Transboundary and Emerging Diseases

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ORIGINAL ARTICLE

Bovine Papillomavirus Type 13 Expression in the Urothelial Bladder Tumours of Cattle

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Summary

Bovine papillomavirus type 13 (BPV-13), a novel Deltapapillomavirus, has been found associated with urothelial tumours of the urinary bladder of cattle grazing on lands infested with bracken fern. BPV-13 was detected in 28 of 39 urothelial tumours. Diagnosis was based on sequencing of L1 and E5 amplicons from tumour samples. The nucleotide sequences generated from these amplicons showed a 100% homology with the sequences of BPV-13 L1 and E5 DNA found in Brazil from a fibropapilloma of the ear in a cow and from equine sarcoids in two horses. GenBank accession number of our representative BPV-13 sequences is JQ798171.1. Furthermore, mRNA encoding BPV-13 E5 oncoprotein was also documented, and its expression was also shown by immunohistochemistry and immunofluorescence in the basal and suprabasal urothelial tumour cells. In twenty-three tumours, BPV-13 was simultaneously found with BPV-2, a Deltapapillomavirus genus, species 4. The latter virus was detected by amplifying and sequencing a 154-bp-sized DNA fragment of BPV-2 E5. In addition, BPV-13 by itself was seen to be expressed in five BPV-2-negative urothelial tumours. This study shows that BPV-13 is present in urothelial tumour cells thus sharing biological properties with BPV-1 and BPV-2. Although further studies are needed, BPV-13 appears to be another worldwide infectious agent responsible for a distressing disease causing severe economic losses in cattle industry.

Introduction

Thirteen bovine papillomavirus (BPVs) types from cattle have so far been characterized and classified into three genera: Deltapapillomavirus (BPV-1, -2 and -13), Xipapillomavirus (BPV-3, -4, -6, -9, -10, -11, and -12), Epsilonpapillomavirus (BPV-5 and -8) (Lunardi et al., 2013a). BPV-7 has been proposed but not yet assigned to Dyoxipapillomavirus (Rector and Van Ranst, 2013).

Although BPVs have been characterized as epitheliotropic, Deltapapillomavirus are able to cause both epithelial and mesenchymal cell infections resulting in benign and malignant lesions of the skin and mucosa; indeed, their biological properties are prevalently characterized by inducing cutaneous fibropapillomas and a transspecies transmission (IARC, 2007).

Despite BPV DNA has been detected in blood and in non-epithelial tissues including reproductive tract of cattle (Campo et al., 1994; Wosiaki et al., 2005; Yaguiu et al., 2008; Lindsey et al., 2009; Santos et al., 2014), only recently a non-neoplastic productive *in vivo* Deltapapillomavirus infection has been shown to occur in peripheral blood mononuclear cells (PBMCs) of cattle (Roperto et al., 2011) and trophoblasts of the placenta of pregnant cows (Roperto et al., 2012).

Moreover, BPV-2 has consistently been associated with cancer of the urinary bladder, and the E5 protein, the major Deltapapillomavirus oncoprotein, is expressed in cancer

cells. Cofactors in pasture bracken fern that have been identified as chemical mutagens and immunosuppressants are prerequisite for the occurrence of cancer. BPV-1, and occasionally BPV-2, causes aggressive and persistent equine sarcoids (IARC, 2007).

BPV-13 is a novel papillomavirus recently found in a fibropapilloma of the ear of a cow (Lunardi et al., 2013a) and in equine sarcoids from two horses (Lunardi et al., 2013b). The phylogenetic analysis identified BPV-13 as the third member of the Deltapapillomavirus genus, species 4 (Lunardi et al., 2013a). Like BPV-1 and BPV-2, BPV-13 is able to infect both epithelial and mesenchymal cells and jumps species. Unlike other bovine Deltapapillomavirus, BPV-13 had not been found to be associated with urothelial tumours.

The aim of this study was to investigate the presence and the expression of BPV-13, alone and in combination with BPV-2 infection, in urothelial tumours of the urinary bladder in cattle grazing on fern-infested lands from southern Italy.

Materials and Methods

Ethics statement

In this study, we did not perform any animal experiments. We collected the samples directly from private and public slaughterhouses; the animals were slaughtered following a mandatory clinical ante-mortem examination as required by European Union (EU) legislation.

Tumour samples

Thirty-nine bovine urothelial tumour samples and five normal (control) bladder samples were collected with the permission of the medical authorities in some slaughterhouses.

Both neoplastic and normal bladder samples were routinely divided into several parts. Some parts were fixed in 10% buffered formalin for microscopic investigations. The remaining parts were immediately frozen in liquid nitrogen and stored at −80°C for subsequent biomolecular analysis.

Histopathology

The tissues fixed in 10% buffered formalin were routinely paraffin embedded. Histologic diagnosis was assessed on 5-μm-thick haematoxylin-eosin (HE)-stained sections using morphologic criteria suggested in the recent report on the new histological classification of urothelial tumours of the urinary bladder of cattle (Roperto et al., 2010).

DNA extraction and PCR for Bovine Papillomavirus type 13

DNA was extracted from 39 urothelial tumours and five normal bladder samples using the DNeasy Tissue kit (Qiagen, Hiden, Germany). The DNA concentration was determined from the 260 : 280 nm absorbance ratio (NanoVue Plus; GE Healthcare Life Sciences, Little Chalfont, UK). The PCRs amplified a 288 base pair (bp) product from BPV-13 L1 gene, (nt 5660 ± 5679, 5′-CCAACCCCAGTAAGCAAGGT-3′; nt 5947 ± 5927, 5′-AAGAGGTTGACCTCGGGAGA-3′) and a 153 bp product from the BPV-13 E5 ORF (nt 3852 ± 3871, 5′-CACTGCCATTTGGTGTTCTT-3′; nt 4004 ± 3983, 5′-AGCAGTCAAAATGATCCCAA-3′); furthermore, a 154 bp product from the BPV-2 E5 ORF was also amplified using the following specific primers: 5′-CACTGCCATTTGTTTTTTTC-3′ (nt 3841 ± 3861); 5′-GGAGCACTCAAAATGATCCC-3′ (nt 3992 ± 3972). A 199 bp product from the beta-actin gene was used to assess DNA extraction and sample integrity. The PCR was carried out in a 25 μl reaction mixture containing 2.5 μl 10× buffer, 2 mM $MgCl_2$, 2.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 480 nM of each primer and 200 mM of each dNTP and 50 ng DNA extract. PCR conditions were as follows: denaturation for 3 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s for BPV-13 L1 and E5 and at 50°C for 30 s for BPV-2 E5, and extension at 72°C for 5 min. In each experiment, a blank sample consisting of reaction mixture without DNA and a positive sample (a kind gift by Dr A.A. Alfieri, Universidade Estadual de Londrina, Brazil) were included. Amplified DNA was purified through silicagel membranes using the QIAquick PCR quantification kit according to the manufacturer's instructions (Qiagen).

PCR products were purified by Qiaquick® PCR purification Kit (Qiagen GmbH) and bidirectionally PCR sequenced with BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) following manufacturer's recommendations. Sequences were dye-terminator removed by DyeEx® 2.0 spin kit (Qiagen GmbH) and therefore run on 3500 Genetic Analyzer (Applied Biosystems). Electropherograms were analysed using Sequencing analysis v5.2 and sequence scanner v1.0 softwares (Applied Biosystems). The sequences obtained were compared to those reported in BLAST (http://blast.ncbi.nlm.nih.org).

RNA extraction and Reverse Transcription PCR for BPV-13 E5

Total RNA was extracted from the tumour and normal bladders using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA quality was

determined by agarose gel electrophoresis and ultraviolet spectrophotometer analysis. The RNA was treated with RNase-free DNase I Fermentas Life Sciences (Dasit, Milan, Italy) to remove potential DNA contamination.

Total RNA was transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and the reaction was incubated at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and then kept at 4°C for 5 min (RT+).

Furthermore, a reverse transcription without the reverse transcriptase enzyme was also performed (RT−).

The synthesized cDNA was analysed by PCR with specific primers for the E5 ORF (as described above). The RT-PCR product was sequenced after separation by gel electrophoresis.

Immunohistochemistry

Immunohistochemistry was performed in parallel on some normal as well as on BPV-2-negative neoplastic samples of the bovine urinary bladder. Briefly, paraffin sections were deparaffinized and blocked for endogenous peroxidase in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed by pre-treating with microwave heating in citrate buffer pH 6.0 (twice for 5 min each at 750 W). The slides were washed three times with phosphate buffered saline (PBS, pH 7.4, 0.01 M) then incubated for 1 h at room temperature with donkey serum (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted at 1 in 20 in PBS. A purified polyclonal sheep anti-BPV-2 E5 primary antibody (a kind gift by Prof. L. Nasir, Glasgow University) diluted at 1 in 5000 in phosphate buffered saline (PBS; pH 7.4, 0.01 M) was applied overnight at room temperature in a humid chamber. Then, the slides were rinsed for 20 min with PBS and incubated for 30 min with a secondary donkey anti-sheep antibody (Santa Cruz, Biotechnology Inc.) diluted at 1 in 100 in PBS. Sections were washed three times with PBS and then incubated with streptavidin-conjugated to horseradish peroxidase (LSAB Kit; DakoCytomation, Denmark). Colour development was obtained by treatment with diaminobenzidine (DakoCytomation, Glostrup, Denmark) for 5–20 min. Sections were counterstained with Mayer's haematoxylin.

Immunofluorescence

An immunofluorescence staining was also performed to detect BPV-13 E5 oncoprotein. The five BPV-2-negative tumour samples were stained, and normal bovine urinary bladder mucosa were tested in parallel as control. Sections were deparaffinized, rehydrated and heated in a microwave oven in citrate buffer pH 6.0 (twice for 5 min each at 750 W) to allow antigen unmasking. Briefly, the sections were rinsed three times in phosphate buffered saline (PBS; pH 7.4, 0.01 M), pre-incubated for 1 h with normal donkey serum (diluted at 1 in 20 in PBS) and then a purified polyclonal sheep anti-BPV-2 E5 primary antibody (a kind gift by Prof. L. Nasir, Glasgow University) diluted at 1 in 500 in phosphate buffered saline (PBS; pH 7.4, 0.01 M) was applied overnight at room temperature in a humid chamber. Before the exposure to the secondary antibody, all the slides were washed for 20 min with PBS. A secondary antibody Alexa Fluor 488 donkey anti-sheep (Invitrogen, Life Technologies, Carlsbad, CA, USA), diluted at 1 in 50 in PBS, was applied for 2 h at room temperature. After washing three times with PBS, the slides were mounted under aqueous medium (Sigma-Aldrich, Milan, Italy).

For immunofluorescence observations and photography, a laser scanning confocal microscope LSM-510 (Zeiss, Göttingen, Germany) was used.

Results

Histologic diagnosis of 39 urothelial bladder tumours was performed, and thirty-three of them (~85%) were found to be associated with papillomavirus infection. A coinfection with BPV-13 and BPV-2 was detected in 23 tumours (~70%). Further five tumours were from bladder samples expressing BPV-13 alone; BPV-2 alone was seen in additional five urothelial tumours (Table 1).

PCR analysis amplified BPV-13 L1 and E5 DNA fragments, length 288 and 153 bp, respectively, both in singly and double infected tumour samples. The presence of BPV-13 L1 and E5 DNA was also confirmed by sequencing, which revealed a 100% homology with BPV-13 L1 and E5 DNA sequences reported in GenBank (accession number is JQ798171.1) (Figs 1 and 2). In addition, BPV-13 DNA was also detected in the urothelium from one apparently healthy cattle.

Furthermore, in tumour samples only, reverse transcriptase PCR analysis demonstrated the presence of mRNA encoding BPV-13 E5 oncoprotein (Fig. 3). E5 was also detected by immunohistochemistry and immunofluorescence. Indeed, the expression of BPV-13 E5 oncoprotein was clearly shown in the five tumour samples which did not harbour any BPV-2 infection. A strong immunoreactivity for E5 protein was present in urothelial cells scattered both across basal and suprabasal layers (Fig. 4). BPV-13 E5 expression was also confirmed by immunofluorescence in the same samples (Fig. 5).

Molecular diagnosis of BPV-2 infection was based on amplifying by PCR analysis a 154-bp-sized DNA fragment known to be specific for BPV-2 E5. The sequence of these amplicons showed a 100% identity with sequence of BPV-2 E5 deposited in GenBank (accession number is M20219.1) (Fig. 6). This DNA fragment was also amplified and sequenced in bladder mucosa from two normal cattle.

Table 1. Deltapapillomavirus infection in bovine urothelial tumours

Microscopic patterns	BPV 2 E5 154 bp	BPV 13 E5 153 bp	BPV 13 L1 288 bp
Invasive carcinoma	–	–	–
Invasive carcinoma	+	–	–
Invasive carcinoma	+	+	+
Invasive carcinoma	+	+	+
Papillary carcinoma	+	+	+
Normal mucosa	–	+	+
CIS	–	–	–
PUNLMP	–	+	+
Papillary carcinoma	–	–	–
Invasive carcinoma	+	+	+
CIS	+	+	+
Papilloma	–	–	–
Papillary carcinoma	+	+	+
Papilloma	+	+	+
Papillary carcinoma	+	–	–
Papillary carcinoma	–	–	–
Papillary carcinoma	–	–	–
Invasive carcinoma	–	+	+
Invasive carcinoma	+	+	+
PUNLMP	+	–	–
Invasive carcinoma	+	+	+
CIS	+	–	–
Invasive carcinoma	+	+	+
Inverted papilloma	+	+	+
Invasive carcinoma	+	+	+
Invasive carcinoma	+	+	+
Invasive carcinoma	+	+	+
PUNLMP	+	+	+
Papillary carcinoma	+	+	+
CIS	+	+	+
Papilloma	+	+	+
CIS	–	+	+
CIS	+	+	+
Papilloma	+	+	+
PUNLMP	+	+	+
Inverted papilloma	+	+	+
Papillary carcinoma	+	+	+
CIS	+	–	–
Papilloma	–	+	+

+: presence of BPV; –: no detection of BPV; PUNLMP, papillary urothelial neoplasm of low malignant potential; CIS, carcinoma *in situ*.

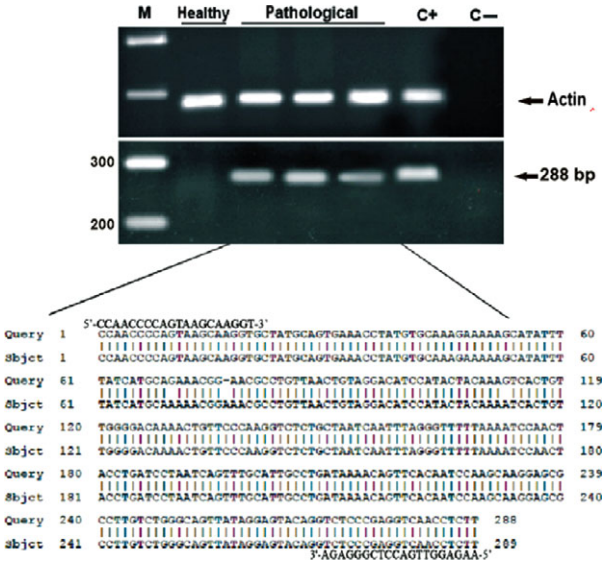

Fig. 1. PCR analysis for detection of BPV-13 L1 DNA. Lane M, DNA marker ladder with 300, 200 bp from top to bottom; Lane 2, PCR product of total DNA from bladder of healthy cow as the template; Lanes 3–5, PCR products of total DNA from pathological bladder as the template; Lane C+, positive control; Lane C–, negative control in which the template was replaced with distilled water.

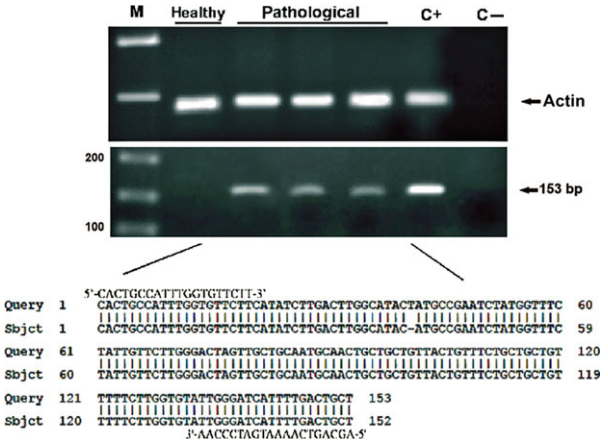

Fig. 2. PCR analysis for detection of BPV-13 E5 DNA. Lane M, DNA marker ladder with 200, 150, 100 bp from top to bottom; Lane 2, PCR product of total DNA from bladder of healthy cow as the template; Lanes 3–5, PCR products of total DNA from pathological bladder as the template; Lane C+, positive control; Lane C–, negative control in which the template was replaced with distilled water.

Discussion

Here, we document the expression of BPV-13 in 28 of 39 (~72%) tumours in cattle. However, BPV-13 was seen in 28 of 33 (~85%) urothelial tumours associated with virus infection. This is the first report of BPV-13 infection associated with urothelial tumours of the urinary bladder in cattle. To date, urothelial tumours in veterinary oncology have been associated exclusively with infection of BPV-1 and BPV-2, agents known to belong to Deltapapillomavirus genus, species 4 (Campo et al., 1992; Borzacchiello et al., 2003; Nasir and Campo, 2008; Roperto et al., 2008, 2013a, b, 2014; Lunardi et al., 2013a). Furthermore, this is the first study which shows the presence of BPV-13 in Europe, thus demonstrating it is a worldwide virus.

BPV-13 has been found to infect urothelial cells prevalently in combination with BPV-2. Indeed, a co-infection

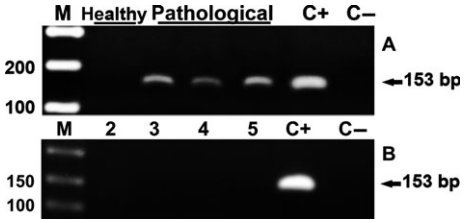

Fig. 3. Reverse transcriptase (RT)-PCR for detection of BPV-13 E5 mRNA. A. (RT+); Lane M, DNA marker ladder with 300, 200, 100 bp from top to bottom; Lane 2, PCR product of cDNA from bladder of healthy cow as the template; Lanes 3–5 PCR products of total DNA from pathological bladder as the template; Lane C+, positive control; Lane C−, negative control in which the template was replaced with distilled water. B. Same tumour samples as in A. Reverse transcription was performed without reverse transcriptase enzyme (RT−); Lane M, DNA marker ladder with 200, 150, 100 bp from top to bottom; Lane 2, cDNA amplicons from bladder of healthy cow are not present; Lanes 3–5, No cDNA amplicons from pathological bladder as the template are detected; Lane C+, positive control; Lane C−, negative control in which the template was replaced with distilled water.

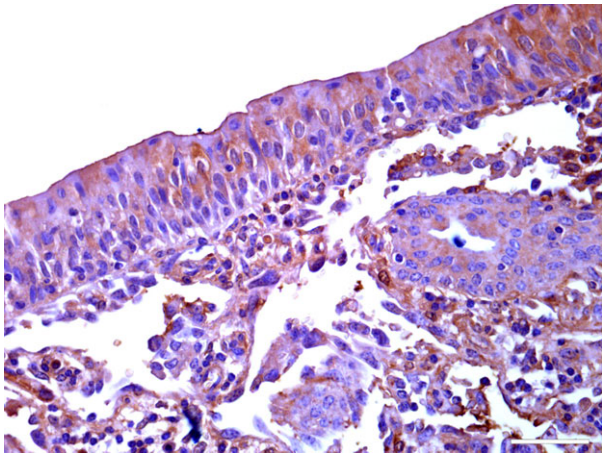

Fig. 4. Immunoreactivity for BPV-13 E5 oncoprotein detected in urothelial tumour cells scattered across basal and suprabasal layers. Scale bar – 100 μm.

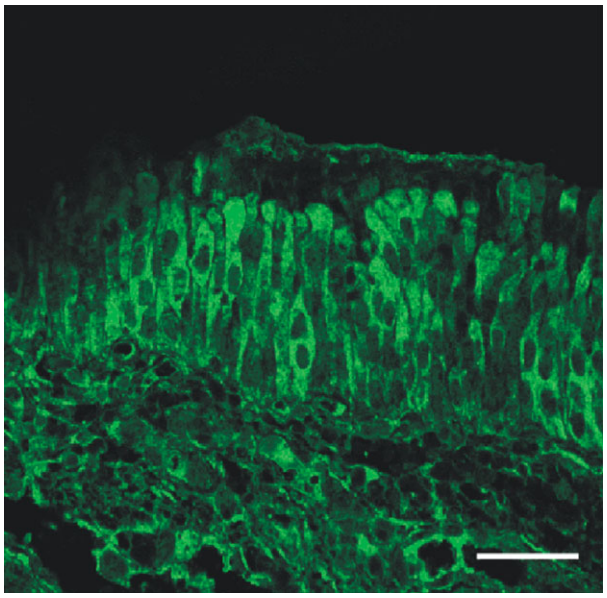

Fig. 5. BPV-13 E5 oncoprotein in urothelial tumour cells detected by immunofluorescence. Scale bar – 100 μm.

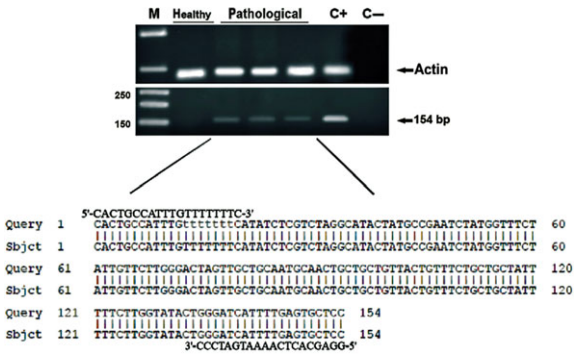

Fig. 6. PCR analysis for detection of BPV-2 E5 DNA. Lane M, DNA marker ladder with 250, 200, 150 bp from top to bottom; Lane 2, PCR product of total DNA from bladder of healthy cow as the template; Lanes 3–5, PCR products of total DNA from pathological bladder as the template; Lane C+, positive control; Lane C−, negative control in which the template was replaced with distilled water.

with BPV-13 and BPV-2 was found in twenty-three of thirty-three urothelial tumour samples (~70%). The significance of this co-infection is to be elucidated but we hypothesize a cooperative interaction between these genetically similar BPV types takes place which leads to cell transformation.

The incidence of BPV-13 infection involved in urothelial tumours appears to be quite common. BPV-13 by itself has been found to infect tumour cells in five BPV-2-negative urothelial tumours, which allows us to suggest that this novel virus is an additional oncogenic agent responsible for naturally occurring bladder carcinogenesis in cattle. We show that E5 expression takes place in urothelial tumour cells both by immunohistochemistry and immunofluorescence. These findings are consistent with the evidence that BPV-13 E5 oncoprotein has a transforming activity. It has been hypothesized BPV-13 E5 oncoprotein has an oncogenic potential. Indeed, the amino acids identified as necessary to maintain the transforming activity of BPV-1 E5 protein are also preserved in the BPV-13 E5 protein (Lunardi et al., 2013a). Furthermore, it has been shown that BPV-13 E5 has a very high (94%) identity in the nucleotide sequence with BPV-2 E5 known to be the major oncogenic protein of Deltapapillomaviruses (Lunardi et al., 2013b).

Our data confirm that bovine Deltapapillomaviruses are the most important biological agents responsible for the great majority of urothelial tumours of the urinary bladder in cattle feeding on bracken fern. In absence of papillomavirus infection and/or bracken fern in the diet, bladder tumours remain an extremely rare carcinogenetic event in cattle accounting for 0.01–0.1 per cent of all malignant bovine neoplasms (Meuten, 2002).

Further studies are needed to define the epidemiology of BPV-13, to better understand its oncogenic potential, and to improve our understanding about the role, if any, in important non-neoplastic infections just as other Deltapapillomaviruses do (Roperto et al., 2012).

Conclusion

An accurate diagnosis of BPV infection and of viral genotypes is needed to achieve prophylactic and therapeutic immunization strategies which should lead to an effective immunotherapeutic management of these important bovine viral pathogens responsible for severe economic losses in the cattle industry.

Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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References

Borzacchiello, G., G. Iovane, M. L. Marcante, F. Poggiali, S. Roperto, and A. Venuti, 2003: Presence of bovine papillomavirus type 2 DNA and the expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows. *J. Gen. Virol.* 84, 2921–2926.

Campo, M. S., W. F. H. Jarrett, R. Barron, B. W. O'Neil, and K. T. Smith, 1992: Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. *Cancer Res.* 53, 1–7.

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

International Agency for Cancer Research (IARC), 2007: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 90. Human Papillomaviruses, pp. 47 and 473. IARC Press, Lyon.

Lindsey, C. L., M. E. Almeida, C. F. Vicari, C. Carvalho, A. Yaguiu, A. C. Freitas, W. Beçak, and R. C. Stocco, 2009: Bovine papillomavirus DNA in milk, blood, urine, semen, and spermatozoa of bovine papillomavirus-infected animals. *Genet. Mol. Res.* 8, 310–318.

Lunardi, M., A. A. Alfieri, R. A. Otonel, B. K. de Alcântara, W. B. Rodrigues, A. B. de Miranda, and A. F. Alfieri, 2013a: Genetic characterization of a novel bovine papillomavirus member of the Deltapapillomavirus genus. *Vet. Microbiol.* 162, 207–213.

Lunardi, M., B. K. de Alcântara, R. A. A. Otonel, W. B. Rodrigues, A. F. Alfieri, and A. A. Alfieri, 2013b: Bovine papillomavirus type 13 DNA in equine sarcoids. *J. Clin. Microbiol.* 51, 2167–2171.

Meuten, D. J., 2002: Tumors of the urinary system. In: Meuten, D. J. (ed.), Tumors in Domestic Animals, 4th edn, pp. 524. Iowa State Press, Blackwell Publishing Company, Ames.

Nasir, L., and M. S. Campo, 2008: Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and equids. *Vet. Dermatol.* 19, 243–254.

Rector, A., and M. Van Ranst, 2013: Animal papillomaviruses. *Virology* 445, 213–223.

Roperto, S., R. Brun, F. Paolini, C. Urraro, V. Russo, G. Borzacchiello, U. Pagnini, C. Raso, C. Rizzo, F. Roperto, and A. Venuti, 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.

Roperto, S., G. Borzacchiello, R. Brun, L. Leonardi, P. Maiolino, M. Martano, O. Paciello, S. Papparella, B. Restucci, V. Russo, G. Salvatore, C. Urraro, and F. Roperto, 2010: A review of bovine urothelial tumours and tumour-like lesions of the urinary bladder. *J. Comp. Pathol.* 142, 95–108.

Roperto, S., S. Comazzi, E. Ciusani, F. Paolini, G. Borzacchiello, I. Esposito, R. Lucà, V. Russo, C. Urraro, A. Venuti, and F. Roperto, 2011: PBMCs are additional sites of productive infection of bovine papillomavirus type 2. *J. Gen. Virol.* 92, 1787–1794.

Roperto, S., G. Borzacchiello, I. Esposito, M. Riccardi, C. Urraro, R. Lucà, A. Corteggio, R. Tatè, M. Cermola, O. Paciello, and F. Roperto, 2012: Productive infection of bovine papillomavirus type 2 in the placenta of pregnant cows affected with urinary bladder tumors. *PLoS One* 7, e33569.

Roperto, S., V. Russo, A. Özkul, A. Corteggio, A. Sepici-Dincel, C. Catoi, I. Esposito, M. G. Riccardi, C. Urraro, R. Lucà, D.

M. Ceccarelli, M. Longo, and F. Roperto, 2013a: Productive infection of bovine papillomavirus type 2 in the urothelial cells of naturally occurring urinary bladder tumors in cattle and water buffaloes. *PLoS One* 8, e62227.

Roperto, S., V. Russo, A. Özkul, A. Sepici-Dincel, P. Maiolino, G. Borzacchiello, L. Marcus, I. Esposito, M. G. Riccardi, and F. Roperto, 2013b: Bovine papillomavirus type 2 infects the urinary bladder of water buffalo (*Bubalus bubalis*) and plays a crucial role in bubaline urothelial carcinogenesis. *J. Gen. Virol.* 94, 403–408.

Roperto, S., V. Russo, G. Borzacchiello, C. Urraro, R. Lucà, I. Esposito, M. G. Riccardi, C. Raso, M. Gaspari, D. M. Ceccarelli, R. Galasso, and F. Roperto, 2014: Bovine papillomavirus type 2 (BPV-2) E5 oncoprotein binds to the subunit D of the V_1-ATPase proton pump in naturally occurring urothelial tumors of the urinary bladder of cattle. *PLoS One* 9, e88860.

Santos, E. U., M. A. Silva, N. E. Pontes, L. C. Coutinho, S. S. Paiva, R. S. Castro, and A. C. Freitas, 2014: Detection of different bovine papillomavirus types and co-infection in bloodstream of cattle. *Transbound. Emerg. Dis.* 63, e103–e108.

Wosiaki, S. R., M. A. Barreiro, A. F. Alfieri, and A. A. Alfieri, 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., M. L. Z. Dagli, E. H. Jr Birgel, B. C. A. Alves Reis, O. P. Ferraz, E. M. Pituco, A. C. Freitas, W. Beçak, and R. C. Stocco, 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.

