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A N I N T E R N A T I O N A L J O U R N A L

A Bovine Teat Papilloma Specimen Harboring *Deltapapillomavirus* (BPV-1) and *Xipapillomavirus* (BPV-6) Representatives

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ABSTRACT

The common occurrence of multiple papillomavirus infections has been shown in several studies involving the human host. However, investigations with the aim of identifying mixed papillomavirus infections in cattle have been conducted only recently. In the current work we describe a co-infection with two different bovine papillomavirus (BPV) types that was identified in a bovine teat papilloma. The skin wart was obtained from a cow belonging to a Brazilian beef herd. A PCR assay was carried out with the FAP primer pair, which amplifies a partial segment of the L1 gene (approximately 478 bp), and the amplicon was submitted to direct sequencing. Because nucleotide sequences with satisfactory quality scores were not obtained, the amplicon was cloned and further sequencing, involving ten selected clones, was performed. The sequence analysis of the cloned inserts revealed the presence of two different BPV types. BPV-1 (Deltapapillomavirus genus) was detected in six clones, while BPV-6 (Xipapillomavirus genus) was detected in four clones. This finding confirms the presence of BPV co-infection associated with cutaneous papillomatosis in cattle.

Key words: cattle**,** cutaneous papillomatosis, BPV, co-infection.

INTRODUCTION

The papillomaviruses (PVs) are a highly diverse group of viruses that infect mucous and cutaneous epithelia and are able to induce hyperplastic lesion in most mammals and birds (Knowles et al., 1996; de Villiers et al., 2004). However, it has been reported that PVs can be detected in healthy skin from both humans and animals as commensal agents (Antonsson and Hansson, 2002; Ogawa et al., 2004).

PVs are small, nonenveloped, circular double-stranded DNA viruses classified in the *Papillomaviridae* family. This viral family is complex and includes 18 genera and more than a hundred PV types. Different genera share <60% nucleotide sequence identity in the L1 ORF, and a distinct PV type is recognized when this identity is

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less than 90% (de Villiers et al., 2004; Bernard, 2005).

More than 100 types of human papillomaviruses (HPVs) have been described while only dozens of types have been confirmed in animal hosts (de Villiers et al., 2004). This is likely a result of a bias in research efforts rather than a true difference in viral diversity (Chan et al., 1997).

Although the number of characterized bovine papillomaviruses is higher than the number of characterized viral types for any other animal species, BPV has been the subject of much less research than has HPV. However, recent studies have suggested that a similar viral diversity is present in cattle. Currently, only 10 types of bovine papillomavirus (BPV-1 to -10) are known, and they are classified in the genera *Deltapapillomavirus* (BPV-1 and -2), *Epsilonpapillomavirus* (BPV-5 and -8), and *Xipapillomavirus* (BPV-3, -4, -6, -9, -10), with the remaining virus (BPV-7) belonging to an unassigned genus (de Villiers et al., 2004; Ogawa et al., 2007; Tomita et al., 2007; Hatama et al., 2008).

In recent years, the strategy of partial amplification of the L1 gene using a PCR assay with degenerate primers followed by sequencing has allowed the identification of a broad range of new and putative new PV types in both human and animal hosts (Forslund et al., 1999; Antonsson and Hansson, 2002). In cattle, these molecular tools led to the identification of four new BPV types in the last few years (Ogawa et al., 2007; Tomita et al., 2007; Hatama et al., 2008). In addition, BPV types previously described and putative new BPV types were identified in both papillomas and healthy skin from cattle herds in Sweden, Japan, and Brazil (Antonsson and Hansson, 2002; Ogawa et al., 2004; Claus et al., 2008; Claus et al., 2009a).

The identification of multiple HPV infections in the same patient or even in the same lesion has been a relatively common finding (Berkhout et al., 1995; Kay et al., 2002). On the other hand, investigations with the aim of detecting mixed BPV infections have been conducted only recently. These works have demonstrated the occurrence of multiple BPV infections in cutaneous lesions as well as in healthy skin from cattle herds (Antonsson and Hansson, 2002; Ogawa et al., 2004; Maeda et al., 2007; Claus et al., 2009b).

The aim of this work is to describe the identification of a co-infection with two different BPVs in the same bovine teat papilloma from a cow in a Brazilian cattle herd.

MATERIALS AND METHODS

Papilloma specimen

A single skin wart was obtained from an adult cow belonging to a beef cattle herd with endemic cutaneous papillomatosis from the northern region of the Paraná state in southern Brazil. To minimize the risk of cross-contamination between different lesions, the following precautions were taken: i) the sample was collected by hand (wearing gloves) and stored individually until the DNA extraction procedure; and ii) all materials used in the analysis were sterilized and disposable. The papilloma specimen was ground in phosphate-buffered saline solution (PBS, pH 7.2), and the suspension (20% w/v) was centrifuged for 15 min at 3000 x *g* at 4°C. An aliquot (250 µL) from the supernatant was treated with lysis buffer [10mM Tris, 1mM EDTA, 0.5% Nonidet P40, 1% SDS, and 0.2 mg/mL proteinase K (Invitrogen, USA)]. After homogenization, the sample was incubated at 56°C for 30 min.

DNA extraction

For DNA extraction, a combination of the phenol/chloroform/isoamyl alcohol and the silica/guanidine isothiocyanate methods was carried out according to Alfieri et al. (2006). Briefly, a fraction of the sample was treated with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), homogenized, and heated at 56°C for 15 min (Sambrook and Russell, 2001). After centrifugation at 10,000 x *g* for 10 min, the aqueous phase was processed according to the silica/ guanidine isothiocyanate method (Boom et al., 1990). DNA was eluted in 50 µL of sterile ultrapure (MilliQ®) water and kept at -20°C until use. An aliquot of sterile ultrapure water was included as a negative control in the DNA extraction procedure.

PCR assay

The PCR assay was carried out using the primer pair FAP59 (forward; 5'-TAACWGTIGGICAYCCWTATT-3') and FAP64 (reverse; 5'-CCWATATCWVHCATITCICCATC-3') according to Forslund et al. (1999), with slight modifications (Claus et al., 2007). A 5 µL aliquot

of the PCR product was analyzed by electrophoresis on a 2% agarose gel in TBE buffer, pH 8.4 (89 mM Tris, 89 mM boric acid, 2 mM EDTA) run at a constant voltage (90V) for approximately 45 min. The gel was stained with ethidium bromide (0.5 µg/mL) and visualized under UV light.

Sequence analysis and cloning

The obtained PCR product was purified using a PureLink Quick Gel Extraction Kit (Invitrogen, USA). Initially, a direct sequencing reaction was performed using the DYEnamic ET dye terminator cycle sequencing kit (GE Healthcare, UK) with the FAP59 and FAP64 primers in a MegaBACE 1000/Automated 96 Capillary DNA Sequencer (GE Healthcare, UK) according to the manufacturer's instructions. Since nucleotide sequences with satisfactory quality scores were not obtained, the amplicon was cloned using the TOPO TA Cloning kit for Sequencing (Invitrogen, USA) according to the manufacturer's instructions. Sequencing reactions employing the plasmid DNA from ten selected clones were performed in both directions using M13 forward and reverse primers.

The obtained sequences were examined with PHRED software for quality analysis of the chromatogram readings. The sequences were accepted if the base quality was equal to or greater than 20. Consensus sequences were determined by CAP3 software, and sequence identity was verified with all sequences deposited in GenBank using BLAST software.

RESULTS AND DISCUSSION

PV DNA could be detected in the papilloma specimen using the PCR assay with the FAP primers, which resulted in a single band of approximately 480 bp in length, with high quality.

Despite several attempts, the FAP amplicon did not produce adequate nucleotide sequences, and the BPV type in the corresponding papilloma could not be determined by direct sequencing. Considering the difficulty of obtaining good sequences from this PCR product, the presence of co-infection with multiple BPV types was suspected.

The sequence analysis of 10 clones revealed that the PCR product was generated from two different BPV types. BPV-1 was detected in six clones, while the BPV-6 was detected in four clones. The obtained nucleotide sequences showed 100% similarity with BPV-1 and BPV-6. This result confirms that the bovine teat papilloma analyzed was harboring two BPV types, one belonging to the *Deltapapillomavirus* genus (BPV-1) and the other to the *Xipapillomavirus* genus (BPV-6). A similar circumstance was experienced by Maeda et al. (2007) when analyzing three teat papilloma specimens; however, all BPV types detected belonged to a unique genus.

Reports of co-infections with different HPV types are frequent in human patients, mainly under immunosuppression conditions. In a study involving renal transplant recipients, more than 30% of the PCR-positive samples were shown to harbor more than one HPV type (Berkhout et al., 1995). Nucleotide sequence analysis of cloned amplicons revealed five different epidermodysplasia verruciformis (EV) - HPV L1 sequences in a premalignant keratosis, and three distinct EV - HPV types were found in a squamous cell carcinoma (SCC) from a renal transplant recipient. Previous analyses of these biopsies identified a single HPV type through direct sequencing of PCR products (Bens et al., 1998). In Brazil, multiple HPV types were detected in 161 (78.9%) specimens collected from a group of 208 immunodeficiency virus-infected women (Levi et al., 2002).

In cattle, Ogawa et al. (2004) described co-infections with different BPV types in herds from Japan. In that study, BPV co-infections could be demonstrated in a considerable number of both teat papilloma and healthy skin samples, with most of them characterized as double infections. In another work that evaluated the BPV types involved in an outbreak of teat papillomatosis, three out of fourteen samples analyzed also revealed double infections (Maeda et al., 2007).

Investigations using healthy skin swabs have also demonstrated the occurrence of double infections in cattle, probably as a latent condition (Antonsson and Hansson, 2002; Ogawa et al., 2004; Maeda et al., 2007). However, the importance of the identification of a multiple infection in healthy skin should be interpreted carefully since a wide variety of different PV types can be detected in normal skin samples and seem to occur in a commensal form.

The main impediment in the characterization of the PV type is the absence of a conventional cell culture system for *in vitro* virus propagation. Thus,

adequate molecular techniques are fundamental for the diagnosis of PV infections and for identification of the viral type.

The partial amplification of the L1 gene by PCR with degenerate primers followed by sequencing has provided a considerable advancement in the knowledge of PV epidemiology. In some situations, the molecular strategy of cloning and sequencing the generated inserts can be an important tool for detection of co-infections.

In some cattle herds, BPV infections are considered an important health problem. However, knowledge of the important epidemiological aspects of BPV infection is still restricted worldwide. Despite bovine species being the most studied animal host for PV infection, this is the first description of a BPV co-infection associated with cutaneous papillomatosis outside of Japan.

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RESUMO

Em seres humanos, as infecções múltiplas pelo papilomavírus têm sido demonstradas em vários estudos. Em bovinos, somente recentemente foram conduzidas investigações com o objetivo de avaliar infecções mistas pelo papilomavírus. O presente trabalho teve como objetivo descrever a co-infecção por dois tipos de papilomavírus bovino (BPV) em um papiloma de teto. A amostra clínica foi obtida de uma vaca pertencente a um rebanho de corte localizado na região norte do estado do Paraná, Brasil. Inicialmente, a técnica de PCR foi realizada com o par de oligonucleotídeos iniciadores FAP, que amplificam um segmento do gene L1, sendo que o amplicon gerado foi submetido ao sequenciamento direto. Entretanto, como as sequências obtidas não apresentaram qualidade aceitável, o amplicon foi clonado e dez clones foram selecionados para um novo sequenciamento. A análise das sequências dos insertos revelou a presença de dois diferentes tipos de BPV. O BPV-1 (gênero *Deltapapillomavirus*) foi detectado em seis clones, enquanto o BPV-6 (gênero *Xipapillomavirus*) foi detectado em quatro clones. Esse resultado confirma a ocorrência da co-infecção pelo BPV associada a papilomas cutâneos em bovinos.

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