

Productive Infection of Bovine Papillomavirus Type 2 in the Urothelial Cells of Naturally Occurring Urinary Bladder Tumors in Cattle and Water Buffaloes

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Abstract

Background: Papillomaviruses (PVs) are highly epitheliotropic as they usually establish productive infections within squamous epithelia of the skin, the anogenital tract and the oral cavity. In this study, early (E) and late (L) protein expression of bovine papillomavirus type 2 (BPV-2) in the urothelium of the urinary bladder is described in cows and water buffaloes suffering from naturally occurring papillomavirus-associated urothelial bladder tumors.

Methods and Findings: E5 protein, the major oncoprotein of the BPV-2, was detected in all tumors. L1 DNA was amplified by PCR, cloned and sequenced and confirmed to be L1 DNA. The major capsid protein, L1, believed to be only expressed in productive papillomavirus infection was detected by Western blot analysis. Immunohistochemical investigations confirmed the presence of L1 protein both in the cytoplasm and nuclei of cells of the neoplastic urothelium. Finally, the early protein E2, required for viral DNA replication and known to be a pivotal factor for both productive and persistent infection, was detected by Western blot and immunohistochemically. Electron microscopic investigations detected electron dense particles, the shape and size of which are consistent with submicroscopic features of viral particles, in nuclei of neoplastic urothelium.

Conclusion: This study shows that both active and productive infections by BPV-2 in the urothelium of the bovine and bubaline urinary bladder can occur *in vivo*.

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Introduction

Papillomaviruses (PVs) are small oncogenic highly epitheliotropic DNA viruses showing a marked tropism for squamous epithelium [1]. Papillomaviruses are known to establish productive infections only within stratified epithelia of the skin, the anogenital tract and the oral cavity [2].

Among the thirteen bovine papillomavirus (BPV) types, (BPV-1 to -13), that have been completely described [3,4], BPV-1/-2 are closely related types [5] and assigned to the genus Deltapapillomavirus, species 4, the biological properties of which include lesions in ungulates, fibropapillomas in the respective host and interspecies-transmission [2,6]. BPV-1/-2 are the only papillomaviruses known to infect both epithelial and mesenchymal cells. Similar to other PVs, BPV-1/-2 replication and virion production

appear to be confined to the epithelial region of the lesions [5]. The viral life cycle is tightly linked to the differentiation of the infected epithelial cells. It has been suggested that production of viral capsid proteins takes place in productive infections only [2], assembly of the virion particles takes place in the granular and cornified layers and virus particles are released as the dead squamous cells disintegrate [2,7]. However, it has been recently shown that other cells such as peripheral blood mononuclear cells (PBMCs) and placental trophoblastic epithelium are additional sites of *in vivo* productive infection of BPV-2 [8,9].

The E5 protein, the major oncoprotein of BPV-2, is responsible for cell transformation through different molecular pathways. It has been shown that in spontaneous bladder carcinogenesis of cattle E5 oncoprotein binds to the activated form of the platelet derived growth factor β receptor (PDGF β -R) [10]. More recently,

a new molecular mechanism via Calpain 3 activation has been studied in bovine urothelial tumors [11].

The papillomavirus E2 early protein is a pivotal factor of both productive and persistent infection as it is the main regulator of viral DNA replication and viral gene transcription [2,12]. It has been suggested that high levels of E2 during the viral life cycle regulate the expression of the late papillomavirus genes L1 and L2 thus facilitating the new virus progeny [2,13]. Novel biological activities of E2 have been recently proposed. It has been suggested that E2 plays an important role in intracellular trafficking as it interacts with a functional family of proteins involved in vesicle-mediated transport mostly between Golgi apparatus and endosomes as well as endosomes and lysosomes [12].

It is well-known that BPV-1/-2 play a role in bladder carcinogenesis of large ruminants as they can infect the urothelium of the urinary bladder in which they usually establish latent and/or abortive infections. BPV-2 has appeared to be responsible for bladder tumors of cattle for several years already [14,15,16]. Although it has been suggested that papillomavirus disease is still little-known in buffalo and urinary bladder pathology is a neglected area of investigations in this species [17,18], however the BPV-2 infection of the urothelial cells of the urinary bladder resulting to urothelial tumors has very recently been shown to occur also in buffalo [19]. High-risk human papillomavirus (HPV) infection has been also proposed as responsible for some urothelial tumors in man [20].

The present report documents the expression of BPV-2 L1 protein and virion assembly in the urothelial cells of naturally occurring urinary bladder tumors. This study is the first to reveal that the neoplastic urothelium of the bovine and bubaline urinary bladder is an additional site of productive BPV-2 infection.

Materials and Methods

Ethics Statement

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

Tissue samples

Neoplastic bladder samples were collected from twenty cows and twenty-one water buffaloes both at public and private Italian and Turkish slaughterhouses, respectively. As far as bovine samples are concerned they were collected, after the permission of the medical authorities, in the slaughterhouses named 'Macello Comunale' of Muro Lucano (PZ), 'Fratelli Peta' of Marcellinara (CZ) and 'Barbara Rocco sas' of Simbario (VV). As far as buffalo samples are concerned they were collected, after the permission of the medical authorities, in the slaughterhouses named 'Bafra Belediye Mezbahas' in Bafra/Samsun and 'Coskun Et ve Mamulleri Sanayi ve Ticaret Anonim irketi et Fabrikas Kesimhanesi, Kagithane' at Istanbul. To prevent possible cross-contaminations, each sample was immediately divided into several parts that were frozen in liquid nitrogen for subsequent molecular biological analysis, or fixed in 10% neutral buffered formalin and in 4% glutaraldehyde in 0.1 M phosphate buffer for microscopical and electron microscopical investigations.

Histopathology

Tissues fixed in 10% neutral buffered formalin were routinely processed for paraffin embedding. Histologic diagnosis of bladder

tumors was assessed on 5- μ m-thick hematoxylin-eosin (HE)-stained sections.

BPV-2 E5 Immunoprecipitation

Tissue samples from urinary bladders of cows and water buffaloes were lysed in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 150 mM NaCl, 2 mM PMSF, 1.7 mg/ml Aprotinin, 50 mM NaF, and 1 mM sodium orthovanadate. The protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Milan, Italy). Proteins derived from bladders (1000 μ g) were immunoprecipitated by using 2 μ g of a polyclonal sheep anti-E5 antibody (a kind gift by Dr. M.S. Campo) and 30 μ l of Protein A/G-Plus Agarose (Santa Cruz Biotechnology, CA, USA). Immunoprecipitates were washed five times in complete lysis buffer (above), finally heated in 1X Laemmli sample buffer at 100°C for 10 min. Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% polyacrylamide) under reducing conditions. After electrophoresis, proteins were transferred on nitrocellulose filter membrane (GE Healthcare Life Sciences, Chalfont St Giles, UK) for 1 h at 300 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 dry milk, incubated with anti-E5 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 Biotechnology, CA, USA) for 60 min at room temperature. Proteins were visualized by enhanced chemiluminescence system (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, CA, USA).

BPV-2 L1 DNA detection, cloning and sequencing

DNA was extracted from urinary bladder samples from 20 cows and 21 water buffaloes with urothelial bladder tumors and from five healthy cows and five water buffaloes using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol. All the samples were lysed using proteinase K. Lysates were loaded onto DNeasy spin columns. After two washings pure DNA was eluted in low salt buffer. PCR analysis was performed using specific primers for BPV-2, L1 region: nt 1318 \pm 1341, 5'-GTTATACCACC-CAAAGAAGACCCT-3', and nt 1490 \pm 1466, 5'-CTGGTTGCAACAGCTCTCTTTCTC-3' [21]. To evaluate the adequacy of the DNA samples, a control PCR for bovine β -actin sequence was performed using a set of primers designed by Primer BLAST software (forward, 5'-GAGCGTGGCTA-CAGCTTAC-3'; reverse, 5'-CATTGCCGATGGTGATGA-3'). Aliquots 50–100 ng of purified DNA were amplified in 25 μ l of reaction mixture containing 1.5 mM MgCl₂ for β -actin primers and 2 mM for BPV-2 L1 primers, 200 mM each dNTP, 480 nM of each primer and 2.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Monza, Italy). The reaction was carried out in a thermocycler (Veriti, Applied Biosystems) with an initial denaturation step of 3 min. Then, 35 cycles of amplification were carried out with a denaturation step at 94°C for 40 sec, an annealing step at 60°C, 30 sec, for β -actin or at 55°C, 40 sec, for BPV-2 L1, and an extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was performed in each PCR assay. Detection of the amplified products was carried out by electrophoresis on ethidium bromide-stained agarose gel. In each experiment, a blank sample consisting of reaction mixture without DNA and a positive sample consisting of cloned BPV-2 (a kind gift by Dr. A. Venuti) were included. The quality of DNA was tested with primers for bovine β -actin gene. To confirm the PCR data, the amplified products were excised from the gel and purified

through silicagel membranes by using the QIAquick PCR quantification kit according to the manufacturer's instructions (Qiagen, Milan, Italy). Then, the amplified DNA was cloned into pGEM-T vector using the pGEM[®]-T Easy Vector Systems (Promega, Milan, Italy), and sequenced in an automated apparatus (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Monza, Italy).

Western blot analysis for L1 and E2

Samples were solubilized at 4°C in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100. Immediately prior to use, the following reagents were added: 1 mM DTT, 2 mM PMSF, 1.7 mg/ml Aprotinin, 25 mM NaF, 1 mM Na₃VO₄ (Sigma-Aldrich, Milan, Italy).

Lysates were clarified at 21,500×g for 30 min. The protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Milan, Italy). For Western blotting, 50 µg of lysate proteins were heated at 100°C in 4X premixed Laemmli sample buffer. Proteins were separated on polyacrylamide gel and transferred to nitrocellulose filter membrane (GE Healthcare Life Sciences, Chalfont St Giles, UK) for 1 h at 350 mA in 192 mM glycine/25 mM Tris-HCl (pH 7.5)/10% methanol. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS, pH 7.5) for 1 h at room temperature, washed with TBS-0.1% Tween. Then, the membranes were probed with a monoclonal mouse anti-HPV-16 L1 (Chemicon International, CA, USA) and a polyclonal rabbit anti-E2 (a kind gift by Dr. E. Androphy) antibodies for an overnight incubation at 4°C. After three washes in Tris-buffered saline, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Milan, Italy) for 1 h at room temperature. After three washing steps, bound antibody was visualized by an enhanced chemiluminescence system (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, CA, USA).

Immunohistochemistry

Neoplastic and normal bladder sections were processed with the same procedures. Briefly, the sections were deparaffinized and then endogenous peroxidase activity was blocked by incubation in 0.3% H₂O₂ in methanol for 20 min. Antigen retrieval was performed by pretreating with microwave heating (twice for 5 min each at 750 W) in citrate buffer pH 6.0. 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 rabbit serum (Sigma-Aldrich, Milan, Italy) diluted at 1 in 10 in PBS. The excess serum was drained off and a polyclonal sheep anti-BPV-2 E5 primary antibody (a kind gift by Dr. M.S. Campo) diluted at 1 in 40,000 in PBS, was applied for 1 h at room temperature in a humid chamber. Following incubation, the sections were rinsed three times for 5 min with PBS before application of the rabbit anti-sheep biotinylated secondary antibody (Santa Cruz Biotechnology, Inc., CA, USA), diluted at 1 in 100 in PBS for 45 min at room temperature. For E2 and L1 detection the slides were washed three times with PBS, pH 7.4, 0.01 M, then incubated for 1 h at room temperature with protein block serum-free (DakoCytomation, Denmark). The anti-BPV-2 E2 primary antibody diluted at 1 in 50/100 in PBS and the anti-HPV-16 L1 diluted at 1 in 200 in PBS were applied overnight at 4°C in a humid chamber. The sections were rinsed three times for 5 min with PBS, incubated for 40 min at room temperature with appropriate biotinylated secondary antibody (labelled streptavidin-biotin (LSAB) Kit; DakoCytomation, Denmark). Finally, all the sections were washed three times with PBS and then incubated with streptavidin-conjugated to horseradish peroxidase (LSAB Kit;

DakoCytomation, Denmark). Color development was obtained by treatment with diaminobenzidine (DakoCytomation, Denmark) for 5–20 min. Sections were counter stained with Mayer's hematoxylin.

Transmission Electron Microscopy

Ten bladder samples from cattle and twelve from water buffaloes, were immediately fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2–3 h. They were washed (20 min 5 times) and post fixed in 1% OsO₄ in the same buffer for 1 h. They were washed again in 0.1 M phosphate buffer (pH 7.4) and then dehydrated in graded alcohol, and embedded in Agar Low Viscosity Resin (Agar Scientific Limited, Essex, England). Semi-thin section (400 nm) were cut on an EM UC6 ultramicrotome (Leica Microsystems) and were stained with 1% toluidine blue in water solution and examined by light microscopy. Ultra thin sections (60–70 nm), obtained from chosen areas, were collected onto 300-mesh grids coated with formvar and counter-stained with lead citrate and uranyl acetate. The sections were observed with a JEOL JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a thermionic tungsten filament and operated at an acceleration voltage of 100 kV. Images were taken using a Morada cooled slow-scan CCD camera (3783×2672 pixels) and micrographs were taken with iTEM software (Olympus Soft Imaging System GmbH, Munster, Germany). The same procedure was used to obtain ultra thin sections from normal bladder of healthy cows and water buffaloes.

Results

Table 1 and Table 2 report histological diagnosis of urothelial tumors of the urinary bladder of cattle and buffaloes, respectively, performed according to recent morphological criteria [22]. In all tumor cases, the BPV-2 E5 oncoprotein was detected by immunoprecipitation (Figure 1). As it has been shown that BPV-2 plays a crucial role in bladder carcinogenesis of large ruminants [14,19] and since the cattle and buffaloes affected with urothelial tumors, here reported, were from Italy and Turkey respectively, we analyzed the sequence of the L1 gene, the most conserved PV gene, to assess any variation in BPV-2 between the two countries. PCR analysis was performed in all bovine and bubaline samples. L1 DNA was amplified and a band of ~55 kD was shown both in benign and malignant tumors (Figure 2). Amplicon sequencing detected a DNA fragment composed of 164 bp (Figure 2) showing an absolute homology (100%) with the known sequences of BPV-2 L1 DNA. There were no differences in L1 DNA sequence between cattle and buffaloes. GenBank accession number of our L1 sequence is M20219.

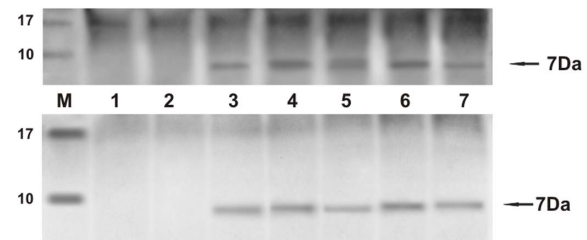


Figure 1. E5 immunoprecipitation. The presence of E5 protein detected by immunoprecipitation. a) Lanes 1–2: urinary bladders from healthy cows. Lanes 3–7: five tumors of the urinary bladders in cows. b) Lanes 1–2: urinary bladders from healthy buffaloes. Lanes 3–7: five tumors of the urinary bladders in buffaloes. doi:10.1371/journal.pone.0062227.g001

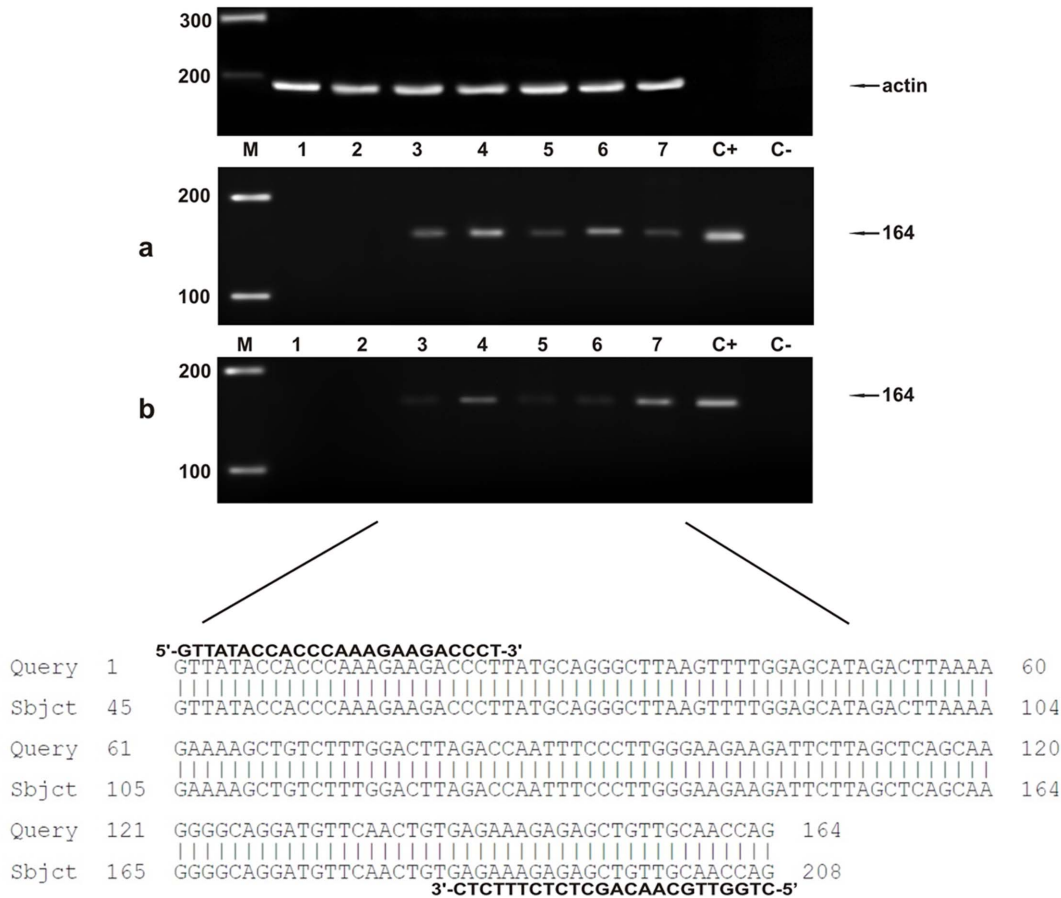


Figure 2. PCR amplification of DNA samples. a) Lane M, molecular mass marker (HyperLadder II Bioline); lanes 1–2 urinary bladder samples from healthy cows without BPV-2 L1 DNA; lanes 3–7 bladder tumors samples from five of twenty cows showing BPV-2 L1 DNA; lane C+, positive control (cloned BPV-2 DNA); lane C-, negative control (no DNA added). The arrow indicates the position of the 164 bp BPV-2 L1 PCR product. b) Lane M, molecular mass marker (HyperLadder II Bioline); lanes 1–2 urinary bladder samples from healthy buffaloes without BPV-2 L1 DNA; lanes 3–7 bladder tumors samples from five of twenty-one buffaloes showing BPV-2 L1 DNA; lane C+, positive control (cloned BPV-2 DNA); lane C-, negative control (no DNA added). The arrow indicates the position of the 164 bp BPV-2 L1 PCR product. The lower part of the figure shows 100% homology between the sequence of the amplicons in lanes 3–7 and the sequence of BPV-2 L1 found in Italy (GenBank M20219). doi:10.1371/journal.pone.0062227.g002

Next, we assessed whether the L1 gene was expressed. We studied the expression of L1 across the spectrum of urothelial neoplastic lesions as it is well known that L1 expression reflects type and grade of tumors [23]. The presence of L1 protein in urothelial tumors of both cows and buffaloes was revealed by Western blot analysis (Figure 3). Its presence was confirmed morphologically. Immunohistochemically, a marked immunoreactivity was evident both in the cytoplasm and nuclei of the urothelial cells of neoplastic nests. Surprisingly, L1 protein expression was also evident in the basal neoplastic urothelial cells lining microcysts containing pale pink secretions (Figure 4A). No L1 protein expression was detected in normal urothelial cells (Figure 4B).

Since it is known that E2 protein is essential for the viral life cycle and plays a part in productive infection, molecular and immunohistochemical studies on the expression of this protein were also carried out. E2 protein expression was detected by Western blot analysis in total protein extracts from both bovine and bubaline urothelial tumors (Figure 5). E2 protein expression was also shown with morphological procedures. Immunohistochemical studies confirmed the presence of E2 protein in the cytoplasm and nuclei of neoplastic urothelial cells (Figure 6A). E2

protein expression was not detected in normal urothelial cells (Figure 6B).

Ultrastructural studies showed electron dense particles, about 50 nm in diameter, the size and shape of which were consistent with the submicroscopic features of viral particles both in the nuclei of bovine and bubaline urothelial cells (Figure 7A and 8A). Similar electron dense particles were not observed in the nuclei of urothelial cells from urinary bladders of healthy cows and buffaloes (Figure 7B and 8B).

Discussion

It has been suggested that papillomavirus L1 protein, the major late capsid protein, is only expressed in productive infections and its presence is considered proof of a complete virus life cycle [2,23]. Its expression depends on the episomal maintenance or the integration of viral genome into the host cell chromosome. In the case of human papillomavirus, and particularly HPV-16, the virus responsible for cervical cancer, the viral DNA needs to exist episomally in order to be productively replicated [24] and many cancer cells represent a “dead end” for the virus when viral DNA becomes integrated into the host cell chromosome, thus failing to produce infectious virions [25]. PV integration is considered to be

Table 1. Tumor histotypes and expression of viral proteins in urinary bladder neoplasias of cattle.

Samples	Microscopic patterns of tumors	BPV-2 E5	BPV-2 L1
T1	Papilloma	IP	WB
T2	Papilloma	IP	WB
T3	Papillary Urothelial Neoplasm of Low Malignant Potential	IP	WB
T4	Papillary Urothelial Neoplasm of Low Malignant Potential	IP	WB
T5	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T6	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T7	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T8	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T9	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T10	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T11	High-Grade Papillary Urothelial Carcinoma	IP	WB
T12	High-Grade Papillary Urothelial Carcinoma	IP	WB
T13	High-Grade Papillary Urothelial Carcinoma	IP	WB
T14	High-Grade Papillary Urothelial Carcinoma	IP	WB
T15	Low Grade Invasive Urothelial Carcinoma	IP	WB
T16	Low Grade Invasive Urothelial Carcinoma	IP	WB
T17	Low Grade Invasive Urothelial Carcinoma	IP	WB
T18	High-Grade Invasive Urothelial Carcinoma	IP	WB
T19	High-Grade Invasive Urothelial Carcinoma	IP	WB
T20	High-Grade Invasive Urothelial Carcinoma	IP	WB

IP: protein detection by immunoprecipitation; **WB:** protein expression shown by Western Blot.
doi:10.1371/journal.pone.0062227.t001

Table 2. Tumor histotypes and expression of viral proteins in urinary bladder neoplasias of buffalo.

Samples	Microscopic patterns of tumors	BPV-2 E5	BPV-2 L1
T1	Papilloma	IP	WB
T2	Papilloma	IP	WB
T3	Papillary Urothelial Neoplasm of Low Malignant Potential	IP	WB
T4	Papillary Urothelial Neoplasm of Low Malignant Potential	IP	WB
T5	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T6	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T7	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T8	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T9	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T10	Low-Grade Papillary Urothelial Carcinoma	IP	WB
T11	High-Grade Papillary Urothelial Carcinoma	IP	WB
T12	High-Grade Papillary Urothelial Carcinoma	IP	WB
T13	High-Grade Papillary Urothelial Carcinoma	IP	WB
T14	High-Grade Papillary Urothelial Carcinoma	IP	WB
T15	Low Grade Invasive Urothelial Carcinoma	IP	WB
T16	Low Grade Invasive Urothelial Carcinoma	IP	WB
T17	Low Grade Invasive Urothelial Carcinoma	IP	WB
T18	High-Grade Invasive Urothelial Carcinoma	IP	WB
T19	High-Grade Invasive Urothelial Carcinoma	IP	WB
T20	High-Grade Invasive Urothelial Carcinoma	IP	WB

IP: protein detection by immunoprecipitation; **WB:** protein expression shown by Western Blot.
doi:10.1371/journal.pone.0062227.t002



Figure 3. L1 expression. The presence of L1 protein detected by Western blot. a) Lanes 1–2: urinary bladders from healthy cows. Lanes 3–7: urinary bladder tumors from five of twenty cows. Lane 8: positive control, (bovine placenta infected with BPV-2). b) Lanes 1–2: urinary bladders from healthy buffaloes. Lanes 3–7: urinary bladder tumors from five of twenty-one buffaloes. Lane 8: positive control (bovine placenta infected with BPV-2). doi:10.1371/journal.pone.0062227.g003

a key event in the progression of persistent virus infection to invasive cancer [26,27]. It is believed that viral integration could facilitate the accumulation of secondary genetic changes in host cell genome via oncoprotein expression. Genetic mutations and increased oncogene expression mediated by E2 are considered to be crucial events in the development of invasive cancer [24,28]. However, it has recently been shown that integration of PV genome may cause no significant increase in oncoprotein expression following E2-disruption; furthermore it is still a matter of debate whether PV DNA integration leads to genomic instability or rather is a consequence thereof [29]. Very recently, it has been shown that over 60% of the cervical cancer cases harbor intact E2 despite the fact that PV E2 protein negatively regulates transcription of PV oncogenes [30].

L1 protein expression has been detected in high percentage in papillomavirus-associated pathological conditions such as inflammation and low grade lesions; it has been found to be very rarely expressed in high grade lesions and in cancers [26,31,32]. It has been suggested that L1 protein expression tends to be turned down with increasing severity of lesions as progression of low grade intraepithelial lesions to cancer appears to be associated with corresponding reduction of L1 protein expression [33,34]. It is believed that the higher the malignant potential of tumor cells, the lower the expression of the L1 protein, until the viral genome becomes integrated and L1 is not detectable anymore [26,35]. Therefore, it has been suggested that the detection of L1 protein could serve as possible prognostic marker useful in predicting the biological behavior of tumors [23,33,34].

Similar to the situation in cervical cancer, L1 expression appears to take place in typical papillomavirus-associated urothelial papilloma of the urinary bladder in man in which the state of the virus is known to be prevalently episomal [36] but it is not expressed in urothelial carcinoma, in whose cells the virus is known to occur integrated [20].

Tumors of the urinary bladder are common in cattle and buffaloes reared on pastures rich in bracken fern [19,22,37,38]. Although an active papillomavirus infection is known to occur in the great majority of urothelial cancer cases in ruminants, neither structural viral antigens nor production of virus have ever been detected in bovine or bubaline bladder tumors [2,19].

Our current molecular, microscopic and submicroscopic investigations provide for the first time evidence that a productive

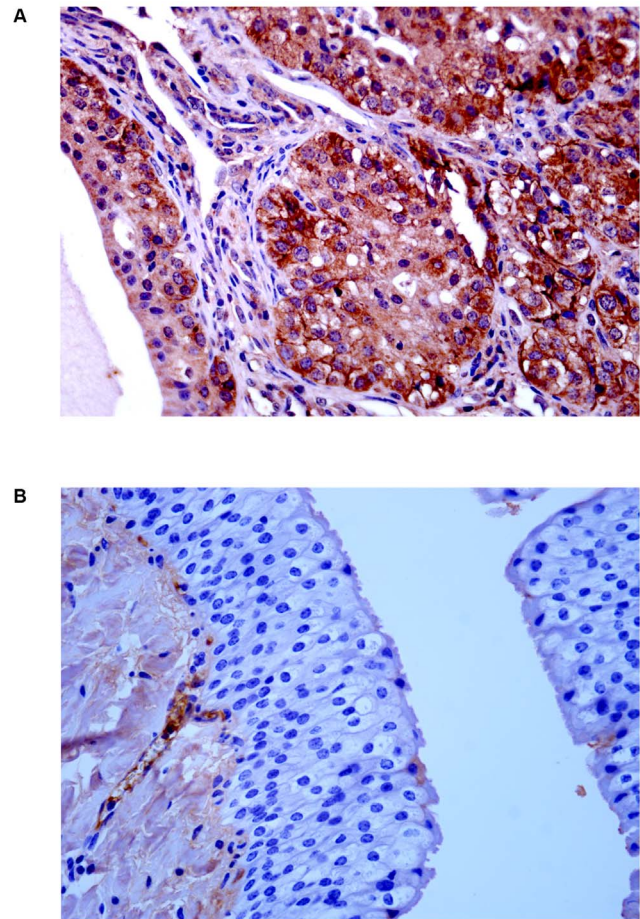


Figure 4. L1 immunohistochemistry. A) Immunohistochemical evidence of L1 protein in urothelial neoplastic cells. B) No L1 protein immunoreactivity was detected in normal urothelial cells. doi:10.1371/journal.pone.0062227.g004

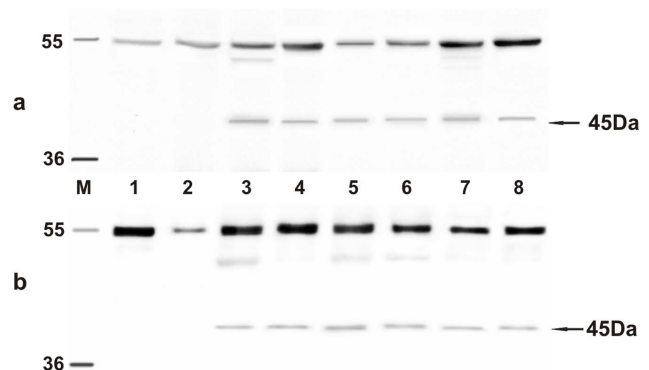


Figure 5. E2 expression. The presence of E2 protein detected by Western blot. a) Lanes 1–2: urinary bladders from healthy cows. Lanes 3–7: urinary bladder tumors from five of twenty cows. Lane 8: positive control, (bovine placenta infected with BPV-2). b) Lanes 1–2: urinary bladders from healthy buffaloes. Lanes 3–7: urinary bladder tumors from five of twenty-one buffaloes. Lane 8: positive control, (bovine placenta infected with BPV-2). doi:10.1371/journal.pone.0062227.g005

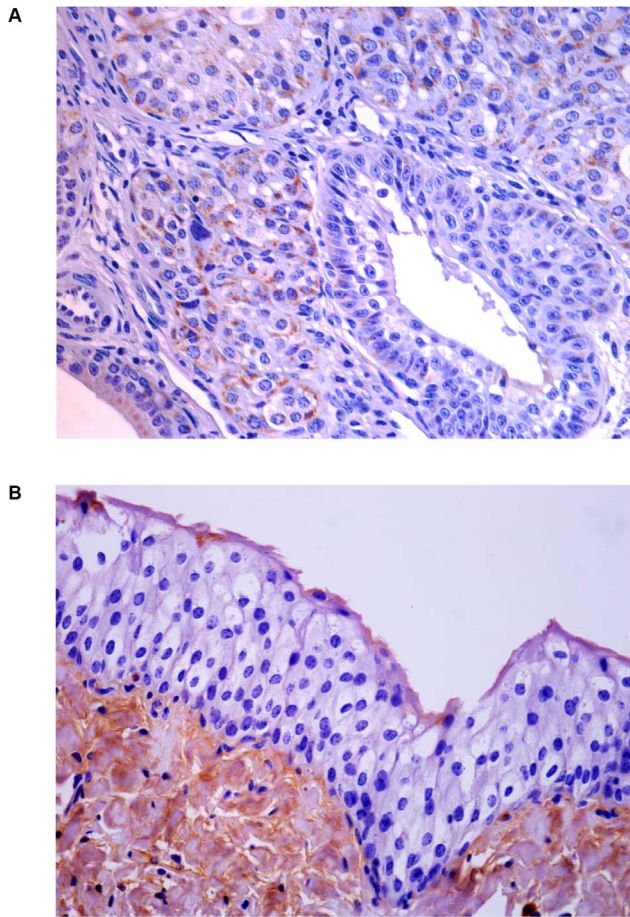


Figure 6. E2 immunohistochemistry. A) Immunohistochemical evidence of E2 protein in urothelial neoplastic cells. B) No E2 protein immunoreactivity was detected in normal urothelial cells. doi:10.1371/journal.pone.0062227.g006

infection of BPV-2 occurs in the urothelial cells of cows and buffaloes suffering from naturally acquired BPV-2-induced bladder tumors.

We have detected the expression of BPV-2 E2 protein in urothelial cancer cells. It is known that E2 protein is necessary for continued maintenance of the viral episome and can enhance encapsidation of the viral genome into infectious particles thus playing a part in a productive infection [24,39]. Furthermore, the expression of BPV-2 L1 protein strongly indicates that a complete life cycle of BPV-2 occurs also both in bovine and bubaline neoplastic urothelial cells. Up to now it has been believed that urothelial tumor cells were not permissive for productive infection by BPV-2. It has been suggested that the permanent episomal state of viral DNA could establish an abortive infection responsible for bladder carcinogenesis in cattle [37,40]. However, we do not exclude that a greater quantity of E2 overexpression-mediated episomal BPV-2 may take place in urothelial cells which could result in increased expression of viral oncogenes thus exposing urothelial cells to a greater quantity of BPV-2 oncoproteins. Similar mechanisms have just been suggested for anal intraepithelial neoplasia (AIN) in human medicine [41].

Further studies are needed to know the infectivity of the viral particles and to better understand whether and/or how L1

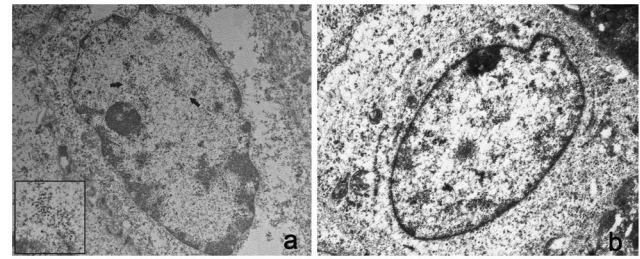


Figure 7. Presence of viral particles in bovine urothelial cells. A) Electron microscopical examination showed numerous intranuclear electron dense particles, 45–50 nm in diameter (black arrows). X 15,000. The size and shape of these particles are consistent with the submicroscopic features of viral particles (insert). X 50,000. B) **Normal urothelial cells.** No electron dense particles are detected in the nucleus of a normal urothelial cell. X 15,000. doi:10.1371/journal.pone.0062227.g007

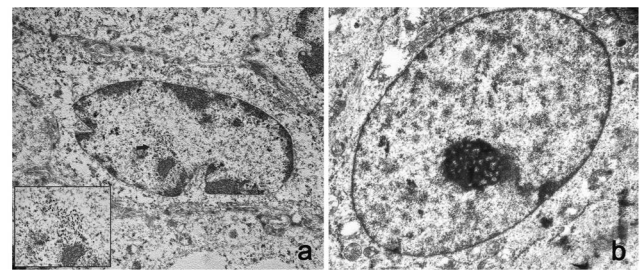


Figure 8. Presence of viral particles in bubaline urothelial cells. A) Numerous electron dense particles, 45–50 nm in diameter (black arrows) were seen in the nuclei. X 15,000. The size and shape of these particles are consistent with the submicroscopic features of viral particles (insert). X 50,000. B) **Normal urothelial cells.** No electron dense particles are seen in the nucleus of a normal urothelial cell. X 15,000. doi:10.1371/journal.pone.0062227.g008

protein, known to play a crucial role in infection and immunogenicity [42], stimulates the adaptive immune responses thus contributing to the chronic inflammation constantly seen in the stroma of bovine urothelial tumors [22,43]. The interrelationship between tumor cells and their inflammatory microenvironments is a matter of debate [44]. Naturally occurring bladder carcinogenesis is quite common in cattle; therefore, we suggest this spontaneous tumor is an appropriate biological model for future research into papillomavirus-associated carcinogenesis.

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Author Contributions

Conceived and designed the experiments: SR VR FR. Performed the experiments: SR VR IE MGR CU RL DMC AC ML. Analyzed the data: SR CO AO ASD CC LM FR. Wrote the paper: SR FR.

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