



## **Bovine papillomavirus in beef cattle: first description of BPV-12 and putative type BAPV8 in Brazil**

**R.P. Araldi<sup>1,2</sup>, R.F. Carvalho<sup>1</sup>, T.C. Melo<sup>1,3</sup>, N.S.P. Diniz<sup>1,2</sup>, T.A. Sant'Ana<sup>1,2</sup>, J. Mazzuchelli-de-Souza<sup>1</sup>, D.D. Spadacci-Morena<sup>4</sup>, W. Beçak<sup>1,5</sup> and R.C. Stocco<sup>1</sup>**

<sup>1</sup>Laboratório de Genética, Instituto Butantan, São Paulo, SP, Brasil

<sup>2</sup>Programa de Pós-Graduação Interunidades em Biotecnologia, Universidade de São Paulo, São Paulo, SP, Brasil

<sup>3</sup>Programa de Pós-Graduação em Morfologia, Universidade Federal de São Paulo, São Paulo, SP, Brasil

<sup>4</sup>Laboratório de Fisiopatologia, Instituto Butantan, São Paulo, SP, Brasil

<sup>5</sup>Departamento de Biologia, Universidade Federal da Integração Latino-Americana, Foz do Iguaçu, PR, Brasil

Corresponding author: R.C. Stocco

E-mail: rita.stocco@butantan.gov.br

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**ABSTRACT.** Bovine papillomavirus (BPV) is an oncogenic virus associated with benign and malignant lesions, which result in notable economic losses. Peripheral blood samples and cutaneous papillomas were obtained from four adult beef cattle. Viral molecular identification was performed using specific primers for BPV-1, -2 and -4 in blood diagnosis and FAP59/FAP64 for skin papillomas. Histopathologic examination was done as a complementary and differential diagnosis. The fragments were purified, sequenced, and compared using BLASTn. The blood diagnosis showed the presence of BPV-2 and the analysis of cutaneous papillomas showed the presence of BPV-4, a new

putative virus type BAPV8, and BPV-12, revealing for the first time the presence of BPV-12 and the putative type BAPV8 in beef cattle in Brazil. The sequences were deposited in the GenBank. Histopathology revealed acanthosis, hyperkeratosis, and koilocytosis in all samples analyzed. The presence of BAPV8 and BPV-12 in Brazil emphasizes the ubiquitous dissemination of BPVs in the herds of Brazil.

**Key words:** Bovine papillomavirus; BPV-12; BAPV8; Diagnosis; Histopathology

## INTRODUCTION

Bovine papillomaviruses (BPVs) are recognized as the causal agents of benign and malignant tumors in cattle, such as cutaneous papillomas, benign fibroplasias, and urinary bladder and esophagus cancer (Bocaneti et al., 2014), causing significant economic losses. These oncogenic viruses, with a double-stranded circular DNA genome of approximately 8 kb, display tropism for squamous epithelial and mucosal tissues, where they are associated with benign and malignant epithelial lesions (Stocco dos Santos et al., 1998; Roperto et al., 2008; Talbert-Slagle and DiMaio, 2009). Currently, 13 types of BPVs are well characterized and classified into three distinct genera - *Delta*, *Epsilon*, and *Xipapillomavirus* - and each BPV has been associated with type-specific lesions (Borzacchiello and Roperto, 2008; Lunardi et al., 2013).

Studies involving techniques of BPV diagnosis have attracted great interest due to the problems caused by the viral infection, such as dermatitis and cancer, resulting in significant economic impacts on livestock (Nascimento et al., 2012). Moreover, the results obtained through diagnostic techniques allow drawing a molecular epidemiology of the infections, which has prophylactic importance, since immunity is species-specific (Claus et al., 2009). The limited number of BPV types described compared to human papillomavirus (HPV) reflects a small sample size analyzed and not a lack of antigenic diversity for BPVs (Claus et al., 2009).

Diagnostic techniques include clinical examination, histopathology - as a differential and complementary diagnosis (Betioli et al., 2012) - and detection of viral DNA sequences by a series of techniques such as Southern blot, dot blot, reverse blot and *in situ* hybridization with radiolabeled probes (Leto et al., 2011). The use of monoclonal antibodies against epitopes of BPV could also provide a diagnosis by immunohistochemistry (Elzein et al., 1991).

However, polymerase chain reaction (PCR) has been shown to be a more sensitive method for the identification and characterization of BPV, by using degenerate primers followed by DNA purification, sequencing and sequence similarity analysis with computer programs and tools such as BLASTn (Borzacchiello et al., 2003; Ogawa et al., 2004; Jelínk and Tachezy, 2005; Monteiro et al., 2008; Leto et al., 2011; Carvalho et al., 2012; Nascimento et al., 2012; Zhu et al., 2012). Molecular diagnosis may also be performed by restriction fragment polymorphism length (RFLP), a technique that employs the fragmentation of the L1 gene by the use of restriction endonucleases (Carvalho et al., 2013).

The L1 gene, which encodes the major capsid protein L1, is the most conserved gene within the papillomavirus (PV) genome and has therefore been used for identifying new viral types over the past 15 years (de Villiers et al., 2004). Divergences in the nucleotide sequence

of the L1 ORF are used as taxonomic classification criteria, and the use of PCR assays with degenerate primers, followed by sequencing, has allowed the identification of several PV types in human and other animal hosts (Forsslund et al., 1999). The use of specific primers has also been successfully employed, mainly for BPV identification in blood (Araldi et al., 2013). The PCR primer pair FAP was designed from two relatively conserved regions found in the L1 gene and has been shown to amplify PV DNA both in papilloma and healthy skin of many animal species, including cattle (Ogawa et al., 2004).

BPVs have been detected in Brazil in the last decades, but the extent of the impact of BPV-associated diseases in both dairy and cattle herds, although dramatic, has not yet been studied epidemiologically. Reports available in different regions of the country indicate a significant diversity of viral types, implying evident disease burden (Yagui et al., 2008; Claus et al., 2009; Carvalho et al., 2012; Lunardi et al., 2013). Thus, it is critical to identify the BPV types and their prevalence, distribution and clinical consequences for the development of prophylactic and/or therapeutic procedures.

Histopathological analysis of the lesion is an important procedure, since it identifies intraepithelial tumors associated with oncogenic viruses, such as those caused by BPV, making this a complementary tool in molecular diagnosis (Turk et al., 2005; Monteiro et al., 2008; Leto et al., 2011). Histopathological analysis also indicates a predilection for anatomical and topographical areas related to specific viral types (Monteiro et al., 2008). The pathological findings include cell hyperplasia of the spinous layer (acanthosis), hyperkeratosis, parakeratosis, papillomatosis and koilocytosis (Turk et al., 2005; Anjos et al., 2010; Marins and Ferreira, 2011). Although koilocytosis is present in BPV-infected tissue, this is not considered a pathognomonic marker (Marins and Ferreira, 2011). Obviously, in the development of therapeutic procedures, the evaluation of the characteristic lesions will be important.

Accordingly, the present study represents an effort to identify viral BPV sequences in cattle herds in São Paulo State, Brazil, describing for the first time the presence of two new virus strains circulating in Brazil and correlating this with histopathology analysis of the lesions.

## MATERIAL AND METHODS

The blood and cutaneous papilloma samples were collected from four adult beef cattle (*Bos taurus*, Red Angus), aged 2 to 3 years, from Avaré, SP, Brazil. The methods used in this study were approved by the Ethics Committee on Animal Use of Instituto Butantan (protocol No. 1035/13).

The peripheral blood was collected using Vacutainer systems with EDTA. Lesions were collected by a veterinarian, using local anesthesia (2% lidocaine). The papilloma samples collected were divided into two portions using a sterile bistoury. One portion was used for histopathological analysis, where it was fixed in 10% formalin, dehydrated in an alcohol series, embedded in paraffin, and sectioned (4-5  $\mu\text{m}$ ). The slides were stained with hematoxylin-eosin and examined under an Axiophot light microscope (Carl Zeiss, Germany) at 5X and 20X magnification, and images were captured using the AxioVision 4.7.2 software.

The DNA from blood was extracted using the Illustra Blood GenomicPrep Mini Spin kit (GE Healthcare, Buckinghamshire, UK), according to manufacturer instructions. The extracted DNA was quantified in an ND-1000 NanoDrop spectrophotometer (Applied Biosystems, Singapore) and subjected to PCR with bovine  $\beta$ -globin primer pair (forward

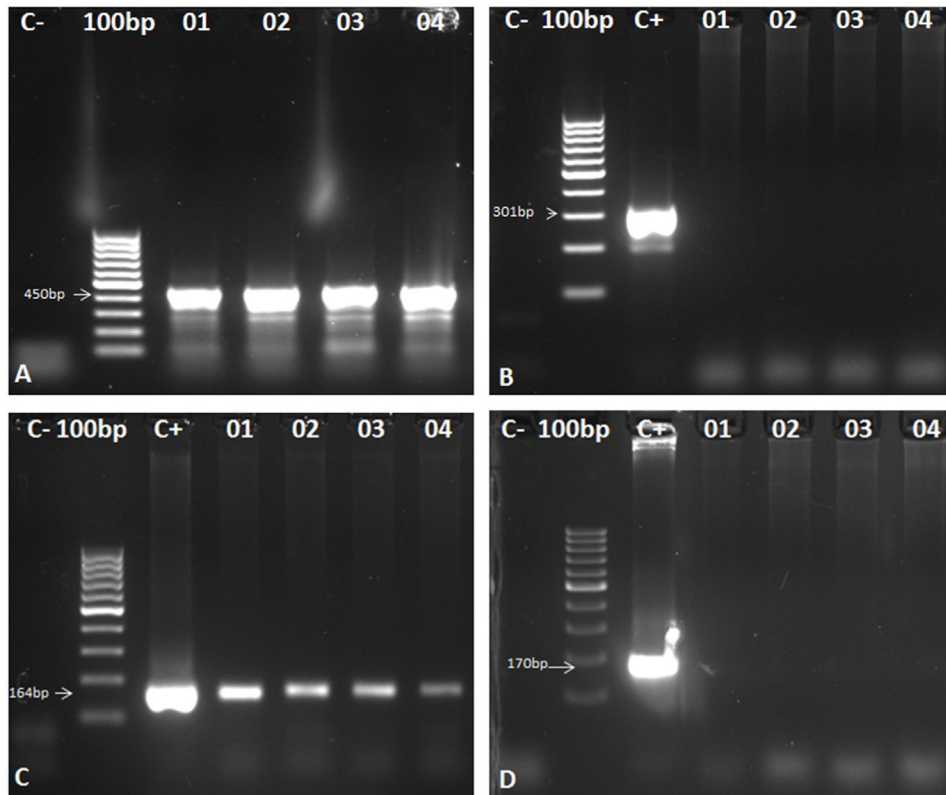
5'-AACCTCTTTGTTACAAAGAG-3', reverse 5'-CAGATGCTTAACCCACTGAGC-3'), to verify quality and integrity (Yagui et al., 2008). Peripheral blood BPV identification was done employing specific primers for BPV-1 (forward 5'-GGAGCGCCTGCTAACTATAGGA-3' and reverse 5'-ATCTGTTGTTTGGGTGGTGAC-3'), which amplify a fragment of the L1 gene, resulting in a 301-bp amplicon, BPV-2 (forward 5'-GTTATACCACCCAAAGAAGACCCT-3' and reverse 5'-CTGGTTGCAACAGCTCTCTTTCTC-3'), which amplify the L2 gene, resulting in a 164-bp amplicon, and BPV-4 (forward 5'-GCTGACCTTCCAGTCTTAAT-3' and reverse 5'-CAGTTTCAATCTCCTCTTCA-3'), which amplify a segment of the E7 gene, resulting in a 170-bp amplicon. These primers were chosen because these BPV types are often observed in Brazil as well as their association with esophageal (BPV-4) and bladder (BPV-1 and 2) cancer. The amplification reactions were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems), with PCR SuperMix (Invitrogen, Carlsbad, CA, USA), under the following conditions: 5 min at 95°C, followed by 35 cycles of 1 min and 30 s at 98°C, 2 min at 52°C and 1 min and 30 s at 72°C and a final extension step of 5 min at 72°C. The PCR products were analyzed by 2% agarose gel electrophoresis and staining with GelRed (Biotium Inc., Hayward, CA, USA) in TAE buffer. Electrophoresis was performed in a Horizon 20:25 tank (Life Technologies, Carlsbad, CA, USA) at 100 V, 400 mA for 120 min, along with a 100-bp DNA Ladder (Invitrogen). The gel was visualized on a MiniBIS Pro transilluminator (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel) and images were captured by the GelCapture software version 7.1 (DNR Bio-Imaging Systems Ltd.). Cloned BPV-1, -2, and -4 were used as positive controls. The other portion from the lesions was used for molecular identification and virus typing, using the degenerate primer pair FAP59/FAP64. DNA tissue extraction was performed using the Illustra Cells and Tissue Genomic Prep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK), and the DNA extracted was kept at -20°C. The quality of DNA samples obtained was verified by PCR amplification of a bovine  $\beta$ -globin gene fragment, according to Yagui et al. (2008). Viral molecular identification was performed using the degenerate primers FAP59 (forward 5'-TAACWGTIGGICAYCCWTATT-3') and FAP64 (reverse 5'-CCWATATCWWHCATITCICCATC-3') (Forsslund et al., 1999), which promote the partial L1 gene amplification, resulting in an amplicon of 478-bp (Ogawa et al., 2004). The PCR reactions were performed in a Veriti 96 Well Thermal Cycler (Applied Biosystems), with PCR SuperMix (Invitrogen), under the following conditions: 10 min at 94°C, followed by 45 cycles of 1 min and 30 s at 94°C, 2 min at 52°C and 1 min and 30 s at 72°C, and a final extension step of 5 min at 72°C, for primer pair FAP59/FAP64. The PCR products were analyzed by 2% agarose gel electrophoresis and staining with GelRed (Biotium Inc.). The fragments were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The purified material was subjected to sequencing in an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The quality of DNA sequences was checked and overlapping fragments were assembled using the BioEdit software 7.0.9.0 (Hall, 1999) and the sequences were compared using the NCBI database and BLAST (<http://blast.ncbi.nlm.nih.gov>).

Homology analyses were performed with the NCBI database and BLAST (Altschul et al., 1997). Nucleotide and amino acid sequences from other BPV types as well from HPV-16 were retrieved from GenBank for comparison with the sequences obtained here. The BioEdit software was used to identify the equivalent amino acid sequences. The sequence alignments were performed with the MEGA 5.0 software (Tamura et al., 2011), using full alignment and 2000

bootstrap replications, to ensure a higher level of confidence in our analysis (Efron et al., 1996). A phylogenetic relationship comparing nucleotide sequences was determined with the MEGA software. Neighbor-joining trees were drawn using TreeView, version 1.6.6 (Page, 1996).

## RESULTS

$\beta$ -globin amplification using peripheral blood DNA showed an amplicon of 450 bp, showing DNA integrity (Figure 1). BPV identification using specific primers showed the presence of BPV-2 in all samples analyzed but did not reveal the presence of BPV-1 or -4 (Figure 1).

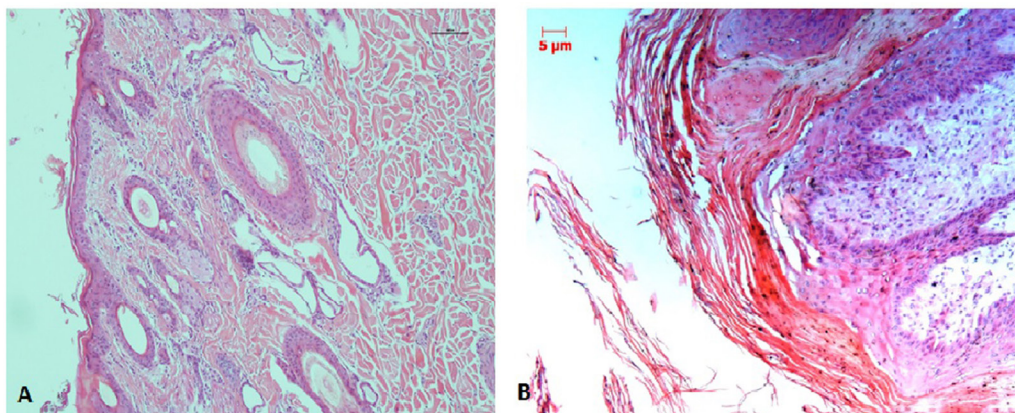


**Figure 1.** A. Amplicons of 450 bp with  $\beta$ -globin primers, B. amplicons of 301 bp with specific primer to BPV-1, only observed amplification in positive control (BPV-1), C. amplicons of 164 bp with specific primer to BPV-2, showing the amplification of all samples, and D. amplicons of 170 bp with specific primer to BPV-4, only in the amplification of positive control (BPV-4).

The analysis of microscopic fragments of skin formations revealed exophytic neoplasms characterized by the proliferation of squamous epithelial cells arranged in fingerlike projections, infiltrative and interconnecting beams that extended into the dermis. Marked hyperkeratosis was observed in the stratum corneum. In the granular layer, there was a large

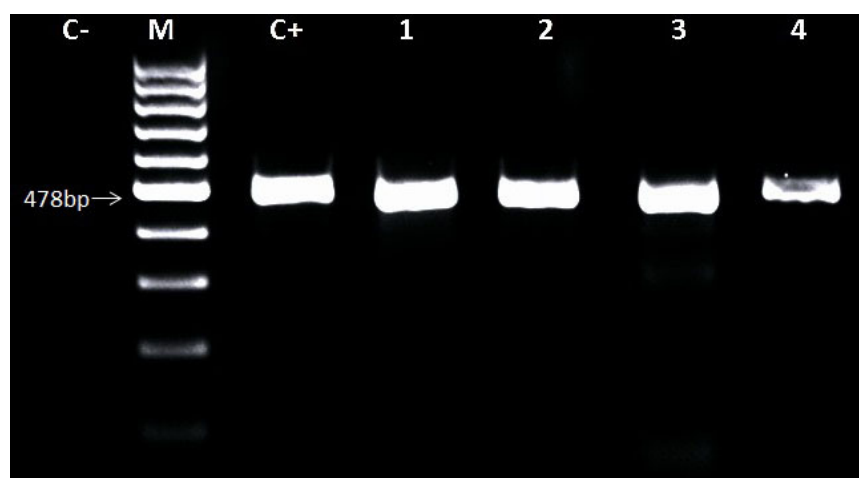


amount of various-sized keratohyalin granules: in the spinosum stratum, it was possible to verify koilocytosis. The stromal layer was composed of fibrovascular tissue, with an intense proliferation of fibroblasts with fascicular arrangement, without evidence of atypical features (Figure 2).



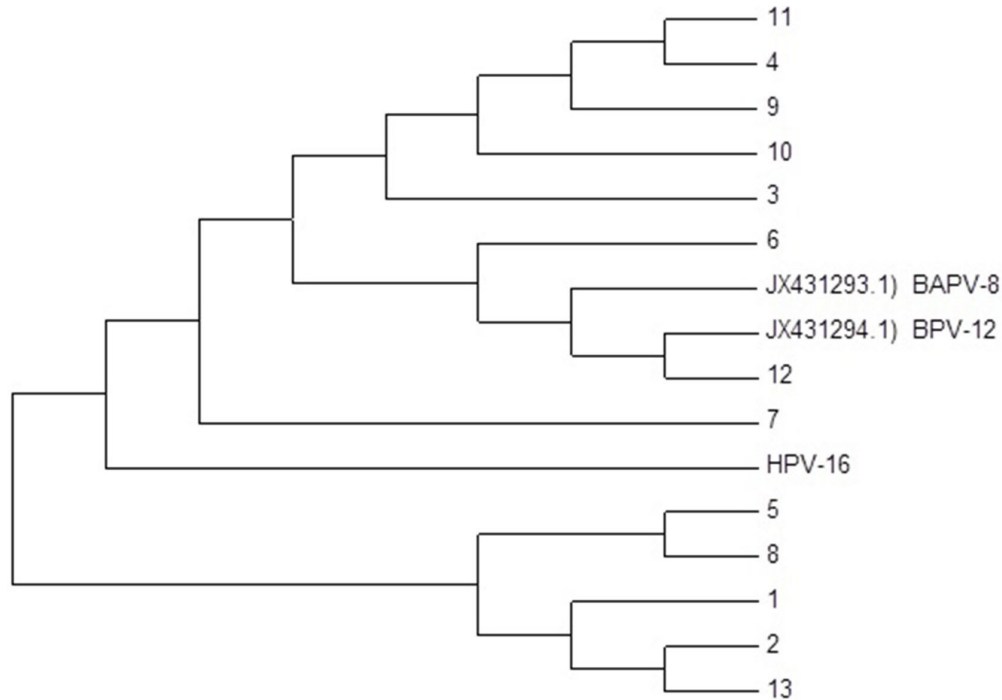
**Figure 2.** A. Histological analysis of normal skin tissue, obtained from an animal not infected with BPV, showing the epidermis and derma. B. Histopathological analysis of cutaneous papilloma, obtained from the adult bovine, infected with BPV-12, showing hyperkeratosis, acanthosis and koilocytosis (20X).

BPV identification using the degenerate primer pair FAP59/FAP64 resulted in amplicons of 478 bp (Figure 3). The amplicon was purified and sequenced. The results of the four cutaneous papilloma samples showed the presence of BPV-4 (X05817.1) with 98% identity in sample 01, putative BAPV8 (AB543507.1) with 100 and 99% identity in samples 02 and 03, respectively, and BPV-12 (AB543507.1) with 100% identity in sample 04. The sequences obtained here were deposited in GenBank (JX431293.1 - BAPV8 and JX431294.1 - BPV-12).



**Figure 3.** Electrophoresis' gel showing 478-bp FAP59/FAP64 amplicons of L1 BPV gene in four clinical sample (lanes 1 to 4). C- = negative control group; C+ = positive control group; Lane M = marker.

The phylogenetic tree (Figure 4) showed that the sequences found in this sample of beef cattle in Brazil belong to the genus *Xipapillomavirus*, where BAPV8 is the closest to BPB-6. These results confirmed the presence of new putative viruses, BAPV8 and BPV-12, in Brazil.



**Figure 4.** Phylogenetic tree, showing the sequences found for the first time in Brazil (JX431293.1 - BAPV8 and JX431294.1 - BPV-12) as belonging to *Xipapillomavirus*.

## DISCUSSION

The presence of BPV in peripheral blood related to cytogenetic aberrations was first described by Stocco dos Santos et al. (1998). The presence of BPV-2 in peripheral blood leads to chromatin changes (Stocco dos Santos et al., 1998; Melo et al., 2011; Araldi et al., 2013), which result in genomic instability, evidenced by chromosomal loss and gain. This event is frequently observed in solid tumors, with it being associated with the oncogenic process (Duensing and Münger, 2002; Toledo, 2011; Córtes-Gutiérrez et al., 2012).

The presence of BPV sequences in peripheral blood has drawn increasing interest over the last years, and studies have demonstrated the presence of the virus in CD4+ and CD8+ leukocytes (Roperto et al., 2011) and BPV blood activity (Araldi et al., 2013). The presence of BPV DNA sequences in these cells has led to discussion about new infection pathways for the virus: viral particles can be transported to injured areas through leukocyte infiltration. Evidence of this pathway has already been reported by Wobeser et al. (2012) and Araldi et al. (2013).

The BPV-2 is an important co-factor in the development of bovine bladder cancer in animals exposed to feeding on bracken fern, *Pteridium aquilinum*. This bracken contains mutagenic, carcinogenic and immunosuppressive compounds such as prunasin, quercetin, ptaquiloside, shikimic acid and thiaminase (an enzyme that decomposes vitamin B1) (Wosiack et al., 2002; Borzacchiello, 2007).

The histopathology of the lesions related to these new viral types found in Brazil was similar to the cutaneous papillomas reported for other BPV types. Hyperkeratosis (orthokeratotic and parakeratotic) is an effect of the increase in keratohyalin granules in the granular layer, because the granules are associated with the keratinization process (Ferraro et al., 2011). The presence of koilocytosis represents a cytopathic effect characterized by papillomavirus infection, showing the presence of degenerating, dying cells, characterized by a prominent halo, which does not stain with eosin or periodic acid-Schiff (Fletcher, 1983).

Genomic instability is the result of the expression of BPV oncogenes E6 and E7. The oncoprotein E6 is responsible for the disruption of the actin cytoskeleton and clastogenicity and affects the DNA repair system, cell proliferation and proteasome degradation through ubiquitination of p53 (Duensing and Münger, 2002; Zhu et al., 2012). The oncoprotein E7 leads to cellular transformation, degradation of the pRb tumor suppressor, deregulation of cell cycle and tumorigenesis (DeMasi et al., 2007; Freitas et al., 2011; Marins and Ferreira, 2011).

The results obtained from the cutaneous papillomas showed the presence of different BPV types, revealing co-infection when compared with the results obtained from peripheral blood. Co-infection was reported by Carvalho et al. (2012) and Araldi et al. (2013). Although there are few discussions about the impact of co-infection, it is known that it does not increase the clastogenic effect of BPV when compared with infection by only one viral type (Araldi et al., 2013). However, this scenario is extremely important in relation to the development of a vaccine against BPV, which requires the development of products that show a spectrum of coverage for more than one viral type.

The phylogenetic tree showed that both sequences found (BAPV8 and BPV-12) belong to the genus *Xipapillomavirus*, with the new putative virus BAPV8 being closest to the well-characterized BPV-6, as proposed by Ogawa et al. (2004). The presence of both BAPV8 and BPV-12 in Brazil points to the ubiquity of BPVs, since these two relatively new viral types, which were first characterized in Japan, were also detected in South America. Thus, this study highlights for the first time the detection of these rare BPV types in beef cattle in Brazil.

The identification of new BPVs in Brazil in beef cattle is of epidemiological importance since the country has approximately 210 million cattle, being major exporter of meat, milk, and leather. Thus, studies concerning diagnosis of BPVs and related clinical aspects in the Brazilian herd are highly relevant for developing new strategies for virus control, both at the national and international level.

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