

Original Research Article

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## Papillomavirus (PV) - Associated Skin Diseases in Domestic and Wild Animals: Animal Nucleotide Sequence Identity of PV Types to their Closest Related PV and HPV Sequences Deposited in the Gen Bank

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### ABSTRACT

The papilloma viruses belong to the *Papilloma viridae* family and new genera were described on the basis of the interspecies phylogenetic similarity. They are DNA viruses that induce the formation of benign and malignant tumors in a vast range of hosts. The main objective of the present study was to detect PV DNA and papilloma virus-associated skin diseases in domestic and wild animals. This paper analyzed nucleotide sequence similarity of animal papilloma virus types to their closest related PV types and HPV sequences deposited in the Gen Bank. Genomic DNA was extracted from 75 blood samples and 18 warts-like lesions from several wild and domestic animals of the 15 following species: *Ara ararauna*, *Ara chloroptera*, *Ara macao*, *Bos taurus*, *Bubalus bubalis*, *Canis familiaris*, *Equus caballus*, *Felis concolor*, *Macaca fascicularis*, *Ovis aries*, *Panthera leo*, *Panthera onca*, *Sus scrofa*, *Sus domesticus* and *Struthio camelus*. Papilloma virus-associated skin diseases as papillomatosis, adenocarcinomas, nodular formations, melanoma and fibrous epulic lesions were diagnosed by histopathology. PV DNA bands of skin biopsy and blood samples from the animals submitted to amplification of the L1 ORF were detected. About 31 biological samples of animals of the eight species *Ara chloroptera*, *Ara macao*, *Bos taurus*, *Canis familiaris*, *Felis concolor*, *Ovis aries*, *Panthera onca*, *Struthio camelus* generated viral DNA fragments PCR amplified using degenerated primer pairs FAP59/FAP64. Nucleotide sequence similarities of animal papilloma virus types to their closest related PV types and HPV sequences deposited in Gen Bank were described suggesting that the sequences share the same ancestry of the genetic evolutionary.

#### Keywords

Animal papillomavirus, Genetic diversity, Nucleotide sequence, *Papillomarividae*, Polymerase chain reaction.

#### Article Info

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### Introduction

Papilloma viruses (PV) are classified in the *Papilloma viridae* family presenting 26 genera, 62 species and 69 known animal papilloma viruses with high genetic variability. New genotypes have been sequenced and classified on the basis in the interspecies phylogenetic similarity. Papilloma viruses are constituted of a closed

circular double-stranded DNA genome, are non-enveloped, non-segmented with approximately 8 kb (Huber *et al.*, 2017).

More than 200 types of PV have been described in humans, while only 20 types have been confirmed in animals. In some host species, multiple types of PV have been

found, and the available data suggest some correlation between each type of PV, pathology and target tissue (epithelial tissue, stratified mucosal epithelium and mesenchymal cells of the dermis) (Chan *et al.*, 1997). Previous studies had described immunological aspects and morphological structures associated HPV-positive in SiHa (HPV-16) and HeLa (HPV-18) cervical carcinoma cell lines (Simões and Barth, 2017).

Human HPV vaccination is the best preventive strategy against cervical cancer, cervical intraepithelial neoplasia and genital warts. Licensed HPV vaccines, being bivalent (Cervarix®) against HPV16/18, quadrivalent (Gardasil®) target HPV16/18/6/11 or nonavalent (Gardasil-9®) target HPV16/18/31/33/45/52/58/6/11, have been applied in many countries (Huber *et al.*, 2017). Several potential benefits of vaccination have been associated in HPV related diseases as anus-genital cancer, head and neck cancer, colorectal tumors, penile cancer and recurring respiratory papillomatosis. Currently, the papillomavirus can be used as viral vectors in the gene therapy and new therapeutic targets have been successfully applied by recombinant DNA methods (Simões and Barth, 2015).

More than 16 putative new types of bovine papillomavirus (BPV) have been isolated, and more of them are continuously being discovered by complete genomic phylogenetic analysis of DNA sequences (Tomita *et al.*, 2006). Cytogenetic studies in bovines detected premature chromosome condensation (PCC) in peripheral blood lymphocytes cultures and skin lesions cultures induced by papillomavirus spontaneous infection (Marins *et al.*, 2010). Chromosome aberrations have been detected in cell cultures from bovines, canines and equines infected with papillomavirus and have been applied as

a biomarker for genomic instability (Simões and Barth, 2016). Furthermore, *bovine papillomavirus* (BPV) virus-like particles (VLP) inside the cell nucleus had been demonstrated from warts bovines by electron microscopy (Simões and Barth, 2017).

*Canine oral papillomavirus* (COPV) induces cauliflower-like warts in the oral mucosa of domestic dogs, as well as in wild canids (Nicholls *et al.*, 1999). Experimental infection with COPV observed during the period of four to eight weeks after inoculation, the development of papillomas followed by immune-mediated spontaneous regression (Nicholls *et al.*, 1999). More than 95% of COPV lesions regress within two to four weeks and only a few papillomas progress to more severe lesions, including oral squamous cell carcinoma (Ghim *et al.*, 2000). Degenerated primers FAP59/FAP64 have been used to detected DNA sequences in canines latent papillomavirus infection (Marins *et al.*, 2012).

According Hanna and Dunn (2003), 1.400 biopsies were performed on felines and, after clinical and microscope analysis tumors were diagnosed as cutaneous fibropapillomas. Previous studies have found cutaneous papillomas in the domestic cat (*Felis domesticus*) and the snow leopard (*Panthera uncia*). Papillomavirus induced intermediate ultrastructural aberrations in the viral assembly were observed inside the intracytoplasmatic inclusions of both animals (Sundberg *et al.*, 2000).

A new papillomavirus type of 8.000bp DNA fragment was detected using Bam HI restriction endonuclease, different from the cutaneous lesions of domestic felines. It was identified as an Asian lion *Panthera leo persica papillomavirus* (PIPV) from a biopsy of the ventral surface of the tongue of free living and captivity animals (Sundberg *et al.*,

1996). Studies involving wild animals are rare. Clinically, small papillomas were found on the foot of an impala (*Aepyceros melampus*) and on the head of a giraffe (*Giraffa melopardalis*) in Kenya, Africa (Karstad and Kaminjolo, 1978).

In monkeys, clinical and pathological findings suggest a positive infection containing *Rhesus papillomavirus type 1* (RhPV-1) DNA integrated into the tumor cell DNA, confirmed by hybridization techniques (Ostrow *et al.*, 1990). Sobestiansky *et al.*, (1999) described papillomatosis in pigs, affecting the skin, upper digestive tract and particularly the genital mucosa of females and males.

There are few studies on bird papillomavirus. Benign and malignant lesions in the mucosal epithelium of chaffinch (*Fringilla coelebs*) and bramling (*Fringilla montifringilla*) have been described in birds (Moreno-Lopez *et al.*, 1984), as well as papilloma lesions of the African gray parrot (*Psittacus erithacus timneh papillomavirus* - PePV) (Terai *et al.*, 2002).

Independent of species, PV types were based on their nucleotide sequence identity and according the Guidelines from the Papillomavirus Nomenclature Committee 1995 (Quebec, Canada), they were defined as a new papillomavirus type if the sequence of L1 ORF displayed less than 90% similarity with the L1 genes of all types known. So, putative new PV types had been amplified using primers FAP 59/FAP64 (Ogawa *et al.*, 2004).

The objective of this study was to detect: (I) amplifications PVDNA using the FAP59/FAP64 primer in samples of healthy domestic and wild animals with no apparent clinical signs, (II) to analyze nucleotide sequence similarity of animal papillomavirus

types to their closest related PV types and HPV sequences deposited in Gen Bank and (III) to diagnose papillomavirus-associated skin diseases in domestic and wild animals by histopathology.

## **Material and Methods**

### **Animals**

#### **Animals free of epithelial lesions**

Specimens (blood samples) were collected from the following domestic and wild animals: *Ara ararauna*, *Ara chloroptera*, *Ara macao*, *Bos taurus*, *Bubalus bubalis*, *Canis familiaris*, *Equus caballus*, *Felis concolor*, *Ovis aries*, *Panthera onca*, *Sus domesticus* and *Struthio camelus*. All animals in the study were free of warts lesions and clinically healthy.

#### **Wild animals and birds**

Two blood samples were collected from each one of wild animals at RioZoo Foundation (*Felis concolor* and *Panthera onca*). Both felines were adult and female.

One *Ara chloroptera*, one *Ara macao*, and eight *Ara ararauna*, all adults belonging to the *Psittaciformes* order, *Psittacidae* family, were mechanically contained for blood collection with an insulin needle containing EDTA.

Blood samples of ten ostriches of *African Black* breed (*Struthio camelus*) from Southern Rio de Janeiro at Saquarema municipality were also included in this study.

#### **Domestic mammals**

Blood samples of ten farm dairy cattle (*Bos taurus*) were selected in the Campos dos Goytacazes municipality at Northern Rio de

Janeiro. Blood samples were collected from 12 buffalos (*Bubalus bubalis*) in the President Kennedy municipality, Espírito Santo State. The farm was dedicated to an extensive system of crossbred buffaloes.

Blood samples of ten ovines from São Francisco do Itabapoana and Campos dos Goytacazes municipality, Rio de Janeiro State, were collected. All animals were adult and female.

Six young swine *Landrace X Large White* breed (*Sus domesticus*) from Northern Fluminense, Rio de Janeiro State, were selected also for blood collection from the ear vein or femoral vein using the anticoagulant *ethylene diamine tetra acetic acid* (EDTA).

Blood samples of twelve equines (*Equus caballus*) of the Rio de Janeiro Jockey Club aged between two and four years old, all thoroughbred, were also selected.

### **Domestic animals with epithelial lesions**

#### ***Canis familiaris***

Three dogs from Southwest of Rio de Janeiro (*Canis familiaris*) were also part of the study. Two dogs were Poodle and one was a Duchound breed showing warts-like lesions.

#### ***Bos taurus***

Ten bovines were selected, six were females and four were males showing papillomas of several morphologies and on different anatomical regions.

### **Wild animals with epithelial lesions**

#### ***Sus scrofa***

Wart-like tumors of the tarsal region (lower limb) of *Sus scrofa* was extracted using sterile scalpel and tweezers after mechanical

containment of the wild pigs in captivity proceeding from the southern region of Pantanal, Mato Grosso do Sul. Blood was also collected in vacutainer tubes containing anticoagulant EDTA.

#### ***Ara ararauna***

Samples of adult macaws, *Ara ararauna*, were obtained from the RIOZOO Foundation Quarantine Center. Surgical removal of the lesion was performed using sterile scalpel and tweezers after chemical restraint with inhalation anesthesia in one of the macaws with lesions on the cloacal mucosa.

#### ***Panthera leo***

One adult *Panthera leo* from the RIOZOO Foundation was sedated with anesthetic darts for therapeutic interventions. After verification of the presence of a papilloma-like cauliflower lesion on the oral mucosa, the lion was sedated with ketamine and xylazine to collect the sample.

#### ***Macaca fascicularis***

During routine visit to the Department of Primatology of the Laboratory Animal Breeding Center of the Oswaldo Cruz Foundation, Rio de Janeiro, one of the captive animals of the species *Macaca fascicularis*, 15 years-old, presented a typical flan papilloma-like clinically oral lesion. The sample was cataloged, extracted after sedation and preserved in formalin buffer for histopathology analysis.

### **Histopathology**

About 18 biopsies from papillomas-like samples were collected and immersed in 10% formalin buffer solution embedded in paraffin (automated tissue processor TP1020, LEICA), micro-cuts of 5 µm thickness (semiautomatic

microtome RM2145, LEICA) and stained with haematoxylin and eosin (H and E).

### **Molecular screening**

DNA extraction was carried out from blood samples and epithelial tissues from animals using QIAamp DNA blood and tissue mini kit following the manufacturer's protocol (Qiagen). The procedures for sample collection followed the standards of local research units according to each analyzed species. Blood collection was carried out using EDTA vacutainers tubes for each species.

### **PCR amplifications**

Specimens DNA was amplified by polymerase chain reaction (PCR) with  $\beta$ -globin primers (sense: 5' - AACCTCTT TGTTACAACCAG - 3') / (antisense: 5' - CAGATGCTTAACCCA CTGAGC - 3') (Yagui *et al.*, 2006). Degenerate primers pairs PCR FAP59 (forward: 5' - TAACWGTIGGICAYCCWTATT - 3') and FAP64 (reverse: 5' - CCWATATCWV HCATITCICCATC - 3') were performed according Ogawa *et al.*, (2004).

PCR was carried out in a volume of 50 $\mu$ L containing 2.5 $\mu$ L DNA sample, 0.5 $\mu$ M of each primer, 200 $\mu$ M dNTP mixture, 1.5mM MgCl<sub>2</sub> and one unit of DNA *Taq* polymerase in (20mmol/L) 1x buffer Tris-HCl pH 8.4. PCR reactions were performed using negative control samples (high purified water) and positive control (HeLa cell DNA). Amplifications were performed with the following cycling profile: initial denaturation at 94°C for 10 min followed by 45 cycles of 1.5-min denaturation at 94°C, 1.5-min annealing at 50°C, and 1.5-min elongation at 72°C. The last cycle was followed by a final extension of 5 min at 72°C (Ogawa *et al.*, 2004). All reactions were done in a PTC-

100 thermocycler. PCR DNA-specific bands were analyzed on a 2% agarose gel stained with ethidium bromide, visualized by UV light and photographed using Bio Doc Analyze (Biometra Ti5).

### **Purification and sequencing**

PCR products were purified by GFX PCR DNA purification kit (GE Healthcare). Subsequently, 2  $\mu$ l of the purified PCR product was quantified and 5 $\mu$ l were used with the same primers (5 $\mu$ M FAP59/FAP64) as in the amplification reaction for sequencing. This was performed using the BigDye® protocol (Applied Biosystems, USA) as described by the manufacturer. The chromatograms were first viewed using the Sequence Analyser software Base Caller Cimarron 3.12.

### **Alignment**

The quality of the sequences obtained was evaluated by Chromas program version 2.33 and/or the *Biological Sequence Alignment Editor* (BioEdit), and the sequences obtained were compared with available sequences of the GenBank database using the *Basic Local Alignment Search Tool* program (BLAST) server (<http://www.ncbi.nlm.nih.gov/blast/>).

## **Results and Discussion**

### **Histopathology**

Histological examination of haematoxylin and eosin stained sections demonstrated epithelial hyperplasia, acanthosis, hyperkeratosis and tissue proliferation. Among the 18 samples microscopically examined, about 12 were diagnosed as papillomatosis (66.66%), comprising bovines and primate; three samples from canine lesions were diagnosed as adenocarcinomas (16.66%); one from

macaw was diagnosed as nodular formations (5.55%); one from the harvest of the wild pig, was diagnosed as melanoma (5.55%); and finally, one was as fibrous epulic of the oral mucosa from lion (5.55%).

Microscopic examination of cutaneous papillomatosis lesions showed alterations by pronounced hyperplasia of the stratum spinosum (acanthosis), associated with hyperkeratosis, sometimes parakeratotic and orthokeratotic and hypergranulosis, due to the exacerbation of the granular layer (with abundance of kerato-hyaline granules). Koilocytes and viral inclusion corpuscles were present also. The stromal, sparse and variably infiltrated by lymphocyte-lymphocytic cells with predominance of lymphocytes emitted papilliform projections.

The papillomatosis lesions in the primate revealed acanthosis, low hyperkeratosis, rare koilocytes and papillary support irregularly infiltrated by lymphocytes and plasmocytes. The absence of granules was awesome. Samples from dogs revealed hyperkeratosis, inflammatory infiltrate of mononuclear cells, proliferation of sebaceous adenomas, sometimes exhibiting degrees of degeneration and cystic formations with presence of corneous pearls. These findings were identified as adenocarcinoma.

### **Molecular analysis**

Based on the partial sequence of the L1 gene, of a total of the 75 blood samples analyzed by PCR from several species of animals, 31 biological specimens were positive to viral DNA amplifications of the eight species below: *Ara chloroptera*, *Ara macao*, *Bos taurus*, *Canis familiaris*, *Felis concolor*, *Ovis aries*, *Panthera onca*, *Struthio camelus*. Skin warts samples from bovines and canines were clinically and histopathologically previously diagnosed as papillomatosis (Figs 1 and 2).

The other species were free of papillomatosis lesions and clinically healthy. Only one of the 12 *Bubalus bubalis* samples showed viable genomic DNA after extraction. So, this species was excluded of the study.

Some positive samples had not enough genetic material for sequencing. Some samples showed noise after the sequencing which generated interference in the data analysis. Although the  $\beta$ -globin gene had been investigated in the domestic swine species, and all tested samples were considered eligible for PCR analysis of PV DNA sequences, bands amplified by PCR reaction using degenerate primers FAP59/FAP64 were not detected in blood samples. PV DNA bands were detected in skin biopsies and blood samples from bovines and canines with papillomatosis. It was possible to demonstrate that the genome of different animal species is strictly related with the genotypes of HPV by the analysis of the nucleotides sequence of the ORF L1.

Additionally, the fact that these strains are from diverse species and originally proceeding from distinct geographic regions indicates the phylogenetic proximity among hosts closely related at different nucleotide positions to papillomavirus types and HPV sequences deposited in Gen Bank. The alignment showed the overlapping of similar stretches of two sequences by Blast. The samples analyzed presented alignments with few nucleotides, varying according to the species investigated. Data about all samples in this study are summarized in tables 1 and 2.

*Bos taurus*: BP28 samples showed alignment with 14 nucleotides of sequences of the *Human papillomavirus* type 97 (HPV-97) isolate W15189 (GenBank accession numbers EF2002168.1 - nt:215-228 and 6529-6516). About 100% identity was detected also with the HPV-97 isolate 624 complete genome

(Access: EF436229.1 - nt:215-228 e 6529-6516), and with the HPV-97 complete genome (Access: DQ080080.1 - nt:215-228 e 6529-6516). 94% similarity was also verified with *Lynx rufus papillomavirus* type 1 (LrPV-1) complete genome (Access: AY904722.1) and 90% genetic similarity with the *Human papillomavirus* type 7 (HPV-7) E1 protein (E1) gene (Access: AF322243.1 - nt:151-170 e 32-12). BP29 samples showed 100% identity with *Erethizon dorsatum papillomavirus* type 1 EdPV-1 (Access: AY684126.1) and *Ovine papillomavirus* 1 (OvPV-1) complete genome (U83594.1).

*Canis familiaris*: In the clinically healthy canine (CP75), 17 nucleotides were aligned from sequences of isolates HPV L1 gene and L1 and L2 ORF of the HPV-5 (15nt), HPV-8 (15nt), -12 (15nt), -14 (21nt), -36 (15nt), -40 (17nt), -47 (15nt) and -82 (15nt). Alignment of the 90% identity was detected with *Equus caballus papillomavirus* type 1, (EqPV-1), complete genome (Access: AF498323.1) (18nt) and *Equinus papillomavirus* (EqPV-1), complete genome (Access: AF394740.1) (18nt). Another sample (CP98) detected 100% identity in nucleotide positions (nt:639-655 and 394-410) of the *Human papillomavirus* vs75-3 DNA, part of L1 ORF (Access: X79945.1), as well as 100% alignment of the nucleotide positions (nt: 642-656 and 133-147) of the *Human papillomavirus* DNA for capsid protein L1 gene (clone vs203-2) (Access: X89880.1).

In the CP95 samples, 100% identity of papillomaviruses of the *European elk papillomavirus* (EPPV) (15nt) and *Reindeer papillomavirus* (RPV) (15nt) were detected also. This sample showed 100% homology with *Human papillomavirus* type 5, complete genome (Access: M17463.1); *Human papillomavirus* type 5 L2 and L1 genes (Access: DQ080001.1); *Human papillomavirus* type 5 DNA and naturally

occurring deletions in the late region of the virus (Access: M22961.1); *Human papillomavirus* type 5b complete genome (Access: D90312.1); *Human papillomavirus* L2 protein (L2) gene, partial (Access: M73052.1); *Human papillomavirus* L2 protein (L2) gene sequence (Access: M73058-1 and M73056.1); *Human papillomavirus* type 8, complete genome (Access: M12737.1); *Human papillomavirus* type 12 genomic DNA (Access: X74466.1); *Human papillomavirus* type 36, complete genome (Access: U31785.1); and *Human papillomavirus* type 47 (Access: M32305.1).

The CP96 sample showed 94% identity with *Human papillomavirus* type 40 genomic DNA (Access: X74478.1). The analyzed sample presented also a close relationship with the *Human papillomavirus* type 82 DNA, complete genome (Access: AB027021.1), showing 100% of alignment (nt:161-175 and 971-985).

The CP97 sample showed 94% identity with the *Human papillomavirus* type 28, complete genome (Access: U31783.1) and 85% with the *Human papillomavirus* type 74 subtype AE10, complete genome (Access: AF436130.1). The similarity with another animal species were 90% with *Trichosurus vulpecula papillomavirus* major capsid protein L1 gene (Access: AF181682.1) and 76% identity with *Bandicoot papillomatosis carcinomatosis virus* type 2, complete genome (Access: EU277647.1).

Adenocarcinomatous cutaneous lesions (CP98 sample), of canines showed similarity with the L1 gene HPV (18nt) and with HPVs-6 (26nt), -7 (21nt), *Human papillomavirus* type 8 (HPV-8) (18nt), complete genome (Access: M12737.1), -28 (18nt), E6ORF *Human papillomavirus* type 31 (HPV-31) (19nt), 88% identity with *Human papillomavirus* type 40 (HPV-40) (22nt) genomic DNA (Access:

X74478.1) and (15nt) *Human papillomavirus type74* (HPV-74) subtype AE10 (Access: AF436130.1). It also showed similarity with marsupial species, such as *Bandicoot papillomatosis carcinomatosis virus 2* (33nt); *Trichosurus vulpecula papillomavirus* (19nt); 90% identify with the dolphin *Tursiops truncatus papillomavirus type 2* (18nt) and 88% with primate *Pygmy Chimpanzee papillomavirus type 1* (22nt) (Access: X62844.1).

In the wild feline research, *Panthera onca* designed PoP46 sample, was aligned with 16 nucleotides of *Human papillomavirus type 7 - HPV-7* (Access: X74476.1- nt: 848-863 and nt: 2319-2274), a viral type of a tropical common skin wart. *Felis concolor* showed a closer relationship (18nt) with *Human papillomavirus type 84 - HPV-84*, complete genome (Access: AF293960.1) and 94% similarity (17nt) with *Human papillomavirus type 87 - HPV-87* (candidate) complete genome (Access: AJ400628.2), respectively. *Struthiocamelus: Struthio camelus* samples identified as SP42 and 43 were selected for sequencing. Nearly 18 nucleotides (nt: 64-81 and 3623-3640) were aligned with *Human papillomavirus type 74* (HPV-74) subtype AE10, complete genome (Access: AF436130.1) and 94% similarity with *Human papillomavirus type 107* (HPV-107) complete genome (Access: EF422221.1), respectively. Genetic identity showed 81% similarity (30nt) with *Human papillomavirus type 34* (HPV-34) genomic DNA (Access: X74476.1) and 88% similarity (22nt) with *Bovine papillomavirus type 3* (BPV-3), complete genome (Access: AF486184.1; AJ620207.1). *Ara macao*: of the *Ara macao* species designated AP45 sample, 16 nucleotides were aligned with *Uncia uncia papillomavirus type 1* (UuPV-1) (Access: DQ180494.1).

Some histopathological samples were diagnosed as other dermatopathies similar to

the cases of inflammatory dermatitis described by Yuan *et al.*, (2007). The findings of papillary lesion were similar to those detected in *Erethizon dorsatum* PV (EdPV-1), with hyperplastic epidermal regions (acanthosis and orthokeratous hyperkeratosis), with coilocytes in the granular stratum and in the corneum stratum (Rector *et al.*, 2005). Similarly, we found the histological findings of PV infection in samples of Asian lions (*Panthera leopersica*) (Sundberg *et al.*, 1996).

Identical microscopic alterations were also found by Maeda *et al.*, (2007) in the epidermal proliferation with hyperplasia and acanthosis in the corneum stratum and cutaneous warts from the teats of cows affected by papillomatosis by histological analysis. As well as the histopathological analysis of the oral lesions of the polar bear (*Ursus maritimus*) revealed epithelial hyperplasia with formation of dermal papillae and orthokeratotic hyperkeratosis (Stevens *et al.*, 2008), similar histological alterations were also found in this study.

Macroscopically, circular and sessile projections, with flat surfaces and pink coloration, as described by Maeda *et al.*, (2007), were also observed in this present study analyzing biopsies fragments of the oral mucosa of the Asian lion (*Panthera leo persica*) and *Cynomolgus* primate (*Macaca fascicularis*). Both lesions showed the same micro- and macroscopic histological changes, independently of epithelial differentiation of the papilloma virus host.

### **Molecular**

The PCR reaction used in this study matches with the molecular data by applying generic primers that amplify the L1 gene, the most conserved and stable region of the viral genome among all types of papillomaviruses.



The pair of generic primers targeted to the gene of interest was the degenerate FAP59 / FAP64, designed by Forslund *et al.*, (1999), from human tumor samples, applied also by Ogawa *et al.*, (2004), to detect cutaneous tumors of HPV types and normal skin samples.

Following the methodology proposed by Antonsson and Hansson (2002) and Ogawa *et al.*, (2004), the main strategy for identification of the present PV types has been the partial amplification of papillomavirus L1 gene of

human and animal origin. These strategies have employed degenerate primers for the amplification by PCR to be able to identify not only the HPV types previously described, but also to detect possible new virus subtype viruses in humans and animal species (Claus *et al.*, 2007). Other researchers as Antonsson and McMillan (2006) and Maeda *et al.*, (2007) detected the presence of PV DNA using the same pair of generic primers FAP59/FAP64, using swab samples from healthy epithelium of Australian animals and warts of cow teats respectively.

**Table.1** Nucleotide sequences identity of animal papillomavirus types from papilloma specimens (wart and/or blood samples) compared to their closest related HPV sequences deposited in Gen Bank

Samples*	Species	Identity	Gen Bank	Accession
BP28	<i>Bos taurus</i>	100%	Human papillomavirus type 97 isolate W15189, complete genome	EF2002168.1
CP75	<i>Canis familiaris</i>	91%	Human papillomavirus isolate HPVX14 L1 protein gene	AF498323.1
CP78	<i>Canis familiaris</i>	100%	Human papillomavirus DNA L1 gene (clone vs203-2)	X89880.1
CP95	<i>Canis familiaris</i>	100%	Human papillomavirus type 5 L2	DQ080001.1
CP95	<i>Canis familiaris</i>	100%	Human papillomavirus type 47	M32305.1
CP95	<i>Canis familiaris</i>	100%	Human papillomavirus type 8, complete genome	M12737.1
CP95	<i>Canis familiaris</i>	100%	Human papillomavirus type 36, complete genome	U31785.1
CP96	<i>Canis familiaris</i>	100%	Human papillomavirus type 82 DNA, complete genome	AB027021.1
CP96	<i>Canis familiaris</i>	94%	Human papillomavirus type 40 genomic DNA	X74478.1
CP97	<i>Canis familiaris</i>	94%	Human papillomavirus type 28, complete genome	U31783.1
CP98	<i>Canis familiaris</i>	100%	Human papillomavirus type 74 AE10, complete genome	AF436130.1
ScP42	<i>Struthio camelus</i>	100%	Human papillomavirus type 74 subtype AE10	AF436130.1
ScP 43	<i>Struthio camelus</i>	94%	Human papillomavirus type 107, complete genome	EF422221.1
PoP46	<i>Panthera onca</i>	100%	Human papillomavirus type 7	X74463.1
FcP47	<i>Felis concolor</i>	94%	Human papillomavirus type 87, complete genome	AJ400628.2
FcP47	<i>Felis concolor</i>	90%	Human papillomavirus type 84, complete genome	AF293960.1

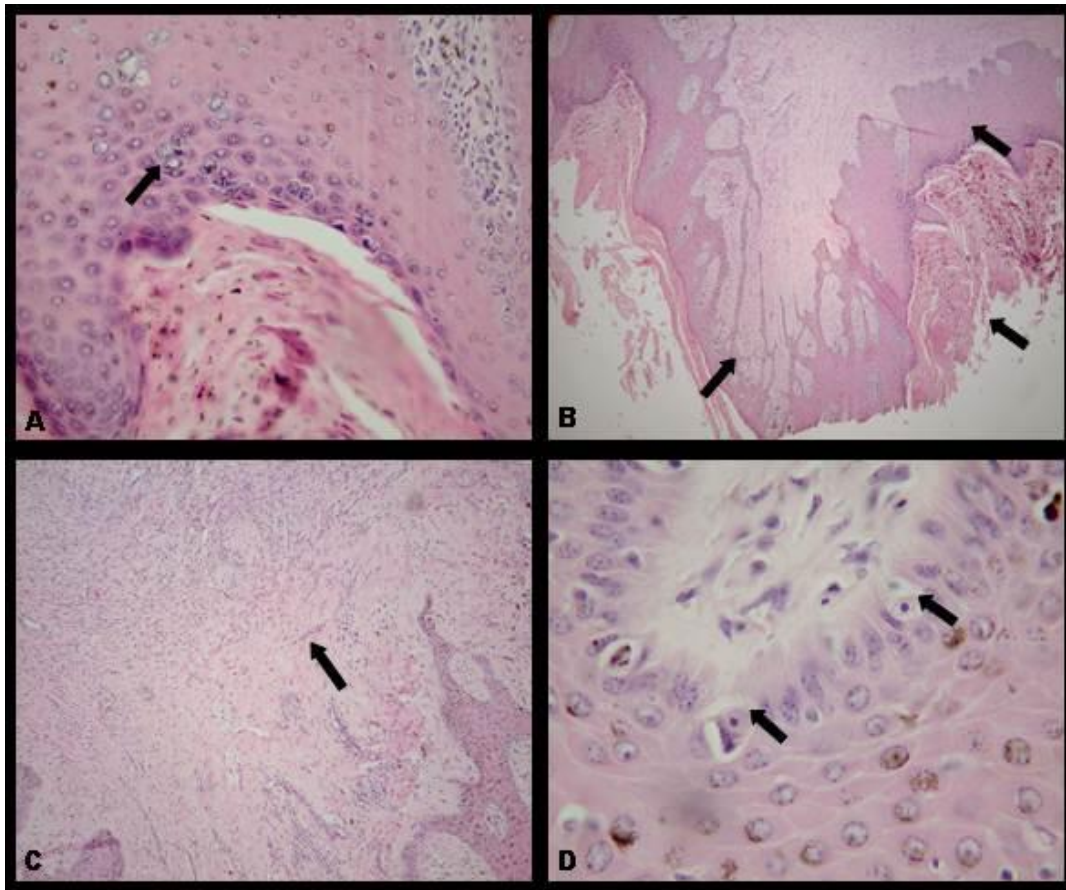
\*Samples: BP: *Bovinepapillomavirus*; CP: *Caninepapillomavirus*; ScP: *Struthio papillomavirus*; PoP: *Panthera onca papillomavirus*; FcP: *Felis concolor papillomavirus*

**Table.2** Nucleotide sequences identity of animal papillomavirus types from papilloma specimens (wart and/or blood samples) compared to their closest related others PV sequences deposited in Gen Bank

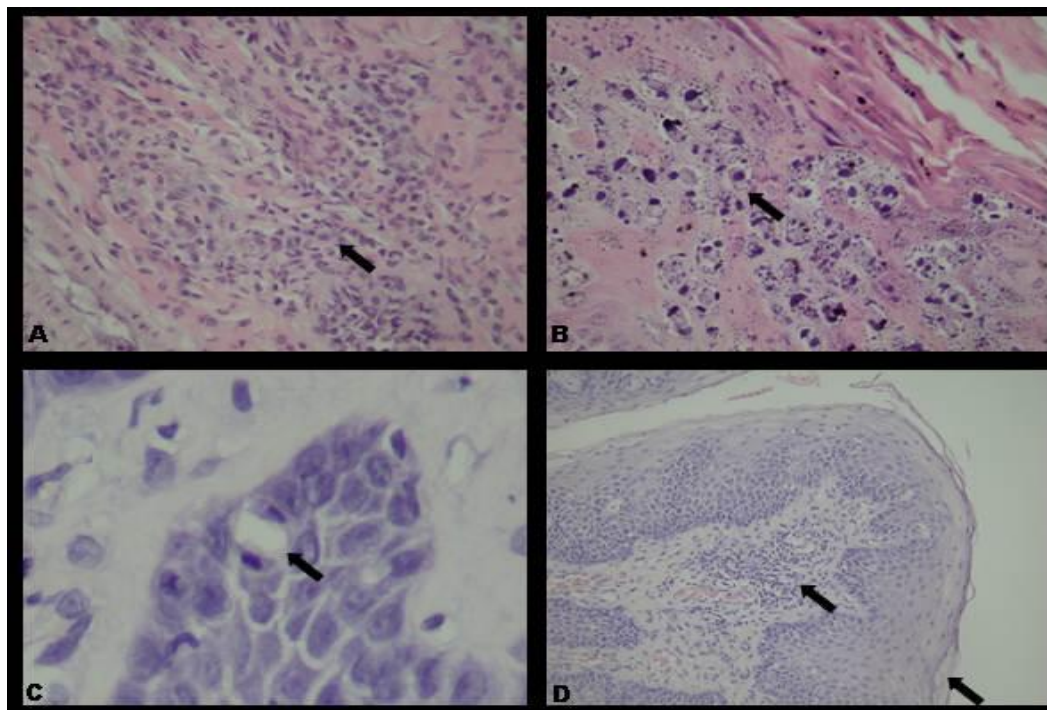
Samples*	Species	Identity	Gen Bank	Acession
BP 28	<i>Bos taurus</i>	94%	<i>Lynx rufus papillomavirus type 1, complete genome</i>	AY904722.1
BP29	<i>Bos taurus</i>	100%	<i>Erethizon dorsatum papillomavirus type 1, complete genome</i>	AY684126.1
BP29	<i>Bos taurus</i>	100%	<i>Ovine papillomavirus 1, complete genome</i>	U8359.1
CP75	<i>Canis familiaris</i>	90%	<i>Equus caballus papillomavirus type 1</i>	AF498323.1
CP75	<i>Canis familiaris</i>	90%	<i>Equinus papillomavirus, complete genome</i>	AF394740.1
CP95	<i>Canis familiaris</i>	100%	<i>European elk papillomavirus</i>	M15953.1
CP95	<i>Canis familiaris</i>	100%	<i>Reindeer papillomavirus</i>	AF443292.1
CP97	<i>Canis familiaris</i>	90%	<i>Trichosurus vulpecula papillomavirus</i>	AF181682.1
CP97	<i>Canis familiaris</i>	76%	<i>Bandicoot papillomatosis carcinomatosis virus type 2</i>	EU277647.1
CP98	<i>Canis familiaris</i>	90%	<i>Tursiops truncatus papillomavirus type 2, complete genome</i>	AY956402.1
CP98	<i>Canis familiaris</i>	88%	<i>Pygmy chimpanzee papillomavirus type 1 DNA</i>	X62844.1
SP43	<i>Struthio camelus</i>	88%	<i>Bovine papillomavirus type 3, complete genome</i>	AF486184.1
AP45	<i>Ara macao</i>	100%	<i>Uncia uncia papillomavirus type 1</i>	DQ180494.1

\*Samples: BP: Bovine papillomavirus; CP: Canine papillomavirus; SP: *Struthio papillomavirus*; AP: *Ara macao papillomavirus*

**Fig.1** Histological changes of cutaneous papillomatosis in bovine, H and E. The arrows indicate: A) Kerato-hyaline granules at the dermo-epidermal junction; B) Formation of interpapillary ridges, presence of acanthosis and hyperkeratosis; C) Proliferation of connective tissue characteristic of fibropapilloma; D) Basophilic Intranuclear Inclusion Corpuscle. Magnifications: A - 200x, B - 50x, C - 100x, D - 400x



**Fig.2** Histological changes of bovine cutaneous papillomatosis (A and B) and papillomatosis of oral mucosa of primate (C and D) by H and E. The arrows indicate: A) Tissue infiltrated by lymphoplasmacytic cells with predominance of lymphocytes; B) Hyperkeratosis and hypergranulosis due to exacerbation of the granular layer by the formation of granulations; C) Vacuolated basal cell in cell division compatible with the initiation of viral replication; D) Low hyperkeratosis evidencing the connective axis by lymphocytes and plasma cells. Magnifications: A - 200x, B - 200x, C - 400x, D - 100x



The findings in canine samples in the present study are in accordance with data described by Tanabe *et al.*, (2000), when identifying viral DNA sequences in cutaneous samples of canines, and are in agreement also with Claus *et al.*, (2007) for amplified fragments from bovine papillomas. Viral DNA sequences have been detected in samples of bovine papillomas (Ogawa *et al.*, 2004; Claus *et al.*, 2007), oral lesions of wild animals (Sundberg *et al.*, 1996; Stevens *et al.*, 2008), intact epithelial swabs from Australian animals (Antonsson and McMillan, 2006) and genital mucosa swabs from non-human primates (Mello, 2005).

This study detected DNA PV in the blood samples of animal species of the Brazilian fauna with and without clinical manifestation

of the papillomavirus infection, which had not yet been investigated. Therefore non-amplification detected from whole blood samples of the same species investigated probably was due to the presence of the virus limits below detection, or the PV present was not detectable by the use of primers for analysis in the region L1, with possible deletions of the gene of the test sample and loss of chromosomal material.

However, the findings of the present study corroborate with Mello (2005) that the PV presence in asymptomatic animals is not always easy to detect. The detection of PV nucleotide sequences in animal species were generally not amplified, emphasizing their non-specificity.

Despite the recent studies of avian papillomatosis, PV infection have been described from samples of squamous papillomas in species of closely related finches, chaffinch (*Fringilla coelebs papillomavirus* - FPV), bramling (*Fringilla montifringilla papillomavirus*) and African gray parrot (*Psittacus erithacus timneh papillomavirus* - PePV) (Moreno-Lopez *et al.*, 1984; Terai *et al.*, 2002). In the present study, PV DNA sequences were amplified from blood samples from clinically healthy birds of species not yet investigated, such as *Ara chloroptera*, *Ara macao* and *Struthio camelus*. So, this is the first record of PV infection in these species.

The similarities of the nucleotide sequences were determined by Ogawa *et al.*, (2004) with putative new PV types using the same pair of primer FAP59/FAP64 applied in this present study. Previous reports showed latent or subclinical infection of PV from healthy skin of humans and animals (Antonsson and Hansson, 2002) as well as the samples tested with latent and subclinical infection of the several species investigated. According to American research, new PV infection was detected in the species *Trichechus manatus latirostris*, amplifying fragment with 478 bp using the MY09 / MY11 oligonucleotide pair (Woodruff *et al.*, 2005). In a Koala sample, identified as KoPV, a band of 440 bp was detected, using the same primers used in the present study (Antonsson and McMillan, 2006).

But the sensitivity of the FAP59/FAP64 PCR was higher than that of the MY09/MY11 PCR for DNA PV samples. Therefore, the samples of this paper were tested only for degenerate primer FAP. Essentially, these alignments should indicate a homology between the sequences. In other words, homology indicates having evolutionary significance with similarity between sequences sharing the same ancestry.

So, this study demonstrated the nucleotide sequence similarity of animal papillomavirus types to their closest related PV types and HPV sequences deposited in Gen Bank indicating a phylogenetic proximity among the investigated hosts.

In addition, healthy domestic and wild animals with no apparent clinical papilloma signs suggest latent or subclinical infections of PV types. Our results showed PV DNA amplifications detected by using FAP59/FAP64. This same primer had been used also by Antonsson and Hansson (2002) and Ogawa *et al.*, (2004) as PCR based on highly conserved regions within the L1 open reading frame (ORF).

Size variations of the amplified fragments within a same species and among different animal species were shown in the present study. These variations were also found by Forslund *et al.*, (1999), when detecting 12 probable new types of HPVs with variations of 434 bp, 437 bp, 440 bp and 446 bp sizes of the generated fragments. The majority of HPVs amplified a 478 bp fragment using the degenerate primer pair FAP59/FAP64. But other types as HPV-40 amplified a 740 bp fragment and HPV-58 amplified 264bp.

Thus, this study showed a high diversity of the *Papillomaviridae* family in diagnosed papillomavirus infections. The amount of nucleotides aligned by identity demonstrated all other possible alignments considering the probability that the alignment has been occurred randomly by genetic similarity comparing closest related PV types and HPV sequences deposited in Gen Bank.

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