Bovine Papillomavirus Type 13 DNA in Equine Sarcoids

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Equine sarcoids are locally aggressive fibroblastic neoplasms considered to be the most common skin tumors of horses worldwide. Bovine papillomavirus types 1 and 2 have typically been associated with sarcoids in equids. Investigations aiming to identify papillomavirus strains, aside from bovine papillomaviruses 1 and 2, which might be associated with sarcoid lesions, have been lacking. The aim of this article is to report the identification of a third bovine papillomavirus type, bovine papillomavirus 13, associated with equine sarcoids. Six sarcoid lesions were collected from diverse anatomical sites on two horses from southern Brazil. To detect a broad spectrum of papillomavirus strains, eight degenerate primer pairs designed to detect conserved regions on the L1 and E1 genes were tested on the DNA samples. Direct sequencing was then performed on the obtained amplicons, and sequence identities were compared with sequences from all bovine papillomavirus types. The FAP59/FAP64, MY09/MY11, and AR-E1F2/AR-E1R4 sequences generated from the sarcoids were shown to present 99 to 100% identity with bovine papillomavirus 13, a new bovine papillomavirus type previously described in cattle. The results from this study suggest that there is a need to identify bovine papillomavirus type 13 and other papillomavirus strains that might be associated with sarcoids in diverse geographical areas; such investigations might establish the frequency of occurrence of this viral type in these common tumors of equids.

Equine sarcoids are locally aggressive fibroblastic neoplasms considered to be the most common skin tumors in horses worldwide. Other equids, such as zebras, donkeys, and mules, are also affected. These tumors rarely regress and very often recur after therapy. Clinically, sarcoids can vary in appearance and are classified into six different clinical types, occult, verrucous, nodular, fibroblastic, mixed, and malevolent (1).

Once equine sarcoids were shown histologically to resemble the fibrotic section of bovine fibropapillomas, the involvement of bovine papillomaviruses (BPVs) in their etiology was suspected (2, 3). The intradermal inoculation of cell-free extract from cattle warts into healthy horses represented the first successful attempt to demonstrate an association between sarcoids and BPVs and resulted in the growth of sarcoid-like lesions (4). More recently, the consistent identification of BPV DNA and the demonstration of the expression of diverse viral genes in sarcoids have corroborated the evidence of a direct involvement of BPV in the pathogenesis of sarcoids (5–11).

BPV types 1 and 2 (BPV1 and BPV2), which are known to cause bovine fibropapillomas, have typically been associated with equine sarcoids. Previous investigations established this association either through DNA-DNA hybridization using BPV1- and BPV2-labeled genomes as probes or by PCR assays with type-specific primers (5, 12, 13).

Variations in the frequencies of recovery of BPV1 and -2 from sarcoids have been demonstrated by studies conducted in diverse geographic areas. While BPV1 has been shown to be more prevalent in affected horses from Europe and Australia (5, 14, 15), BPV2 has been more commonly reported in lesions from horses in the western United States and Canada (10, 16).

Currently, 13 BPV types from cattle have been identified and characterized. These BPVs are classified in the genera *Deltapapillomavirus* (BPV1, -2, and -13), *Xipapillomavirus* (BPV3, -4, -6, -9, -10, -11, and -12), and *Epsilonpapillomavirus* (BPV5 and -8), with the exception of BPV7, which belongs to an as-yet-undesigned

Papillomavirus genus (17, 18). In addition to the fully sequenced BPV types, numerous putative new BPV types from diverse geographical regions have been detected in cattle herds through PCR assays employing consensus or degenerate primers, which amplify partial fragments of the L1 gene (19, 20). An investigation using PCR with degenerate primers revealed notable diversity among the BPVs detected in papillomas from Brazilian cattle herds. The study identified four putative new BPV types designated BPV/BR-UEL2 to BPV/BR-UEL5 (21). Recently, the sequencing of the complete genome of the BPV/BR-UEL4 strain (also named BPV type 13) enabled its classification as a third member of the *Deltapapillomavirus* genus (18).

Investigations aiming to detect and characterize papillomavirus (PV) strains, aside from BPV1 and -2, which might be associated with sarcoids, have been lacking. The aim of this article is to report the identification of a third BPV type in equine sarcoids.

MATERIALS AND METHODS

Sarcoid lesions. Six sarcoid lesions were individually and surgically collected from diverse anatomical locations of two horses from a farm located in the northern region of Parana state in southern Brazil (Table 1). A portion of each lesion was fixed in 10% buffered formalin and routinely processed for histopathological evaluation. The remaining fragment of each tumor was kept at –20°C for molecular analyses.

DNA extraction. Fragments from each specimen were ground in liquid nitrogen before the DNA extraction procedure. The DNA extraction

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TABLE 1 Anatomical distribution and pathological classifications of evaluated sarcoids

Animal	Age (mo)	Gender	Breed	Location of sarcoid	Pathological classification
A	20	Male	American quarter horse	Ear Muzzle Cheek	Verrucous Verrucous Fibroblastic
B	17	Female	American quarter horse	Ear Neck Cheek	Verrucous Verrucous Fibroblastic

was performed with the protocol for purification of total DNA from animal tissues in the DNeasy blood and tissue kit (Qiagen, Hilden, Germany).

PCR amplification of partial fragments of the L1 and E1 genes. To detect potential additional PV strains that might have been associated with equine sarcoids, eight degenerate primer pairs designed to detect the conserved regions of the L1 and E1 genes of a broad spectrum of diverse cutaneous PV strains were tested on all of the DNA samples. Table 2 shows the sequences and features of the primers employed.

PCR mixtures consisted of 2 μ l of the extracted DNA and 48 μ l of PCR mix, which included 1 μ M each primer, 200 μ M each deoxynucleoside triphosphate (dNTP) (Invitrogen, Life Technologies, Carlsbad, CA), 2.5 U of Platinum *Taq* DNA polymerase (Invitrogen, Life Technologies, Sao Paulo, Brazil), 1 $\times$ PCR buffer (20 mM Tris-HCl [pH 8.4] and 50 mM KCl), 1.5 mM MgCl₂, and ultrapure sterile water to make up the final volume. Amplification was performed with the following cycling profile: an initial step of 5 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at an optimal temperature for primer annealing, and 1 min at 72°C, and a final extension step of 5 min at 72°C. Aliquots from the PCR-amplified products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.5 mg/ml), and examined under UV light.

PCR amplification of the entire L1 gene. To amplify the entire sequence of the L1 gene from PV strains isolated from evaluated equine sarcoids, the sequences of the L2 genomic region and long control region (LCR) of the recently reported BPV13, obtained from a specimen collected from a cow, were used to select a primer set able to target the complete L1 nucleotide sequence of equine sarcoid PV strains.

PCR mixtures consisted of 2 μ l of the extracted DNA and 48 μ l of PCR mix, which included 1 μ M each BPV13 L1 forward primer (5'-GCCACA CCATTGACCTCTA-3') and BPV13 L1 reverse primer (5'-GCCAGCAA AGCGATTATTC-3'), 200 μ M each dNTP (Invitrogen, Life Technologies, Carlsbad, CA), 2.5 U of Platinum *Taq* DNA polymerase (Invitrogen, Life Technologies, Sao Paulo, Brazil), 1 $\times$ PCR buffer (20 mM Tris-HCl [pH 8.4] and 50 mM KCl), 1.5 mM MgCl₂, and ultrapure sterile water to make up the final volume. Amplification was performed with the following cycling profile: an initial step of 5 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min 30 s at 72°C, and a final extension step of 10 min at 72°C.

Sequencing and sequence analyses. Initially, all PCR products were purified using the illustra GFX PCR DNA and gel band purification kit (GE Healthcare, Little Chalfont, United Kingdom). Direct sequencing was then performed using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA) with the corresponding forward and reverse primers in the 3500 genetic analyzer (Applied Biosystems), according to the manufacturer's instructions. The sequences obtained were examined with the Phred application for quality analysis of chromatogram readings. The sequences were accepted if the base quality was ≥ 20 . The consensus sequences were determined by CAP3 software, and the sequence identities were compared with those of all sequences deposited in GenBank using the BLAST program.

Phylogenetic analysis. Pairwise and multiple sequence alignments at the nucleotide and amino acid levels and sequence similarities were calculated using ClustalW in MEGA v5 software (26). The L1 nucleotide sequences of all previously sequenced BPV types, of other even-toed ungulate PV types, and of the new viral type BPV13 were included in the analysis. Phylogenetic trees were reconstructed from L1 nucleotide sequence alignment by the maximum-likelihood method with the general

TABLE 2 Sequences and features of degenerate primers used for PCR amplification of partial sequences of BPV type 13

Primer	Genomic region target	Polarity	Sequence (5'-3') ^a	Nucleotide position ^b	Degree of degeneracy	Expected amplicon length (bp)
AR-E1F1 ^c	E1	Forward	CAGGGVMWTTCCCTGBARYTGTTYC	962–986	288	836
AR-E1R2 ^c	E1	Reverse	TCATANGCCCACTGNACCAT	1797–1778	16	
AR-E1F2 ^c	E1	Forward	ATGGTNCAGTGGGCNTATGA	1778–1797	16	552
AR-E1R4 ^c	E1	Reverse	ATTNCCATCHADDGCATTCT	2329–2309	108	
AR-L1F1 ^c	L1	Forward	TTDCAGATGGCNGTNTGGCT	5425–5444	48	974
AR-L1R5 ^d	L1	Reverse	CCATRTTHWKDCCYTG	6398–6382	144	
AR-L1F8 ^c	L1	Forward	GGDGAYATGDGKGAMATWGG	6016–6035	144	704
AR-L1R9 ^c	L1	Reverse	GGRCATTTKGTWGCWADGGA	6719–6697	48	
AR-E1F2 ^c	E1	Forward	ATGGTNCAGTGGGCNTATGA	1778–1797	16	371
AR-E1R3 ^d	E1	Reverse	TTNCCWSTATTTNGGNGGNCC	2148–2129	1,024	
AR-L1F1 ^c	L1	Forward	TTDCAGATGGCNGTNTGGCT	5425–5444	48	600
AR-L1R3 ^c	L1	Reverse	CATRTCHCCATCYTCWAT	6024–6007	24	
FAP59 ^e	L1	Forward	TAACWGTNGGNCAYCCWTATT	5558–5578	128	480
FAP64 ^e	L1	Reverse	CCWATATCWVHCATNTCNCCATC	6035–6013	576	
MY09 ^f	L1	Forward	GCMCAGGGWCATAAAYAATGG	6379–6398	8	450
MY11 ^f	L1	Reverse	CGTCCMARRGGAWACTGATC	6830–6811	16	

^a Degenerate nucleotides: B = T, C, or G; D = A, T, or G; H = A, T, or C; K = T or G; M = A or C; N = A, G, C, or T; R = A or G; S = C or G; V = A, C or G; W = A or T; Y = C or T.

^b Position relative to the sequence of human papillomavirus type 1a (HPV1a).

^c From reference 22.

^d From reference 23.

^e From reference 24.

^f From reference 25.

FIG 1 Histological features characteristic of equine sarcoids observed in the lesion collected from the ear of animal A and macroscopically classified as the verrucous type. Discrete epidermal hyperplasia and inward projections of the epidermis (rete pegs, arrow) into the dermis can be observed, as can the proliferation of fibroblasts arranged in short interlacing bundles. Hematoxylin and eosin (H&E) staining was used.

time reversible model using MEGA v5 software. Bootstrap support values were determined for 1,000 replications.

The GenBank accession numbers of the sequences we used were M15953 (AaPV1), X02346 (BPV1), M20219 (BPV2), AF486184 (BPV3), X05817 (BPV4), AF457465 (BPV5), AJ620208 (BPV6), DQ217793 (BPV7), DQ098913 (BPV8), AB331650 (BPV9), AB331651 (BPV10), AB543507 (BPV11), JF834523 (BPV12), JQ798171 (BPV13), EF680235 (CcaPV1), HQ912790 (CdPV1), HQ912791 (CdPV2), U83594 (OaPV1), U83595 (OaPV2), M11910 (OvPV1), and AF443292 (RtPV1).

Gene sequence accession numbers. The partial E1 gene sequence and the complete L1 gene sequence of a selected equine sarcoid lesion were deposited in the GenBank database, developed by the National Center for Biotechnology Information (NCBI), with the accession numbers [KC763354](#) and [KC763355](#), respectively.

RESULTS

Using the pathological classification of equine sarcoids in which six different gross morphological types can be recognized (1), we categorized the evaluated lesions as fibroblastic or verrucous (Table 1).

The six skin lesions were confirmed to be equine sarcoids by histopathological evaluation. Characteristic features such as epithelial extensions projecting into the underlying dermis (rete pegs) and proliferating dermal fibroblasts forming whorls or interlacing bundles were observed in the sarcoid tumor specimens. In addition, epidermal hyperplasia and hyperkeratosis, particularly in verrucous tumors, and perpendicularly arranged fibroblasts at the dermoepidermal junction (picket fence) were identified (Fig. 1).

PCRs employing the primer pairs FAP59/FAP64, MY09/MY11, and AR-E1F2/AR-E1R4 yielded amplicons of the expected molecular size for DNA samples from all lesions evaluated.

To amplify the entire sequence of the L1 gene, the primer pair BPV13 L1 forward/BPV13 L1 reverse was tested on all DNA samples; an amplicon of approximately 1,800 bp in length, consisting of part of the L2 gene, the complete L1 gene, and a partial fragment of the LCR, was generated.

Using the BLASTN program, we showed that all of the sequences generated from the sarcoids demonstrated 99 to 100% identity with BPV13, a new BPV type isolated from a bovine cutaneous wart (18).

The partial nucleotide sequences of the putative E1 gene obtained from all equine lesions presented identities of 90.7% and 91.5% with the deltapapillomaviruses (Delta-PVs) BPV1 and -2, respectively, which are classically associated with sarcoids. The corresponding predicted amino acid sequences exhibited similarities of 97.7 and 96.6% with the same viral types. The identities shared with other representatives of the *Deltapapillomavirus* genus varied from 64.3 to 68.9% (for nucleotide sequences) and from 61.9 to 71.6% (for amino acid sequences).

The complete L1 nucleotide sequences obtained from the equine samples presented identities of 85.3 and 88.7% with the BPV1 and -2 nucleotide sequences, respectively, whereas the corresponding predicted amino acid sequences exhibited similarities of 93.9 and 94.8%, respectively. Similarities with sequences of other Delta-PVs ranged from 65.5 to 68.4% (for nucleotide sequences) and from 64.7 to 76.2% (for amino acid sequences).

Phylogenetic analysis based on the complete L1 nucleotide sequence from BPV13 obtained from equine sarcoid DNA samples and the corresponding sequences from all other previously described BPV types and from other even-toed ungulate PV types confirmed that this viral strain can be grouped with other *Deltapapillomavirus* representatives (Fig. 2).

DISCUSSION

In this study, we described the identification of BPV type 13, the most recently classified viral type in the *Deltapapillomavirus* genus, in equine sarcoids from two horses from southern Brazil.

The *Deltapapillomavirus* genus consists of papillomaviruses that are known to infect diverse species of wild and domestic even-toed ungulates. Currently, six different viral species are classified in this genus: (i) Delta-PV1 contains viral strains from *Alces alces* (European elk) and *Rangifer tarandus* (reindeer); (ii) Delta-PV2 contains the isolate identified in *Odocoileus virginianus* (American white-tailed deer); (iii) Delta-PV3 clusters PV types obtained from *Ovis aries* (domestic sheep); (iv) Delta-PV4 groups together viral types that infect *Bos taurus* (domestic cow); (v) Delta-PV5 includes a virus isolated from *Capreolus capreolus* (Western roe deer); and (vi) Delta-PV6 consists of two PV strains isolated from *Camelus dromedarius* (dromedary camel). In addition to causing fibropapillomas in their respective hosts, some representatives of this genus are also capable of transspecies transmission. BPV1 and -2, which were first isolated from cattle, have been detected in equine sarcoids worldwide (17, 27).

The phylogenetic tree obtained using the complete L1 nucleotide sequences from BPV type 13 isolated from equine sarcoids, from other previously described BPV types, and from other even-toed ungulate PV types indicated that this viral strain can be grouped with representatives from Delta-PV4 species. In addition, the highest level of identity encountered with BPV1 and -2 in the L1 pairwise alignment, the histopathological alterations similar to those verified in BPV1- and BPV2-induced sarcoids, and the capacity to infect cattle and horses confirm the current classification of this strain in the *Deltapapillomavirus* genus. Because BPV type 13 has been classified in the same genus as all viral types initially isolated from cutaneous warts from cattle, which have been consistently associated with sarcoids (the *Deltapapillomavi-*

FIG 2 Maximum-likelihood phylogenetic tree reconstructed from L1 nucleotide sequences of all previously sequenced BPVs, of other even-toed ungulate PVs, and of BPV type 13 obtained from equine sarcoid DNA samples. The tree is divided into the previously determined genera *Deltapapillomavirus* (δ), *Epsilonpapillomavirus* (ϵ), and *Xipapillomavirus* (ξ) and an undesignated *Papillomavirus* genus (BPV7). The numbers at the internal nodes represent the bootstrap support values (percentages) determined for 1,000 replications. The scale bar indicates the number of nucleotide substitutions per site.

rus genus), the identification of this virus in sarcoid lesions is to be expected biologically. However, the participation of this virus in the pathogenesis of sarcoids still has to be clarified experimentally.

Many studies have confirmed the presence of PVs in sarcoids from diverse geographical areas using PCR assays with BPV1/2-specific primers (5, 10, 14–16). To evaluate whether BPV type 13 could be amplified by PCR assays widely employed in previous epidemiological investigations, DNA samples from the six lesions were tested with primers designed previously (14) (data not shown). Interestingly, an amplicon of the expected size (approximately 250 bp), representing a partial sequence of the E5 open reading frame (ORF), was obtained from each sample evaluated. The nucleotide sequence generated from these amplicons had 94% identity with the corresponding E5 fragment of BPV2, confirming the occurrence of an infection with a different BPV type (in this case, BPV13). This was confirmed by differentiation between BPV1 and -2 based on the presence (BPV1) or absence (BPV2) of the BstXI restriction site in a fraction of the previously obtained PCR products. Thereafter, an *in silico* analysis of the sequences obtained suggested that BPV13 could be misclassified as BPV2 if this diagnostic system is used without direct sequencing of the obtained amplicons.

The detection of the BPV/BR-UEL4 strain through amplification and sequencing of a partial L1 gene fragment in a lesion obtained from an affected horse from another geographical area of Brazil (southeastern region, Rio de Janeiro state) confirms our finding (28).

Because there were approximately 40 papillomatosis-affected

cattle on the farm where the two horses were kept, we believe that these sarcoid-affected horses were likely infected by direct or indirect contact with infected cattle (29). The previous identification of the BPV/BR-UEL4 strain in cattle warts collected from two other farms in nearby cities corroborates this speculation (21).

Although the occurrence of equine sarcoids is reported in equids worldwide, investigations with the aim of genotyping PV strains associated with these lesions in South American herds are virtually absent. According to the findings presented in this study, investigations aiming to identify BPV type 13 and other PV strains that might be associated with sarcoids collected from herds from diverse geographical areas are needed to evaluate the frequency of occurrence of this new viral type in this common tumor of equids.

The identification of BPV13 in the sarcoids examined in this study gives rise to the hypothesis that a fraction of PV isolates previously diagnosed from sarcoids worldwide by PCRs using BPV1/2 E5-specific primers might have been misclassified as BPV2, especially as direct sequencing of numerous amplicons was not performed (10, 15, 29–31). To avoid the misidentification of PV types, the use of general PCRs with broad-spectrum primers and sequencing is recommended.

The utility of our findings relies on the capability of investigators to make adjustments to the diagnostic tools currently employed for the genotyping of PV strains found in association with sarcoid tumors. Furthermore, once the use of protective and curative vaccines based on BPV1 and -2 virus-like particles (VLPs) have been shown to be promising alternatives for preventing or treating equine sarcoids, the identification of a third BPV type (the

BPV13 strain) in equine sarcoids will be relevant for designing an immunogen against this common equid disease.

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