

Short Communication

Teat papillomatosis associated with bovine papillomavirus types 6, 7, 9, and 10 in dairy cattle from Brazil

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Abstract

This study describes the clinical, histopathological, and virological characterization of teat papillomatosis from Brazilian dairy cattle herds. Four types of bovine papillomavirus were identified (BPV6, 7, 9, and 10); one of these (BPV7) is being detected for the first time in Brazilian cattle.

Key words: papilloma, BPV, genotyping.

Papillomaviruses (PVs) are small (52-55 nm), non-enveloped, double-stranded DNA oncoviruses that replicate in the nucleus of squamous epithelial cells and can induce warts in the skin and mucosal epithelia of most higher vertebrate species. Some specific viral types have the potential to cause malignant progression in papillomatous lesions of animals and humans (Antonsson *et al.*, 2002; de Villiers *et al.*, 2004). In cattle, bovine papillomavirus (BPV) is the etiological agent of cutaneous and teat papillomatosis, cancers of the upper gastrointestinal tract and urinary bladder (Campo 2002; Borzacchiello *et al.*, 2003; Wosiacki *et al.*, 2006).

BPV produces characteristic gross lesions that are either exophytic (proliferating outwards) or endophytic (inverted) and are composed of a hyperplastic epithelium supported by a discrete dermal tissue containing dilated capillaries (Ginn *et al.*, 2007; Hargis *et al.*, 2012).

Currently, the genomes of thirteen BPVs (BPV1 to 13) have been fully characterized. They are classified into four genera based on their genome relatedness and biological properties (de Villiers *et al.*, 2004). These genera are *Deltapapillomavirus* (BPV1, 2, and 13), *Xipapillomavirus* (BPV3, 4, 6, 9, 10, 11, and 12), *Epsilonpapillomavirus*

(BPV5 and 8), and a yet unnamed PV genus (BPV7) (Bernard *et al.*, 2010; Hatama *et al.*, 2011; Zhu *et al.*, 2012; Lunardi *et al.*, 2013). Moreover, the occurrence of numerous additional viral types has been proposed based on partial nucleotide sequence analysis of the major capsid protein L1, obtained from both benign cutaneous lesions and swab samples of healthy skin from cattle (Antonsson and Hansson 2002; Maeda *et al.*, 2007; Ogawa *et al.*, 2007; Claus *et al.*, 2008; Claus *et al.*, 2009c).

Teat papillomatosis has been reported in dairy herds worldwide as a cattle health problem resulting in economic losses (Campo 2003). While milking can become difficult in markedly affected individuals, ulceration and rupture of established cutaneous lesions might predispose dairy cattle to mastitis and distortion of the milk ducts. Additionally, the maintenance of affected cows with alteration in mammary gland shape and/or even of herds with a high number of affected animals may prevent economic profits in the dairy industry (Campo 2002; Borzacchiello *et al.*, 2008).

Although papilloma lesions found in teats and udders can be caused by diverse BPV types, the BPV6 has often been identified in this anatomical location. Therefore, the association of BPV1, 3, 5, 7, 8, 9, 10, 11, and other types

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still to be characterized, have been confirmed in mammary glands of cattle (Campo *et al.*, 1981; Jarrett *et al.*, 1984; Ogawa *et al.*, 2004; Claus *et al.*, 2007; Maeda *et al.*, 2007; Claus *et al.*, 2008).

Despite the high number of cattle herds affected by cutaneous papillomatosis in several geographical regions of Brazil, studies investigating BPV diversity associated with different clinical outcomes are sporadic (Stocco dos Santos *et al.*, 1998; Freitas *et al.*, 2003; Wosiacki *et al.*, 2005; Wosiacki *et al.*, 2006; Claus *et al.*, 2008; Claus *et al.*, 2009b; Claus *et al.*, 2009c; Lunardi *et al.*, 2010).

The aim of this report is to describe the identification of BPVs 6, 7, 9, and 10 in association with cases of teat papillomatosis from two dairy cattle herds of Brazil. This study represents the first detection of the BPV7 in Brazilian cattle.

Seven exophytic teat papillomas were individually collected from three dairy cows belonging to two different cattle herds from the Northern region of Parana state in Southern Brazil. These lesions were observed on different parts of the teat and coded for identification. A portion of each lesion was fixed in 10% buffered formalin solution and routinely processed for histopathological evaluation. Fragments from each papilloma specimen were grounded in phosphate-buffered saline solution (PBS pH 7.2) and suspensions (10–20% w/v) were centrifuged for 15 min at 3,000 \times g at 4 °C. Aliquots (250 μ L) from supernatant were treated with lysis buffer (10 mM Tris; 1 mM EDTA; 0.5% Nonidet P40; 1% SDS; and 0.2 mg/mL proteinase K) (Invitrogen, Life Technologies, Carlsbad, CA, USA). After homogenization, the samples were incubated at 56 °C for 30 min. For DNA extraction, a combination of the phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate methods was performed as previously described (Alfieri *et al.*, 2006). DNA was eluted in 50 μ L of ultrapure sterile water and kept at -20 °C until used. Aliquots of ultrapure sterile water were included as negative controls in the DNA extraction procedures.

The PCR assay was carried out by using the primer pair FAP59 (forward: 5'-TAACWGTIGGICAYCCWTA TT-3') and FAP64 (reverse: 5'-CCWATATCWWHCAT ITCICCATC-3') (Forslund *et al.*, 1999), with modifications (Claus *et al.*, 2007). Aliquots from the PCR products were analyzed by electrophoresis in a 2% agarose gel in TBE buffer, pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA) at constant voltage (90 V) for approximately 45 min, stained with ethidium bromide (0.5 μ g/mL), and visualized under UV light.

PCR bands suggestive of PV amplification were excised from the agarose gel and purified by using illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont, UK). Direct sequencing was then performed in the 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, USA), with the FAP59 and FAP64 primers. The obtained sequences were examined with the

PHRED software for quality analysis of chromatogram readings. The sequences were accepted if the base quality was equal to or higher than 20. Consensus sequences were determined by using the CAP3 software, and the sequence identity was verified with all sequences deposited in the GenBank by using the BLAST software. The guidelines of the Papillomavirus Nomenclature Committee 1995 (14th International Papillomavirus Conference, Quebec City, Quebec, Canada) were followed to identify PV types (de Villiers *et al.*, 2004).

Grossly, all cutaneous lesions were exophytic and were histologically classified as benign squamous cell neoplasms (cutaneous papilloma) irrespective of the type of virus isolated. Microscopically, all tumor growths demonstrated similar histological features; being characterized by varying degrees of hyperkeratosis or parakeratosis with elongated digital-like proliferation of the squamous epithelium (Figure 1A). In all tumors fragments evaluated, most keratinocytes within the stratum spinosum demonstrated clear perinuclear halo, some having pyknotic nucleus (characterized as koilocytes) others revealed discrete ballooning degeneration; in some areas, two or more adjacent degenerated cells fused to produce microvesicles (Figure 1B-D). Further, there was acanthosis, reduced mitotic index (1–2 per 40x Obj.), foci of apoptosis of squamous epithelium, and severe accumulations of irregular keratohyalin granules within cells of the stratum granulosum. However, characteristic basophilic intranuclear viral inclusion bodies were not observed.

Amplicons of the expected length, approximately 480 bp, were obtained from each of the seven teat papilloma DNA samples, via the FAP PCR assay. Negative controls were not amplified.

Through direct sequencing of the FAP products, BPV6, 7, 9, and 10 were identified in the examined papilloma specimens. Two cows with more than one teat papillomas evaluated were coinfecting by different BPV types (BPV7 and 10, and BPV6 and 9, respectively). The BPV strains identified were designated as BPV6/BR-UEL, BPV7/BR-UEL, BPV9/BR-UEL, and BPV10/BR-UEL (GenBank accession numbers: HM245430, HM245431, HM245433, and HM245432, respectively).

The gross characteristics of the cutaneous lesions as well as the PV type classification and GenBank accession numbers of the obtained sequences are indicated in Table 1. However, an association between the macroscopic characteristics and the viral type present in the cutaneous teat lesions was not observed in our analysis.

BPV types 7, 9, and 10, in addition to BPV type 6, were identified as causative agents of warts in dairy cow teats. The histopathological findings of this study are consistent with those lesions associated with BPV (Ginn *et al.*, 2007; Hargis and Ginn 2012). Although viral inclusions were not observed, the characteristic cytopathic effects in-

duced by this virus (Ginn *et al.*, 2007; Hargis and Ginn 2012) was identified in all tissue fragments evaluated.

The current taxonomic classification system for the *Papillomaviridae* family has recently proposed the introduction of the taxonomic terms “species” and “genus” for this viral family, establishing the relationship between

phylogenetic assemblages and biological and pathological properties shared by viruses grouped together (de Villiers *et al.*, 2004). The three *Xipapillomavirus* representatives (BPV6, 9, and 10) identified in our study were found determining true papillomas in teat skin. These histological features are in agreement with the biological properties de-

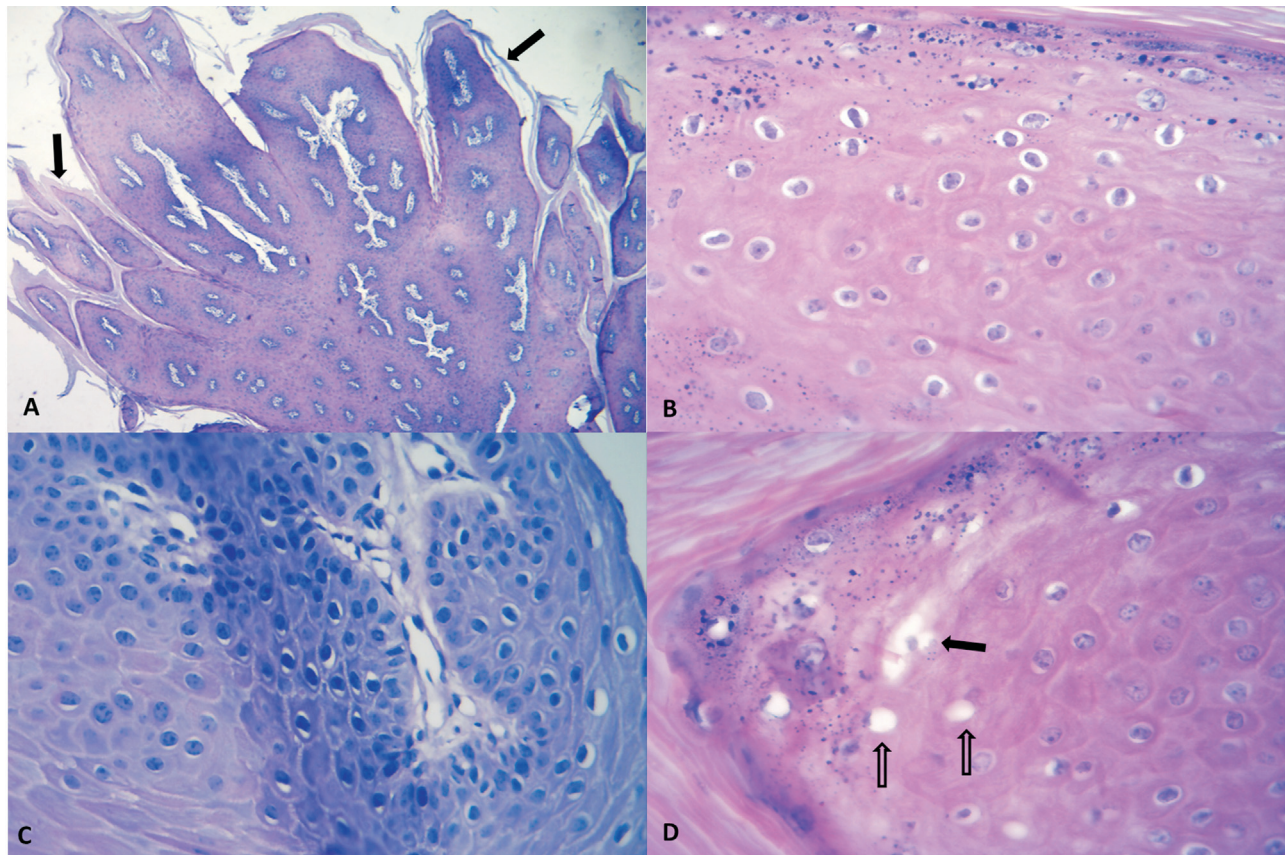


Figure 1 - Histopathological features of teat papillomas; skin, mammary gland, cow. There is outward digital-like proliferation of the squamous epithelium with hyperkeratosis (arrows) of the stratum corneum (A). Observe that the nuclei of most keratinocytes are surrounded by a clear halo (B) and the severely condensed basophilic-staining (pyknotic) nucleus (C) of the koilocytes. There are several degenerated keratinocytes (open arrows) and observe some that adjacent swollen cells are coalesced into a larger microvesicle (closed arrow) (D). (A, Hematoxylin and eosin, 4 x Obj.; B-D, Hematoxylin and eosin, 40 x Obj.).

Table 1 - Lesion characteristics and classification of BPV strains identified in teat lesions from dairy cattle herds.

| Herd | Animal | Lesion | Macroscopic characteristics of true papillomas | Nucleotide sequence | |
|------|--------|--------|--|---------------------|---------------------------|
| | | | | BPV classification | GenBank accession number* |
| 1 | A | A1 | Filiform | 7 | HM245431 |
| | B | B1 | Cauliflower | 7 | - |
| | | B2 | Cauliflower | 10 | HM245432 |
| | | B3 | Cauliflower | 10 | - |
| 2 | C | C1 | Cauliflower | 6 | HM245430 |
| | | C2 | Flat and round | 6 | - |
| | | C3 | Flat and round | 9 | HM245433 |

*A single representative of each BPV type had its sequence deposited at the GenBank database.

scribed for this specific genus (de Villiers *et al.*, 2004). Regarding the histological characteristics of BPV7-induced cutaneous lesions, this viral type also gave rise to true epithelial papilloma at teat skin. The histological analyses of cutaneous lesions determined by BPV7 were lacking in the study that established its phylogenetic position and characterized its genome (Ogawa *et al.*, 2007). Therefore, based on our findings, it is likely that future isolates that are to be grouped with BPV7 in its yet unnamed genus should also be able to induce true cutaneous papillomas in cattle.

The identification of four different viral types in teat warts herein evaluated demonstrates the high diversity in BPV types involved in this specific condition. Although BPV6 is the viral type that is classically associated with teat papilloma worldwide, our findings of diverse BPV types are in agreement with the results obtained by other studies investigating viral diversity in Japanese cattle herds affected by teat papillomatosis. Such investigations have confirmed the high BPV diversity that occurs in benign teat lesions and healthy teat skin, in a commensal form (Campo 2002; Ogawa *et al.*, 2004; Maeda *et al.*, 2007). To the best of the authors' knowledge, this study represents the first identification of the BPV type 7 in cattle from South America. The identification of this BPV type in a geographical region not related to the location where it was first isolated, Japan, supports the hypothesis that this viral type is widespread and affect dairy cattle herds worldwide (Ogawa *et al.*, 2007; Hatama *et al.*, 2008). Previous studies involving the identification of BPV types 9 and 10 have documented that these viral types are easily detected from naturally occurring teat papilloma but not from healthy teat skin (Ogawa *et al.*, 2004; Maeda *et al.*, 2007; Hatama *et al.*, 2008). This observation correlates well with our findings that confirm the specific association of these viral types with teat papillomatosis. More recently, the tumorigenic potential of BPV9 was tested through experimental infection of the teat skin of heifers, resulting in the development of papillomatosis (Hatama *et al.*, 2009). Conversely, BPV7 has been detected mainly from swab samples of healthy teat skin, and besides the identification herein reported, it was identified in only a few teat lesions from dairy cattle in Japan (Ogawa *et al.*, 2004).

The identification of BPV7 in teat papillomas from Brazilian cattle coupled with data obtained in a recent study involving genotyping of viral strains associated with cutaneous warts from other anatomical sites from cattle from the northeastern region, clearly indicates that almost all fully characterized BPV types (BPV1 to 11) are circulating in Brazilian herds (Carvalho *et al.*, 2012). Besides the high diversity verified in affected herds, the presence of multiple viral types in different lesions evaluated from a single individual was demonstrated in this study. Previously, other investigations showed that the occurrence of coinfections in a single lesion, adding up two to six viral types, is a common

field event (Claus *et al.*, 2009a; Schmitt *et al.*, 2010; Carvalho *et al.*, 2012). Surprisingly, we found that BPV6 and 7 produced lesions with different gross appearances. Whether the differences observed are associated with the presence of undetected viral types establishing coinfections is yet to be clarified.

The limited number of recent studies aiming to identify BPV types associated with lesions of the mammary gland of cattle has reported a considerable diversity of the viral types involved. Therefore, the viral type (BPV6) that has been classically associated with this anatomical location is not the only causative agent of these lesions in affected herds.

Detailed epidemiological investigations conducted in different geographical areas are required to define the most prevalent BPV types associated with this disease. Since the host immunity against PV infections is type-specific, the availability of such information would allow the elaboration of a multivalent immunogen based on antigens from the viral types capable of causing cutaneous papillomas. It is noteworthy to remember that currently there are no commercially available alternatives for the effective treatment or prevention of teat papillomatosis.

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