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EcPV2 DNA in equine genital squamous cell carcinomas and normal genital mucosa

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ABSTRACT

Squamous cell carcinoma (SCC) represents the most common genital malignant tumor in horses. Similar to humans, papillomaviruses (PVs) have been proposed as etiological agents and recently *Equine papillomavirus type 2* (EcPV2) has been identified in a subset of genital SCCs.

The goals of this study were (1) to determine the prevalence of EcPV2 DNA in tissue samples from equine genital SCCs, penile intraepithelial neoplasia (PIN) and penile papillomas, using EcPV2-specific PCR, (2) to examine the prevalence of latent EcPV2 infection in healthy genital mucosa and (3) to determine genetic variability within EcPV2 and to disentangle phylogenetic relationships of EcPV2 among PVs.

EcPV2 DNA was detected in all but one penile SCC (15/16), in all PIN lesions (8/8) and penile papillomas (4/4). Additionally, EcPV2 DNA was demonstrated in one of two metastasized lymph nodes, one contact metastasis in the mouth, two vaginal and one anal lesion. In healthy horses, EcPV2 DNA was detected in 10% (4/39) of penile swabs but in none of vulvovaginal swabs (0/20). This study confirms the presence of EcPV2 DNA in equine genital SCCs and shows its involvement in anal lesions, a lymph node and contact metastases. Latent EcPV2 presence was also shown in normal male genital mucosa. We found that different EcPV2 variants cocirculate among horses and that EcPV2 is related to the Delta + Zeta PVs and is only a very distant relative of high-risk human PVs causing genital cancer. Thus, similar viral tropism and similar malignant outcome of the infection do not imply close evolutionary relationship.

1. Introduction

Squamous cell carcinomas (SCC) represent 20% of all equine tumors, making it the second most common neoplasm in horses. SCC is a malignant epithelial tumor and is most often associated with the eyes and the external male genitals (Fig. 1), but it can develop in any epithelial tissue of the body (MacFadden and Pace, 1991). In many horses, penile SCCs are accompanied by large, confluent, pink to yellowish plaques (Fig. 1), often referred to as "precancerous lesions" (Brinsko, 1998). Similar to the frequently described penile intraepithelial neoplasia (PIN) in men, the same term can be used in horses. Furthermore, according to our own clinical observations, many genital SCCs are accompanied by genital papillomas, which can also be considered as precancerous lesions. Similarly, in female horses, SCC of the vulval region can be accompanied by vulval intraepithelial lesions (VIN) and papillomas.

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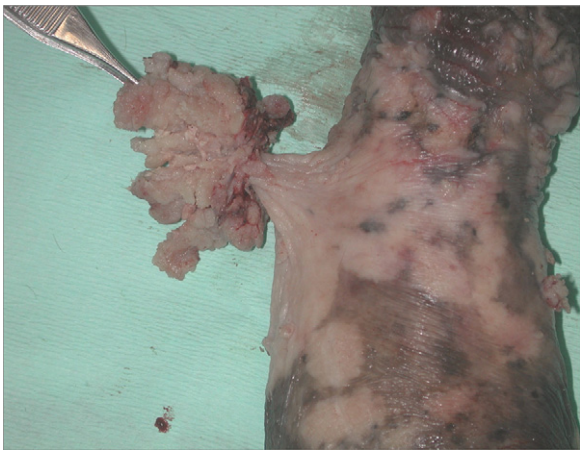

Fig. 1. Penile squamous cell carcinoma with PIN lesions in a 22 years old Arabian horse (patient 10).

Papillomaviruses (PVs) are small, epitheliotropic, non-enveloped double-stranded circular DNA viruses. In humans, more than 150 different PVs have been fully sequenced, some of them associated with both benign and malignant clinical conditions, ranging from spontaneously regressing cutaneous and genital warts, to invasive anogenital and skin cancer (de Villiers et al., 2004). Similar to humans, certain PVs have been proposed as etiological agents of cancer in animals. In horses, *Bovine papillomavirus type 1* (BPV1) and less commonly type 2 (BPV2) are associated with equine sarcoids, a common fibroblastic skin tumor in horses. The presence of PVs in equine SCCs was first investigated in 1984, but could not be demonstrated at that time (Junge et al., 1984). In 2004 the complete nucleotide sequence of the first equine PV, *Equus caballus papillomavirus 1* (EcPV1), isolated from a cutaneous papilloma, was determined (Ghim et al., 2004). In 2007 EcPV1 DNA was demonstrated in a high proportion of cutaneous papillomas but could not be isolated from genital papillomas (Postey et al., 2007). However, the existence of a different PV already suggested in 1986 by Obanion et al., was strongly supported since both PCR and immunohistochemical analysis respectively indicated the presence of PV DNA and PV antigens in genital lesions (Postey et al., 2007). Recently a novel equine papillomavirus (EcPV2) has been identified in a subset of genital SCCs and papillomas, but not in ocular SCCs (Scase et al., 2010; Lange et al., 2011). The first goal of this study was to confirm the presence and determine the prevalence of EcPV2 DNA in tissue samples from equine genital SCCs (male and female), genital papillomas and PIN/VIN lesions.

Humans are exposed since early life to PV infection, and most of these PV infections in humans are asymptomatic (Antonsson et al., 2000). The same appears to be true in several animal species, including virtually all mammals (Antonsson and Hansson, 2002; Bravo et al., 2010). Since latent PV infections have also been shown in cattle and horses (Campo et al., 1994; Antonsson and Hansson, 2002; Bogaert et al., 2008) the second goal of this study was to investigate the possibility of latent EcPV2 infections in the genital mucosa of healthy horses.

Finally, the third goal of our study was to analyze the phylogenetic relationships among the EcPV2 sequences isolated from different specimens and to reconstruct the phylogenetic relationships of EcPV2 within the *Papillomaviridae* family.

2. Methods

2.1. Study population and sample collection

2.1.1. Affected horses

Thirty-four tissue samples derived from 18 horses with one or more genital SCC, PIN/VIN lesions or papillomas were included in the study. These horses were patients referred to the Department of Surgery and Anaesthesiology of Domestic Animals of Ghent University (Belgium) for treatment of these lesions between 2005 and 2011. The samples enclosed sixteen penile SCCs (P1-P16), eight PIN lesions (PIN1-PIN8) and four penile papillomas (PPA1-PPA4). Additionally, one vulval SCC (V1), one VIN lesion (VIN1), one anal SCC (A1), one contact metastasis in the mouth of a horse with a penile SCC (M1, Fig. 2) and two inguinal lymph nodes with histologically confirmed metastases from a horse with a penile SCC (L1-L2) were collected. Breed, age and sex of the horses are summarized in Table 1. The median age of the horses was 17.5 years (range: 9–28 years). All horses with penile lesions were geldings. The group consisted of nine ponies, six warmbloods, one Arabian, one Standardbred and one Haflinger horse.

Samples were collected after surgical tumor excision or after debulking of the tumoral mass before cryosurgery, immunotherapy or chemotherapy. All samples were collected by excising a representative part of the mass using a sterile scalpel and forceps. When several samples were obtained from the same horse, each sample was processed with a different set of instruments. Samples were stored dry at −18 °C. Six samples were obtained from formalin-fixed, paraffin-embedded tissue samples: PPA1, PPA2, PPA4, L1, L2 and M1.

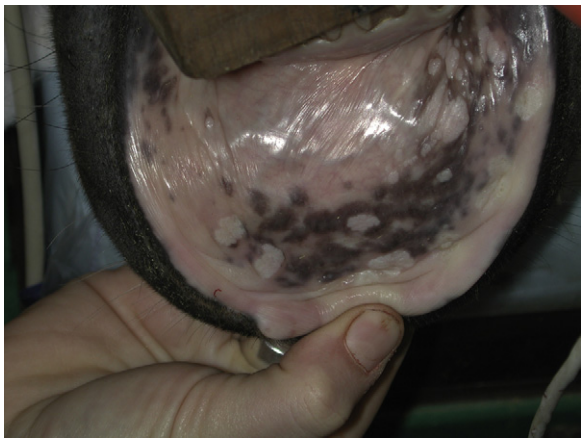

Fig. 2. Contact metastasis in the mouth of a 9 year old pony with penile squamous cell carcinoma (patient 2).

Table 1
Overview of the samples, sex, age and breed of affected horses.

Patient	Samples	Sex	Age (years)	Breed
1	P1, PPA1, L1, L2	Gelding	24	Shetland pony
2	P2, M1	Gelding	9	Pony
3	P3, PIN1	Gelding	22	Warmblood
4	P4, PPA2	Gelding	13	Pony
5	P5, PIN2	Gelding	11	Warmblood
6	P6, PIN3	Gelding	20	Shetland pony
7	P7, P8, PIN4	Gelding	20	Warmblood
8	PIN5	Gelding	22	Pony
9	P9, PIN6, PPA3	Gelding	17	Pony
10	P10, PIN7, PPA4	Gelding	22	Arabian horse
11	P11	Gelding	12	Warmblood
12	P12, PIN8	Gelding	18	Warmblood
13	P13	Gelding	28	Pony
14	P14	Gelding	14	Warmblood
15	P15	Gelding	9	Shetland pony
16	P16	Gelding	16	Pony
17	V1	Mare	20	Standardbred
18	A1, VIN1	Mare	12	Haflinger

P, penile SCC; PPA, penile papilloma; L, lymph node; M, mouth lesion; PIN, penile intraepithelial neoplasia; V, vulval SCC; A, anal SCC, VIN, vaginal intraepithelial neoplasia.

2.1.2. Healthy horses

The stallions ($N = 26$), geldings ($N = 13$) and mares ($N = 20$) were patients referred to the Department of Surgery and Anaesthesiology of Domestic Animals of Ghent University (Belgium) for non-oncologic surgery in February and March 2011. From each of them a penile or vulvovaginal swab was obtained while under general anaesthesia. A cotton-tipped swab was used to sample the genital region: in males the penis was protruded and the distal part of the urethra, the sinus urethralis and fossa glandis, the glans penis, the penile shaft and the preputium were sampled with the same swab. In females a cotton-tipped swab was used to sample the vulva, the glans clitoris and the fossa clitoridis. All precautions were taken to avoid cross contamination and samples were stored dry at −18 °C. The group consisted of 31 warmbloods, four Arabians, one Anglo-Arab, three Thoroughbreds, two Standardbreds, three Friesian horses, one Lusitano, one Quarter horse, two Irish Cobs, one Fjord horse and one Belgian draft horse. The males had a median age of four years (range: 1–20 years) and the females of 4.5 years (range: 1–22 years).

2.2. DNA isolation and PCR

DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. For detection of EcPV2 DNA, PCR was performed using three different EcPV2 specific primer sets in two independent laboratories: Belgium (BE) and Spain (ESP). All penile SCCs and PIN lesions were tested in both laboratories. All normal mucosa samples found positive in the BE laboratory as well as 20% of the negative normal mucosa samples were also tested in the ESP laboratory. The other lesions were analyzed in the BE but not in the ESP laboratory. All samples were analyzed blinded. Correlations between results in both laboratories were calculated using a Cohen's Kappa test. The first primer set (used in BE) amplified a 679 base pair (bp) fragment of the E1 gene (position 215–893) in the genomic sequence of EcPV2 (GenBank accession number EU503122), using EcPV2-NB forward primer 5′-GCGGACTGCGCGTCACAAGAGGGGC-3′ and reverse primer 5′-ACGCAAGCACCACCCACTGCTTGGCA-3′. The second primer set (used in BE) amplified a 57 bp fragment of the L1 gene using the EcPV2-SP1 forward primer 5′-CGTGCA-CAGGGGCAAAAC-3′ PCR and the EcPV2-SP1 reverse primer 5′-AACTGTAACATACACCTTGTC-3′ (Scase et al., 2010). For both primer sets PCR was performed in a 10 μL reaction mixture, containing 200 μM of each dNTP, 0.5 μM of forward and reverse primer, 0.5 U Fast Start *Taq* DNA polymerase (Roche) and 1.5 mM $MgCl_2$ with 2.5 μL of template DNA (undiluted or 1:10 diluted). Amplification was performed as follows using the EcPV2-NB primers: 95 °C for 5 min, 35 cycles at 95 °C for 20 s, 68 °C for 40 s, 72 °C for 1 min and finally 72 °C for 10 min. For the EcPV2-SP1 primers the following conditions were used: 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 56.5 °C for 1 min, 72 °C for 1 min and finally 72 °C for 10 min. All samples were confirmed for presence of sufficient DNA by routine equine GAPDH PCR (Bogaert et al., 2006). The third primer set used in ESP (VLC primers) amplified a 1643 bp fragment of the URR-E6-E7 region using the EcPV2_URRE6E7 forward primer 5′-CCCCACCAAGAAAAGGAAGGCAT-3′ and EcPV2_URRE6E7 reverse primer 5′-TAGTCCCCTCCCTTGGGC-TAGTA-3′. PCR was carried out in a 50 μL volume containing 2 μL of extracted DNA, 5 μL of 10× reaction buffer with 20 mM $MgCl_2$, 500 μM of each dNTP, 1 U of *Taq* DNA polymerase (Biotools), 2.5 μM of forward and reverse primers. PCR profile was 94 °C for 30 s, 30 cycles at 94 °C for 20 s, 60 °C for 20 s and 72 °C for 2 min, followed by a final extension step at 72 °C for 5 min. When the amplicon of the first PCR did not contain enough DNA for sequencing, a nested PCR was subsequently performed using 2 μL of the previous PCR product and the following primers: EcPV2_URRE6E7_nested 5′-CAAGAAAAGGAAGGCATAAGCAA-3′ (forward) and EcPV2_URRE6E7_nested 5′-TTGGGCTAG-TACCTGGGTCATTG-3′ (reverse). All other conditions and PCR profile were the same as for the first PCR.

Negative extraction controls with DNA from equine sarcoids (with confirmed BPV-1 infection) and equine intestine as well as a no-template controls with H_2O, were included in each experiment. PCR products were separated by electrophoresis on a 0.8% or 2% agarose gel and visualized by ethidium bromide staining.

The URR-E6-E7 region was sequenced with the following primers: 465_F 5′-AGCTCTGATGCTGTGCCAAATC-3′ (forward); 1049_F 5′-GCCATCTTTACCAAAGCTCCAGA-3′ (forward); 248_R 5′-CCCGGTGTCAAGATCAGATTAGG-3′ (reverse); 837_R 5′-GGGGCCTCCTGCATATCTTAATG-3′ (reverse) and 1456_R 5′-AACTCTGAATTGAGGATGCTCCC-3′ (reverse). The number in the name represents the position of the primer within the amplicon. Amplicon purification and Sanger sequencing were performed at GenoScreen (Lille, France). Accession numbers for sequences from the URR-E6-E7 region derived in this study are JN664026–JN664042.

2.3. Phylogenetic analysis

Phylogenetic analysis of EcPV2 diversity in the upstream regulatory region (URR) and the E6 gene was

performed with 17 sequences generated in this study and with seven additionally sequences added from the GenBank database (HM153764, HM153758, HM153762, HM153759, EU503122, HM153760 and HM461973). Sequences of the E6 gene were aligned at nt level with MAFFT v.6.717b (Katoh et al., 2005). Maximum Likelihood (ML) based phylogenetic analysis was conducted using RAxML v7.2.8 (Stamatakis, 2006) with the GTR + Γ4 substitution model and 1000 bootstrap cycles. Figures were produced using Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

To analyze the phylogenetic relationships of EcPV2 within the *Papillomaviridae* family, a selection of 96 phylogenetically representative PVs was assembled based on previous studies (Bravo et al., 2010; Gottschling et al., 2011b) (Table S1 available online/on request). From this selection the aa and nt sequences of the E1, E2, L2 and L1 genes were analyzed. Individual genes were aligned with MAFFT v.6.717 (Katoh et al., 2005) at the aa level and back translated to nt with PAL2NAL. The alignments were filtered with GBLOCKS (Castresana, 2000) and concatenated at aa and nt level using a custom perl script. For the genes E1, E2, L2 and L1 as well as for the concatenate, the LG protein substitution model was identified as the best suited evolutionary aa model using ProtTest v.2.4 (Abascal et al., 2005). ML-based phylogenetic analysis were conducted using RAxML v7.2.8 (Stamatakis, 2006), under the GTR + Γ4 substitution model for the nt alignments using twelve partitions (three for each gene corresponding to each codon position). The concatenated E1-E2-L2-L1 aligned nt sequence data consisted of 5247 positions containing the following distinct alignment site patterns in the three partitions of each gene: 604, 567 and 636 for E1; 288, 281 and 288 for E2; 296, 285 and 300 for L2; and 433, 395 and 476 for L1. The trees were rooted using the sequences of PVs retrieved from aves and testudines, as described (Gottschling et al., 2011b). Figures were produced using Figtree. With the individual best-scoring ML trees for each gene a supernetwork was constructed using Splits Tree 4 (Huson and Bryant, 2006).

3. Results

3.1. EcPV2 DNA is consistently detected in equine anogenital SCCs, PIN/VIN lesions, papillomas and in contact and lymph node metastases

Amplicons of the expected size were detected in all but one penile SCC (15/16), in all PIN lesions (8/8) and penile papillomas (4/4), one of the lymph node metastases (1/2), the mouth lesion and all vaginal and anal lesions (3/3). Sample P13 was negative in all three PCR reactions, PIN1 was positive with the NB and SP1 primer sets, but negative with the ESP primer set. PIN2 was positive with the SP1 and VLC primer sets, but PCR with the NB primers turned out negative. L2, M1 and PPA4 were positive using SP1 PCR, but negative with NB PCR. These samples however were not tested in the ESP laboratory. All samples were confirmed to have PCR grade DNA after extraction using routine GAPDH PCR. Cohen's Kappa value for the results between the two laboratories was 0.949. Control DNA of equine sarcoids and intestine and H_2O were successfully used as negative controls.

Table 2
Summary of the EcPV2 DNA analysis of samples obtained from healthy horses.

Sample group	Penile swabs		Vulvovaginal swabs	
	N	Pos (%)	*N*	Pos (%)
Stallions	26	2 (7%)		
Geldings	13	2 (15%)		
Mares			20	0 (0%)

N, number of samples examined. Pos (%), number (percentage) of samples positive for EcPV2 DNA.

3.2. Ten percent of healthy males but none of the healthy females carry EcPV2 DNA in the genital mucosa

Thirty-nine penile swabs and 20 vulvovaginal swabs were obtained from healthy horses. Ten percent (4/39) of penile swabs tested positive in males: a four-year-old warmblood stallion, an eight-year-old Friesian gelding, a six-year-old Arabian stallion and a 20-year-old warmblood gelding. No EcPV2 DNA was detected in any of the 20 vulvovaginal samples. Results are summarized in Table 2. Absolute concordance was obtained between both laboratories, BE and ESP (Cohen's Kappa = 1).

3.3. Resequencing of E1 gene corrects reference EcPV2 sequence

A DNA fragment of ca. 680 bp that partially spans the E1 gene was amplified in all cases using primers designed based on the reference EcPV2 sequence (GenBank EU503122). After sequencing, this amplicon was identified without ambiguity as belonging to this PV. However, the EcPV2 sequence deposited in the database lacks the N-terminus of the E1 open reading frame, which is otherwise highly conserved among PVs. In all of the amplicons sequenced in this study there was a consistent single nucleotide polymorphism compared with the deposited EcPV2 sequence, which restored the E1 frame. We interpret thus that the original sequence could have been deposited with a mismatch. Reinforcing this view, comparison with a second re-sequencing of EcPV2 in another study (Lange et al., 2011) also points in this direction. The corrected sequence generated here has been deposited under GenBank accession number GU809241.

3.4. Several EcPV2 variants circulate in the analyzed horse population

Seventeen sequences encompassing the URR-E6-E7 region (1594 bp) were obtained from penile SCC, PIN lesions or normal mucosa. Among these sequences nine variants were identified, but no particular variant could be assigned exclusively to a specific specimen or lesion (data not shown). Although this region is highly variable in PVs (Garcia-Vallve et al., 2006), nucleotide identity among

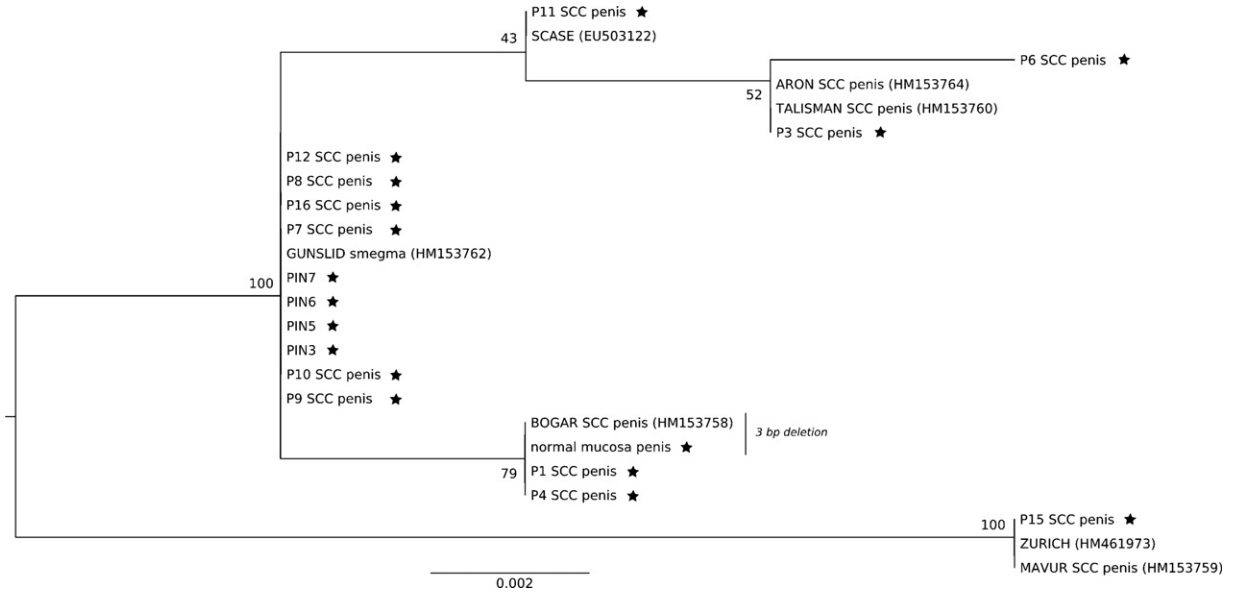

Fig. 3. Maximum likelihood phylogenetic tree for the E6 gene based on 17 sequences obtained in this study (indicated with a star symbol) along with seven sequences retrieved from GenBank. Sample name corresponds to samples showed in Table 1 except for the sequence from a normal mucosa. GenBank accession numbers are shown for retrieved sequences. Bootstrap support values (>40%) are shown at the nodes. The scale bar represents genetic distance. The tree is rooted at midpoint. (SCC, squamous cell carcinoma; PIN, penile intraepithelial neoplasia.)

these 17 sequences was rather high, with values ranging from 98.8 to 100%.

The alignment of the E6 gene of our 17 sequences along with the seven homologous EcPV2 sequences retrieved from GenBank consisted of 327 positions, with nucleotide identities ranging from 98.1 to 100%. In this reduced fragment, that spans the N-terminus of the E6 oncogene, six different variants could be defined. All genetic strains except one characterized in this study exactly match to any of the retrieved sequences. The best scoring ML tree of these 24 strains is shown in Fig. 3. Remarkably, sample P15 (accession number JN664040) coming from a penile SCC is relatively distant from the rest of the sequences. Last, two sequences showing a three nt deletion in the E6 gene clustered along with sequences without this deletion (Fig. 3).

3.5. EcPV2 is related to other equine PVs, and they could be distant relatives of the Delta + Zeta PV crowngroup

The best scoring ML tree for the nt sequences used (Fig. 4) showed that PVs segregated into four well supported crown groups, plus eight PVs (TmPV1, EcPV1, EcPV2, EcPV3, RaPV1, OaPV3, MnPV1, FdPV2, ZcPV1, CPV3, CPV4 and CPV5) whose detailed phylogenetic relationships could not be disentangled. Support values suggest that the three equine PVs could be distantly related to the Delta + Zeta PV crowngroup (Bravo and Alonso, 2007; Gottschling et al., 2011b) (Fig. 4), which encompasses PVs infecting different hosts within Laurasiatheria: horse (EcPV1, EcPV2 and EcPV3), cow (BPV1, BPV2, BPV5 and BPV8), camel (CdPV1 and CdPV2), sheep (OaPV1 and OaPV2), roe deer (CcaPV1), deer (OvPV1), European elk (AaPV1) and reindeer (RtPV1). Within this crowngroup, PVs infecting camelids, cervids and infecting horses were respectively monophyletic, i.e., they share a recent common ancestor (Fig. 4). However, PVs that infect bovids are not monophyletic, i.e., BPV1/2 and BPV5/8 are not sister taxa.

As an alternative to concatenated genes, phylogenetic relationships were also reconstructed using the individual best scoring ML trees for each of the analysed genes to build a supernetwork (Fig. 5). The four large PV crowngroups could again be defined (Bravo et al., 2010; Gottschling et al., 2011b), but conflicting information between the individual trees prevented to infer with confidence the relationships of EcPV2 and of the rest of equine PVs with the rest of the members of the *Papillomaviridae* family.

4. Discussion

In the present study EcPV2 DNA was detected in all but one (para-)genital SCCs, PIN/VIN lesions and genital papillomas, as well as in contact and lymph node metastases. This reinforces the hypothesis of an etiological role of EcPV2 in the development of these genital lesions (Scase et al., 2010), comparable to the role of high-risk human PVs in anogenital cancers. In humans, high-risk-HPV infection is a necessary but not sufficient cause of cervical cancer. Other cofactors, such as long-term use of oral contraceptives, high parity, smoking, immunosuppression, or other sexually transmitted infections, are necessary for progression from high-risk HPVs infection to cancer (Munoz et al., 2006). A similar situation may occur in horses, since we observed a rate of 10% healthy EcPV2 carriers in males. Known cofactors in the development of SCCs in horses are repeated trauma, retention of smegma (Burney et al., 1992), solar radiation, breed, hair color and age (Valentine, 2006). Functional interactions of EcPV2

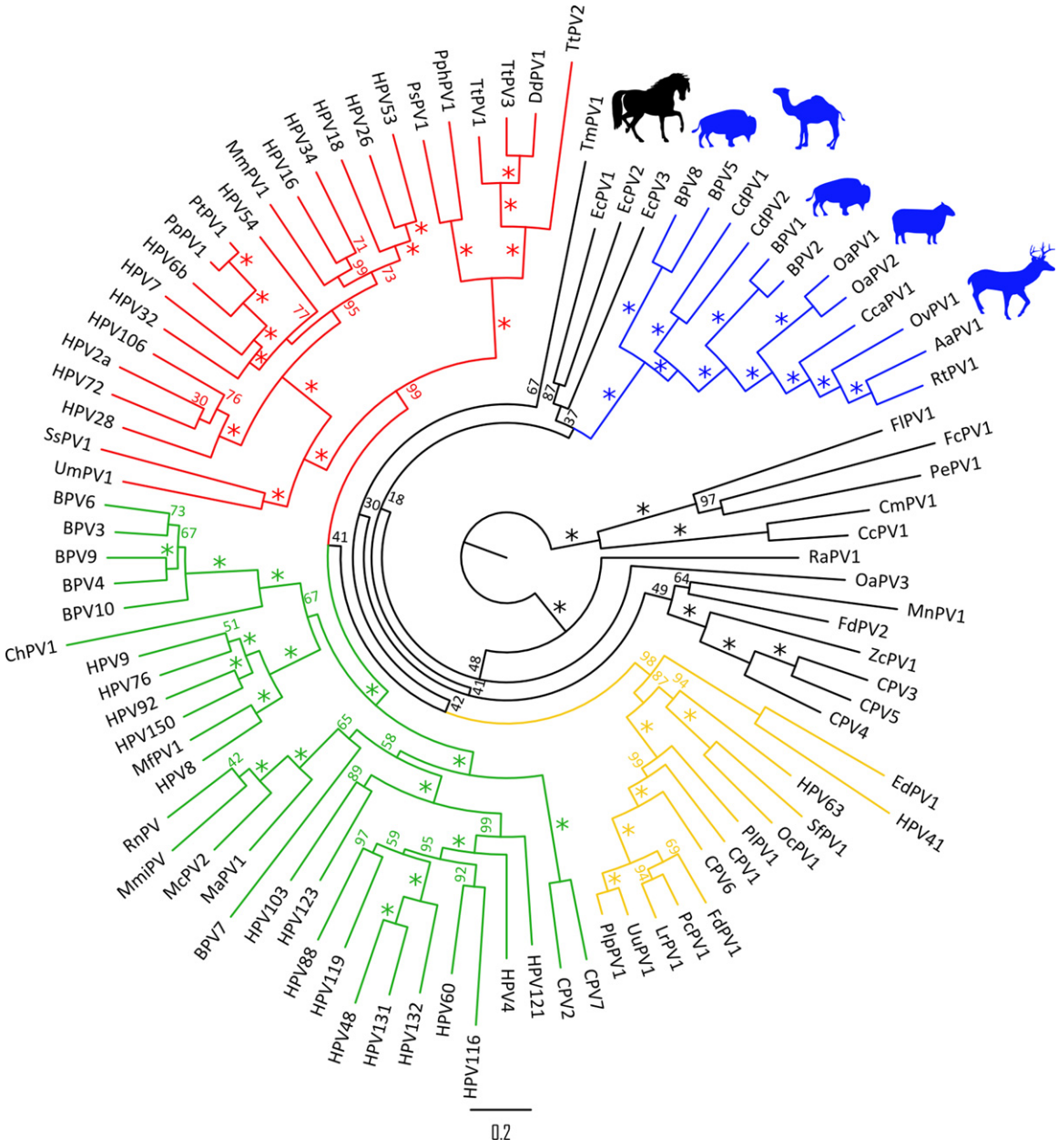

Fig. 4. Best-known maximum likelihood phylogenetic tree for the nucleotide concatenate E1E2L2L1. The different groups are colored red (Alpha + Omikron-PVs), green (Beta + Xi-PVs), blue (Delta + Zeta-PVs), and yellow (Lambda + Mu-PVs). Numbers above and below the nodes correspond to ML bootstrap support values, asterisks indicate maximal support values. For EcPV2 and its close related viruses the hosts are indicated with its corresponding silhouettes. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

proteins with cellular targets should be analysed in order to establish the precise role of EcPV2 in tumor development.

Our study demonstrates EcPV2 DNA in a lymph node with metastatic lesions from a horse with penile SCC. Earlier, EcPV2 DNA has been detected in a lymph node of a horse with penile SCC (Scase et al., 2010), though it was not mentioned in that study whether there were signs of metastasis. In humans, the relationship between high-risk human PVs DNA in lymph nodes and metastases has not yet been elucidated. The presence of human PVs DNA in histologically tumor-free lymph nodes could be a sign of (early) tumoral involvement and therefore an important prognostic factor (Lukaszuk et al., 2007). Other researchers hypothesise that PV DNA detected in histologically tumor-free lymph nodes originates either from immune cells that have taken up PV virions or from free migrating virions, and therefore do not attribute a prognostic value to PV DNA in lymph nodes (Fule et al., 2006). Our data suggest that the potential for the presence of PV DNA in lymph nodes to be a marker for early tumor involvement should be explored.

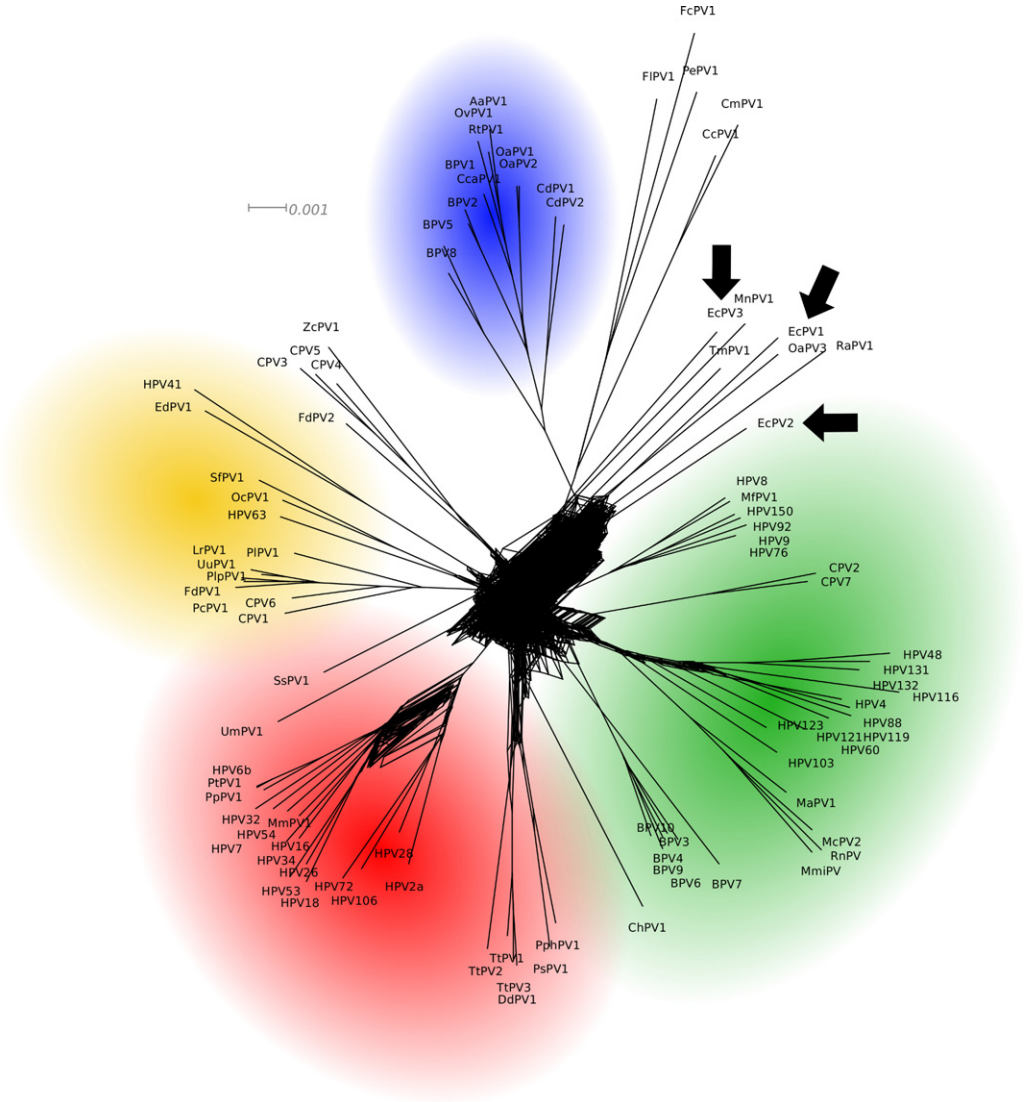

Fig. 5. Supernetwork constructed from the best trees of the nt genes E1, E2, L2 and L1. The different crowngroups are colored red (Alpha + Omikron-PVs), green (Beta + Xi-PVs), blue (Delta + Zeta-PVs), and yellow (Lambda + Mu-PVs). (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

EcPV2 DNA was also detected in a contact lesion in the mouth of a horse with a penile SCC. According to its owners, the horse was often licking and biting his penile SCC and a few months later the mouth lesion appeared. Histological examination of this mouth lesion showed focal epithelial hyperplasia. The detection of EcPV2 DNA in this lesion suggests a broad tropism of EcPV2, able to productively infect histologically different mucosal tissues in different anatomical locations, as has been also reported for COPV, infecting dogs and causing both oral and ocular lesions (Brandes et al., 2009).

The possibility of transmission from infected to non-infected horses is unknown. In women, cervical cancer is strongly associated with genital high-risk HPV infections in their male sexual partners and vice versa (Barrasso et al., 1987). In horses, sexual transmission could also be possible since EcPV2 DNA was detected in both penile and vulvovaginal SCCs. However, also horses that had never been used as breeding animals and were included in this study resulted positive for EcPV2 DNA, which suggests transmission routes additional to sexual contact. In this context, the possibility of both intra- and inter-individual PV inoculation among different anatomical locations (e.g. penis, scrotum, anus, cervix or hand) has also been demonstrated in humans (Hernandez et al., 2008). An alternative hypothesis is that the virus could be spread by insects, which is a suggested transmission route of BPV in the pathogenesis of equine sarcoids (Finlay et al., 2009). Finally, vertical transmission from mare to foal, before, during of directly after delivery, could be an alternative transmission route, as has also suggested in cattle and in humans (dos Santos et al., 1998; Rombaldi et al., 2008).

In healthy horses, EcPV2 DNA was detected in 10% (4/39) of penile swabs but none of vulvovaginal swabs (0/20),

which indicates the existence of latent EcPV2 infections. This is comparable to humans where the majority of the population undergoes subclinical infections by different HPVs at least once in their life, while only a small part shows progression to clinical lesions (Koutsky et al., 1988). We interpret therefore that, as in humans, spontaneous clearance will happen in most horses, while evolution to precarcinogenic lesions will only occur in a limited number of horses, of which only a small percentage will evolve to SCCs. A long-term follow-up study of infected but clinically normal horses is required to gain insight in the evolution of latency.

Sequence analysis regarding the URR-E6-E7 region, identified an amplicon with an in-frame three nt deletion coming from penile normal mucosa. Different annotations of the E6 gene with different putative start codons are deposited on the GenBank database (e.g. accession numbers EU503122 and HM461973) and it is not clear whether the three nt deletion resides in the URR or in the E6 region. However, since this deletion corresponds to a codon, we interpret that it belongs to the E6 coding region thus supporting that the start of the gene is the most upstream annotated one. The presence of this three nt deletion in a sequence (accession number HM153758) derived from a penile SCC (Scase et al., 2010), as well as the report of the most upstream E6 annotation in another study (Lange et al., 2011) further sustain our interpretation.

Phylogenetic analysis based on sequences from this study as well as sequences from other studies (Scase et al., 2010; Lange et al., 2011) indicate that closely related EcPV2 variants circulate in the horse population. From the ML tree based on an alignment that compress the E6 gene it may be concluded that more than a variant may be associated to different lesions, although PIN lesions seem to be related to a specific EcPV2 linage. Further research with a more exhaustive sampling will be needed to establish a possible association between specific lesions and viral strains. Adequate specimens to test this would comprise, ideally, samples from normal and affected tissues collected from both males and female horses.

Evolutionary analysis of the sequences using the concatenated supergene approach showed that the three equine PVs may be monophyletic and that they could be close relatives to the Delta + Zeta crowngroup. Reconstruction based on the L1 sequence and performed with less powerful methods had previously suggested that EcPV2 could be a relative of the Alpha-PVs (Scase et al., 2010). However, independent analyses using Bayesian approaches (Lange et al., 2011) further confirm our results. To complement the view of the supergene approach we have additionally combined the individual phylogenies of the individual PV genes into a phylogenetic supernetwork. Our results show that the conflicting topologies between the individual genes hindered the inference of deep relationships among groups, and prevented to assign with confidence the position of all three equine PVs among the *Papillomaviridae*. Our results reinforce the idea that the evolutionary histories of the different PV genes could only be partially shared (Rector et al., 2008; Gottschling et al., 2011a), and that the evolution of PVs has been driven by multiple mechanisms (Gottschling et al., 2007, 2011b).

Finally, an evolutionary explanation for the non-existence of cervical cancer in mares despite EcPV2 involvement in several other genital cancers might be that the virus–host interaction mechanisms between high-risk HPVs and those of EcPV2 may be essentially different, even though they infect anatomically similar host cells. High-risk HPVs involved in cervical cancer are phylogenetically very distant from EcPV2. Members of the Delta + Zeta PVs crowngroup, the distant relatives of EcPV2, are associated mainly to cutaneous papillomas and fibropapillomas. This is the case of EcPV1, which was isolated and characterized from cutaneous lesions (Obanion et al., 1986; Ghim et al., 2004) but also that of PVs infecting Bovidae and Cervidae, and also classified in this crowngroup (Bravo and Alonso, 2007; Gottschling et al., 2007, 2011b). Thus, the most parsimonious explanation is that the tropism of the common ancestor to the whole clade was cutaneous, whereas the mucosal tropism of EcPV2 is derived, appeared and developed after viral speciation, and is not shared by the rest of the members of the superclade. On the other hand, high-risk HPVs belong to the Alpha + Omicron PV crowngroup, where the predominant tropism is mucosal, and only a few PV, such as HPV3 or SsPV1, have subsequently developed a cutaneous tropism. The last common ancestor to both crowngroups could have existed before the radiation within mammals, around 95 millions of years ago (Gottschling et al., 2011b). The ancestors of both crowngroups specialised in different tropisms, and only later, EcPV2 colonised separately the genital mucosal niche.

5. Conclusion

The present study establishes the prevalence of EcPV2 DNA to be 94% in equine genital SCCs and 100% in precancerous lesions; and is the first to demonstrate its putative involvement in contact and lymph node metastases. Moreover, presence of EcPV2 DNA in normal equine penile mucosa was demonstrated in 10% of healthy horses. Our results suggest that the oncogenic potential of different PVs is a derived trait that can appear in different PV lineages as a result of convergent evolution.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2012.02.005.

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