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**Original Full Paper**

# PCR Amplification of DNA Sequence of Bovine Papillomavirus Type 2 in Urinary Bladder of Cattle with Enzootic Hematuria in Espirito Santo, Brazil

Dyeime Ribeiro de Sousa[1], Anderson Barros Archanjo[2], Mariana Drummond Costa Ignacchiti[2], Olavo dos Santos Pereira Júnior[2], Ana Paula Madureira[3], Louisiane de Carvalho Nunes[1]*

[1]Universidade Federal do Espírito Santo (UFES), Alegre, ES, Brazil;
[2]Departamento de Farmácia e Nutrição, Universidade Federal do Espírito Santo (UFES), Alegre, ES, Brazil;
[3]Departamento de Engenharia de Biossistemas. Universidade Federal de São João Del Rei, UFSJ, São João Del Rei, Minas Gerais, Brazil.
*** Corresponding Author:** Depto de Medicina Veterinária, CCA, Universidade Federal do Espírito Santo, PO Box 16, 29500-000 Alegre, Espírito Santo, Brazil Tel.: +55 (28) 3552 8643. Email address: louisiane.nunes@ufes.br

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**Abstract**

The goal of this study was to investigate the presence of bovine papillomavirus type 2 (BPV-2) genomic DNA using polymerase chain reaction (PCR) assays, in the urinary bladder of cattle with bovine enzootic hematuria (BEH), in the neoplastic and non-neoplastic lesions in Espírito Santo, Brazil. Thus, 46 bladders with gross BEH lesions were collected from adult cattle at slaughterhouses. The samples were divided into two portions: one for use in histopathological analysis and the other one for PCR. Microscopic analysis showed that 56.52% of the bladders presented neoplastic lesions, of which 50% were benign, 23.08% were malignant and 26.92% presented both types of neoplasms. The remaining 43.48% of the bladders did not show any neoplasms. Presence of BPV-2 was confirmed in 43.48% of the bladders: 65% of these were found in neoplastic lesions and 35% in non-neoplastic lesions. Among the neoplastic lesions, detection of BPV-2 was more frequent in benign lesions (53.84%), followed by malignant lesions (23.08%) and by those presenting both benign and malignant lesions (23.08%). These results confirm the presence of BPV-2 in both neoplastic and non-neoplastic conditions in urinary bladders of cattle with enzootic hematuria from the studied geographic area.

**Key words**: bovine enzootic hematuria, polymerase chain reaction, bovine papillomavirus type 2

## Introduction

Bovine enzootic hematuria (BEH) is a chronic disease associated with poisoning by *Pteridium* sp. This is the only plant that has been confirmed to be a natural cause of cancer in animals. Such occurrences are due to the carcinogenic and mutagenic agents that this plant contains, which lead to neoplasm formation. Clinically, BEH causes intermittent hematuria and death due to anemia (20).

In Brazil, plants of the genus *Pteridium* has been responsible for losses in cattle rearing in regions where this plant is widespread (6,7). Moreover, in areas of BEH occurrence, such as in the states of Espírito Santo and Rio de Janeiro, the resulting economic losses are significant (8, 17). The association between bovine papillomavirus type 2 (BPV-2), ingestion of bracken fern and bladder tumors in cattle has already been documented (3,7,9,11). Jarrett et al. (7) also suggested that *Pteririum* sp. might have an immunosuppressive effect, thus contributing to tumor development.

BPVs are small viruses with double-stranded DNA (12) that are associated with neoplasm formation on the skin, teats, penis and bladder. They infect basal cells, which leads to formation of tumor lesions that are generally benign, with a tendency to regress in immunocompetent animals due to a cell-mediated immune

 

response (3). Although bladder tumors in cattle are rare, with a prevalence of 0.01 to 0.1% (18), prolonged ingestion of bracken fern increases the prevalence of neoplasms in up to 80% of the adult animals (2,3).

Considering the economic importance of BEH (20), especially in dairy cows and in different geographical regions worldwide (2,8), studies that contribute towards defining the etiology of neoplastic and non-neoplastic lesions are necessary and of great importance (21). On this basis, the objective of the present study was to investigate the presence of BPV-2, using PCR assays, in neoplasms and non-neoplastic lesions of the urinary bladder of animals with BEH.

## Material and Methods

*Sample collection and anatomical-pathological characterization of the urinary bladders*

Forty-six bladders presenting macroscopic lesions were collected at a slaughterhouse, in the state of Espírito Santo (Brazil), from adult cattle coming from farms with occurrences of *Pteridium* sp. or BEH. The urinary bladders were identified according to their origin, individually placed in plastic bags and then into cool boxes containing ice, for transportation to the Animal Pathology Section of the Federal University of Espírito Santo (UFES). Urine was collected in sterile tubes for confirmation of macro- or microhematuria, in accordance with the method of Falbo et al. (5).

Each bladder was divided into four quadrants (A, B, C e D), which corresponded to the cranial (A and B) and caudal (C e D) portions of the bladder, and segments containing gross lesions in each quadrant were collected. Each fragment was divided into two portions: one for histopathological analysis and the other one for PCR. A total of 184 samples were obtained for each evaluation technique. The samples were histologically processed by means of the routine method of paraffin embedment. The overall evaluation of morphological alterations and classification of the lesions as neoplastic or non-neoplastic was done in accordance with the methodology described by Peixoto et al. (11).

*Polymerase chain reaction (PCR)*

Extraction of total DNA was performed form all 46 urinary bladder samples in accordance with the CTAB/proteinase K method described by Sambrook et al. (15), modified as described below. DNA samples were suspended using 30 µL of TE/RNase (10 mM of Tris-HCl, pH 7.5; 1 mM of EDTA; and 40 µg/mL of RNase, pH 8.0), in a water bath at 70°C for 60 minutes. DNA quantification was carried out using a spectrophotometer (Femto, 700 Plus), at the wavelength $\lambda = 260$ nm.

Specific primers, as described by Wosiacki et al. (21), were used to amplify a fragment of the L1 gene of BPV-2. The nucleotide sequences were 5'-ATTCTAAAGGAGGACACG-3' (forward) and 5'-TGTTCCCAAAGTGTCTG-3' (reverse), and this pair of primers was used to amplify a 386-bp DNA fragment of the gene L1.

PCR was performed in a reaction mixture of 50 µL, containing 200 ng of total DNA in 1 Taq buffer/KCl, 2 mM of $MgCl_2$, 25 µM of each primer (Bioneer Inc, Alameda, CA, USA), 10 µM of dNTP and 1 U of Taq DNA polymerase (recombinant, Fermentas). The amplifications were done in a thermocycler (TC-3000 Barloworld Scientific Techne) programmed for 40 cycles, in which each cycle was composed of an initial step of denaturation at 94°C for 3 min, followed by 1 min at 94°C, 1 min at 48°C for primer annealing, 1 min for extension at 72°C and a final extension at 72°C for 7 min.

The reagent purity control (no DNA template) was used to test for the presence of a nonspecific reaction. The amplification products were analyzed by means of gel electrophoresis in a 1.5% (w/v) agarose gel stained with ethidium bromide (0.5 µL/mL). The images were viewed and recorded using a photo documentation system (L-PIX HE Loccus Biotecnologia). Normal bladder samples without micro or macroscopic injury were used as the negative control for viral DNA amplification. These were collected at a slaughterhouse in a BEH non-endemic area, under the same conditions.

*Statistical analysis*

The data were organized using the SAS statistical software package, version 8 (Statistical Analysis System; SAS Institute Inc., Cary, NC, USA: SAS Institute Inc., 2000), and were presented using descriptive statistics (absolute and percentage distributions) and analytical statistics. Associations among variables were estimated by means of Spearman's correlation. The chi-square and Fisher's exact tests were used to assess the significance of the proportions. Fisher's exact test was used when the expected frequencies of each cell in the cross tabulation were less than five. The tests were performed at a significance level of 0.05.

## Results

*Epidemiologic data*

The 46 urinary bladders evaluated in this study were obtained from cattle from various regions of the states of Espírito Santo (ES) and Minas Gerais (MG): 50% (23/46) from the municipality of Guaçuí, ES; 19.56% (9/46) from Muniz Freire, ES; 13.04% (6/46) from Alegre, ES; 4.35% (2/46) from Afonso Cláudio, ES; and 4.35% (2/46) from Mutum, MG.

*Clinical parameters and histopathological evaluation*

Urine analysis revealed macrohematuria in 50% (23/46) and microhematuria in 50% (23/46) of the samples. Microscopic evaluation of the bladders showed that 56.52% (26/46) presented some kind of neoplastic lesion. Of these, 50% (13/26) were benign (mainly hemangiomas and myxomas), 23.08% (6/26) were malignant (urothelial carcinoma, carcinoma *in situ*, adenocarcinoma and hemangiossarcoma) and 26.92% (7/26) presented benign and malignant neoplasms in the same sample. In 43.48% (20/46) of the urinary bladders, no neoplasms were detected. However, inflammatory, vascular, metaplastic and/or dysplastic lesions were observed.

The analysis of the presence of neoplasms per quadrant revealed 51 lesions among the 184 fragments obtained and, of these, 3.8% (7/184) were located in quadrant A, 4.34% (8/184) in quadrant B, 9.24% (17/184) in quadrant C and 10.33% (19/184) in quadrant D. There was no significant difference in the presence of neoplasms per quadrant.

*Evaluation by PCR*

PCR evaluation of BVP-2 enabled detection of the viral DNA in 43.48% (20/46) of the bladders. The viral DNA was found in neoplastic lesions in 65% (13/20) of the positive samples, and in non-neoplastic lesions in 35% (7/20). There was no significant difference regarding to viral DNA detection between the analyzed bladder with or without neoplasms ($P > 0.3767$, Fisher's exact tests). In the neoplastic lesion group, it was found that BPV-2 DNA was most commonly present in benign lesions alone (53.84%; 7/13), followed by presence in malignant lesions alone (23.08%; 3/13) and in samples presenting both benign and malignant lesions (23.08%; 3/13). Analysis of viral DNA presence in the bladders per quadrant, in all 184 fragments, showed that 4.9% (9/184) were detected in quadrant A; 4.34% (8/184) in quadrant B; 3.8% (7/184) in quadrant C; and 3.8% (7/184) in quadrant D. There was no significant difference in detection of BVP-2 DNA in relation to the location per quadrant in the bladders.

**Discussion**

The origin of cattle with bladder lesions utilized in this study was mostly from endemic areas for BEH and had high infestation with *Pteridium* sp. (17). In the municipalities of Mutum (MG) and Afonso Claudio (ES), the presence of BEH and contamination of pastures with bracken fern had previously been described by Silva et al. (16).

The microhematuria data collected in the present study were similar to those obtained by Falbo et al. (5). These data point out to the importance of carrying out urinalysis tests on animals living in areas endemic for BEH, since macroscopic observation of blood in the urine is not always possible. Oliveira (10) reported that there was a high correlation between macrohematuria and the presence of vascular tumors, considering also the occurrence of microhematuria in some cases. Silva et al. (16) reported that both macrohematuria and microhematuria could be present or absent with gross bladder lesions and histological changes could neoplastic or non-neoplastic conditions.

This study revealed that most of the bladders evaluated had neoplastic lesions, the varying histogenesis in neoplastic processes of bladder lesions of cattle with BEH, epithelial or mesenchymal of origin, benign or malignant, were also observed by other authors (7,11,20). The presence of non-neoplastic bladder changes reinforces that the histopathological findings in cattle with BEH is variable (5,11,19).

The BVP-2 evaluation showed that viral DNA was present in 43.48% of the bladders, thus suggesting the relationship between the viruses and fern bracken ingestion, with clinical hematuria, as previously described by Campo et al. (3), Borzacchiello et al. (2), and Wosiacki et al. (22). Although the absence of viral DNA was observed in 56.52% in this study, Wosiacki et al (22) reported that false negative results may occur due to the presence of inhibitors of the PCR reaction. When the benign lesions persist and progress to malignant lesions, the viral DNA is usually integrated into the host cell genome, and there is only one viral DNA copy for each host cell. The integration of papillomavirus DNA is considered an important step in tumour progression (3). It is believed that the absence of viral DNA in samples may also be the result of DNA modifications within the amplified region or primer binding site.

In non-neoplastic lesions, BPV-2 was confirmed in 35% of the samples. Somvanshi et al. (19) found this in 68% of the bladder sample and Resende et al. (13) found a positive viral DNA in 15.3%. Considering that, so far, no relationship has been found between the presence of viral DNA and a particular tumor type, the presence of different histological characteristics may be related to other factors in urinary bladders with BEH (1). There is a need to clarify the relationship between infections with BPV-2 and non-neoplastic changes. The mucosa of the urinary bladder is not considered to be a target organ for virus proliferation (3). However, it has been reported that BPV-2 can persist in a latent form in a variety of tissues (4,14). Moreover, when animals are immunosuppressed or the epithelium is damaged, the virus is activated, possibly through production of cytokines, which stimulate cell proliferation and induce expression of the viral genes, thereby leading to tumor formation (2,3).

 

## Conclusion

This study demonstrated that BPV-2 genomic DNA was present in urinary bladders of cattle with BEH, from the studied geographic area.

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## References

1. BALCOS LGF., BORZACCHIELLO G., RUSSO V., POPESCU O., ROPERTO S., ROPERTO F., Association of bovine papillomavirus type-2 and urinary bladder tumours in cattle from Romania. **Vet. Res. Sci.**, 2008, 85, 145-148.
2. BORZACCHIELLO G., IOVANE G., MARCANTE ML., POGGIALI F., ROPERTO F., ROPERTO S., VENUTI A. Presence of bovine papilomavirus type 2 DNA and expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows. **J. Gen. Virol.**, 2003, 84, 2921-2926.
3. CAMPO MS., JARRETT WFH., BARRON R., O'NEIL BW., SMITH KT. Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. **Cancer Res.**, 1992, 52, 6898-6904.
4. DINIZ N., MELO TC., SANTOS JF., MORI E., BRANDÃO PE., RICHTZENHAIN LJ., FREITAS AC., BEÇAK W., CARVALHO RF., STOCCO RC. Simultaneous presence of bovine papillomavirus in blood and in short-term lymphocyte cultures from dairy cattle in Pernambuco, Brazil. **Genet. Mol. Res.**, 2009, 8, 1474-1480.
5. FALBO MK., REIS ACF., BALARIN MRS., BRACARENSE APFRL., JR ARAÚJO JP., WERNER O., SANDINI IE. Alterações hematológicas, bioquímicas, urinárias e histopatológicas na intoxicação natural em bovinos pela samambaia *Pteridium aquilnum* (L.) Kühn. **Ciên. Agr. londrina.**, 2005, 26, 547-558.
6. FURLAN FH., MENDES ERS., DUCATTI KR., MARCON GC., DOMBROSKY T., AMORIM TM., RIET-CORREA F. Intoxicação aguda por *Pteridium aracnoideum* e *Pteridium caudatum* em bovinos e distribuição das plantas em Mato Grosso. **Pesq. Vet. Bras.**, 2014, 34, 343-348.
7. GABRIEL AL., KOMMERS GD., MASUDA EK., FIGHERA RA., PIAZER JVM., BARROS CSL., MARTINS TB., ROSA FB. Aspectos clínico-hematológicos e lesões vesicais na intoxicação crônica espontânea por *Pteridium aquilinum* em bovinos. **Pesq. Vet. Bras**, 2009, 29, 515 – 525.
8. GALVÃO A., BRITO MF., ARAGAO AP., YAMASAKI EM., PEIXOTO PV., TOKARNIA CH. Sobrevivência/viabilidade de bovinos com hematúria enzoótica após transferência para região livre de *Pteridium arachnoideum*. **Pesq. Vet. Bras**, 2012, 32, 887-902.
9. JARRETT WF., MCNEIL PE., GRIMSHAW WT., SELMAN IE., MCINTYRE WI. High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papilloma virus. **Nature**, 1978, 20, 215-217.
10. OLIVEIRA LGP. **Novos Aspectos Patológicos e Patogenéticos da Hematúria Enzoótica Bovina.** Dissertação, Mestrado em Medicina Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, Brasil. 2009.
11. PEIXOTO PV., FRANÇA TN., BARROS CSL., TOKARNIA CH., Histopathological aspects of bovine enzootic hematuria in Brazil. **Pes. Vet. Bras.**, 2003, 23, 65-81.
12. QUINN PJ., MARKEY BK., CARTER ME., DONNELLY WJC., LEONARD FC. **Microbiologia Veterinária e Doenças Infecciosas,** Editora Artmed, Porto Alegre, 2005: 512 p.
13. RESENDE AR., ROPERTO S., TRAPAN F., URRARO C., RODRIGUES A., ROPERTO F., BORZACCHIELLO G. Association of bovine papillomavirus type 2 (BPV-2) and urinary bladder tumours in cattle from the Azores archipelago. **Res. Vet. Sci.**, 2011, 90, 526-529.
14. ROPERTO S., BRUN R., PAOLINI F., URRARO C., RUSSO V., BORZACCHIELLO G., PAGNINI U., RASO C., RIZZO C., ROPERTO F., VENUTI A., Detection of bovine papillomavirus type 2 in the peripheral blood of cattle with urinary bladder tumours: possible biological role. **J. Gen. Virol.**, 2008, 89, 3027-3033.
15. SAMBROOK J., FRITSCH EF., MANIATIS T., **Molecular cloning: a laboratory manual**, Cold Spring Harbor Laboratory, New York, 1989.
16. SILVA MA., SOUSA DR., MADUREIRA AP., NUNES LC. Caracterização histopatológica de bexigas associadas à hematúria enzoótica bovina, **Rev. Bras. Med. Vet**, 2012, 34, 319-326.
17. SILVA MA., SCÁRDUA CM., DÓREA MD., NUNES LC., MARTINS IVF., DONATELE DM. Prevalência de hematúria enzoótica bovina em rebanhos leiteiros na microrregião do Caparaó, Sul do Espírito Santo, entre 2007 e 2008. **Ciên. Rur.**, 2009, 39, 1847-1850.
18. SMITH BL., The toxicity of bracken fern (*genus Pteridium*) to animals and its relevance to man. In: Felix D'Mello, J.P. (Ed.), **Handbook of Plant and Fungal Toxicants**, CRC Press, Boca Raton, Florida, USA, 1997, p. 705-773.
19. SOMVANSHI R., PATHANIA S., NAGARAJAN N., PANGTY K., KUMAR P. Pathological study of non-neoplastic urinary bladder lesions in cattle and

buffaloes: a preliminary report. **Trop. Anim. Health. Prod.**, 2011, 44, 855-861.

20. TOKARNIA CH., BRITO MF., BARBOSA JD., PEIXOTO PV., DÖBEREINER J. **Plantas Tóxicas do Brasil para Animais de Produção**. Helianthus, Rio de Janeiro, 2012: 566 p.
21. WOSIACKI SR., BARREIROS MAB., ALFIERI AF., ALFIERI AA. Seminested- PCR for detection and typing of bovine papilomavirus type 2 in urinary bladder and whole blood from cattle with enzootic haematuria. **J. Virol. Meth.**, 2005, 126, 215-219.
22. WOSIACKI SR., CLAUS MP., ALFIERI AA., ALFIERI AF., Bovine papillomavius type 2 detection in the urinary bladder of cattle with chronic enzootic haematuria**. Mem. Inst Oswaldo Cruz**, 2006, 101, 635-638.

 