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Brief Communication

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 tumors

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ABSTRACT

The consistent and specific presence of *Equus caballus* papillomavirus type 2 (EcPV2) DNA and mRNA in equine genital squamous cell carcinoma (gSCC) is suggestive of an etiological role in tumor development.

To further validate this concept, EcPV2-neutralizing serum antibody titers were determined by an EcPV2 pseudovirion (PsV) neutralization assay. Furthermore, an EcPV2 L1 virus-like particle (VLP)-based vaccine was generated and its prophylactic efficacy evaluated *in vivo*.

All 6/6 gSCC-affected, but only 3/20 tumor-free age-matched animals revealed EcPV2-neutralizing serum antibody titers by PsV assay. Vaccination of NZW rabbits and BalbC mice with EcPV2 L1 VLP using Freund's or alum respectively as adjuvant induced high-titer neutralizing serum antibodies (1600–12,800). Passive transfer with rabbit EcPV2–VLP immune sera completely protected mice from experimental vaginal EcPV2 PsV infection.

These findings support the impact of EcPV2 in equine gSCC development and recommend EcPV2 L1 VLP as prophylactic vaccine against EcPV2 infection and associated disease in equids.

Introduction

Papillomaviruses (PV) are a large group of epitheliotropic DNA viruses that ubiquitously infect vertebrates, causing mostly benign papillomas (warts), and rarely malignant intraepithelial neoplasia and invasive cancer. More than 60 animal PV and 160 human PV (HPV) genotypes have been completely characterized thus far (Bernard et al., 2010). In humans, 15 to 20 oncogenic (high-risk) HPV types are causally associated with development of squamous cell carcinomas (SCC) of the anogenitalia – most notably the uterine cervix – and the oropharynx, which together contribute 5% to the human cancer cases worldwide (de Martel et al., 2012; Parkin, 2006; zur Hausen, 2009).

SCC represents the most common type of horse cancer (~20% of all equine malignant tumors), which can affect any body site, yet predominate at sites with muco-cutaneous transition such as the external genitalia (~57%), the head and ocular region (~43%) (Knowles et al., 2015; Strafuss, 1976). Equine SCC usually emerges from non-invasive precursor lesions including plaques, papillomas and *in situ* carcinomas. There is growing evidence for *Equus caballus* papillomavirus type 2 (EcPV2) being causally involved in the pathogenesis of equine genital SCC (gSCC) and its precursor lesions (Knight et al., 2011; Scase et al., 2010; Sykora et al., 2012). Viral DNA and transcripts were consistently found in lesions, but not in normal adjacent tissue or non-genital SCC (Scase et al., 2010), and only sporadically in genital swabs or smegma of tumor-free horses (Bogaert et al., 2012; Fischer et al., 2014; Sykora et al., 2012). In previous studies EcPV2 DNA was detected in 45–90% of all penile SCC (Bogaert et al., 2012; Knight et al., 2011). Little is known about the natural route of EcPV2 transmission, although anecdotal reports indicate that virus can be transmitted via direct contact (Sykora et al., 2012). Moreover, the assumed EcPV2 infection-related mechanisms underlying tumor pathogenesis remain to be elucidated, and information on the seroprevalence of EcPV2 in gSCC-affected horses is still lacking. Addressing these issues is of importance, as similar pathological features, the

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presumed direct route of transmission, and external genitalia as preferential sites of infection could establish EcPV2-positive gSCC as a correlative naturally occurring PV-associated genital tumor.

For humans three multivalent HPV vaccines have been introduced that target mucosal PV infections. These subunit vaccines comprise virus-like particles (VLPs) self-assembled from the L1 major capsid proteins of high-risk HPV16/18 (bivalent), HPV6/11/16/18 (quadrivalent), or HPV6/11/16/18/31/33/45/52/58 (nonvavalent) adsorbed to adjuvant. VLPs are empty capsids that morphologically and immunologically mimic authentic virions and induce high-titers of type-restricted neutralizing antibodies. Animal vaccination trials with VLP-based immunogens have been conducted with CRPV (cottontail rabbit PV) in rabbits (Breitburd et al., 1995), COPV (canine oral PV) in dogs (Suzich et al., 1995), BPV4 in cattle (Kirnbauer et al., 1996), and, more recently, BPV1/2 in horses (Hainisch et al., 2012). A murine challenge model based on vaginal infection with PV pseudovirions (PsV), i.e. PV L1/L2 capsids harboring a reporter plasmid, mimics the natural situation *in vivo*. Following infection of mucosal keratinocytes and expression of the reporter protein (Luciferase), the latter can be detected *in vivo* by bioluminescence imaging (Roberts et al., 2007). This model has proven more sensitive in the detection of protective antibodies induced by PV vaccines compared to *in vitro* PsV neutralization assays (Longet et al., 2011).

In a serological pilot study, we have developed an EcPV2 neutralization assay and evaluated the immune response of horses to natural exposure with EcPV2. Furthermore, given the severity of equine gSCC disease and current limitations of therapy, we have generated a prophylactic EcPV2 L1 VLP vaccine and addressed its protective efficacy in a murine experimental EcPV2 PsV challenge model.

Results

EcPV2 seroprevalence in gSCC-affected and clinically tumor-free horses

EcPV2 pseudovirions (PsV), i.e. viral capsids harboring the alkaline phosphatase-expressing reporter plasmid pYSEAP, were generated as virion surrogate for the development of an EcPV2 neutralization assay. Codon-modified synthetic genes encoding EcPV2 L1 major and L2 minor capsid proteins (Scase et al., 2010) were synthesized and transferred into 293TT cells, PsV were purified on Optiprep gradients and a PsV-based neutralization assay (PBNA) was established according to Buck et al. (2004). Using this assay, sera of six horses with histologically confirmed, EcPV2 DNA-positive gSCCs (Sykora et al., 2012), and of 29 apparently tumor-free control horses were assessed for the presence of EcPV2-neutralizing antibodies (Table 1).

PBNA revealed EcPV2-neutralizing antisera in 6/6 (100%) horses diagnosed with penile or vulvar SCC confirmedly harboring EcPV2 DNA, with titers ranging from 50 to 3200. From 20 age-matched control animals, three (15%; #12, 14 and 21) were seropositive, with titers ranging between 50 and 400. Interestingly, genital swabs/smegma obtained from these three horses had also scored positive for EcPV2 DNA. Furthermore, two of the three seropositive control horses (#12 and #14) had been co-stabled for many years with animals bearing gSCC (an Icelandic gelding not included in this study and mare #6). No information on the stabling history was available for #21. In contrast to EcPV1-induced juvenile papillomas, gSCC mainly affect older animals (Junge et al., 1984) thus it is noteworthy that the small number of younger horses enrolled in this study ($n=9$; medium age 3.8 years; maximum age 8 years) tested seronegative for EcPV2. This finding indicates that either infection occurs later in life (by increasing cumulative number of close or sexual contacts), or that seroconversion is rare or short-lived in transiently infected animals but may increase with persistent infection.

Generation of EcPV2 L1 virus-like particles (VLP)

To develop a prophylactic vaccine for preventing infection and ensuing disease, EcPV2 L1 VLP was generated. The EcPV2 L1 ORF was PCR amplified from DNA isolated from an equine penile carcinoma, sub-cloned into baculovirus transfer vector pEVmod (Kirnbauer et al., 1992), and verified by bidirectional sequencing. Two clones were isolated, one harboring a wild-type (wt) L1 sequence (Scase et al., 2010), and one containing a mutated (mut) form of L1. The latter comprised an unintentionally introduced point mutation (A1110G) predicted to change the highly conserved amino acid (aa) 370 Glutamic acid to Glycine (E370G). Two recombinant baculoviruses were isolated and L1 proteins expressed in Sf9 insect cells (Schellenbacher et al., 2009). SDS-PAGE and Coomassie-staining of crude cell lysates and gradient-purified protein preparations (Shafti-Keramat et al., 2003) revealed EcPV2 wt L1 VLP and mut L1 migrating as ~50 KD proteins (Fig. 1A, *). Negatively stained preparations were visualized by transmission electron microscopy (TEM), demonstrating assembly into spherical particles with the expected diameter of ~50–55 nm for EcPV2 wt L1, whereas EcPV2 mut L1 aggregated into irregular complexes (Fig. 1B).

Immunization with EcPV2 wt L1 VLP and EcPV2 mut L1 protein

To analyze immunogenicity of EcPV2 wt L1 VLP in comparison to non-assembled mut L1 protein, one New Zealand White (NZW) rabbit each was immunized on days 0, 21 and 42 with 50 μg of the respective protein using complete or incomplete Freund's adjuvant (CFA/IFA). In addition, five Balb/C mice were subcutaneously immunized on days 0, 14 and 28 with 10 μg of EcPV2 wt L1 VLP using clinically relevant (less reactogenic) aluminum hydroxide adjuvant. All sera were drawn two weeks after the final boost. Rabbit antisera were tested in serial log dilutions (10^{-2} to 10^{-7}) for L1-specific antibody responses by ELISA (Fig. 2A), using either wt L1 VLP or mutated L1 protein as ELISA antigens. Under native ELISA conditions, post-immune but not pre-immune sera revealed antibody titers of 100,000 against L1 VLP, and of 1,000,000 against mut L1 protein. Similar titers were observed when using denatured preparations of L1 VLP (10,000) or mut L1 protein (1,000,000) as antigens (not shown).

Vaccination with EcPV2 wt L1 VLP, but not mut L1 protein, induced high-titer neutralizing antisera in rabbits and mice

Neutralizing antisera raised to PV virions are directed to conformation-dependent epitopes present on assembled VLP (or pentamer subunits) and correlate with vaccine efficacy (Harro et al., 2001). Therefore rabbit immune sera were tested in PBNA using EcPV2 PsV consisting of assembled wt EcPV2 L1 and L2, enclosing the reporter plasmid pYSEAP (Fig. 2B). Rabbit antiserum to L1 VLP was neutralizing at high titers (6400), whereas antiserum to mut L1 protein or pre-immune serum did not neutralize EcPV2 PsV at the highest concentration tested (dilution of 1:50). Each of 5 mouse antisera against EcPV2 wt L1 VLP also neutralized PsV with titers ranging between 1600 and 12,800 (Fig. 2B).

Passive transfer of neutralizing antiserum raised against VLP protected mice against vaginal infection with EcPV2 PsV

To evaluate efficacy of prophylactic vaccination with EcPV2 L1 VLP, we used a well-established murine genital challenge model with EcPV2 PsV (Roberts et al., 2007). Mice were passively transferred (i.v.) with 20 μl of rabbit pre-immune or immune serum to EcPV2 wt L1 VLP, EcPV2 mut L1 protein, or BPV1 L1 VLP and challenged with luciferase-encoding EcPV2 PsV. Mice (5 per group) that had received EcPV2 VLP immune serum were completely protected against vaginal

Table 1
Demographics and neutralizing antibody titers against EcPV2 pseudovirions (PsV) in horses bearing vulvar/penile SCC, and tumor-free horses.

Neutralizing antibody titers against ECPV-2 pseudovirions in horses by age and genital SCC							
Vulvar/Penile SCC ($n=6$)				**Tumor-free ($n=29$)**			
N°	**Sex**	**Age (y)**	**Neutralization titer**	**N°**	**Sex**	**Age (y)**	**Neutralization titer**
#1	m	24	**3200**	#7	m	30	**0**
#2	f	24	**3200**	#8	f	30	**0**
#3	f	22	**100**	#9	m	24	**0**
#4	m	>20*	**400**	#10	f	22	**0**
#5	f	>20*	**50**	#11	m	21	**0**
#6	f	20	**50**	#12	m	20	**200**
EcPV2 Seropositivity (>20 years)			***6/6 (100%)***	#13	m	20	**0**
				#14	f	20	**50**
				#15	m	19	**0**
				#16	m	19	**0**
				#17	m	19	**0**
				#18	f	19	**0**
				#19	m	18	**0**
				#20	m	18	**0**
				#21	m	17	**400**
				#22	m	17	**0**
				#23	m	17	**0**
				#24	f	17	**0**
				#25	f	16	**0**
				#26	m	15	**0**
				EcPV2 Seropositivity (≥15 years)			***3/20 (15%)***
				#27	f	s	**0**
				#28	m	7	**0**
				#29	f	7	**0**
				#30	m	4	**0**
				#31	m	4	**0**
				#32	m	1	**0**
				#33	m	1	**0**
				#34	f	1	**0**
				#35	f	1	**0**
				EcPV2 Seropositivity (≤8 years)			***0/9 (0%)***

Antisera of 29 healthy horses ($n=20$ ≥15 years; $n=9$ ≤8 years), and 6 equids suffering from vulvar or penile SCC (≥20 years), were analyzed for neutralizing antibodies against EcPV2 PsV using endpoint 2-fold serial dilutions (starting at 1:50 up to 1:25,600). Titers <50 are scored as 0.

infection with EcPV2 PsV (Fig. 3A and B). In contrast, bioluminescence imaging detected genital EcPV2 PsV infection in mice transferred with antiserum raised against EcPV2 mut L1 protein, heterologous BPV1 L1 VLP, or the respective pre-immune sera, confirming conformational dependence and type specificity of prophylactic EcPV2 VLP vaccination (Fig. 3B).

Discussion

Knowledge about PV infections in horses and other equids, e.g. donkeys, zebras and mules, had been limited to trans-species infection by sarcoid-inducing BPV1 and 2, until EcPV1 could be established as the causative agent of juvenile papillomas (Chambers et al., 2003; Ghim et al., 2004; O'Banion et al., 1986). In recent years, additional EcPV types 2 to 7 have been identified and substantial evidence has accumulated for causal contribution of EcPV2 in the development of equine gSCC (Bogaert et al., 2012; Kainzbauer et al., 2012; Scase et al., 2010; Scott and Miller, 2003; Sykora et al., 2012). The advanced age of horses bearing EcPV2-positive genital lesions (Junge et al., 1984; Lange et al., 2013), the description of EcPV2-positive gSCC and precursor lesions, and the occasional detection of EcPV2 DNA from genital swabs (and most notably smegma) from apparently healthy animals living in close contact with gSCC-bearing horses (Sykora et al., 2012) are features shared by high-risk HPV-induced genital disease. Although only a small number of gSCC-affected horses ($n=6$) were available for this pilot study, 100% (6/6) of these animals tested seropositive to EcPV2 L1, as compared to 15% (3/20) of age-matched, or 10.3% (3/29) of all apparently tumor-free control horses enrolled in this study.

Although human cervical cancer correlates stronger with seropositivity to the early viral oncoprotein E6 than to the viral capsid proteins L1 and L2 (Kirnbauer et al., 1994; Viscidi et al., 1993), the seroprevalence of HPV16 capsid antibodies in cervical cancer patients was significantly higher than in control groups (Kim et al., 1999). Even if shown in a limited number of cases and controls the strong association of EcPV2 L1 seropositivity (100%) with equine genital lesions harboring EcPV2 DNA further strengthens the concept of a causal involvement of EcPV2 infection in the development of equine gSCC and corresponding precursor lesions.

The clinical relevance of EcPV seropositivity in lesion-free horses remains less well-defined. A recent study from Switzerland enrolling 50 clinically healthy horses reported 10% EcPV2 DNA positivity without corresponding antibody titers, an even higher EcPV2 seropositivity of 28% without DNA detection, and 8% of animals positive for both EcPV2 DNA and antibodies (Fischer et al., 2014). Seropositivity against EcPV2 L1 is a positive marker for past or present productive EcPV2 infections and may therefore detect possibly contagious animals, whereas the negative predictive value of EcPV2 seronegativity is limited. In humans 40–60% of women seroconvert following cervical HPV DNA detection and antibody titers are significantly lower than those raised by HPV vaccination. Whether discordances are due to different stages of infection were not clearly answered neither in humans nor equids.

Seropositivity was also observed for three tumor-free control horses, i.e. one Warmblood mare, and two geldings of Icelandic

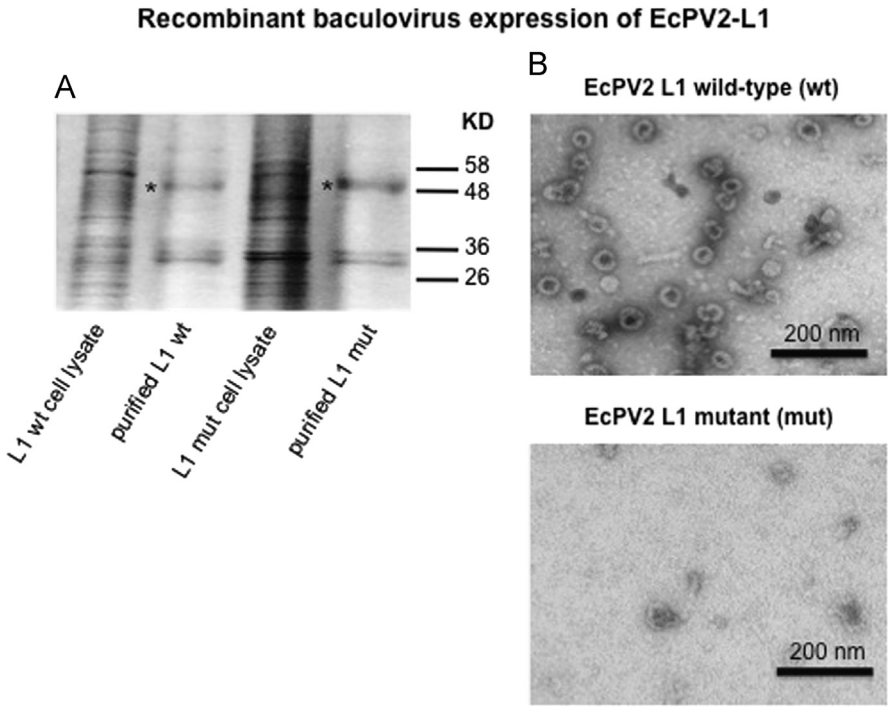


Fig. 1. Recombinant wild-type EcPV2 L1 (A) but not mutated EcPV2 L1 (B) assembles into virus-like particles (VLP). Following PCR-cloning of EcPV2 L1 ORF into transfer vector pEVmod, DNA sequencing identified both a wild-type (wt) and a mutated (mut) L1 clone (E370G). Following expression by recombinant baculoviruses in insect cells, protein bands specific for the full length L1 protein migrated at ~50 KD (indicated by *) by SDS-PAGE and Coomassie-staining following density gradient purification for both wt and mut L1. Transmission electron microscopy of negatively stained preparations revealed assembly into VLP for wt L1, and irregular aggregates for mut L1 protein (B).

breed. The seropositive mare #14 had been co-stabled with the SCC-bearing mare #6 for many years but remained free from disease within a six-year observation period (2007–2012). Interestingly, mare #14 tested negative for EcPV2 L1 neutralizing antibodies six years later (2012) indicating transient infection. Similarly, one of the EcPV2 L1-seropositive geldings (#12) had lived for several years in close contact with an Icelandic horse bearing an untreated EcPV2-positive penile SCC, before being moved to another place. No history is available for the third seropositive Icelandic gelding (#21). The two seropositive geldings have been clinically examined for three years since blood collection and have since remained tumor-free.

Together with the fact that EcPV2-associated genital lesions are predominantly encountered in older horses, these observations indicate that EcPV2 infection is cleared in a subset of younger, immunocompetent horses, whereas it may persist in other individuals and cause progressive lesions at advanced age. Genital SCC-affected, EcPV2 L1-seropositive gelding #2 was imported from Iceland before 1997 and developed a penile SCC after 2010. Interestingly, sequencing of intralesional viral DNA revealed an 'Iceland-specific' EcPV2 variant (Kainzbauer et al., 2012; Scase et al., 2010; Sykora et al., 2012); (unpublished data), indicating that infection had occurred before 1997 and persisted before tumor development, possibly by age-related changes in the immune-status, and/or hormone, cytokine and/or growth factor levels, as proposed by Maglennon and Doorbar (2012).

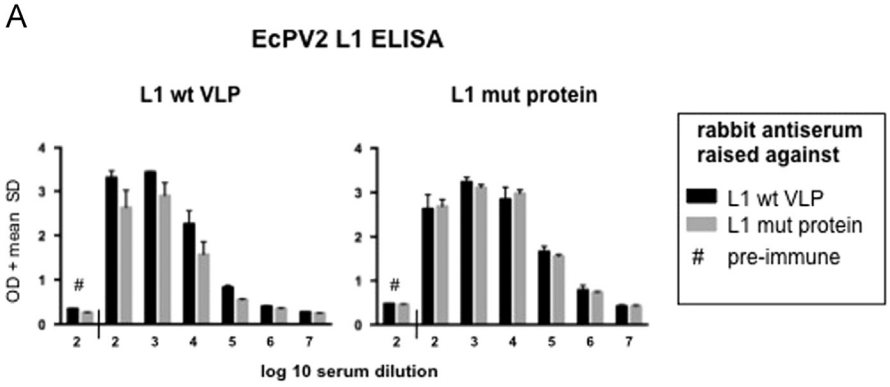
Seropositivity may identify potentially contagious horses with productive benign lesions or disease preceding tumor development. Before introduction of valuable animals into stables with cases of gSCC or with a significant percentage of seropositive animals, susceptible horses may benefit from prophylactic EcPV2 vaccination.

In agreement with previously reported EcPV2 DNA detection rates for clinically healthy horses (~0–10%) (Bogaert et al., 2012; Sykora et al., 2012), a recent study from Switzerland reported 10% EcPV2 DNA positivity for genital samples, and EcPV2 L1-specific seropositivity for 28% of 50 healthy animals tested (Fischer et al., 2014). This higher EcPV2 seroprevalence obtained by ELISA when compared to 15% seropositivity determined by the herein described PBNA possibly reflects the pronouncedly higher specificity of the latter method (Pastrana et al., 2004). As observed for L1-VLP ELISA, it is reasonable to assume that non-assembled L1-GST ELISA results in higher overall but less specific L1 detection rates when compared to the more stringent PBNA, which specifically detects neutralizing antibodies. The EcPV2 PBNA herein thus represents a more stringent method for analysis of protective serum antibodies following natural infection (Zhao et al., 2014), and for seroepidemiological studies on the prevalence of EcPV2 and age distribution in different equid populations. Such studies would also guide informed decisions on the ideal age for future EcPV2 vaccinations in equid stocks.

As demonstrated for the L1 proteins of many other PV types including EcPV1 (Ghim et al., 2004), recombinantly expressed EcPV2 wt L1 protein efficiently assembled into VLP. Assembly was disrupted by a single aa point mutation (E370G) targeting a highly conserved motif in the beta1 sheet structure of L1, as identified in small $T=1$ HPV16 L1 capsids (Bishop et al., 2007; Kirnbauer et al., 1993). Immunization with EcPV2 wt L1 VLP (and mut L1 protein) induced high-titers of antibodies against L1 epitopes when assessed by ELISA using wt L1 VLP or mut L1 protein as antigen in native or denatured form, suggesting that a significant proportion of antibodies induced by L1 VLP (and mut L1) recognizes linear epitopes. In contrast, neutralizing antibodies were only generated against conformational epitopes of the assembled immunogenic wt L1 VLP capsid, supporting the importance of the native structure of the L1 immunogen for induction of protective antibody responses (Kirnbauer et al., 1992).

The establishment of a murine cervico-vaginal challenge model (Roberts et al., 2007) has facilitated investigations of the mechanisms of genital PV infection and of vaccine efficacy in vivo. EcPV2 PsV enclosing a luciferase reporter gene efficiently infected the genital murine mucosa, comparable to mucosal high-risk HPVs (Schellenbacher et al., 2013). As expected, passive transfer of EcPV2 wt L1 VLP antiserum completely protected mice from experimental EcPV2 infection. Based on previous studies on HPV VLP vaccines,

A



B

EcPV2 L1 Pseudovirion Neutralization Assay

Rabbit antiserum	Neutralization titer
EcPV2 L1 wt	6,400
EcPV2 L1 mut	0
Mouse antiserum	**Neutralization titer**
EcPV2 L1 wt #1	6,400
EcPV2 L1 wt #2	1,600
EcPV2 L1 wt #3	6,400
EcPV2 L1 wt #4	12,800
EcPV2 L1 wt #5	6,400

Fig. 2. Immunization with EcPV2 L1 VLP raises neutralizing antibodies in rabbits and mice. New Zealand White (NZW) rabbits were immunized (A) with 50 μg of EcPV2 wt L1 VLP or mut L1 subcutaneously (week 0, 3, 6) using CFA/IFA adjuvant. BalbC ($n=5$) were vaccinated (B) with 10 μg of EcPV2 wt L1 subcutaneously (week 0, 2, 4) using aluminum hydroxide adjuvant. Antisera in indicated dilutions (10^{-2} to 10^{-7}) were tested for L1-specific antibodies using EcPV2 wt L1 VLP and EcPV2 mut L1 protein in ELISA (A). Immune sera of both rabbits and mice were analyzed for neutralizing antibodies (dilutions 1:50 to 1:25,600) in EcPV2 PsV-based neutralization assays (PBNA) (B). Titers <50 are scored as 0.

protection against PsV-infection in this model is a valuable surrogate of prophylactic L1 VLP vaccine efficacy in preventing persistent viral infection and consequent disease (Jagu et al., 2009) as determined in randomized controlled clinical trials (Day et al., 2012).

SCC and sarcoids are the two most common tumor types diagnosed in equids (Knowles et al., 2015; Sundberg et al., 1977) which pronouncedly impair the health, welfare and material value of equids. If the causal role of EcPV2 infection in penile and vulvar cancer development can be firmly proven, half to two thirds of horse tumors, i.e. gSCC and sarcoids, could be prevented by a vaccine that protects against infection by BPV1/2 and EcPV2. We have previously demonstrated that BPV1 L1 VLPs efficiently protect horses from experimental challenge with BPV1 virions (Hainisch et al., 2012; Kirnbauer et al., 1996), and that anti-BPV1 L1 antibodies cross-neutralize BPV2 (Shafti-Keramat et al., 2009). Herein, we show that passive transfer of rabbit antiserum to EcPV2 L1 VLP efficiently protects mice from EcPV2 PsV challenge. Consequently, similar to licensed HPV vaccines, a bivalent BPV1/EcPV2 L1 VLP vaccine has the potential to significantly reduce the incidence of PV-associated (semi-)malignant diseases in horses and other equids.

Material and methods

Animals

Tumor bearing (minimum age of 20 years as documented for equids #1, #2, #3, #6, or estimated by owners for #4 and #5) and control horses (age ≥ 15 years, mean 19,9) were owned by, and routinely treated at, the Equine Clinic at the Veterinary University of Vienna. SCC were surgically removed, verified by routine histopathology, serum samples obtained and stored at $-20\,^{\circ}C$. Demographics: Information about age, sex and genital (penile or vulvar) cancers is shown in Table 1.

EcPV2 pseudovirions (PsV)

Production of PsV and neutralization assays (PBNA) were performed as described by Buck (http://home.ccr.cancer.gov/lco/protocols.asp) with few minor modifications. EcPV2 PsV were generated by codon modification "as-different-as-possible" of NCBI reference sequence NC_012123 (Scase et al., 2010). (http://home.ccr.cancer.gov/lco/codonmodification.htm). Altered L1 and L2 genes were synthesized (Geneart-Invitrogen, Germany) and cloned into expression vector pVITRO1-neo-mcs (Invivogen) using restriction enzyme sites Bgl2 and Nhe1, or BamH1 and Avr2, respectively. The EcPV2 L1/L2 expression vector was co-transfected with reporter plasmid pYSEAP (encoding for secreted-alkaline for in-vitro assays), or pCLucf (encoding for firefly luciferase for in vivo infection of mice) into 293TT cells.

Recombinant EcPV2-L1 baculovirus expression vectors

EcPV-2 DNA was isolated from an equine penile SCC (Sykora et al., 2012). To express recombinant EcPV2 L1 protein, the L1 ORF was PCR amplified using a mixture of Taq and high-fidelity polymerase and primers complementary for EcPV2 sequence nucleotides (nt) 5673–5695, additionally introducing restriction enzyme (RE) site BglII (5′-CGATAGATCTACCTATAAATATGGCTTTCTGGACAATGAACA-

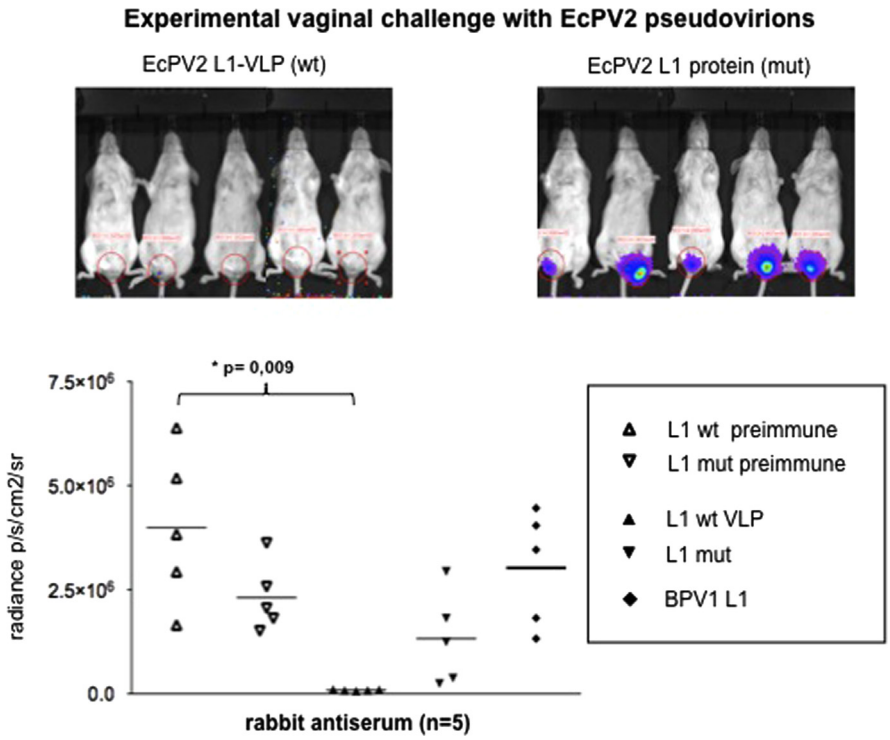


Fig. 3. Passive transfer with EcPV2 L1 VLP antiserum completely protects mice from experimental vaginal challenge with EcPV2 PsV. Groups of mice ($n=5$) were passively transferred with 20 μl of pre-immune or immune sera to EcPV2 wt L1 VLP, mut L1 protein, or BPV1 L1 VLP into the tail vein and challenged with EcPV2 PsV encapsidating Luciferase reporter plasmid. Three days later, PSV infection was determined by detection of Luciferase activity by bioluminescence imaging (*Y*-axis: average radiance; p/s/cm^2/sr), with the respective background luminescence (unvaccinated mice challenged with CMC only) subtracted from all data points.

3′), and (nt) 7174–7153 plus EcoRI RE site (5′-CCTGGAATTCGCTTATGCCTTCCTTTTCTTGG-3′). Subsequently, the amplimer was cloned into the baculovirus transfer vector pEVmod (Kirnbauer et al., 1992) and verified by RE digest and bidirectional sequencing (VBC-Biotech, Vienna, Austria). Two clones were identified containing either wt or mut L1 (a single nt point mutation (A1110G) changing a codon for highly conserved aa 370 Glutamic Acid (E) to Glycine (G)). Recombinant baculoviruses were generated by cotransfection of Sf-9 insect cells with transfer vectors and linearized baculovirus DNA (BaculoGold; BD Biosciences). Expression in SF9 insect cells and purification of VLP on sucrose and CsCl density gradients were performed as described previously and purified VLP were visualized by negative stain and TEM at 30,000 × magnification (Schellenbacher et al., 2009).

Immunization

Following extensive dialysis of EcPV2 L1 proteins against PBS containing 0.5 M NaCl, 1 mM $CaCl_2$, and 0.02% Tween 80, NZW rabbits were immunized with 50 μg of either wt or mut L1 protein in CFA followed by boosts at weeks 3 and 6 in IFA. Balb/C mice were immunized with 10 μg of wt L1-VLP at weeks 0, 2, and 4 using aluminum hydroxide as adjuvant (Alhydrogel 2%, Invivogen, San Diego, CA). Blood samples were obtained 2 weeks after the third immunization and sera stored at −20 °C. NZW rabbits were immunized at Charles River Laboratories, Germany. Murine studies have been approved (BMWF-66.009/0173–1I/3b/2011) and animal care was in accordance to the guidelines of the Austrian Federal Ministry for Science and Research.

EcPV2 enzyme-linked immunosorbent assay (ELISA)

Polyclonal antisera raised against EcPV2 L1-VLP (wt), or L1 protein (mut), were tested for antibodies against both EcPV2 L1-VLP (wt) and L1 protein (mut). Native proteins diluted in PBS (150 ng/100 μl PBS/1% Non Fat Dry Milk) were coated onto 96-well microtiter plates (Nunc MaxiSorp) overnight at 4 °C. Additionally, proteins were plated under denaturing conditions ($NaHCO_3$ pH 10.6, 0.01 M DTT) overnight at 37 °C. After blocking with PBS/1% milk for 30 min, the antigen was incubated with rabbit antisera serially diluted 10^{-2} to 10^{-7} in PBS/1% milk for 1 h at room temperature. Finally a 1:5000 dilution of peroxidase-conjugated goat anti rabbit-Ab (Bio-Rad) was added and the plate was developed by adding the substrate ABTS (Bohringer Mannheim). The optical density (OD) at 405 nm was determined using an ELISA reader (Dynatech).

EcPV2 pseudovirion based neutralization assay (PBNA)

Expression vectors for EcPV2 codon-modified L1 and L2 ORFs were co-transfected with reporter plasmid pYSEAP or pCLucF. Neutralization assays were performed according to an adapted protocol (http://home.ccr.cancer.gov/lco/neutralizationassay.htm). PsV were pre-incubated with 2-fold serial dilutions of horse sera or EcPV2-L1 vaccinated rabbits or mice from 1:50 up to 1:25,600.

EcPV2 murine vaginal challenge

The intravaginal PsV challenge model (Roberts et al., 2007) was adapted using microtrauma induced by a cytobrush to prime vaginal mucosa for infection (Karanam et al., 2010). Twenty-five female Balb/C mice in groups of five animals were pretreated with subcutaneous injection of progesterone (3 mg Depo-Provera) four days prior to vaginal challenge. Three days later mice were passively transferred with 20 μl of rabbit serum: 1, EcPV2 L1 wt preimmune 2, EcPV2 L1 mut preimmune 3, EcPV2 L1 wt immune 4, EcPV2 L1 mut immune 5, BPV1 L1 immune. 24 h later mice were intraperitoneally anaesthetized with Ketamine/Xylazine, and a mechanical microtrauma of the vaginal epithelium was set by 30 × rotating a cervical cytobrush. Luciferase-encoding EcPV2 PsV in 3% carboxymethylcellulose (CMC) (1:1) were inserted into the

vaginal vault, and 72 h later infection was analyzed by detection of luciferase-induced bioluminescence produced in the vagina upon intravaginal application of 20 μl luciferin (Caliper; 7 mg/ml) into anaesthetized animals. Bioluminescence was imaged in the vaginal area (IVIS 50, Caliper). An identical region of interest (ROI) was selected for all animals and luminescence data are given minus the matching background signal (mice challenged with CMC only). Statistical analysis was performed using the Microsoft Excel Software and a two-tailed unpaired *t*-test (heteroscedastic) used to calculate *p*-values.

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