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Análise molecular de Papilomavírus canino identificado em cães naturalmente infectados no estado de Sergipe - Brasil

São Cristóvão
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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Parasitária da Universidade Federal de Sergipe como parte dos requisitos necessários para obtenção do grau de Mestre em Biologia Parasitária na área de concentração de Biologia Molecular.

Orientador: Prof. Dr. Marcus Vinicius de Aragão Batista

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RESUMO

REIS, J. D. R. **Análise molecular de Papilomavírus canino identificado em cães naturalmente infectados no estado Sergipe - Brasil.** 2017. Dissertação (Mestrado), Programa de Pós-graduação em Biologia Parasitária, Universidade Federal de Sergipe – UFS. São Cristóvão, 2017.[1]

A papilomatose é uma doença infecciosa causada pelos papilomavírus pertencentes a família *Papillomaviridae*. Em cães, a espécie *Canis familiaris* papilomavírus (CPV) é o agente etiológico da doença e possui até o momento vinte tipos identificados. Estes vírus podem levar a lesões neoplásicas benignas na pele e epitélio da mucosa, que são os papilomas exofíticos, endofíticos e as placas pigmentadas, podendo ocorrer a progressão para o câncer carcinoma de células escamosas (CCEs). A patogenicidade do vírus relacionada aos tipos, subtipos e variantes pode influenciar diferentes tipos de lesões nos animais. Com isso, percebe-se que há uma necessidade de identificar e caracterizar os vírus existentes para um melhor entendimento da doença e adoções de medidas tanto preventivas quanto de tratamento. Nesse estudo o objetivo geral é analisar a diversidade genética de papilomavírus canino presentes em cães infectados no estado de Sergipe - Brasil. Estratégias de diagnóstico foram utilizadas para a identificação de papilomavírus canino, em que o exame histopatológico foi utilizado para a caracterização da lesão e o PCR juntamente com a eletroforese para a confirmação da presença do DNA viral, depois foi realizado o sequenciamento para subsequente genotipagem das amostras e ferramentas de bioinformática foram utilizadas para análise das sequências de nucleotídeos e aminoácidos do gene L1. Como resultado, dezoito amostras de possíveis lesões papilomatosas foram coletadas, nove amostras tiveram a presença do DNA de CPV, com lesões exofíticas orais e uma cutânea, confirmadas pelo histopatológico. Foi possível observar na amostra oito a presença de CCEs oral. O CPV1 foi identificado em nove amostras deste estudo. Oito amostras eram variantes de CPV1 com 100% de identidade com uma variante já descrita e uma amostra foi identificada como uma nova variante de CPV1, possuindo uma diferença de 0,8% quando comparada com a sequência do gene L1 de referência de CPV1 e uma diferença de 0,2% com a variante já existente de CPV1. As mutações das variantes e a alteração na estabilidade da estrutura proteína foram analisadas e pôde-se observar que a mutação V204A da nova variante do isolado oito é altamente desestabilizadora com alteração de função da proteína. Esse estudo corrobora com a caracterização do CPV1 como o principal causador da papilomatose oral em cães, observando-se a variabilidade genética de CPV1 com variantes de diferentes potenciais patogênicos, com uma putativa nova variante associada ao câncer com uma mutação que pode desestabilizar a estrutura proteica de L1 de CPV1 alterando sua função.

Palavras-chave: Papilomatose canina; Papilomavírus canino; Diversidade genética; variantes; câncer.

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[1] Orientador: Prof. Dr. Marcus Vinicius de Aragão Batista. Docente do Programa de Pós-graduação em Biologia Parasitária da Universidade Federal de Sergipe.

ABSTRACT

REIS, J. D. R. **Molecular analysis of Canine Papillomavirus identified in naturally infected dogs from Sergipe State - Brazil**. 2017. Thesis (Master's degree), Postgraduate program in Parasitic Biology, Federal University of Sergipe - UFS. São Cristóvão, 2017.

The papillomatosis is an infectious disease caused by papillomaviruses which belongs to the *Papillomaviridae* family. In dogs, *Canis familiaris* papillomavirus (CPV) species is the causative agent of the disease and it has so far twenty identified types. These viruses can lead to benign neoplastic lesions of the skin and mucosal epithelium that are exophytic and endophytic papilloma and pigmented plaques which may occur progression to carcinoma squamous cell (SCCs) cancer. The pathogenicity of the virus related to the types, subtypes and variants can influence deafferents lesions on the animal. Thus, there is a need to identify and characterize the existing virus to a better understanding of the disease and adoptions of both preventive measures as treatment. In this study, the general objective is to analyze the genetic diversity of canine papillomavirus present in infected dogs in the state of Sergipe - Brazil. We used diagnostic strategies for the identification of canine papillomavirus; wherein the histopathology was used to the characterization of the lesion as papilloma and the PCR with electrophoresis for confirm the viral DNA, then sequencing performed for subsequent genotyping of samples and bioinformatics tools was used for analysis of the nucleotide and amino acid sequences of the L1 gene. As a result, eighteen samples of possible papillomatous lesions were collected and nine samples had the presence of CPV DNA with oral and cutaneous exophytic lesions confirmed by histopathology. It was possible observe in one sample the presence of oral SCCs. The Canine papillomavirus type 1 (CPV1) was identified in nine samples of this study. Eight samples were CPV1 variants with 100% identity with the already described variants and one sample was identified as a new CPV1 variant having a difference of 0.8% when compared with the reference sequence of the CPV1 L1 gene and a difference of 0.2% with the existing CPV1 variant. The mutations of the variants and the change in stability of the protein structure were analyzed and it was observed that the V204A mutation of the new variant of isolate eight is highly destabilizing with alteration of protein function. This study corroborates the characterization of CPV1 as the main cause of oral papillomatosis in dogs, observing the genetic variability of CPV1 with variants of different pathogenic potentials, with a putative new variant associated with cancer with a mutation that can destabilize the L1 protein structure of CPV1 altering its function.

Keywords: Canine papilomatosis; Canine Papillomavirus; Genetic diversity; variants; cancer.

SUMÁRIO

LISTA DE ABREVIATURAS E SÍMBOLOS

% - Porcentagem

AlphaPVs - *Alphapapilomavírus*

CCE - Carcinoma de células escamosas

ChiPVs - *Chipapilomavírus*

cm - Centímetros

CPV - *Canis familiaris* papilomavírus

DNA - Ácido desoxirribonucleico

et al. - et allii (e colaboradores)

HPV - Papilomavírus humano

ICTV - Comitê internacional de taxinomia dos vírus

LambdaPVs - *Lambdapapilomavírus*

mm - Milímetro

nm - Nanômetro

ORFs - Open read frames

pb - Pares de base

PCR - Reação em cadeia polimerase

PVs - Papilomavírus

TauPvs - *Taupapilomavírus*

1 INTRODUÇÃO

Os papilomavírus (PVs) fazem parte da família *Papilomaviridae,* que infectam hospedeiros vertebrados, como os mamíferos, as aves e os reptéis. Esta família pode ser considerada uma das mais antigas e extensas, com uma grande diversidade genética, sendo o *Papilomavírus humano* (HPV) o que possui mais tipos identificados, com 205 tipos descritos até o momento no banco de dados *Papillomavirus Episteme* (https://pave.niaid.nih.gov/) (RECTOR; VAN RANST, 2013; VAN DOORSLAER, 2013).

Com um genoma circular de aproximadamente 8000 pb e DNA fita-dupla, os PVs são caracterizados por possuírem um capsídeo de até 55 nm, com simetria icosaédrica. As informações genéticas destes estão contidas em seis a oito ORFs (Open Read Frames): os genes de expressão precoce (E1, E2, E4, E5, E6 e E7) e os genes de expressão tardia (L1 e L2) que são estruturais (LANGE; FAVROT, 2011).

No ciclo de infecção dos PVs, que acompanha o ciclo celular dos queratinócitos, primeiramente, os genes iniciais do vírus são expressos no núcleo das células na camada basal da epiderme e há a replicação do DNA viral; depois a expressão destes genes fica baixa e o genoma do vírus episssomal, ocorrendo centenas de amplificações do DNA viral por célula (LANGE; FAVROT, 2011; LANGE et al., 2013; YHEE et al., 2010).

Os genes tardios são expressos no núcleo dos queratinócitos na camada espinhosa e granular, no final da diferenciação celular. Quando os genes tardios se expressam no estrato granuloso e córneo, ocorre a montagem dos vírions no núcleo e estas partículas virais são liberadas (LANGE; FAVROT, 2011; LANGE et al., 2013).

O gene L1 codifica a principal proteína do capsídeo viral, a proteína L1, que é responsável pela produção de partículas virais que são utilizadas para produção de vacinas. Além disso, o gene L1 é utilizado para a classificação dos PVs e construção de árvores filogenéticas (BERNARD et al., 2010).

Para a classificação dos PVs leva-se em consideração as similaridades entre as sequências de nucleotídeos de L1, onde diferentes gêneros devem

possuir menos de 60% de identidade e as espécies de um mesmo gênero devem compartilhar entre 60% a 70% de identidade (DE VILLIERS et al., 2004).

Para identificação de um novo tipo, deve-se ter uma diferença de mais de 10% em relação ao tipo mais próximo já conhecido. Diferenças entre 2 e 10% entre as sequências definem um novo subtipo e menos de 2% uma nova variante (DE VILLIERS et al., 2004).

O Comitê Internacional de Taxonomia dos Vírus (ICTV) (http://www.ictvonline.org/index.asp) classifica os PVs quanto ao gênero de acordo com o alfabeto grego. Já as espécies possuem o mesmo nome do gênero mais uma numeração arábica ao final deste nome, o que vai diferenciá-las.

Os gêneros de CPV aprovadas pelo ICTV são: *Chipapilomavírus* (*ChiPVs*), *Lambdapapilomavírus* (*LambdaPVs*) e *Taupapilomavírus (TauPVs)*. E as espécies aprovadas são: *ChiPV 1*; *ChiPV 2*; *ChiPV 3*; *TauPV 1*; *TauPV 2*; *LambdaPV 2;* e *LambdaPV 3*.

Na comunidade científica os PVs são conhecidos pela nomenclatura baseada no nome científico do hospedeiro e o tipo de papillomavírus identificado. Para o papilomavírus canino é utilizado o nome "*Canis familiaris* papilomavírus" (CPV) acrescentando ao final do nome, a numeração da identificação do tipo, com exceção para o CPV1 que também é conhecido como "*Canis familiaris* oral papilomavírus 1" (BERNARD et al., 2010).

De acordo com o banco de dados *Papillomavirus Episteme*, existem 20 tipos do CPV. Estes estão distribuídos nos três gêneros: *Lambdapapilomavírus (LambdaPVs)* (CPV 1 e 6); *Taupapilomavírus (TauPVs)* (CPV 2, 7, 13, 17 e 19); *e Chipapilomavírus (ChiPVs)* (CPV 3-5, 8-12, 14-16, 18 e 20).

Os tipos identificados por fazerem parte de cada espécie, até o momento, são: *ChiPV 1 (*CPV3, 5, 9,11 e 12); *ChiPV 2 (*CPV4); *ChiPV 3 (*CPV8, 10, 14 e 15); *TauPV 1 (*CPV2, 7, 17); *TauPV 2* (CPV13); *LambdaPV 2* (CPV1); *LambdaPV 3* (CPV6) (RECTOR; VAN RANST, 2013; MUNDAY et al., 2016).

Os tipos, subtipos e variantes dos PVs podem afetar sua patogenicidade e consequentemente a forma em que a doença se apresenta no hospedeiro (LANGE; FAVROT, 2011). Em cães, sabe-se que as lesões causadas por CPV podem diferir de acordo com o tipo viral infectante (ZHOU et al., 2014). O CPV1, por exemplo, é conhecido por ser o tipo que causa a papilomatose oral (SANCAK et al., 2015).

Em relação ao estudo das variantes do papilomavírus canino, só existe um estudo que foi realizado no Brasil, onde foram identificadas duas variantes de CPV1, mas o estudo não destaca a ocorrência da variante e sim o tipo viral (ALCÂNTARA et al., 2014). Diferentemente do HPV, que observa-se em vários estudos sua importância, com algumas variantes do gênero *AlphaPVs* relacionadas ao câncer cervical de humanos, como variantes do HPV16 que estão associadas ao alto risco para essa neoplasia (BERNARD et al., 2010; VAN DOORSLAER, 2013).

Além da patogenicidade do vírus, a imunidade do animal é um fator determinante para desenvolvimento e agravamento da doença. Após a infecção, a imunidade celular do animal vai atuar para a erradicação do vírus e a imunidade humoral vai proteger o organismo contra uma reinfecção (LANGE; FRAVOT, 2011).

A transmissão do vírus dar-se-á através do contato com a pele ou mucosa e é facilitada pela ocorrência de algum trauma (SYKES, 2013). O alvo da infecção, comumente, são os cães jovens, mas os adultos imunossuprimidos também são infectados pelo CPV. O período de incubação do vírus é até 2 meses. E o período que as lesões permanecem no animal é de 4 meses a 1 ano, depois deste período as lesões podem regredir ou o vírus pode persistir e a doença se agravar (BIANCHI et al., 2012; HNILICA, 2012; SYKES, 2013).

O tempo de remissão natural, na infecção pelo CPV1, pode ser de 1 mês a 1 ano, destacando os altos títulos de anticorpos na fase de recuperação clínica e a proteção de uma reinfecção pelo mesmo tipo de vírus, enfatizando que devido a este fato as vacinas podem ser utilizadas para prevenção do tipo de vírus circulante (SANCAK et al., 2015).

O vírus tem tropismo pela camada basal da epiderme e epitélio escamoso da mucosa, levando às lesões neoplásicas benignas, como os papilomas exofíticos orais e cutâneos, endofíticos e as placas pigmentadas; com possível progressão para a neoplasia maligna, o carcinoma de células escamosas (CCE). Como também, o hospedeiro pode se manter assintomático (LANGE et al., 2011; SARDON et al., 2015; ZHOU et al., 2014).

A papilomatose oral é a forma mais descrita da doença e caracteriza-se pela presença de papilomas exofíticos na cavidade oral e junções mucocutâneas, que ao se multiplicarem podem levar a uma obstrução da faringe e disfagia

(FERNANDES et al., 2009). Apresenta-se, clinicamente, na forma de verruga, de consistência dura, com o formato de couve-flor, mas também pode ter a forma de franja ou nodular (LANGE; FAVROT, 2011).

Normalmente o tipo mais associado a papilomatose oral é o CPV1, mas outros tipos também já foram identificados na cavidade oral, como o CPV2, CPV13, CPV17 e CPV19. O CPV13 foi identificado na boca de um cão com sintomas de papilomatose oral; o CPV17 foi identificado no CCE oral; o CPV2 e CPV19 foi um caso de coinfecção em papilomas orais (LANGE et al., 2012; MUNDAY et al., 2016; TISZA et al., 2016).

Além de infectar a região oral, o CPV1 também já foi relatado em lesões hiperplásicas da conjuntiva ocular e em papilomas cutâneos exofíticos e endofíticos (BRANDES et al., 2009; SANCAK et al., 2015).

Os papilomas exofíticos cutâneos apresentam-se na forma de massas pedunculadas, lisas ou folhosas, de cor da pele a pigmentadas, encontrando-se mais na face e extremidade das patas (HNILICA, 2012). Os tipos de CPV descritos nesta forma foram o CPV 1, 2, 6, 7, 9 e 12. O CPV9 foi detectado em um caso de *verrucosis generalizada* e é o único tipo do gênero *ChiPVs* a estar relacionado nesta forma da doença. E o CPV12 foi isolado em um calo cutâneo de cães da raça Greyhound na região do coxim (ANIS et al., 2016; CAVANA et al, 2015; LANGE; FAVROT, 2011).

O papiloma endofítico cutâneo ocorre quando há um crescimento para dentro da pele, resultando em nódulos cinzas planos ou em relevo de 1 ou 2 cm com forma de copo e um poro central de queratina (LANGE et al., 2009). Esta lesão tem o formato que lembra um umbigo e localiza-se na região ventral do abdômen e inguinal (HNILICA, 2012). Outras formas relatadas foram a de cúpula com 4 mm, as pápulas pretas de 2 mm e lesões no coxim. Os tipos de vírus isolados na forma endofítica foram o CPV1, CPV2 e CPV6 (LANGE; FAVROT, 2011).

As placas virais pigmentadas são placas hiperqueratóticas e hiperpigmentadas que podem medir até 3 cm de diâmetro e encontram-se na maioria das vezes, nos membros e abdômen. Todos os tipos de CPV que foram isolados de placas pigmentadas são do gênero *ChiPVs* (LANGE et al., 2013; MUNDAY; KIUPEL, 2010). Neste tipo de lesão, suspeita-se da predisposição genética da raça Pug, que teve os tipos CPV4 e CPV18 isolados, em que o

CPV18 é um tipo novo isolado nessa raça, mas o CPV4 já foi encontrado várias vezes (LANGE et al., 2016).

O CCE é um câncer comum em cães, principalmente o CCE oral que é a segunda neoplasia mais encontrada da cavidade oral de cães. O papilomavírus canino como agente etiológico desta neoplasia maligna é considerado raro, a pesar de existirem vários trabalhos associando os tipos virais de CPV a esse tipo de câncer. Mas muitos trabalhos não provam a progressão da lesão causada pelo CPV para o câncer. Os tipos de CPV associados ao CCE foram: CPV1 e 3 (invasivo); CPV3, 4 e 7 (*in situ)*; CPV12 e 16 (metastático); e o CPV1 e 17 (oral) (GOLDSCHMIT et al., 2006; LANGE; FAVROT, 2011; LUFF et al., 2016; MUNDAY et al., 2015; MUNDAY et al. 2016; TEIFKE; LÖHR; SHIRASAWA, 1998).

Para se chegar ao diagnóstico da papilomatose viral, os métodos utilizados de maior frequência, juntamente com a análise das características clínicas e epidemiológicas da doença, são o exame histopatológico para caracterizar a lesão da epiderme e a Reação em Cadeia Polimerase (PCR) para identificação do DNA viral (LANGE; FAVROT, 2011).

Na histologia, as alterações encontradas nas lesões exofíticas são: hiperplasia epidérmica, hiperqueratose ortoqueratótica, corpos de inclusão, grânulos de querato hialina no estrato espinhoso, células claras e coilócitos (LANGE; FAVROT, 2011). Já as lesões endofíticas são caracterizadas na histologia por projeções papilares da epiderme estendendo-se para a derme, podendo ocorrer células paraqueratóticas, grânulos de querato hialina, coilócitos, corpúsculos de inclusão intranuclear basofílicos e eosinofílicos, como também inclusões citoplasmáticas eosinofílicas (LANGE et al., 2009). E nas placas pigmentadas ocorrem: hiperplasia do epitélio, hiperpigmentação, hiperqueratose ortoqueratótica e grânulos de querato hialina (LUFF et al., 2016).

Nota-se que os diferentes tipos de CPV causam desordens patológicas específicas nos cães, podendo levar a dermatopatias e também a condições clínicas graves, dependendo da multiplicação da lesão ou sua progressão. Percebe-se, também, que existe uma grande diferença entre a quantidade de tipos descobertos em humanos e animais, além disso, há poucos estudos de diversidade genética dos CPV caracterizando seus tipos e variantes existentes, que se acredita terem graus diferentes de patogenicidade.

O estudo sobre diversidade genética em cães limita-se principalmente a descoberta de novos tipos, mas é importante ampliar este tipo de estudo, com descrições de subtipos e variantes encontrados e caracterizá-los de acordo com a patologia do animal.

Nesse contexto, este trabalho tem a proposta de estudar a diversidade genética do CPV no estado de Sergipe e colaborar com caracterização do mesmo visando a identificação de isolados virais, o estudo de suas sequências de nucleotídeos e aminoácidos do gene L1, assim como relacionar estes achados com as lesões encontradas no hospedeiro, para um maior entendimento da multicausalidade das patologias associada a este vírus, estendendo estes dados para ajuda futura em ações de medidas de profilaxia e tratamento.

2 OBJETIVOS

2.1 Objetivo geral

Analisar a diversidade genética de Papilomavírus canino identificados em cães naturalmente infectados no estado de Sergipe, Nordeste do Brasil.

2.2 Objetivos específicos

- Descrever o perfil clínico dos cães infectados com o papilomavírus canino e dos cães com suspeita clínica, mas que não estavam infectados com o vírus.
- Caracterizar histologicamente as lesões epiteliais encontradas nos isolados positivos para o papilomavírus canino no estado de Sergipe.
- Analisar a nível molecular as sequências de nucleotídeos e aminoácidos do gene L1 dos isolados de Papilomavírus canino do tipo 1 (CPV1) do estado de Sergipe.
- Observar as alterações na estabilidade da estrutura proteica de L1 de CPV1 devido as mutações encontradas nos isolados deste estudo.
- Associar as variantes de CPV1 e as alterações na proteína L1 devido suas mutações com o perfil de lesões clínicas e histológicas dos isolados de Sergipe.

3 ARTIGO CIENTÍFICO

Artigo 1. REIS, J.D.R.; de OLIVEIRA, L.B.; SOARES, R.C.; BATISTA, M.V.A. Genetic diversity of Canine papillomavirus in naturally infected dogs and the identification of a putative high-risk new viral variant in Northeastern Brazil. **Transboundary and Emerging Diseases.** Em preparação.

GENETIC DIVERSITY OF CANINE PAPILLOMAVIRUS IN NATURALLY INFECTED DOGS AND THE IDENTIFICATION OF A PUTATIVE HIGH-RISK NEW VIRAL VARIANT IN NORTHEASTERN BRAZIL

Canis familiaris papillomavirus 1 variants isolated from dog lesions

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SUMMARY

Canine papillomavirus (CPV) is the causative agent of viral papillomatosis in dogs. These viruses can lead to benign exophytic or endophytic papillomas, or malignant squamous cell carcinoma (SCC). Although CPV types present different pathological aspects, it is known that genetic variants in human viruses could have increased pathogenicity. However, knowledge on CPV variants and their association with clinical aspects is still incipient. Therefore, the aim of this study was to assess the CPV genetic variability in canine lesions from Northeastern Brazil and their association with clinical aspects. Histopathological and molecular analysis were carried out to detect papillomas and viral DNA. Sequencing and bioinformatics tools were used for CPV identification, genotyping, and diversity analysis. Mutations were characterized based on their impact on the L1 protein structure and function. Samples tested positive for CPV DNA with oral and cutaneous exophytic lesions. It was possible to observe an oral SCC in one sample. CPV1 was the most prevalent type, and we could identify different variants. A new CPV1 variant was identified, with the genetic distance of 0.8% when compared to the reference CPV1. The detected mutations were non-synonymous, and was predicted to disturb the L1 protein structure and function, which could be associated with a more aggressive infection development. The putative new CPV1 variant was present in a malignant oral neoplastic lesion, which raises the hypothesis that this variant could be related to a possible progression to this type of cancer. Although novel studies with a wider epidemiological approach is important to confirm this association, this study has demonstrated the diversity of CPV isolates from Brazil, and a new CPV1 variant with novel protein structure changing mutations associated with oral SCC, which is important for the understanding of the papillomavirus pathogenesis, and for the development of more effective diagnostic and treatment methods.

Keywords: canine papillomavirus; CPV1 variants; DNA mutational analysis; genetic diversity; squamous cell carcinoma.

INTRODUCTION

Canis familiaris papillomavirus (CPV) is a double-stranded DNA virus that belongs to the *Papillomaviridae* family, which contains a circular genome with Early (E1, E2, E4, E5, E6 and E7) and Late (L1 and L2) genes (Lange & Favrot, 2011). L1 is the most conserved gene, being used to classify all *Papillomavirus* (PVs), as well as to construct phylogenetic trees in genetic diversity studies (Bernard et al., 2010).

Compared with the *Human papillomavirus* (HPV), which presents more than 200 described types, CPV has, so far, 20 characterized types distributed in three genera, deposited in public databases *Papillomavirus Episteme* (https://pave.niaid.nih.gov/): *Lambdapapilllomavirus* (*LambdaPVs*) (CPV 1 and 6); *Taupapillomavirus* (*TauPVs*) (CPV 2, 7, 13, 17, 19); and *Chipapillomavirus* (*ChiPVs*) (CPV 3-5, 8-12, 14-16, 18 and 20),

CPV1 is known to be the most frequent CPV type found in oral papillomas with the classical cauliflower-like form (Sancak et al., 2015). It is also able to cause exophytic and endophytic cutaneous papillomas, such as pigmented plaques and squamous cell carcinoma (SCC) (Lange et al., 2016). In addition, CPV1 has already been identified in asymptomatic hosts (Lange et al., 2012).

Other CPV types have also been identified in the oral cavity: CPV2, CPV13, CPV17 and CPV19 (Lange et al., 2012; Munday et al., 2016; Tisza et al., 2016). CPV 1, 2, 7 and 9 were also found in cutaneous exophytic papillomas (Cavana et al., 2015; Gil da Costa et al., 2016). CPV1, CPV2 and CPV6 have been isolated in the endophytic form (Lange & Favrot, 2011). Only CPV types that belongs to *ChiPV* were isolated in pigmented plaques (Lange et al., 2013)

Canine papillomavirus as the etiologic agent of oral and cutaneous SCC is considered rare. However, some CPV types have already been reported in this type of cancer: CPV 1, 2, 3, 7, 12, 16 and 17 (Goldschmidt et al., 2006; Lange & Favrot, 2011; Luff et al., 2016; Munday et al., 2016; Teifke et al., 1998).

Specific CPV types are involved with the pathogenicity in the host that results in different skin and oral disorders (Zhou et al., 2014). But there is no data characterizing CPV variants and their pathogenesis although it is known that

different HPV variants present different pathogenicity degree with some variants related to cancer (Burk et al., 2013). Therefore, studies that aim at understanding the role of CPV variants in cancer development are important. In this context, this study has the objective to analyze the genetic diversity of canine papillomavirus identified in naturally infected dogs and its pathologies.

MATERIALS AND METHODS

Sample collection

Eighteen samples (ten cutaneous and eight oral) of 11 dogs clinically characterized as papillomas were collected with the procedure of skin biopsy, respecting all protocols of animal welfare. The samples were obtained at different veterinary clinics in the state of Sergipe, Northeastern Brazil.

All papilloma samples were divided in two, and stored. One half was placed in a tube fixed with 10% formalin for histopathological examination, and the other half was stored in a 1.5 ml microtube at -20°C until DNA extraction for molecular analysis.

Several animal epidemiological data were obtained: city, age, sex, breed, habitat, lesion sites, lesion evolution, and lesion description. In the age analysis, dogs up to one year were considered young but from one to eight years old were considered adults. Elderly dogs were the ones with more than eight years of age (Souza et al., 2006).

Histopathological analysis

The histopathological examination was used to confirm the papilloma lesions. Histological diagnosis was carried out following the guidelines laid down by the World Health Organization (WHO) for the histological classification of epithelial and melanocytic tumors of the skin of domestic animals (Goldschmidt, 1998). Tissues were embedded in paraffin, which were cut and stained by the Hematoxylin-Eosin (HE) method.

Molecular analysis

DNA extraction was performed using the Wizard® Genomic DNA Purification kit (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. DNA quantification was carried out in a NanoDrop Lite spectrophotometer (Thermo Scientific).

PCR reactions were performed using Taq DNA Polimerase Master Mix Red (Ampliqon) with high-fidelity DNA polymerase (GE) and the degenerate primer pair FAP59 (5'-TAACWGTIGGICAYCCWTATT-3') and FAP64 (5'-CCWATATCWVHCATITCICCATC-3'), which amplifies a fragment of the L1 gene, generating an amplicon of approximately 480 bp (Forslund et. al., 1999). The used PCR conditions were: initial denaturation at 94°C for 10 min, followed by 40 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min (Claus et al., 2007). The final extension of 72°C was modified to 10 min. PCR products were subjected to 1.5% agarose gel electrophoresis and visualized in UV transilluminator.

The purification of the amplified products was performed with the Wizard SV Gel and PCR Clean-Up System kit (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. PCR products were cloned into the pGEM-T vector (Promega) and transformed into competent DH5α bacteria. Bacterial clones were randomly selected for confirmation. At least two different positive clones were sequenced twice, in both directions, with the BigDye Terminator Cycle Sequencing v3.1 kit in a 3500 Genetic Analyzer (Applied Biosystems).

DNA sequence analysis

The quality of the sequences was evaluated by reading the chromatogram in the Staden 2.0 program package (Staden, 1996), with Phred 30. The sequence identity was then determined using the Blastn program (Altschul et al., 1990) with the default parameters.

A multiple sequence alignment was performed including all isolates from this study and the CPV sequences available in GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide/) (Supplementary Table S1) using the Muscle algorithm, incorporated into MEGA software 6.0 (Tamura et al., 2016). A distance matrix was also generated in MEGA software 6.0 (Tamura et al., 2016).

The phylogenetic tree was reconstructed using the Maximum Likelihood method in PhyML 3.1 program (Guindon et al., 2010). The model of nucleotide substitution was GTR+I+G, which was the evolutionary model that most fit the data, determined by jModelTest 2.1.7 program (Darriba et al., 2012). Branch support in the phylogenetic tree was determined by performing 1,000 bootstrap replicates.

The DNA sequences of all variants determined in this study were deposited in the GenBank database under the following accession numbers: CPV1UFSBR-01 (MF321769); CPV1UFSBR-02 (MF321770); CPV1UFSBR-03 (MF321771); CPV1UFSBR-04 (MF321772); CPV1UFSBR-05 (MF321773); CPV1UFSBR-06 (MF321774); CPV1UFSBR-07 (MF321775); CPV1UFSBR-08 (MF321776); CPV1UFSBR-09 (MF321777).

Protein structure analysis

In order to assess the impact of the mutations on the L1 protein structure and function, the tertiary structure of L1 protein of CPV1 was predicted. The structure model was determined by using an homology-based approach with Modeller 9.18 software (Sali, 1993). First, the L1 amino acid sequence from CPV1 (NP_056819.1) was used as the query sequence. Then, Blastp tool was used with the default parameters to identify a homologous template structure in Protein Data Bank (1DZL_A).

The obtained models were refined by energy minimization by using ModRefiner (Xu & Zhang, 2011). Procheck program (Laskowski et al., 1993) was used to assess the stereochemical quality of the protein model, which was visualized in The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC (Delano, 2004). In order to predict the possible effects of the mutations on the CPV1 L1 protein stability by a statistical potential energy function, the Site Directed Mutator (SDM) method was used (Topham & Srinivasan, 1997).

RESULTS

Eighteen lesions clinically characterized as papillomas were collected, of which 50% (9/18) tested positive for histology and PCR. However, the samples were collected from eleven dogs which 54.5% (6/11) of the dogs tested positive for CPV. In total, 54.55% (6/11) of dogs belonged to the following breeds: Labrador (1), Yorkshire (1), Poodle (3), and Pit bull (1). In addition, 45.45% (5/11) were mixed breed dogs. Most positive dogs for CPV were mixed breed dogs [66.6% (4/6)]. Negative dogs for CPV prevailed within a defined breed [80% (4/5)].

When it comes to the sex of the animal, 63.64% (7/11) of all dogs were males and 36.36% (4/11) were females. However, 50% (3/6) of the positive dogs for CPV were male and 50% (3/6) were female. Among the negative dogs for CPV, 80% (4/5) were male. In this study, 45.45% (5/11) of dogs were old, 45.45% (5/11) were young and 9.1% (1/11) was adult. Positive dogs for CPV were mostly young [83.33% (5/6)], and negative dogs for CPV were mostly old [60% (4/5)].

Most of the animals were domiciled [63.63% (7/11)]. However, most of positive dogs for CPV [66.67% (4/6)] lived in a place with a high flow of animals (such as the street, shelter, or kennel), and only 33.33% (2/6) of positive dogs for CPV were domiciled. Cutaneous lesions were more frequent with 54.5% (6/11) of affected dogs, while 45.5% (5/11) of all dogs presented oral lesions. When assessing only the dogs that tested positive for CPV, oral lesions were prevalent [83.3% (5/6)] with a different profile for negative dogs that had cutaneous lesions in 100% of the cases (5/5).

Regarding the evolution of the lesion in the animal, 54.54% (6/11) of the animals had lesions for up to one year, and 27.27% (3/11) of them had lesions for more than one year. Two animals had no data available. In positive dogs for CPV, 66.7% (4/6) had lesions that evolved within one year and 33.3% (2/6) had no data available. On the other hand, 60% (3/5) of negative animals for CPV had lesions that evolved for more than one year. All lesions were classified as warts, being neoplasms with characteristics described as nodular, fringe, mass, and cauliflower form. All data acquired from the animals are shown in Table 1.

Samples of oral lesions (CPV1UFSBR-01 to CPV1UFSBR-06, CPV1UFSBR-08 and CPV1UFSBR-09) presented common histopathological alterations for exophytic lesions, such as epidermal hyperplasia, koilocytes and hyperkeratosis. One oral lesion (CPV1UFSBR-08) was also classified as SCC and hyperpigmentation (Figure 1). The skin lesions (CPV1UFSBR-07) were classified as papillomas based on histopathological analysis.

Molecular analyses of distance matrix among the sequences of CPV have shown that all sequences from this study are variants when compared to the CPV1 L1 reference sequence (CPV1a) (Table 2). However, the isolate CPV1UFSBR-08 is characterized as a new CPV1 variant, having 99.2% identity to the reference sequence CPV1a, and differs 0.2% from the isolate CPV1UFSBR-01 and other CPV1 variants already described such as CPV1BRUEL1 and CPV1BRUEL2 (Supplementary Table S2).

In the phylogenetic tree, it was observed that all CPV types were grouped together based on their genus. Isolates from Northeastern Brazil in this study group together with CPV1, in *LambdaPV* genus. However, differently from the reference sequence, our isolates group together with CPV1BRUEL1 and CPV1BRUEL2 isolates, from Southern Brazil. These results evidences that all isolates from the Northeast region of Brazil are CPV1 variants. In addition, the phylogenetic analysis confirms that CPV1UFSBR-08 is a putative new CPV1 variant (Figure 2).

The variant CPV1UFSBR-01 have a mutation in site 141 of nucleotide sequence where “c” changed for “t” and mutation in site 603 present among all CPV variants where changed “a” for “c”. These mutations didn’t change any amino acid in L1 sequence. However, mutations in almost all variants (CPV1UFSBR-01 to CPV1UFSBR-08) occurs in site 146 of L1 nucleotide sequence that change “t” for “a” which change the L1 amino acid sequence in site 49 an F (Phenylanine) for Y (Tyrosine) (Table 3). The variant CPV1UFSBR-08 have a mutation in site 611 where “t” changed for “c” which it has an important alteration in the site 204 of L1 amino acid sequence where V (Valine) has changed for A (Alanine) (Table 3). All observed mutations have changed an essential amino acid for a non-essential one, which could have an impact in the CPV1 L1 protein structure and function.

It was possible to obtain a reliable CPV1 L1 protein model, which resembles the natural conformation of stability. According to the stereochemical parameters, the best CPV1 L1 protein model presented 91.5% of residues in most favorable regions in the Ramachandran plot. The CPV1 L1 protein has 503 amino acids residues, with several central beta-sheets domains, surrounded by alpha-helices and turns (Figure 3). The two non-synonymous mutations found in the CPV1 variants described in this study are located in beta sheet (V204A) and loop region (F49Y).

In the evaluation of the impact of these mutations on the stability of the CPV1 L1 protein, we could predict that the mutation F49Y has ΔΔG = - 0.97, which indicates that this mutation is slightly destabilizing, and it is not associated with changes in protein function. On the other hand, the mutation V204A has ΔΔG = -2.73, indicating that it is highly destabilizing and associated with changes in protein function (Supplementary Table S3).

DISCUSSION

This is the first study in the Northeast region of Brazil that has molecularly identified CPV isolates, with CPV1 being the most prevalent viral type, which was identified both in oral and cutaneous papillomas. In addition, several CPV1 variants was found, which demonstrates the genetic variability of CPV samples from Brazil. One of these CPV variants has never been described.

CPV1 is known worldwide to cause oral papillomatosis (Regnard et al., 2016). Cutaneous papillomas are less frequent and are usually solitary lesions, but extensive cutaneous papillomatosis may occur (Teifke et al., 1998). In Londrina - Southern Brazil, CPV1 has already been identified in oral and cutaneous lesions (Alcântara et al., 2014). Papillomas from different cities in Southern Brazil were found: on the lips, nose, mucous membrane of the oral cavity, inguinal region, thoracic and hind limbs, prepuce, and eyelid (Alcântara et al., 2014; Bianchi et al., 2012). This study, in agreement, papillomas were found in that same regions. It is noticed that CPV can cause oral lesions in specific locals, as well as extensive

cutaneous lesions, with the same pattern of occurrence in different areas from Brazil.

The morbidity of papillomatosis is reported as high in kennels, hospitals, or environments that have high flow and turnover of animals (Fernandes et al., 2009). As expected, most of the animals with viral papillomatosis found in this study lived in places with high flow of animals. This is important to highlight the necessity of the prophylaxis measures, which should be adopted in these places in order to prevent the spread of papillomatosis, such as diseased animal isolation.

Canine papillomatosis has been described as a disease predominant in young dogs (Souza et al., 2006). In studies carried out in Korea and Southern Brazil, CPV1 infection was also more frequent in young dogs and affected mixed breed dogs (Alcântara et al., 2014; Bianchi et al., 2012; Yhee et al., 2010). In this study, papillomatosis was prevalent in young mixed breed dogs, without gender preference, as previously described (Bianchi et al., 2012). This data can support the establishment of a clinic profile from dogs with CPV1 infection in Brazil, which is relevant for the development of control measures.

It was possible to observe that animals tested negative for CPV presented a different profile in race, sex, age, habitat, contact with other animals, lesion sites, and time of evolution. In general, they did not show characteristic changes of papillomas in histology, the lesions were predominantly cutaneous, and they were most frequent in older dogs. However, these lesions were clinically characterized like warts, which occurs in viral papillomatosis, showing the importance of histological analysis to described if the epithelial lesions were caused by canine viral papillomatosis or not.

The presence of hyperkeratosis, epidermal hyperplasia, and koilocytes characterize a lesion as exophytic papillomas (Lange & Favrot, 2011). In this study, all samples that tested positive for CPV had epidermal lesions of exophytic papillomas, but no inclusion bodies were observed, the same characteristics found in other studies (Alcântara et al., 2014; Bianchi et al., 2012).

Interestingly, an isolate viral (CPV1UFSBR-08), in addition to the common findings for exophytic papillomas, also was diagnosed in histology for oral Squamous Cell Carcinoma (SCC) and hyperpigmentation. The oral SCC

associated with CPV1 is considered rare (Porcellato et al., 2014). Hyperpigmentation is a common finding in pigmented plaques, being observed in oral SCC associated with CPV17 and in a case of cutaneous exophytic papillomas associated with CPV9 (Cavana et al., 2015; Lange & Favrot, 2011; Munday et al., 2015; Narama et al., 2005). This result is important because it demonstrates the possibility of different lesions caused by CPV1, which may be associated with the pathogenicity of the variant viral. Another possibility could be a coinfection by diverse types of CPVs, but further investigation is required.

Nevertheless, to the best of our knowledge, this is the first study that identified a novel CPV1 variant associated with an oral SCC. The oral SCC associated with CPV1 has been demonstrated, but the genetic variability of CPV1 and the presence of oral SCC has never been assessed (Porcellato et al., 2014; Teifke et al., 1998). The new CPV1 variant (CPV1UFSBR-08) found in this study associated with oral SCC demonstrates the possibility that some CPV1 variants may influence the progression of virus infection to cancer.

In this study, nine CPV1 variants were identified in Northeastern Brazil, which evidences the genetic diversity of this virus in the country. Eight of these CPV1 variants have already been identified in Southern Brazil (Alcântara et al., 2014), with the same mutations in L1 gene sequences and with similar infection profile causing oral and cutaneous exophytic lesions in oral cavity, lips, as well as the hind limbs in multiple cutaneous papillomas. On the other hand, one CPV1 variant found in this study was associated with a more aggressive and malignant epithelial lesion (oral SCC), which could be a consequence of the mutations found in this isolate. In this context, this isolate had a mutation V204A that was predicted to destabilize the protein, and it could be associated with changes in protein function. This is an important result because this mutation could increase the virulence of this CPV1 variant, supporting the hypothesis that it could be associated with oral SCC.

Although more studies are needed to confirm this association, this study has shown the importance of the genetic diversity of CPV, identifying for the first time the presence of a new CPV1 variant with novel protein structure changing mutations, potentially pathogenic, associated with oral SCC. In addition, CPV isolates from the Northeastern Brazil have been molecularly characterized for the

first time, increasing the knowledge on CPV genetic variability and circulation in Brazil, which is relevant for the development of more effective prevention and treatment strategies.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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Figure legends:

Figure 1. a. Sample 09: Histology, HE, 100X. Blue arrow indicates hyperkeratosis, the yellow arrows indicate the koilocytes. Figure shows epithelial hyperplasia and digitiform aspects; **b.** Sample 08: Histology, HE, 400X. Blue arrows indicate cellular pleomorphism and yellow, the koilocytes. The delimited area indicates loss

of epithelial stratification, characteristic of SCC. Brown spots of melanocyte granules related to hyperpigmentation.

Figure 2. Maximum Likelihood phylogenetic tree based on L1 gene sequences of CPV reference types and variants and the isolates from this study. Scale bar represents the genetic distance of 0.2 nucleotide substitutions per site. Only bootstrap values above 50% are shown.

Figure 3. Protein regions wherein mutations were found. a. Delimitation of the mutation V204A in beta sheet region and mutation F49Y in loop region; b. Zoom of the protein structure with the mutations regions.

Figure 1.

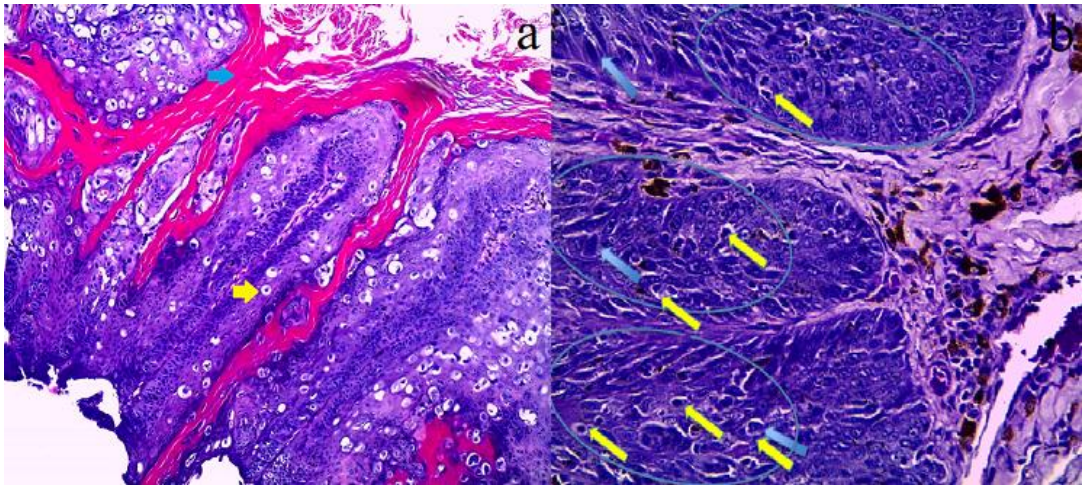

Figure2.

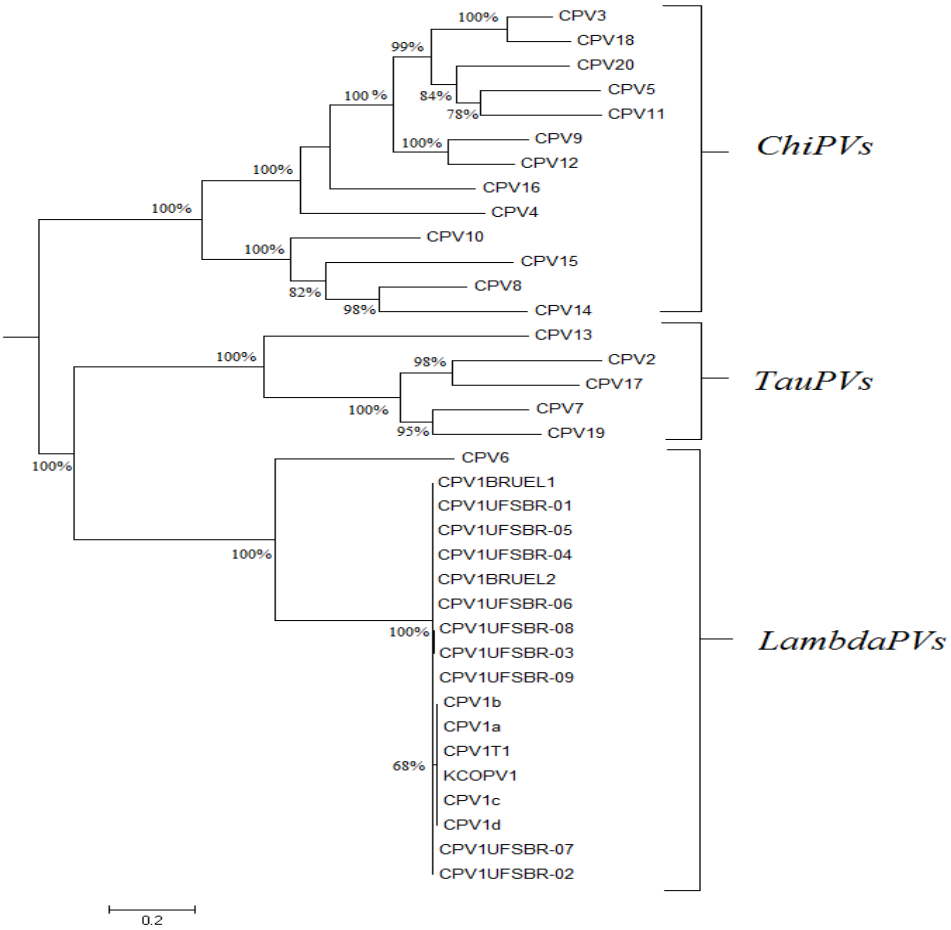
CPV3
CPV18
CPV20
CPV5
CPV11
CPV9
CPV12
CPV16
CPV4
CPV10
CPV15
CPV8
CPV14
CPV13
CPV2
CPV17
CPV7
CPV19
CPV6
CPV1BRUEL1
CPV1UFSBR-01
CPV1UFSBR-05
CPV1UFSBR-04
CPV1BRUEL2
CPV1UFSBR-06
CPV1UFSBR-08
CPV1UFSBR-03
CPV1UFSBR-09
CPV1b
CPV1a
CPV1T1
KCOPV1
CPV1c
CPV1d
CPV1UFSBR-07
CPV1UFSBR-02
100%
99%
100 %
84%
78%
100%
100%
100%
100%
82%
98%
100%
98%
100%
95%
100%
100%
100%
68%
0.2
ChiPVs
TauPVs
LambdaPVs

Figure 3.

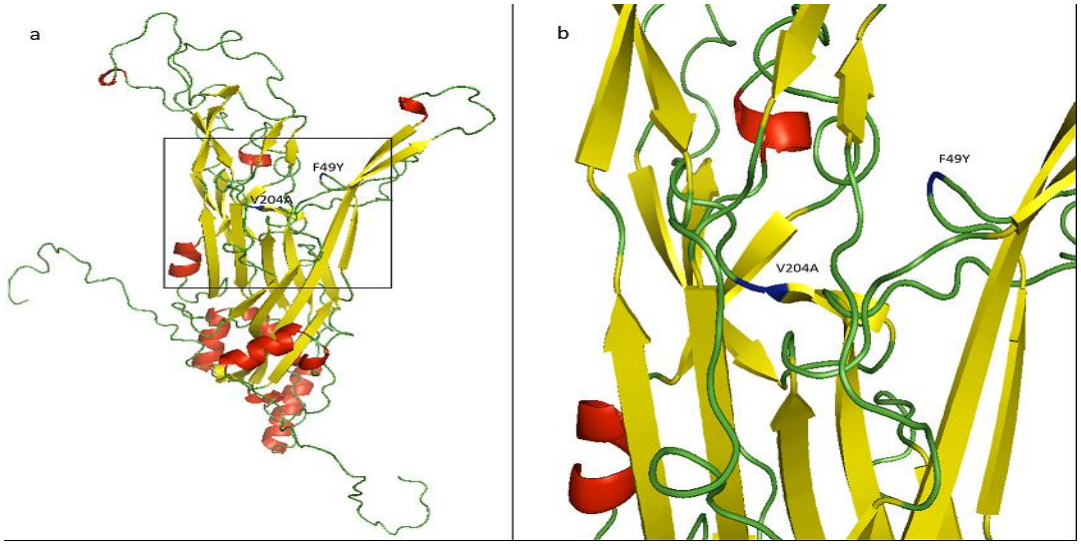
a
F49Y
V204A
b
F49Y
V204A

Table 1. Epidemiologic information about the samples from this study.

Dogs	Samples	Breed	Sex	Age	Habitat	Evolution	Characteristics	Site of lesions
1	CPV1UFSBR-01	Mix breed	F	9 months	Residence	3 weeks	Nodular, pink	Oral cavity
1	CPV1UFSBR-02	Mix breed	F	9 months	Residence	3 weeks	Nodular, pink	Lips
1	CPV1UFSBR-03	Mix breed	F	9 months	Residence	3 weeks	Nodular, pink	Oral cavity
2	CPV1UFSBR-04	Mix breed	M	Young	Street	-	Nodular, pink	Oral cavity
3	CPV1UFSBR-05	Labrador	F	9 years	Residence	4 months	Fringe, pink	Oral cavity
3	CPV1UFSBR-06	Labrador	F	9 years	Residence	4 months	Fringe, gray	Oral cavity
4	CPV1UFSBR-07	Pit bull	M	7 months	Kennel	5 months	Nodular, pink	Skin
5	CPV1UFSBR-08	Mix breed	F	Young	Street	-	Mass, gray	Oral cavity
6	CPV1UFSBR-09	Mix breed	M	Young	Shelter	3 months	Cauliflower, gray	Oral cavity
7	UFSBR-10	Poodle	M	15 years	Residence	3 years	Cauliflower, pink	Skin
8	UFSBR-11	Poodle	F	11 years	Residence	1 year	Nodular, pink	Skin
9	UFSBR-12	Poodle	M	8 years	Residence	2 years	Mass, pink	Skin
9	UFSBR-13	Poodle	M	8 years	Residence	2 years	Mass, pink	Skin
9	UFSBR-14	Poodle	M	8 years	Residence	2 years	Mass, pink	Skin
10	UFSBR-15	Yorkshire	M	10 years	Residence	4 years	Mass, black	Skin
10	UFSBR-16	Yorkshire	M	10 years	Residence	4 years	Mass,black	Skin
11	UFSBR-17	Mix breed	M	6 years	Residence	2 months	Mass, black	Skin
11	UFSBR-18	Mix breed	M	6 years	Residence	2 months	Mass,black	Skin

† CPV: Canine Papillomavirus, ‡ F: Femeal, § M: Male

Table 2. Histology, PCR and genotyping of samples tested positive for papillomavirus.

Samples	Histology	PCR	Genotyping by Blastn	CPV1 variants
CPV1UFSBR-01	Exophytic papilloma	+	CPV1	99,4% of identity with CPV1
CPV1UFSBR-02	Exophytic papilloma	+	CPV1	99,6% of identity with CPV1
CPV1UFSBR-03	Exophytic papilloma	+	CPV1	99,6% of identity with CPV1
CPV1UFSBR-04	Exophytic papilloma	+	CPV1	99,4% of identity with CPV1
CPV1UFSBR-05	Exophytic papilloma	+	CPV1	99,4% of identity with CPV1
CPV1UFSBR-06	Exophytic papilloma	+	CPV1	99,4% of identity with CPV1
CPV1UFSBR-07	Exophytic papilloma	+	CPV1	99,4% of identity with CPV1
CPV1UFSBR-08	Exophytic papilloma, SCC	+	CPV1	99,2% of identity with CPV1
CPV1UFSBR-09	Exophytic papilloma	+	CPV1	99,8% of identity with CPV1

†PCR: Polimerase chain reaction; ‡ CPV: Canine papillomavirus; §: Carcinoma squamous cell.

Table 3. Mutations in the L1 gene sequences found in CPV1 variants from this study.

Mutations					
Nucleotide site	**141**	**146**	**603**	**606**	**611**
Reference sequence	**c**	**t**	**a**	**t**	**t**
Variant sequences					
CPV1UFSBR-01	t	a	c	.	.
CPV1UFSBR-02	.	a	c	.	.
CPV1UFSBR-03	.	a	c	.	.
CPV1UFSBR-04	.	a	c	c	.
CPV1UFSBR-05	.	a	c	c	.
CPV1UFSBR-06	.	a	c	c	.
CPV1UFSBR-07	.	a	c	c	.
CPV1UFSBR-08	.	a	c	c	c
CPV1UFSBR-09	.	.	c	.	.
Amino Acid site		**49**			**204**
Reference sequence		**F**			**V**
CPV1UFSBR-01	.	Y	.	.	.
CPV1UFSBR-02	.	Y	.	.	.
CPV1UFSBR-03	.	Y	.	.	.
CPV1UFSBR-04	.	Y	.	.	.
CPV1UFSBR-05	.	Y	.	.	.
CPV1UFSBR-06	.	Y	.	.	.
CPV1UFSBR-07	.	Y	.	.	.
CPV1UFSBR-08	.	Y	.	.	A
CPV1UFSBR-09	.	.	.	.	.

† c (citosina); ‡ t (timina); § a (adenina); † F (Phenylanine); ‡ Y (Tyrosine); § V (Valine); ¶ A (Alanine).

Supplementary Table S1. CPV sequences used in this study.

CPV strains	Country	Accession Number
CPV1a	Not available	NC_001619
CPV1BRUEL1	Brazil	KF199909
CPV1BRUEL2	Brazil	KF199910
CPV1T1	China	HM054511
KCOPV1	South Korea	FJ479789
CPV1b	Not available	D55633
CPV1c	Not available	D26115
CPV1d	Not available	L22695
CPV2	Not available	AY722648.1
CPV3	Switzerland	NC_008297.1
CPV4	Not available	NC_010226.1
CPV5	Germany	FJ492743
CPV6	Switzerland	NC_013237.1
CPV7	Scotland	FJ492742
CPV8	Switzerland	HQ262536.1
CPV9	USA	JF800656.1
CPV10	USA	NC_016075.1
CPV11	USA	JF800658
CPV12	USA	JQ754321
CPV13	Switzerland	NC_023852.1
CPV14	Switzerland	NC_019852.1
CPV15	USA	JX899359
CPV16	USA	NC_026640.1
CPV17	New Zealand	KT272399
CPV18	USA	KT326919
CPV19	USA	KX599536
CPV20	USA	KT901797

Supplementary Table S2. Distance matrix among the CPV sequences from this study and reference CPV sequences.

Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
CPV1UFSBR01																	
CPV1UFSBR02	0.000																
CPV1UFSBR03	0.000	0.000															
CPV1UFSBR04	0.000	0.000	0.000														
CPV1UFSBR05	0.000	0.000	0.000	0.000													
CPV1UFSBR06	0.000	0.000	0.000	0.000	0.000												
CPV1UFSBR07	0.000	0.000	0.000	0.000	0.000	0.000											
CPV1UFSBR08	0.002	0.000	0.000	0.000	0.000	0.000	0.000										
CPV1UFSBR09	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000									
CPV1BRUEL1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000								
CPV1BRUEL2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000							
CPV1T1	0.006	0.004	0.004	0.006	0.006	0.006	0.006	0.008	0.002	0.008	0.005						
KCOPV1	0.006	0.004	0.004	0.006	0.006	0.006	0.006	0.008	0.002	0.008	0.005	0.000					
CPV1a	0.006	0.004	0.004	0.006	0.006	0.006	0.006	0.008	0.002	0.008	0.005	0.000	0.000				
CPV1b	0.006	0.004	0.004	0.006	0.006	0.006	0.006	0.008	0.002	0.008	0.005	0.000	0.000	0.000			
CPV1c	0.006	0.004	0.004	0.006	0.006	0.006	0.006	0.008	0.002	0.008	0.005	0.000	0.000	0.000	0.000		
CPV1d	0.006	0.004	0.004	0.006	0.006	0.006	0.006	0.008	0.002	0.008	0.005	0.000	0.000	0.000	0.000	0.000	

Supplementary Table S3. Stability score for mutant proteins and how the single nucleotide polymorphisms (SNPs) could affect the structure or function of CPV1 L1 protein.

Residue position	Wild-type residue	Mutant residue	Predicted ΔΔG	Outcome
49	F	Y	- 0.97	slightly destabilizing and not associated with changes in protein function
204	V	A	-2.73	highly destabilizing and associated with changes in protein function

4 CONCLUSÃO

Este estudo foi o primeiro do Nordeste-Brasil a identificar o CPV1 a partir de lesões epiteliais exofíticas orais e cutânea de cães, destacando que todos os isolados eram variantes.

As variantes de CPV1 circulantes no Brasil foram isoladas de hospedeiros com perfis semelhantes de infecção, afetando mais cães jovens e SRD com papilomas na cavidade oral, lábios, membros posteriores e múltiplos papilomas cutâneos. E pela primeira vez no mundo destaca-se a presença de uma nova variante de CPV1 potencialmente patogênica relacionada ao CCE oral. Já os cães que apresentam lesões clínicas semelhantes a papilomas e descritas como verrugas, mas que nos exames histopatológico e PCR foram constatados que não possuíam a papilomatose viral, tiveram um perfil diferenciado, acometendo cães idosos, machos, domiciliados com evolução de lesões a mais de um ano e apresentando neoplasias exclusivamente cutâneas.

Cães com infecções mais brandas foram os que tiveram variantes de CPV1 associadas às mutações nas sequências de aminoácidos de L1 que desestabilizaram levemente a estrutura proteica sem possível alteração na função da proteína que pudessem levar ao adoecimento. No entanto, a mutação da nova variante CPV1UFSBR-08 que foi associada ao câncer possivelmente causou um prejuízo a função da proteína, pelo fato de que esta mutação desestabilizou altamente a estrutura da proteína L1 de CPV1, o que provavelmente levou a características de lesão mais grave.

Sabendo-se da existência de variantes circulantes no mundo que podem induzir diferentes lesões em cães, pode-se montar estratégias de prevenção como criação de vacinas para tipos mais patogênicos que podem levar ao câncer e adotar medidas diferenciadas para o tratamento a partir de uma diferenciação dos potenciais patogênicos de tipos e variantes de CPV de cães infectados.

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APÊNDICE

Instrumento de coleta de dados

FICHA DE COLETA Nº ____ AMOSTRA Nº _____
1- **Clínica/Médico Veterinário:**
2- **CRMV:**
3- **Cidade:** **UF:**
4- **Data da coleta:**
5- **Nome do Animal:**
6- **Raça:** **Sexo:** **Idade:**
7- **Habitat: ( ) domiciliado ( ) outros _____.**
8- **Tipo da lesão: Oral ( ) Cutânea ( ) Placas Pigmentadas ( ) Outras ( ) _____.**
9- **Tempo de evolução das lesões:**
10- **Descrição das Lesões:**
11- **Descrição dos Procedimentos realizados para biópsia das lesões:**

ANEXO

Normas de Publicação

Author Guidelines

Original Articles: should not exceed 30 typewritten pages, including illustrations, tables and references.

All manuscripts should be double spaced with a font size of 11 points or larger.

PREPARING YOUR SUBMISSION

Manuscripts must be submitted as a Word or rtf file and should be written in English. The manuscript should be submitted in separate files: main text file; figures.

Text File: The text file should be presented in the following order:

(i)Title;(ii)a short running title of less than 70 characters; (iii)the full names of the authors;(iv)the author's institutional affiliations at which the work was carried out, (footnote for author's present address if different to where the work was carried out);(v)summary and keywords;(vi)main text;(vi)acknowledgements;(vii)conflict of interest statement;(viii)references;(ix)tables (each table complete with title and footnotes);(x)figure legends;(xi) appendices (if relevant);Figures and supporting information should be supplied as separate files.

Title: The title page should contain a short informative title that contains the major key words. The title should not contain abbreviations (see Wiley's best practice SEO tips)

Authorship: Please refer to the journal's authorship policy the Editorial Policies and Ethical Considerations section for details on eligibility for author listing.

Acknowledgements: Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section. See section on Authorship for more detail. Financial and material support should also be mentioned Thanks to anonymous reviewers are not appropriate.

Conflict of Interest Statement: You will be asked to provide a conflict of interest statement during the submission process. See the section 'Conflict of Interest' in the Editorial Policies and Ethical Considerations section for details on what to include in this section. Please ensure you liaise with all co-authors to confirm agreement with the final statement. The Conflict of Interest statement should be included within the main text file of your submission.

Summary: Please supply a summary of up to 300 words for all articles. A summary should be a concise summary of the whole paper, not just the conclusions, and it must be understandable without reference to the rest of the paper. It should contain no citation to any other published work.

Keywords: Include up to six keywords that describe your paper for indexing purposes and list them in alphabetical order. Keywords should be taken from those recommended by the US National Library of Medicine's Medical Subject Headings (MeSH) browser list at https://www.nlm.nih.gov/mesh/.

Main Text: Where possible, the text should be divided into the following sections: Summary, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, Conflict of Interest Statement and References.

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Journal article

Beers, S. R., & De Bellis, M. D. (2002). Neuropsychological function in children with maltreatment-related posttraumatic stress disorder. The American Journal of Psychiatry, 159, 483–486.*doi*:10.1176/appi.ajp.159.3.483

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Bradley-Johnson, S. (1994). *Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school* (2nd ed.). Austin, TX: Pro-ed.

Internet Document

Norton, R. (2006, November 4). How to train a cat to operate a light switch [Video file]. Retrieved from http://www.youtube.com/watch?v=Vja83KLQXZs

Tables: Tables should be self-contained and complement, but not duplicate, information contained in the text. They should be supplied as editable files, not pasted as images. Legends should be concise but comprehensive – the table, legend and footnotes must be understandable without reference to the text. All abbreviations must be defined in footnotes. Footnote symbols: †, ‡, §, ¶, should be used (in that order) and *, **, *** should be reserved for P-values. Statistical measures such as SD or SEM should be identified in the headings.

Figure Legends: Legends should be concise but comprehensive – the figure and its legend must be understandable without reference to the text. Include definitions of any symbols used and define/explain all abbreviations and units of measurement.

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Appendices: Appendices will be published after the references. For submission, they should be supplied as separate files but referred to in the text. Supporting Information