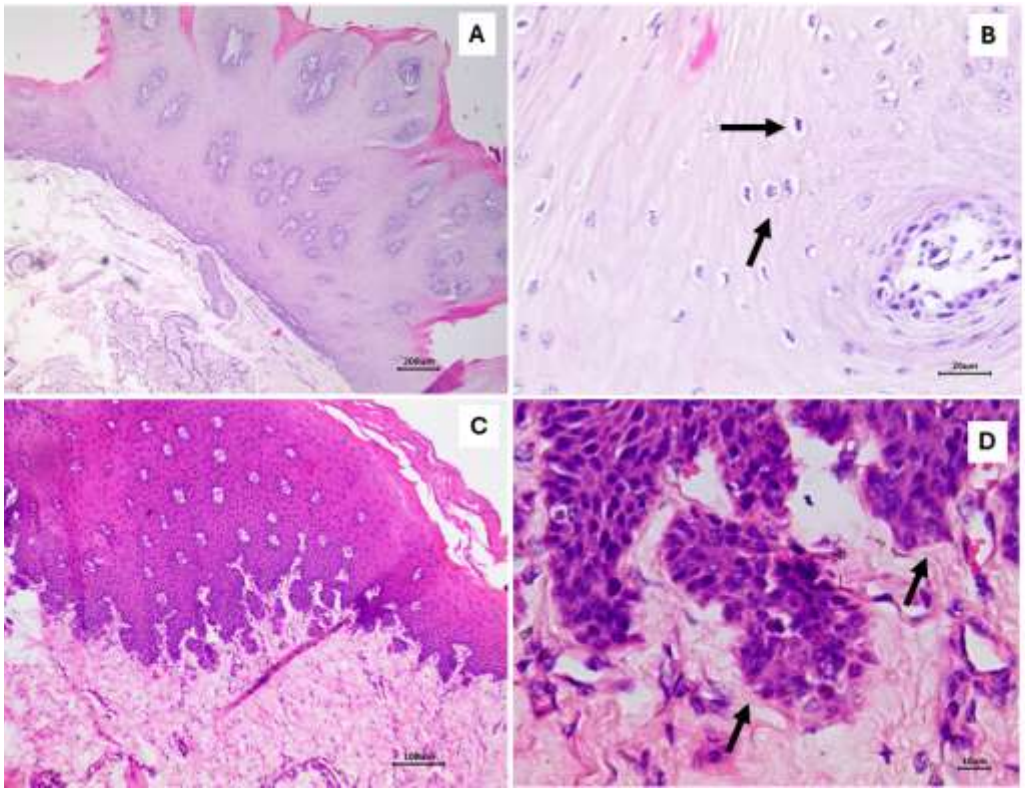

Figure 1. Esophageal mucosa of a Girolando dairy bull. (**a**) Marked epithelium proliferation and parakeratotic hyperkeratosis resulting in exophytic folding. Scale bar 200 μm; (**b**) Acanthosis and koilocytosis (arrows). Scale bar 20 μm; (**c**) Proliferation of disorganized epithelial cells without invasion through the basement membrane into the submucosa. Scale bar 100 μm; (**d**) Epithelial proliferation without invasion of the basement membrane (arrows). Scale bar 10 μm. Figures a to d – Hematoxylin-Eosin.

The selected PCR using the primer pairs FAP59/FAP64 and AR-E1F2/AR-E1R4, whose amplicons represented partial fragments of the BPV L1 and E1 genes, successfully yielded PCR products with the expected lengths for DNA samples from both lesions evaluated. In contrast, PCR with MY primer pair resulted in absence of amplification in either DNA samples. No amplicons were obtained from the negative controls included in the PCR assays. Direct sequencing of the FAP amplicon obtained from the squamous papilloma DNA sample generated a partial L1 nt sequence shown to belong to BPV4 by BLASTn evaluation (99.32% identity; accession number: OP682875). Moreover, the partial nt sequence of E1, which was obtained from the same lesion, showed the highest identity (98.96%) with a BPV4 strain available in GenBank (accession number: MW436424). Contrastingly, direct sequencing of the amplicon representing a partial fragment of the L1 gene obtained from the CIS lesion revealed the highest similarity of this amplicon with BPV2, with an identity of 99.78% (accession number: KF284153). Similarly, for the

esophageal CIS DNA sample, sequencing of the PCR product obtained using the primer pair targeting the E1 gene generated a partial nt sequence highly similar to the same viral type, exhibiting 99.51% identity (accession number: MF045490) through BLASTn-based analysis.

Sequencing analysis of the library using the RCA product of the esophageal squamous papilloma generated a total of 1,796,770 reads. After trimming, 1,127,632 high-quality reads were retained for the sequence analysis. These sequences were *de novo* assembled into contigs that were further compared with the GenBank database using BLASTn and BLASTx. For the squamous papilloma-derived DNA sample, only one contig was characterized as PV, representing the complete genome sequence of BPV4 (7,274 nt, GC content of 41.9%, mean coverage of 4,081, 504,788 used reads, and 40,038 unused reads). Through BLASTn analysis, the highest identity obtained by comparison with other BPV complete genomic sequences deposited in GenBank was 99,11% with the BPV4 strain 4827RS16-BR node 9 recovered from a teat papilloma in cattle in Brazil in 2016 (accession number: MW436424).

In contrast, the library obtained from the RCA product amplified from the CIS lesion generated a total of 1,867,724 reads, with 1,310,390 high-quality reads remaining after trimming. From this esophageal lesion, *de novo* assembly generated one contig representing the whole-genome sequence of the BPV2 strain (7,947 nt, GC content of 45.8 %, mean coverage of 2,177, 266,312 used reads, and 388,883 unused reads). BLASTn analysis of this sequence revealed that the BPV2 strains E170428-1 and E181025 (accession numbers: LC510376 and LC510384), identified from equine sarcoids in Japan in 2017 and 2018, respectively, exhibited the closest similarity to the BPV2 genome obtained (nt identity of 99.94%). Additionally, *de novo* assembly generated three other contigs corresponding to BPV subgenomic sequences. These sequences were highly similar to BPV4 and represented partial fragments of the L1 (223 nt, mean coverage of 0.946, and 2 used reads), L2 (334 nt, mean coverage of 1.799, and 7 used reads), and E7 (208 nt, mean coverage of 0.878, and 2 used reads) genes (Table 2). When compared with the BPV4 genome sequenced from the esophageal squamous papilloma, the partial nt sequences of the L1, L2, E7 genes generated from the CIS by *de novo* assembly exhibited identities of 100, 99.7, and 100% respectively.

Through the reference guided assembly employing the BPV4 reference genome, the reads obtained from the CIS lesion generated 12 different contigs that mapped to most genes of the BPV4. These nucleotide sequences represented partial fragments of the E7 (263 nt, mean coverage of 99.619, 2 used reads, and 1,817,830 unused reads), E1 (194 nt, mean coverage of 100, and 4 used reads; 913 nt, mean coverage of 100, and 10 used reads; 149 nt, mean coverage of 100, and 1 used read), E2 (147 nt, mean coverage of 100, and 1 used read; 341 nt, mean coverage of 100, and 4 used reads), L2 (627 nt, mean coverage of 100, and 11 used reads; 148 nt, mean coverage of 100, and 1 used read), and L1 (216 nt, mean coverage of 100, 2 used reads; 147 nt, mean coverage of 94.23, and 1 used read; 519 nt, mean coverage of 100, and 8 used reads; 166 nt, mean coverage of 100, and 3 used reads) genes. The comparison of these CIS BPV4 partial sequences with the BPV4 strain sequenced from the squamous papilloma revealed identities varying from 99.4 to 100%.

Table 2. Types of bovine papillomavirus identified in esophageal lesions with distinct histopathological classifications using different amplification and sequencing strategies.

Histological type	Amplification and sequencing methods			
	PCR / Sanger sequencing		RCA / High throughput sequencing	
	Partial L1 gene[a]	Partial E1 gene[b]	Complete genome	Partial BPV genes
Squamous papilloma	BPV4	BPV4	BPV4[c]	–
Carcinoma *in situ*	BPV2	BPV2	BPV2[d]	BPV4

Primers: [a]FAP; [b]AR-E1F2/ AR-E1R4.
GenBank accession Nos.: [c]PQ140660 ; [d]PQ140661.

Phylogenetic reconstruction based on the complete L1 nt sequences of all PVs, already characterized through the complete genome sequencing and classified in the *Xipapillomavirus* genus, confirmed that the BPV4 genome derived from esophageal squamous papilloma belongs to the *Xipapillomavirus 1* viral species (Figure 2). Additionally, the phylogenetic tree of the complete L1 nt sequences of PVs fully sequenced and classified in the *Deltapapillomavirus* genus revealed that the Brazilian BPV2 strain present in the CIS lesion on the same organ belonged to the *Deltapapillomavirus 4* viral species, with high statistical support (Figure 3).

Figure 2. Phylogenetic analysis of the Brazilian bovine papillomavirus strain identified in a squamous papilloma developed in the esophagus of a dairy bull from Southern Brazil using complete L1 gene nucleotide sequences. The evolutionary history was inferred using the Maximum Likelihood method and General Time Reversible model. The tree with the highest log likelihood (-18441.70) is shown. Numbers at internal nodes represent the bootstrap support values (percentages) determined for 1,000 replications (values <50 are not shown). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. We included 18 nucleotide sequences in this analysis. There were a total of 1580 positions in the final dataset. Evolutionary analyses were conducted using MEGA11 [21]. The Brazilian bovine papillomavirus strain sequenced in this study is indicated using a black-filled circle. Representatives of the viral PV types belonging to the five species of the genus *Xipapillomavirus*, recognized by the International Committee on Taxonomy of Viruses (ICTV), are shown. The GenBank accession numbers of the PV types included in the phylogenetic analysis are indicated.

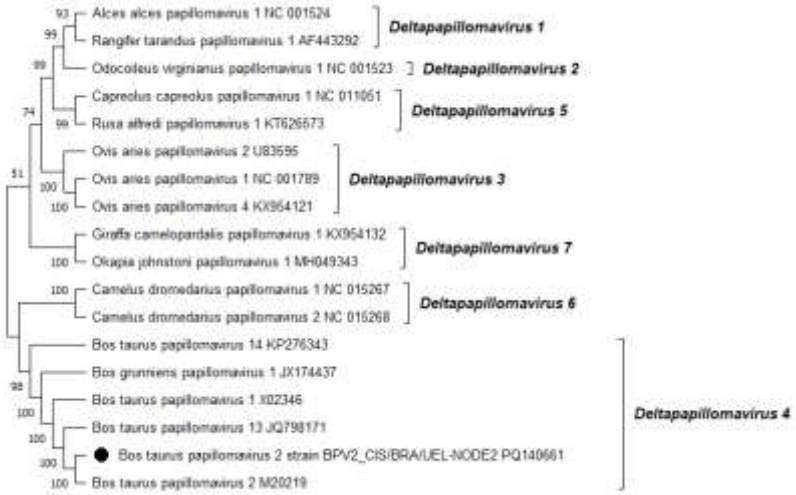

Figure 3. Phylogenetic analysis of the Brazilian bovine papillomavirus strain identified in a carcinoma *in situ* lesion developed in the esophagus of a dairy bull from Southern Brazil using complete L1 gene nucleotide sequences. The evolutionary history was inferred using the Maximum Likelihood method and General Time Reversible model. The tree with the highest log likelihood (-17206.33) is presented. Numbers at internal nodes represent the bootstrap support values (percentages) determined for 1,000 replications (values <50 are not shown). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 18 nucleotide sequences; the final dataset comprised a total of 1537 positions. Evolutionary analyses were conducted in MEGA11 [21]. The Brazilian bovine papillomavirus strain sequenced in this study is represented by a black-filled circle. Representatives of the viral PV types belonging to the seven species of the genus *Deltapapillomavirus*, recognized by the International Committee on Taxonomy of Viruses (ICTV), are shown. The GenBank accession numbers of the PV types included in the phylogenetic analysis are indicated.

The complete genomic sequence of the BPV4 strain characterized from the esophageal squamous papilloma was deposited in the GenBank database under the accession number PQ140660. The whole genome of the BPV2 strain sequenced from the esophageal CIS lesion has been submitted under the accession number PQ140661, while the nt sequences obtained from the CSI lesion by the *de novo* assembly, and representing partial fragments of the BPV4 L2, E7, and L1 genes, were deposited under the accession numbers PQ140662 to PQ140664, respectively. In addition, the BPV4 subgenomic sequences generated by the reference guided assembly of the CIS lesion were deposited under the accession numbers PQ365677 to PQ365688. Summary data of the predicted proteins identified in the BPV4 and BPV2 complete genomes through the ORF Finder program are presented in Table 3.

Table 3. Features of the predictive ORFs identified in BPV genomes sequenced from bovine esophageal lesions through high-throughput sequencing of rolling circle amplification products.

BPV strain	ORF	ORF location	Length (nt)	Length (aa)	Molecular mass (KDa)	pI
BPV4[a]	E5	333-461	129	42	4.995	4.21
	E8	448-585	138	45	5.312	3.58
	E7	716-1,012	297	98	10.993	4.41
	E1	1,002-2,831	1,830	609	69.506	5.98
	E2	2,773-4,005	1,233	410	46.416	9.96
	E4	3,299-3,739	441	146	16.728	6.31
	L2	4,020-5,597	1,578	525	57.244	4.84
	L1	5,608-7,128	1,521	506	57.575	7.14
BPV2[b]	E6	935-1,348	414	137	15.867	8.97
	E7	1,323-1,706	384	127	13.601	9.08
	E1	1,693-3,513	1,821	606	68.043	6.10
	E8	2,048-2,278	231	76	8.710	9.88
	E2	3,455-4,693	1,239	412	45.681	6.15
	E4	4,038-4,379	342	113	12.688	10.75
	E5	4,735-4,869	135	44	5.210	4.35
	L2	5,038-6,441	1,404	467	49.433	4.94
	L1	6,454-7,947	1,494	497	55.610	8.44

[a]GenBank accession No.: PQ140660.

[b]GenBank accession No.: PQ140661.

4. Discussion

In this study, BPV2 and BPV4 were identified in lesions with different histological classifications developed in the esophagus of an asymptomatic dairy bull in Southern Brazil. The viral types were detected by PCR assays using degenerate primers that amplify a broad range of PV sequences and through a untargeted amplification strategy involving the RCA of BPV genomes followed by sequencing via HTS. Additionally, deep sequencing of the RCA product derived from the esophageal CIS lesion revealed mixed infection by BPV2 and 4; the BPV2 complete genomic sequence and partial nucleotide sequences from most genes of the BPV4 were obtained.

Although BPV4 is the viral type mostly associated with tumor lesions that develop in the mucous membrane lining the upper digestive canal of cattle, both BPV4 belonging to the genus *Xipapillomavirus* and BPV2 belonging to the genus *Deltapapillomavirus*, serve as causal agents of hyperplastic lesions of the bovine upper alimentary tract, as previously reported [10, 11, 27]. The BPV4 infection was associated with progression to malignancy [28, 29], however, BPV2 infections seems to rarely lead to fibropapillomas at this anatomical site [11]. As only BPV4 was identified in alimentary cancers of cattle, a link between BPV2 infection of the upper digestive tract and the development of malignancy has not been established in cattle [11].

In this study, the BPV2 complete genomic sequence was amplified from an esophageal CIS DNA sample and sequenced. To the best of our knowledge, we have identified this viral type in a premalignant lesion of the bovine upper alimentary tract for the first time. Additionally, the results reflect that a mixed infection with BPV4 can be

characterized in the CIS lesion through RCA amplification followed by HTS. A recent investigation carried out in the midwestern region of Brazil revealed the occurrence of a mixed infection caused by BPV4 and BPV2 in a squamous papilloma developed in the esophagus of a dairy cow; viral types were identified using conventional PCR employing the degenerate primer pairs FAP59/FAP64 and AR-E1F2/AR-E1R4, followed by cloning and sequencing of the amplified L1 and E1 gene fragments [30]. Mixed infection by BPV2 and BPV4 has been previously described in a squamous papilloma of the esophagus of a dairy cow; however, for the first time, this study presents its occurrence in a CIS in the same organ of cattle.

The RCA and HTS of the complete genome of BPV4 in the squamous papilloma of the esophagus of the dairy bull are comparable to the previous findings. Previously, BPV4 was reported in papillomatous lesions without malignant progression developed in cattle raised in Scotland and England. The conventional PCR-based molecular analyses of these biological samples confirmed the viral type, whereas, histopathological analyses revealed papillomatous changes throughout the upper alimentary tract [31]. In Italy, through histopathological evaluation, esophageal papilloma (inverted and exophytic) was detected in 147 cattle among a total of 1,133 animals slaughtered between 2000 and 2001; BPV4 was present in > 60% of the examined samples [32].

In addition to the viral types commonly detected in tumors in the upper digestive tract, studies carried out in India revealed BPV5 in lesions from this anatomical site of cattle for the first time. This viral type was detected in six hyperproliferative lesions acquired from the rumen, reticulum, and esophagus of cattle and was histologically diagnosed to be fibropapillomas and papillomas [33]. In the present study, we did not detect BPV5 in the lesions developed in the upper alimentary tract of the dairy bull via broad-spectrum PCR assays using consensus primers or untargeted amplification by RCA, combined with deep sequencing.

Although BPV4 was associated with the emergence of papillomas in the upper alimentary tract of cattle, a retrospective survey was conducted using 47 papillomas in the mouth and esophagus of 30 animals with upper digestive canal carcinomas, collected from Southern Brazil between 2003 and 2014; PCR assays conducted using both the FAP primer pair and specific primers targeting the BPV4 L1 gene did not reveal BPV DNA in these lesions [34]. In the present study, RCA of the BPV genomes followed by HTS successfully identified BPV2 and 4 sequences in the CIS lesion, as well as BPV4 in a squamous papilloma in the upper alimentary tract of a dairy bull, which indicates that BPV4 is not the only BPV type present in the CIS developed in the bovine esophageal mucous membrane. This molecular strategy potentially allows the identification of diverse BPV types, even in mixed infections, in different hyperproliferative lesions of cattle, deepening the research on BPV. In the northern region of Brazil, cutaneous papillomas collected from cattle were reported to contain three viral types previously characterized in one sample and BPV24, a novel type of BPV, in another sample; notably, both samples were collected from the skin lesions of a single animal [35]. In an investigation carried out on cows slaughtered in another state of Southern Brazil, RCA and HTS-based assay using 23 teat warts revealed seven known viral types (BPV3, 4, 6, 8, 9, 12, and 27); moreover, 14 new types (BPV30–BPV43) were first characterized [14].

In conclusion, the present study, for the first time, demonstrates the association of BPV2 with a CIS lesion in the bovine upper alimentary tract and reveals the occurrence of mixed infection by BPV2 and BPV4 in the tumor. Since mixed infection in esophageal CIS could not be detected by common molecular tools, such as PCR using degenerate primers followed by Sanger sequencing of the amplicons, the use of untargeted molecular techniques, such as RCA and HTS, is essential for advancement in PV research. This is the first study that revealed the association between BPV and proliferative lesions in the bovine upper digestive canal using this molecular strategy.

Author Contributions: Conceptualization, M.L., B.F.M. and A.A.A.; methodology, M.L., B.F.M., L.A.V. and A.A.A.; validation, M.L. B.F.M., and A.A.A.; formal analysis, M.L., B.F.M., K.C.B.G., and

E.G-B.; investigation, M.L., B.F.M., A.P.F.B., L.F.C.C.F. and A.F.A.; resources, A.A.A., B.F.M., L.F.C.C.F., L.A.V. and A.P.F.B.; data curation, K.C.B.G., E.G-B. and L.A.V.; writing—original draft preparation, B.F.M. and M.L.; writing—review and editing, M.L., B.F.M., A.A.A. and A.P.F.B.; visualization, M.L., B.F.M., A.A.A., A.F.A., L.A.V. and L.F.C.C.F..; supervision, A.A.A.; project administration, M.L.; funding acquisition, A.A.A. All authors have read and agreed to the published version of the manuscript.

Funding: The authors thank the following Brazilian Institutes for their financial support: the National Council of Technological and Scientific Development (CNPq); the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES); and the Financing of Studies and Projects (FINEP). This work was supported by the National Institute of Science and Technology of Dairy Production Chain (CNPq/INCT-Leite) [Grant No. 465725/2014-7]. EG-B acknowledges funding from EU Horizon 2020 grants MOOD (H2020-347 874850). AAA is a recipient of CNPq Fellowships.

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee for Animal Use of the Universidade Estadual de Londrina (protocol number 050.2021).

Data Availability Statement: The original data presented in the study are openly available in GenBank database under the following accession numbers: PQ140660-PQ140664; PQ365677 to

PQ365688.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Doorbar, J.; Quint, W.; Banks, L.; Bravo, I.G.; Stoler, M.; Broker, T.R.; Stanley, M.A. The biology and life-cycle of human papillomaviruses. *Vaccine* **2012**, *30*, 55-70. DOI: 10.1016/j.vaccine.2012.06.083
2. Daudt, C.; da-Silva, F.R.C.; Lunardi, M.; Alves, C.B.D.T.; Weber, M.N.; Cibulski, S.P.; Alfieri, A.F.; Alfieri, A.A.; Canal, C.W. Papillomaviruses in ruminants: an update. *Transbound. Emerg. Dis.* **2018**, *00*, 1-15. DOI: 10.1111/tbed.12868
3. Schwarz, E.; Freese, U. K.; Gissmann, L.; Mayer, W.; Roggenbuck, B.; Stremlau, A.; Hausen, H. Z. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **1985**, *314*, 111-114. DOI: 10.1038/314111a0
4. Campo, M.S. Bovine papillomavirus and cancer. *Vet. J.* **1997**, *154*, 175-188. DOI: 10.1016/s1090-0233(97)80019-6
5. ICTV International Committee on Taxonomy of Viruses. Available online: https://ictv.global (accessed on 15 March 2024).
6. PaVE Papillomavirus Episteme. Available online: https://pave.niaid.nih.gov (accessed on 13 March 2024).
7. Munday, J.S. Bovine and human papillomaviruses: a comparative review. *Vet. Pat.* **2014**, *51*, 1063-1075. DOI: 10.1177/0300985814537837
8. Jarret, W.F.H.; McNeil, P.E.; Grimshaw, W.T.R.; Selman, I.E.; McIntyre, W.I.M. High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papilloma virus. *Nature* **1978**, *274*, 215-217. DOI: 10.1038/274215a0
9. Borzacchiello, G.; Roperto, F. Bovine papillomavirus, papillomas and cancer in cattle. *Vet. Res.* **2008**, *39*, 45. DOI: 10.1051/vetres:2008022
10. Campo, M.S.; O'Neil, B.W.; Barron, R.J.; Jarret, W.F.H. Experimental reproduction of the papiloma-carcinoma complex of the alimentary canal in cattle. *Carcinogenesis* **1994**, *15*, 1597-1601. DOI: 10.1093/carcin/15.8.1597
11. Jarret, W.F.H.; Campo, M.S.; Blaxter, M.L.; O'Neil, B.W.; Laird, H.M.; Moar, M.H.; Sartirana, M.L. Alimentary fibropapilloma in cattle: a spontaneous tumor, nonpermissive for papillomavirus replication. *J. Natl. Cancer Inst.* **1984**, *73*, 499-504. DOI: 10.1093/jnci/73.2.499
12. Rector, A.; Bossart, G.D.; Ghim, S.; Sundberg, J.P.; Jenson, A.B.; Ranst, M.V. Characterization of a novel close-to-root papillomavirus from a Florida manatee by using multiply primed rolling-circle amplification: Trichechus manatus latirostris papillomavirus type 1. *J. Virol.* **2004**, *78*, 12698-12702. DOI: 10.1128/JVI.78.22.12698-12702.2004
13. Daudt, C.; da-Silva, F.R.C.; Streck, A.F.; Weber, M.N.; Mayer, F.Q.; Cibulski, S.P.; Canal, C.W. How many papillomavirus species can go undetected in papiloma lesios? *Sci. Rep.* **2016**, *6*, 36480. DOI: 10.1038/srep36480
14. Sauthier, J.T.; Daudt, C.; da-Silva, F.R.C.; Alves, C.D.B.T.; Mayer, F.Q.; Bianchi, R.M.; Driemeier, D.; Streit, R.S.A.; Staats, C.C.; Canal, C.W.; Weber, M.N. The genetic diversity of "papillomavirome" in bovine teat papiloma lesions. *Anim. Microbiome* **2021**, *3*, 51. DOI: 10.1186/s42523-021-00114-3
15. Araldi, R.P.; Melo, T.C.; Neves, A.C.; Spadacci-Morena, D.D.; Magnelli, D.G.; Modolo, D.G.; de-Sá-Júnior, P.L.; Mazucchelli-de-Souza, J.; Carvalho, R.F.; Beçak, W.; Stocco, R.C. Hyperproliferative action of bovine papillomavirus: genetic and histopathological aspects. *Genet. Mol. Res.* **2015**, *14*, 12942-12954. DOI: 10.4238/2015
16. Manos, M.M.; Ting, Y.; Wright, D.K.; Lewis, A.J.; Broker, T.R.; Wolinsky, S.M. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cell* **1989**, *7*, 209-214.

17. Forslund, O.; Antonsson, A.; Nordin, P.; Stenquist, B.; Hansson, B.G. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. *J. Gen. Virol*. **1999**, *80*, 2437-2443. DOI: 10.1099/0022-1317-80-9-2437
18. Rector, A.; Van-Doorslaer, K.; Bertelsen, M.; Barker, I.K.; Olberg-Lemey, R.A.P.; Sundberg, J.P.; Van-Ranst, M. Isolation and cloning of the raccoon (Procyon lotor) papillomavirus type 1 by using degenerate papillomavirus-specific primers. *J. Gen. Virol*. **2005**, *86*, 2029-2033. DOI: 10.1099/vir.0.80874-0
19. Rector, A.; Tachezy, R.; Van Ranst, M. A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. *J. Virol*. **2004**, *78*, 4993–4998. DOI: 10.1128/jvi.78.10.4993-4998.2004
20. Gurevich, A.; Saveliev, V.; Vyahhi, N.; Tesler, G. QUAST: A quality assessment tool for genome assemblies. *Bioinformatics* **2013**, *29*, 1072-1075. DOI: 10.1093/bioinformatics/btt086
21. Ewels, P.; Magnusson, M.; Lundin, S.; Kaller, M. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics.* **2016**, *32*, 3047-3048. DOI: 10.1093/bioinformatics/btw354
22. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* **2014**, *30*, 2114-2120. DOI: 10.1093/bioinformatics/btu170
23. Li, H.; Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* **2009**, *25*, 1754-1760. DOI: 10.1093/bioinformatics/btp324
24. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R.; 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. *Bioinformatics.* **2009**, *25*, 2078-2079. DOI: 10.1093/bioinformatics/btp352
25. Danecek, P.; Bonfield, J.K.; Liddle, J.; Marshall, J.; Ohan, V.; Pollard, M.O.; Whitwham, A.; Keane, T.; McCarthy, S.A.; Davies, R.M.; Li, H. Twelve years of SAMtools and BCFtools. *Gigascience.* **2021**, *10*, giab008. DOI: 10.1093/gigascience/giab008
26. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol*. **2021**, *38*, 3022-3027. DOI: 10.1093/molbev/msab120
27. Araldi, R.P.; Assaf, S.M.R.; de-Carvalho, R.F; de-Carvalho, M.A.C.R.; de-Souza, J.M.; Magnelli, R.F.; Módolo, D.G.; Roperto, F.P.; Stocco, R.C.; Beçak, W. Papillomaviruses: a systematic review. *Genet. Mol. Bio*. **2017**, *40*, 1-21. DOI: 10.1590/1678-4685-GMB-2016-0128
28. Campo, M.S.; Moar, M.H.; Sartirana, M.L.; Kennedy, I.M.; Jarret, W.F.H. The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. *EMBO J*. **1985**, *4*, 1819-1825. DOI: 10.1002/j.1460-2075.1985.tb03856.x.
29. Jackson, M.E.; O'Brien, V.; Morgan, I.M.; Grindlay, G.J.; Campo, M.S. Bovine papillomavirus type 4: neoplastic cell transformation and control of infection by vaccination (review). *Int. J. Oncol*. **1996**, *9*, 1189-1199. DOI: 10.3892/ijo.9.6.1189
30. Fernandes, K.P.; Alfieri, A.A.; Darold, G.M.; Boabaid, F.M.; Agnol, A.M.D.; Lunardi, M. Case report: mixed infection of bovine papillomaviruses associated with squamous papiloma of the upper alimentary tract in a dairy cow. *Front. Vet. Sci*. **2022**, *9*, 1020166. DOI: 10.3389/fvets.2022.1020166
31. Tsirimonaki, E.; O'Neil, B.W.; Williams, R.; Campo, M.S. Extensive papillomatosis of the bovine upper gastrointestinal tract. *J. Comp. Pathol*. **2003**, *129*, 93-99. DOI: 10.1016/s0021-9975(03)00007-0
32. Borzacchiello, G.; Ambrosio, V.; Roperto, S.; Poggiali, F.; Tsirimonakis, E.; Venuti, A.; Campo, M.S.; Roperto, F. 2003. Bovine papillomavirus type 4 in oesophageal papillomas of cattle from the south of Italy. *J. Comp. Pathol*. **2003**, *128*, 203-206. DOI: 10.1053/jcpa.2002.0626
33. Kumar, P.; Nagarajan, N.; Saikumar, G.; Arya, R.S.; Somvanshi, R. Detection of bovine papilloma viruses in wart-like lesions of upper gastrointestinal tract of cattle and buffaloes. *Transbound. Emerg. Dis*. **2013**, *62*, 264-271. DOI: 10.1111/tbed.12127
34. Faccin, T.C.; Cargnelutti, J.F.; Rodrigues, F.S.; de-Menezes, F.R.; Piazer, J.V.M.; de-Melo, S.M.P.; Lautert, B.F.; Flores, E.F.; Kommers, G.D. Bovine upper alimentary squamous cell carcinoma associated with bracken fern poisoning: Clinical-pathological aspects and etiopathogenesis of 100 cases. *PLoS ONE* **2018**, *13*, e0204656. DOI: 10.1371/journal.pone.0204656
35. Daudt, C.; da-Silva, F.R.C.; Cibulski, S.P.; Streck, A.F.; Laurle, R.E.; Munday, J.S.; Canal, W.C. Bovine papillomavirus 24: a novel member of the genus Xipapillomavirus detected in the Amazon region. *Arch. Virol*. **2019**, *164*, 637-641. DOI: 10.1007/s00705-018-4092-3

8 CONCLUSÕES

- Neste estudo, tanto tipos de papilomavirus bovino anteriormente descritos (BPVs 1, 2 e 4), quanto tipos virais previamente não associados com lesões hiperproliferativas do trato gastrointestinal superior (BPVs 9, 39 e o provável novo tipo de BPV similar ao BPV12), foram identificados em papilomas localizados em lesões esofágicas dos animais avaliados. Estes achados demonstraram diversidade viral superior a estimada nos estudos iniciais envolvendo o TGIS de bovinos;
- O uso de uma combinação de primers degenerados capazes de anelar com sequências dos genes conservados L1 e E1 de papilomavírus, associado ao sequenciamento direto dos fragmentos amplificados pelo método Sanger, permitiu a detecção de tipos virais adicionais nas lesões do TGIS de bovinos;
- A utilização de ferramentas moleculares inovadoras, como a RCA associada ao HTS, proporcionou a caracterização genômica completa de tipos virais presentes em duas lesões esofágicas de um bovino afetado. O BPV4 foi detectado na lesão classificada como papiloma escamoso, enquanto coinfecção pelos BPVs 2 e 4 foi demonstrada pela primeira vez em um CIS esofágico.

ANEXOS

ANEXO A

Protocolos e técnicas

- O protocolo completo utilizado para extração das amostras teciduais está descrito em anexo A;

- PCR utilizando MY09/MY11

- 2,5 µL de solução tampão para PCR10x;

- 0,5 µL de cada dNTP (Invitrogen, Carlsbad, CA, USA);

- 1,0 µL de $MgCl_2$;

- 1,0 µL de cada primer;

- 0,125 µL da enzima *HotStar Taq* DNA Polimerase (Qiagen, Hilden, Alemanha);

- 2,5 µL de DNA;

- Adiciona-se a água em quantidade suficiente para completar o volume final de 25 µL.

- Etapas para amplificação:

- Desnaturação inicial: 15 min a 95°C;

- Seguida de 45 ciclos de: i) desnaturação: 1 min a 94°C; ii) anelamento: 1 min a 55°C; iii) extensão: 1 min a 72°C;

- Extensão final: 10 min a 72°C.

- PCR utilizando FAP59/FAP64

- 2,5 µL de solução tampão para PCR10x;

- 0,5 µL de cada dNTP (Invitrogen, Carlsbad, CA, USA);

- 0,5 µL de cada primer;

- 0,125 µL da enzima HotStar Taq DNA Polimerase (Qiagen, Hilden, Alemanha);

- 2,5 µL de DNA;

- Adiciona-se a água em quantidade suficiente para completar o volume final de 25 µL.

- Etapas para amplificação:

- Desnaturação inicial: 15 min a 95 °C;

- Seguida de 45 ciclos de: i) desnaturação: 1 min a 94 °C; ii) anelamento: 1 min a 50 °C; iii) extensão: 1 min a 72 °C;

- Extensão final: 10 min a 72 °C.

- PCR utilizando AR-E1R2/E1R4

- 2,5 µL de solução tampão para PCR10x;

- 0,5 µL de cada dNTP (Invitrogen, Carlsbad, CA, USA);

- 1,0 µL de $MgCl_2$;

- 1,25 µL de cada primer;

- 0,125 µL da enzima HotStar Taq DNA Polimerase (Qiagen, Hilden, Alemanha);

- 2,5 µL de DNA;

- Adiciona-se a água em quantidade suficiente para completar o volume final de 25 µL.

- Etapas para amplificação:

- Desnaturação inicial: 15 min a 95 °C;

- Seguida de 45 ciclos de: i) desnaturação: 1 min a 94 °C; ii) anelamento: 1 min a 50 °C; iii) extensão: 1 min a 72 °C;

- Extensão final: 10 min a 72 °C.

- Gel de agarose 2%

- Purificação dos produtos de PCR excisados do gel.

- Quantificação dos produtos de PCR.

- RCA

- Adicione 5 µL de *sample buffer*;

- Adicione 1 µL de DNA;

- Desnature a amostra por 3 min a 95 °C;

- Resfrie a amostra a 4 °C;

- Em banho de gelo, adicione:

- 5 µL de *reaction buffer;*

- 0,2 µL do mix me enzima;

- 0,1 µL de dNTP mãe;

- Adicione 5 µL do mix acima para a amostra desnaturada e resfriada;

- Incube por 18 h;

- Inative a enzima por 10 min a 65 °C;

- Resfrie a 4 °C.