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Detection of bovine papillomavirus type 2 in the peripheral blood of cattle with urinary bladder tumours: possible biological role

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Bovine papillomavirus type 2 (BPV-2) infection has been associated with urinary bladder tumours in adult cattle grazing on bracken fern-infested land. In this study, we investigated the simultaneous presence of BPV-2 in whole blood and urinary bladder tumours of adult cattle in an attempt to better understand the biological role of circulating BPV-2. Peripheral blood samples were collected from 78 cattle clinically suffering from a severe chronic enzootic haematuria. Circulating BPV-2 DNA was detected in 61 of them and in two blood samples from healthy cows. Fifty of the affected animals were slaughtered at public slaughterhouses and neoplastic proliferations in the urinary bladder were detected in all of them. BPV-2 DNA was amplified and sequenced in 78 % of urinary bladder tumour samples and in 38.9 % of normal samples as a control. Circulating episomal BPV-2 DNA was detected in 78.2 % of the blood samples. Simultaneous presence of BPV-2 DNA in neoplastic bladder and blood samples was detected in 37 animals. Specific viral E5 mRNA and E5 oncoprotein were also detected in blood by RT-PCR and Western blot/immunocytochemistry, respectively. It is likely that BPV-2 can persist and be maintained in an active status in the bloodstream, in particular in the lymphocytes, as a reservoir of viral infection that, in the presence of co-carcinogens, may cause the development of urinary bladder tumours.

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INTRODUCTION

Bovine papillomaviruses (BPVs) are species-specific, double-stranded DNA viruses responsible for cutaneous and mucosal neoplastic lesions. They are small non-enveloped viruses with an icosahedral capsid. Their open reading frames (ORFs) 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 forming the capsid. Bovine papillomavirus type 2 (BPV-2) is classified in the genus *Deltapapillomavirus*, species 4, the biological properties of which are characterized by the induction of fibropapillomas in cattle and sarcoids in equids (Brandt *et al.*, 2008; Chambers *et al.*, 2003; de Villiers *et al.*, 2004). BPV-2 infection in the presence of environmental carcinogens, such as ptaquiloside (PT) of bracken fern (*Pteridium aquilinum*), has been associated with urinary bladder neoplastic lesions in adult cattle, in which chronic enzootic haematuria (CEH) is the most important clinical sign (Campo, 1997; Campo *et al.*, 1992; Hopkins, 1986).

The effect of the route of viral infection, and the synergistic relationship between BPV-2 and immunosuppressive and oncogenic compounds present in the bracken fern in the malignant progression of bladder lesions (Campo, 1997; Campo *et al.*, 1992; Jarrett *et al.*, 1978; Reddy & Fialkow, 1983; Stocco dos Santos *et al.*, 1998) are not well-known, thus deserving further investigations.

To date, the BPV-2 genome has been detected in lymphocytes during latent papillomavirus infection in cattle (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 haematuric animals (Stocco dos Santos *et al.*,

A supplementary figure showing sequence data is available with the online version of this paper.

1998). More recently, BPV-2 DNA was detected in seven of 12 urinary bladders and 10 of 14 blood samples obtained from Brazilian cattle suffering from CEH. No histological diagnosis of any tumours was performed. In addition, BPV-2 was also detected in one urinary bladder and in one whole blood sample from asymptomatic cattle (Wosiacki *et al.*, 2005).

In an attempt to gain insights into understanding the role and significance of BPV-2 presence in the blood stream, an analysis of blood samples was carried out on a large number of haematuric cattle grazing on bracken fern-infested lands, where urinary bladder tumours occur endemically (Borzacchiello *et al.*, 2003).

METHODS

Blood and bladder samples. Blood samples from 78 cows with clear clinical signs of chronic haematuria and from 14 healthy 4- to 8-year-old control cows were collected; both groups were the same Podolica breed and shared grazing lands. A post-mortem examination was carried out on 50 of the haematuric animals following slaughter at public slaughterhouses. Most of them appeared to be cachectic, showing a metabolic state characterized by weight and muscle loss as well as anaemia and they were not fit for human consumption. Eighteen bladder samples from healthy animals were also collected in the same way.

Samples of neoplastic and control bladder mucosa were split in two. One half was immediately fixed in 10 % neutral formalin and embedded in paraffin. Ticked sections (5 μm) were cut and stained with haematoxylin and eosin. Neoplastic lesions were classified according to the criteria reported in the recent World Health Organization (WHO) Blue Book on the pathology and genetics of tumours of the urinary system and male genital organs in humans (Lopez-Beltran *et al.*, 2004; Sauter *et al.*, 2004). Tumours of the urinary bladder in cattle share many morphological similarities to their human counterparts, and the WHO histological classification appears to encompass all microscopic patterns of the urinary bladder tumours observed in cattle (Roperto *et al.*, 2007). The other half of the sample was immediately frozen in liquid nitrogen and stored in dry ice.

BPV-2 DNA detection and sequencing. Unfractionated whole blood samples and frozen samples of bladder tumours were analysed by PCR as described previously (Borzacchiello *et al.*, 2003). Briefly, venous blood was collected in heparinized vacutainers (BD Biosciences) and then stored at 4 °C; DNA was extracted with QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Frozen bladder samples were minced and incubated at 55 °C overnight with 60 μg proteinase K ml^{-1} in the appropriate buffer (Manos *et al.*, 1989) and then at 95 °C for 10 min to inactivate the enzyme. Each sample (10 μl) was amplified in 50 μl reaction mixture containing 3 mM $MgCl_2$, 1 U Platinum *Taq* (Invitrogen), 25 pmol each primer and 200 μM dNTPs. The reaction was carried out in an iCycler (Bio-Rad Laboratories) using forward (5′-TTGCTGCAATGCAACTGCTG-3′) and reverse (5′-TCATAGGCACTGGCACGTT-3′) primers that amplify a DNA fragment encompassing part of the E5 and L2 open reading frame (ORF) of BPV-1 (311 bp, from nt 3915 to 4226) and BPV-2 (306 bp, from nt 3919 to 4225) (Otten *et al.*, 1993). PCR conditions were as follows: denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 1 min. The final PCR products were electrophoresed in 2 % agarose gel and visualized by ethidium bromide staining.

To confirm the PCR data, the amplified band was excised from the gel and purified through silicagel membranes by using the QIAquick PCR quantification kit, according to the manufacturer's instructions (Qiagen). Then, the amplified DNA was subjected to direct sequencing in an automated apparatus (Biogen).

Detection of circular BPV-2 DNA. BPV-2-positive DNA samples from tissues and blood were analysed by rolling-circle amplification (RCA) according to the method developed by Rector *et al.* (2004). The multiple-primed RCA is a method that utilizes the Φ29 DNA polymerase with random hexamer primers to amplify the complete circular genome of papillomaviruses without the need for prior knowledge of their DNA sequences. Briefly, multiple-primed RCA was performed with the TempliPhi 100 amplification kit (Amersham Biosciences) according to the manufacturer's instructions. Extracted DNA (2 μg) was transferred into a 0.5 ml tube with 5 μl TempliPhi sample buffer containing 450 μM extra dNTPs, and 0.2 μl TempliPhi enzyme mix containing the Φ29 DNA polymerase and exonuclease-protected random hexamers in 50 % glycerol. The samples were denatured at 95 °C for 3 min and then placed on ice. The reaction mixtures were incubated overnight (approx. 16 h) at 30 °C. Afterwards, the reaction mixtures were placed on ice, subsequently heated to 65 °C for 10 min to inactivate the Φ29 DNA polymerase, and stored at −20 °C until further analysis. The multiply primed RCA products (2 μl) were digested with 10 U *Eco*RI, a single cutter of the BPV-2 complete genome, and resolved in an ethidium bromide-stained agarose gel. As a negative control, water was amplified by multiple primed RCA and 2 μl was digested with *Eco*RI.

BPV-2 mRNA in blood samples. Blood samples were collected from 15 animals with cancer and stored in PAXgene blood RNA tubes (Qiagen) containing a proprietary reagent that immediately stabilizes intracellular RNA. Purification of total RNA from bovine whole blood was performed with the PAXgene blood RNA kit (Qiagen) according to the manufacturer's instructions. RNA (300 ng) was reverse transcribed using the Superscript III First Strand kit (Invitrogen) in a final volume of 20 μl. To rule out the presence of contaminating DNA in RNA samples, all the assays were performed both with and without reverse transcriptase. The synthesized cDNA was analysed by PCR with specific primers for the E5 ORF (forward primer, 5′-CACTGCCATTTGTTTTTTTC-3′; reverse primer, 5′-GGAGCAC TCAAAATGATCCC-3′) using the PCR conditions described above, but using an annealing temperature of 48 °C. The final amplified products were electrophoresed in a 2 % agarose gel and visualized by ethidium bromide staining. The amplified band was excised from the gel and subjected to direct sequencing as above.

Protein isolation and E5 expression. Fifteen bladder samples stored in dry ice were homogenized in lysis buffer containing 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 % Triton X-100. Immediately prior to use, the following were added: 1 mM DTT, 2 mM PMSF, 1.7 mg Aprotinin ml^{-1}, 25 mM NaF, 1 mM Na_3VO_4 (Sigma-Aldrich) by Ultra-turrax T8 Ika-Werke. The proteins were removed by centrifugation at 12 300 ***g*** for 30 min at 4 °C. The protein concentration was measured using the Bradford assay (Bio-Rad).

Blood samples (30 ml) were harvested from cattle suffering from chronic enzootic haematuria. An equal volume of 1 × PBS was added to the blood and the resulting solution was transferred on a 2 : 1 Ficoll gradient (Biochrom AG). After centrifugation at 352 ***g*** for 20 min at room temperature, the recovered lymphocytes were homogenized with 50 μl lysis buffer (described above for bladder samples). The protein concentration was measured using the Bradford assay (Bio-Rad).

Proteins derived from tissues (1 mg) or from lymphocytes (500 μg) were immunoprecipitated by using 2 μg antibody anti-E5 (a kind gift from Dr M. S. Campo, University of Glasgow, Scotland) and 30 μl G-Sepharose (GE Healthcare). Immunoprecipitates were washed four times in complete lysis buffer (above), finally we added 4× LDS loading buffer (Invitrogen) and then heated at 70 °C for 10 min. Immunoprecipitates were separated on 4–12 % polyacrylamide gels and transferred to nitrocellulose filter membranes (Bio-Rad) for 16 h at 25 mA in 192 mM glycine/25 mM Tris-HCl (pH 7.5)/10 % methanol. Membranes were blocked for 1 h at room temperature in 5 % non-fat dried milk and incubated with primary antibody overnight at 4 °C. After three washes in Tris-buffered saline, membranes were incubated with rabbit anti-sheep IgG–horseradish peroxidase (HRP) (Santa Cruz) for 30 min at room temperature. Proteins were visualised by enhanced chemiluminescence (Amersham Biosciences).

Cytospin preparations and immunocytochemistry. Blood from cows with haematuria was collected in heparin-coated tubes. Blood (100 μl) was lysed with 700 μl erythrocyte lysis buffer containing 0.8 % ammonium chloride and then leukocytes were washed twice by centrifugation at 250 ***g*** in 1 % PBS. A 100 μl aliquot of each sample was put into the appropriate well of a cytospin chamber (Thermo Scientific) and centrifuged at 164 ***g*** for 5 min at 4 °C. After fixing in methanol, slides were blocked for endogenous peroxidase activity in 0.3 % H_2O_2 in methanol for 20 min. Slides were then incubated overnight at room temperature in a humidified chamber with a 1 : 500 dilution of polyclonal sheep anti-E5 antibody (kindly provided by Dr M. S. Campo). The slides were washed three times with PBS, then incubated for 30 min with a 1 : 100 dilution of the appropriate biotinylated rabbit anti-sheep IgG (Santa Cruz). They were washed three times with PBS and then incubated with streptavidin-conjugated HRP (LSAB kit; DakoCytomation). Colour was developed by treatment with diaminobenzidine (DakoCytomation) for 5–20 min. Sections were counterstained with Mayer's haematoxylin.

RESULTS

Microscopic patterns

Neoplastic lesions were detected in the urinary bladder of all of the slaughtered animals. Flat lesions were observed in six animals, papillary proliferations were detected in 25 animals and the remaining 19 cattle had invasive tumours. In the last group, polypoid, ulcerated and/or sessile mucosal lesions were also manifest. Five of the flat lesions showed histological patterns of carcinoma *in situ* and one of urothelial dysplasia; endoluminal proliferations included three urothelial papillomas, four papillary urothelial neoplasms of low malignant potential, nine low-grade urothelial carcinomas and six high-grade urothelial carcinomas. In addition, two adenocarcinomas (enteric type) and only one squamous cell carcinoma were seen. Invasive tumours showed the histological features of low-grade invasive carcinomas (six cases), high-grade invasive carcinomas (ten cases), the nested variant of urothelial carcinomas (two cases) and a sarcomatoid carcinoma (one case).

BPV-2 DNA detection

PCR analysis, validated by direct sequencing of the amplified product, demonstrated the presence of true BPV-2 sequences; only the sequenced samples were defined as being positive. BPV-2 sequences were detected in 39 of the 50 tumour samples (78 % of examined cases) and in seven of 18 normal bladder control samples (39 % of the examined cases). In contrast, none of the analysed samples showed the presence of BPV-1 sequences. The difference in the presence of BPV-2 DNA between pathological and normal samples was highly significant (Fisher's exact test, $P<0.004$), suggesting a strong association of BPV infection with bladder tumours. Direct sequencing detected some false-positive samples arising from bovine genomic sequences (about 2 % of all analysed samples), as reported previously (Borzacchiello *et al.*, 2003).

BPV-2 sequences were detected in 61 of 78 blood samples of haematuric animals (78.2 % of the examined samples) and in two of 14 healthy samples (~14.2 % of the examined samples) (Fig. 1a). The difference in the presence of BPV-2 DNA between pathological and normal blood samples was highly significant (Fisher's exact test, $P<0.001$).

The data from the PCR and direct sequencing showed a high (94.87 %) concordance between positive blood and bladder samples in the 50 animals suffering from bladder tumours

Sequence analysis

Sequence analyses of the positive bladder and blood samples from the same animal were done using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and BLAST [http://

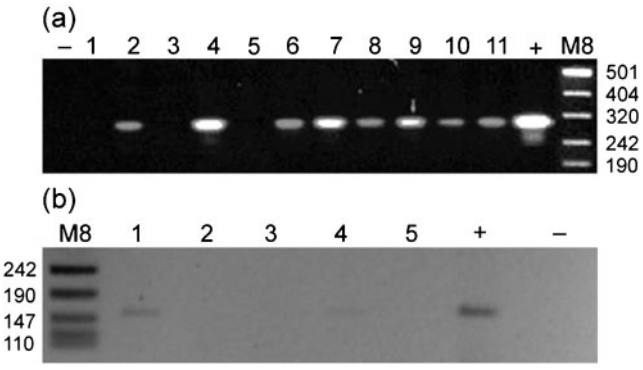

Fig. 1. PCR of blood samples. (a) DNA extracted from whole blood samples from healthy animals (lanes 1–5) and those with cancer (lanes 6–11) was subjected to PCR amplification with primers for BPV. The 310 bp amplification products were visualized in an ethidium-bromide-stained 2 % agarose gel. +, DNA from a recombinant plasmid for BPV-2; –, no DNA; M8, molecular mass marker type VIII (Roche), sizes given to the right (bp). (b) RT-PCR of total RNA from whole blood of healthy animals (lanes 2 and 5) or those with BPV-2-positive bladder tumours (lanes 1, 3 and 4). The length of the E5 amplification product was 150 bp. +, DNA from a BPV-2-positive bladder tumour; –, no cDNA; M8, molecular mass marker type VIII (Roche), sizes given to the left (bp).

www.ncbi.nlm.nih.gov/BLAST (Altschul *et al.*, 1997)] programs. This revealed the presence of DNA sequences matching the BPV-2 complete genome from the GenBank database, and in one case revealed the presence of two mutations in the non-coding region between the E5 and L2 genes (Supplementary Fig. S1, available in JGV Online). Alignment of this DNA sequence with those in the database revealed that the same mutations were present in the DNA sequence of a BPV-2 isolate from Hungarian equine sarcoids (GenBank accession number AF102551).

BPV-2 physical status and mRNA expression

Episomal (circular) forms of the BPV DNA were detected in 39 bladder tumour and blood samples collected from the same animals by using the multiple primed RCA (Fig. 2). Samples were digested with *Eco*RI, which recognizes a single cut site in the BPV-2 genome, and the detection of a single ~8000 bp band indicated the presence of episomal forms. Despite the fact that this was a non-quantitative method, it is noteworthy that the band intensity of circular BPV DNA recovered from blood samples was always lower than that detected in tumour samples. Unfortunately, RNA from these samples was not available for the detection of viral transcripts, so we were unable to verify the expression of the viral genes. Nevertheless, the presence of viral transcripts for the E5 ORF was detected in two of three blood samples from other animals with BPV-2-positive bladder tumours (Fig. 1b).

Presence of the BPV-2 E5 oncoprotein

Western blot analysis showed the presence of E5 protein in neoplastic tissues (Fig. 3a) and in peripheral blood cells from animals with cancer (Fig. 3b). Analysis of cytospin preparations of peripheral blood leukocytes from infected animals demonstrated a strong cytoplasmic E5 immunoreactivity in numerous lymphocytes (Fig. 4). No E5 oncoprotein expression was detected in healthy animals.

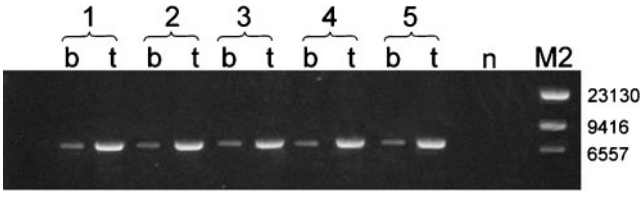

Fig. 2. Detection of circular BPV-2 DNA in bladder and blood samples by RCA. DNA was processed as in Methods and the amplified products were digested with *Eco*RI. Lanes: 1–5, DNA from BPV-2 infected animals; b, DNA from blood sample; t, DNA from bladder sample; M, molecular mass marker type II (Roche), sizes given to the right (bp); n, negative control consisting of water amplified by multiple primed RCA that was digested with *Eco*RI.

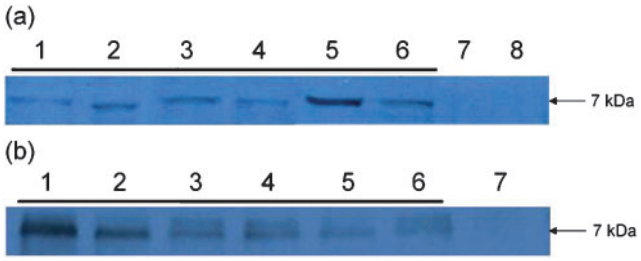

Fig. 3. Western blot analysis of E5 protein from neoplastic bladder tissues and peripheral blood cells. (a) Lanes 1–6, neoplastic tissues; lanes 7–8 healthy animals. (b) Lanes 1–6, blood from animals with cancer; lane 7, blood from a healthy animal.

DISCUSSION

This study focused on the presence of BPV-2 in the blood of a large number of haematuric cattle suffering from urinary bladder tumours. The results of PCR analyses, confirmed by direct sequencing, also revealed the presence of false positive samples, probably because the primers represent consensus sequences devised to amplify genomic sequences of different BPVs (i.e. BPV-1 and BPV-2). It has been suggested that the use of consensus primers may

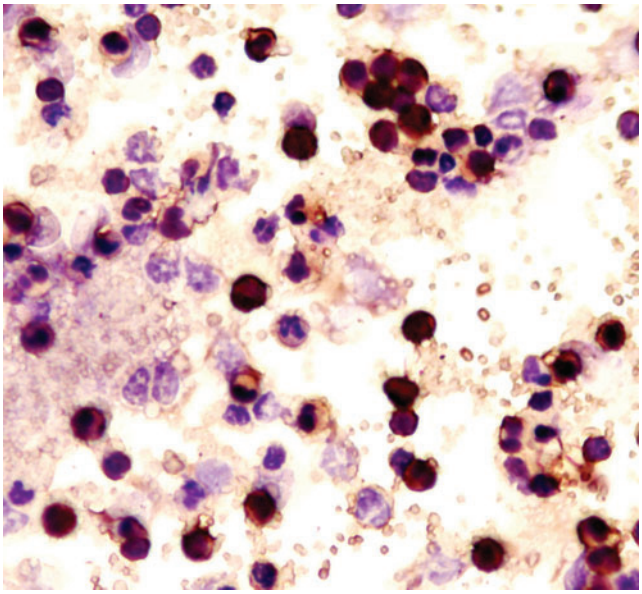

Fig. 4. Detection of cytoplasmic E5 in lymphocyte cells from cytospin preparations of PBMCs. Samples were incubated with sheep anti-E5, then rabbit anti-sheep IgG followed by streptavidin-conjugated HRP. Diaminobenzidine was used for colour development and samples were stained with haematoxylin. Magnification, ×550.

increase the possibility of amplifying non-specific sequences, as is the case for the MY09/11 primers for human papillomavirus (Fernandez-Contreras *et al.*, 2000). These facts should be taken into account when evaluating a positive result from a PCR using consensus primers if this is the only diagnostic method used. In this analysis, only sequenced samples were considered; this revealed the presence of BPV-2 in whole blood samples. Moreover, RCA analysis suggested that the complete genome of BPV-2 was present in circular form in blood samples as well as in bladder tumours.

Our data on a large number of animals with histologically confirmed neoplastic disease not only clearly corroborate previous reports (Campo *et al.*, 1994; Stocco dos Santos *et al.*, 1998; Wosiacki *et al.*, 2005) but also raise a number of questions about the source, role and significance of the presence of BPV DNA and viral oncoproteins in blood.

The simultaneous presence of BPV-2 DNA sequences in most of the blood and bladder tumour samples suggests that BPV-2 DNA could be derived from common sources, thus implying that the virus might spread via a haematogenous route. Recently, human papillomavirus (HPV) DNA was detected in peripheral blood mononuclear cells (PBMCs) obtained from paediatric patients and healthy blood donors. It has been speculated that PBMCs may serve as a source of HPV in the infection of epithelial cells (Bodaghi *et al.*, 2005). If this is also the case in cattle, BPV-2 could reach the urothelial tissue not only from the paragenital area, as already suggested (Campo, 2002), but also via the blood stream, and could become involved in urinary bladder tumour development independently or jointly with several biological and/or chemical co-factors. PT, the major carcinogen of the bracken fern, is activated in alkaline environments such as the urine of herbivores (Borzacchiello *et al.*, 2006; Prakash *et al.*, 1996), it then appears to act synergistically with BPV-2, thus resulting ultimately in tumours of the urinary bladder in cattle. Recently, BPV DNA was simultaneously detected in different tissues of the same animals, thus suggesting a haematogenous virus spread (Freitas *et al.*, 2007). In our study, the detection of circulating BPV DNA in only a limited number of healthy cattle that share grazing lands with animals with cancer suggests the plausibility of this hypothesis. We not only detected circulating BPV DNA sequences but also showed the presence of episomal DNA by RCA analysis. The presence of circular BPV DNA implies that at least E1 and E2 proteins are expressed, as they are absolutely required for the maintenance and replication of the episomal BPV DNA (Tonon *et al.*, 2001). Moreover, mRNAs encoding the E5 gene were detected in a number of blood samples from animals suffering from BPV-positive bladder tumours. In addition to this, the E5 oncoprotein was detected in the blood of 13 of 15 cows suffering from urothelial tumours, in which E5 expression was documented in immunocytochemical analysis. Taken together, these data may indicate a biological activity of BPV-2 in the blood of these animals, reinforcing the hypothesis that the bloodstream may work as reservoir for BPV infection. In particular, it appears that lymphocytes are infected by BPV-2, as clearly demonstrated by the strong immunocytochemical positivity for the E5 protein.

An alternative explanation for circulating BPV DNA is that it might, at least in part, originate from primary bladder tumours. The presence of viral DNA in the peripheral blood fraction (PBF), plasma and serum is not an unusual event in tumour patients. Recently, HPV DNA was detected in serum and/or PBF of individuals with cervical, head/neck or schistosomiasis-associated bladder cancer (Yang *et al.*, 2005) and women suffering from cervical carcinomas (Ho *et al.*, 2005; Widschwendter *et al.*, 2003). It has been postulated that HPV DNA is involved in carcinogenesis in breast tissue in some patients suffering from cervical cancer (Widschwendter *et al.*, 2004). Very recently, sequences of the HPV types 16 and 18, known to be involved in cervical carcinogenesis, were detected in 86 and 48 % of breast cancer samples, respectively (de Villiers *et al.*, 2005; Kan *et al.*, 2005). It is noteworthy that HPV DNA has been detected both in lung and breast cancers of patients with a history of cervical cancer, thus suggesting that HPV DNA might be transported from the original site of infection to pulmonary and breast tissues by the bloodstream and subsequently be involved in secondary tumour development (Cheng *et al.*, 2001; Widschwendter *et al.*, 2004). However, it is well-known that urinary bladder cancers in cattle are characterized by a relatively low incidence of apparent metastases (about 8–10 %) (Pamukcu *et al.*, 1976; Roperto *et al.*, 2005). In addition, non-metastatic tumours in other organs of cattle with a history of urothelial cancer are known to occur very rarely. Therefore, it is reasonable to suggest that if the presence of BPV-2 E5 oncoprotein in PBMC is the result of a passive spread, the oncoprotein doesn't appear to play an important role in neoplastic events in distant organs. However, detection of E5 in blood may be utilized as an additional diagnostic and/or prognostic marker and as a target for introducing immunotherapeutic procedures to reduce deaths and economic losses due to BPV activity.

In conclusion, we provide evidence that BPV-2 may persist and be maintained in a replicative status in the bloodstream, in particular in the lymphocytes, and act as a reservoir of viral infection that in the presence of biological and/or chemical co-carcinogens can be involved in bladder tumour development.

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REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Bodaghi, S., Wood, L. V., Robby, G., Ryder, C., Steiberg, S. M. & Zeng, Z. M. (2005). Could human papillomavirus be spread through blood? *J Clin Microbiol* **43**, 5428–5434.

Borzacchiello, G., Iovane, G., Marcante, M. L., Poggiali, F., Roperto, F., Roperto, S. & Venuti, A. (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.

Borzacchiello, G., Russo, V., Gentile, F., Roperto, F., Venuti, A., Nitsch, L., Campo, M. S. & Roperto, S. (2006). Bovine papillomavirus E5 oncoprotein binds to the activated form of the platelet-derived growth factor β receptor in naturally occurring bovine urinary bladder tumours. *Oncogene* **25**, 1251–1260.

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

Campo, M. S. (1997). Papillomavirus and cancer. *Vet J* **154**, 175–188.

Campo, M. S. (2002). Animal models of papillomavirus pathogenesis. *Virus Res* **89**, 249–261.

Campo, M. S., Jarrett, W. F. H., Barron, R. J., O'Neil, B. W. & Smith, K. T. (1992). Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. *Cancer Res* **52**, 6898–6904.

Campo, M. S., Jarrett, W. F. H., O'Neil, B. W. & Barron, R. J. (1994). Latent papillomavirus infection in cattle. *Res Vet Sci* **56**, 151–157.

Chambers, G., Ellsmore, V. A., O'Brien, P. M., Reid, S. W., Love, S., Campo, M. S. & Nasir, L. (2003). Association of bovine papillomavirus with the equine sarcoid. *J Gen Virol* **84**, 1055–1062.

Cheng, Y. W., Chiou, H. L., Sheu, G. T., Hsich, L. L., Chen, J. T., Chen, C. Y., Su, J. M. & Lee, H. (2001). The association of human papillomavirus 16/18 infection with lung cancer among nonsmoking Taiwanese women. *Cancer Res* **61**, 2799–2803.

de Villiers, E. M., Fauquet, C., Broker, T. R., Bernard, H. U. & zur Hausen, H. (2004). Classification of papillomaviruses. *Virology* **324**, 17–27.

de Villiers, E. M., Sandstrom, R. E., zur Hausen, H. & Buck, C. E. (2005). Presence of papillomavirus sequences in condylomatous lesions of the mamillae and in invasive carcinoma of the breast. *Breast Cancer Res* **7**, R1–R11.

Fernandez-Contreras, M. E., Sarria, C., Nieto, S. & Lazo, P. A. (2000). Amplification of human genomic sequences by human papillomaviruses universal consensus primers. *J Virol Methods* **87**, 171–175.

Freitas, A. C., Silva, M. A. R., Carvalho, C. C. R., Birgel, E. H., dos Santos, J. F., Beçak, W. & Stocco dos Santos, R. C. (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*, vol. 2, pp 697–703, Edited by A. Méndez-Vilas. Badajoz: Formatex.

Ho, C. M., Yang, S. S., Chien, T. Y., Huang, S. H., Jeng, C. J. & Chang, S. F. (2005). Detection and quantitation of human papillomavirus type 16, 18 and 52 DNA in the peripheral blood of cervical cancer patients. *Gynecol Oncol* **99**, 615–621.

Hopkins, N. C. G. (1986). Aetiology of enzootic haematuria. *Vet Rec* **118**, 715–717.

Jarrett, W. F. H., McNeil, P. E., Grimshaw, W. T. R., Selman, I. E. & McIntyre, W. I. M. (1978). High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papillomavirus. *Nature* **274**, 215–217.

Kan, C. Y., Iacopetta, B. J., Lawson, J. S. & Whitaker, N. J. (2005). Identification of human papillomavirus DNA gene sequences in human breast cancer. *Br J Cancer* **93**, 946–948.

Lopez-Beltran, A., Sauter, G., Gasser, T., Hartmann, A., Schmitz-Dräger, B. J., Helpap, B., Ayala, A. G., Tamboli, P., Knowles, M. A. & other authors (2004). Infiltrating urothelial carcinoma. In *Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs*. Edited by J. N. Eble, G. Sauter, J. I. Epstein & I. A. Sesterhenn. Lyon: IARC Press.

Manos, M. M., Ting, J., Wright, D. K., Lewis, A. I., Broker, T. R. & Wolinsky, S. M. (1989). Use of polymerase chain reaction amplification for the detection of genital human papillomavirus. *Cancer Cells* **7**, 209–214.

Otten, N., von Tscharner, C., Lazary, S., Antczak, D. F. & Gerber, H. (1993). DNA of bovine papillomavirus type 1 and 2 in equine sarcoids: PCR detection and direct sequencing. *Arch Virol* **132**, 121–131.

Pamukcu, A. M., Price, J. M. & Bryan, G. T. (1976). Naturally occurring and bracken fern-induced bovine urinary bladder tumors. Clinical and morphological characteristics. *Vet Pathol* **13**, 110–122.

Prakash, A. S., Pereira, T. N., Smith, B. L., Shaw, G. & Seawright, A. A. (1996). Mechanism of bracken fern carcinogenesis: evidence for H-ras activation via initial adenine alkylation by ptaquiloside. *Nat Toxins* **4**, 221–227.

Rector, A., Tachezy, R. & Van Ranst, M. (2004). A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. *J Virol* **78**, 4993–4998.

Reddy, A. L. & Fialkow, P. J. (1983). Papillomas induced by initiation promotion differ from those induced by carcinogen alone. *Nature* **304**, 69–71.

Roperto, S., Ambrosio, V., Borzacchiello, G., Galati, P., Paciello, O., Russo, V. & Roperto, F. (2005). Bovine papillomavirus type-2 (PBV-2) infection and expression of uroplakin IIIb, a novel urothelial cell marker, in urinary bladder tumors of cows. *Vet Pathol* **42**, 812–818.

Roperto, S., Borzacchiello, G., Cesellato, R., Galati, P., Russo, V., Sonnino, S. & Roperto, F. (2007). Sialic acid and GM3 ganglioside expression in papillomavirus-associated urinary bladder tumours of cattle with chronic enzootic haematuria. *J Comp Pathol* **137**, 87–93.

Sauter, G., Algaba, F., Amin, M. B., Busch, C., Cheville, J., Gasser, T., Grignon, D. J., Hofständer, F., Lopez-Beltran, A. & Epstein, J. I. (2004). Non-invasive urothelial tumours. In *Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs*. Edited by J. N. Eble, G. Sauter, J. I. Epstein & I. A. Sesterhenn. Lyon: IARC Press.

Stocco dos Santos, R. C., Lindsey, C. J., Ferraz, O. P., Pinto, J. R., Mirandola, R. S., Benesi, F. J., Birgel, E. H., Bragança Pereira, C. A. & Beçak, W. (1998). Bovine Papillomavirus transmission and chromosomal aberrations: an experimental model. *J Gen Virol* **79**, 2127–2135.

Tonon, S. A., Picconi, M. A., Bos, P. D., Zinovich, J. B., Galuppo, J., Alonio, L. V. & Teyssie, A. R. (2001). Physical status of E2 human

papilloma virus 16 viral gene in cervical preneoplastic and neoplastic lesions. *J Clin Virol* **21**, 129–134.

Widschwendter, A., Blassnig, A., Wiedemair, A., Müller-Holzner, E., Müller, H. M. & Marth, C. (2003). Human papillomavirus DNA in sera of cervical cancer patients as tumor marker. *Cancer Lett* **202**, 231–239.

Widschwendter, A., Brunhuber, T., Wiedemair, A., Müller-Holzner, E. & Marth, C. (2004). Detection of human papillomavirus DNA in breast cancer of patients with cervical cancer history. *J Clin Virol* **31**, 292–297.

Wosiacki, S. R., Barreiro, M. A. B., Alfieri, A. F. & Alfieri, A. A. (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.

Yang, H., Yang, K., Khafagi, A., Tang, Y., Carey, T. E., Opipari, A. W., Lieberman, R., Oeth, P. A., Lancaster, W. & other authors (2005). Sensitive detection of human papillomavirus in cervical, head/neck, and schistosomiasis-associated bladder malignancies. *Proc Natl Acad Sci U S A* **102**, 7683–7688.