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Bovine Papillomavirus Type 1 in Brains of Cattle with a Neurological Syndrome: Pathological and Molecular Study

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Abstract

Bovine papillomavirus (BPV) infection is endemic in Brazilian herds. Papillomaviruses are oncogenic, with a trophic response in squamous epithelial and mucosal tissues, and are associated with asymptomatic infections, proliferative benign skin lesions (papillomas), and malignant epithelial lesions (carcinomas). The presence and expression of BPV in the blood of healthy and papillomatosis-affected cattle has been demonstrated. Experimental inoculation of Bovine papillomavirus (BPV) into calf meninges can result in meningiomas and papillomatosis, but it´s not known if its natural infection causes neoplasia and neurological syndrome in cattle. We assessed the frequency of BPV in 300 Central Nervous System (CNS) samples from cattle with neurological syndrome from several Brazilian regions obtained from surveillance of neurological syndrome. Samples were negative for rabies, Neospora caninum, BoHV-1 and BoHV-5, bovine leukemia virus, and catarrhal malignant fever (PCR). Samples were fixed in 10% buffered formalin and submitted to macroscopic examination. For histological analysis, slides were submitted to a staining protocol using hematoxylin and eosin. PCR for BPV detection was applied in CNS frozen samples using generic primers *FAP59* and *FAP64* (*L1* gene). Thirteen (4.3%) samples were positive for BPV by PCR, with 11 of these showing no pathological changes in microscopy, and two exhibiting nonspecific non-purulent meningoencephalitis. No CNS samples showed neoplasia. Nine of the 13 BPV positive samples (69.2%) came from females and four (30.8%) from males. The 13 positive animals were age 5 to 168 months with seven over 36 months (53.8%). Five were dairy cattle, four crossbred, and three beef cattle. Only one of the 13 positive samples provided sufficient BPV DNA for sequencing, which emonstrated 99% identity to samples of BPV-1 obtained from cutaneous papillomas in cattle in Brazil. The small quantity of BPV DNA in the CNS and the low number of PCR-positive samples may be associated with low neurotropism, unspecific inflammation, or BPV-infected lymphocytes in CNS tissues or bloodstream. Natural BPV-1 infection was not associated with cerebral neoplasia or neurological syndrome.

Keywords: BPV; Central nervous system; Meningoencephalitis; Histopathology; PCR; DNA sequencing; Phylogeny

Introduction

The Brazilian epidemiological surveillance of bovine neurological syndromes comprises programs to combat rabies, bovine spongiform encephalopathy, and other diseases with characteristic progressive neural symptoms (Ministry of Agriculture, Livestock and Supply) [1] and several regional surveys of cattle with central nerve system (CNS) disorders have been conducted [2-10]. Analysis conducted by the Biological Institute, São Paulo, Brazil of samples from 131 cattle with a CNS neurological syndrome confirmed the etiologic agent in only Bovine papillomavirus 1 in brains of cattle with a neurological syndrome 38.9% [8]. In the remaining samples, the agent could not be identified by isolation or molecular techniques, indicating the importance of investigating other potential etiological agents and standardizing new diagnostic techniques. The histological evaluation of 2,603 CNS samples from cattle with neurological syndrome in Brazil revealed no tissue changes in 2,130, and, of the 473 with lesions, 395 exhibited non-specific non-purulent meningoencephalitis (NPME) [4]. Non purulent menigoencephalitis is characterized by mononuclear leukocyte infiltration and is commonly attributed to viral infections, but may also be associated with protozoa, non-pyogenic bacterial agents [11,12], and neoplastic retroviruses including bovine leukemia virus [13]. The etiology of neurological disorders with minimal or no histopathology of the CNS is speculative and may be metabolic disease, intoxication, or toxinfection [10]. In Brazil, investigation of neurologic syndrome in cattle revealed less than 1% with neoplasms in the CNS [5,7,9,10,13]. These results confirm the importance of

investigating, in addition to the usual agents that affect the CNS of cattle, other pathogens prevalent in bovines in which epidemiology and pathogenicity are unknown.

Bovine papillomavirus (BPV) is a cosmopolitan disease, independent of the level of proficiency in livestock husbandry. The absence of epidemiological studies of BPV distribution may lead to underestimation of infection rates, presenting a challenge to developing vaccines, since we do not know the prevalent virus types in each country [14].

BPV infection is endemic in both dairy and beef cattle, but is more common in dairy cattle. It preferentially affects young cattle, but can occur in all ages. The viruses are widespread in Brazilian herds, with an estimated 60% infected [15], and although this rate may be higher when asymptomatic individuals are included [16,17]. BPV belongs to the Papillomaviridae [14,18]. They are classified into four genera, five species, and 15 types (http://pave.niaid.nih.gov) based on the degree of nucleotide similarity of the major capsid gene, *L1*.

Papillomaviruses are oncogenic, with a trophic response in squamous epithelial and mucosal tissues [16], and are associated with asymptomatic infections, proliferative benign skin lesions (papillomas), and malignant epithelial lesions (carcinomas) [14,16,19,20]. The presence and expression of BPV in the blood of healthy and papillomatosis-affected cattle has been demonstrated [17]. The occurrence of papillomas in the CNS has been reported. Papilloma of the choroid plexus has been described in cattle and horses, but its occurrence is higher in dogs as a tumor located in the third or fourth ventricle [11,12,21]. Microscopically, papillomas of the choroid plexus are organized into arboriform vascular connective tissue, covered by an epithelial layer cuboid or columnar in structure [11,12,21]. The occurrence of edema, hemorrhage, necrosis, and mineralization in the neuropil has been associated with this tumor [21]. Papillary meningioma is a tumor derived from cells of the arachnoid membrane and the pia mater of the CNS. No predisposing factor to its spontaneous appearance has been identified. It is the most common primary CNS tumor in dogs and is rare in cattle and horses [21]. The aforementioned studies included macroscopy and microscopy but no investigation of BPV DNA in the CNS lesions.

Meningiomas have been experimentally induced in calves via scarification the meninges and inoculation with BPV [22], and viral DNA has been detected in the consequent tumor tissue [23]. Fibromatous tumors have been observed in the meninges of calves sacrificed at 90 and 145 days post-inoculation with bovine cutaneous papilloma suspension. Tumors extended to the brain along the blood vessels; however, metastases were not observed [24]. Studies associating natural BPV infection with CNS lesions have not been conducted. The CNS originates from the embryonic ectoderm [11]. Epithelial BPV tropism is well known, but it is necessary to investigate whether BPV presents neurotropism or if infection in the CNS of naturally infected cattle is nonspecific. Given that BPV is widespread in Brazilian cattle herds, that it has been demonstrated that BPV experimental infection in CNS can cause papilloma and meningioma in calves, and that there is a high rate in Brazilian herds of cattle with neurological syndrome with no causal agent identified, the aim of this study was to determine whether BPV is a cause of neurological syndrome in naturally infected animals.

Material and Methods

Three-hundred CNS samples (convenience sampling) from several Brazilian regions obtained from surveillance of neurological syndrome from January 2012 to September 2015 were submitted to differential diagnosis. Samples were negative according to laboratory protocols described for rabies [25], *Neospora caninum* [26], BoHV-1 and BoHV-5 [27], bovine leukemia virus [28], and catarrhal malignant fever (PCR) [29]. Samples were fixed in 10% buffered formalin and submitted to macroscopic examination. They were cut into fragments [30], placed in histology cassettes, and processed using a standard protocol [31] with a Leica TP 1020 automatic tissue processor (Leica Microsystems, Germany). The CNS fragments were embedded in paraffin in histology cassettes using a Leica EG 1160 paraffin embedding center (Leica Microsystems, Germany). Samples were cut into 3 µm sections on a Leica RM 2255 rotary microtome (Leica Microsystems, Germany), extended in a Leica HI1210 waterbath (60°C) (Leica Microsystems, Germany), placed on glass slides previously treated with albumin to facilitate adhesion, and placed in an oven at 63°C until the paraffin was melted (approximately 1 hour). The slides were submitted to a staining protocol using hematoxylin and modified eosin [31]. Synthetic resin (Entellan, Merck) was used to assemble the slide and coverslip. The slides were analyzed using a Leica DM 2000 trinocular optical microscope (Leica Microsystems, Germany).

The 300 CNS samples were submitted to PCR to detect BPV by the Laboratory of Bovid Viruses of the Biological Institute. For BPV analysis, DNA was extracted from refrigerated samples by Trizol (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. The amplification of the DNA fragment was conducted using generic primers *FAP59* and *FAP64* (478 bp) with nucleotide sequence of the gene that encodes the L1 protein of the BPV capsid *FAP59* (5'-TAA CWG TIG GIC AYC CWT ATT-3') and *FAP64* (5'-CCW ATA TCW VHC ATI TCI CCA TC-3') [32].

The PCR protocol was optimized according laboratory conditions and reagents [32]. We used 12.5 µL Master Mix Promega, 3.75 µL nuclease-free water (NFW), 0.75 M of each primer diluted to 10 ρmol/µL, and 5 µL of DNA sample. The PCR cycle program (Mastercycler gradient, Eppendorf, Germany) consisted of initial denaturation at 94°C for 5 min; 45 cycles of amplification 94°C for 1 min, 49.3°C for 1 min, and 72°C for 1 min; and a final extension 72°C for 5 min. The negative BPV PCR control was NFW, and the positive PCR control was BPV-1 from a bovine skin papilloma diluted 1:10. The analysis of the amplified products was performed by electrophoresis (100V/60 min) (PowerPac, Bio-Rad, EUA) in 1.5% agarose gel in 1X TBE buffer and confirmed with Gel Red (Biotium, EUA) at a dilution of 1:150 in NFW. The image of the gel under UV light was recorded on a photodocumentor (GelDoc-It UVP, Germany) coupled to a computer. The samples were considered positive when they presented aband corresponding to 478 bp equal to, or stronger than, the positive control. Phylogenetic analysis was constructed with MEGA version 6.0 using the maximum likelihood method [33], with bootstrap values based on 1000 replicates, general time reversible model. Only values above 70% were accepted. Bovine papillomavirus PCR positivity was assessed relative to presence of histological lesions and epidemiological factors including age, sex, breed, and region of origin of the sample.

Results

Thirteen of the 300 CNS samples were found BPV positive by PCR. Of these 13 BPV PCR-positive animals, 11 showed no histological lesions, while inflammatory lesions typical of non-specific non-purulent meningoencephalitis were observed in two from São Paulo state: a 24-month-old male Nelore and a female of indeterminate age and breed. No papillomatosis lesions were observed macro- or microscopically in any of the 300 CNS samples. Nine of the 13 positive cattle were female and four were male, aged from 5 to 168 months, with seven over 36 months. Five were dairy cattle, four were cross-breeds, and three were beef cattle. In one, the breed and age were unknown. Eleven of the positive BPV-1 cattle came from São Paulo State, one from Rio de Janeiro State, and one from Minas Gerais State.

Bovine papillomavirus genome sequencing was possible in only one of the 13 PCR positive CNS samples (LVB/13 25441-TA 2297/13) and was identified as BPV-1 genotype. The results of sequencing of the *L1* gene revealed 99% identity with the BPV-1 isolate KU 736826.1 from bovine cutaneous papilloma in other Brazilian herds (Figure 1). The LVB/13 25441-TA 2297/13 sample showed 100% of identity with other BPV-1 genotypes from Brazil (KC 595244.2 and KU 728468.1), Croatia (JX 046508) and Poland (KF 284141) in GenBank (Figure 1). There was no similarity to the *BPV-6* previously identified in Brazil (Figure 1).

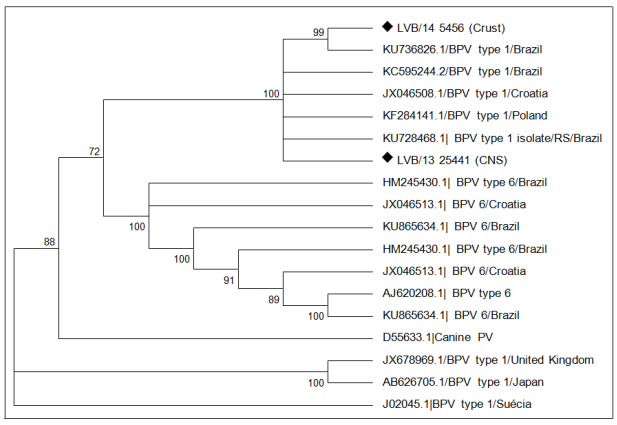

Figure 1: The nucleotide sequences obtained in the present study were aligned to the BPVsequences available in GenBank and used for the construction of the phylogenetic tree, maximum likelihood, with a bootstrap value of 1000 replicates (MEGA 6.0). ♦Indicates the CNS sample positive for BPV (LVB/13-25441) and the positive control (LVB/14-5456 - cutaneous papilloma).

The alignment of the amino acid sequences (Figure 2) showed a difference of a single amino acid at position 152: D (aspartic acid) and F (phenylalanine) in CNS sample LVB/13 25441 and the Brazilian BPV-1 group, respectively.

Discussion

The low number of samples positive for BPV (13) did not allow statistical analysis to determine whether there was a significant difference in the frequency of occurrence relative to histological lesions, age, sex, breed, or place of origin. As this study represents the first report of BPV in the CNS of cattle, the scientific literature provides no epidemiological studies to compare with our results.

Non-specific non-purulent meningoencephalitis is the most frequent lesion observed in animals with neurological syndrome [3,4]. This type of inflammatory infiltrate is characterized by mononuclear cells and occurs mainly with viral agents that cause encephalitis such as rabies, BoHV-5, malignant catarrhal fever, *Neospora caninum* [3,4], and bovine leukemia virus [28], whose possibilities were excluded by the differential diagnosis.

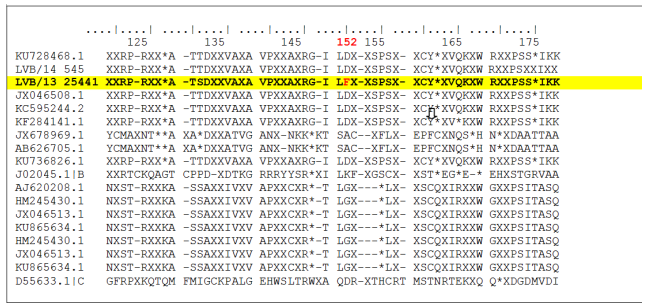

Figure 2: Amino acid sequence of BPV-1 from sample LVB/13 25441 of BPV-1 identified in bovine CNS with neurological syndrome based on the nucleotide sequences of the *L1* gene. A difference was observed in a single amino acid at position 152 of D (aspartic acid) and F (phenylalanine) in CNS sample LVB/13 25441 and F (phenylalanine) in the Brazilian BPV-1 group.

In the single sample with sufficient DNA to perform sequencing, the BPV genotype 1 (*Deltapapillomavirus*) was identified [18,20]. This study shows the importance of better understanding the role of BPV in non-epithelial tissues and its consequences to health and the epidemiology of BPV in cattle.

The BPV-1 genotype in the CNS was identified by the same set of primers (*FAP59* and *FAP64*) used by Claus et al. [34]. These authors studied BPV genotypes in cutaneous papilloma, and in addition to BPV-1 and BPV-6, they found genotype 2 demonstrating the BPV diversity in Brazilian cattle and the possibility of concomitant infection with multiple genotypes (BPV-1 and BPV-2) in the same animal. Phylogenetic classification of papillomavirus is based on the L1 open reading frame (ORF) sequence homology, since this is the most conserved ORF among the papillomavirus types [35-38]. According to this system, a new type of papillomavirus is recognized when L1 ORF sequencing demonstrates a nucleotide identity difference greater than 10%. Differences of identity of 2-10% define a subtype, and <2% characterizes a viral variant [35,36,39]. Forslund et al. [32], used the same degenerate primers *FAP59* and *FAP64* for partial amplification of the *L1* gene and subsequently sequenced the amplified product, describing 12 supposed new types of human papillomavirus (HPV). The strategy allowed the identification of 16 new types of BPV from both skin lesions and healthy skin [40-42]. These findings suggest that the molecular diversity characterized in HPV, in which more than 200 viral types are currently recognized, may also occur in BPV [39].

Meningioma can be induced in calves by intra-meningeal inoculation of BPV [22,23], and fibromatous tumors were induced in the meninges of calves by inoculation with bovine cutaneous papilloma suspension [24], but studies associating natural BPV infection with CNS lesions have not been conducted.

Bovine papillomatosis may be a herd problem, in that the virus is easily transmitted by animal-to-animal contact and by fomites. Morphologic and biological features of lesions may differ depending on the anatomic site and papillomavirus type [43]. BPV-2 DNA has been detected in the reproductive tract (ovaries, uterus, cumulus, and oocytes) of cows, with authors suggesting that this infection may not be as specific, since BPV-2 may be transported in the blood to these tissues [44].

Research has shown BPV-2 expression in the blood of healthy and papillomatosis-affected cattle: active and inactive virus was found in papillomatosis affected and asymptomatic cattle, and activation of the virus in blood was independent of productive infection in epithelial tissue, suggesting that environmental and genetic factors could contribute to the activation of BPV in blood [17]. Active BPV-containing blood cells are suggested to be responsible for spreading the infectious agent to various organs [45-47]. BPV DNA was detected in $CD4^+$ and $CD8^+$ lymphocytes from the peripheral blood mononuclear cells [47]. The detection of BPV in different tissues and cells, including reproductive sites such as oocytes, the ovary, uterus, cumulus cells, and uterine lavage, corroborates the idea that active BPV in blood cells of cattle could facilitate virus dissemination to non-epithelial sites of asymptomatic and symptomatic animals [45,48-50]. The presence of BPV in bloodcells may explain the non-specific BPV-1 infection in the CNS of the 13 positive animals of the present study, since this BPV genotype has not been associated with meningoencephalitis. However, it was not possible to infer the existence of other BPV genotypes in twelve of the PCR-positive samples, because we did not obtain sufficient DNA for genomic sequencing. BPV-1 is a recognized agent causing fibropapilloma and is commonly found in cutaneous papillomatosis in bovine herds in Brazil, which leads to the conclusion that its finding in the CNS indicates a carrier state and is not associated with neurotropism or neuropathogenesis.

Conclusion

The occurrence of BPV-1 due to natural infection in the CNS of cattle was not associated with neoplasia and neurological syndrome.

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Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Approval

This research was approved by the Bioethics Committee in Animal Research of the Biological Institute (CETEA-IB), registry number 134/14.

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