Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Simultaneous presence of bovine papillomavirus in blood and in short-term lymphocyte cultures from dairy cattle in Pernambuco, Brazil

N. Diniz[1], T.C. Melo[1], J.F. Santos[1], E. Mori[2], P.E. Brandão[2], L.J. Richtzenhain[2], A.C. Freitas[1], W. Beçak[3], R.F. Carvalho[3] and R.C. Stocco[3]

[1]Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, Brasil
[2]Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brasil
[3]Laboratório de Genética, Instituto Butantan, São Paulo, SP, Brasil

Corresponding author: R.C. Stocco
E-mail: ritastocco@butantan.gov.br or ritastocco@yahoo.com

Genet. Mol. Res. 8 (4): 1474-1480 (2009)
Received September 3, 2009
Accepted September 30, 2009
Published December 15, 2009

ABSTRACT. Bovine papillomaviruses (BPV) are the causal agents of benign and malignant lesions; they can cause dramatic economic losses in cattle. Although 10 virus types have been described, three types are most common in tumors, namely BPV-1, -2 and -4. Previous studies have reported BPV in blood cells and the possibility of blood acting as a latent virus site and/or transmission agent of virus dissemination. We studied a Holstein dairy herd in Pernambuco, Brazil, in which several animals showed severe cutaneous papillomatosis, without previous determination of BPV types. Blood samples and short-term lymphocyte cultures were collected from 54 cows. We compared the BPV types detected in peripheral blood to those identified in the respective lymphocyte cultures: BPV-1 was detected in 74% and BPV-2 in 87% of the whole blood samples. Simultaneous virus presence (BPV-1 and BPV-2) was found in 65% of the blood samples. BPV-1 or BPV-2 were detected in the lymphocyte cultures in 93% of the samples, and both in

 ©FUNPEC-RP www.funpecrp.com.br

89%. The detection of viral DNA in whole blood and in lymphocyte cultures is evidence that this virus is carried by lymphocytes.

Key words: Bovine papillomavirus; Cutaneous papillomatosis

INTRODUCTION

Bovine papillomaviruses (BPVs) are species-specific, double-stranded DNA viruses responsible for cutaneous and mucosal neoplasic lesions. They are small non-enveloped viruses with an icosahedral capsid. Their open reading frames are divided into early (E) and late (L) regions. The early region encodes non-structural proteins E1 to E7, of which, E5, E6 and E7 are known to be oncoproteins. The late region encodes structural proteins L1 and L2, which form the capsid (Campo, 2006). Bovine papillomavirus types 1 and 2 (BPV-1 and BPV-2) are classified in the genus Deltapapillomavirus, defined as fibropapillomavirus. BPV-4 is included in Xi-papillomavirus and induces epithelial papillomas. In cattle feeding on bracken fern, BPV-4 is associated with upper alimentary tract cancer. BPV-1 and BPV-2 are associated with both fibropapillomas in cattle and sarcoids in equids (Brandt et al., 2008; Nasir and Campo, 2008). BPV-2 infection in the presence of environmental carcinogens, such as ptaquiloside, a toxic principle of bracken fern (*Pteridium aquilinum*), has been associated with urinary bladder neoplasic lesions in adult cattle, in which chronic enzootic hematuria is the most important clinical sign (Hopkins, 1986; Campo et al., 1992; Campo, 1997). The effect of the route of viral infection and the synergistic relationship between BPV and immunosuppressive and oncogenic compounds present in bracken fern in the malignant progression of the lesions is not well known, thus deserving further investigations (Jarrett et al., 1978; Reddy and Fialkow, 1983; Campo et al., 1992; Campo, 1997; Stocco dos Santos et al., 1998).

To date, the BPV-2 genome has been detected in lymphocytes during latent papillomavirus infection in cattle (Stocco dos Santos et al., 1998; Campo et al., 1994). In addition, the occurrence of horizontal transmission of BPV-2 has been reported in healthy cattle experimentally inoculated with peripheral blood from hematuric animals (Stocco dos Santos et al., 1998). BPV-2 was also detected in one urinary bladder and in one whole blood sample from asymptomatic cattle (Wosiacki et al., 2005). The presence of BPV-2 in the blood stream was verified in cattle with hematuria, grazing on bracken fern (Moura et al., 1988; Borzacchiello et al., 2003). Roperto et al. (2008) compared the presence of BPV-2 in blood cells and bladder lesions, and found that 37 of the 78 animals studied showed BPV-2 in the blood and in bladder tumors.

In the present study, we investigated BPV-1, -2 and -4 in fresh whole blood samples and in corresponding short-term lymphocyte cultures. Our aim was to corroborate our pioneer data about the importance of blood cells as carriers of the virus throughout the host body, and as critical elements for virus transmission.

MATERIAL AND METHODS

Animal selection and collected samples

Fifty-four female Holstein, six years old, were selected at random, with and without cutaneous papillomatosis and 20 mL blood was collected from each animal, 10 mL with heparin for short-term lymphocyte cultures and 10 mL with EDTA for DNA extraction.

Short-term lymphocyte cultures

Short-term lymphocyte cultures were used for virus detection and for cytogenetic studies evaluating the frequencies of chromosome aberrations (Melo et al., 2009), according to previously described protocols (Stocco dos Santos et al., 1998; Yaguiu et al., 2008).

DNA extraction

Venous blood samples were collected in heparinized vacutainers and vacutainers with EDTA. DNA was extracted from whole blood samples and from lymphocyte cultures with the QIAamp DNA mini kit (Qiagen) according to manufacturer instructions.

BPV detection

DNA samples obtained (whole blood and lymphocyte cultures) were evaluated to verify the possibility of their use in polymerase chain reactions (PCR). The bovine β-globin gene was used according to Yaguiu et al. (2008). BPV-1, -2 and -4 types were verified following protocols described by Yaguiu et al. (2008) and Lindsey et al. (2009). The amplified products were electrophoresed on a 2% agarose gel, visualized by GelRed™ and documented in Biometra by BioDocAnalyse™. The PCR products were analyzed by enzymatic digestion and sequencing.

Restriction analysis

The PCR products were digested with specific restriction enzymes, selected by the pDRAW32 program: *Dde*I (N E Biolabs$_{inc}$) for BPV-1-positive samples, *Hin*fI (N E Biolabs$_{inc}$) for BPV-2-positive samples and *Mae*III (N E Biolabs$_{inc}$) for samples positive for BPV-4.

The reactions were performed as follows: 1 µL enzyme (5 U/µL), 2 µL buffer, 8 µL of the PCR product, and 9 µL water in a final volume of 20 µL; incubated for 5 h at 37° and at 65°C for 10 min. Digestion products were submitted to electrophoresis on a 2% agarose gel, visualized by GelRed™ and documented in Biometra by BioDocAnalyse™.

Sequencing

The positive samples were purified for sequencing using Invisorb® Fragment Clean-Up (Invitek), following manufacturer instructions. The sequencing reactions used BigDye™ Terminator (Applied Biosystems™) in ABI 377 equipment (Applied Biosystems™). The sequencing analysis was performed with the Bioedit software (version 7.0).

RESULTS

Of the 108 DNA samples obtained from whole blood and lymphocyte cultures, 95 were positive for β-globin amplification, which were thus available for BPV PCR analyses. PCR showed BPV-1 in 74% (40/54) and BPV-2 in 87% (47/54) of the whole blood samples. Simultaneous virus presence (BPV-1 and BPV-2) was found in 65% (35/54) of

the blood samples. With regard to the lymphocyte cultures, BPV-1 and BPV-2 were independently detected in 93% of the samples (50/54), and were simultaneously present in 89% (48/54) (Figure 1, Table 1).

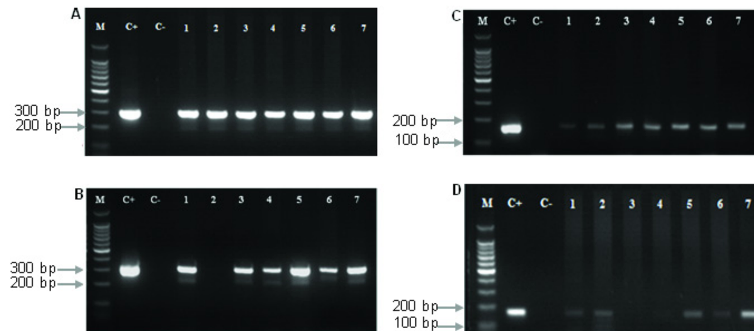

Figure 1. PCR products on 2% agarose gel visualized by GelRed™. **A.** Whole blood. **B.** Lymphocyte culture positive for BPV-1. **C.** Whole blood. **D.** Lymphocyte culture positive for BPV-2. *Lanes 1-7* are the respective samples; M = 100-bp ladder; C+ = positive control; C- = negative control.

Table 1. BPV-1 and BPV-2 detected in whole blood and peripheral lymphocytes.

Virus type	Whole blood	Peripheral lymphocytes
BPV-1	40/54 (74%)	50/54 (93%)
BPV-2	47/54 (87%)	50/54 (93%)
BPV-1 and BPV-2	35/54 (65%)	48/54 (89%)

Data are reported as number/total animals with percent in parentheses.

Using the specific enzyme *Dde*I, the BPV-1-positive samples showed the two expected fragments of 63 and 238 bp. PCR products of BPV-2-positive samples, when digested with *Hin*fI, yielded fragments of 60 and 104 bp (Figure 2).

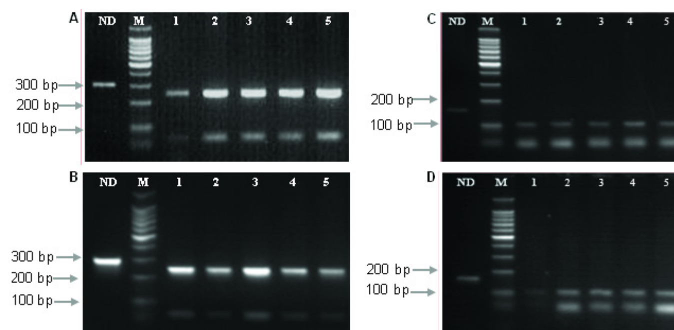

Figure 2. Enzymatic digestion products on 2% agarose gel visualized by GelRed™. **A.** Whole blood. **B.** Lymphocyte culture positive for BPV-1. **C.** Whole blood. **D.** Lymphocyte culture positive for BPV-2. *Lanes 1-5* are the respective samples; M = 100-bp ladder; ND = not digested.

 ©FUNPEC-RP www.funpecrp.com.br

The sequencing procedure in the samples analyzed showed homology corresponding to the BPV-1 and BPV-2 types.

BPV-4 was not detected in any of the samples studied, fresh blood or lymphocyte cultures.

DISCUSSION

Bovine papillomaviruses have been extensively studied due to the related diseases: cutaneous papillomatosis, benign and malignant lesions in the esophagus and bladder, which cause extremely dramatic losses in infected herds. In addition, these diseases provide a very important model for studies on the mechanisms of infection and interaction of papillomavirus and host cell (Lindsey et al., 2009).

This study describes the presence of two BPV types in a dairy herd in Pernambuco, Brazil: BPV-1 and BPV-2. BPV-4 was not detected.

The animals evaluated were selected at random (Figure 3), with and without cutaneous papillomatosis. The protocol included the analysis of blood samples and their respective lymphocyte cultures. The aim was the detection of viral sequences in blood and mainly in lymphocyte cultures. No concern was directed to clinical aspects of the animals or specific analysis of their eventual lesions.

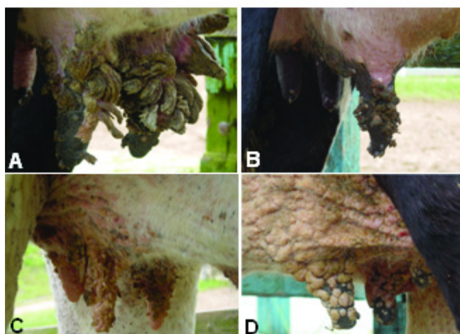

Figure 3. Cutaneous papillomatosis in the animals evaluated (A-D).

The simultaneous presence of BPV-1 and BPV-2 viruses could be related to different sources, as undetected infected tissues, considering that the virus can spread via a hematogenous route throughout the host body. Human papillomavirus DNA was detected in peripheral blood mononuclear cells obtained from afflicted children and from non-infected blood donors (Bodaghi et al., 2005). Almeida et al. (2000) detected human papillomavirus sequences in blood samples of women treated for cervical and breast cancer, and correlated with the presence of virus in whole blood with the frequencies of chromosomal aberrations in corresponding short-term cultured lymphocytes.

In cattle, viral DNA has been found in blood (Campo et al., 1994; Stocco dos Santos et al., 1993, 1998; Wosiacki et al., 2006) and in plasma (Freitas et al., 2007).

In this study, the focus was also directed to the *in vitro* environment, specifically short-term lymphocyte cultures. The simultaneous presence of viral sequences in blood and

corresponding culture provides further evidence of the importance of blood as a virus carrier and lymphocytes as active virus carriers in the blood. To investigate this hypothesis, a cytogenetic analysis was performed on the cultivated cells, and an increased level of chromosomal aberrations was found, clearly supporting this notion (Melo et al., 2009); the alterations of host chromatin strongly suggest the occurrence of virus-host genome interaction.

However, virus DNA was not detected in all the samples. We cannot reject the possibility of different virus loads in the different samples and cultures. Virus load studies are necessary to understand the variability of the data obtained and the consequences for virus spread in the tissue-blood-tissue route.

In conclusion, we provided evidence that BPV-1 and BPV-2 may persist in blood and remain active in lymphocytes, acting as a source of viral infection and interacting with the host chromatin. As the selection of animals did not include clinical evaluations, the absence of BPV-4 in the samples analyzed could be due to the low incidence of this virus type in this region or to the animals harboring this virus type in tissues other than blood. Investigations of mechanisms of virus dissemination through different tissues are important for the appropriate use of vaccines to be developed.

ACKNOWLEDGMENTS

The authors thank Carolina da Paz Sabino for her editorial support, MCT/CNPq, DECIT/MS, Fundo Setorial de Biotecnologia (CT-Biotecnologia) e Saúde (CT-Saúde) and FAPESP for fellowships (Projects #554816/2006-7, #559043/2008-2 and #2006/02439-6, respectively).

REFERENCES

Almeida ME, Carvalho C, Stocco dos Santos RC and Beçak W (2000). Bovine papillomaviruses (BPV 2 and BPV 4) in body fluids and semen of bovines. In: 18th International Papillomavirus Conference, Barcelona, 492 (Abstract).

Bodaghi S, Wood LV, Roby G, Ryder C, et al. (2005). Could human papillomaviruses be spread through blood? *J. Clin. Microbiol.* 43: 5428-5434.

Borzacchiello G, Iovane G, Marcante ML, Poggiali F, et al. (2003). Presence of bovine papillomavirus type 2 DNA and expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows. *J. Gen. Virol.* 84: 2921-2926.

Brandt S, Haralambus R, Schoster A, Kirnbauer R, et al. (2008). Peripheral blood mononuclear cells represent a reservoir of bovine papillomavirus DNA in sarcoid-affected equines. *J. Gen. Virol.* 89: 1390-1395.

Campo MS (1997). Bovine papillomavirus and cancer. *Vet. J.* 154: 175-188.

Campo MS (Editor) (2006). Bovine Papillomavirus: Old System, New Lessons? In: Papillomavirus Research: from Natural History to Vaccine and Beyond. Caister Academic Press, Wymondham, 1-31.

Campo MS, Jarrett WF, Barron R, O'Neil BW, et al. (1992). Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. *Cancer Res.* 52: 6898-6904.

Campo MS, Jarrett WF, O'Neil W and Barron RJ (1994). Latent papillomavirus infection in cattle. *Res. Vet. Sci.* 56: 151-157.

Freitas AC, Silva MAR, Carvalho CCR, Birgel EH Jr, et al. (2007). Papillomavirus DNA Detection in Non-Epithelial Tissues: a Discussion about Bovine Papillomavirus. In: Communicating Current Research and Educational Topics and Trends in Applied Microbiology (Méndez-Vilas A, ed.). Formatex, Badajoz, 697-704.

Hopkins NC (1986). Aetiology of enzootic haematuria. *Vet. Rec.* 118: 715-717.

Jarrett WF, McNeil PE, Grimshaw WT, Selman IE, et al. (1978). High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papillomavirus. *Nature* 274: 215-217.

Lindsey CL, Almeida ME, Vicari CF, Carvalho C, et al. (2009). Bovine papillomavirus DNA in milk, blood, urine, semen, and spermatozoa of bovine papillomavirus-infected animals. *Genet. Mol. Res.* 8: 310-318.

Melo TC, Diniz N, Campos SRC, Ferraz OP, et al. Cytogenetic studies in bovines infected by BPV: first evaluation in

Pernambuco, Brazil. *Vet. Dermatol.* (in press).

Moura JW, Stocco dos Santos RC, Dagli ML, D'Angelino JL, et al. (1988). Chromosome aberrations in cattle raised on bracken fern pasture. *Experientia* 44: 785-788.

Nasir L and Campo MS (2008). Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and equids. *Vet. Dermatol.* 19: 243-254.

Reddy AL and Fialkow PJ (1983). Papillomas induced by initiation-promotion differ from those induced by carcinogen alone. *Nature* 304: 69-71.

Roperto S, Brun R, Paolini F, Urraro C, et al. (2008). Detection of bovine papillomavirus type 2 in the peripheral blood of cattle with urinary bladder tumours: possible biological role. *J. Gen. Virol.* 89: 3027-3033.

Stocco dos Santos RC, Ferraz O, Castro N, Pinto JR, et al. (1993). Chromosome Aberrations in Cattle Inoculated with Blood of Hematuric Bovines. In: Proceedings of the Seventeenth International Congress of Genetics. August 15-21, Birmingham, 213.

Stocco dos Santos RC, Lindsey CJ, Ferraz OP, Pinto JR, et al. (1998). Bovine papillomavirus transmission and chromosomal aberrations: an experimental model. *J. Gen. Virol.* 79 (Pt 9): 2127-2135.

Wosiacki SR, Barreiro MA, Alfieri AF and Alfieri AA (2005). Semi-nested PCR for detection and typing of bovine Papillomavirus type 2 in urinary bladder and whole blood from cattle with enzootic haematuria. *J. Virol. Methods* 126: 215-219.

Yaguiu A, Dagli ML, Birgel EH Jr, Alves Reis BC, et al. (2008). Simultaneous presence of bovine papillomavirus and bovine leukemia virus in different bovine tissues: *in situ* hybridization and cytogenetic analysis. *Genet. Mol. Res.* 7: 487-497.

 ©FUNPEC-RP www.funpecrp.com.br