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Detection and expression of bovine papillomavirus in blood of healthy and papillomatosis-affected cattle

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ABSTRACT. Papillomaviruses (PV) are double-stranded DNA viruses that can cause benignant and malignant tumors in amniotes. There are 13 types of bovine papillomavirus (BPV-1 to -13); they have been found in reproductive tissues and body fluids. Normally these viruses are detected in epithelial tissue. We looked for BPV in the blood of healthy cattle and cattle with papillomatosis, using PCR and RT-PCR. BPV types 1 and 2 were detected in 8/12 blood samples of asymptomatic bovines and in 8/9 samples from cattle with papillomatosis. Six of 8 asymptomatic samples positive for BPV also showed expression for BPV. Five of 6 samples were positive for E2 expression, while 3/6 samples were positive for E5 expression. Five of 8 symptomatic samples positive for BPV also showed BPV expression. Five of 5 were positive for E2 expression, while 1/5 was positive for E5 expression. Two of 6 blood samples of asymptomatic cattle and 1/5 symptomatic blood samples scored positive for both E2 and E5 expression. This is the first

study showing expression of BPV genes in the blood of asymptomatic and papillomatosis-affected animals.

Key words: BPV; Blood; Viral gene expression; Cattle

INTRODUCTION

Papillomaviruses (PVs) are double-stranded DNA tumor viruses that have been identified in various animals, including humans (Bernard et al., 2010). Their open reading frame is divided into early (E) and late (L) regions. The E region encodes non-structural proteins E1 to E7. E1 and E2 control the replication and transcription of the genome, and the known oncoproteins are E5, E6, and E7. The L region encodes structural proteins L1 and L2, which form the capsid (Campo, 2006). Bovine papillomavirus (BPVs) can infect basal epithelial cells and subepithelial fibroblasts leading to the formation of tumors known as papillomas or warts (Nasir and Campo, 2008). The lesions are usually benign and tend to regress as a result of the cell-mediated immune response (Knowles et al., 1996). However, as a result of environmental co-factors, they can turn into malignant tumors (Campo, 2006; Borzacchiello and Roperto, 2008). Some recent studies describe the co-infection among BPVs in skin tumors (Pangty et al., 2010; Schimitt et al., 2010; Carvalho et al., 2012) and the cross-species infection caused by BPV (Bogaert et al., 2008; Silvestre et al., 2009; van Dyk et al., 2011; Freitas et al., 2011). PVs are described as epitheliotropic, although their presence has been detected in different tissues and cells (Freitas et al., 2003; Yaguiu et al., 2006; Lindsey et al., 2009; Silva et al., 2011). Recently, human and bovine PV DNA have been shown to be detectable in peripheral blood mononuclear cells (Freitas et al., 2003; Bodaghi et al., 2005; Roperto et al., 2008, 2011) and plasma and serum (Widschwendter et al., 2003; Freitas et al., 2007). The expression of structural and non-structural genes, as well as their corresponding proteins, has been demonstrated in the lymphocytes of cattle with bladder cancer (Roperto et al., 2008, 2011). Few studies have contributed to improving the understanding of PV transmission; however, it has been hypothesized that lymphocytes can harbor the virus in the bloodstream (Stocco dos Santos et al., 1998; Freitas et al., 2003; Diniz et al., 2009), which can spread through non-epithelial tissues and fluids (Freitas et al., 2007). Previous data showing that the lymphocytes may be PV carriers and the blood a potential new route of PV transmission seem to support this hypothesis (Bodaghi et al., 2005; Roperto et al., 2008, 2011). The presence of PV in the blood of asymptomatic individuals has been described in the literature, but the significance of this finding still remains to be fully clarified (Stocco dos Santos et al., 1998; Wosiacki et al., 2005; Lindsey et al., 2009).

Although previous studies have shown viral expression in the blood of BPV-infected cattle with bladder cancer (Roperto et al., 2008, 2011), it is not clear if BPV transcription occurs in animals with only benign tumors such as cutaneous papillomas. This study aimed to evaluate the presence and expression of BPV in the blood of healthy and papillomatosis-affected cattle.

MATERIAL AND METHODS

Animals

Twenty-one cattle were selected for this study. The animals belonged to 6 different

farms located in Bahia State, in northeast Brazil. All the farms had papillomatosis-affected and healthy animals. Some animals of each farm, both symptomatic and asymptomatic for papillomatosis, were selected.

Blood samples

Blood (5 mL) was collected from the animals in EDTA-containing tubes, and 200 µL total blood was used for DNA and RNA extraction.

DNA and RNA extraction and cDNA synthesis

All samples were submitted to DNA and RNA extraction using the Invirsob® Spin Universal RNA Mini kit (Invitek, Germany), in accordance with the manufacturer protocol. The quality of the purified DNA was checked by the β-globin gene PCR, as described by Freitas et al. (2003). Amplification was carried out in a final volume of 25 µL containing 100 ng DNA, 1X Master Mix (Promega, USA), and 0.2 µM specific primers. Subsequently, the RNA samples were digested with RNase-free DNase (Promega) and the first strand of cDNA was synthesized with the Oligo(dT) 15 primer (Promega) and ImProm-II™ Reverse Transcriptase (Promega). Two negative controls, one without the reverse transcriptase and another without RNA [no-template control (NTC)] were used during the synthesis of cDNA. β-actin transcript was amplified from cDNA with primers previously described by Robinson et al. (2007) to verify the cDNA quality. PCR was carried out using 0.2 µM of each primer, 5 µL cDNA, and 1X Master Mix (Promega). PCR consisted of 40 cycles of denaturation for 45 s at 94°C, annealing for 40 s at 52°C, and extension for 40 s at 72°C.

BPV detection by PCR

The presence of BPV1/2 DNA was assessed with primers targeting the E5 and L2 BPV genes, as previously described by Roperto et al. (2008). Two negative controls were used, an NTC, and a control with DNA of Madin-Darby bovine kidney (MDBK; ATCC-CCL22) cells. BPV1 and 2 genomes cloned into pAT153 plasmid were used as positive control. PCR products were electrophoresed on 2% agarose gel with TAE buffer and run at a constant voltage (100 V) for approximately 35 min. DNA was visualized under UV light after being stained with ethidium bromide.

Determination of BPV expression

The presence of BPV transcripts was evaluated for two different BPV genes, E2 and E5. The two sets of primers used for the reactions were described by Roperto et al. (2008) and Silva et al. (2011). PCR was carried out according to the conditions described by Silva et al. (2011). To confirm the presence of BPV transcripts, the purified amplicon from PCR was cloned into the pGEM-T Easy vector (PromegapGEM-T Easy Vector System, Promega) and transformed into competent JM 109 *Escherichia coli* cells (Promega). The DNA of recombinant clones was isolated with the PureYeld™ Plasmid Miniprep System (Promega), and direct sequencing was conducted with an ABI PRISM® 3100 Genetic Analyzer (Applied

Biosystems, USA) with the primers for BPV1/2. The DNA sequences were analyzed with the Staden Package software (http://staden.sourceforge.net) for the quality analysis of chromatogram readings and the formation of the consensus sequences. The identified sequences were analyzed by means of the BLAST tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

RESULTS

Presence of BPV

The presence of BPV1/2 DNA was assessed in all blood samples that scored positive for β-globin PCR. The NTC and virus-free DNA from MDBK cells tested negative, thus confirming the absence of cross-contamination and the reliability of the method employed. The blood samples from asymptomatic animals scored positive in 8 of 12 (66%) isolates, while 8 of 9 symptomatic individuals (88%) showed a positive signal (Figure 1).

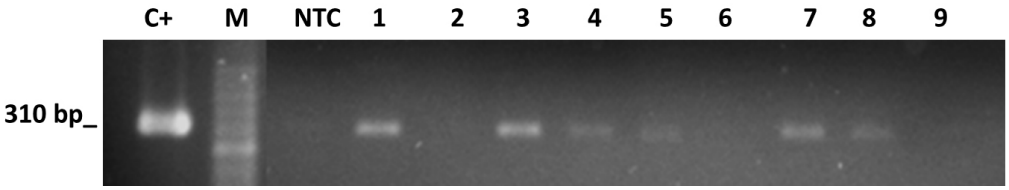

Figure 1. Representation of bovine papillomavirus (BPV)1/2 detection in cattle blood. PCR for E5L2 BPV2 genes. *Lane C+* = positive control; *lane M* = 50-bp weight marker; NTC = no template control; *lanes 1* to *8* = blood cattle samples; *lane 9* = MDBK sample.

Expression of BPV

The cDNA samples were assessed for β-actin gene transcripts. The samples scoring positive were evaluated for the presence of viral transcripts for the E2 and E5 BPV genes. In blood samples, 10 of 11 (91%) samples positive for BPV showed amplified E2 transcripts, while 4 of 11 samples (36%) were positive for E5 expression. Three samples (27%) were positive for concomitant E2 and E5 expression. In the asymptomatic blood group, 5 of 6 samples expressing BPV genes (83%) were positive for E2 expression, while 3 of 6 (50%) scored positive for E5 expression. The blood of papillomatosis-affected animals scored positive for E2 expression in 5 of 5 (100%) samples expressing BPV, and 1 of 5 (20%) isolates expressed the E5 gene. These results are summarized in Table 1 and shown in Figures 2 and 3, and they were confirmed by direct sequencing of the E2 and E5 genes obtained by RT-PCR.

Table 1. Viral expression of E2 and E5 bovine papillomavirus (BPV) genes in bovine blood positive for BPV detection.

Samples	BPV2 expression	E2 expression	E5 expression	E2 and E5 expression
Asymptomatic	6/8 (75%)	5/6 (83%)	3/6 (50%)	2/6 (33%)
Symptomatic	5/8 (63%)	5/5 (100%)	1/5 (20%)	1/5 (20%)
Total	11/16 (69%)	10/11 (91%)	4/11 (36%)	3/11 (27%)

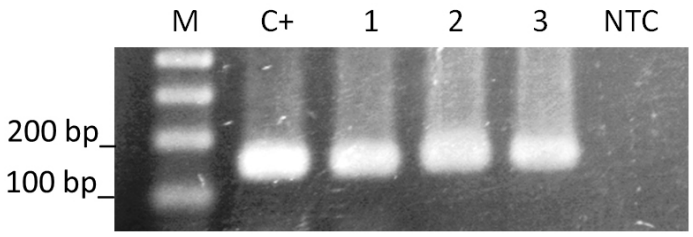

Figure 2. Representative E5 bovine papillomavirus (BPV) expression in cattle blood. RT-PCR for E5 BPV gene. *Lane M* = 100-bp molecular weight marker; *lane C+* = positive control; *lanes 1* to *3* = positive blood samples; NTC = no template control.

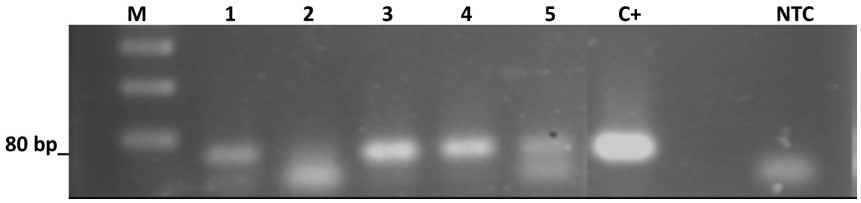

Figure 3. Representative E2 bovine papillomavirus (BPV) expression in cattle blood. RT-PCR for E2 gene. *Lane M* = 100-bp molecular weight marker; *lanes 1* to *5* = samples; *lane C+* = positive control; NTC = no template control.

DISCUSSION

This is the first study, to the best of our knowledge, describing the expression of BPV2 in the blood of healthy and papillomatosis-affected cattle. We found a significant level of blood samples positive for expression of BPV. E2 and E5-BPV2 transcripts were found in the blood of healthy and papillomatosis-affected cattle. A previous study has also shown the expression of E5 oncogene in the blood cells of animals with bladder cancer (Roperto et al., 2008). Moreover, E and L proteins were found in white blood cells (Roperto et al., 2011). However, this is the first study showing BPV expression in healthy and papillomatosis-affected cattle. In humans, HPV-specific RNA is known to occur in the blood cells of papillomavirus-infected cancer patients (Pao et al., 1991).

Some samples showed the presence of virus, but with no virus expression. In these samples the blood probably acts as a site of virus latency. However, in other samples, the virus was active in blood. As active and inactive virus was found in papillomatosis-affected and asymptomatic cattle, the activation of the virus in blood was independent of productive infection in epithelial tissue. It is suggested that environmental and genetic factors could contribute to the activation of BPV in blood as observed for PV in epithelial tissues (Campo, 2006). Active PV-containing blood cells are suggested to be responsible for spreading the infectious agent to various organs (Freitas et al., 2003, 2007; Roperto et al., 2011). The detection of BPV in different tissues and cells, including reproductive sites such as oocytes, the ovary, uterus, cumulus cells, and uterine lavage could corroborate this idea (Freitas et al., 2003; Yaguiu et al., 2006; Lindsey et al., 2009). We think that active BPV in blood cells of cattle could facilitate virus dissemination to non-epithelial sites of asymptomatic and symptomatic animals.

The presence of BPV in the blood of newborn calves has been detected (Stocco dos Santos et al., 1998; Freitas et al., 2003; Yaguiu et al., 2008), suggesting the vertical trans-

mission of BPV. In humans, it has been shown that HPV-infected women can transmit the infection to the fetus by transplacental mechanisms (Rombaldi et al., 2008). More studies are needed to understand the possible importance of active BPV-containing blood in vertical transmission and the role of this mechanism in asymptomatic and papillomatosis-affected animals with regard to the dissemination of BPV in the herd.

Blood could also be a source for horizontal BPV transmission. Stocco dos Santos et al. (1998) demonstrated that the peripheral blood of animals affected by papillomatosis can act as a vehicle for the transmission of BPV to healthy cattle. It is believed that flies can be a vector for BPV and transmit the virus between cattle and horses (Nasir and Campo, 2008; Finlay et al., 2009). Up to this moment, information about this virus-vector-host system is nonexistent; however, we should not exclude this possibility. The presence of active BPV in the blood cells of asymptomatic individuals could represent a source of horizontal transmission on farms negative for papillomatosis.

This study shows the importance of better understanding the role of BPV in non-epithelial tissues, and its consequences to health and epidemiology of BPV in cattle.

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