Virology & Mycology
ISSN: 2161-0517

Ertelt et al., Virol-mycol 2017, 6:2
DOI: 10.4172/2161-0517.1000169

Research Article OMICS International

Investigation of a Putative Novel Papillomavirus that causes Inverted Papillomas in Horses

Antonia Ertelt[1*], **Olivia Kershaw**[2], **Christine Förster**[3], **Matthias König**[3] **and Heidrun Gehlen**[1]

[1]*Equine Clinic, Internal Medicine, Free University of Berlin, Germany*

[2]*Department of Veterinary Pathology, Free University of Berlin, Germany*

[3]*Institute of Virology, Faculty of Veterinary Medicine, Justus Liebig University Giessen, Germany*

[*]**Corresponding author**: Ertelt A, Equine Clinic, Internal Medicine, Free University of Berlin, Germany, Tel: +49 30 838 62299; Fax: +49 30 838 62529; E-mail: antonia.ertelt@fu-berlin.de

Received date: August 17, 2017; **Accepted date:** October 12, 2017; **Published date: October 19**, 2017

Abstract

The Papillomaviridae family comprises a large number of viruses that can infect a broad range of hosts giving rise to benign lesions of the skin or mucosal membranes. Until today, seven viruses termed EcPV1-7 were identified in equine papillomas.

Two trotter stallion siblings at the age of two were referred to the Equine Clinic of Free University of Berlin, because of erythema, alopecia and crusting. After a diagnosis of immune-mediated skin disease, the horses underwent long-term treatment with glucocorticoids and azathioprine. Two months later, both horses developed hundreds of small wart-like proliferations on the head, neck, chest and trunk. Six healthy horses, housed in the same stable, were used to assess the occurrence of the virus in healthy skin.

Biopsies were obtained for histopathology and for amplification and cloning of the papillomavirus genome as well as for sequence analysis.

Histopathology revealed well demarcated cup-shaped epidermal proliferations. Foci were covered by parakeratotic, or abnormally formed, keratin. In deeper layers, numerous enlarged cells with cytoplasmic eosinophilic inclusion-like structures and occasional intranuclear basophilic inclusion bodies were striking, existing beside cells with a peripheral cytoplasmic clearing (koilocytes). Histopathological findings were consistent with endophytic papilloma similar to the Le Net-type described in immunosuppressed dogs caused by a distinct canine papillomavirus. Furthermore, papillomavirus DNA was detected in tissue samples using a broad range PCR analysis. Preliminary phylogenetic analysis of a partial sequence from the *L1* gene indicated a putative new papillomavirus that has not been described in horses so far. Biopsies of the control horses showed no abnormalities in histopathology and were tested negative for the putative novel papilloma virus DNA.

Keywords: EcPV; Horse; Warts; Papilloma virus; Skin disease; Autoimmune diseases

Introduction

Papillomas are common equine neoplasms that affect several cutaneous sites, as well as oral, ocular and genital mucous membranes [1-6]. To date, almost 200 distinct papilloma viruses have been cloned, sequenced, and allocated to various genera, including seven viruses termed EcPV1-7 that were identified in equine papillomas [7-9]. The papillomavirus genome is organized in 3 regions: a) a region coding for early (E) and functional proteins (E1–E7), b) a region encoding the late (L) capsid proteins (L1 and L2), and c) a region required for genome replication and transcription (LCR (long control region)) [10]. Papillomavirus group-specific antigens have been detected in papillomas by the peroxidase-antiperoxidase (PAP) technique in retrospective studies on equine tumors [11,12]. A virus known as equine papillomavirus type 1 (EcPV1) was detected, characterized, and its DNA cloned from equine cutaneous papillomas [8]. EcPV2 has been identified from equine papillomas affecting the genital area and may involve in the development of penile squamous cell carcinomas [9,13,14]. EcPV4 was found in genital plaques, EcPV2, EcPV3, EcPV4, EcPV5 and EcPV6 in aural plaques, and EcPV7 in penile masses [3,6,9]. EcPV2 has a distinct restriction endonuclease digestion pattern and showed only moderate cross-hybridization with EcPV1 on low stringency Southern blot hybridization [7,8]. While EcPV1 has been assigned to the genus ZetaPV, EcPV2 to the genus DyoiotaPV and EcPV3 to the genus DyorhoPV, classification of the remaining EcPVs is unclear. EcPV4 and 5 may represent novel species within the genus Dyoiota, while EcPVs 6 and 7 might fit into the genus DyorhoPV and belong to the same genus as EcPV3 [9]. This report describes the clinical and pathological manifestations as well as molecular detection of a putative novel papillomavirus in two trotter siblings with cutaneous lesions. Furthermore, an epidemiological analysis of the putative novel papillomavirus in the horse population was done.

Materials and methods

Horses

Two trotter stallion siblings at the age of two years were referred because of erythema, alopecia and crusting. After a diagnosis of

immune-mediated vasculitis of unknown origin, the horses underwent long-term treatment with glucocorticoids and azathioprine. The horses were housed in stables side by side with small paddocks, where direct contact was possible. Two months after treatment initiation, both horses had developed wart-like proliferations on the skin. Furthermore, six healthy clinic owned horses (3 warm bloods, 1 trotter, 1 tinker, 1 pony), 3 geldings and 3 mares, in the age between 5-26 years (mean age 16 years), housed in the same stable with no direct contact to the stallions, were used to examine occurrence of the virus in healthy skin.

Biopsy sampling

From the two trotter stallion siblings multiple skin punch biopsies were taken from the neck, chest and trunk, where papillomas were obvious. In the six healthy horses skin biopsies were taken from the neck applying the same procedure as in the stallions. The biopsies were fixed in 10% formalin for histopathological examination, in addition to isotonic saline added with penicillin for virus detection and phylogenetic analysis.

Amplification and cloning of genomes

Individual DNA was prepared from crushed sample material using the QI Amp DNA mini-kit (Qiagen, Hilden, Germany) according to the recommendations of the supplier. A fragment from the *L1* gene of the papillomavirus genome was amplified using pan-papillomavirus-specific primers PAP1F and PAP1R [15]. DNA fragments of the expected size were excised from agarose gels and purified using the QIAEX II Gel Extraction Kit (Qiagen) and cloned into the Plasmid pCR® 2.1 using the TOPO®TA cloning kit (Fisher Scientific, Darmstadt, Germany). Positive clones from each sample were sent in for nucleic acid sequencing (Seqlab, Gottingen, Germany).

Sequence analysis

Sequence comparison was conducted using the HUSAR software packages [DKFZ, Heidelberg]. Sequences were compared to GenBank entries using the BLASTN program. Related sequences were identified and included in for phylogenetic analyses together with members of the 7 EcPVs. Phylogenetic distances were calculated [Kimura-2-parameter method] and trees generated based on the neighbor-joining method. A bootstrap analysis with 1000 replicates was included to test statistical significance.

Results

Clinical findings

The two stallions showed hundreds of small wart-like proliferations on the head, neck, chest and trunk (Figures 1 and 2).

The proliferations appeared as white to gray colored tiny rounded and slightly rough nodules. The skin of the six healthy clinic owned horses revealed no abnormalities in the clinical dermatological examination.

Histopathological findings

In the two trotter stallions, papillomas were characterized by well demarcated cup-shaped epidermal proliferations with centrifugal arrangement of the rete ridges (Figure 3).

Figure 1: Wart-like proliferations on the head of a trotter stallion.

Figure 2: Wart-like proliferations on the chest of a trotter stallion.

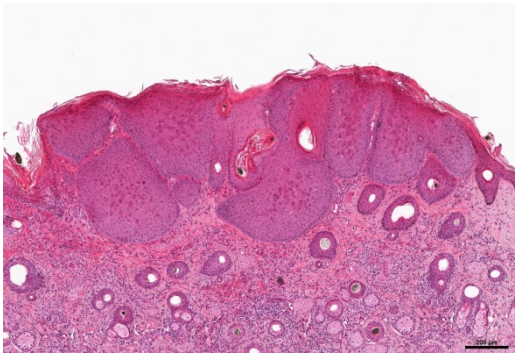

Figure 3: Skin from neck: cutaneous masses were characterized by epidermal cup-shaped proliferation invaginating into the dermis (endophytic growth), covered by a superficial layer of parakeratotic, or abnormally formed, keratin.

Proliferative foci were covered by a hyperplastic stratum corneum generating a superficial layer of parakeratotic or abnormally formed keratin. In the sub-corneal layers, multifocally enlarged cells with a peripheral cytoplasmic clearing (koilocytes) were present (Figures 4 and 5).

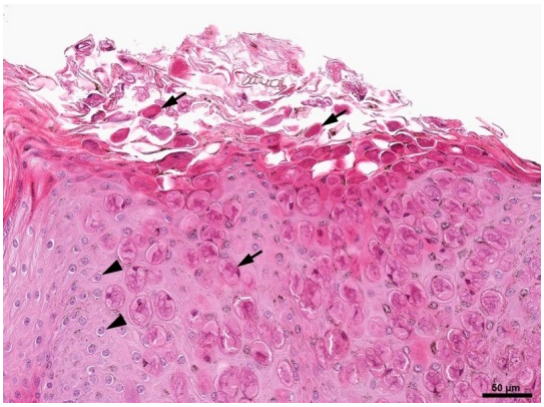

Figure 4: Histopathology of affected skin: In the thickened superficial layers of the thickened epidermis, koilocytes (arrowheads) and enlarged cells with cytoplasmic eosinophilic inclusion-like structures (keratin tonofilaments, arrows) were present.

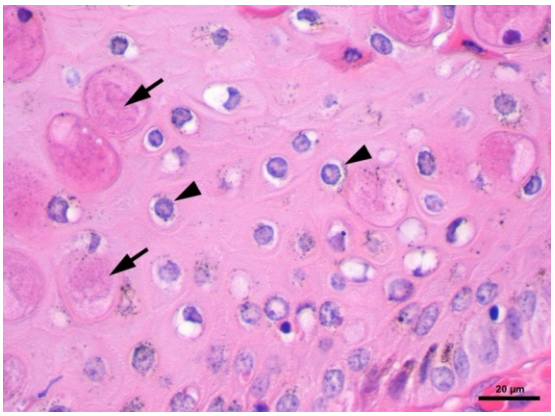

Figure 5: Histopathology of affected skin: In the thickened superficial layers of the thickened epidermis, koilocytes (arrowheads) and enlarged cells with cytoplasmic eosinophilic inclusion-like structures (keratin tonofilaments, arrows) were present.

Additionally, numerous cells were distended by eosinophilic inclusion-like structures (keratin tonofilaments) in the cytoplasm, surrounding or marginalizing the nuclei, giving the cells a signet-ring-like appearance (Figures 4 and 5). Occasionally, nuclei contained one or a few basophilic inclusion bodies (Figure 6).

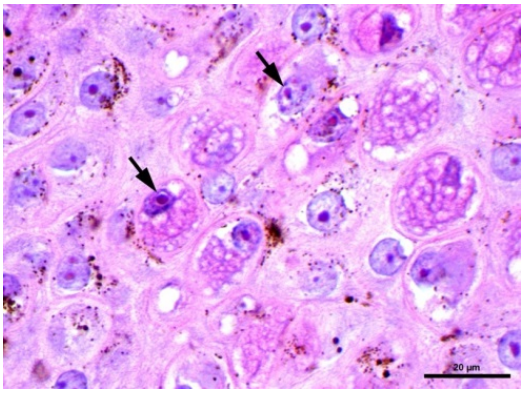

Figure 6: Occasionally, nuclei contained basophilic inclusion bodies (arrows).

These cells were still detectable in the upper stratum corneum, often containing basophilic inclusion bodies contrasted by the eosinophilic cornified mass (Figure 4). Basal layers were hyperplastic with few mitotic figures. Histologic findings were consistent with inverted (endophytic) papilloma and were similar to the Le Net-type described in immunosuppressed dogs, caused by a distinct canine papillomavirus. However, no histopathologic abnormalities were found in the skin of the six healthy horses.

Sequence analysis of the papillomavirus

Papillomavirus DNA was detected in all samples from the two trotter stallions. Sequence analysis showed 100% identity between the obtained viral sequences from both horses (GenBank accession numbers: KX575663, KX575664). Comparison of the newly obtained 473 bp fragment from the *L1* gene to existing GenBank entries showed that the closest relationship was with unclassified papillomaviruses found in the European mole (Talpa europaea, TePV), deer mouse (Peromyscus maniculatus, PmPV), Californian sea otter (Enhydra lutris, ElPV) and human papillomavirus type 63 (genus Mupapillomavirus) (Table 1).

	EcPV7	EcPV3	EcPV4	EcPV5	EcPV2	CPV	ElPV	HPV63	TePV	9081/2	PmPV	EcPV1
EcPV6	32.41	32.53	61,16	71.4()	6S.69	71),66	58.82	7S.31	77.25	82.14	66.64	66.75
EcPV7		34.73	73.61	76.19	67.4')	13.08	66.63	74.39	70.66	78.45	68.57	71.41
EcPV3			68. 12	67.99	67.09	69.33	62.7'2	78.78	71.4	71).96	6S.21	61.67
EcPV4				45.02	46.5	64,32	64.08	69.33	62.96	81.54	65.5	62.32
EcPV5					55.03	59.45	61.31	72.7	56.05	74.47	65.85	64.65
EcPV2						68.48	60.41	69.86	70.28	80.73	57.99	63.2
CPV							41.35	45.95	49.12	54.95	54.79	61.73

ElPV								47.72	49.73	51.8	SS,47	54.03
TePV									50.34	S3.88	62.89	61.02
HPV63										48 95	52.91	55,65
9081/2											53.6	67.34
PmPV												63.36

Table 1: Pairwise genetic distances calculated using the Kimura-2-parameter correction. Members of the equine PV (EcPV1 (nc_003248, ZetaPV), EcPV2 (nc_012123, DyoiotaPV), EcPV3 (nc_017862, DyorhoPV), EcPV4 (nc_020085, unclassified), EcPV5 (nc_020084, unclassified), EcPV6 (nc_020500, unclassified), EcPV-7 (nc_020501, unclassified)), canine oral PV (CPV, nc_001619, LambdaPV), Talpa europaea PV (TePV, kc460987, unclassified), human PV type 63 (HPV63, x70828, MuPV), Enhydra lutris PV (ElPV, kj410351, unclassified), Peromyscus maniculatus PV (PmPV, jf755418, unclassified) and the newly obtained sequences (9081 & 9082) were included.

Phylogenetic analyses including members of the seven established equine analyses also demonstrated the relationship of the new virus with HPV 63 and PmPV, albeit with low bootstrap values (Figure 7 or data not shown).

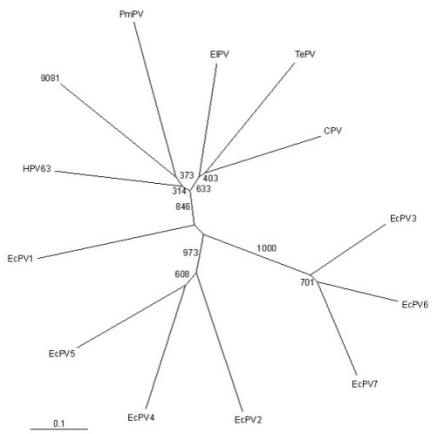

Figure 7: Phylogenetic tree based on partial sequences from the *L1* gene. Members of equine PV (EcPV1-7), canine oral PV (CPV), human PV type 63 (HPV 63), Talpa europaea PV (TePV), Enhydra lutris PV (ElPV), Peromyscus maniculatus PV (PmPV) and the newly obtained PV sequences (9081 and 9082) were included. Branch lengths are proportional to the genetic distances. Numbers within the tree represent Bootstrap values.

All samples taken from the control group were tested negative for the putative novel papilloma virus DNA.

Discussion

Viral papillomatosis is common in horses and frequently observed in young horses of less than 3 years [10]. The resulting warts are most common on the muzzle, genitalia, ears and distal legs. Inverted papillomas have never been observed in horses so far. However, they are infrequently observed in humans and dogs [16-22]. As the name implies, inverted papilloma is characterized by an endophytic growth pattern. In humans, most of them occur in the nasal cavity, although cutaneous involvement has also been described [22]. Several papilloma viruses have been associated with the development of these lesions and neoplastic transformation often occurs when high-risk human papilloma viruses are involved [21,23-25]. HPV 6/11 and HPV 16 were present in human inverted papillomas and research suggests that HPV 16 may be involved in malignant transformation [23,25]. Likewise the presence of papilloma virus DNA has been demonstrated in inverted papillomas of dogs. As is the case in humans, it is suggested that the virus is the causative agent of these lesions [25]. Indeed, the histological findings clearly indicate viral replication taking place and consequently, causality is likely [26]. Similarly, in the present cases, we found histopathological changes, such as the koilocytes, consistent with a papilloma virus infection. The presence of eosinophilic cytoplasmic inclusion-like structures as well as intranuclear basophilic inclusion bodies are indicative of degenerative cellular processes, which might be induced due to viral infection and the latter one are the result of viral replication itself. In the literature, it is suggested that more than one papilloma virus may be involved due to phenotypic differences in detected virus. The identified virus DNA in dogs matched with COPV and CPV2 [19,26]. In addition, the authors found in two dogs viral DNA that appears to belong to two novel and distinct canine PVs. The sequences of these two viruses suggest that they may belong to the genus Lambda PVs and species COPV [25].

The isolated viruses in both stallions were identical and did not show a close relationship to the viruses detected in humans and dogs with endophytic papilloma.

In these cases presumably immune suppression was induced by an autoimmune skin disease and long-term treatment with dexamethasone and azathioprine. This leads to infection with this novel papillomavirus. Similarly, people who have weakened immune systems are at greater risk of HPV infections. Immune systems can be weakened by HIV/AIDS or by immuno-suppressing drugs used after organ transplants. The prevalence of HPV in HIV-infected patients is higher than in non-HIV-infected individuals and varies over time and with the degree of immunosuppression. In people with immunosuppression, warts may become extensive, may frequently relapse after treatment, and are more likely to be dysplastic [27-31].

Most papillomavirus infections are subclinical and will not cause clinical signs. To investigate if this novel virus may be present in normal healthy skin of horses, healthy horses were examined. The healthy horses consisted of clinic owned horse from the same stable. Also, these horses were also present, but with no direct contact, at the time the papillomavirus infection evolved in the trotter stallions. Despite a possible contact between diseased and healthy horses, novel papillomavirus DNA could not be detected in the control group.

Nevertheless, a ubiquitous occurrence of the novel papillomavirus is still possible taken into consideration that the number of horses tested was very small. Transmission via contact with infected humans, small animals, rodents, birds and insects is also possible.

Because the viruses cannot actively penetrate the skin of their hosts, abrasion is one of the prerequisites for PV infection [10]. The autoimmune skin disease of the stallion led to abundant skin lesions and provided an appropriate primary surface for viral infection. Once the underlying disease was cured, spontaneous regression of the warts was observed in one stallion. The other stallion died of unrelated reasons before a regression could be observed. Spontaneous regression is commonly observed in cutaneous papillomatosis in young horses [32,33]. In equine aural plaques spontaneous regression rarely occurs [6]. In contrast to human genital warts, genital papillomas in horses have not been reported to spontaneously resolve [34,35]. Recently, a prophylactic vaccine containing EcPV 2 L1 virus-like particles for EcPV 2-associated genital papillomas in equids has been developed [36] but the effectiveness has yet to be proven.

Conclusion

In conclusion, it is most likely that immunosuppression as well as the skin lesions lead to a clinically apparent infection with the newly detected papillomavirus and that this infection caused the papillomas seen in both horses.

References

1. Le Net JL, Orth G, Sundberg JP, Cassonnet P, Poisson L, et al. (1997) Multiple pigmented cutaneous papules associated with a novel canine papillomavirus in an immunosuppressed dog. Vet Pathol 34: 8-14.
2. Scott DW, Miller WH (2003) Epithelial neoplasms. Equine Dermatology St. Louis: Saunders pp: 700–731.
3. Newkirk KM, Hendrix DV, Anis EA, Rohrbach BW, Ehrhart EJ, et al. (2014) Detection of papillomavirus in equine periocular and penile squamous cell carcinoma. J Vet Diagn Invest 26: 131-135.
4. Lange CE, Vetsch E, Ackermann M, Favrot C, Tobler K, et al. (2013) Four novel papillomavirus sequences support a broad diversity among equine papillomaviruses. J Gen Virol 94: 1365-1372.
5. Nasir L, Brandt S (2013) Papillomavirus associated diseases of the horse. Vet Microbiol 167: 159-167.
6. Zakia LS, Olivo G, Basso RM, Herman M, Araujo JP, et al. (2016) Imiquimod treatment for equus caballus papillomavirus infection in equine aural plaques. Vet Dermatol 27: 175-e44.
7. O`Banion MK, Reichmann ME, Sundberg JP (1986) Cloning and characterization of an equine cutaneous papillomavirus. Virol 152: 100-109.
8. Sundberg JP, Van Ranst M, Burk RD (1996) The nonhuman (animal) papillomaviruses: Host range, epitope conservation and molecular diversity, in: G. von Krogh, G. Gross (Eds.), Human Papillomavirus Infections in Dermatovenereology, CRC Press, Boca Raton 47-68.
9. Lange CE, Tobler K, Lehner A, Grest P, Welle MM, et al. (2013) EcPV2 DNA in equine papillomas and in situ and invasive squamous cell carcinomas supports papillomavirus etiology. Vet Pathol 50: 686-692.
10. Torres SMF, Koch SN (2013) Papillomavirus-Associated Diseases. Vet Clin North Am: Equine Pract. 29: 643-655.
11. Sundberg JP, Junge RE, Lancaster WD (1984) Immunoperoxidase localization of papillomaviruses in hyperplastic and neoplastic epithelial lesions of animals. Am J Vet Res 1441-1446.
12. Junge RE, Sundberg JP, Lancaster PID (1984) Papillomas and squamous cell carcinomas of horses. J Am Vet Med Assoc 185: 656-659.
13. Knight CG, Dunowska M, Munday JS, Peters-Kennedy J, Rosa BV, et al. (2013) Comparison of the levels of equus caballus papillomavirus type 2 (EcPV-2) DNA in equine squamous cell carcinomas and non-cancerous tissues using quantitative PCR. Vet Microbiol. 166: 257-262.
14. Scase T, Brandt S, Kainzbauer C, Sykora S, Bijmholt S, et al. (2010) Equus caballus papillomavirus-2 (EcPV-2): An infectious cause for equine genital cancer? Equine Vet J 42: 738-745.
15. Forslund O, Antonsson A, Nordin P, Stenquist B, Hansson BG, et al. (1999) A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J Gen Virol 80: 2437-2443.
16. Scott DW, Miller WH, Griffin CE (2001) Neoplastic and non-neoplastic tumors. Kirk and Muller's Small Animal Dermatology, WB Saunders Philadelphia. pp: 1236-1413.
17. Campbell KL, Sundberg JP, Goldschmidt MH, Knupp C, Reichmann ME, et al. (1988) Cutaneous inverted papillomas in dogs. Vet Pathol 25: 67-71.
18. Shimada A, Shinya K, Awakura T, Narama I, Maeda H, et al. (1993) Cutaneous papillomatosis associated with papillomavirus infection in a dog. J Com Pathol 108: 103-107.
19. Goldschmidt MH, Kennedy JS, Kennedy DR, Kennedy DR, Yuan H, et al. (2006) Severe papillomavirus infection progressing to metastatic squamous cell carcinoma in bone marrow-transplanted X-linked 240 SCID dogs. J Virol 80: 6621-6628.
20. Gross TL, Ihrke PJ, Walder EJ (2005) Epidermal tumors. Veterinary dermatolopathology. A macroscopic and microscopic evaluation of canine and feline skin disease. Blackwell Publishing, Oxford 245: 562-603.
21. Kim JY, Yoon JK, Citardi MJ, Batra PS, Roh HJ, et al. (2007) The prevalence of human papilloma virus infection in sinonasal inverted papilloma specimens classified by histological grade. Am J Rhinol 273: 664-669.
22. Bakhshaee M, Talebmehr M, Taiebi Meibod N, Razmara N (2007) A case of neglected inverted papilloma with invasion to the skin. Dentomaxillofacial Radiol 36: 245-248.
23. Arndt O, Nottelman K, Brock J, Neumann OG (1994) Inverted papilloma and its association with human papillomavirus (HPV). A study with polymerase chain reaction (PCR). HNO 42: 670-676.
24. Beck J, Mc Clatchey KD, Lesperance MM, Esclamado R, Carey T, et al. (1995) Human papillomavirus types important in progression of inverted papillomas. J Otolaryngolo Head and Neck Surg 113: 558-563.
25. Piroozmand A, Mostafavi Zadeh SM, Madani A, Soleimani R, VNedaeinia R, et al. (2016) The association of high risk human papillomaviruses in patients with cervical cancer: An evidence based study on patients with squamous cell dysplasia or carcinoma for evaluation of 23 human papilloma virus genotypes. Jundishapur J Microbiol 9: e32728.
26. Lange CE, Tobler K, Brandes K, Breithardt K, Ordeix L, et al. (2010) Canine inverted papillomas associated with DNA of four different papillomaviruses. Vet Dermatol 21: 287-291.
27. De Villiers EM, Fauquet C, Broker TR, Bernard HU, Hausen HZ, et al. (2004) Classification of papillomaviruses. Virol 324: 17-27.
28. Van Aardt MC, Dreyer G, Snyman LC, Richter KL, Becker P, et al. (2016) Oncogenic and incidental HPV types associated with histologically confirmed cervical intraepithelial neoplasia in HIV-positive and HIV-negative south african women. S Afr Med J 106: 617-622.
29. Piesiaków ML, Imko-Walczuk B, Osiecka K, Kiełbowicz M, Dębska-Ślizień A (2016) Carcinogenic viruses in etiopathogenesis of skin cancers in patients after organ transplantation. Postepy Hig Med Dosw 70: 86-94.
30. Nicol AF, Brunette LL, Nuovo GJ, Grinsztejn B, Friedman RK, et al. (2016) Secretory leukocyte protease inhibitor expression and high-risk HPV infection in anal lesions of HIV positive patients. J Acquir Immune Defic Syndr 73: 27-33
31. Palefsky JM, Holly EA, Ralston ML, Costa DM, Greenblatt RM, et al. (2001) Prevalence and risk factors for anal human papillomavirus infection in human immunodeficiency virus (HIV)-positive and high risk HIV-negative women. J Infect Dis 183: 383-391.

32. Lange CE, Tobler K, Ackermann M, Favrot C (2011) Identification of two novel equine papillomavirus sequences suggests three genera in one cluster. Vet Microbiol 149: 85-90.

33. Ghima SJ, Rectorb A, Deliusc H, Sundberg JP, Jenson AB, et al. (2004) Equine papillomavirus type 1: Complete nucleotide sequence and characterization of recombinant virus-like particles composed of the EcPV-1 L1 major capsid protein. Biochem Biophys Res Commun 324: 1108-1115.

34. Baker DA, Ferris DG, Martens MG, Fife KH, Tyring SK, et al. (2011) Imiquimod 3.75% cream applied daily to treat anogenital warts: Combined results from women in two randomized, placebo-controlled studies. Infect Dis Obstet Gynecol 2011: 1-11.

35. Arany I, Tyring SK, Stanley MA, Tomai MA, Miller RL, et al. (1999) Enhancement of the innate and cellular immune response in patients with genital warts treated with topical imiquimod cream 5%. Antiviral Res 43: 55-63.

36. Schellenbacher C, Shafti-Keramat S, Huber B, Fink D, Brandt S, et al. (2015) Establishment of an in vitro equine papillomavirus type 2 (EcPV2) neutralization assay and a VLP-based vaccine for protection of equids against EcPV2-associated genital tumor. Virol 486: 284-290.