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# THE ASSOCIATION BETWEEN EQUINE PAPILLOMAVIRUS TYPE 2 AND EQUINE SQUAMOUS CELL CARCINOMAS

A thesis presented in partial fulfilment
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# ABSTRACT

Squamous cell carcinomas (SCCs) are malignant epithelial neoplasms affecting most species. Equine SCCs are most common on the penis, where they result in significant welfare and economic costs and frequently necessitate euthanasia. In humans, half of penile SCCs are caused by infection with papillomaviruses (PVs). The research described in this thesis investigated whether PVs similarly cause equine penile SCCs (EPSCCs).

Testing of equine penile samples using conventional PCR and consensus primers amplified PV DNA significantly more frequently from SCCs than from non-SCC lesions. Sequencing of the amplified DNA showed that there was just one PV type present, and that it was a newly-discovered PV called equine papillomavirus type 2 (EcPV-2). *In situ* hybridization and immunohistochemistry localized PV DNA and antigen to neoplastic cells but not to adjacent tissue. These results suggested that EcPV-2 could influence the development of EPSCCs.

A quantitative PCR assay was then developed to test for EcPV-2 presence and load in a large number of equine samples from the penis and from other SCC-prone body sites. This showed that EcPV-2 is present significantly more frequently, and at significantly higher loads, in EPSCCs than in non-SCC tissues. Furthermore, some equine pharyngeal SCCs contained low EcPV-2 loads. However, as EcPV-2 was also sometimes present in grossly normal pharyngeal samples, the significance of this was uncertain. EcPV-2 DNA was only rarely detectible in grossly normal vulvovestibular mucosal samples and never in nictitating membrane samples.

To help determine whether EcPV-2 causes cancer or is an incidental bystander, immunostaining for three cellular regulatory proteins (transformation-related protein 53 (p53), retinoblastoma protein (pRb), and cyclin-dependent kinase inhibitor 2A) was performed. This showed that, unlike high-risk human PVs, the presence of EcPV-2 DNA within a SCC was not associated with degradation of the tumor suppressor proteins

p53 or pRb. While these results do not support a causative association between EcPV-2 and equine SCCs, the possibility that EcPV-2 causes cancer by changing other cell regulatory proteins cannot be excluded.

Overall, evidence from our and others' research strongly suggests that EcPV-2 is involved in EPSCCs, but does not prove unequivocally that it causes these neoplasms.

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Finally, in chapter three I wrote, "*One veterinarian contacted me after seeing Horse A, a 9-year-old Standardbred cross gelding ...*" The veterinarian was my wife, Brielle. Her observation, curiosity and willingness to biopsy on my behalf provided me with New Zealand's first recognized case of EcPV-2 infection, which in many ways allowed this thesis to progress past chapter 3. That alone would have been enough, but it was her endless and loving support that let me reach the end, and I need to thank her more than anyone else. I could never have done this without her. While I worked on this thesis, she finished her own PhD, gave birth to and reared our two fantastic children, moved us to Canada, and looked after me more than I deserve. She never stops smiling, and she brightens life for everyone around her. Thank you, Brielle.

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# CHAPTER ONE

# Background and Literature Review

## 1.1 Introduction

Papillomaviruses (PVs) are established to cause a defined subset of human cancers, especially squamous cell carcinomas (SCCs) of the cervix, genitals, and throat. The research described in this thesis was carried out to investigate whether PVs similarly cause SCCs in horses.

In the following literature review SCCs are defined and briefly discussed. Then, the current knowledge about SCCs in horses is reviewed, with a particular emphasis on SCCs of those body sites investigated in this thesis. Next, the biology of PVs is discussed, and information about the relationship between PV infection and SCC development in horses, humans and other species is reviewed. Methods that can be used to judge the significance of detecting a PV in a cancer are also discussed. Finally, other risk factors for SCC development in animals are discussed in order to highlight gaps in knowledge and to justify this investigation into PVs as possible agents in the development of equine SCCs.

## 1.2 Overview of Squamous Cell Carcinomas

Squamous cell carcinomas are malignant epithelial neoplasms that are a significant cause of morbidity and mortality in humans and animals.[101,190] In humans, SCCs are distressing and potentially disfiguring even if treated successfully and may be fatal if treatment is unsuccessful. In production animals, SCCs have a significant economic and welfare cost and, in companion animals, euthanasia for humane or economic reasons is a frequent outcome.

Although SCCs may originate from any epithelial surface of the body, they most commonly arise from the stratified squamous epithelium of the skin and body openings. They have a varied clinical appearance, but the majority appear as plaque-like, crusting to ulcerated masses. Secondary bacterial infection of the ulcerated tumor surface may occur, resulting in an adherent purulent exudate. As SCCs progress, islands, cords and trabeculae of neoplastic epithelial cells infiltrate underlying tissue. This often causes a significant desmoplastic (fibrous) response, resulting in a firm texture and thickening of the affected area. In addition, SCCs appear to have a tropism for dermal and subcutaneous nerves, possibly explaining the tenderness on palpation described by human patients.[158]

Within SCCs, neoplastic cells show a variable degree of squamous differentiation and keratinization. Well-differentiated cutaneous SCCs typically contain characteristic islands (keratin “pearls”) formed by concentric layers of keratinized neoplastic cells, but these are absent from poorly differentiated SCCs. The number of mitotic figures seen is variable, but tends to be higher in poorly differentiated tumors.

The biological behavior of any particular SCC is partly dependent on the species affected and the location of the neoplasm. However, most SCCs are locally invasive and may metastasize to regional lymph nodes late in the course of the disease.[101,158] Treatment options include surgical excision and adjunctive therapy, such as radiation therapy, immunotherapy, chemotherapy, cryotherapy, hyperthermia, and

brachytherapy.[100,158,269] Complete surgical excision is often curative; however, poor surgical access or the need to preserve function or appearance may prevent complete excision. Incomplete excision is associated with more frequent recurrence.[158]

## 1.3 SCCs in Horses

Overall, SCC is the second most frequent cutaneous and mucocutaneous neoplasm in horses, after sarcoids.[152,233,268] Although any horse may be affected, the prevalence of SCC has been reported to be higher in lightly pigmented and draft breeds,[70,268] and in middle-aged to older horses.[132,152,268] Equine SCC predilection sites are the penis, the eye and adnexa, the vulva and vestibule, and the stomach. Other less commonly affected sites include the mouth, pharynx and larynx, haired skin, the paranasal sinuses and nasal passages, the urinary bladder, and the hoof.

In the following sections the current information on equine SCCs is discussed for each anatomic location.

### 1.3.1 Equine Penile SCCs (EPSCCs)

The equine penis is an important predilection site for SCCs, with 25-50% of all cutaneous SCCs affecting the male external genitalia.[152,245] Not surprisingly, SCCs are the most common neoplasms of the equine penis.[34,269,270] Equine penile SCCs (EPSCCs) affect older horses, with the average age at diagnosis reported to be between 10 and 18 years.[83,119,132,156]

Squamous cell carcinomas may develop anywhere on the male external genitalia but are more common on the free part (including the glans) of the penis than the prepuce or urethra.[83,84,119,132,152,156] The majority of horses present due to a malodorous, blood-stained and purulent preputial discharge,[49,119] although other reported clinical signs include preputial edema, dysuria, hematuria, weight loss, and urinary incontinence.[152] Rarely, urethral SCCs have also been reported to cause hemospermia[14] and bladder rupture.[159]

EPSCCs begin as small, plaque-like or papillary lesions that progress to larger cauliflower-like or pedunculated masses with areas of necrosis and ulceration.[49,152] Histologically, they are usually well differentiated and keratinization is almost always present. Infiltration of inflammatory cells into the tumor is common, and foci of necrosis and mineralization are frequent.[83]

EPSCCs are typically slowly progressive with a low metastatic potential. However, the lack of specific clinical signs and difficulty in examining the external genitalia mean that EPSCCs may be advanced at the time of diagnosis, increasing the risk of local or metastatic spread by the time that treatment is started.[156] Metastasis is usually first to local lymph nodes, with pulmonary metastasis also reported.[9,41,52,84,269,270]

Treatment options for EPSCCs range from minimally invasive (e.g., topical chemotherapy) to radical surgical procedures (e.g., penile amputation and removal of inguinal lymph nodes),[271] with the choice depending primarily on the size and site of the lesion, the presence of metastasis and the owner's willingness to opt for surgery.[271] There is no standardized approach to treatment of EPSCCs and reported outcomes vary.[82,119,156,271] Tumors may recur after incomplete excision and the long-term prognosis for non-resectable EPSCCs is poor.[269] The largest study to date comparing different surgical treatment modalities reported an overall success rate (as determined by absence of tumor recurrence) of just over 50%.[269] EPSCCs cause significant discomfort and pain in affected horses, both before and after treatment, and also cause emotional and financial distress to owners, who may opt for expensive but unsuccessful treatment or may be unable to afford treatment and so euthanize a potentially curable horse on humane grounds.

Currently, the causes of EPSCCs are poorly understood. As discussed briefly here and in greater detail in later sections, there is evidence to support PV infection and exposure to ultraviolet radiation as possible risk factors for EPSCC development.

Penile squamous papillomas and precancerous plaques are both PV-induced lesions that are recognized as potential precursors of EPSCCs. Squamous papillomas are

keratinized exophytic epithelial tumors with a sparse, branching fibrous stroma.[153] They occur on the male genitalia of many domestic species but are most common in horses,[83] in which they have been reported to contain PV antigens and are assumed to be PV-induced.[132] Approximately one third of equine male genital squamous papillomas are found in proximity to an EPSCC, suggesting that they make have the potential for neoplastic transformation.[83,119] Precancerous plaques are unique to horses and are intermediate in appearance between squamous papillomas and SCCs and cannot be grossly distinguished from either. Because they frequently progress to invasive SCC, they are also considered premalignant.[34,83,119] Like squamous papillomas, equine genital precancerous plaques are reported to contain PV DNA sequences.[132,277] Histologically, precancerous plaques consist of a hyperplastic to dysplastic epithelium, with or without koilocytes, in which cells of the basal layer exhibit an increased mitotic rate and loss of polarity but no invasion of the basement membrane.[119,277] While evidence suggests both squamous papillomas and precancerous plaques can progress to SCCs, the proportion of EPSCCs that develop from these and the proportion that develop *de novo* are currently unknown. More detail on PVs as a cause of EPSCCs is provided in section 1.5.2 *Papillomaviruses and SCCs in Horses.*

Evidence supporting ultraviolet (UV) radiation as a cause of EPSCCs includes the inconsistent observation that horse breeds with unpigmented genitalia (especially appaloosa and American paint) more frequently develop EPSCCs than do breeds with pigmented genitalia. As pigment protects against UV-induced cell damage, the predisposition to EPSCCs seen in unpigmented breeds suggests a role of UV-damage in disease development.[84,268] However, unlike SCCs in UV-exposed skin in other body regions, there is no evidence that EPSCCs develop from pre-existing areas of actinic keratosis (solar damage). The potential role of UV light in development of EPSCCs is discussed in more detail in section 1.7.1.2 *UV Exposure and SCCs in Horses*.

### 1.3.2 Equine Ocular or Adnexal SCCs (EOSCCs)

The eye (referring to the globe and its adnexa) is the most common site of SCC development in horses, and 43-57% of equine SCCs develop at this location.[232,245] Squamous cell carcinomas are the most common neoplasms of the eye and adnexa in

horses,[147,245] and the average age of horses with EOSCCs is around 10 years.[232,245] Females were affected more frequently than males in one study, [232] another study found the opposite[70] and a third found no difference,[137] suggesting that there is no strong gender predisposition to EOSCCs.

Equine ocular SCCs most commonly affect the nictitating membrane and medial canthus.[99,232] However, they also develop in the limbus, bulbar conjunctiva, and eyelid.[71,147] Unilateral disease is most common, but bilateral involvement has been reported in 11% to 20% of cases.[137,175,232] Clinical signs reflect anatomic location, with ocular discharge, ulceration, blepharospasm, and visual impairment being common.

The gross appearance of EOSCCs varies based on anatomic location.[99,115] Limbic SCCs are typically cauliflower-like pink masses, beginning at the lateral limbus and extending to the cornea. In contrast, SCCs arising on the cornea may appear as flattened opaque plaques that resemble scars. Nictitating membrane SCCs are typically pink or white, proliferative and ulcerative and generally arise at the free margin of the membrane. Eyelid SCCs have a less predictable appearance, ranging from non-ulcerated thickening of the lid to exophytic, ulcerated masses with abundant mucopurulent exudate. Regardless of location, EOSCCs may eventually progress to large, fleshy masses with variable ulceration, necrosis, and inflammation.[99] Histologically, EOSCCs have been subdivided into four basic types: carcinoma *in situ*, papillomatous, non-invasive and invasive.[206] Cellular features and desmoplasia seen in EOSCCs are typical for SCCs that develop at other body sites and do not need further description.

As with EPSCCs, EOSCCs are locally invasive but slow to metastasize.[36,50,100] The rate of metastasis is reported to range from 6-15%,[232] with metastasis reported in local lymph nodes, salivary glands, brain and lungs.[76,91,133] SCCs of the equine eyelid are considered to be more aggressive and to have a less favorable prognosis than other EOSCCs.[71,137]

Treatment options for EOSCCs include surgical excision, with or without adjunct therapy (including cryotherapy, hyperthermia, immunotherapy, chemotherapy, laser ablation, and radiation therapy).[100,137,175] Complete surgical excision of an invasive

ocular mass can be difficult to achieve and 25-67% of EOSCCs are reported to recur after treatment.[99,137,175] Local invasion by the tumor often causes ulceration, necrosis and inflammation; enucleation or euthanasia on humane grounds are frequent outcomes of EOSCC.[100,232]

Currently, the causes of EOSCCs are not known. Like EPSCCs, PV infection and UV light exposure are both plausible causes of neoplasia and so are introduced briefly here and discussed in detail later. DNA sequences from an unspecified PV were amplified from 45 of 57 EOSCCs but only 3 of 20 normal eyelids in one study, but this has so far not been pursued further.[136] Draft breeds (Belgian, Suffolk, Clydesdale and Percheron) and Appaloosas appear to be predisposed to EOSCC development, possibly due to a lack of protective ocular pigmentation in these breeds.[70,137,232] This is discussed further in section 1.7.1.2 *UV Exposure and SCCs in Horses*.

### 1.3.3 SCCs of the Oral Cavity and Pharynx

Horses rarely develop neoplasia of the oral cavity or pharynx. However, of neoplasms in these locations, SCCs are the most common type and around 5% of equine SCCs develop in the oral cavity or pharynx.[68,228] No breed or sex predilection has been identified, however this may be due to the small number of reported cases.[131,228] Oral or pharyngeal SCCs occur in older horses and the average age reported in two retrospective studies was 15 years.[131,228]

Equine oral or pharyngeal SCCs have been reported to develop on the tongue, gingiva, hard palate, pharynx, and larynx with clinical signs including dysphagia, ptyalism, halitosis, dyspnea, stridor, cough, and nasal discharge.[90,122,131,228,266,272,282] The neoplasms typically appear as ulcerated, multilobular, exophytic masses and often invade adjacent bony and lymphatic structures.[228] As with other equine SCCs, they tend to metastasize late in the course of the disease, initially to draining lymph nodes and then to the lungs.[228] Preneoplastic lesions for oral or pharyngeal SCCs are not described, likely because tumors are advanced before clinical signs are noticed and the oral cavity and pharynx examined.

Most SCCs of the equine oral cavity and pharynx are not detected until late in the clinical course. Therefore, many have bony involvement at the time of diagnosis and treatment is often not attempted due to the difficulty of surgical excision.[131,152,228] As most affected horses are euthanized at diagnosis[112,131] detailed data on survival times of horses with oral or pharyngeal SCCs are not reported.

### 1.3.4 SCCs of the Vulva and Vestibule

It is estimated that 5-12% of all equine SCCs affect the female external genitalia,[152] suggesting that genital SCCs are one half to one quarter as frequent in females as in males.[36,49] Squamous cell carcinomas are the most frequent neoplasms of the female external genitalia,[162,249] with the mean age of affected horses reported to be 12-19 years.[245,268] In one study, 4 of 7 mares with vulvovestibular SCCs were Pintos,[245] suggesting either a breed predisposition or a susceptibility to UV exposure in light-colored breeds, although numbers are insufficient to draw conclusions.

There is little published information describing the precursor lesions, gross appearance or behavior of equine female genital SCCs. The vulva is most commonly affected,[132] although SCCs of the clitoral fossa are also described.[245] One report describes development of an *in situ* vulvar SCC at the site of prior surgical excision of papillomaviral-induced vulvar papillomas.[238] Treatment options reported for female genital SCCs include surgical debulking and chemotherapy, [49,257] but detailed data on survival times of affected horses are not reported.

### 1.3.5 SCCs in Other Locations in Horses

SCCs of the haired skin are relatively uncommon in horses, although they have been reported to arise in lightly pigmented skin of the muzzle, lips, pinnae and perineum.[38,173,245] While most are thought to be caused by exposure to UV light, three SCCs were reported in sites of previous skin trauma, including an injection site abscess,[11] a neck laceration that had been treated topically with various chemicals for 18 months[80] and a non-healing thermal burn suffered 8 years previously.[230]

Gastric SCCs are the most common neoplasms of the equine gastrointestinal tract,[36,111] although only a small proportion of equine SCCs develop at this location. The mean age of horses with gastric SCCs is approximately 12 years.[50,200,256] The reported incidence of equine gastric SCCs is increasing, although it is not known whether this is due to a true increase in neoplasm frequency or due to improved diagnostic techniques.[111] No breed or sex predilections for gastric SCCs have been established. Most gastric SCCs originate in the proximal part of the stomach, which is lined by stratified squamous epithelium.[50,111] Clinical signs are often vague and can include anorexia, weight loss, fluid abdominal distension, dysphagia, ptyalism, choke, and regurgitation.[50,111,200,256] Most equine gastric SCCs form cauliflower-like, ulcerated masses that extend from the luminal surface and also infiltrate the deeper layers of the wall. Metastasis to draining gastric and esophageal lymph nodes is common.[111,256] Additionally, direct extension to the distal esophagus, and transcoelomic spread to the diaphragm, liver, spleen, greater omentum, and peritoneum are also common.[256] Non-specific clinical signs and the inability to visualize the neoplasm mean that, by the time of diagnosis, the overwhelming majority of equine gastric SCCs are advanced at diagnosis and are untreatable.[36,111,168]

Sinonasal tumors are rare in horses, but SCCs are the most common neoplasms in the region.[26,110,283] Sinonasal SCCs are rarely treated due to advanced disease at the time of diagnosis and difficulty of surgical access. Most affected horses are over 15 years old at diagnosis[283] but there is no reported breed or sex predilection. Although SCCs of the upper respiratory tract may originate in the paranasal sinuses, nasal cavity, nasopharynx or guttural pouch,[152,161] they most commonly arise in the caudal maxillary sinus.[26,110] In some cases, paranasal sinus SCCs have arisen in the oral cavity and spread to the maxillary sinuses.[50,110,112] Clinical signs include nasal discharge, dysphagia, facial swelling, anorexia, and dyspnea.[152] Neoplasms are typically ulcerated cauliflower-like masses covered with necrotic debris.[152] Local invasion may eventually affect the upper respiratory tract, digestive tract or central nervous system.[26,90,142] However, despite their invasive growth, equine sinonasal SCCs rarely metastasize.[66,110] Like SCCs of the oral cavity and pharynx, sinonasal SCCs are usually diagnosed after

invasion has occurred and surgical removal is difficult.[66,110,152] Most affected horses are euthanized.[26,66]

Although rare, SCCs are reported to be the most common primary bladder tumor of horses.[49,169,262] The majority of horses are older (12-23 years of age) and the most common clinical signs are hematuria and stranguria.[89] Treatment is difficult, although successful surgical resection was reported in one case.[234]

Additional sites of SCC development that have been reported in horses include the esophagus,[22,111] guttural pouch,[161,264] middle ear,[161] external ear canal,[242] and hoof wall.[18,72]

## 1.4 Papillomaviruses

The research in this thesis investigated the association between papillomaviruses (PVs) and SCCs in horses. In this section of the literature review an overview of PV biology is given. This is followed in section 1.5 by a discussion of the relationship between PVs and SCCs in various species.

### 1.4.1 Overview

Papillomaviruses are small, circular, non-enveloped, double-stranded DNA viruses that replicate in stratified squamous epithelium. They were formerly grouped with polyomaviruses into the family Papovaviridae, but are now recognized to form their own distinct family, Papillomaviridae. Important features of PVs include their epitheliotropism, their species specificity, their ability to establish chronic infection and stimulate abnormal cell growth, and their preferential infection of certain anatomic sites.

As PVs are DNA viruses they replicate in the cell nucleus. A PV viral particle (virion) is approximately 50 nm in diameter and consists of a single circular molecule of double-stranded DNA approximately 8000 base pairs long and contained within a spherical protein coat (capsid). The capsid consists of two virally coded structural proteins

named L1 and L2. The major capsid protein (L1) represents about 80% of the total viral protein.[121]

Papillomaviruses are considered to be highly host-specific, and examples of a PV from one species resulting in a productive infection in another are extremely rare.[121,180] Cross-species infection by bovine PV type 1 (BPV-1) and BPV-2 is believed to cause cutaneous mesenchymal neoplasms (sarcoids) in horses and is possible experimentally in laboratory rodents.[86,203] The unclassified PV associated with feline sarcoids is also likely of bovine origin.[183,185,201] However, in all these cases there is no evidence of BPV replication and these cross-species infections are considered dead ends in the abnormal host species.

### 1.4.2 Genome

The complete genomes of many human and non-human PVs have been sequenced, and the genomic organization of these viruses shown to be remarkably conserved. All open reading frames (ORFs) are located on one strand of the viral DNA, indicating that all genetic information is also located on this strand; the other viral DNA strand does not serve as a template for transcription. Up to 10 ORFS may be present, depending on the specific PV type. These are classified as either early (E) or late (L) ORFs based on their location in the genome. The early (E1 to E8) region of the PV genome encodes viral regulatory proteins, including those necessary for initiating viral replication. The early region is expressed in non-productively infected cells and in transformed cells.[113] The late (L1 and L2) region of the PV genome encodes capsid proteins and is only expressed in productively infected cells.[12] The PV genome also contains a regulatory region in which there are no ORFs. This is known as the long control region (LCR) or upstream regulatory region (URR).[121]

The functions of PV ORFs are complex but may be divided into four broad groups that are discussed briefly here and in more detail later. First, *E1* and *E2* are regulatory genes that are important regulators of viral DNA transcription and replication. *E1* is directly involved in DNA replication, whereas *E2* has an auxiliary role by greatly enhancing the ability of *E1* to initiate DNA replication.[160,212,239] Second, *E5*, *E6*, and *E7*

are oncogenes that are involved in disruption of cell cycle regulation.[160,188] As these three genes are important in the development of cancer they are discussed in detail later, in section 1.5.1.1 *Oncogenic Mechanisms of PVs*. Third, *L1* and *L2* are structural genes that code for the viral capsid.[12] Finally, *E3*, *E4* and *E8*, when present, code for proteins of unknown or less well understood function.[116,192] The E4 protein may sustain cells that are amplifying viral DNA in the S phase of the cell cycle[58] and may assist virion escape from cells by weakening structural proteins.[35,67] Little is known about the functions of the E3 and E8 proteins.

### 1.4.3 Classification

The position, size, and function of the E1, E2, L1, and L2 ORFs are well conserved among all PV types. However, as the L1 ORF is the most highly conserved it is used for the classification of PVs. Within the family Papillomaviridae, PVs of the same genus share greater than 60% nucleotide sequence homology in the L1 ORF. Types share between 70% and 90% nucleotide homology. A subtype has 90-98% homology and a variant greater than 98% homology.[62]

PV genera are named using the Greek alphabet (for example alphaPV and betaPV. With the family Papillomaviridae now containing more genera than there are letters in the Greek alphabet, the prefix “dyo” is added and the alphabet restarted, continuing after the omegaPVs with dyodeltaPVs.[17]

In 2010, the 29 known PV genera contained 189 fully sequenced and classified PV types. This consisted of 120 human PV types (contained within 5 genera), 64 from non-human mammals (within 20 genera), 3 from birds (in 3 genera), and 2 from reptiles (in 1 genus).[17] By 2013, known PV genera had increased to 35, and PV types to 282, with 170 from humans and 112 from non-human species.[61,214] Given the speed with which new PV types have been described, many new types will inevitably continue to be identified. The large number of human PV types suggests that non-human species are likely to be infected with similar numbers, implying that only a small fraction of all non-human PV types are currently recognized.[62]

In addition to dividing PVs into genera, PVs may be also grouped by the species they infect, by their ability to cause cancer, or by the mucosal surface they infect. First, PVs may be named according their host species, and numbered sequentially (e.g., *Bos taurus* PV type 1 (BPV-1), BPV-2 and so on). Second, certain human PVs are associated with malignant progression from benign papillomatous proliferations. These PVs are known as "high-risk" PVs. Finally, most PVs are anatomically site-specific and so can be broadly divided into "cutaneous-adapted" and "mucosal-adapted" types.

### 1.4.4 Life cycle and replication

PVs have a specific tropism for stratified squamous epithelia, which they infect through microabrasions or wounds. Their life cycle and replication are closely linked with the differentiation sequence of epithelial cells. Because the basal cell is the only cell in a stratified squamous epithelium capable of dividing, PVs must infect basal cells to induce a persistent infection.[227,244]

The replication of PVs can be divided into three phases (figure 1.1). In the first phase, after entry to the basal cell and transportation to the nucleus, the viral genome is amplified to around 10-50 copies per cell.[45] Little is known of this process. The second phase is one of genome maintenance. Papillomaviral DNA is maintained in the nucleus of the dividing basal cells as circular extrachromosomal elements (plasmids) that are replicated at the same time as host cellular DNA during normal cell division. This results in the presence of PV plasmids in each daughter cell and indefinite maintenance of the PV genome.[150] This is controlled by the early (E) genes, particularly E1 and E2.[160,212,239] The third phase is one of vegetative DNA amplification, in which capsid protein synthesis and virion assembly occur in the upper, terminally differentiated cells of the epithelium. As the keratinocyte progresses toward terminal differentiation an induction of viral DNA replication occurs, with an increase in the viral genome from 10-50 copies per cell to around 10,000 copies per cell.[265] Keratinocytes containing assembled virions are destined to be sloughed as squames from the epithelial surface, with infective PV particles released as the squame degrades. The absence of PV-induced cell lysis minimizes the biologic cost to the host and reduces the inflammatory response to viral infection.

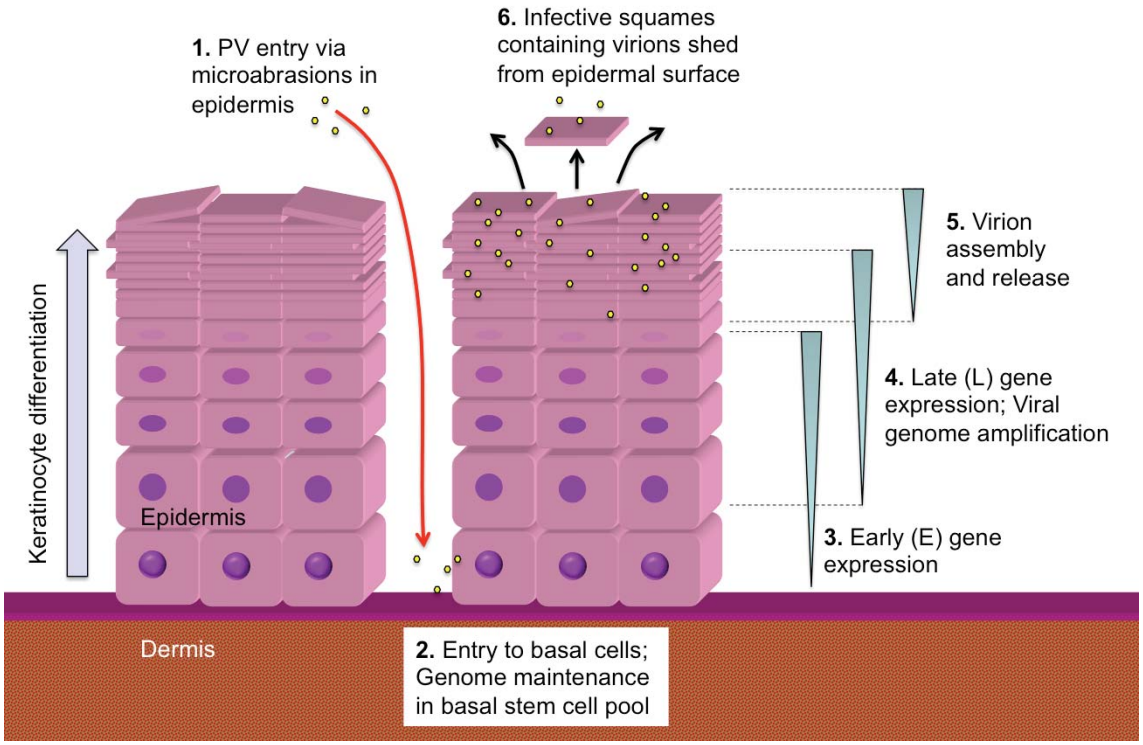


*Figure 1.1. Schematic diagram of papillomaviral life cycle within the epidermis. Refer to section 1.4.4 Life Cycle and Replication for details. PV, papillomavirus. (Figure modified from Moody et al.: Human papillomavirus oncoproteins: pathways to transformation. Nature Reviews Cancer 10: 550-560, 2010)*

The strategy of minimizing the host response by restricting viral DNA amplification and the synthesis of large amounts of viral antigens to the superficial layers of an epithelium enables long-term persistent PV infection.[160,240] However, suprabasal cells are post-mitotic, resulting in loss of the cellular enzymes necessary for PV DNA replication. To prevent loss of this essential cellular machinery, PVs disrupt the normal epithelial differentiation process and maintain epidermal cells in an S phase-like state of the cell cycle. This is largely achieved by expression of the PV E5, E6 and E7 genes. As these genes disrupt normal cell cycle regulation they also have the potential to promote neoplastic transformation (discussed later in section 1.5.1.1 *Oncogenic mechanisms of PVs*).[160]

*In vitro*, PVs are difficult to propagate because their replication is dependent on differentiation and maturation within a stratified epithelium, which is not mimicked by monolayer cell cultures that are commonly used.[150] Techniques producing a multilayer epithelium resembling a stratified squamous epithelium have been developed, but they are not practical for molecular or biochemical analysis of virus replication.[120]

While monolayer cultures do not permit complete viral replication they can be useful to study certain aspects of the PV life cycle, including non-structural viral genes and viral DNA replication.[247]

### 1.4.5 Diagnosis of PV Infection

When determining whether a lesion is caused by a PV, a combination of microscopic examination and molecular testing is often the most accurate approach. These techniques include light and electron microscopy, immunohistochemistry (IHC), polymerase chain reaction (PCR), and *in situ* hybridization (ISH).

Light microscopy can be used to detect the histologic changes that are induced by some PV infections. These changes include thickening and folding of an epithelium, elongated dermoepidermal interdigitations, and cellular dysplasia. Keratinocytes, especially of the stratum spinosum, may show viral cytopathic effects, which generally take two forms. First, keratinocytes may be swollen, with abundant pale grey-blue cytoplasm or, second, they may have dark, pyknotic nuclei and clear perinuclear haloes (koilocytosis; fig 3.2 in chapter 3). Amphophilic to eosinophilic intranuclear viral inclusion bodies may be present, but are not consistently found.[109] Electron microscopy can be used to demonstrate viral particles. However, these will only be present when productive viral infection is present and the presence of viral particles may not be consistently distributed within a lesion.[150]

Immunohistochemistry relies on the development of antibodies against viral or host proteins. The PV protein most commonly targeted by IHC is the L1 capsid protein. However, L1 proteins are only produced during productive infections, when viral capsids are assembled. Therefore, a disadvantage of using IHC is that PV infections being maintained within the basal stem cell pool will not be detected by IHC. Another disadvantage of using IHC to detect PVs is the uncertainty of cross-reactivity between the antibody and a PV of a different genus or type than the PV of interest.

Rather than detecting PV proteins, IHC may instead be used to detect certain host cellular proteins whose levels are altered by PV infection. Immunostaining of these

host cellular proteins can be used as an indirect marker of PV infection or activity within a lesion (discussed in section 1.6.2 *Immunohistochemical Evidence of PV Oncogenic Activity Within Lesions*).

Polymerase chain reaction amplifies segments of PV DNA from a lesion. This technique has the advantage of being highly sensitive and specific, and able to detect fewer than 10 copies of a viral genome, depending on the technique used. Several different PCR techniques exist, and those used in this thesis are briefly discussed here. The first method uses degenerate consensus primers capable of amplifying a segment of the highly conserved PV *L1* gene from a large number of different PV types. Amplicons may then be sequenced and the exact PV type(s) within a lesion identified. The major advantage of this method is that it is capable of detecting a wide variety of PV types in a single amplification reaction. For this reason it is useful as a screening test when the types of PV present in a lesion are unknown. Disadvantages of consensus primers include the requirement to sequence PCR products and the fact that a PV type with low primer affinity may be obscured by a PV type with greater primer affinity. A second PCR technique involves designing specific primers that amplify only one type of PV. The advantage of this is that only the PV of interest is amplified, making this method more sensitive than when consensus primers are used. In addition, using multiple specific primers allows multiple PV types to be amplified from a single lesion. Another advantage is that primers need not be targeted only at segments of the *L1* gene, and primers that amplify other genes of interest, for example *E1* or *E6*, may be used. Furthermore, sequencing of the PCR products is not required when using specific PCR primers. A third PCR method is quantitative PCR (qPCR), which allows the number of viral copies to be determined and expressed either in terms of copies per host cell or copies per nanogram of host template DNA. This is useful as it is increasingly recognized that viral load rather than simple PV presence correlates with risk for malignant progression.[287]

A disadvantage of PCR is that the location of PV DNA within a lesion cannot be determined, making the significance of its presence uncertain. *In situ* hybridization (ISH) may be used instead of PCR to both detect PV DNA and demonstrate its location

within a lesion. Demonstrating that PV DNA is located within lesional cells but not in adjacent normal cells can be important when investigating the role of the PV in lesion development. However, due to a lack of an amplification stage, ISH has the disadvantage of being less sensitive than PCR. Additionally, the PV type cannot be determined by ISH if consensus PV probes are used.

### 1.4.6 Host Response to PV Infection

Healthy humans and non-human species are generally infected by a large number of PV types with no clinical disease or immune response.[5,6] PVs evade recognition or minimize the immune response by the host by infecting keratinocytes, which are poor antigen presenting cells.[265] Additionally, PVs are not lytic, so cell death (and, hence, inflammation) is not induced.[240] Furthermore, capsid proteins are only produced in superficial layers of epithelium and are thus distant from normal immune surveillance. Finally, there is no viremic stage of infection and therefore little opportunity for systemic antigen presentation.

Nevertheless, the host's immune response plays an important role in modulating the severity of PV-associated disease, and most PV infections are transient and resolve within several months of infection. T-cell (cell-mediated) immune response is most important for controlling the size and duration of lesions induced by PVs after infection, and resolution is considered to be due to this T cell response.[150,240] The onset of this response is highly variable, with marked variation among individuals in the time taken to resolve a PV infection.[240] Delayed development of an adequate T-cell response can result in PV lesion persistence, florid infection or neoplastic transformation.[174,194]

B-cell (humoral) immune response to PV capsid proteins is also important. Despite the low immunogenicity of PVs, most infections do result in the development of small quantities of circulating antibodies.[93,240] While these do not influence lesion resolution, they can help prevent subsequent reinfection by that particular PV type.[93,150,194,240] As immune protection is PV type-specific these antibodies do not offer any protection from infection with other PV types.[130,240,258]

Considerable variation in lesion duration, progression, host response and rate of regression is seen. This variation has been studied in both naturally and experimentally infected dogs and rabbits and may be linked to individual variation in MHC-II haplotype or antigen presentation.[33,48,108,223]

### 1.4.7 Vaccination

Animals that recover from papillomas are immune to reinfection with that PV type,[93,150] and the ability of prophylactic vaccination to prevent PV infection was first reported in 1937.[235] However, the inability to produce large amounts of PV in the laboratory, the large number of PV types, and the type-specificity of the immune response have made production of a PV vaccine that protects against more than one or two PV types challenging.

Previously, PV vaccines have been made from homogenized wart extracts or purified virus and inoculated into non-epithelial sites. Heterogenous PV vaccines, using homogenized wart extract from another individual, have been shown to effectively prevent wart formation in cattle, dogs and rabbits.[195] Both "live" and formalin-inactivated wart extract are effective in dogs.[15,44] However, the former has been occasionally associated with development of SCC and other tumors at the injection site.[31] Purified PV virions, rather than wart suspension, have been shown to be effective prophylactic vaccines against infection with the same PV type in calves.[129,130] While these types of vaccine may be useful for preventing warts, they are not suitable for preventing cancer. This is because injecting an oncogenic virus into a healthy individual carries the risk of causing cancer, rather than preventing it. For this reason live or inactivated PV vaccines are not used for cancer prevention.

The discovery of virus like particles (VLPs) was a major advance in the development of prophylactic vaccines against PV infections that cause cancer. VLPs are empty PV capsids, structurally identical to PV virions, but without the viral DNA core. They are produced when recombinant non-PV viral vectors expressing PV capsid genes are inoculated into cultured cell lines. The infected cells express PV capsid proteins, which

self-assemble into VLPs that are antigenically almost identical to native virions.[167] VLP technology is now used to produce L1 VLP subunit vaccines that prevent infection with the high-risk PVs associated with human cervical cancer. Currently, no L1 VLP subunit vaccines are used routinely in animals. However, animal models were used to demonstrate the efficacy of VLPs as models for human HPV vaccine development. Systemic immunization with L1 VLPs conferred high protection against experimental challenge with the native virus when tested by infecting the skin with *Sylvilagus floridanus* PV (SfPV; formerly cottontail rabbit PV)[32,46] or the oral mucosa with either *Canis familiaris* PV type 1 (CPV-1; formerly canine oral papillomavirus)[241,252] or BPV-4.[138] The use of CPV-1 L1 VLP subunit vaccination to treat, rather than prevent, canine oral papillomatosis has also been investigated. In one report, the use of a CPV-1 L1 VLP subunit vaccine did not result in lesion resolution in a dog, and the animal was subsequently euthanized.[194] In contrast, another report describes lesion resolution shortly after CPV-1 L1 VLP subunit vaccination.[145] However, since these lesions almost invariably spontaneously resolve, determining whether or not the vaccine was the cause of the resolution is difficult.

Historically, some studies have suggested that autogenous vaccines, prepared by injection of homogenized PV lesion into the original patient, could cause lesion resolution. This has been investigated in humans, horses, cattle, dogs, goats, parrots and rabbits, with mixed results.[77,157,195,210,251,284] In reports where lesions have resolved following autogenous vaccination it is not possible to prove that lesions would not have resolved spontaneously, and the use of autogenous vaccination has not been supported by any adequately controlled study.

Injection of DNA plasmids coding for capsid proteins or other PV genes (DNA vaccination) into patients is a novel approach to vaccination. It has been shown to prevent SfPV infection in rabbits[248] and CPV-1 infection dogs.[195] In addition, DNA vaccines could potentially be used for therapeutic, as well as prophylactic vaccination. This is possible because they stimulate an immune response against PV E6 and E7 antigens, and therefore target any PV-infected cell, even those in which viral integration into the host genome has occurred.[172] This is in contrast to VLP-based PV

vaccines, which target the PV L1 capsid proteins and thus require viral replication to occur in order to be effective.[118]

Overall, there are currently no therapeutic vaccinations for people or animals already infected by PVs, although several approaches are under investigation.[124,172] In humans, vaccines must be administered prophylactically, prior to first exposure to a particular PV as they have no therapeutic value after infection.[118] The same is likely to be true for animals.

## 1.5 Papillomaviruses and SCCs

In this section, the evidence that PVs cause SCCs in humans and non-human species is reviewed. Humans are discussed first because the epidemiology of infection and oncogenic mechanisms have been the focus of intense research in our species. Human PV-induced SCCs illustrate many of the methods that were used to investigate equine SCCs in this thesis.

While some research suggests an association between PVs and certain types of SCCs in some non-human species, definitive evidence supporting a causative association between PVs and SCCs is lacking for most. In this literature review, the associations that have been identified between PV infection and SCCs in non-human species will be discussed, beginning with horses and ending with other domestic and non-domestic mammals.

### 1.5.1 Papillomaviruses and SCCs in Humans

It is estimated that nearly 18% of human cancers globally in 2002 were attributable to infectious agents.[204] Of these, nearly one third were due to HPV infection.[204]

Cervical cancer is the second most common cause of cancer-related death in women globally.[288,295] A connection between HPV infection and cervical SCCs in women was first proposed in the 1970s and established unequivocally in the 1990s.[293,295] It is now known that nearly 100% of cervical cancers are associated with HPV infection. The

high-risk alphaPVs, especially HPV-16 and HPV-18, are considered the primary cause of human cervical SCCs, making these HPVs among the most important human carcinogens.[24,295]

Although cervical cancer is the most thoroughly studied form of HPV-associated SCC, infection with high-risk alphaPVs has also been associated with a proportion of vulvar, vaginal, penile, anal, perianal, oral and pharyngeal SCCs. The rate of diagnosis of these non-cervical SCCs has been increasing and, in the USA, the number of HPV-associated non-cervical SCCs diagnosed annually now roughly equals the number of cervical cancers.[95] The HPV-induced non-cervical SCCs are evenly split among men and women.[95]

Approximately 50% of vulvar SCCs,[155] 30-50% of penile SCCs,[220] and 60-90% of vaginal[155] and anal[87] SCCs (collectively called anogenital cancers) are currently thought to be caused by high-risk alphaPV infection. The causes of the HPV-negative human anogenital cancers are not known. Since the prognosis for HPV-positive vulvar and penile SCCs is better than that for HPV-negative SCCs,[149] it is important to determine whether or not a genital SCC is HPV-induced.

One third to one quarter of SCCs of the oral cavity, pharynx and tonsils (grouped together as head and neck SCCs or HNSCCs) are caused by the same high-risk alphaPV types that cause cervical cancers.[97,144,295] The remainder of HNSCCs are thought to be caused by tobacco or alcohol. Similar to penile and vulvar SCCs, the prognosis for disease-free survival in HPV-induced HNSCCs is much more favorable than for HNSCCs caused by tobacco or alcohol.[96] In fact, the behavior of these two subsets of HNSCCs is so different that HPV-induced HNSCCs are considered to be a distinct molecular, clinical, and pathologic disease entity from HNSCCs caused by tobacco or alcohol exposure.[96]

While the alphaPVs are generally well accepted to be important causes of genital and HNSCCs, whether HPVs also cause human cutaneous SCCs remains uncertain. In contrast to other HPV-associated SCCs, cutaneous neoplasms do not contain alphaPV

DNA. Instead they typically contain evidence of infection by betaPVs. Evidence supporting a role for betaPVs in the development of skin cancer is provided by two groups of immunosuppressed patients: epidermodysplasia verruciformis (EV) sufferers and transplant recipients. Epidermodysplasia verruciformis is a rare, heritable condition that prevents individuals clearing or controlling infection with betaPVs.[202] People with EV develop multiple cutaneous viral plaques in childhood, particularly at sun-exposed sites. Some of these progress to SCCs after several decades of persistence, providing evidence for a role of PVs in development of SCCs. Chronically immunosuppressed solid organ transplant recipients have a higher rate of cutaneous PV infection, wart formation and SCC development than immunocompetent people,[27,79] although it cannot be proven that the PV infection in these patients causes the increased risk for SCC.

While observations of immunosuppressed people suggest a possible role of betaPVs in SCC development, their role in cutaneous SCCs in immunocompetent patients is harder to determine. Studies of cutaneous SCCs from immunocompetent people have revealed HPV DNA in a higher proportion than in normal skin. However, numerous betaPV types are commensal inhabitants of healthy skin,[5] making the significance of their detection in tumors difficult to interpret. It is possible that the higher rates of PV detection in the SCCs are simply due to a more permissive microenvironment within a neoplasm favoring persistence or proliferation, rather than being evidence of causation. In addition, skin is exposed to solar UV radiation, which is an established cause of genetic mutation and skin cancer, and controlling for this confounding variable in epidemiologic studies is difficult.[8] Most cutaneous SCCs in immunocompetent people begin as actinic (solar-induced) keratoses rather than as viral plaques, which suggests that UV radiation has a greater role than PVs in cutaneous SCC development.[171] It is even possible that that sunlight and certain PVs have a syncarcinogenic effect, with tumor suppressor gene mutations induced by UV radiation[59] preventing apoptosis of PV-infected cells, allowing unchecked expression of papillomaviral oncoproteins.[246]

#### *1.5.1.1 Oncogenic Mechanisms of PVs*

PV infections are frequent, and the overwhelming majority of them are asymptomatic. For this reason, the detection of a PV within a SCC could be evidence that the PV caused the SCC or could simply be due to coincidental infection of the neoplasm by the PV. When determining whether or not PVs cause neoplasia an important piece of evidence is whether or not mechanisms by which the PV can cause neoplasia can be demonstrated. The mechanisms by which HPV-16 and HPV-18 cause cancer have been extensively studied and are well established.[166,288] These high-risk alphaPVs are thought to cause cancer through expression of the PV oncogenes *E6*, *E7* and, to a lesser extent, *E5*.[288,294] The PV E6 and E7 proteins have numerous effects,[165,166,288] but those most relevant to this thesis are the inactivation of the tumor suppressor proteins p53 and pRb, discussed later this section.

It is important to recognize that the PV *E6* and *E7* oncogenes are *normally* expressed during any type of PV infection (both low-risk and high-risk PVs). They disrupt the epithelial differentiation process and maintain epithelial cells in an S phase-like state; this is a normal part of the PV life cycle and does not necessarily result in gross lesions. However, PV DNA may also become integrated accidentally into the host genome, typically in such a way that the PV *E2* gene is disrupted. This releases the viral promoters of the PV oncogenes *E6* and *E7* and leads to overexpression of the E6 and E7 proteins.[54,73] Integration of HPV DNA into the host genome is seen in many HPV-associated cancers, and the HPV *E6* and *E7* oncogenes are consistently expressed at higher levels in HPV-infected malignant neoplasms.[294] In addition, their efficiency at transforming cells is increased when they are expressed together.[163,189]

The PV E6 oncoprotein complexes with the tumor suppressor protein p53, inducing its rapid degradation through ubiquitin-dependent proteolysis.[224] In normal cells, expression of the p53 gene is upregulated in response to any cellular stresses that threaten genomic stability. The p53 protein then regulates the transcription of several downstream genes that are involved in cell cycle arrest, DNA repair, and apoptosis. Loss of p53 protein due to E6-mediated degradation permits cells with DNA damage to advance, unchecked, through the cell cycle.[121,294] In addition to p53 degradation, E6

also degrades the pro-apoptotic protein BAK, which results in resistance to apoptosis and subsequent chromosomal instability.[127] Furthermore, E6 activates telomerase[140,276] and inhibits degradation of SRC-family tyrosine kinases,[199] both promoting cell division. Finally, HPV E6 binds to the XRCC1 protein (required for the repair of DNA single-strand breaks and genetic stability), promoting genetic instability of infected cells.[126]

The PV E7 oncoprotein inactivates the retinoblastoma tumor suppressor protein (pRb) through its sequestration[74] or degradation.[29] In normal non-dividing cells, pRb complexes with the members of the E2F transcription factor family, repressing their activity and preventing cell cycling. E7-mediated inactivation of pRb releases E2F and permits transcriptional activation of the genes involved in cellular DNA synthesis, thereby promoting cell cycle progression.[74] In addition, E7 stimulates production of the regulatory molecules cyclins E and A, and blocks functions of the cyclin-dependent kinase inhibitors p21 and p27.[88,289] These actions promote progression through the cell cycle by uncoupling cyclin-dependent kinases from their inhibitors.[25] Papillomaviral E6 and E7 proteins additionally cooperate to induce centrosome duplication and genomic instability.[69]

The PV E5 protein is important in the early course of infection and stimulates cell growth by forming complexes with certain cell receptors involved in proliferation, including epidermal growth factor receptor, platelet-derived growth factor beta receptor, and colony-stimulating factor-1 receptor.[121,125] It may also prevent cellular apoptosis following DNA damage.[291] However, as HPV-induced lesions progress to invasive cancer, the E5 coding sequence is frequently deleted.[231] Thus, E5 is not necessary for HPV-induced carcinogenesis, although it may participate in its initiation.[121] Interestingly, the E5 gene is highly conserved among the PVs that induce fibropapillomas in ungulates and sarcoids in horses. The E5 gene of these PVs is believed to be responsible for the proliferation of dermal fibroblasts in fibropapillomas[121,188] and equine sarcoids.[42]

### 1.5.2 Papillomaviruses and SCCs in Horses

In 2009, when I started this research project, bovine PVs were established as a cause of sarcoids in horses.[43,203] However, at that time, only one equine PV (*Equus caballus* PV type 1, or EcPV-1) had been fully classified. EcPV-1 causes self-limiting warts on the muzzle, face and limbs of predominantly young horses.[209,233] A second, unknown PV was suspected to cause equine aural plaques (ear warts).[78,209] Squamous papillomas of the penis and vulva had been described but the causative virus was unknown.[34,83,119] While some preliminary studies had investigated equine SCCs for a possible association with PVs, in 2009 there was no good evidence that equine PVs could cause neoplasia.

I decided to investigate a potential involvement of PVs in equine genital SCCs for three reasons. First, squamous papillomas of the equine penis and vulva, which are assumed to be PV-induced, do not always resolve spontaneously and can progress to invasive SCCs.[34,83,119] This could suggest that equine genital SCCs arising from squamous papillomas could are caused by a PV infection. Second, UV light, believed to be the cause of equine cutaneous and periocular SCCs, seemed a less plausible cause for EPSCCs given the UV-protected position of the penis. Third, by 2009 there was increasing recognition of an association between PV infection and SCCs in other non-human species (section 1.5.3).

The following paragraphs summarize the investigation into PVs and equine SCCs that took place prior to the start of my research in 2009. The increasing evidence for the existence of a mucosal equine PV distinct from those causing skin or ear warts is also discussed. It should be noted that during the course of my own research reports of an association between PVs and SCCs in horses began to be published by other groups. As these reports had similar methods and results and were reported around the same time as the studies in this thesis, they are discussed in the relevant chapters in the thesis rather than in the section below.

1. In 1984 PV antigens were detected by IHC in 12 of 45 equine papillomas (7 cutaneous and 5 genital), but in 0 of 90 SCCs from all predilection sites (including 23 male and 11 female genital).[132] As previously discussed, PV-

induced neoplasms in people rarely contain productive PV infections so the failure to detect PV antigen in these equine SCCs does not exclude a causative association. In another study in the same year PV antigens were also not detected by IHC in any of 32 equine SCCs (3 cutaneous, 1 oral, 1 penile, 2 vulvovaginal and 25 ocular), although they were detected in 11 of 35 papillomas (7/16 cutaneous, 2/12 penile, 2/2 vulvovaginal and 0/5 ocular).[250]

2. In 1986 the PV in equine cutaneous papillomas was characterized using Southern blot hybridization, IHC and electron microscopy.[198] In the study PV-specific sequences were also demonstrated within two penile papillomas. These had a different restriction pattern to the PV found in cutaneous papillomas (termed EqPV at the time) and hybridized to the EqPV probe only under non-stringent conditions. This suggested that penile papillomas contained a PV distinct from that in cutaneous papillomas.
3. In 2004 the complete genome of EqPV was sequenced from cutaneous papillomas, and the virus renamed EcPV-1.[94] The authors speculated that, based on the earlier studies above, a second mucosotropic equine PV existed.
4. In 2007 PCR was used for the first time to investigate PVs in equine papillomas.[209] When using primers designed to amplify EcPV-1 DNA sequences, 3 of 6 genital papillomas produced a weak band of the expected size. However, nucleotide sequencing of the PCR products and a BLAST search did not yield any matches. This again suggested that the PV infecting the genitalia of horses was distinct from that causing papillomas in haired skin.
5. In 2009 ten penile and three vulvar precancerous plaques (precursors of equine genital SCCs) were shown by PCR to contain PV DNA sequences.[277] These sequences were not characterized further. In the same year a case of vulvar carcinoma *in situ* arising within squamous papillomas was reported.[238] PV antigen was demonstrated within the papillomatous lesions by IHC, but the PV was not investigated further.
6. In 2009 equine periocular SCCs were investigated for the presence of PVs.[136] A novel PV sequence was amplified by PCR from 45 of 57 ocular SCCs but from only 3 of 20 normal eyelids. The amplified DNA sequence was most similar to

human PV type 21 but its full genome was never sequenced. No further investigation into this PV has been reported since.

In summary, at the time that research for this PhD was started, there was strong evidence that a mucosotropic strain of an equine PV existed, and that it was distinct from the PV that causes typical cutaneous papillomas. Using a variety of techniques, unknown PV types had been detected in genital papillomas and precancerous plaques, a single case of vulvar carcinoma *in situ* and a proportion of ocular SCCs.

### 1.5.3 Papillomaviruses and SCCs in Other Species

In cats, viral plaques are uncommon cutaneous lesions that are believed to be caused by *Felis catus* PV type 2 (FcaPV-2).[187] A small proportion of these may progress to Bowenoid *in situ* carcinomas (BISCs), then invasive SCCs,[104,179,281] and both these neoplasms have been reported to contain FcaPV-2 DNA.[178,179,182] In a study of feline cutaneous SCCs, FcaPV-2 DNA and increased cyclin-dependent kinase inhibitor p16 (p16) immunostaining were detected significantly more frequently in the SCCs from UV-protected skin than in those from UV-exposed skin.[179] This suggested that the cause of the SCCs in UV-protected skin could be FcaPV-2. In another study of feline cutaneous SCCs, lesions that contained PV DNA more frequently had reduced pRb immunostaining and increased p16 immunostaining, suggesting that FcaPV-2 could induce SCC development through inactivation of pRb. These results all support an association between the presence of FcaPV-2 and cutaneous SCCs, and also suggest an oncogenic mechanism for this virus.

Oral SCCs in cats have also been investigated due to the strong association between human HNSCCs and HPV infection. However no association between feline oral SCCs and PV infection has been shown.[4,181,184]

In dogs, reports of an association between PV infection and development of SCCs are relatively rare. PV-induced cutaneous pigmented viral plaques have occasionally been reported to progress to *in situ*, and invasive, SCCs.[37,186,243] Transformation of PV-induced cutaneous papillomas to SCCs was reported in 2006 in 4 of 24 bone marrow-

transplanted severe combined immunodeficiency dogs; 3 of these developed metastatic SCCs, an extremely rare outcome in dogs.[102] Administration of live CPV-1 vaccine was reported in 1987 to result in invasive cutaneous SCCs at the injection sites in 9 of 4500 dogs.[31] Overall, even though a single SCC has been reported adjacent to an area of oral papillomatosis and PV DNA sequences have been occasionally detected in canine oral SCCs there is currently little evidence to suggest that CPV-1 causes anything other than self-resolving and well-recognized oral papillomas.[255,279]

In cattle grazed on bracken-infested pasture, papillomas of the digestive tract caused by BPV-4 infection have been reported to progress to SCCs.[39,128] However, mutagens within bracken are believed to be necessary co-factors for cancer development and BPVs alone are not sufficient to cause SCCs.[23,39] A possible association between BPV infection and ocular SCCs has also been investigated. Using various hybridization assays and electron microscopy, BPV DNA or particles were not detected in any of 23 bovine ocular SCC-derived cell lines.[221]

In sheep, PVs have been detected in perineal and periocular SCCs.[2,259,275] PVs have also been detected in precancerous lesions of the pinna.[263] The significance of detection of PVs in SCCs and precancerous lesions typically attributed to sun exposure is not clear, as has already been discussed in section 1.5.1 *Papillomaviruses and SCCs in humans*.

PVs have been associated with *in situ* or invasive cutaneous SCCs in rabbits,[33] western barred bandicoots,[286] multimammate mice,[253] and ferrets.[218] PVs have also been associated with basosquamous carcinoma of the skin in bats[164] and invasive cervical SCCs in cynomolgus macaques.[285]

## 1.6 Evidence Supporting a Causative Association between PVs and SCCs

A PV infection within a lesion can be detected using various methods, including light and electron microscopy, PCR, ISH and IHC. However, the simple detection of a PV within a SCC does not prove that the PV influenced neoplasm development.

Historically, fulfillment of Koch's postulates was used to establish a standard for evidence of causation in infectious disease. As originally written, these postulates require that: (1) a microbe occurs in every instance of the disease in question; (2) the microbe does not occur in any other disease and; (3) after being fully isolated from the body and repeatedly grown in pure culture, the microbe can induce the disease anew.

Fulfilling Koch's postulates regarding the relationship between PVs and neoplasia is not possible for five reasons. First, integration of PV DNA into the host genome, and increasing dysplasia within a neoplasm decreases the ability of a PV to replicate. If no viral replication is present, the PV cannot be detected using histology, IHC, or electron microscopy. Therefore, a PV may not be detectible in a neoplasm even if that neoplasm had been initiated by PV infection.[81] Second, PVs are often present commensally in healthy tissues or in tissues affected by unrelated disease. Third, PVs cannot be grown successfully in culture. Fourth, the interval between infection by a PV and the appearance of lesions can be very long, up to several decades. Finally, as integration of PV DNA into the host DNA is considered a chance event, only a subset of individuals infected by a PV will subsequently development neoplasia.

However, despite these challenges, it is now confidently stated that nearly 100% of human cervical SCCs and a proportion of anogenital and HNSCCs are caused by high-risk alphaPVs.[293] The following sections summarize the different types of evidence and methods that, in combination, may be used to prove that PVs can cause cancer. This section is included because several of these methods were used for the research in this thesis.

### 1.6.1 Evidence Supporting a Causative Association between PVs and SCCs: Detection of PV DNA in Lesions

The emergence of nucleic acid amplification techniques such as PCR and *in situ* hybridization has permitted sensitive detection of microbial DNA sequences in tissues, even when target molecules are present in extremely low numbers. However, the sensitivity of these techniques has presented a new problem: how to interpret the significance of a small quantity of microbial DNA within a tissue.

For example, HPV DNA is detectible in more than 95% of human cervical cancers.[293] However, without further information it cannot be determined whether HPV caused the cervical cancer, is an irrelevant commensal, or is present as changes occurring within the neoplastic cells permitted an opportunistic infection.

Given the degree to which DNA sequence-based microbial detection methods are used today, and the frequency with which questions of causality arise, Koch's original postulates have been modified. The following molecular guidelines for establishing microbial causality have been proposed to accommodate both modern technology and the improved understanding of disease pathogenesis:[85]

1. A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease. Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased (i.e., with anatomic, histologic, chemical, or clinical evidence of disease) and not in those organs that lack lesions.
2. Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease.
3. With resolution of disease (for example, with clinically effective treatment), the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur.
4. When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship.
5. The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms. When lesions (e.g., pathology, microbial morphology, and clinical features) are predicted by sequence-based phylogenetic relationships, the meaningfulness of the sequence is enhanced.
6. Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific *in situ* hybridization of microbial sequences to

areas of tissue lesions, to visible microorganisms, or to areas where microorganisms are presumed to be located.

7. These sequence-based forms of evidence for microbial causation should be reproducible.

The authors of these guidelines note that strict adherence to every one of these guidelines may not be required to establish causation. They emphasize the importance of relative numbers (not simply presence or absence) of sequence copies and the biologic plausibility of the microorganism in question causing disease. It is interesting to note that all seven criteria cannot be fulfilled in many recently defined human diseases including cervical SCCs, Whipple's disease, Kaposi's sarcoma, hepatitis C and hantavirus pulmonary syndrome. In the experimental chapters of this research thesis I have followed as many of these guidelines as practical when investigating the connection between PVs and equine SCCs.

### 1.6.2 Evidence Supporting a Causative Association between PVs and SCCs: Immunohistochemical Evidence of PV Oncogenic Activity Within Lesions

As viral replication is rarely present within invasive SCCs, immunohistochemical detection of PV capsid antigens is of limited value when investigating the role of PVs in cancer. However, IHC can be useful to investigate a PV etiology by studying the effects of viral gene expression on host cell proteins. Papillomaviral oncogene expression can change the presence of certain host proteins either by mediating their destruction or upregulating their expression. Therefore, immunostaining for host proteins may be used as indirect markers of PV oncogene expression within a lesion. Immunostaining for pRb, p16, and p53 are most frequently investigated and are briefly discussed below, and again in chapter 6. It is important to note that alteration in expression of any one of these three host proteins is not typically pathognomonic for PV infection. Instead, their expression is assessed along with molecular techniques that evaluate PV presence and oncogene expression. As such, IHC forms part of an algorithm that permits accurate diagnosis and interpretation of the significance of the presence of HPV DNA within a neoplasm.[237]

### *1.6.2.1 pRb*

The retinoblastoma protein prevents cell replication by inhibiting progression from the G1 to S phases of the cell cycle and thus functions as a tumor suppressor. In a quiescent (non-dividing) cell, pRb is hypophosphorylated and active, meaning it prevents the cell from proceeding through the G1-S checkpoint. When pRb is phosphorylated it is inactivated, and this braking effect on the G1-S checkpoint is released, promoting progression through the cell cycle. The PV E7 oncoprotein inactivates pRb through its sequestration[63,74,123] or degradation,[29,290] thus promoting cell cycling. Loss of, or reduction in pRb immunostaining may be seen in HPV-associated anogenital and HNSCCs in humans.[3,151,222] In cats, loss of pRb immunostaining was found in one study to be associated with the presence of PV DNA within a series of feline cutaneous pre-neoplastic and neoplastic lesions.[176]

### *1.6.2.2 p16$^{CDKN2A}$*

Phosphorylation of Rb is promoted by enzymes called cyclin-dependent kinases (CDKs). As their names suggest, CDKs function only when complexed with cyclins, which are proteins involved in cell cycle regulation. When a CDK is complexed with a cyclin molecule then the pRb checkpoint is released, promoting progression through the cell cycle. CDKs are, in turn, regulated by enzymes called CDK inhibitors. The function of the CDK inhibitors is ultimately to prevent cell cycling by preventing phosphorylation of pRb.

The p16$^{CDKN2A}$ protein (p16) is an example of a CDK inhibitor. When the PV E7 oncoprotein degrades pRb this increases cell cycling. In a compensatory effort to prevent cell cycling, the cell increases expression of p16. While the loss of pRb means that p16 can no longer control cell cycling, the increased expression of p16 can be detected immunohistochemically. Increased immunostaining of p16 is so consistently found in human PV-induced HNSCCs that it strongly suggests a PV etiology for that cancer.[55,57,139,141,151] Increased immunostaining for p16 has recently been shown to be significantly associated with loss of cellular pRb and the presence of PV DNA in a series of cutaneous preneoplastic and neoplastic feline lesions.[177-179,184]

#### *1.6.2.3 p53*

The p53 protein functions as a tumor suppressor, maintaining genetic integrity and promoting apoptosis in response to irreparable DNA damage. In normal cells, p53 is rapidly turned over and removed within minutes of its production.[219] For this reason, wild-type p53 is not usually detectable in normal cells using IHC.

Immunostaining for p53 can be useful when trying to distinguish between SCCs caused by PVs and those caused by UV light. Whereas the PV E6 oncoprotein complexes with p53 and induces its rapid degradation through ubiquitin-dependent proteolysis,[224] UV light may induce *p53* gene mutations[30,292] that result in production of a dysfunctional p53 protein that is ineffective but at the same time abnormally stable and persistent.[106,292] Thus, a high level of p53 immunostaining in a solar-exposed neoplasm supports a UV etiology. As lack of p53 immunostaining within a cell could either be normal or due to PV-induced degradation, the absence of p53 immunostaining does not necessarily indicate a PV etiology. However, the presence of p53 immunostaining would suggest that the SCC was unlikely to be caused by PV infection. This is relevant when investigating equine tissues from SCC-prone body sites such as eyelids and external genital openings, which are exposed to both sunlight and PV infection.

### 1.6.3 Evidence Supporting a Causative Association between PVs and SCCs: Expression of Oncogenic PV mRNA Transcripts

Overexpression of PV E6 and E7 proteins disrupts normal tumor suppression and cell cycling, sometimes leading to development of neoplasia. It is therefore logical that detection and quantification of PV E6 and E7 mRNA transcripts could provide more mechanistic information than simple detection of PV DNA within a neoplasm. This approach has been investigated in human cervical cancers with mixed results. Some studies have shown that HPV mRNA expression increases in human cervical lesions as they progress from dysplastic lesions to invasive carcinomas.[143,225,278] Others have not shown this, possibly due to the instability of stored mRNA, the lack of consistency among detection techniques used by different investigators and the lack of longitudinal clinical data available (reviewed in reference [57]). A similar approach has

also been used in human HNSCCs.[19,267] In those studies the presence of HPV E6/E7 mRNA as detected by ISH was well correlated with p16 expression and PV DNA ISH.

In non-human species, PV oncogene mRNA expression has been rarely investigated. One study measured bovine PV *E2, E5, E6* and *E7* mRNA expression in equine sarcoids and found a positive correlation between BPV viral load and mRNA expression. However, the same study found no correlation between either measurement and cellular proliferation.[21] The authors' hypothesis that the clinical behaviour of different types of equine sarcoid could be explained by differences in BPV activity could not be demonstrated. At the time that the work for this thesis was started, mRNA expression had never been investigated for any equine PV.

### 1.6.4 Evidence Supporting a Causative Association between PVs and SCCs: Progression of PV-Infected Lesions to SCCs

Some SCCs develop at sites of PV-induced precursor lesions such as squamous papillomas, dysplastic lesions, precancerous plaques and *in situ* carcinomas. The precursor lesion typically contains evidence of PV infection such as viral cytopathic change, PV antigens (detected by IHC) and PV DNA (detected by PCR and ISH). However, this evidence of infection may be lost as precursor lesions progress to SCCs because viral replication decreases.[81]

This progression of PV-associated precursor lesions to invasive SCCs is reported in humans, cats, dogs and horses. Human cervical SCCs may occur after progression of increasingly dysplastic mucosal lesions, which are graded CIN I (cervical intraepithelial neoplasia level 1) to CIN III. These dysplastic lesions are associated with PV infection, and high-risk PV types are found with increasing frequency in the higher-grade CIN lesions. Lesions that have fully progressed to CIN III constitute the greatest risk for invasive SCC development.[53] Human penile SCCs typically begin as carcinomas *in situ*, the majority of which are associated with HPV infection.[56] In horses, genital SCCs are often preceded by PV-induced squamous papillomas or precancerous plaques,[34,83,119] In cats, Bowenoid *in situ* carcinomas caused by FcaPV-2 may develop an invasive phenotype and progress to SCCs.[10,104] In dogs, cutaneous pigmented viral plaques,

which are caused by multiple canine PV types,[261] have been reported to progress to *in situ* or invasive SCCs.[37,186,191,243,260]

Progression of an obviously PV-induced lesion to an invasive neoplasm may suggest that the PV is involved in the transformation of cells and the promotion of neoplasia. However, it must also be remembered that the observation that a PV-induced lesion can progress to an invasive SCC does not necessarily prove that the PV had any significant role in the process of neoplastic transformation.

### 1.6.5 Evidence Supporting a Causative Association between PVs and SCCs: Success of Vaccination

Possibly the most definitive evidence for a causal association between a microbe and a cancer is the ability to prevent that cancer by preventing infection by that microbe. In humans, vaccination programs to prevent high-risk HPV infection were implemented in several countries beginning in the late 2000s. As HPV-induced cancers take many years to develop, it is currently too early to confirm that these vaccines will prevent cervical cancer and HNSCCs. However, if the predicted sharp drop in cancer rates is observed in the future, this will be definitive proof that these HPVs play an essential role in SCC development.[105,117,197,217,274] In non-human species there has never been a similar trial using vaccination to reduce the incidence of a PV-induced cancer.

## 1.7 Other Risk Factors for Development of SCCs

Papillomavirus infection is not the only risk factor for SCC development. Ultraviolet radiation, certain chemicals and even chronic inflammation are also associated with the development of neoplasia. These non-PV causes of SCC are discussed briefly in the following sections. They are included in this literature review to highlight areas in which conclusive evidence of causation is lacking, in order to justify my investigation into an association between PV infection and SCC development in horses.

In humans and many non-human species, the most accepted cause of cutaneous SCCs is UV exposure. Correspondingly, there is a widespread belief that genital and

periocular SCCs in horses are also primarily caused by UV light. This has been investigated in several studies, the findings of which are interpreted below.

In addition to UV radiation, human SCCs have also been associated with several other factors. Some of these, for example tobacco and alcohol use, are not relevant to horses. However, other factors, such as chronic inflammation, immunosuppression, preputial smegma accumulation, and parasitism occur in both humans and horses and so are discussed.

### 1.7.1 Ultraviolet Radiation

Ultraviolet radiation is the most abundant environmental carcinogen and the skin is equipped with effective defenses against UV-induced damage. Nevertheless, strong epidemiologic evidence suggests that UV exposure causes skin cancer in humans and domestic mammals. While the effect of UV radiation on skin cannot be investigated experimentally in humans, it has been investigated in several animal models, including mice, fish, and opossums.[7]

#### *1.7.1.1 UV Exposure and SCCs in Humans*

Solar radiation is the main source of UV exposure, and there is abundant epidemiologic and mechanistic evidence that UV exposure predisposes to human cutaneous SCCs.[7,216] The epidemiological evidence includes the observation that the highest incidence rates of cutaneous SCCs are seen in light-skinned populations living in regions of high UV exposure.[216] The use of UV-emitting tanning lamps is associated with increased risk for cutaneous SCCs,[134] and treatment for psoriasis with UV light therapy is also associated with increased risk for cutaneous SCCs, even decades after cessation of therapy.[135,196]

Neoplasms that are caused by UV radiation contain certain characteristic “signature” DNA mutations. These are most frequent in the *p53* tumor suppressor gene, and are present in the majority of UV-induced SCCs.[30,47,216] As already discussed, mutations in *p53* caused by UV light may result in production of dysfunctional but stabilized p53 protein, whose accumulation can be detected using IHC. However, p53 mutations are common in many human neoplasms, including some in locations not exposed to UV

light. To investigate whether p53 accumulation is due to a UV-induced mutation the *p53* gene is sequenced and compared to the p53 gene from a normal cell from that individual. Mutations in *p53* caused by UV radiation damage leave distinctive "fingerprints" consisting predominantly of C to T transitions at certain specific codons, known as hotspots.[292] As these hotspot mutations are not seen in non-UV-induced cancers their presence is considered good evidence that a SCC was caused by UV exposure.[103]

#### *1.7.1.2 UV Exposure and SCCs in Horses*

While there is good evidence associating sun exposure with cutaneous SCCs in humans, the relationship between UV light and SCCs of external body sites in horses is less clear. Evidence supporting a role for UV light in SCC development in horses includes the observation that the prevalence of SCCs on external body sites increases with increased mean annual solar radiation, increased altitude, and decreased latitude.[70,233,268] Additionally, the predilection sites for external equine SCCs (including eyelids, external genitalia, perineum and muzzle) are generally sparsely haired. As hair protects against UV damage, this has led to the assumption that skin at sparsely haired sites receives greater UV exposure, predisposing it to SCC development.[75]

The observations that SCCs appear to be more common in equine skin that is exposed to greater amounts of UV light has led to the frequent assertion in the veterinary literature that UV light is the main cause of all cutaneous SCCs in horses, including SCCs of the external genitalia and periocular tissues. However, few studies have specifically examined these important SCC subtypes and the results of those studies have been inconclusive.

Equine penile SCCs are reported anecdotally to be most common in breeds with non-pigmented genitalia, such as appaloosas and American paint horses,[36,229] the assumption being that those breeds lack the UV protection afforded by the melanin of pigmented breeds. However, of three large scale surveys of EPSCCs,[156,268,270] only one supported a predisposition in breeds with non-pigmented genitalia. In that particular survey, conducted in the USA Pacific northwest, 29 horses with EPSCCs were included.

The breed distribution was: 14 (48.3%) appaloosa; 4 (13.8%) paint; 4 (13.8%) quarter horse; 3 (10.3%) mustang; 3 (10.3%) Arabian; and 1 (3.4%) morgan. However, the author acknowledged that the overall makeup of the equine population in the Pacific northwest was not investigated,[268] and it is possible that the apparent breed predilections demonstrated may have simply reflected higher numbers of appaloosas and paints in the area in which the study took place.[270] Additionally, the genitalia of each horse were not examined so the presence or absence of pigment in each case was not determined. In contrast to the Pacific northwest survey, two other studies of EPSCCs have found no breed predilection.[156,270] Of 114 horses with EPSCCs, the breed distribution was: 19 (16.6%) Dutch warmblood; 16 (14%) Welsh; 13 (11.4%) new forest; 12 (10.5%) Haflinger; 12 (10.5%) Icelandic; 8 (7%) Arabian; and 34 (29.8%) horses or ponies of other or unknown breed.[270] As in the first study, no effort was made to determine the color of the genitals in each case. In another study of 45 horses with EPSCCs, the breed distribution was: 17 (37.8%) ponies; 10 (22.2%) cobs; 6 (13.3%) crossbreds/hunters; 4 (8.9%) thoroughbred crosses; 2 (4.4%) Irish draughts; 2 (4.4%) warmblood crosses; 2 (4.4%) Arabians; 1 (2.2%) American quarter horse; and 1 (2.2%) warmblood.[156] Currently, there is no strong evidence that EPSCCs are more common in breeds of horses with non-pigmented genitalia.

Another factor that should be considered when assessing the relationship between EPSCCs and UV light is the exposure of the male genitalia to sunlight. The ventral and predominantly retracted location of the equine penis means it is not directly exposed to UV light for long periods. This may suggest that UV exposure is a less plausible cause for development of PSCCs. However, it is also possible that the lack of hair on the equine penis and the relatively thin layer of protective keratin covering the mucosal surface may result in this area being particularly sensitive to the effect of UV light so that only a brief period of exposure is required for carcinogenesis. It has also been suggested that reflected UV radiation could cause the development of EPSCCs,[36] however grassland pasture does not effectively reflect UV light, unlike snow and sand.[70]

Only 6 EPSCCs have been evaluated for the presence of p53 immunostaining.[254] In that study, immunostaining was present in 5 of the EPSCCs, suggesting the presence of p53 gene mutations. While this could indicate a role of UV light in neoplasia development, no DNA sequencing was performed to determine if p53 hotspot mutations characteristic of UV damage were present. Additionally, the frequency at which p53 mutations are present in non-UV induced equine SCCs, such as gastric SCCs, is unknown.[254] Therefore, it is difficult to interpret the importance of p53 immunostaining results in such a limited number of horses.

Squamous cell carcinomas of the eye and adnexa of horses (EOSCCs) have also been suggested to be caused by chronic exposure to solar UV radiation.[70,100] Evidence supporting this includes the observation that horse breeds with non-pigmented mucocutaneous junctions, such as appaloosa, paint and pinto, appear to be at increased risk for EOSCC development,[70,268] even to the point that prophylactic tattooing of eyelids is practiced although with no conclusive evidence of effectiveness.[98] Two studies partially support this link between lightly pigmented breeds and development of EOSCCs, but contain additional, conflicting information. In one survey of horses from the U.S. Pacific northwest, of 36 horses with EOSCCs the breed distribution was: 14 (38.9%) paints; 9 (25%) quarter horses; 4 (11.1%) draft breeds; 3 (8.3%) appaloosas; 3 (8.3) Arabians; 2 (5.6%) Tennessee walking horses; and 1 (2.8%) thoroughbred.[268] However, the breed distribution of healthy horses in this region was not reported. In another study of 1006 horses with EOSCCs submitted to 14 U.S. veterinary colleges, several breeds had a significantly ($P < 0.001$) higher prevalence of EOSCCs when ranked relative to one another (based on the adjusted odds ratio).[70] In that study, the adjusted odds ratio for different breeds were: 21.7 for draft breeds (Belgian, Clydesdale and Shire); 7.9 for appaloosas; 4.5 for paints and pintos; 3.1 for mixed breeds; 1.8 for ponies; 1.2 for other breeds; and 1.0 or less for quarter horses, thoroughbreds and Arabians. It is significant that draft breeds, in particular Belgians, which often have pigmented ocular adnexa, are reported to have an increased risk for not just EOSCCs but all primary tumors, suggesting a multifactorial basis for SCC development.[70,211] It should also be noted that no effort was made to determine which individual horses had pigmented ocular adnexa and which did not.

As for EPSCCs, it is possible that UV radiation alone does not account for all cases of equine ocular SCC.

In three separate studies using IHC, p53 immunostaining was observed in 5 of 5,[236] 6 of 6[254] and 1 of 1[1] EOSCCs, which could suggest that UV radiation was the cause. In one EOSCC, sequencing of the p53 gene from neoplastic cells revealed C to T transitions at hotspots, suggesting the mutation could have been due to UV light.[205] The evidence provided by p53 immunostaining that EOSCCs are UV-induced is therefore stronger than that for EPSCCs. However, no study has ever assessed p53 immunostaining of non-UV-induced SCCs, making it difficult to determine the relationship between UV exposure and p53 IHC.

Equine vulvar SCCs are less common and less well-studied than either EOSCCs or EPSCCs. Although lack of pigmentation and UV exposure are risk factors proposed by some authors, there are insufficient data to either support or refute this. In one study p53 immunostaining was observed in 2 of 3 vulvar SCCs.[254] Additionally, p53 hotspot mutations were identified in an equine vulvar SCC, consistent with development due to UV exposure.[205]

Cutaneous non-genital SCCs are reported to be more common in horses with lightly pigmented skin and hair (white, grey-white and Palomino), especially around the mucocutaneous junctions.[36,233] In contrast to EPSCCs or EOSCCs, they occur most frequently in sun-damaged skin and typically develop within areas of actinic (solar) keratosis,[38,233] which is good evidence that they are UV-induced.

In summary, the evidence that UV light is responsible for neoplasm development is stronger for periocular and cutaneous SCCs than it is for EPSCCs. However, investigations to date have been relatively few and have often had small sample sizes; this applies particularly to investigations into UV-induced *p53* mutation. The assertion that UV light is responsible for equine SCCS based on the fact that unpigmented breeds develop these neoplasms more frequently than pigmented breeds is not borne out by all studies. In addition, equine oral and pharyngeal SCCs are well recognized and their

cause, although unknown, cannot be UV exposure. Given the proportion of human cancers that are believed to be due to HPV infection, investigation into possible non-UV causes of equine SCCs is justified.

### 1.7.2 Miscellaneous Risk Factors for Development of SCCs

In addition to PVs and UV light, other factors have been proposed as possible causes of SCC development in humans and horses. Some of these factors, such as smegma accumulation, immunosuppression and chronic inflammation are common to both species. For these risk factors the majority of research has focused on humans, and less information on horses is available. Other factors, such as tobacco or alcohol use, occupational exposure to carcinogens, and certain rare genetic conditions are not significant factors in horses, but are included for completeness.

#### *1.7.2.1 Miscellaneous Risk Factors for Development of SCCs in Horses*

Smegma is a preputial secretion composed principally of desquamated epithelial cells. It may accumulate in the equine prepuce and form inspissated concretions within the urethral sinus that cause discomfort or interfere with urination. In addition, smegma accumulation in horses has been reported anecdotally to be associated with EPSCCs.[14,41,50,107] Equine smegma was reported to be carcinogenic in a 1947 study, in which it was applied to the skin of mice.[208] The findings from that study have never been replicated and in 2006 doubts regarding the methods and statistical analyses used were reported.[273] Currently, whether or not smegma promotes EPSCC development is uncertain.

Age, breed, and castration status may influence the development of SCCs in horses. SCCs of all body regions are mainly diseases of middle-aged to older horses, with reported mean ages of 8-12 years.[132,152,245,268] The breeds with the highest incidence of SCC overall are draft breeds (Clydesdales, Belgians, Shires), appaloosas, American paints, pintos and mixed breeds.[36,70,245] When just EPSCCs are considered, geldings are reported to develop neoplasia more frequently than stallions. This has been suggested to be due to greater amounts of smegma in the prepuces of geldings due to less frequent cleaning compared to valuable breeding stallions.[34,49,50,119] However, an

increased prevalence in geldings is not supported by all studies, and some authors suggest that any difference in the numbers of penile SCCs in geldings and stallions may be biased by the low number or younger age of intact males in the equine population.[83,107,156,49,119]

Sites of chronic inflammation, irritation or previous injury may be at greater risk for metaplastic transformation and development of SCCs. Proposed irritants causing chronic inflammation include topically applied chemicals, smegma (discussed above), and flies.[41] Ocular SCCs can occur at sites of previous ocular injury, particularly if healing is delayed.[92] Although three reports exist of cutaneous SCCs arising at sites of previous injury,[11,80,230] skin trauma is still not considered a significant risk factor for SCC development. Gastric infestation with larvae of *Gasterophilus* species has been considered as a possible cause for gastric SCCs.[50,51] However, the ubiquity of the parasite is in contrast to the rarity of gastric SCCs and there is little evidence to support a causal relationship.[111,280]

#### *1.7.2.2 Miscellaneous Risk Factors for Development of SCCs in Humans*

Human penile SCCs commonly arise at sites of chronic inflammation caused by such diverse conditions as lack of circumcision, phimosis and urethral stricture.[65] While smegma retention has been previously described as a risk factor for penile SCCs, this is now disputed.[20,154,208,215,273] Currently in humans it is uncertain whether it is the chronic inflammation that accompanies smegma accumulation or the smegma itself that predisposes to neoplasia.[20,65]

Immunosuppressed patients have a markedly higher risk of many different cancers including genital, oral, pharyngeal, and cutaneous SCCs. The number of people living with chronic immunosuppression is increasing due to the antirejection drugs used after organ transplantation, the immunosuppressive drugs used to treat autoimmune and rheumatologic diseases, and the prevalence of AIDS.[216] In organ transplant recipients there may be up to a 65-fold increase in incidence of SCCs,[16,170,213] and HIV infection is associated with development of SCCs that are more aggressive and lethal than SCCs in the general population.[193] The connection between immunosuppression

and development of SCCs is not fully understood, however in people it is hypothesized to be due to a reduced ability to resolve HPV infection.[216]

In addition to the previously discussed epidermodysplasia verruciformis, other heritable or genetic risk factors exist for development of cutaneous SCCs. Development of cutaneous SCCs in parents is associated with an increased incidence of invasive SCCs in their children, suggesting an underlying hereditary susceptibility.[114] Certain specific variants of the melanocortin-1 receptor (those known to be associated with red hair, fair skin, increased UV sensitivity, and melanoma risk) are also associated with increased risk for cutaneous SCC development.[28] Individuals with a particular glutathione-S-transferase genotype are at increased risk for development of solar keratosis due to a reduced ability to detoxify certain carcinogens and mutagens produced by UV radiation.[40] Some genetic syndromes (e.g., xeroderma pigmentosum and albinism) can lead to increased risk for cutaneous SCCs by reducing the skin's ability to tolerate UV radiation.[64]

An association between smoking and SCCs of the mouth, pharynx, larynx, esophagus, cervix, penis and bladder is well established.[207] Smoking is also a risk factor for cutaneous SCCs, with a relative risk twice that of control subjects.[60] The use of chewing tobacco, betel quid and areca nut is also strongly associated with development of oral and oropharyngeal SCCs.[146,148] Similarly, alcohol is classified as a carcinogen, and its use is associated with development of oral and pharyngeal SCC.[146,148]

Chemical carcinogens such as arsenic, coal-tar products and psoralens can promote the development of cutaneous SCCs.[64] Occupational exposure to phenoxy herbicides and dioxins may be associated with an elevated risk for oral and pharyngeal SCCs.[13,226]

## 1.8 Conclusion

As reviewed, there is abundant epidemiologic and mechanistic evidence showing that PVs cause cervical, anogenital and HNSCCs in humans. In horses, several non-viral causes for the development of SCCs have been previously proposed; however, as

described in this literature review, the evidence supporting these causes is often inconclusive. This suggests a possible role of PVs in the development of equine SCCs, especially male genital, periocular, and oral/pharyngeal SCCs.

In the research described in subsequent chapters of this thesis, tissue samples from SCC-prone sites from a large number of horses, both neoplastic and disease-free, will be screened for PV DNA using PCR. If a PV is present significantly more frequently in neoplastic than non-neoplastic tissues then the oncogenic activity of the PV will be investigated in an attempt to establish a causal relationship between infection and development of neoplasia. In these ways I will test the hypothesis that, like human cervical, anogenital and HNSCCs, some equine SCCs are caused by infection by PVs. Proving a papillomaviral cause for a subset of equine SCCs would be beneficial in developing strategies to prevent SCC development.

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# CHAPTER TWO

## Investigation into Papillomaviral Presence in Penile Lesions

## 2.1 Introduction

Squamous cell carcinomas (SCCs) comprise approximately one fifth of all cutaneous neoplasms in horses.[25] Nearly half of equine SCCs affect the penis,[22] and SCCs are by far the most common neoplasm of the equine external male genitalia.[26] Equine penile SCCs (EPSCCs) cause significant discomfort and pain in affected animals. As they commonly recur after treatment they cause emotional and financial distress to owners, who may opt for expensive but unsuccessful treatment, or may choose to euthanize a potentially curable horse on humane or financial grounds.

Currently, a definitive cause of EPSCCs has not been identified. Previous factors that have been suggested to predispose to EPSCC development include UV light exposure[12,25] and smegma accumulation.[8,15] While UV exposure is a well-recognized cause of SCCs in sun-exposed skin in some species, the ventral and predominantly internal location of the equine penis likely reduces its exposure to sunlight, making UV exposure a less plausible cause of EPSCCs. Additionally, recent evidence suggests that smegma may be less of a factor in EPSCC development than previously thought.[9,27]

In humans, approximately half of penile SCCs are caused by high-risk alphaPVs,[4,6,20] with the remainder being of unknown cause. This led me to hypothesize that PVs may similarly influence the development of a proportion of EPSCCs. Therefore, the aim of the work described in this chapter was to investigate a series of EPSCCs and non-SCC penile lesions for the presence of PV infection. More frequent detection of PVs in EPSCCs than in non-neoplastic equine penile samples would support an association between PV infection and EPSCC development.

## 2.2 Materials and Methods

### 2.2.1 Case Material

Cases of equine penile disease were identified by searching the tissue archives of the Department of Biomedical Sciences, Cornell University, College of Veterinary Medicine, Ithaca, New York. The original diagnosis for each case was confirmed by examination of the original hematoxylin and eosin-stained sections. Signalment information for each horse was recorded, when available.

Samples of the identified penile lesions had been formalin-fixed and embedded in paraffin blocks for histologic sectioning and long-term storage. From each formalin-fixed, paraffin-embedded (FFPE) tissue block two types of samples were collected. First, a 10 µm thick section was cut from each block and placed into a labeled 1.5 ml Eppendorf snap-lock microcentrifuge tube for DNA extraction and subsequent PCR. Second, three 5 µm sections were cut from each block and mounted, unstained, on

three charged glass slides for subsequent immunohistochemistry (IHC) and *in situ* hybridization (ISH). A new microtome blade was used to cut sections from each case to prevent cross-contamination of samples.

### 2.2.2 DNA Extraction from Samples

Total genomic DNA was extracted from all FFPE tissue sections. Sections were initially deparaffinized with xylene and then washed twice with ethanol. Template DNA was then extracted using a commercially available kit according to the manufacturer's instructions (DNeasy blood and tissue kit, Qiagen GmbH, Hilden, Germany). The quantity and quality of extracted DNA in each sample were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

### 2.2.3 PCR

Conventional PCR was used to amplify PV DNA sequences from the samples. Two different consensus primer sets were used for each sample; both primer sets have been shown to amplify conserved regions of the PV L1 gene in samples from a variety of human and non-human species.[3,7,11] The primers used were MY09 (5'-CGT CCM ARR GGA WAC TGA TC-3'), MY11 (5'-GCM CAG GGW CAT AAY AAT GG-3'), FAP59 (5'-TAA CWG TNG GNC AYC CWT ATT-3'), and FAP64 (5'-CCW ATA TCW VHC ATN TCN CCA TC-3') where M indicates A or C, R indicates A or G, W indicates A or T, Y indicates C or T, V indicates A, C or G, H indicates A, C or T, and N indicates A, G, C or T. The MY09/MY11 primer set was designed to amplify DNA from mucosal-adapted PVs and amplifies a 451 base pair segment of the PV L1 gene. The FAP59/FAP64 primer set was designed to detect DNA sequences from cutaneous-adapted PVs and amplifies a 478 base pair segment of the PV L1 gene.

The PCR reactions consisted of 3 µl of template DNA and 2 µl of primer to make a final concentration of 0.25 µM, and 25 µl of HotStarTaq master mix (Qiagen GmbH, Hilden, Germany) that contained 250 units HotStarTaq DNA polymerase, PCR buffer with 3 mM $MgCl_2$, and 400 µM of each dNTP. Amplification was carried out under the following cycling conditions: 94 °C for 10 minutes followed by 45 cycles of 94 °C for 1.5 minutes, 50 °C for 1.5 minutes, and 72 °C for 1.5 minutes. The final extension step was

at 72 °C for 5 minutes. Amplicons underwent electrophoresis in a 1% agarose gel containing 0.64 µg/ml ethidium bromide and bands containing the amplified fragment were visualized under UV light. All PCR reactions were carried out in duplicate. DNA extracted from a bovine cutaneous fibropapilloma known to contain BPV-1 was used as a positive control and template DNA was replaced by an equivalent volume of water in the negative controls.

### 2.2.4 Sequencing and Sequence Analysis

Each band from a PCR-positive sample was cut from the gel under UV light using a new blade and placed into a 1.5 ml Eppendorf tube. DNA was extracted from the gel band using a commercially available kit according to the manufacturer's instructions (Qiaex II 150 Gel Extraction Kit, Qiagen GmbH). For sequencing, a 15 µl reaction volume containing 20 ng DNA and 3.2 picoM of primer was submitted to the Massey Genome Service, where it was subjected to automatic dye-terminator cycle sequencing with the BigDye Terminator 3.1 Ready Reaction Cycle Sequencing Kit using an ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing results were compared with known sequences from GenBank using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).[1]

### 2.2.5 *In Situ* Hybridization

*In situ* hybridization for PV DNA was only performed on samples in which PV DNA had been detected using PCR. The ISH was performed using the Discovery automated slide-processing system (Ventana Medical Systems Inc., Tucson, AZ, USA). The slides were pre-treated by mild cell conditioning using citrate buffer–based RiboCC reagent (Ventana Medical Systems) and enzyme pretreatment with Protease 3 for 12 minutes (Ventana Medical Systems). The sections were then incubated for hybridization with a generic antisense probe for PVs at 200 ng per slide for 1 hour at 37°C after 4 minutes of denaturation at 95°C. Three stringency wash steps using 0.5X Ribo-Wash (Ventana Medical Systems; equivalent to 0.5X saline sodium citrate) for 4 minutes at 42°C were followed by incubation with anti-rabbit anti-digoxigenin antibody (Sigma, St Louis, MO, USA) for 32 minutes at 42°C. After streptavidin–alkaline phosphatase conjugate UMap anti-rabbit AP (Ventana Medical Systems) incubation for 16 minutes at 42°C, the signal

was detected with the BlueMap NBT/BCIP substrate kit (Ventana Medical Systems) for 2 hours at 42°C. Finally, the sections were counterstained with Red Stain II (Ventana Medical Systems) for 4 minutes before coverslipping. The positive control specimen was a sample of a canine oral papilloma known to be infected with *Canis familiaris* PV type 1 (CPV-1). Probes were omitted in the negative control specimens.

### 2.2.6 Immunohistochemistry

Immunohistochemistry (IHC) to detect the presence of PV antigens was only performed on samples that had been shown to contain PV DNA using PCR. The primary antibody used in IHC had been generated against the L1 antigen of human PVs 1, 6, 11, 16, 18, and 31 (Lifespan Biosciences, Seattle, WA, USA). Immunostaining was performed with an automated staining system (Benchmark, Ventana Medical Systems) that incorporated a commercial detection system (Enhanced V-Red Detection Kit, Ventana Medical Systems). Antigen retrieval was achieved by incubating slides in a high-pH antigen retrieval solution (CC2 Std, Ventana Medical Systems) for 60 minutes. The primary antibody was applied at a concentration of 1:100 for 32 minutes and sections were then counterstained with hematoxylin. Positive control specimens included tissues known to be infected with CPV-1. For negative control specimens, the primary antibody was replaced with Tris buffered saline (Dako, Carpinteria, CA, USA).

### 2.2.7 Statistical Analysis

To compare the ages of the horses in each group, standard tests for normality and variance were conducted using SAS 9.1 (SAS Institute, Cary, NC, USA). Between-group differences in PV detection rates were assessed by Fisher's exact test using SPSS 15.0 (IBM Corporation, Armonk, NY, USA).

## 2.3 Results

### 2.3.1 Case Material

Forty cases of equine penile disease were identified. These included 20 EPSCCs and 20 cases of non-SCC penile disease (Table 2.1). The ages were available for 18 of 20 horses from the EPSCC group and for 14 of 20 horses from the non-SCC group. Horses with EPSCCs had a mean age of 23.9 years, which was significantly older than horses with non-SCC penile lesions (13.3 years; p=0.004). All horses in the study originated from the northeastern United States with the exception of one (horse number 740), which was from the southern United States (Virginia). Differentiation between samples from geldings and stallions was not possible as this information was not recorded in the signalment information.

### 2.3.2 PCR

Each sample was confirmed to contain at least 17 ng/µl of DNA using spectrophotometry (range: 17.4 – 41.9). Using the MY09/MY11 consensus primer set, PV DNA sequences were amplified from 10 of 40 (25%) penile samples included in the study (Table 2.1). Samples that contained PV DNA included 9 of 20 (45%) EPSCCs and 1 of 20 (5%) samples of non-SCC penile disease. DNA was amplified significantly more frequently from samples of EPSCC than non-SCC penile lesions ($p<0.05$). The non-SCC lesion that contained PV DNA was a case of chronic ulcerative and granulomatous balanoposthitis from a 14-year-old horse. As expected, PV DNA was amplified from the bovine fibropapilloma positive control, but not from non-template negative controls. The average age of the horses in which PV DNA was detected (22.4 years) was not significantly different from the average age of the horses in which PV was not detected (17.9 years; $P = 0.147$).

No PV DNA was amplified from any of the 40 test samples or from the negative control by the FAP59/FAP64 consensus primers. PV DNA was amplified from the positive control using these primers.

| Horse ID | Age (years) | Diagnosis | PV DNA amplified | ISH for PV DNA | IHC for PV Antigen |
|---|---|---|---|---|---|
| 901 | 20 | EPSCC | EcPV-2 | Intense | Yes |
| 502 | 30 | EPSCC | EcPV-2 | Intense | No |
| 951 | 20 | EPSCC | EcPV-2 | Intense | No |
| 242 | 19 | EPSCC | EcPV-2 | Moderate | No |
| 931 | 21 | EPSCC | EcPV-2 | Moderate | No |
| 078 | 20 | EPSCC | EcPV-2 | Moderate | No |
| 531 | 22 | EPSCC | EcPV-2 | No | No |
| 767 | 38 | EPSCC | EcPV-2 | No | No |
| 861 | 20 | EPSCC | EcPV-2 | No | No |
| 059 | 29 | EPSCC | No | - | - |
| 101 | 20 | EPSCC | No | - | - |
| 262 | 25 | EPSCC | No | - | - |
| 831 | 30 | EPSCC | No | - | - |
| 406 | 10 | EPSCC | No | - | - |
| 591 | 28 | EPSCC | No | - | - |
| 651 | ? | EPSCC | No | - | - |
| 652 | 22 | EPSCC | No | - | - |
| 803 | 27 | EPSCC | No | - | - |
| 353 | 30 | EPSCC | No | - | - |
| 911 | ? | EPSCC | No | - | - |
| 979 | 14 | Chronic ulcerative and granulomatous balanoposthitis | EcPV-2 | No | No |
| 125 | 4 | Granulation and ulceration | No | - | - |
| 695 | ? | Chronic balanitis | No | - | - |
| 871 | 0.1 | Lymphocytic and granulomatous posthitis | No | - | - |
| 359 | ? | Inflammatory polyp | No | - | - |
| 787 | 16 | Inflammatory polyp | No | - | - |
| 335 | 17 | Eosinophilic granuloma | No | - | - |
| 461 | ? | Eosinophilic granuloma | No | - | - |
| 602 | 13 | Eosinophilic dermatitis with lymphoid hyperplasia | No | - | - |
| 160 | 8 | Habronemiasis | No | - | - |
| 256 | 14 | Focal neutrophilic vasculitis | No | - | - |
| 561 | "Old" | Melanoma | No | - | - |
| 631 | 14 | Melanoma | No | - | - |
| 021 | ? | Amelanotic melanoma | No | - | - |
| 373 | 25 | Undifferentiated sarcoma or amelanotic melanoma | No | - | - |
| 442 | 18 | Lymphosarcoma | No | - | - |
| 768 | 12 | Lymphosarcoma | No | - | - |
| 853 | 14 | Mastocytoma | No | - | - |
| 225 | "Mature" | Tricholemmoma | No | - | - |
| 740 | 17 | Ductal apocrine adenoma | No | - | - |

*Table 2.1. Identification number, age, diagnosis, PCR result, ISH result, and IHC result for each of 40 horses with penile disease. EPSCC, equine penile squamous cell carcinoma. EcPV-2, Equus caballus papillomavirus type 2. PCR was performed using the MY09/MY11 consensus primer set, ISH was done using a generic antisense probe for PVs, and IHC was performed using an antibody against the L1 antigen of several human PV types. '-' indicates that the sample was not tested.*

### 2.3.3 Sequencing

The DNA from the ten penile lesions in which PV DNA was amplified by PCR was sequenced. In all cases the sequence was between 98% and 100% similar to *Equus caballus* papillomavirus type 2 (EcPV-2; GenBank accession No. EU503122). The mismatching of small numbers of bases within the sequences was considered likely to be the result of errors in the sequencing process rather than evidence of multiple PV types.

### 2.3.4 *In Situ* Hybridization

The consensus PV DNA probes hybridized within 6 of the 10 samples in which PV DNA had been amplified by PCR. In these cases, the labeled PV DNA was present in the nuclei of scattered neoplastic epithelial cells within lesions but not in adjacent, histologically normal tissue (Figure 2.1). Three of the cases exhibited intense labeling within numerous cells superficially within the neoplasm. The remaining three exhibited less intense labeling in a smaller proportion of neoplastic cells. All six cases in which PV DNA was detected by ISH were EPSCCs and no PV DNA was detected by ISH within the inflammatory lesion that had been PV positive by PCR. Cell nuclei were labeled as expected in the positive control sample, and no labeling was visible within the negative controls.

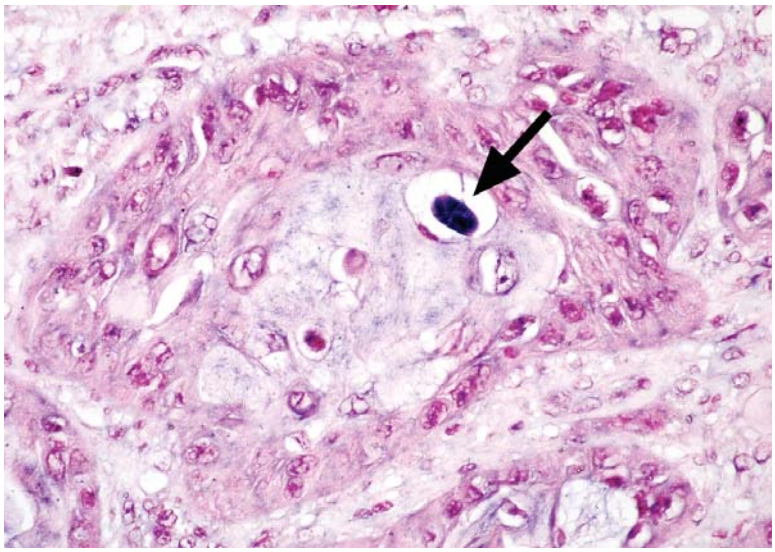

*Figure 2.1. In situ hybridization, penile squamous cell carcinoma; horse 901. In situ hybridization using a generic antisense probe for papillomaviruses shows papillomaviral DNA in the nucleus of a neoplastic epithelial cell (arrow). Counterstained with Red Stain II. 40X objective.*

### 2.3.5 Immunohistochemistry

Immunostaining using anti-PV-antibodies was only visible in 1 of the 10 samples in which PV DNA had been amplified by PCR. This case was an EPSCC that had also shown intense labeling by ISH. Immunostaining was rare within the neoplasm and was most often present in small clusters of cells close to the surface of the neoplasm (Figure 2.2). The cells that immunostained were typically larger cells with enlarged nuclei and increased quantities of lightly-stained cytoplasm. While this cell morphology is consistent with descriptions of keratinocyte changes typically observed in PV-induced lesions, the changes in cell morphology were subtle and had not been observed during examination of routinely-stained histological slides. Immunostaining was confined to the neoplastic cells and was not visible in the non-neoplastic epithelium surrounding the EPSCCs. As expected, immunostaining was present within the positive control sample, and was not visible in the negative control.

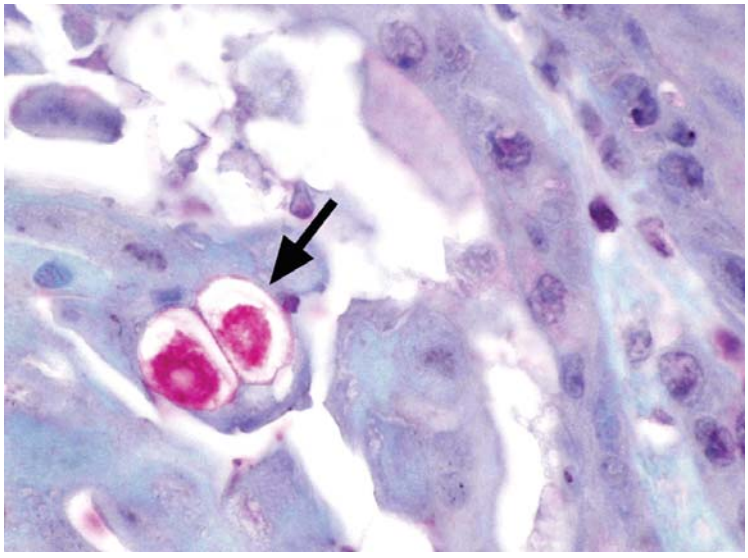

*Figure 2.2. Immunohistochemistry, penile squamous cell carcinoma; horse 901. Immunohistochemistry shows papillomaviral antigen in the nuclei of two neoplastic epithelial cells (arrow).The cells showing immunostaining have large nuclei with increased quantities of pale cytoplasm. Immunohistochemistry using an antibody generated against the L1 protein of human papillomavirus with hematoxylin counterstain. 40X objective.*

## 2.4 Discussion

Papillomaviral DNA was detected by PCR significantly more frequently in EPSCCs than in non-SCC penile lesions. These results confirm that the presence of PV DNA is associated with EPSCCs and provides evidence that PV infection might cause the development of these cancers. However, since PV infections are typically ubiquitous

and asymptomatic, the possibility cannot be excluded that PVs were detected more frequently in EPSCCs than in non-SCC samples simply because these cancers provide a more permissive environment for infection.

No PV DNA sequences were detected in 11 of the 20 EPSCCs. This could suggest that infection by PVs influences the development of only a proportion of EPSCCs. However, although two sets of consensus primers were used in this experiment, it is possible that other PV types were present in some of the EPSCCs but were not amplified by the primers used.

The PV DNA sequences detected in all 10 cases that contained PV DNA were from EcPV-2. The complete genome of this PV was first determined in 2008 (GenBank accession No. EU503122) and, at the time that our experiment was performed, this PV had not been reported to be associated with any disease. This PV is classified as a dyoiotaPV, the only member of this genus.[5]

The detection of a single PV type within all the EPSCCs supported the hypothesis that this PV type influenced cancer development. If the high prevalence of EcPV-2 in EPSCCs was simply due to a more permissive environment for PVs within the neoplasms, it would also be expected that EPSCCs might contain other equine PV types, which was not the case. However, at the time of this experiment only one other equine PV type (EcPV-1) was recognized.[13] As PV types tend to be restricted to specific body locations,[24] it is unknown whether EcPV-1 can even infect the penis. Additionally, it is possible that novel equine PV types were present within the EPSCCs but were not detected by the PCR primers used in this study. Overall, the detection of EcPV-2 exclusively does not provide additional evidence of a causative association between infection and cancer development, although it does suggest that this is the PV type that is most likely to cause EPSCCs if these cancers are caused by PV infection.

Although our results provide some evidence of a causative role of EcPV-2 in EPSCC development, we were only able to amplify EcPV-2 PV DNA from 9 of the 20 EPSCCs. The apparent absence of EcPV-2 in over half of the EPSCCs tested could be due to a

number of reasons. First, it is possible that EPSCCs, like human penile SCCs, have multiple causes. It has been reported that only half of human penile SCCs are caused by PV infection.[4,6,20] Second, it is possible that EcPV-2 was actually present within a higher proportion of the EPSCCs than we report, but was not detected by the methods used. While the results of our experiment confirm that the MY09/11 primers amplify EcPV-2, the sensitivity of these primers for this PV is unknown. If some of the EPSCCs contained only small amounts of EcPV-2, it might have escaped detection. To maximize the sensitivity of PCR detection of EcPV-2 DNA, PCR primers specific for EcPV-2 should be designed and used; this is described in later chapters. Third, DNA damage by formalin fixation may have prevented PCR amplification of EcPV-2 in some or all of the 11 EPSCC samples that were PV negative. A shortcoming of our experiment was that, although the quantity of the extracted DNA was measured, no internal 'housekeeping' gene was amplified to confirm the presence of amplifiable DNA within the sample. This limitation was addressed in our subsequent work (described in later chapters) in which housekeeping genes were amplified as internal controls to confirm the presence of amplifiable DNA within the samples.

A disadvantage of PCR is that DNA is extracted from the entire sample. This means that when PV DNA is amplified from a neoplasm, it cannot be determined whether the DNA was present within the neoplastic cells or within the surrounding non-neoplastic tissue. Because of this, we used ISH to demonstrate that the PV DNA was located within the lesions. Our ISH results confirmed that PV DNA was present within the neoplastic cells, but was not present within the surrounding normal epithelium. This strengthens the association between PV infection and EPSCCs; however, it still remains possible that the location of the PV DNA was due to a more permissive environment within the neoplastic cells and the available evidence is not sufficient to implicate EcPV-2 as a cause of EPSCCs. The use of ISH in this study also confirmed that the PV DNA detected in the 6 samples in which ISH was successful was not due to cross-contamination during the preparation of the samples for PCR.

We used IHC to detect the PV L1 antigen, which is produced late in the viral life cycle and is present in cells that contain actively replicating virus. In our experiment, one

EPSCC was found to contain anti-PV L1 protein immunostaining. This immunostaining was confined to the neoplastic cells, which indicates that viral replication can occur within an EPSCC. In human PV-induced cancers, viral replication is typically prevented by integration of the PV DNA into the host DNA.[16] Therefore, the presence of PV replication suggests that, if EcPV-2 does cause EPSCCs, it does so by a mechanism that is not dependent on viral integration. This would be similar to equine sarcoids, which are PV-induced skin tumors of horses that have been shown to develop without integration of the PV DNA into the host cell.[2] It is also interesting to note that immunostaining highlighted viral cytopathic changes in a small number of cells. Viral cytopathic changes also occur with active viral replication and further confirm that viral replication was occurring in one of the EPSCCs. The presence of viral replication indicates active infection, but does not suggest either a causative or an 'innocent bystander' role for PVs in EPSCC development.

No viral replication was detected by IHC in 8 EPSCCs that contained detectible PV DNA. This suggests that non-replicative latent PV infections can exist within EPSCCs. In human cervical cancers, evidence suggests that PV replication decreases as infected cells become less differentiated.[10] Therefore, it may be that the one EPSCC that contained replicating PV had remained better differentiated than the other EPSCCs in the study, although this neoplasm did not demonstrate different or additional features on histological examination. Alternatively, the ability to detect antigens by IHC is dependent on the condition of the sample. It is possible that PV replication was present in other EPSCCs, but the PV antigens had been damaged by different fixation conditions and so were not detected using IHC.

A disadvantage of both the ISH and the IHC techniques used in our study was that neither were specific for EcPV-2. Although we detected PV DNA and PV antigen within the EPSCCs, we cannot confirm that these were from EcPV-2. Therefore, it remains possible that a PV type that was not amplified by our PCR primers was detected by these additional tests. Furthermore, the ISH probes and the antibody we used have not been shown to detect EcPV-2; therefore, if neither the ISH probes nor the antibody used for IHC had sufficient affinity for the EcPV-2 DNA or EcPV-2 L1 antigen, then we

may have failed to detect EcPV-2 in some samples. Also, neither ISH nor IHC were performed on samples that did not contain PV DNA amplifiable by the PCR primers used. This was due to the high cost of performing these tests.

In addition to the nine EPSCCs, EcPV-2 DNA was also amplified from a sample of chronic ulcerative and granulomatous balanoposthitis of unknown cause. The presence of EcPV-2 in a non-SCC lesion suggests two possibilities. One is that the ulcerative and granulomatous balanoposthitis was caused by EcPV-2 infection and may be a precursor lesion to EPSCC, similar to a precancerous plaque. Alternatively, the EcPV-2 in this horse may have been an asymptomatic infection unconnected to the inflammatory lesion. Further investigation of the role of EcPV-2 in non-cancerous lesions, and of its ability to cause asymptomatic infection, will help to clarify this issue.

Papillomaviral DNA sequences and PV antigens have previously been detected in small numbers of equine penile squamous papillomas and precancerous plaques. [17-19,23] These are considered likely to be precursor lesions to EPSCCs. One study reported that the PV sequences detected in three equine genital papillomas were from a PV type other than EcPV-1.[19] The consistent detection of EcPV-2 in the our own study suggests that the PV type detected in earlier studies was also likely EcPV-2; however, as the sequences detected in the previous studies were not deposited in GenBank they cannot be compared to the sequences detected in our study.

Three methods were used to detect the presence of PV infections within the samples in this experiment. Of these, PCR detected PV DNA in 10 samples, ISH in 6 samples, and IHC detected PV antigen in only one. The apparent difference in sensitivity between PCR and ISH may be due to the use of template DNA for PCR that is extracted from the entire 10 µm section of tissue. In contrast, only PV DNA that is present on the surface of the section will be exposed to the ISH probes used in ISH. Therefore, it may be that the four EPSCCs that contained amplifiable PV DNA that was not detected by ISH only contained small quantities of PV DNA, which may have been present within the 10 µm section from the block used for PCR but was not present on the exposed surface of the slide used for ISH. Our results also suggest that IHC is less sensitive than

PCR for the detection of PV. This is likely because IHC depends on the presence of viral replication and intact PV antigens. Overall, while ISH and IHC allow localization of PVs within a lesion, the evidence from our study suggests that PCR is a more sensitive method for detecting PV DNA. Of the two consensus primer sets used for PCR, only the MY09/11 set amplified PV DNA. Our observations suggest that the FAP59/64 primer set has a low affinity for EcPV-2 and so is of limited use for investigating this PV.

The average age of the horses with EPSCCs was higher than the average age of the horses with non-SCC penile lesions. This is unsurprising as neoplasia is generally a disease of old age. Since the horses with EPSCCs were older than those without EPSCCs, the possibility that these neoplasms more frequently contained PV DNA because PV infections are more common in older horses had to be considered. However, the age of the horses with PV infection did not differ from the age of the horses without PV infection. Thus, the more frequent detection of PV DNA in horses with EPSCCs was not simply because PV infections are more common in older horses.

## 2.5 Chapter Summary

In the experiments described in this chapter we detected PV DNA significantly more frequently in EPSCCs than in non-SCC penile lesions. *In situ* hybridization revealed that the PV DNA was located in the nuclei of the neoplastic cells, and immunohistochemistry revealed that viral replication was rarely present within the EPSCCs. EcPV-2 was the only PV type detected within the EPSCCs. These results suggest a possible role for EcPV-2 in the development of equine penile cancer. Such a causative association is plausible, particularly given that approximately half of human penile SCCs are caused by PV infection,[4,6,20] but it is not yet proven. Based on the currently available evidence, the alternative possibility that EPSCCs contain more PV than non-SCC penile lesions because they provide a more permissive environment for PVs cannot be excluded.

The results and conclusions from this chapter were published in the article:

*Knight CG, Munday JS, Peters J, Dunowska M:* ***Equine Penile Squamous Cell Carcinomas Are Associated With the Presence of Equine Papillomavirus Type 2 DNA Sequences****. Veterinary Pathology 48: 1190-1194, 2011.*

The manuscript was submitted in May, 2010 and, during the revision process, two manuscripts describing similar findings were published.[21,28] These were:

1. *Scase T, Brandt T, Kainzbauer C, Sykora S, Bijmholt K, Hughes K, Sharpe S, Foote A:* ***Equus caballus papillomavirus-2 (EcPV-2): An infectious cause for equine genital cancer?*** *Equine Veterinary Journal 42: 738-745, 2010*

2. *Vanderstraeten E, Bogaert L, Bravo IG, Martens A:* ***EcPV2 DNA in equine squamous cell carcinomas and normal genital and ocular mucosa.*** *Veterinary Microbiology 147: 292-299, 2011*

All three manuscripts report an association between EcPV-2 and equine genital cancer. The additional findings reported by these two papers will be discussed in later chapters of this thesis.

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# CHAPTER THREE

# Refinement of Diagnostic Techniques for EcPV-2

## 3.1 Introduction

The experiments described in chapter 2 showed that EcPV-2 is significantly associated with equine penile SCCs (EPSCCs). These findings, described in the resulting publication,[19] were supported by similar reports from two other research groups.[26,33] To investigate the nature of this association further, a quantitative PCR assay that would allow us to correlate lesion severity and viral load was desirable. However,

before a qPCR assay could be developed several diagnostic and experimental techniques needed refinement. This was the focus of the work described in this chapter.

In chapter 2, EcPV-2 was detected in archived formalin-fixed and paraffin-embedded (FFPE) tissue samples obtained from the United States. The use of FFPE samples allowed us to obtain sufficient numbers of equine neoplastic penile lesions to test the hypothesis that EPSCCs are associated with PV infection. However, prolonged formalin-fixation causes fragmentation of the DNA within tissues,[2] which may reduce or prevent amplification by PCR. These effects are partly due to the absence of intact template molecules of sufficient integrity and partly due to inhibition of DNA polymerase by fragmented DNA in FFPE tissue.[9] Unfixed (fresh or frozen) tissues should, therefore, be used whenever possible to provide the best samples for PCR amplification. This is especially true of any EcPV-2-positive sample that is used as a positive control. Obtaining and preparing a high quality EcPV-2 positive control was problematic because EcPV-2 had never been identified in New Zealand. The first objective of the work described in this chapter was identification of an EcPV-2-infected horse that could provide fresh tissue samples that could be used to generate positive controls suitable for both conventional and quantitative PCR-based experiments. In addition, identification of an EcPV-2 positive horse in New Zealand would facilitate importation of potentially EcPV-2-containing tissues from other countries in the future because this virus would no longer be exotic to New Zealand.

A second objective of the work described in this chapter was to develop an EcPV-2-specific PCR primer set to replace the use of consensus primer sets. Although consensus primers were used in chapter 2, their use had both advantages and disadvantages. The advantage of using consensus primers in PV diagnosis is that they allow detection of both known and potentially novel PV sequences.[15] This was beneficial in the work described in chapter 2 since the genome of EcPV-2 was unknown. However, consensus primers also have certain disadvantages. First, they are less sensitive than specific primers, and a PV present at low levels may not be detected.[7] Second, they require sequencing of PCR products for identification of the

specific PV type(s) present, which can be costly and time-consuming. Third, if infection is mixed, a PV type present at low levels in a lesion may be overshadowed during amplification by a PV type present at high levels.[7] Alternatively, a PV present at low levels but with a high affinity for the consensus primers may overshadow a PV that is present at high levels but has a lower primer binding affinity.[7] In contrast, specific primers are exactly complementary to regions of the genome of interest and therefore have a higher binding affinity. This improves sensitivity and, in addition, reduces non-specific binding or binding to PVs that are not of interest. Only one PV type (EcPV-2) was amplified from the 40 equine penile samples tested in chapter 2. This suggested that EcPV-2 was the only PV present in these tissues, and that further use of consensus primers was unnecessary.

The third objective of the work described in this chapter was the identification of a high quality EcPV-2 positive control sample suitable for use in upcoming experiments. This would replace the bovine fibropapilloma sample that we had used previously.

Development of non-invasive methods of diagnosis of EcPV-2 infection was the final objective of the work described in this chapter. A means of diagnosing EcPV-2 that did not require biopsy or necropsy would facilitate obtaining the large numbers of samples needed to compare the prevalence of EcPV-2 infection in healthy and SCC-affected horse populations. Since owner and veterinarian compliance would be necessary in future prevalence studies, in addition to being non-invasive the method would also need to be inexpensive and easy to administer.

The materials, methods and results for each of the four objectives achieved in the work described here are presented next (sections 3.2 to 3.5), and a combined discussion (section 3.6) follows at the end of the chapter.

## 3.2 Objective 1: Identification of a Horse Infected with EcPV-2

Identification of a New Zealand horse infected with EcPV-2 was important for several reasons. First, identification of an infected horse would demonstrate that the virus is not exotic to New Zealand, which would simplify future importation of case material from other countries. Second, it would allow collection of fresh samples and avoid the DNA degradation caused by formalin fixation and prolonged storage. Third, identification of a living horse infected with EcPV-2 would permit repeated sampling and observation of lesions.

### 3.2.1 Materials and Methods

To identify an EcPV-2-infected horse, local equine practitioners were invited to a presentation describing the results presented in chapter 2 and asking for their help in identification of horses with penile lesions that could potentially be caused by PVs. These included SCCs, precancerous plaques, and papillomas. One veterinarian contacted me after seeing "Horse A", a 9-year-old Standardbred cross gelding with a several-month history of excessive preputial debris and infrequent protrusion of the penis from the sheath.

*Initial examination*

The horse was sedated routinely and the penis exteriorized. Extending distally from the preputial ring, both the free part of the penis and the glans penis were covered by approximately 100 discrete to confluent, raised, smooth-surfaced, broad-based masses up to 2 cm in diameter (figure 3.1). These were largest and most numerous proximally, leaving no normal skin between masses, but were discrete and smaller distally. Ulceration was not present. Multifocally, the masses produced hard, keratinized horns up to 1.5 cm in length (arrow in figure 3.1).

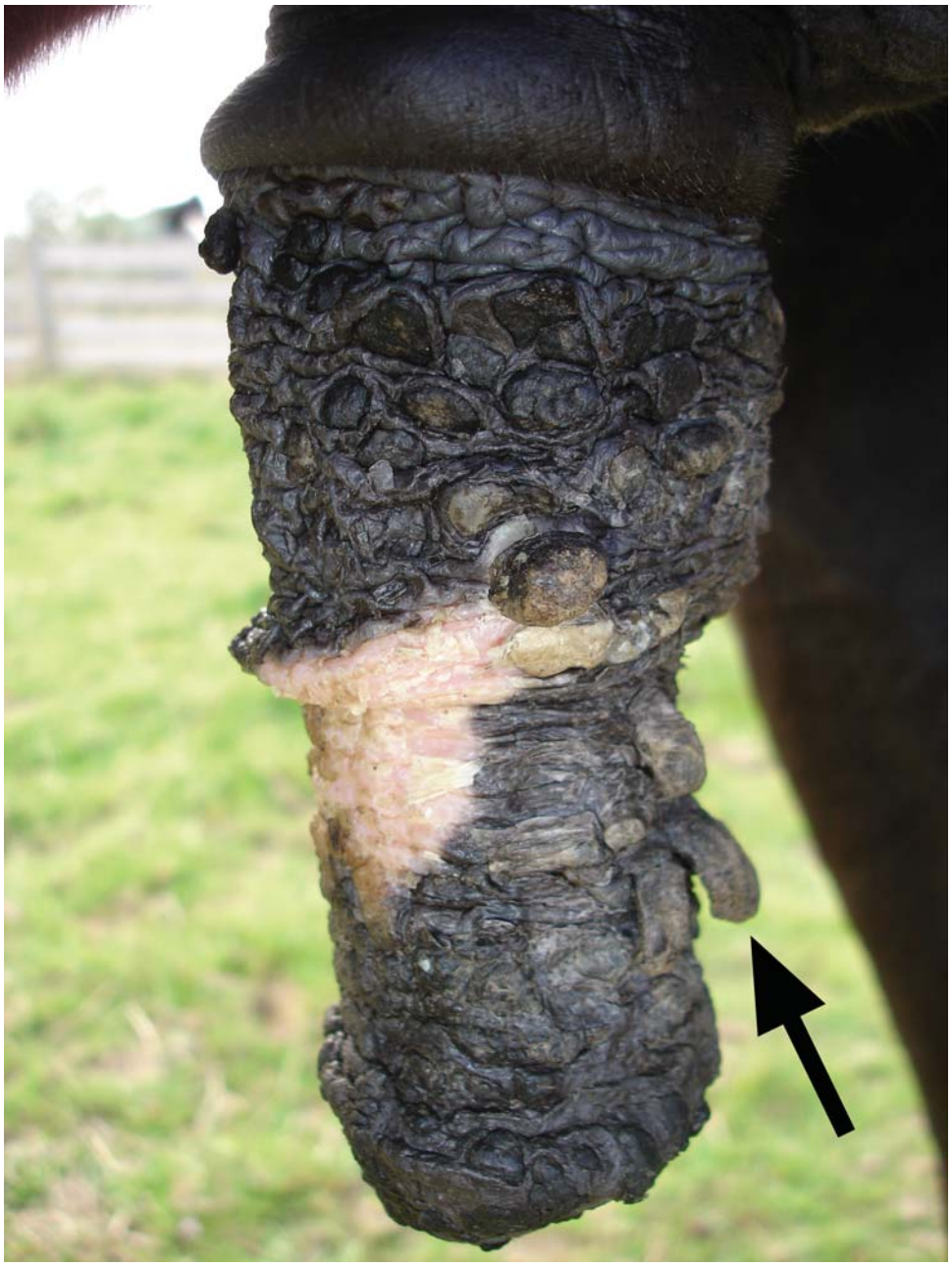

*Figure 3.1. Penile papillomatosis in a 9 year old gelding. Distal to the preputial ring the free part of the penis and glans penis are covered by several dozen discrete to confluent masses up to 2 cm in diameter. The arrow indicates a keratinized horn.*

Biopsy of three representative masses was performed under local anesthesia. Samples were fixed in formalin and processed routinely for histological examination.

Biopsy samples were tested for EcPV-2 using the methods described in chapter 2. DNA was extracted from all three FFPE samples and PCR was used to amplify PV DNA. As in chapter 2, two consensus primer sets were used: the MY09/MY11 primer set, which detects mucosal-adapted PVs and the FAP59/FAP64 primer set, which detects

cutaneous-adapted PVs. Amplified PV DNA was sequenced and results were compared with known sequences from GenBank using the basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov).

*Revisit*

Sixteen months after the original diagnosis, Horse A was revisited to evaluate progression of disease and obtain further tissue samples. The horse was sedated and examined as before.

Three representative papillomas were chosen for biopsy. Samples were then taken in the same way as described previously, with one exception: prior to each biopsy, Scotch tape was used to remove surface squames and debris ("stripping"), according to a previously published method used in humans.[10] Briefly, a strip of tape was attached 5 times to the surface of the papilloma to be biopsied, then discarded. The procedure was repeated with a second strip of tape. After two tape stripping sessions per lesion the three papillomas were biopsied. Following biopsy, each papilloma was bisected using a sterile blade. Half was frozen at -80°C for later use, and the remainder was fixed in 10% formalin. All three formalin-fixed papillomas were later processed for routine histologic examination and IHC and ISH were performed on one papilloma using the methods described in chapter 2.

### 3.2.2 Results

*Initial examination*

All three of the biopsied masses had a similar histologic appearance. Each consisted of well-demarcated areas of epidermal hyperplasia and hyperkeratosis, with retention of orderly keratinocyte maturation and no penetration of the underlying basement membrane (figure 3.2a). Keratinocytes of the stratum granulosum frequently contained giant keratohyalin granules and increased amounts of wispy grey-blue cytoplasm, consistent with viral cytopathic change (figure 3.2b). The nuclei of these cells were pale and vesicular and occasionally contained eosinophilic intranuclear bodies consistent with viral inclusions. Deeper within the epidermis, numerous keratinocytes had dark, condensed nuclei surrounded by a clear cytoplasmic halo

(koilocytosis). A diagnosis of papillomatosis with a presumed papillomaviral etiology was made.

Papillomaviral DNA sequences were amplified from all three penile biopsy samples using the MY09/MY11 consensus primer set. As in chapter 2, no PV DNA sequences were amplified using the FAP59/FAP64 consensus primer set. Both primer sets amplified PV DNA sequences from the bovine fibropapilloma positive control but not from the non-template control. Papillomaviral DNA from all three penile biopsy samples was 100% identical to EcPV-2 (GenBank accession number EU50312) using BLAST.

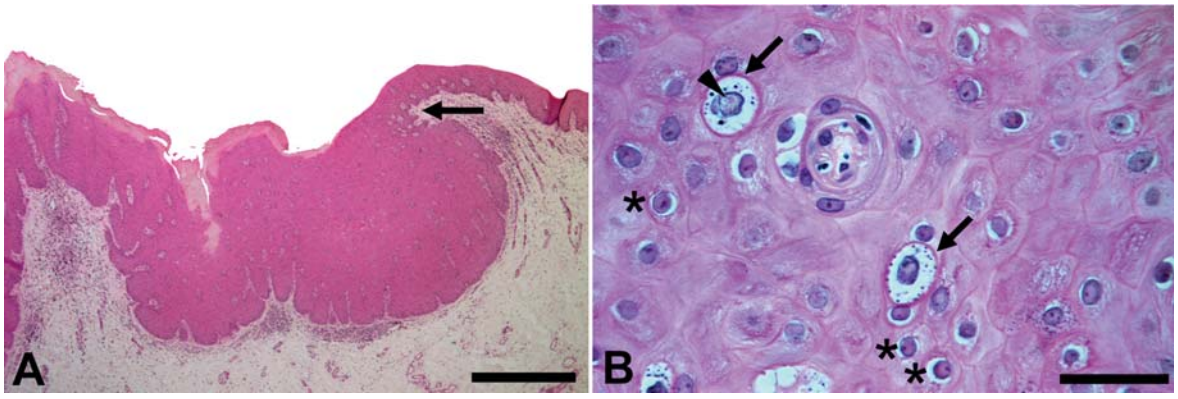


*Figure 3.2. (A) Photomicrograph of equine penile papilloma showing a well-demarcated focus of epidermal hyperplasia. The junction of normal and hyperplastic epidermis is sharply defined (arrow). The basement membrane is intact, and keratinocyte maturation is orderly. Hematoxylin & eosin. Scale bar represents 200 µm. (B) Photomicrograph of equine penile papilloma. Keratinocytes within the stratum spinosum and stratum granulosum contain evidence of viral effects, including expanded, pale cytoplasm with giant keratohyalin granules (arrows), koilocytosis (asterisks) and occasional intranuclear inclusions (arrowhead). Hematoxylin & eosin. Scale bar represents 30 µm.*

*Revisit*

The penile lesions had neither regressed nor progressed to SCC in the 16 months between the initial examination and the revisit (figure 3.3).

Histologically, all three penile papillomas were similar to those biopsied 16 months earlier. There was no microscopic evidence of either regression or progression of lesions, and viral cytopathic changes had remained the same.

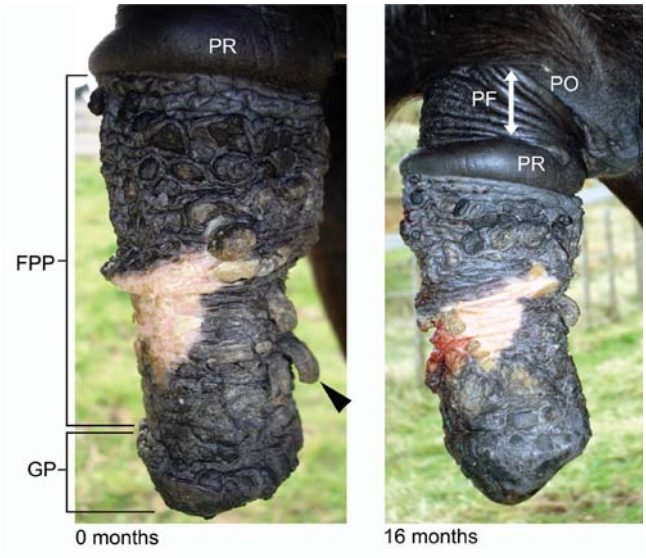


*Figure 3.3. Penis of horse with penile papillomatosis. Photographs are taken 16 months apart and demonstrate lack of either ulceration or lesion progression. The hemorrhage in the right hand photograph is due to biopsy. Multifocally, keratinized horns protrude up to 1.5 cm from the skin surface (arrowhead). FPP, free part of the penis; GP, glans penis; PF, preputial fold; PO, preputial orifice; and PR, preputial ring.*

Using IHC, PV antigen was demonstrated within the nuclei of several hypertrophied keratinocytes, predominantly those of the stratum granulosum (figure 3.4a). Papillomaviral DNA was also visualized within hypertrophied keratinocytes using ISH. The PV DNA appeared confined to the hypertrophied cells and was not detected within keratinocytes in the deeper layers of the epidermis (figure 3.4b).

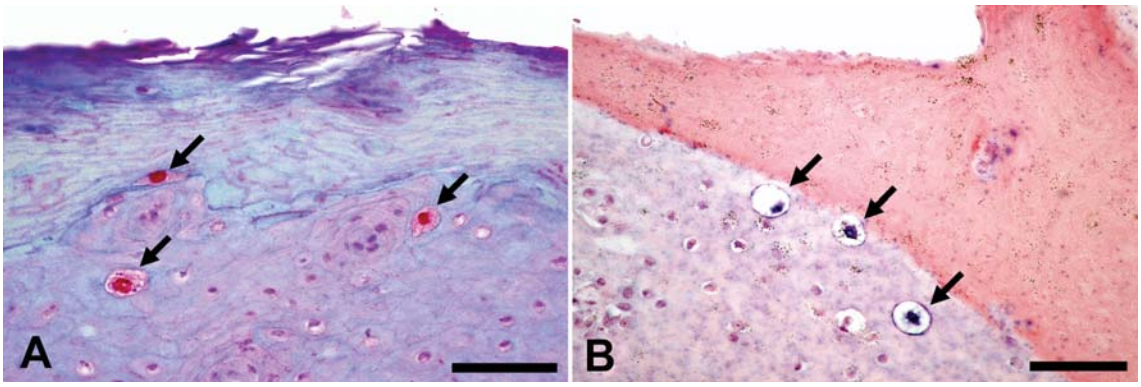


*Figure 3.4. Photomicrograph of equine penile papilloma. (A) Immunohistochemistry demonstrates papillomaviral antigen within the nuclei of several enlarged keratinocytes (arrows). Mouse monoclonal antibody raised against sodium dodecyl sulfate-disrupted bovine PV type 1 that detects multiple papillomavirus types. Hematoxylin counterstain. Scale bar represents 60 µm. (B) In situ hybridization demonstrates papillomaviral DNA within the nuclei of several enlarged keratinocytes (arrows). Hybridization with a generic antisense probe for papillomaviruses. Red Stain II counterstain. Scale bar represents 60 µm.*

## 3.3 Objective 2: Designing and Testing EcPV-2-Specific PCR Primers

### 3.3.1 Materials and Methods

As discussed at the end of chapter 2, two articles describing very similar findings regarding EcPV-2 infection and EPSCCs were published in 2010.[26,33] The specific primer set used in one of these studies (Vanderstraeten *et al.*[33]) was used in the work described here, along with a new primer set that we designed, which also specifically amplifies EcPV-2. The reason for using two primer sets was to confirm that the newly designed primer set performed as well as a set already confirmed to work.

The primer pair used by Vanderstraeten *et al*. (VDS.E1.F and VDS.E1.R) amplifies a 679 base pair segment of the EcPV-2 E1 gene. The sequences of the forward and reverse primers were 5'-GCG GAC TGC GCG TCA CAA GAG GGG C-3' and 5'-ACG CAA GCA CCA CCC ACT GCT TGG CA-3', respectively. For our study, a commercial 2X master mix (FastStart; Roche) was used to prepare 10 µl reaction volumes with a final concentration of 0.8 µM for each primer. Amplification was carried out under the following cycling conditions: 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 20 seconds, 65°C for 20 seconds, and 72 °C for 1 minute. The final extension step was at 72 °C for 10 minutes. Amplicons underwent electrophoresis in a 1% agarose gel containing ethidium bromide and bands containing the amplified fragment were visualized under UV light. Amplicons were sequenced, as described previously.

The specific primer set that we designed (named MD.L1[A]) was designed using commercially available bioinformatics software (Primer 3 version 2.3.4).[32] It amplifies a 104 base pair sequence of the EcPV-2 L1 gene. A commercial 5X master mix (AccuMelt™ HRM SuperMix; Quanta Biosciences) was used to prepare 10 µl reaction volumes with a final concentration of 0.4 µM for each primer. Amplification was carried out under the following cycling conditions: 95 °C for 10 minutes, followed by 35 cycles of 95 °C for 10 seconds, 65°C for 10 seconds, and 72 °C for 10 seconds. The final extension step was at 72 °C for 10 minutes. Amplicons underwent electrophoresis in a

1.5% agarose gel containing ethidium bromide and bands containing the amplified fragment were visualized under UV light. Amplicons were sequenced as previously described.

Using the two primer sets described above, several samples were tested and the results compared. All PCR reactions were carried out in duplicate or triplicate. Four samples that had been previously determined to contain EcPV-2 DNA using the MY09/MY11 consensus primer set and sequencing (data not shown) were used as positive controls. One of these was DNA extracted from a frozen penile papilloma from Horse A, collected in section 3.4.1 of this chapter. The other three were DNA extracted from samples from three additional EcPV-2 positive horses that were identified with the help of local practitioners (a laryngeal SCC lesion, a penile papilloma, and a vulvar swab from a mare with coital exanthema-like lesions). Negative controls were DNA extracted from several equine genital samples that had been previously shown to be PV negative using the MY09/MY11 consensus primer set. As an additional negative control, DNA extracted from a bovine cutaneous fibropapilloma known to contain BPV-1 was used. For non-template controls, template DNA was replaced by an equivalent volume of water.

### 3.3.2 Results

PV DNA sequences were amplified from all four positive control samples using both specific primer sets (VDS.E1 and MD.L1[A]). When visualized under UV light, there was no appreciable difference between primer sets in the intensity of the bands obtained. All amplified DNA sequences were sequenced as previously described and confirmed to be 98-100% similar to EcPV-2 (GenBank accession number EU50312). Papillomaviral DNA sequences were not amplified from any negative control or non-template control sample.

## 3.4 Objective 3: Creation of a Positive Control Sample for EcPV-2 Testing

Sixteen months after the original diagnosis of EcPV-2 infection, Horse A was revisited to assess the progression of the disease and obtain further tissue samples. During PCR of these samples, it was noted that the band of amplified DNA from the papilloma biopsy sample taken 16 months earlier was fainter than the bands of DNA amplified from newly acquired-samples from the same horse (lanes 2 and 7, respectively, in figure 3.6). This difference was attributed to DNA degradation caused by repeated freezing and thawing of the older sample. Thus, we decided it was necessary to create a high quality positive control sample with known DNA content that could be divided into aliquots to avoid the need for repeated freezing and thawing.

### 3.4.1 Materials and Methods

DNA from a frozen penile papilloma biopsy sample and from a frozen laryngeal SCC lesion was amplified by PCR using the MD.L1[A] primer set as described in section 3.3.1. Both samples had been previously shown to be EcPV-2 positive. Bands containing the amplified EcPV-2 DNA sequences were cut from the agarose gel under UV light using a sterile scalpel blade. The time of exposure to UV light was kept to a minimum to reduce fragmentation of amplified DNA. Commercially available DNA gel extraction spin columns (Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Column) were used to collect the DNA from the excised bands following the manufacturer's instructions. Excess agarose was trimmed from all six sides of the excised gel bands and the bands were then placed into the filter cup of the column. The column with the gel band inside was placed in a cup of liquid nitrogen for 1-2 minutes until the gel was frozen. The column was then centrifuged at 13,000 x g for 3 minutes at room temperature, which transferred the purified DNA into a collection tube, leaving residual agarose debris within the filter cup. The DNA content of the purified samples was analyzed using a spectrophotometer (Nanodrop; Thermo scientific instruments). Samples were divided into 10 µl aliquots and frozen stored in 0.2 ml PCR tubes.

### 3.4.2 Results

The DNA content and 260/280 ratios of the purified EcPV-2 DNA sequences from the two tissues used were:

| Sample source | DNA content (ng/µl) | A260 | A280 | 260/280 |
|---|---|---|---|---|
| Equine penile papilloma | 99.14 | 1.983 | 1.688 | 1.17 |
| Equine laryngeal SCC | 8.04 | 0.161 | 0.166 | 0.97 |

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Based on these values, the sample obtained from the equine penile papilloma was chosen for use as a positive control in future experiments.

## 3.5 Objective 4: Testing a Non-Invasive Method for Diagnosis of EcPV-2 Infection

### 3.5.1 Materials and Methods

When Horse A, the EcPV-2-positive horse described in section 3.2, was revisited 16 months after the initial EcPV-2 diagnosis to confirm continued infection we also used this opportunity to test a non-invasive method of diagnosis. While the horse was sedated for examination, but prior to biopsy, three swabs from different locations on the papillomatous surface of the penis were collected in the following way: the tip of a sterile cotton-tipped swab was moistened in sterile saline and drawn back and forth 15 times over an affected area of approximately 5 cm x 5 cm. The swab tip was then cut off and placed into 500 µL of sterile saline in an Eppendorf tube and frozen at -20°C until used.

NOTE: For the following paragraphs, the letters a-g in parentheses refer to figure 3.5. In all cases, where DNA was extracted, a DNeasy blood and tissue kit (Qiagen, GmbH) was used according to the manufacturer's instructions.

To evaluate the effectiveness of the non-invasive sampling techniques, DNA was extracted from one frozen papilloma as an internal control (a). All three penile swabs were then evaluated as follows: After thawing, each swab tip was removed from the saline in which it was stored and placed into 500 µl of phosphate-buffered saline in a 1.5 ml Eppendorf tube (b). The tube and tip were vortexed for 15 seconds to dislodge cells from the swab tip and resuspend them, then the swab tip was removed and discarded (c). Following this step the resulting cell suspension was processed in three different ways for comparison:

1. 50 µl of cell suspension were removed and added to a new Eppendorf tube (d) and 2 µl of this suspension were used directly (without DNA extraction) as template DNA in the PCR.
2. 200 µl of the original cell suspension were removed and added to a new Eppendorf tube, and 20 µl of proteinase K added (e). After 10 minutes digestion at 56°C, DNA was extracted from the swab suspension and used in the PCR.
3. The remaining 250 µl of original cell suspension were centrifuged at 5900 g for 3 minutes and the supernatant aspirated and discarded (f). The residual tissue pellet was resuspended by vortexing in 200 µl of phosphate-buffered saline and 20 µl of proteinase K added (g). After 10 minutes digestion at 56°C, DNA was extracted from the swab suspension and used in the PCR.

PCR was performed using the MY09/MY11 consensus primer set and all PCR reactions were carried out in duplicate. For positive controls, DNA extracted from the EcPV-2 positive papilloma biopsy sample taken 16 months earlier from the same horse was used. For negative controls, template DNA was replaced by an equivalent volume of water. Amplified PV DNA was sequenced and compared with known sequences from GenBank using BLAST, as previously described.

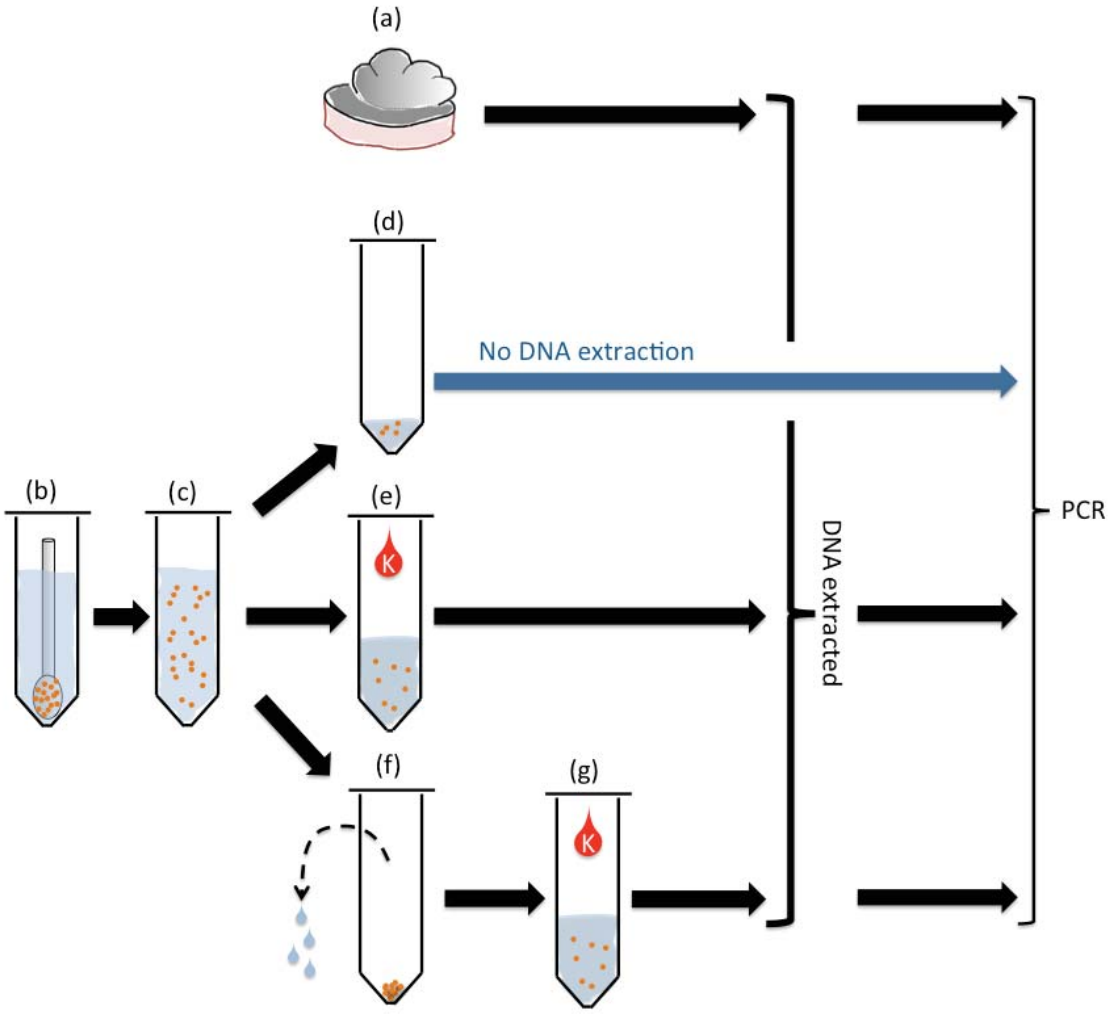


*Figure 3.5. Four different PCR template preparation methods used for penile lesions from a horse with EcPV-2-induced penile papillomatosis.* ***(a)*** *DNA was extracted from a frozen papilloma biopsy sample;* ***(b)*** *Each thawed swab was suspended in 500 µl PBS solution;* ***(c)*** *The tube was vortexed and the swab tip discarded;* ***(d)*** *50 µl of cell suspension were placed in a new tube and 2 µl used directly for PCR;* ***(e)*** *200 µl of cell suspension were placed in a new tube and 20 µl of proteinase K added;* ***(f)*** *The remaining 250 µl of cell suspension were centrifuged and the supernatant discarded;* ***(g)*** *The tissue pellet was resuspended in 200 µl of PBS solution.* ***(K)*** *Proteinase K.*

### 3.5.2 Results

Papillomaviral DNA sequences were amplified from DNA extracted from the frozen papilloma and frozen swabs. Regardless of the type of template DNA used, gel bands were strongly positive for PV DNA sequences (figure 3.6). The PV DNA sequences from all samples were between 98 and 100% similar to the published genome of EcPV-2 (GenBank accession number EU50312).

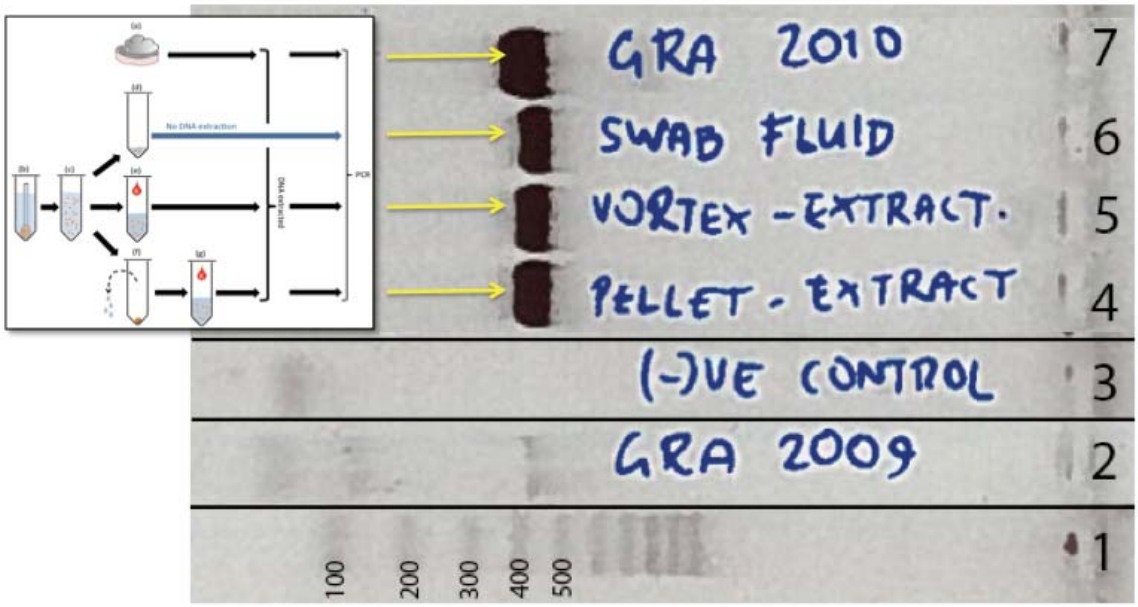


*Figure 3.6. Representative PCR results from samples taken from a horse with EcPV-2-induced penile papillomatosis. Using the MY09/MY11 consensus primer set PV DNA is strongly amplified from swabs prepared using three different methods (lanes 4-6) and from a penile papilloma biopsy sample (lane 7). All bands are strongly positive for PV DNA and all lie at the expected 450 base pair height on the gel. The inset shows the template preparation methods from figure 3.5 that correspond to each lane in this figure. See figure 3.5 for more detail.*

***Lane 1**: DNA ladder; **Lane 2**: Positive control; **Lane 3**: Negative control; **Lane 4**: Cell suspension made from penile swab, with cells concentrated by centrifugation and DNA extracted; **Lane 5**: Cell suspension made from penile swab, with DNA extracted; **Lane 6**: Cell suspension made from penile swab, with no DNA extraction process used; **Lane 7**: DNA extracted from penile papilloma biopsy sample.*

## 3.6 Discussion

The work described in this chapter resulted in the identification of a living EcPV-2-positive horse in New Zealand, the development of EcPV-2-specific PCR primers, and the creation of a high quality positive control sample for use during PCR. In addition, we verified that several different non-invasive methods could be used to diagnose EcPV-2 infection in living horses. This work laid the foundation for the development of a quantitative PCR assay for EcPV-2, which is described in chapter 4. Specific considerations relative to each of the objectives of the work described here are discussed in the following paragraphs.

### 3.6.1 Objective 1: Identification of a Horse Infected with EcPV-2

A living EcPV-2-infected horse (Horse A) was identified in Palmerston North, New Zealand. Histologic and gross features of this horse's penile lesions were consistent

with the clinical classification of papillomatosis, which is defined as a disease state characterized by the development of multiple papillomas.[1] To our knowledge, equine penile papillomatosis had never been described previously and therefore, before being able to confirm this diagnosis, it was necessary to rule out other proliferative lesions of the equine penis. These included fibropapilloma, sarcoid, precancerous plaque, and SCC. Each of these was ruled out based on gross, behavioral, and microscopic differences from the lesions in Horse A. First, the lesions in this horse were multifocal to confluent. While fibropapillomas may also have a multifocal distribution,[12] lesions of the other three diseases are typically present singly or in low numbers. Second, the lesions in the EcPV-2-infected horse did not change over 16 months of observation, while over a similar time period precancerous plaques would be expected to progress to SCCs, and SCCs would be expected to become invasive and cause ulceration and desmoplastic thickening of the penis.[3,11,16] Finally, the histologic features of the lesions of Horse A were inconsistent with those of the other diseases considered. Unlike SCCs, there was retention of orderly keratinocyte maturation and no penetration of the underlying basement membrane. In contrast to penile fibropapillomas[12] and sarcoids[21] there was a lack of dermal spindle cell proliferation or epithelial rete ridge formation. Although histologic characteristics of the lesions in Horse A did share certain features with those of precancerous plaques, their number did not. For these reasons the diagnosis of penile papillomatosis was supported.

The presence of EcPV-2 in lesions was confirmed by PCR and sequencing. In order to show that the virus was not a simple contaminant or commensal, ISH and IHC were also performed. Both tests localized PV DNA or antigen to the nuclei of keratinocytes within lesions, predominantly within the stratum granulosum. These findings showed that the PCR results were not due to contamination. In addition, the location of the PV DNA or antigen was the same as the location of the viral cytopathic changes and inclusions seen on H&E-stained slides. This showed that these viral changes were caused by a PV rather than by some other virus and suggested that EcPV-2 was involved in lesion development, rather than being a simple bystander.

Horse A was the first reported case of equine penile papillomatosis.[20] As such, this case also represents a novel clinical manifestation of EcPV-2 infection. This adds to the limited knowledge about the spectrum of EcPV-2-associated diseases; prior to this report the only lesions reported to be associated with EcPV-2 infection were SCCs,[19,26] solitary papillomas,[26] and a single case of balanoposthitis.[19]

This case also adds to the evidence that equine genital papillomas are caused by EcPV-2. Although an unknown PV had been demonstrated several times since 1984 in equine genital papillomas by IHC,[17,27,28] Southern blot hybridization,[22] and PCR,[23,34] the specific PV in these papillomas was not identified until 2010.[26] Our case is, therefore, only the second time in which the specific PV type has been identified in equine genital papillomas. Because the same PV was amplified from genital papillomas in both our report and the 2010 study[26] it is likely that EcPV-2 is the virus that causes these lesions. Since consensus primers were used in our study, any type of PV present should, in theory, have been amplified; therefore, it is significant that EcPV-2 was the only PV found in the samples. This supports earlier hypotheses that EcPV-1 is not associated with genital papillomas in horses.[13,23]

Additionally, this is the first time that EcPV-2 has been detected in repeated samples taken from the same horse over an extended time period. Both previous EcPV-2 studies used tissues taken at a single time point, with no follow-up.[19,26] In contrast, Horse A was followed for 16 months, during which lesions neither regressed nor progressed, and EcPV-2 infection remained present. This shows that EcPV-2 is able to infect some horses persistently instead of being cleared by the immune system. It also suggests that chronic EcPV-2 infection does not inevitably lead to SCC development, although this cannot be confirmed without following the horse for a longer period. In women, it may take decades for cervical neoplasms to develop following infection with high-risk HPVs[36] and it is possible that a similar situation exists in horses following EcPV-2 infection. Horse A's lesions and EcPV-2 infection status should be monitored regularly.

The distribution of the lesions in this horse suggests that EcPV-2 has a specific anatomic tropism. The proximal extent of the papillomatosis was sharply demarcated by the preputial ring (figures 3.1 and 3.3). The preputial ring (figure 3.7) is unique to equids and is distinct from the preputial orifice, which is present in all male domestic mammals.

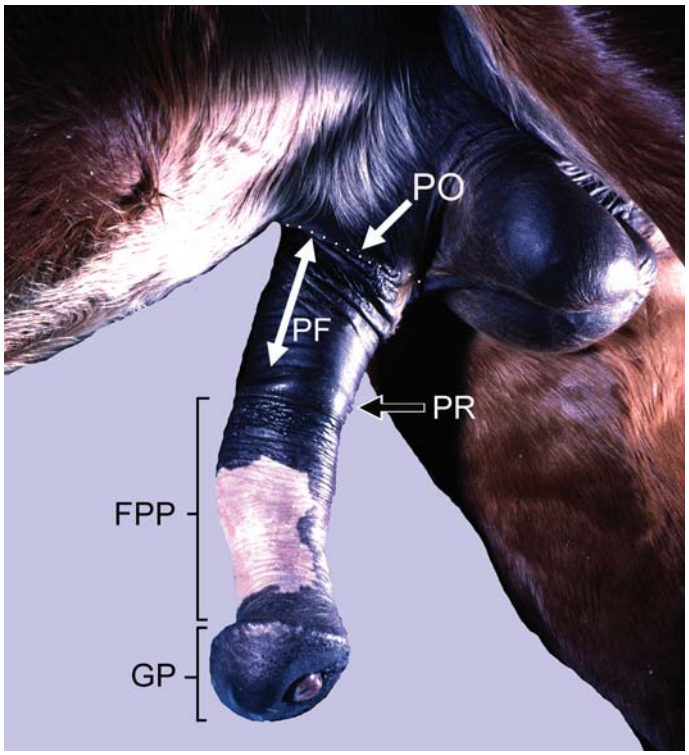


*Figure 3.7. Anatomy of the normal equine penis for comparison with figures 3.1 and 3.3. Note that the preputial ring is the junction between the preputial fold and the free part of the penis. Abbreviations: FPP, free part of the penis; GP, glans penis; PF, preputial fold; PO, preputial orifice; and PR, preputial ring. Image courtesy of Cornell University College of Veterinary Medicine.*

In the retracted equine penis, the skin proximal to the preputial ring is in constant, intimate contact with the skin distal to the ring and, presumably, each is exposed to any infectious agent carried by the other. Therefore, the restriction of the papillomatosis to the skin distal to the ring suggests that EcPV-2 has a specific tropism for the free part and glans of the penis. It also suggests that skin proximal to the preputial ring may have different properties to the more distal skin of the penis, despite there being no gross or histological difference. On a practical level, it suggests that sampling the preputial orifice of a retracted equine penis may be of limited value in the diagnosis of EcPV-2 infection, and that the glans and free part of the penis need

to be exteriorized for sampling if false negatives are to be avoided. This is an important consideration when designing future prevalence studies using live horses.

Horse A was the first horse with EcPV-2 infection identified in New Zealand. Previously EcPV-2 had only been reported in the USA, England, Austria, and Australia. [19,26] While worldwide distribution of EcPV-2 is likely because of international transport of horses, it is still worthwhile confirming its presence in a particular country. This knowledge eases barriers to importation of EcPV-2-infected tissues into New Zealand for research. It also allows large-scale epidemiologic surveys to be conducted and permits investigation of geographic variation in the EcPV-2 genome sequence.

Finally, this case raises the question of how Horse A became infected by EcPV-2. Papillomaviral infections are typically spread between individuals through close contact, with infective PV particles from sloughed squames gaining entry to an uninfected individual via microabrasions or wounds. For example, the HPVs associated with human vulvar, cervical, and penile cancers are typically transmitted venereally,[29] and this could be expected to be the case in genital PV infection in horses also. However, Horse A is a gelding with no known history of sexual contact with other horses. It is unlikely that this horse's penis came into close contact with another horse, and alternate routes of infection should also be considered. Possibilities include PV transmission via fomites, which is reported in humans and cattle,[4,25] by insect vectors, which is reported in rabbits,[8] and transplacental or perinatal PV transmission, which is reported in humans.[24,30]

### 3.6.2 Objective 2: Designing and Testing EcPV-2-Specific PCR Primers.

In order to remove the time and expense of sequencing associated with the use of consensus primers and allow simpler and more rapid diagnosis of EcPV-2 infection we designed PCR primers specific for EcPV-2. Although a previously published specific primer set that amplifies a 679 base pair fragment of the EcPV-2 genome existed (VDS.E1),[33] we chose to design a new set of EcPV-2-specific primers. The primers that we designed (MD.L1[A]) amplify a shorter DNA fragment (104 base pairs), which was desirable for three reasons. First, formalin fixation degrades DNA over time,[2,14] which

reduces the number of long stretches of DNA that remain intact in a tissue sample. This decreases their availability for amplification by PCR, and the sensitivity of the assay is reduced when long products are amplified. Use of primers that amplify a 104 base pair product rather than a 679 base pair product would thus be expected to increase the sensitivity of detection of EcPV-2 in tissues. Second, amplification of a relatively short DNA fragment allows a short (10 second) extension step, which greatly decreases PCR cycling times. Third, a goal of future experiments is the use of quantitative real time PCR (qPCR). When using qPCR, amplification of shorter stretches of DNA (typically 100 base pairs or fewer) is desirable for the reasons already stated, and also because assembly of a shorter PCR product consumes fewer reagents. In summary, by designing a primer set that amplifies a short PCR product and can be used in both conventional and qPCR assays we hope to streamline future experiments, speed up the testing of samples by reducing cycling times, reduce the consumption of reagents, and increase the sensitivity of conventional PCR assays.

The previously published specific primer set amplifies a fragment of the EcPV-2 E1 gene;[33] however, we chose to design primers that amplify a fragment of the L1 gene instead. The L1 gene is the most highly conserved part of the PV genome and specific primers for the PV L1 gene are commonly used in other studies, including those investigating human PVs. Their use is therefore widely accepted. In addition, papillomaviral E genes (as amplified by the VDS.E1 primers[33]) may be deleted during integration into the host genome, which makes them unavailable for amplification by PCR.[5,6,18,35] We are not aware of any studies that suggest the PV L1 gene is deleted during integration into the host genome and therefore assume that it would remain present whether a PV is present in episomal or integrated form. It is thus possible that the use of L1 primers could increase the sensitivity of EcPV-2 detection in PCR assays when compared with the use of E primers. When our newly designed primers were compared with the VDS.E1 primers, we found them to be equally sensitive and specific.

### 3.6.3 Objective 3: Creation of a Positive Control Sample for EcPV-2 Testing.

In chapter 2, where consensus primers and sequencing were used to amplify and identify EcPV-2, the positive control was DNA extracted from a bovine cutaneous fibropapilloma known to contain BPV-1. The disadvantage of this is that use of a bovine PV positive control precludes the use of EcPV-2-specific primers in any future experiments. In the current chapter, EcPV-2 DNA was used as a positive control, which allowed the design and testing of EcPV-2-specific primers. This DNA was extracted from a fresh penile biopsy sample from Horse A, known to be infected with EcPV-2.

For the PCR-based experiments planned in upcoming chapters a new positive control sample was required. A good positive control should contain a known concentration of EcPV-2 DNA, as measured by spectrophotometry. In addition, it should be of high quality and free of contaminants, as indicated by the 260/280 absorbance ratio, also measured by spectrophotometry. Finally, after being produced the sample should be divided into aliquots to avoid the need for repeated freezing and thawing, which degrade and fragment DNA. Using our newly designed MD.L1[A] specific primer set and freshly collected, unfixed tissue from Horse A, a high quality positive control sample was created that met these conditions. This sample is suitable for future use in experiments using conventional PCR.

### 3.6.4 Objective 4: Testing a Non-Invasive Method for Diagnosis of EcPV-2 Infection.

To facilitate future EcPV-2 prevalence studies we developed and demonstrated the efficacy of a simple swab-based method for diagnosis of EcPV-2 infection in equine skin and mucosa. This method permits infection to be diagnosed in live horses without the need for biopsy. Advantages of this include the lack of pain for the horse, reduced expense for the owner and decreased time and technical skill required by the sampler, all of which are likely to increase compliance in sampling studies.

At the diagnostic level, a potential advantage of swabbing intact stratified squamous epithelium (such as penile skin) to diagnose EcPV-2 infection is that the majority of cells collected are surface squames. In addition to their ease of collection, surface squames are ideal samples because they contain many more copies of a PV genome

than do the basal layers of the epithelium. During productive PV replication, the PV genome is amplified from 10-50 copies per cell to around 10,000 copies per cell during the process of keratinocyte differentiation.[31] In addition, since surface squames have generally lost their nuclei as a normal part of squamous differentiation, their genomic equine DNA content is lower than in deeper keratinocyte layers. This means that in swabs of PV-induced lesions the proportion of PV DNA to equine genomic DNA is relatively high, which is advantageous for PCR-based amplification since high levels of host genomic DNA can inhibit primer annealing. Furthermore, when comparing fresh tissues and archival tissues used for PV studies, the DNA obtained from a swab is likely to be superior to that obtained from formalin-fixed, paraffin embedded tissue samples because of the lack of formalin-induced DNA degradation.

Biopsy samples of papillomas from Horse A had been previously shown by PCR to contain EcPV-2 sequences. Horse A was therefore used as a positive control when developing a swab-based method for EcPV-2 diagnosis. As a baseline for comparison of potential swabbing methods, the penis of Horse A was initially swabbed using a kit designed to diagnose HPV infection in women (Broom-like device protocol; Hologic, Bedford, Massachusetts, USA). EcPV-2 sequences were strongly amplified from samples obtained using this method (data not included); however, human kits are not suitable for use in prevalence studies in horses because of their expense. Therefore, we sought an alternate, cheaper technique that would provide an optimal compromise between convenience, cost, and successful PCR amplification of EcPV-2 DNA.

The technique of drawing a sterile, moistened cotton-tipped swab back and forth over a lesion and then freezing the swab tip in saline was adapted from a human study on HPV infection of skin.[10] It was chosen because of its simplicity, low cost, and efficacy in detecting human PV skin infection. Most veterinarians have access to the supplies required and to a freezer for temporary storage of samples, which is important if practitioners are to be involved in collection and submission of samples from horses.

Once swabs were collected, three different methods were used to prepare template DNA for PCR. One involved no extraction of DNA from the swab suspension and was

the method used in the study of human PVs on which the swabbing technique was based.[10] The other two extracted DNA from suspended squames prior to PCR; these were tested because all of our previous experiments had a DNA extraction step prior to PCR. The reason for comparing extraction and non-extraction methods was to determine whether the time and expense involved in DNA extraction were necessary when diagnosing EcPV-2 infection from swabs. The reason for comparing two different DNA extraction methods, one in which a squame suspension was used directly, and the other in which squames were pelleted by centrifugation, was to determine whether it was necessary to concentrate the squames in order to amplify sufficient PV DNA.

As shown in figure 3.6, PV DNA sequences were strongly amplified from samples regardless of which of the three template preparation methods was used. Subjectively, the gel bands for the two samples from which the DNA was extracted prior to PCR (lanes 4 and 5) were slightly stronger than the band for the sample from which DNA was not extracted (lane 6). However, because the DNA content of each sample was not measured prior to PCR it is possible that this apparent increased amplification of DNA sequences was simply due to a larger amount of DNA present in the initial sample.

Our results indicate that simple swabbing using cheap and readily available materials can be used to diagnose EcPV-2 infection in lesions. Although none of the three different template preparation methods was shown to be superior in this trial, the use of centrifugation and DNA extraction would be expected to produce the largest amount of template PV DNA. Centrifugation ensures that a higher proportion of sampled squames are tested and DNA extraction releases the maximum amount of PV DNA from possible protection by the keratinocyte cell membrane. This is important when diagnosing PV infections with a low viral load.

Although we demonstrated that swabbing can be used to diagnose EcPV-2 infection in horses, the presence or absence of EcPV-2 in surface squames must be interpreted with care. PV DNA is maintained in the dividing basal cells of a stratified squamous epithelium as circular plasmids that are replicated during normal cell division at the

same time as the host cell's DNA. Because of this, a non-productive EcPV-2 infection is unlikely to be diagnosed by surface swabbing, which does not collect basal cells. Therefore, failure to detect EcPV-2 in a surface swab does not necessarily rule out infection *per se*, just productive infection. Also, it is possible that PVs detected by surface swabbing of a neoplasm may simply represent contamination of the tumor surface by PV-containing squames from skin elsewhere in the body. If this happens, a commensal PV shed from healthy body locations may be misinterpreted as pathogenic if detected within a neoplasm but not identified as a contaminant. This is of particular concern in studies of human PVs, where contaminating squames may even originate from the person taking swabs or biopsy samples, rather than from the patient. To remove surface squames and potential external contamination, a method used in human studies involves "tape-stripping" the surface of a neoplasm using adhesive tape prior to biopsy. This increases the probability that a PV detected by PCR in a biopsy sample from a neoplasm is a true inhabitant of the neoplasm. In one human study, for example, HPV DNA was amplified by PCR in 69% of swab samples collected from skin tumors, yet in only 12% of biopsy samples of the same tumors taken after removal of superficial cell layers by tape-stripping.[10] While this may simply be due to the amplification of the PV genome that takes place as keratinocytes progress toward terminal differentiation, it may also suggest that surface contamination inflates detection of HPV in swabs. Furthermore, in the same study, the HPV DNA prevalence in swab samples from the surface of tumors (69%) was similar to that of swab samples from healthy skin taken at perilesional sites (78%), foreheads (84%), and buttocks (73%). This suggests that the HPV detected in tumors in that study were commensal, rather than causative. For these reasons, I recommend tape-stripping the surface of suspect EcPV-2-induced lesions before biopsy, and this method was used during the revisit sampling of Horse A in this chapter. Surface swabs were collected from unstripped penile skin, but the sites chosen for biopsy were tape-stripped prior to sampling. As shown in figure 3.6, PV DNA sequences were strongly amplified from the biopsy sample even after tape stripping, suggesting that EcPV-2 in this case was a true inhabitant of the lesion, rather than a surface contaminant.

In summary, although we found that surface swabbing is a valid screening test for EcPV-2 infection, biopsy of tape-stripped lesions followed by histology, PCR, ISH and IHC are still important to provide more information about the role of EcPV-2 in tumor development.

## 3.7 Chapter Summary

The experiments described in this chapter resulted in development of convenient, accurate and reliable methods for diagnosis of EcPV-2 infection. In addition, a higher quality positive control sample than used in earlier experiments was generated. Finally, EcPV-2 was detected for the first time in New Zealand and a novel clinical manifestation of EcPV-2 infection described. These findings are reported in the article:

> *Knight CG, Munday JS, Rosa BV, Kiupel M:* ***Persistent, widespread papilloma formation on the penis of a horse: a novel presentation of equine papillomavirus type 2 infection****. Veterinary Dermatology 22: 570-574, 2011*

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# CHAPTER FOUR

# Development of a Quantitative PCR Assay for EcPV-2

## 4.1 Introduction

The results of the experiments described in chapters 2 and 3, as well as the work of another research group,[31] showed an association between equine papillomavirus type 2 (EcPV-2) infection and equine penile squamous cell carcinomas (EPSCCs). Given the significant role played by PVs in other cancers,[24] these results suggest that EcPV-2 may play a role in the development of EPSCCs. However, no previous studies have used a quantitative technique to assess the EcPV-2 load in normal and cancerous tissues. All previous EcPV-2 research[19,20,31] has used conventional PCR, which is a qualitative technique that simply identifies samples as positive or negative for the presence of

target DNA sequences. In order to investigate the role of EcPV-2 in development of EPSCCs further, a quantitative PCR (qPCR) technique is desirable.

When compared with conventional PCR, qPCR offers several advantages.[6] First, qPCR allows more rapid testing of samples by removing the need for post-amplification gel electrophoresis before results can be interpreted. Second, qPCR removes the need for gel extraction and sequencing because melting curve analysis provides an inbuilt confirmation of the presence of the specific PCR products. Third, and most importantly, qPCR permits quantification of pathogen load within tissues. This is a considerable advantage over conventional PCR, since it is increasingly recognized that pathogen load, and not simply the presence or absence of a pathogen, influences the development of disease.[14,37]

The development of a qPCR assay for EcPV-2 was the aim of the work described in this chapter. Initially, a specific primer set (MD.L1[A], designed in chapter 3) was used and various qPCR assay conditions were tested for their suitability for EcPV-2 detection. However, the assay performed suboptimally under all conditions. Therefore, a new set of EcPV-2-specific primers was designed (named MD.L1[B]), a new positive control was created, and a qPCR assay for EcPV-2 was optimized.

Several specialized terms are used to describe qPCR assays and results. Those most relevant to this chapter are explained briefly in the glossary (section 4.6) at the end of the chapter and illustrated in figures 4.8 and 4.9.

## 4.2 Optimization of a qPCR Assay for EcPV-2 using the MD.L1[A] Primer Set

### 4.2.1 Overview

A disadvantage of the positive control samples prepared in earlier chapters was that they consisted an unknown ratio of equine genomic DNA and EcPV-2 DNA. These controls were thus not suitable for use in qPCR assays that aimed to assess PV copy numbers in tested samples. Therefore before a qPCR assay could be developed a

suitable positive control had to be created, one in which the exact PV DNA content, expressed as copy numbers of the EcPV-2 target DNA sequence per µl, was known. This positive control was created using a recombinant plasmid. After production of a suitable positive control sample, the qPCR assay for detection and quantification of the specific EcPV-2 DNA sequence was tested. Assay parameters such as annealing temperatures and times were varied in trial assays in an attempt to achieve acceptable efficiency and $R^2$ values.

## 4.2.2 Materials and Methods

### *4.2.2.1 Cloning*

EcPV-2 DNA sequences were amplified from genomic DNA previously extracted from an EcPV-2-infected penile papilloma. This was performed using conventional PCR and the MD.L1[A] primer set, which amplifies a 104 base pair segment of the EcPV-2 L1 gene. The PCR conditions have been described in chapter 3 (section 3.3.1). After electrophoresis of the PCR product, the bands containing the amplified EcPV-2 DNA sequence were cut from the agarose gel and the DNA extracted using spin columns, as described in chapter 3. This procedure produced a sample containing a 104 base pair segment of the EcPV-2 L1 gene with a DNA content of 99.14 ng/µl.

The EcPV-2 PCR product was then ligated into a plasmid vector using a commercially available kit according to the manufacturer's instructions (TOPO TA Cloning Kit; Invitrogen). The ligation mixture used to insert the EcPV-2 target sequence into the plasmid vector was prepared as shown in table 4.1. After incubation for 5 minutes at room temperature the mixture was placed on ice until the next step.

| Reagent | Volume |
|---|---|
| EcPV-2 DNA | 1 µl |
| Salt solution | 1 µl |
| Water | 3 µl |
| TOPO Vector | 1 µl |
| Total volume | 6 µl |

*Table 4.1. Preparation of the ligation mixture for insertion of the 104 bp EcPV-2 target sequence into plasmid vectors*

Two µl of the ligation mixture were then added to a vial of chemically competent Top10 *E. coli*. The bacteria were transformed using a heat shock step of 30 minutes on ice followed by 30 seconds at 42°C. Next, after addition of 250 µl of SOC (super optimal broth with added glucose) growth medium, the vial of transformed *E. coli* was incubated with gentle shaking at 37°C for 1 hour to allow recovery from heat shocking and expression of ampicillin resistance genes by the introduced plasmids. The growth medium/*E. coli* mix was then plated onto two LB (lysogeny broth) agar plates impregnated with 100 µg/µl ampicillin and cultured overnight at 37°C.

#### *4.2.2.2 Screening by Colony PCR*

After overnight culture, eight randomly selected colonies were picked from the agar using sterile toothpicks. Each colony was inoculated into 50 µl of LB broth with ampicillin (50 µg ampicillin/ml) and incubated at 37°C for 4 hours.

Following incubation, conventional PCR with the MD.L1[A] primer set was performed on all eight colonies to confirm insertion of the 104 bp segment of the EcPV-2 L1 gene. A commercial 5X master mix (FIREPol Master Mix; Solis Biodyne) was used to prepare 10 µl reaction volumes with 0.5 µl of template and a final concentration of 0.4 µM for each primer. Amplification was carried out under the following cycling conditions: 95 °C for 10 minutes followed by 35 cycles of 95 °C for 10 seconds, 65°C for 10 seconds, and 72 °C for 10 seconds. The final extension step was at 72 °C for 10 minutes. Amplicons were subjected to electrophoresis through a 1.5% agarose gel containing 0.64 µg/ml ethidium bromide. Amplified PCR products were visualized under UV light.

#### *4.2.2.3 Plasmid Isolation*

Two of the eight *E. coli* colonies screened by PCR in the section above (colonies 1 and 2) were arbitrarily selected for plasmid isolation. Both colonies were grown to a larger volume by adding 49.5 µl of each colony to 4 ml of LB broth containing 100 µg/ml ampicillin and incubating overnight at 37°C. After incubation, the bacteria were pelleted by centrifugation at 6000 x g for 30 seconds and the supernatant discarded. The plasmids were then isolated using a commercially available plasmid isolation kit (High Pure Plasmid Isolation Kit; Roche) according to the manufacturer's instructions.

In brief, the *E. coli* pellets were resuspended in 250 µl of suspension buffer + RNase, lysed with lysis buffer, and washed with wash buffer. Plasmid DNA was eluted into 100 µl of elution buffer. Eluted plasmid DNA was refrigerated until the next step.

#### *4.2.2.4 Confirmation of the Inserted EcPV-2 Sequence Within the Plasmid*

To confirm the presence and insertion of the target EcPV-2 L1 sequence into the plasmids from colonies 1 and 2, two different PCR assays were performed. In addition, the plasmids were sequenced.

Assay #1 used the MD.L1[A] primer set. A commercial 5X master mix (AccuMelt) was used to prepare 10 µl reaction volumes containing 1 µl of recombinant plasmid/insert DNA and 0.4 µM concentrations of each primer. The positive control was the sample of EcPV-2 DNA extracted from a horse with penile papillomatosis, as described in section 4.2.2.1. For the non-template control, template DNA was omitted and replaced with 1 µl of water. Amplification was carried out under the following cycling conditions: 95 °C for 10 minutes followed by 35 cycles of 95 °C for 10 seconds, 65°C for 10 seconds, and 72 °C for 10 seconds. The final extension step was at 72 °C for 10 minutes. Amplicons were subjected to electrophoresis through a 1.5% agarose gel containing 0.64 µg/ml ethidium bromide and bands containing the amplified fragment were visualized under UV light.

Assay #2 used a universal M13 forward primer, which hybridizes with plasmid DNA, and the MD.L1[A] reverse primer, which hybridizes with the insert, to produce a 280 bp DNA sequence spanning the junction between the insert and the plasmid (figure 4.1). Amplification conditions, reaction mixes, and primer concentrations were the same as those used for assay #1. No positive control was used and the non-template control was the same as described previously. As for assay #1, electrophoresis was through a 1.5% agarose gel containing 0.64 µg/ml ethidium bromide, and bands containing the amplified fragment were visualized under UV light.

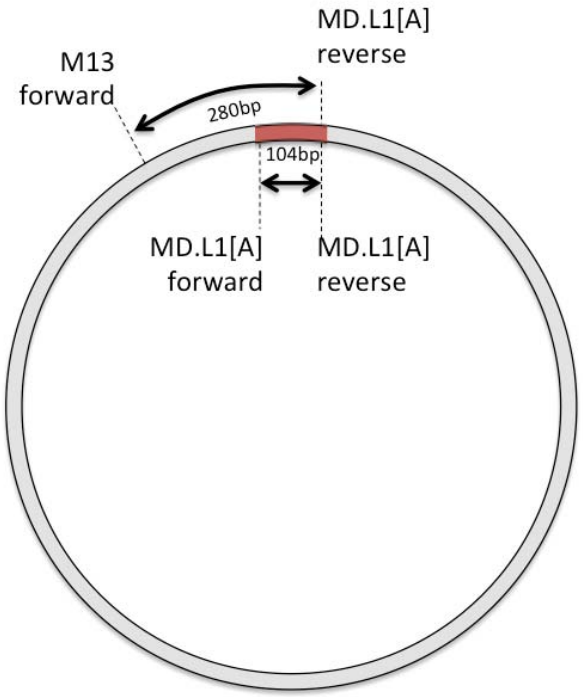


*Figure 4.1. Schematic diagram of the 3931 bp plasmid containing a 104 bp EcPV-2 insert. Two primer sets were used to confirm the presence of the insert in the plasmid.*

Finally, to confirm the correct sequence of the 104 bp EcPV-2 L1 insert within the colony 1 plasmid, 450 ng of DNA were submitted to the Massey Genome Service for sequencing. Results were compared with known EcPV-2 gene sequences using the BLAST algorithm to analyze the nucleotide sequence.[1] The recombinant plasmid produced by the work described in this section was designated "EcPV-2 plasmid".

#### *4.2.2.5 Creation of a Stock Solution of a Linearized Plasmid Containing an EcPV-2 Insert*

The DNA content and 260/280 ratio of the EcPV-2 plasmid were measured using a spectrophotometer (Nanodrop; Thermo). An online DNA copy calculator (thermoscientificbio.com) was used to calculate the dilution necessary to produce a stock solution containing $10^9$ copies of the EcPV-2 L1 target sequence per µl.

In order to use the EcPV-2 plasmid as a positive control it was also necessary to linearize the circular plasmid to prevent supercoiling and open it for primer binding. This was achieved using a restriction enzyme, which cuts the circular plasmid once at a specific location outside the inserted DNA fragment. Linearization and dilution were

achieved simultaneously using a commercially available Pst1 restriction enzyme and buffer (Thermo Scientific). The reaction mix was prepared as shown in Table 4.2 and incubated overnight at 37°C.

| Reagent | Volume |
|---|---|
| Recombinant plasmid | 8.99 µl (equivalent to 1.35 X $10^{10}$ copies of plasmid based on Nanodrop results] |
| 10X Buffer O | 10.00 µl |
| Pst1 Restriction enzyme (10 U/µl) | 5.00 µl (equivalent to 50 U) |
| Water | 76.01 µl |
| Total volume | 100 µl |

*Table 4.2. Preparation of the linearization and dilution reaction mix for circular plasmid vectors containing an inserted 104 bp EcPV-2 target sequence.*

The following day, 2 µl of 0.5 µM EDTA were added to stop the reaction. This produced a stock solution containing $10^9$ copies of the linearized recombinant plasmid per µl. Each copy consisted of a 104 bp segment of the EcPV-2 L1 gene inserted within an approximately 4 kbp plasmid. The purity and linearization of the plasmid were checked by gel electrophoresis. Ten µl of the stock were subjected to electrophoresis through a 1% agarose gel containing 0.64 µg/ml ethidium bromide and bands were visualized under UV light along with an O'GeneRuler 1 kb DNA Ladder (Thermo Scientific).

#### *4.2.2.6 qPCR Assay Optimization*

Serial dilutions of the positive control stock ($10^9$ copies/µl) were made to produce test dilutions from $10^8$ to $10^0$ copies per µl. To make dilutions, 5 µl of the $10^9$ stock were mixed with 45 µl of water to create a solution containing $10^8$ copies/µl. This was repeated using the $10^8$ stock to create a $10^7$ copies/µl dilution and so on. Water was used for the first two dilutions ($10^8$ and $10^7$) to dilute any EDTA remaining from the linearization step, and elution buffer (Roche) dilutions.

The original $10^9$ stock solution was then frozen at -80°C for long term storage. It was not used for subsequent qPCR assays in order to conserve stock and prevent repeated freeze-thaw cycles. The $10^8$ and $10^7$ dilutions were kept refrigerated and re-used for each subsequent qPCR assay. The $10^6$ to $10^0$ dilutions were made fresh for each qPCR assay because dilute DNA solutions are not stable when stored.[15,34]

Quantitative PCR assays were conducted using a real-time qPCR machine (Eco; Illumina), and results analyzed using the machine's proprietary data analysis software (EcoStudy software version 4.0.4420). A commercial SYTO 9-based 2X master mix was used (AccuMelt HRM SuperMix; Quanta Biosciences). Reactions were set up in 10 µl volumes and loaded in 48 well plates (Eco; Illumina), which were covered with optically clear adhesive optical seals (Eco; Illumina). The MD.L1[A] primer set, which amplifies a 104 bp segment of the EcPV-2 L1 gene, was used for all assays.

At the start of this experiment the conditions necessary to achieve an efficiency of 90-110% and an $R^2$ value of > 0.99 were unknown. Using tenfold dilutions of the positive control stock these were investigated in a series of approximately 20 trial assays. The different primer concentrations and amplification conditions tested in these trials are summarized in tables 4.3a and 4.3b.

For each 10 µl reaction volume:

| **Reagent** | **Volume (Concentration)** |
|---|---|
| AccuMelt 2X Master Mix | 5.0 µl |
| **MD.L1[A] Forward Primer** | **0.05-0.25 µl (= 0.1 – 0.5 µM)** |
| **MD.L1[A] Reverse Primer** | **0.05-0.25 µl (= 0.1 – 0.5 µM)** |
| **Water** | **As needed to bring reaction volume to 10 µl** |
| Template DNA | 2.0 µl |
| Total Volume | 10 µl |

*Table 4.3a. Different primer concentrations tested to optimize an EcPV-2 qPCR assay using the MD.L1[A] primer set. Parameters that were varied between assays in order to achieve desired efficiency and $R^2$ are in bold type.*

For each qPCR assay:

| | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| **Polymerase Activation** | 95 | **3-15 min** | N/A |
| **Denaturation** | 95 | **1-5 sec** | **35-45** |
| **Annealing** | **63-66** | **5-15 sec** | |
| **Extension** | 72 | **10-25 sec** | |
| Melt | 95 | 15 sec | N/A |
| | 55 | 15 sec | N/A |
| | 95 | 15 sec | N/A |
| Hold | 4 | ∞ | N/A |

*Table 4.3b. Different amplification conditions tested to optimize an EcPV-2 qPCR assay using the MD.L1[A] primer set. Parameters that were varied between assays in order to achieve desired efficiency and $R^2$ are in bold type.*

#### *4.2.2.7 Investigation of Primer Design*

Secondary structures formed by the 104 bp DNA sequence amplified by the MD.L1[A] primer set were predicted using a web-based program (mfold.rna.albany.edu).[38]

### 4.2.3 Results

#### *4.2.3.1 Screening by Colony PCR*

Approximately 300 colonies of transformed *E. coli* were grown overnight. Eight colonies were screened by PCR for the presence of a 104 base pair segment of the EcPV-2 L1 gene. All eight colonies contained DNA sequences of the expected 104 bp length (figure 4.2).

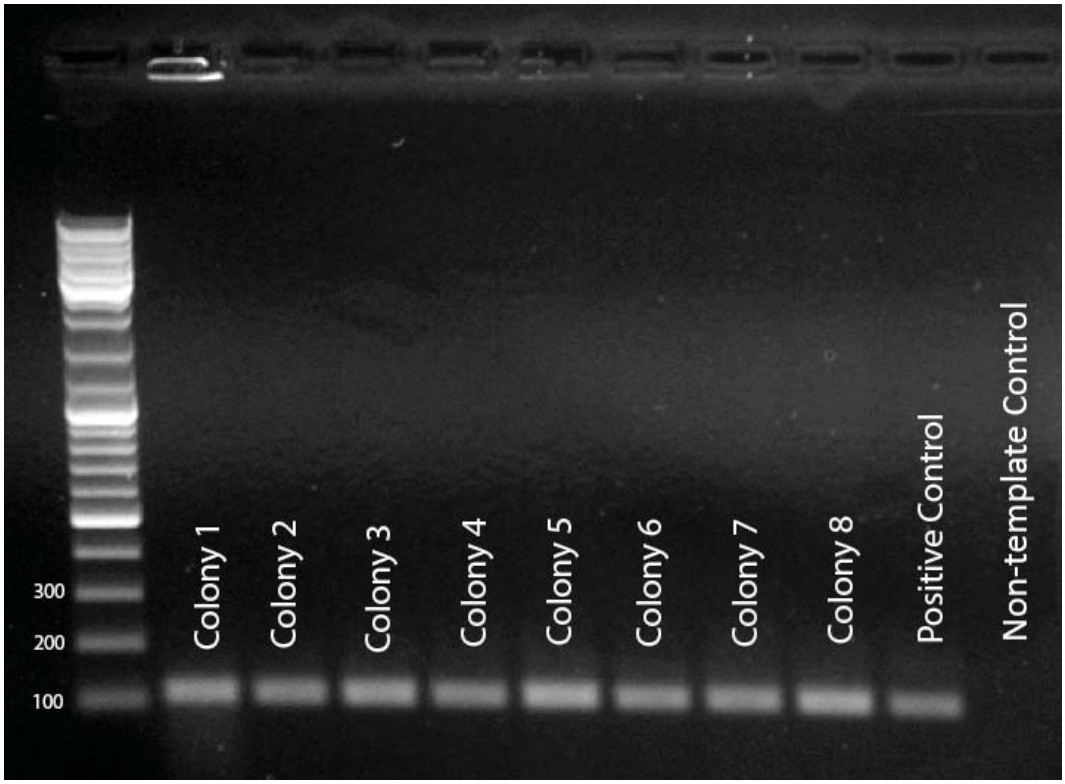


*Figure 4.2. Gel electrophoresis results from eight E. coli colonies screened by PCR for the presence of a recombinant plasmid vector containing an inserted 104 bp segment of the EcPV-2 L1 gene. Using the MD.L1[A] specific primer set the EcPV-2 sequence was amplified from all eight colonies. A sample of EcPV-2 DNA extracted from an equine penile papilloma was used as a positive control (prepared as described in section 4.2.2.1). For the non-template control, template DNA was omitted and replaced with water.*

#### *4.2.3.2 Confirmation of Inserted EcPV-2 Sequence Within the Plasmids*

Both *E. coli* colonies selected for plasmid isolation (colonies 1 and 2) contained EcPV-2 DNA products of the expected lengths (figure 4.3), confirming that the target EcPV-2 sequence was present within the plasmid. Based on extraneous bands in the gel, the plasmid isolated from colony 2 appeared to also contain *E. coli* genomic DNA and was therefore not used for subsequent steps.

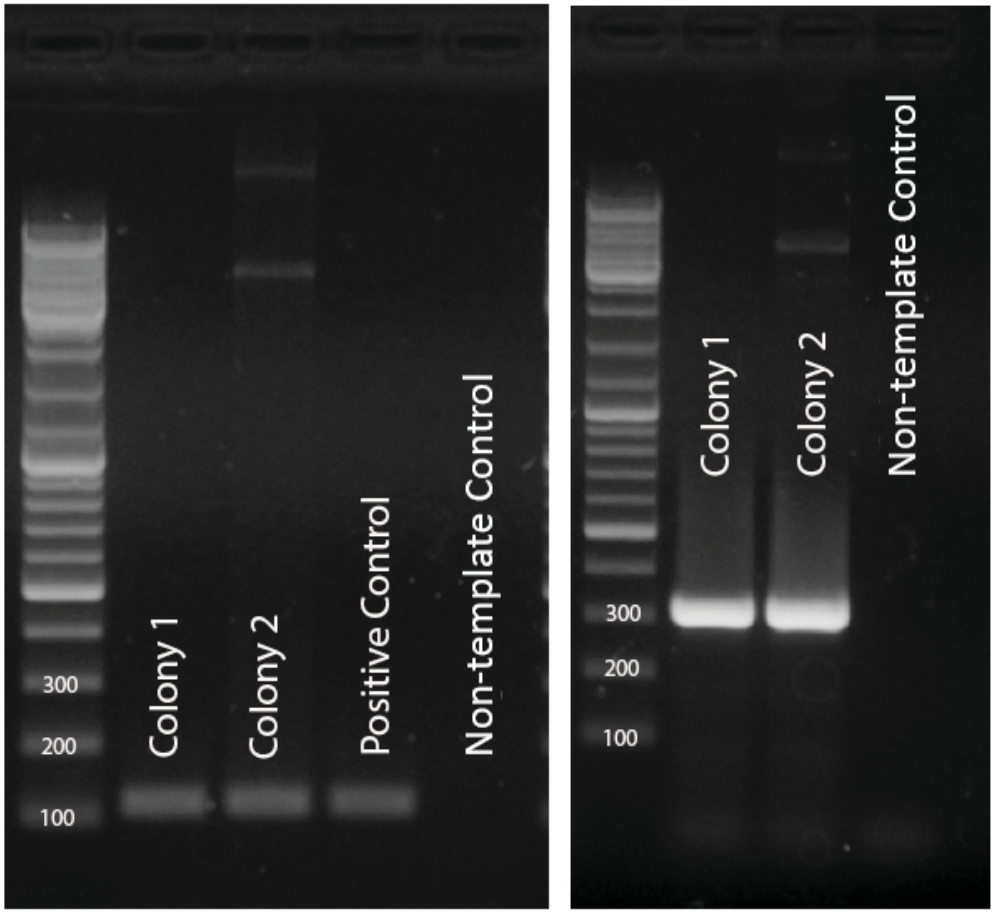


*Figure 4.3. Gel electrophoresis results from two PCR assays confirming the presence of the EcPV-2 L1 insert within plasmids. The left hand assay used the MD.L1[A] primer set, which amplifies a 104 bp sequence of the EcPV-2 L1 gene. The right hand assay used a universal M13 forward primer and the MD.L1[A] reverse primer, producing a 280 bp DNA sequence spanning both the EcPV-2 insert and the plasmid. Both assays confirmed the presence of the EcPV-2 insert in both E. coli colonies selected for plasmid isolation. For the left hand assay the positive control was EcPV-2 DNA extracted from an equine penile papilloma. For the right hand assay no positive control was used. For a non-template control, template DNA was omitted and replaced with an equivalent volume of water.*

When sequence results from the Massey Genome Service were compared using BLAST, the 104 bp product amplified using the MD.L1[A] primers was >99% identical to known EcPV-2 genome sequences.

### 4.2.3.3 Creation of a Stock Solution Containing a Linearized Plasmid

After electrophoresis of the linearized plasmid stock through agarose gel containing ethidium bromide a single band was visualized of the expected 4000 bp size (figure 4.4), which confirmed the purity and linearization of the recombinant plasmid.

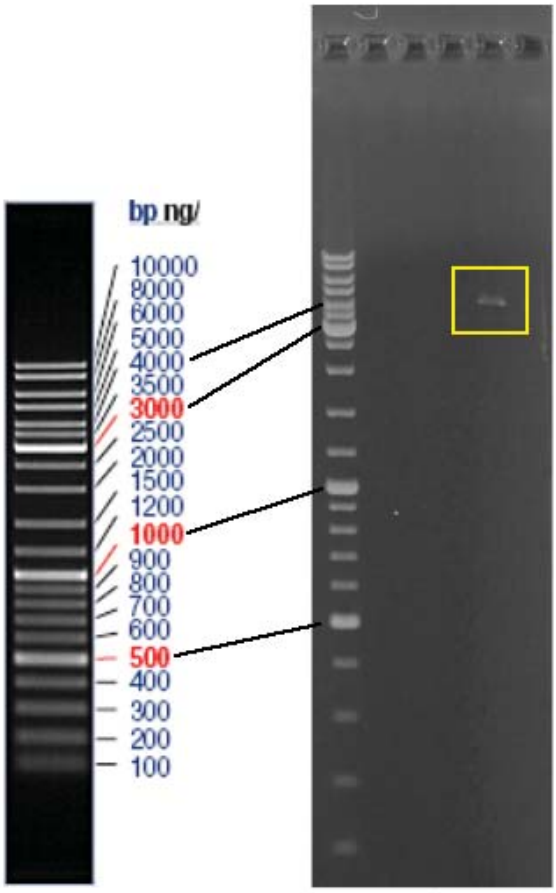


*Figure 4.4. Gel electrophoresis results from a PCR assay confirming that the EcPV-2 control stock solution contains the ~4000 bp plasmid construct. A band is present at the expected 4000 bp height (yellow box). Ladder: O'GeneRuler 1 kb DNA Ladder (Thermo Scientific)*

### 4.2.3.4 qPCR Assay Optimization

In the trial assays, the conditions that achieved the best efficiency and $R^2$ values were: primer concentration 0.5 µM; polymerase activation for 15 minutes; 45 cycles; denaturation for 1 second; annealing at 65°C for 5 seconds; extension for 25 seconds. The $R^2$ value of 0.998 was acceptable, but the highest efficiency that was achieved using these conditions was 84.8%, which did not lie within the acceptable range of 90-110%.

#### *4.2.3.5 Investigation of Primer Design*

The poor efficiency of the assay, in spite of attempts at optimization, suggested that the MD.L1[A] primers might not be suitable for quantitative PCR. Closer examination of the primers revealed that the PCR product amplified by the MD.L1[A] primer set contained two stem and loop secondary structures ("hairpin loops") spanning positions 29-42 and 86-98 (figure 4.5). The second of these loops affected the binding site for the MD.L1[A] reverse primer. Because of the presence of these hairpin loops and the inability to produce an efficiency within the acceptable range, the MD.L1[A] primer set was abandoned for future experiments.


*Figure 4.5. Schematic diagram of secondary structures predicted by mfold (mfold.rna.albany.edu) within the 104 bp PCR product amplified by the MD.L1[A] primer set. Two hairpin loops are present spanning positions 29-42 and 86-98 of the product.*

## 4.3 Development of a qPCR Assay for EcPV-2 using the MD.L1[B] Primer Set

### 4.3.1 Overview

The initial attempt to develop a qPCR assay for EcPV-2 using the MD.L1[A] primer set was abandoned after we found that the PCR product contained secondary structures that reduced assay efficiency. Since the EcPV-2 plasmid created was specific for the MD.L1[A] primer set it could not be used for any future experiments and was discarded. This section describes the design of a new primer set and positive control and subsequent optimization of a qPCR assay for EcPV-2.

### 4.3.2 Materials and Methods

#### *4.3.2.1 Design of the MD.L1[B] Primer Set*

A new primer set (designated MD.L1[B]) was designed using commercially available bioinformatics software (Primer 3 version 2.3.4).[36] This primer set amplified a 118 bp

segment of the EcPV-2 L1 gene. The forward primer sequence was 5'-GCG GTG TCG AGG TGT CAC GG-3' and the reverse primer sequence was 5'-CGC TCC CCT GCA GCT TCC TG-3'. The existence of secondary structures in the PCR product was investigated using mfold (mfold.rna.albany.edu), and none were predicted.[38] Primer matches with the EcPV-2 genome were investigated using BLAST.[1] The forward and reverse primers had 100% sequence identity with the published genome for EcPV-2 (figure 4.6). There were no similarities to any other papillomavirus or virus.

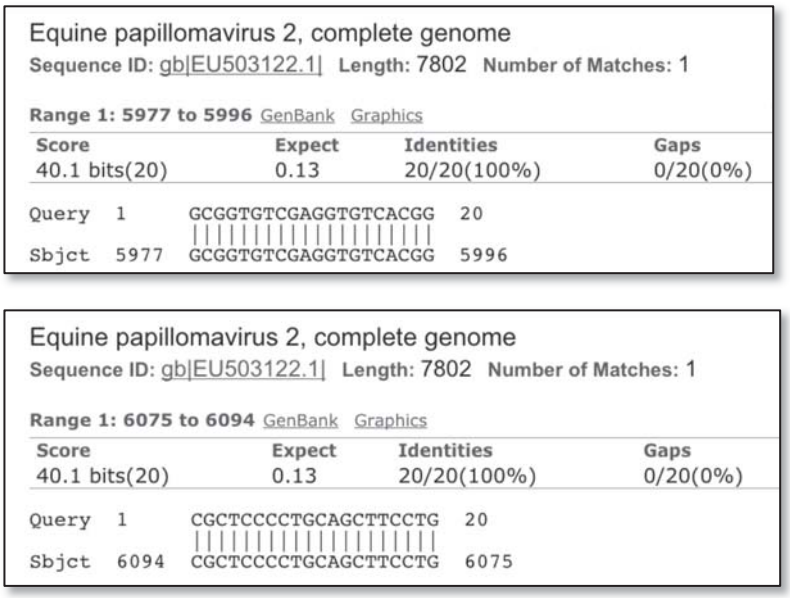


*Figure 4.6. BLAST results for the MD.L1[B] forward and reverse primers.*

#### *4.3.2.2 Initial Comparison of the MD.L1[A] and MD.L1[B] Primer Sets*

A pilot trial was performed to determine whether the new primer set was suitable for use in future experiments. In this trial, serial dilutions of DNA extracted from an equine pharyngeal SCC known to be EcPV-2 positive were amplified by qPCR using both the new and old primer sets and the results compared. The reaction mixes and amplification conditions are described in tables 4.4a and 4.4b. The qPCR assay was conducted using an Eco real-time qPCR machine and AccuMelt master mix, as described earlier. Dilutions of the test sample were $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$. All samples were run in duplicate and water was used for a non-template control. The amplification conditions that had been shown to give the best efficiency and $R^2$ values in section 4.2.3.4 were used.

For each 10 µl reaction volume:

| Reagent | Volume (Old Primer Set) | Volume (New Primer Set) |
|---|---|---|
| AccuMelt 2X Master Mix | 5.0 µl | 5.0 µl |
| MD.L1[A] Forward Primer | 0.25 µl (= 0.5 µM) | N/A |
| MD.L1[A] Reverse Primer | 0.25 µl (= 0.5 µM) | N/A |
| MD.L1[B] Forward Primer | N/A | 0.25 µl (= 0.5 µM) |
| MD.L1[B] Reverse Primer | N/A | 0.25 µl (= 0.5 µM) |
| Water | 2.5 µl | 2.5 µl |
| Template DNA | 2.0 µl | 2.0 µl |
| Total Volume | 10 µl | 10 µl |

*Table 4.4a. Reaction mixes used to compare the MD.L1[A] and MD.L1[B] primer sets.*

For each qPCR assay:

| Step | Temp. (°C) | Time | Cycles |
|---|---|---|---|
| Polymerase Activation | 95 | 15 min | N/A |
| Denaturation | 95 | 1 sec | 45 |
| Annealing | 65 | 5 sec | |
| Extension | 72 | 25 sec | |
| Melt | 95 | 15 sec | N/A |
| | 55 | 15 sec | N/A |
| | 95 | 15 sec | N/A |
| Hold | 4 | ∞ | N/A |

*Table 4.4b. Amplification conditions used to compare the MD.L1[A] and MD.L1[B] primer sets.*

#### *4.3.2.3 Amplification of an EcPV-2 DNA Fragment for Use as a Positive Control.*

To create a specific positive control for use with the new MD.L1[B] set we used a simpler and less time-consuming technique than that described in section 4.2. DNA extracted from an equine pharyngeal SCC known to be EcPV-2 positive was amplified using conventional PCR. Conditions and reagents are listed in tables 4.5a and 4.5b. The reverse primer was from the original MD.L1[A] set, and the forward primer was from the newly designed MD.L1[B] set. These primers amplified a 591 bp fragment of the EcPV-2 genome (figure 4.7). After PCR, amplicons were subjected to electrophoresis through a 1% agarose gel containing 0.64 µg/ml ethidium bromide and visualized using UV light.

For each 10 µl reaction volume:

| Reagent | Volume |
|---|---|
| HotFire 5X Master Mix | 2.0 µl |
| MD.L1[B] Forward Primer | 0.2 µl (= 0.4 µM) |
| MD.L1[A] Reverse Primer | 0.2 µl (= 0.4 µM) |
| Water | 7.1 µl |
| Template DNA | 0.5 µl |
| Total Volume | 10 µl |

*Table 4.5a. Reaction mix used to amplify a 591bp segment of the EcPV-2 L1 gene.*

For each qPCR assay:

| Step | Temp. (°C) | Time | Cycles |
|---|---|---|---|
| Polymerase Activation | 95 | 10 min | N/A |
| Denaturation | 95 | 20 sec | 40 |
| Annealing | 65 | 20 sec | |
| Extension | 72 | 1 min | |
| Final Extension | 72 | 10 min | N/A |
| Hold | 4 | ∞ | N/A |

*Table 4.5b. Amplification conditions used to amplify a 591bp segment of the EcPV-2 L1 gene.*

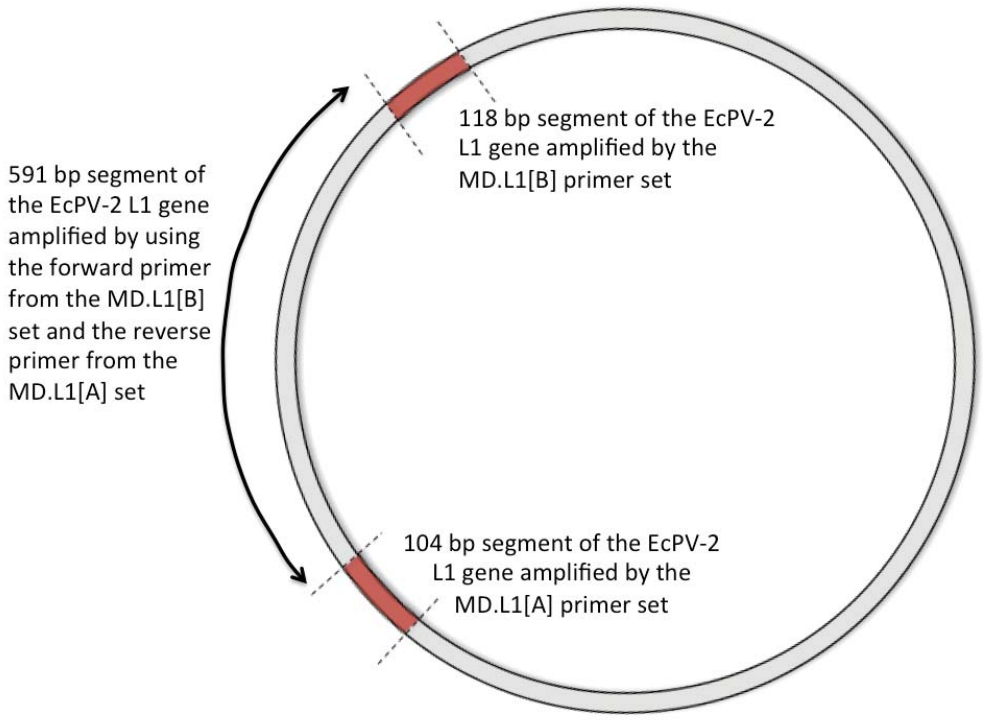


*Figure 4.7. Schematic diagram of the circular EcPV-2 genome showing the 591 bp fragment of the L1 gene amplified by using the reverse primer from the MD.L1[A] set and the forward primer from the MD.L1[B] set.*

#### *4.3.2.4 Confirmation and Quantification of the EcPV-2 DNA Fragment*

Bands containing the 591 bp PCR product were cut from the gel and DNA was extracted using a commercially available gel extraction kit (Qiagen). The DNA content and 260/280 ratio were measured using a spectrophotometer (Nanodrop; Thermo) and the DNA content was confirmed using a fluorometer (Qubit; Invitrogen). A sample was submitted to the Massey Genome Service for sequencing and results compared with the known EcPV-2 genome using BLAST.[1]

An online DNA copy calculator (thermoscientificbio.com) was used to calculate the dilution factors necessary to produce 500 µl of a stock solution containing $10^9$ copies of the EcPV-2 DNA fragment per µl. Elution buffer from the gel extraction kit was used as the diluent.

The 591bp EcPV-2 L1 gene fragment was designated "EcPV-2/L1-591" and is referred to as such in later sections.

#### *4.3.2.5 qPCR Assay Optimization and Generation of a Standard Curve*

A pilot qPCR optimization trial was carried out using the EcPV-2/L1-591. In order to conserve the $10^9$ copies/µl stock solution we did not use it in the pilot trials. Instead, the original solution containing 21 ng/µl of the EcPV-2/L1-591 was used, which corresponds to $3.33 \times 10^{10}$ copies per µl. Serial dilutions were made by adding 5µl of this to 45 µl elution buffer. The process was repeated to produce tenfold dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$. The qPCR assay was conducted using an Eco real-time qPCR machine and AccuMelt master mix, as described in section 4.2.2.6. For a positive control, DNA extracted from an equine pharyngeal SCC previously shown to be EcPV-2-positive was used. For a negative control, DNA extracted from an equine pharyngeal SCC previously shown to be EcPV-2-negative was used. For a non-template control, template DNA was omitted and replaced with an equivalent volume of water. All samples were run in duplicate or triplicate.

The first pilot assay used the primer concentration and PCR conditions that had achieved the best efficiency and $R^2$ values with the MD.L1[A] primer set (section

4.2.3.4). Over several trials, assay parameters were changed until efficiency and $R^2$ values within acceptable levels (90-110% and > 0.99, respectively) were repeatedly obtained (tables 4.6a and 4.6b).

For each 10 µl reaction volume:

| Reagent | Volume (Concentration) |
|---|---|
| AccuMelt 2X Master Mix | 5.0 µl |
| **MD.L1[B] Forward Primer** | **0.15-0.25 µl (= 0.3– 0.5 µM)** |
| **MD.L1[B] Reverse Primer** | **0.15-0.25 µl (= 0.1 – 0.5 µM)** |
| **Water** | **As needed to bring reaction volume to 10 µl** |
| Template DNA | 2.0 µl |
| Total Volume | 10 µl |

*Table 4.6a. Different primer concentrations tested to optimize an EcPV-2 qPCR assay using the MD.L1[B] primer set. Parameters that were varied between assays in order to achieve desired efficiency and $R^2$ are in bold type.*

For each qPCR assay:

| | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| Polymerase Activation | 95 | 15 min | N/A |
| **Denaturation** | 95 | **1-2 sec** | 45 |
| **Annealing** | 65 | **5-10 sec** | |
| Extension | 72 | 25 sec | |
| Melt | 95 | 15 sec | N/A |
| | 55 | 15 sec | N/A |
| | 95 | 15 sec | N/A |
| Hold | 4 | ∞ | N/A |

*Table 4.6b. Different amplification conditions tested to optimize an EcPV-2 qPCR assay using the MD.L1[B] primer set. Parameters that were varied between assays in order to achieve desired efficiency and $R^2$ are in bold type.*

After showing in the pilot assays that optimal qPCR assay efficiency and $R^2$ values could be obtained using the parameters listed in tables 4.6a and 4.6b, the qPCR assay was repeated using the $10^9$ copies/µl EcPV-2/L1-591 stock solution as a standard. Tenfold dilutions of the standard were made in 50 µl volumes by adding 5 µl of the standard to 45 µl of elution buffer to produce serial dilutions from $10^8$ to $10^0$ copies/µl of EcPV-2/L1-591.

The performance of the assay was assessed several times. Each assessment run was performed using an Eco real-time qPCR machine, AccuMelt master mix, a primer concentration of 5 µM, and 2 µl of template DNA. Amplification conditions are listed in table 4.7. Assays were run in duplicate unless otherwise stated. For an additional

positive control, DNA extracted from an equine pharyngeal SCC previously shown to be EcPV-2-positive was used. For a negative control, DNA extracted from an equine pharyngeal SCC previously shown to be EcPV-2-negative was used. For a non-template control, template DNA was omitted and replaced with an equivalent volume of water.

For each qPCR assay:

| | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| Polymerase Activation | 95 | 15 min | N/A |
| Denaturation | 95 | 2 sec | 45 |
| Annealing | 65 | 5 sec | |
| Extension | 72 | 25 sec | |
| Melt | 95 | 15 sec | N/A |
| | 55 | 15 sec | N/A |
| | 95 | 15 sec | N/A |
| Hold | 4 | ∞ | N/A |

*Table 4.7. Amplification conditions for an EcPV-2 qPCR assay using the MD.L1[B] primer set.*

Precision was evaluated by calculating intra-assay variability based on the distribution of Cq values for 5 replicates of each standard in a single qPCR run (assay 1). Reproducibility (inter-assay variability) was evaluated by comparison of the Cq values obtained for the same standards in three separate qPCR runs (assays 2-4). The intra- and inter-assay variability were expressed as coefficients of variation (CV), which were calculated by dividing the Cq value of each replicate of the standard by the mean Cq value for that standard. A master standard curve was created by combining results all four assays (assays 1-4) using EcoStudy software (Illumina). This master standard curve was used to estimate viral copy numbers in clinical samples, which is described in the next chapter.

### 4.3.3 Results

#### *4.3.3.1 Initial Comparison of the MD.L1[A] and MD.L1[B] Primer Sets*

The MD.L1[B] primer set was superior to the MD.L1[A] set (figure 4.8). The relative increase in fluorescence was higher and occurred at an earlier cycle for the MD.L1[B] primer set when compared with the MD.L1[A] set.

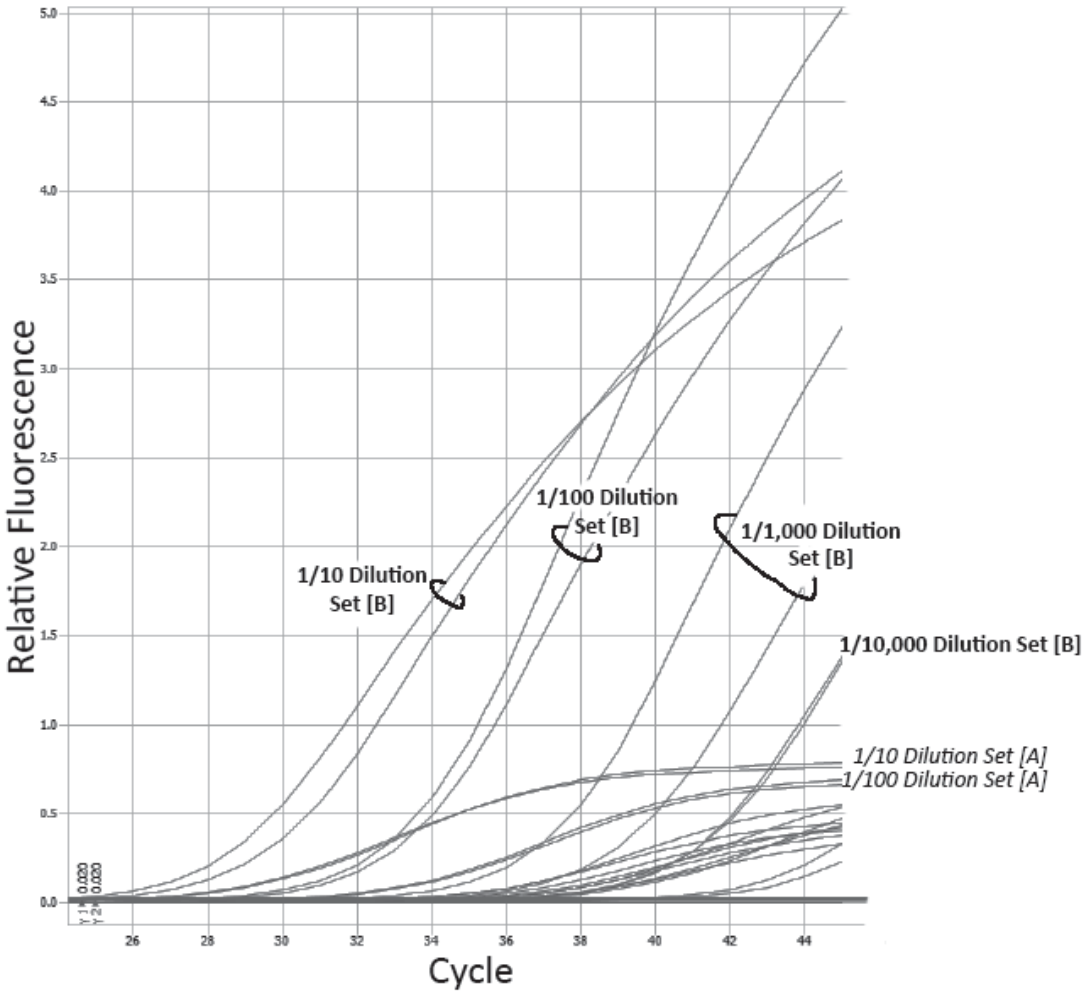


*Figure 4.8. Comparison of results from a qPCR assay using two different primer sets and serial dilutions of DNA extracted from an EcPV-2 positive tissue as test samples. When using primer set [B] the increase in fluorescence was higher and occurred at an earlier cycle than when using primer set [A]. Set [A] is the MD.L1[A] primer set, which amplifies a 104 bp sequence of the EcPV-2 L1 gene. Set [B] is the MD.L1[B] primer set, which amplifies a 118 bp sequence of the EcPV-2 L1 gene*

#### *4.3.3.2 Creation of a Stock Solution Containing an EcPV-2 DNA Fragment*

Sequence analysis showed the amplified 591bp PCR fragment was 100% identical to published EcPV-2 genome sequences (data not shown). Using a spectrophotometer the DNA content was 20.9 ng/µl, and the 260/280 ratio was 1.96, indicating high quality of the DNA. Using a fluorometer the DNA content was 21 ng/µl, which correlated with the spectrophotometer results.

A 500 µl stock solution containing $10^9$ copies of the 591 bp EcPV-2 L1 gene fragment per µl was created by adding 15.2 µl of EcPV-2 amplicon to 484.8 µl of diluent. The resulting stock solution was divided into aliquots, and frozen for future use.

#### *4.3.3.3 qPCR Assay Optimization and Generation of a Standard Curve*

The conditions that achieved the best efficiency and $R^2$ values were: primer concentration 0.5 µM; denaturation for 2 seconds; and annealing for 5 seconds. Using these parameters, efficiency and $R^2$ values of approximately 100% and 1, respectively, were repeatedly obtained (figure 4.9). A single melting peak between 85.3°C and 85.6°C was observed in all runs. As expected, EcPV-2 DNA was amplified from positive controls but not from any negative or non-template controls.

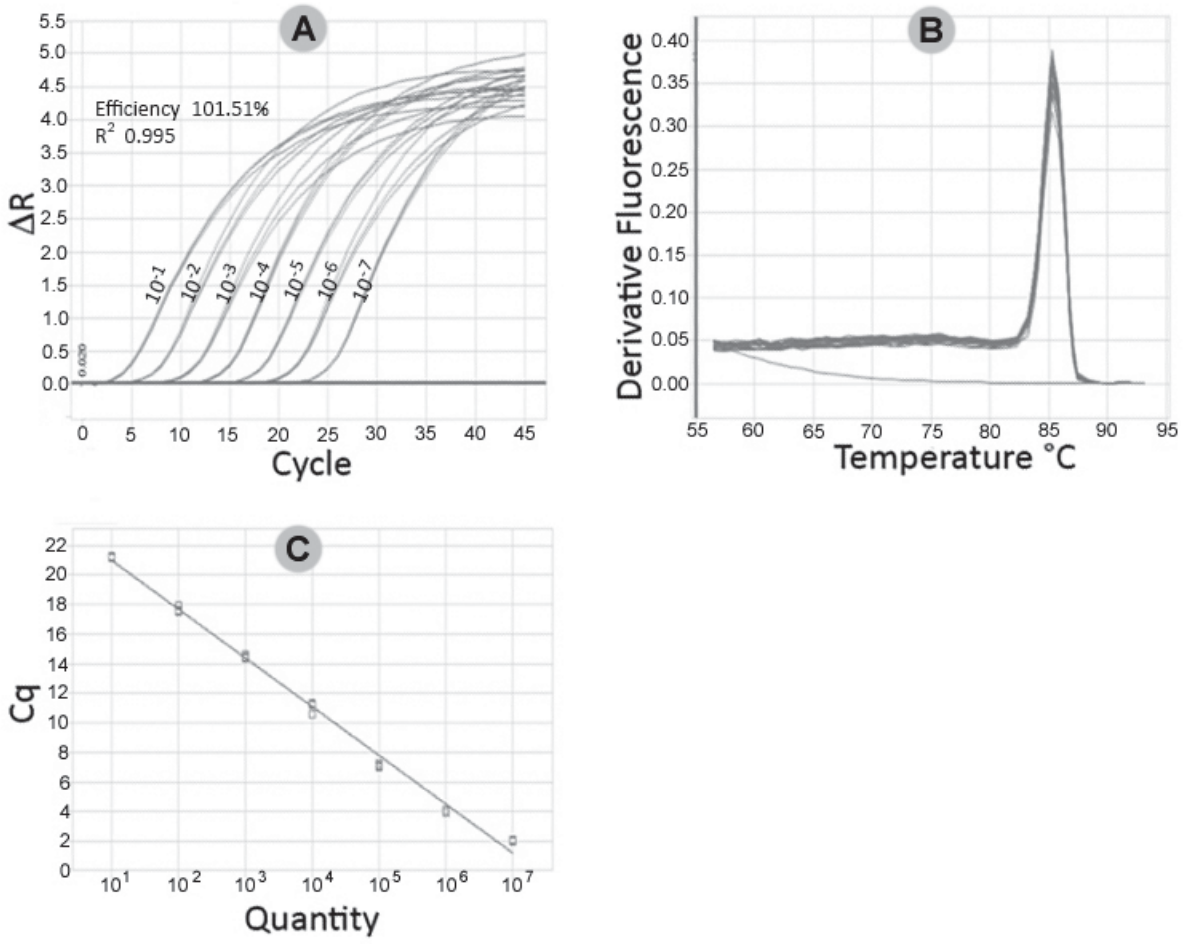


*Figure 4.9. An example of qPCR results using the MD.L1[B] primer set to generate a standard curve using serial dilutions of a test sample. The efficiency of the assay shown is 101.51% and the $R^2$ value is 0.995, and there is a consistent melting peak between 85.3 and 85.6°C. The standards consist of tenfold serial dilutions of a stock solution containing 3.3 x $10^{10}$ copies/µl of the EcPV-2/L1-591 DNA fragment. $10^{-1}$ indicates a 1/10 dilution of the stock solution, $10^{-2}$ indicates a 1/100 dilution and so on. (A) amplification plot; (B) melting peaks; (C) standard curve.*

The efficiency and $R^2$ values for each assay were within acceptable limits (90-110% and > 0.99, respectively). When the four assays were combined the results were linear

within the tested range from 1 to $10^8$ target copies per µl, with an efficiency of 94.66% and an $R^2$ of 0.999 (figure 4.10).

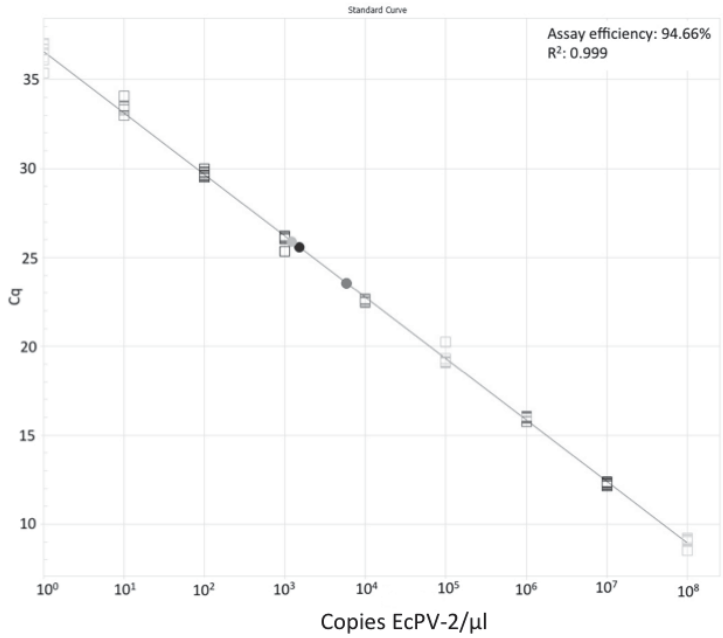


*Figure 4.10. EcoStudy results combining four qPCR assays for EcPV-2. The assay is linear within the tested range from 1 to $10^8$ target copies per µl, with an efficiency of 94.66% and an $R^2$ of 0.999.*

The coefficient of variation (CV) of the mean Cq values obtained in three separate runs of the assay ranged from 0.4% to 2.9%, indicating high reproducibility (table 4.8a). The CV of the mean Cq values obtained using five replicates of each dilution of the standard in a single test run ranged from 0.4% to 3.3%, indicating excellent assay precision (table 4.8b). The assay was able to detect the equivalent of 1 copy of EcPV-2 genomic DNA, indicating good analytical sensitivity.

| Target Copies/µl | Mean Cq | Standard Deviation | Coefficient of Variation |
|---|---|---|---|
| $10^8$ | 9.02 | 0.26 | 2.88 |
| $10^7$ | 12.27 | 0.09 | 0.73 |
| $10^6$ | 15.99 | 0.12 | 0.75 |
| $10^5$ | 19.33 | 0.45 | 2.33 |
| $10^4$ | 22.6 | 0.09 | 0.40 |
| $10^3$ | 25.97 | 0.36 | 1.39 |
| $10^2$ | 29.73 | 0.18 | 0.61 |
| $10^1$ | 33.43 | 0.37 | 1.11 |
| $10^0$ | 36.44 | 0.7 | 1.92 |

*Table 4.8a. Inter-assay variation over three runs using duplicates of each dilution of the standard. The coefficient of variation of the mean Cq values obtained in 3 separate runs of the assay range from 0.4% to 2.9%, indicating high reproducibility.*

| Target Copies/µl | Mean Cq | Standard Deviation | Coefficient of Variation |
|---|---|---|---|
| $10^8$ | 5.28 | 0.023 | 0.44 |
| $10^7$ | 8.66 | 0.12 | 1.39 |
| $10^6$ | 12.37 | 0.09 | 0.73 |
| $10^5$ | 15.7 | 0.14 | 0.89 |
| $10^4$ | 19.11 | 0.16 | 0.84 |
| $10^3$ | 22.49 | 0.14 | 0.62 |
| $10^2$ | 26.04 | 0.26 | 1.00 |
| $10^1$ | 29.69 | 0.41 | 1.38 |
| $10^0$ | 32.85 | 1.08 | 3.29 |

*Table 4.8b. Intra-assay variation in a single run using 5 replicates of each dilution of the standard. The coefficient of variation of the mean Cq values obtained with 5 replicates of each dilution of the standard in a single test run ranged from 0.4% to 3.3%, indicating excellent assay precision.*

## 4.4 Discussion

### 4.4.1 Overview

The focus of this chapter was the design and validation of a quantitative PCR assay for diagnosis of EcPV-2 infection and quantification of viral load within infected tissues. The following sections discuss in turn the general components of an effective qPCR assay and then focus specifically on the EcPV-2 qPCR assay designed in this chapter.

### 4.4.2 Primer Design

#### *4.4.2.1 Optimal Primer Design*

Our initial attempt to develop a qPCR assay for EcPV-2 was unsuccessful due to poor primer design. Although the MD.L1[A] primer set had been used successfully in conventional PCR for EcPV-2, the presence of secondary structures within its product prevented it from achieving an efficiency suitable for qPCR. The following list outlines common rules for good primer sequence design.[16,35]

1. Primers should be roughly 20 (17-28) bases in length. This is generally sufficient because the chance of a perfect match for either primer at an unintended site in the genome is low. The chance that both primer sequences occur at unintended sites in close proximity to one another is extremely low.
2. Base composition should be 40-60% G+C because GC pairing is stronger and thus more stable than AT pairing.

3. Primers should end (3') in a G or C, or CG or GC, which increases the efficiency of priming because of the stronger GC pairing.
4. Melting temperatures between 55-80°C are preferred.
5. Runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided.
6. 3'-ends of primers should not be complementary, as primer dimers will be synthesized preferentially.
7. The GC difference between reverse and forward primers should be ≤5%.
8. The melting temperature difference between reverse and forward primers should be ≤5°C.
9. Primers should not self-hybridize. After designing primers, it should be verified that the primers will not self-hybridize or dimerize. Candidate primers that show undesirable self-hybridization should be discarded.

With regard to the first eight criteria, both the MD.L1[A] and MD.L1[B] primer sets used in this chapter fulfilled all requirements (table 4.9). However, the MD.L1[A] amplicon was not initially assessed for secondary structures. When we discovered that it was not possible to achieve a reaction efficiency greater than 85%, the amplicon was then checked for the presence of secondary structures using mfold,[38] and two stem and loop secondary structures were found ("hairpin loops"; figure 4.5). Hairpin loops can greatly reduce the efficiency of a PCR reaction by limiting a primer's ability to bind to its target site.[33] Because of the presence of these hairpin loops in the MD.L1[A] amplicon, the MD.L1[A] primer set was discarded and a new primer set was designed. The predicted product of the new primer set was assessed using mfold and shown to be free of secondary structures. This highlights the need for early assessment of predicted PCR products for secondary structures before proceeding with experiments.

| Desired Primer Design Criteria | MD.L1[A] Primer Set: Forward, Reverse | MD.L1[B] Primer Set: Forward, Reverse |
|---|---|---|
| 1. Primer length is 17-28 bases | 20, 20 ☑ | 20, 20 ☑ |
| 2. GC composition is 40-60% | 70%, 65% | 70%, 70% |
| 3. Primer end (3') is G, C, GC or CG | GC, GC ☑ | G, G ☑ |
| 4. $T_m$ is 55-80° | 59.97°, 60.04° ☑ | 59.77°, 60.05° ☑ |
| 5. C or G runs at 3' end not present | Not present in either primer ☑ | Not present in either primer ☑ |
| 6. Primer ends (3') not complementary | C, C (not complementary) ☑ | G, G (not complementary) ☑ |
| 7. Fwd & rev primer GC difference ≤ 5% | Yes (5%) ☑ | Yes (0%) ☑ |
| 8. Fwd & rev primer $T_m$ difference ≤ 5° | Yes (0.07°) ☑ | Yes (0.28°) ☑ |

*Table 4.9. Primer design criteria as they apply to the MD.L1[A] and MD.L1[B] primer sets.*

#### *4.4.2.2 Choice of PV Primer Target*

For the assay described in this chapter it was necessary to design EcPV-2-specific primers that amplify a certain region of the PV genome. We chose to design a primer set that amplifies a segment of the EcPV-2 L1 gene.

At the time the work described in this chapter began there were six publications describing the use of PCR to detect EcPV-2.[4,17,19,20,30,31] Primers designed to amplify segments of the following PV genes had been used: E1 gene (4 papers),[4,17,30,31] L1 gene (4 papers),[4,19,20,31] E6 gene (4 papers)[4,17,30,31] and E7 gene (1 paper).[4] Among these papers there was no justification provided for the choice of primer and, therefore, little guidance as to which gene to target when designing our new primers since all performed similarly well. However, two papers described discrepancies in banked E1, E6 and E7 gene sequences from EcPV-2.[4,31] In addition, E genes may be deleted during integration into the host genome in humans.[8,9,39] This has not been investigated for EcPV-2, but one paper speculated about deletion of the EcPV-2 E1 gene as a possible explanation for lack of detection of EcPV-2 DNA in samples when using primers directed at the EcPV-2 E1 gene.[17] We are not aware of any studies that suggest the PV L1 gene is deleted during integration into the host genome in any species. Therefore, because of its conserved nature, its preservation during integration into the host genome, and our familiarity with PV L1 consensus primers we chose to design a primer set that amplified a segment of the EcPV-2 L1 gene.

### 4.4.3 Standards

An effective qPCR assay requires the creation of a set of standards that contain a target DNA sequence specific to the primer set used. Additionally, these standards should be free of potential inhibitors of PCR and other contaminants. Finally, if absolute quantification of EcPV-2 copy numbers is the aim then the number of copies of the target DNA sequence per µl must be known. This allows generation of a standard curve; test samples are compared to this standard curve in order to quantify their target DNA content.

In the experiments described in this chapter we prepared two different types of standard for use in generation of standard curves. The first was a linearized recombinant plasmid containing an inserted 104 bp segment of the EcPV-2 L1 gene. The second was a 591 bp fragment of the EcPV-2 L1 gene generated by conventional PCR amplification. The use of a recombinant plasmid to generate a positive control did have several advantages. Controls created in this way are inexhaustible, unlike those generated by extraction of DNA from tissue samples. Transformed bacterial colonies containing recombinant plasmids with the correct EcPV-2 insert could be stored at -80°C, then thawed and re-cultured to generate further stocks when necessary. Another advantage was that the positive control could be used as template DNA when optimizing a qPCR assay. Using the plasmid-based positive control meant that assays could be optimized without the need to use potentially irreplaceable clinical samples. A third advantage was that serial dilutions of the positive control could be used to determine the analytical sensitivity (limit of detection) of the assay in terms of the smallest number of target DNA sequences able to be detected. However, using conventional PCR to generate a positive control instead of using a plasmid was easier, less time-consuming, and less expensive than the recombinant plasmid method. Because the molecular weight of a single copy of the control molecule (whether recombinant plasmid or PCR product) was known, both techniques allowed preparation of a stock solution containing $10^9$ copies per µl. As such, both techniques were equally suitable for generation of a standard curve and both have been used in previous studies. [3,13,25,37]

### 4.4.4 Assay Performance

#### *4.4.4.1 Assay Efficiency*

An efficiency of 100% for a qPCR assay means that there is an exact doubling of the number of template molecules in each PCR cycle. Acceptable efficiencies range from 90-110% and imply that reaction conditions are optimal, primers are well designed, and pipetting of reagents and samples is accurate. This ensures that results obtained are reliable and repeatable. Reactions with poor efficiency have poor sensitivity, particularly for samples with low copy numbers of the target sequence.[25]

During our trial assays using the MD.L1[A] primer set (section 4.2) we obtained an acceptable $R^2$ value but were never able to achieve an efficiency higher than 85%. Efficiencies less than 90% may be due to poor primer design (secondary structures or inappropriate melting temperatures), poor choice of region to be amplified (secondary structures), or suboptimal reagent concentration. Efficiencies above 110% are most commonly caused, paradoxically, by the presence of PCR inhibitors in concentrated samples. If inhibitors are present in concentrated samples, more cycles are needed to reach the threshold of detection than would be needed in the absence of inhibitors. However, as inhibitors are diluted out the amplification becomes more efficient. Consequently, the difference between Cq values of concentrated and diluted sample is smaller than predicted and the amplification appears (artificially) more efficient.

Primer design is most commonly at fault in poorly performing assays and was therefore the first factor that we investigated when we failed to obtain good assay efficiency. Mfold analysis of predicted PCR products indicated the presence of a secondary structure within the binding site for the reverse MD.L1[A] primer (figure 4.5). In one study a secondary structure at the forward primer binding site prevented efficiency from ever rising higher than 80%, despite trying various annealing temperatures and magnesium concentrations.[25] In that particular study, the efficiency was raised to 95% simply by moving the forward primer 14 bases downstream so that the primer no longer annealed to the region with the secondary structure, emphasizing the importance of primer design on assay efficiency. This was not possible

for our reverse primer, however, because of the presence of an additional secondary structure within the target sequence. Therefore, the primers were abandoned and replaced with a new set, after which acceptable efficiency and $R^2$ values were easily obtained.

#### *4.4.4.2 Assay Sensitivity*

All laboratory tests or assays have two kinds of sensitivity: analytical and diagnostic.

Analytical sensitivity indicates the smallest amount of substance in a sample that can accurately be measured by an assay.[29] Typically, this is expressed as the limit of detection (LOD), which is the minimum concentration that can be detected with reasonable certainty using the procedure. The qPCR assay for EcPV-2 described in this chapter was able to detect a single copy of the EcPV-2 target sequence, meaning it had very high analytical sensitivity.

Diagnostic sensitivity, on the other hand, is the proportion of individuals with a given condition who are identified by the assay as positive for that condition.[29] It is typically expressed as a percentage: the number of individuals with the condition *and* a positive test divided by the number of individuals with the condition. Therefore a high diagnostic sensitivity for our EcPV-2 qPCR assay would mean that any horse infected with EcPV-2 would be positive by qPCR. A low diagnostic sensitivity would mean that many EcPV-2-infected horses would not be detected using our qPCR assay. At this stage, discussion of diagnostic sensitivity for our EcPV-2 qPCR assay is not possible because no clinical samples have been tested. This is discussed in the next chapter, which describes screening of normal and diseased equine tissues.

Diagnostic sensitivity often has more to do with whether a particular target molecule is actually included in a sample from a patient with a condition than with the ability to detect very low concentrations of that molecule. For example, an exquisitely sensitive (high analytical sensitivity) PCR-based blood test for HIV-1 may not detect a proportion of patients who are confirmed seropositive, simply because the virus is not present in the particular sample of blood analyzed (reduced diagnostic sensitivity).[5] Similarly,

equine herpesvirus type 1 may not be detected by PCR in a seropositive horse because it remains latent at such low numbers within ganglia that it may not be included in a sample, despite high analytical sensitivity of the PCR assay.[12] Thus, diagnostic and analytical sensitivity are distinct, and high analytical sensitivity does not guarantee high, or even acceptable, diagnostic sensitivity.

*Analytical Sensitivity of the EcPV-2 Assay*

In theory, a sample containing even a single copy of the EcPV-2 genome would be detected by our assay. No other EcPV-2 qPCR assay has ever been described and so it is not possible to compare this analytical sensitivity with that of alternative EcPV-2 assays. Even for other non-human PV assays analytical sensitivities are rarely reported. A possible reason for this is that it is the *lesions* resulting from PV infection in individuals or small numbers of animals that tend to be the focus of investigation (e.g., equine aural plaques, feline Bowenoid *in situ* carcinomas). Usually, it is the presence and type of PVs in lesions, not the copy number, that are of primary interest. Because of this, the analytical sensitivity of the assay is often not calculated. An exception to this is equine cutaneous sarcoid, in which a proportion of clinically normal horses are also infected by the putatively causative bovine PVs. Viral load is an important consideration in determining why some BPV-infected horses develop sarcoids and some do not. In one study of equine sarcoids the analytical sensitivity was calculated to be 20 copies of bovine PV DNA per sample.[2]

Although the analytical sensitivity of non-human PV assays is rarely considered, in human PV assays it is an issue of great concern because a proportion of HPV-infected people will go on to develop anogenital or head and neck cancer. Therefore, accurate diagnosis of not just HPV infection, but also viral load and type, is essential. Currently, PCR using consensus primers is the most commonly used technique for the diagnosis of HPV infection.[21] Numerous studies have investigated the analytical sensitivities of HPV PCR assays and some are discussed here because of the scarcity of information for non-human PV PCR assays.[18,23,32] One study comparing three common PCR-based assays for HPV detection in cervical samples found a limit of detection of 191 copies of the HPV genome per sample.[32] According to the authors this demonstrated high

analytical sensitivity, implying that our EcPV-2 assay, which can detect a single copy of the EcPV-2 genome per sample, is more than acceptable.

There are two likely reasons why our EcPV-2 assay appears to have such a high analytical sensitivity when compared with either the HPV or BPV assays mentioned in the previous paragraphs. The first is that HPV screening by PCR uses consensus primers, which have a lower analytical sensitivity than specific primers.[10] For example, one study compared detection of HPV-16 using consensus and specific primers and found specific primers to be 50 times more sensitive.[18] Consensus primers are used because HPV-associated disease can be due to infection with any of several types of HPV (HPV-16, -18, -31 and so on) and all must be screened for. This is in contrast to our work on EcPV-2, in which a single PV type is of interest and can therefore be screened for using specific primers. The second reason for our high analytic sensitivity is that clinical samples such as equine sarcoids or human cervical neoplasms contain a mixture of PV and host genomic DNA rather than consisting, like our EcPV-2 standard, of a pure solution of the target sequence. Host genomic DNA, when present at a high concentration in a test sample, can reduce the sensitivity of qPCR assays. This may be due to competitive inhibition of primer-template hybridization or simply to a decreased rate of diffusion of reaction components in the presence of large amounts of long strands of host DNA.[7,26] A useful addition to the work in this chapter would have been to mimic clinical samples by "spiking" our control sample with serially diluted amounts of equine DNA and determining the level at which the added DNA caused any loss of analytical sensitivity (i.e., PCR inhibition). This inhibitory level could then have been used as the upper threshold for the amount of template DNA that could be added to a PCR reaction. Determination of this threshold has been reported for various microorganisms and ideally would have been performed for our EcPV-2 assay.[7,22,26]

In addition to host genomic DNA, clinical samples may also include other potential inhibitors of PCR, such as: fragmented DNA debris in formalin-fixed tissue; blood, bacteria and microorganisms collected at the time of sampling; reagents used in the extraction and purification of nucleic acids; melanin in skin; and even glove

powder.[11,27]

#### *4.4.4.3 Assay Specificity*

As with sensitivity, all laboratory tests also have two kinds of specificity: analytical and diagnostic.[29]

Analytical specificity is the ability of an assay to exclusively identify the target of interest rather than similar molecules in the sample. Therefore, a high analytical specificity for our EcPV-2 qPCR assay would mean that a sample containing EcPV-2 would be positive by qPCR, but not a sample containing EcPV-1 or -3 or any other PV, such as those associated with sarcoids.

The meaning of diagnostic specificity is less clear. An assay with poor diagnostic specificity is one in which the target molecule is amplified but there is no evidence of disease. This may occur with happen in three ways. Firstly, it may occur through external contamination (e.g., from previous amplification of another sample). Secondly, it may be due to the target sequence truly being present but the sequence not representing an intact organism capable of reproducing or causing disease (i.e., analytical specificity is still good, but the results are diagnostically incorrect). Thirdly, and most importantly in the case of PV infection, it may represent a true infection, but one that is either latent or takes many years to cause clinical disease. Whatever the reason, a clinical dilemma may arise when the only indication of a particular infection is a positive result by PCR.

The usual way to confirm the analytical specificity of an assay is to screen samples known to contain sequences similar to, but different from, the target sequence. An assay with high analytical specificity should detect the target sequence exclusively and should not amplify even closely related sequences. In the case of our EcPV-2 assay, PVs known to infect horses (EcPV-1 and -3 and BPV-1 and -2) would have been suitable targets to confirm specificity of the primers. Other suitable targets would have been PVs phylogenetically closely related to EcPV-2. These tests were not performed, which might be considered a shortcoming of the assay developed in this chapter. However, we used an alternative method to check the analytical specificity of our assay, which

was to check primer matches using BLAST.[1] Each of the MD.L1[B] primers had 100% sequence identity with the published genome for EcPV-2 (figure 4.6). There was no similarity to any other papillomavirus or virus, suggesting that this primer set had high analytical specificity for EcPV-2.

## 4.5 Chapter Summary

This chapter described the development of a qPCR assay for EcPV-2. The assay performed well in multiple trial runs, and had excellent analytical sensitivity and specificity. Thus, this assay is suitable for the assessment of the EcPV-2 load in clinical samples.

The results and conclusions from this chapter have been published in the article:

> *Knight CG, Dunowska M, Munday JS, Peters-Kennedy J, Rosa BV:* ***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****. Veterinary Microbiology 166: 257-262, 2013*

## 4.6 Glossary

The following highlights the PCR-specific terms used in this chapter, and provides an explanation of their meaning.

- In a qPCR reaction, DNA is amplified and tracked using a fluorescent **reporter dye**. The reporter dye used in this chapter is **SYTO 9** which binds to double stranded DNA (dsDNA). It fluoresces when bound to dsDNA but not when dissociated and in solution. SYTO 9 was chosen for use because it is a saturating dye that does not inhibit PCR, even when used at high concentrations. In contrast, first generation reporter dyes such as SYBR Green I can be problematic because high dye concentrations inhibit PCR. Because of this they must be used at sub-saturating concentrations.[28]
- The **threshold** is a level set above the background "noise" generated during the early cycles of qPCR when low-level non-specific fluorescence signals are generated (typically cycles 3-15).

- The **Cq** (quantification cycle) is the cycle number at which the fluorescent signal crosses the threshold. The Cq value assigned to a sample reflects the number of cycles needed for a sufficient number of amplicons to accumulate to raise fluorescence above the background "noise". This is used to estimate the amount of DNA present at the start of the reaction.
- Robust and precise qPCR assays are correlated with high **efficiency**. An efficiency of 100% for a qPCR assay means that there is an exact doubling of the number of template molecules in each PCR cycle. Acceptable efficiencies for publication range from 90-110%. Reasons for efficiencies outside this range include incorrect dilution causing errors in the standard curve, poor primer design, and reaction inhibition caused by the addition of excessive template DNA.
- The $\mathbf{R^2}$ value (coefficient of correlation) of a qPCR assay is a measure of how closely plotted data points fit the standard curve. It measures correlation between Cq values and DNA concentrations. The closer to 1 the value, the better the fit. $R^2$ is ideally > 0.99.
- The $\mathbf{T_m}$ (melting temperature) is the temperature at which 50 percent of dsDNA in a sample is single-stranded or "melted". The $T_m$ for a DNA fragment is specific for that fragment and depends on its length and sequence, including the G:C content. At the end of a qPCR assay the temperature is gradually raised (generally from 65°C to 95°C). As the fragment is heated, a sudden decrease in fluorescence is detected when $T_m$ is reached due to denaturation of dsDNA strands and subsequent dissociation of the **SYTO 9** dye. In practical terms, $T_m$ can be used to infer the presence and identity of an amplified DNA fragment. $T_m$ is calculated by plotting temperature (x axis) versus the derivate of fluorescence with respect to temperature (-dF/dT) (y axis), producing a **melting curve**. The maximum amplitude of a melting curve is the **melting peak**, which occurs at $T_m$. Only one amplicon is expected in a sample so there should be a single melting peak. Multiple melting peaks or peaks at unexpected temperatures indicate contamination, primer dimers or mispriming.

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# CHAPTER FIVE

# Investigation of EcPV-2 Load and Presence in Equine Tissues using Quantitative PCR

## 5.1 Introduction

The findings presented in chapters 2 and 3 suggested that the presence of equine papillomavirus type 2 (EcPV-2) DNA sequences was associated with equine penile squamous cell carcinomas (EPSCCs) and papillomas. To allow further investigation of this association, in chapter 4 we developed a sensitive qPCR assay for EcPV-2 that would allow quantification of EcPV-2 viral load. In this chapter we report the results of

an experiment comparing EcPV-2 load in equine squamous cell carcinomas (SCCs) and non-cancerous tissues using qPCR.

This study had three objectives. The first was to compare the viral DNA loads of EcPV-2 in EPSCCs with those in (1) clinically normal equine penile mucosal samples and (2) non-SCC equine penile lesions. If EcPV-2 infections were rare in non-SCC penile mucosa but common in EPSCCs this would support its causal role in EPSCC development, analogous to the role of high-risk HPVs in human penile cancers.[38] Conversely, if EcPV-2 infection were as common in clinically normal penile mucosa and non-SCC equine penile lesions as in EPSCCs this would suggest that the virus is a harmless and ubiquitous commensal that does not play a role in the development of EPSCCs. Since the overall hypothesis of this thesis is that EcPV-2 infection can lead to SCC development, this information was essential.

The second objective was to investigate the viral DNA load of EcPV-2 in other equine body sites that have a predilection for SCC development. These include the vulva,[31] oral cavity and pharynx (collectively, the "throat"),[14,40,50] and nictitating membrane.[30,41,44] It is plausible that if EcPV-2 plays a role in EPSCC development then it may also have a role in SCC development in these other body sites, analogous to high-risk PVs causing cancer of the cervix, external genitalia, and throat in people.[5,18,32,38] However, it was impractical to obtain biopsy samples from living horses due to the need for owner consent and animal restraint, sedation and analgesia. In addition, the glans penis, throat and nictitating membrane are difficult sites to access and sample. For these reasons, freshly killed abattoir horses were used as the source of clinically normal tissues, and archival fixed tissues were used as the source of SCC and non-SCC lesions for the work described in this chapter.

The third objective was to further assess the performance of the qPCR assay developed in chapter 4, this time using clinical samples.

## 5.2 Materials and Methods

### 5.2.1 Sample Collection

Fresh tissues were obtained from 75 clinically normal horses slaughtered on one day at an abattoir in southern New Zealand. Samples of nictitating membrane, pharyngeal mucosa and genital mucosa were taken from each horse as follows. The horse was decapitated during processing, leaving the larynx and proximal trachea attached to the head. Using rat-tooth forceps and scissors, one nictitating membrane was grasped and excised. The excised nictitating membrane was then bisected, with half placed into a plastic specimen bag on ice, and the other half placed into 10% neutral buffered formalin. The pharynx was then exposed by dissection, and an approximately 2 x 2 x 0.5 cm section of oropharyngeal mucosa collected from one side. The specimen was bisected, and half was frozen and half placed into formalin as described above. Finally, a disc of genital mucosa measuring approximately 2 x 2 x 0.5 cm was collected. From males the section was collected from the junction of the glans penis and the free part of the penis. From females the section was collected from the vestibular floor lateral to the clitoral fossa. Each genital mucosal sample was bisected, and half was frozen and half placed into formalin as described above. To avoid cross-contamination between tissues from the same horse, or between horses, instruments were wiped clean with a paper towel, soaked in ethanol, and flamed for several seconds twice after each use. Because of the slaughtering and butchery sequence it was not possible to determine which head belonged with which genitalia. After collection, samples were shipped to Palmerston North overnight on ice and transferred to a minus 80°C freezer for storage.

Formalin-fixed, paraffin-embedded (FFPE) tissues containing SCC and non-SCC disease lesions of the penis, oral cavity and pharynx were obtained by searching the archives of the Department of Biomedical Sciences, Cornell University, College of Veterinary Medicine, Ithaca, New York. Archived tissues had been embedded in paraffin blocks for histologic sectioning and long-term storage. Penile non-SCC lesions excluded sarcoids or precursor lesions of SCC, such as papillomas and precancerous plaques. Oral or pharyngeal SCC samples excluded any from anatomic locations that could have

been exposed to UV light (i.e., haired skin and mucocutaneous junctions). The original diagnosis for each case was recorded and then reconfirmed using the original hematoxylin and eosin-stained sections. The signalment of each horse was recorded, when available. Three 10 μm thick sections were cut from each FFPE tissue block and placed into three labeled 1.5 ml Eppendorf snap-lock microcentrifuge tubes for DNA extraction and subsequent PCR.

### 5.2.2 Age Estimation

The ages of slaughtered horses were estimated based on incisor teeth eruption and wear according to a previously published method.[10] Horses were assigned to the following categories: 0-5 years old; 6-10 years old; 11-15 years old; 16-20 years old; >21 years old.

The ages of the horses providing the FFPE lesional tissue were recorded from patient records, when available.

### 5.2.3 DNA Extraction

Total DNA was extracted from fresh tissues using a commercially available extraction kit according to the manufacturer's instructions (Roche High Pure PCR Template Preparation Kit). The ''Isolation of Nucleic Acids from Mammalian Tissues'' protocol was followed and 1 $mm^3$ of tissue used.

Total DNA was extracted from FFPE tissue shavings using the same Roche extraction kit but following the "Isolation of Nucleic Acids from Formalin-Fixed Paraffin-Embedded Tissue Sections" protocol.

The quantity and quality of extracted DNA were assessed for all samples using a Nanodrop spectrophotometer (Thermo Scientific).

### 5.2.4 Confirmation of Amplifiable DNA in Samples

Each sample was checked for the presence of amplifiable DNA by qPCR using previously published equine beta actin primers.[3] These primers amplify a 219 bp

fragment of the equine beta actin gene, ACTB (GenBank accession number AF035774), which encodes a constitutively expressed cytoskeletal structural protein. Each qPCR reaction was performed using AccuMelt™ HRM SuperMix (Quanta Biosciences, Gaithersburg, Maryland, USA), a final concentration of 0.5 µM of each primer (forward: 5'-CCA GCA CGA TGA AGA TCA AG-3' and reverse: 5'-GTG GAC AAT GAG GCC AGA AT-3') and 2 µl of template DNA in a total volume of 10 µl. The cycling conditions consisted of a 5 minute activation step followed by 45 cycles of denaturation (95°C for 5 s), annealing (59°C for 5 s) and elongation (72°C for 20 s). The cycling was followed by a melting step from 55°C to 95°C.

### 5.2.5 qPCR Assay for EcPV-2

Quantitative PCR assays were run using an Eco real time instrument (Illumina Inc., San Diego, CA, USA). The qPCR was designed to amplify a 118 bp fragment of the EcPV-2 L1 gene. Each qPCR reaction was performed using AccuMelt™ HRM SuperMix (Quanta Biosciences, Gaithersburg, Maryland, USA), a final concentration of 0.5 µM of each primer (MD.L1(B). Forward: 5'-GCG GTG TCG AGG TGT CAC GG-3' and MD.L1(B). Reverse: 5'-CGC TCC CCT GCA GCT TCC TG-3') and 2 µl of template DNA in a total volume of 10 µl. The cycling conditions consisted of a 15 minute denaturation step followed by 45 cycles of denaturation (95°C for 2 s), annealing (65°C for 5 s) and elongation (72°C for 25 s). The cycling was followed by a melting step from 55°C to 95°C.

The numbers of viral copies per µl of template DNA were calculated from quantification cycle (Cq) values using either Eco (single run) or EcoStudy (multiple runs) software (Illumina Inc.) based on the standard curve generated from serial dilutions ($10^8$–$10^0$ copies/µl) of a stock solution containing a known concentration of a 591 bp fragment of the EcPV-2 L1 gene. The copy number values were then normalized to the amount of template DNA in each reaction and expressed as viral copies per nanogram of template DNA. Values lower than 1 copy/µl were extrapolated. Automatic threshold detection settings were used. Samples were considered positive if the amplification curve crossed the threshold and there was a corresponding melting peak between 85.3°C and 85.6°C.

All standards and samples were run in duplicate. A non-template control (water) was included in each run. For analysis of samples from multiple runs, the master standard curve was used within the EcoStudy software (version 4.0.4420). One standard ($10^6$ copies/µl) was designated as a plate control and included on each contributing plate. Plates that showed a Cq variation between the master and control standards of ≤ 0.5 were considered valid.

### 5.2.6 Statistical Analysis

Data were analyzed using SAS 9.2. Fisher's exact test was used to compare the frequency of detection of EcPV-2 in tissues with and without SCC. Differences in mean viral loads were compared using the Wilcoxon–Mann–Whitney test. Differences were considered statistically significant if $p < 0.05$.

## 5.3 Results

### 5.3.1 Sample Collection

In total, 222 fresh tissue samples were collected from 75 slaughtered horses. These were: penile mucosa (*n* = 32); vulvar vestibular mucosa (*n* = 40); pharyngeal mucosa (*n* = 75); nictitating membranes (*n* = 75). Gross lesions were not present in any sample collected. The genital mucosa from three of 75 horses was not available for sampling.

In total, 60 FFPE tissue samples were obtained. These were penile SCCs (*n* = 20), oral or pharyngeal SCCs (*n* = 20), and penile non-SCC lesions (*n* = 20). Penile non-SCC lesions included four samples of granulomatous balanoposthitis, four melanomas, three eosinophilic granulomas, two inflammatory polyps, two lymphomas, and single samples of habronemiasis, vasculitis, mast cell tumor, tricholemmoma, and apocrine adenoma. Penile non-SCC lesions did not include sarcoids or precursor lesions of SCC, such as papillomas and precancerous plaques.

### 5.3.2 Age Estimation

Of the 74 horses whose ages were estimated, 32 (43.2%) were less than 5 years old, 16 (21.6%) were 6–10 years old, 14 (18.9%) were 11–15 years old, 10 (13.5%) were 16–20 years old, and 2 (2.7%) were over 21 years old. The mandibles and lower incisor teeth from one of 75 horses were not available for age estimation.

Of the 60 horses providing FFPE lesional tissue the ages were available for 18 of 20 from the EPSCC group, 14 of 20 from the non-SCC penile lesion group, and 18 of 20 from the oral or pharyngeal SCC group. The mean ages of the horses whose ages were available were: 23.9 years for the EPSCC group; 13.3 years for the non-SCC penile lesion group; and 19.2 years for the oral or pharyngeal SCC group.

### 5.3.3 DNA Extraction and Confirmation of Amplifiable DNA

DNA was extracted from all 282 samples (222 fresh and 60 FFPE). The presence of amplifiable DNA was confirmed in 281 of 282 by qPCR using equine beta actin primers. The single sample in which the presence of amplifiable DNA was not confirmed was a penile non-SCC lesion, and this sample was excluded from the subsequent EcPV-2 qPCR. The average DNA content of the remaining 281 samples was 20 ng/µl (range 6-98 ng/µl).

### 5.3.4 qPCR Assay for EcPV-2 and Statistical Analysis

Results of qPCR testing are presented in table 5.1 and figure 5.1. Overall, EcPV-2 DNA was detected in 27 of 281 samples (9.1 %). This included 19 of 40 (47.5%) SCC lesions, 3 of 19 (15.8%) non-SCC lesions and 5 of 222 (2.3%) non-lesional mucosal samples taken from grossly normal horses. The EcPV-2 copy numbers ranged from 0.55 to 308.01 copies per ng of template DNA. The mean viral DNA load in the 19 EcPV-2 positive SCCs was significantly higher than that in the 8 EcPV-2 positive samples without SCC (49.18 and 2.29 copies/ng template DNA, respectively; $p = 0.0006$).

| Anatomic site | Tissue | Total sample number | Number of samples containing EcPV-2 DNA (%) | Mean EcPV-2 copy number per ng template DNA (range) |
|---|---|---|---|---|
| Penis | | | | |
| | Non-lesional mucosa[a] | 32 | 2 (6.3%) | 7.40 (0.32–14.65) |
| | Non-SCC lesion[b] | 19 | 3 (15.8%) | 1.10 (0.03–3.17) |
| | SCC lesion[b] | 20 | 16 (80.0%) | 55.79 (0.55–308.01) |
| Oral cavity or pharynx | | | | |
| | Non-lesional mucosa[a] | 75 | 2 (2.7%) | 0.02 (0.01–0.02) |
| | SCC lesion[b] | 20 | 3 (15.0%) | 13.92 (1.51–24.08) |
| Vulva | | | | |
| | Non-lesional mucosa[a] | 40 | 1 (2.5%) | 0.006 |
| Nictitating membrane | | | | |
| | Non-lesional mucosa[a] | 75 | 0 (0%) | Not applicable |

*Table 5.1. Summary of results of quantitative PCR evaluation of equine tissues for the presence of EcPV-2 DNA sequences. [a] DNA was extracted from fresh frozen tissues. [b] DNA was extracted from formalin-fixed paraffin-embedded tissues. EcPV-2: equine papillomavirus type 2. SCC: squamous cell carcinoma*

EPSCCs contained EcPV-2 DNA significantly more frequently than non-SCC penile samples ($p < 0.0001$). The viral DNA load in the 16 EcPV-2-positive EPSCC lesions was significantly higher than in the 5 EcPV-2-positive non-SCC penile samples (mean 55.79 and 3.66 copies/ng template DNA, respectively; $p = 0.01$). EcPV-2 DNA was not detected significantly more frequently in penile non-SCC lesions than in grossly normal penile mucosa ($p = 0.36$).

EcPV-2 was detected within 5 of 95 samples of oral or pharyngeal mucosa, confirming that the virus can infect these epithelial surfaces. However, EcPV-2 was not detected significantly more frequently in SCCs from the oral cavity or pharynx (3/20 samples) than in grossly normal pharyngeal mucosa (2/75 samples). In addition, the mean viral load within the 3 EcPV-2-positive oral or pharyngeal SCCs (13.92 EcPV-2 copies/ng template DNA) was not significantly higher than the viral load detected in the grossly normal EcPV-2-positive pharyngeal mucosal samples (0.02 EcPV-2 copies/ng template DNA; $p = 0.2$).

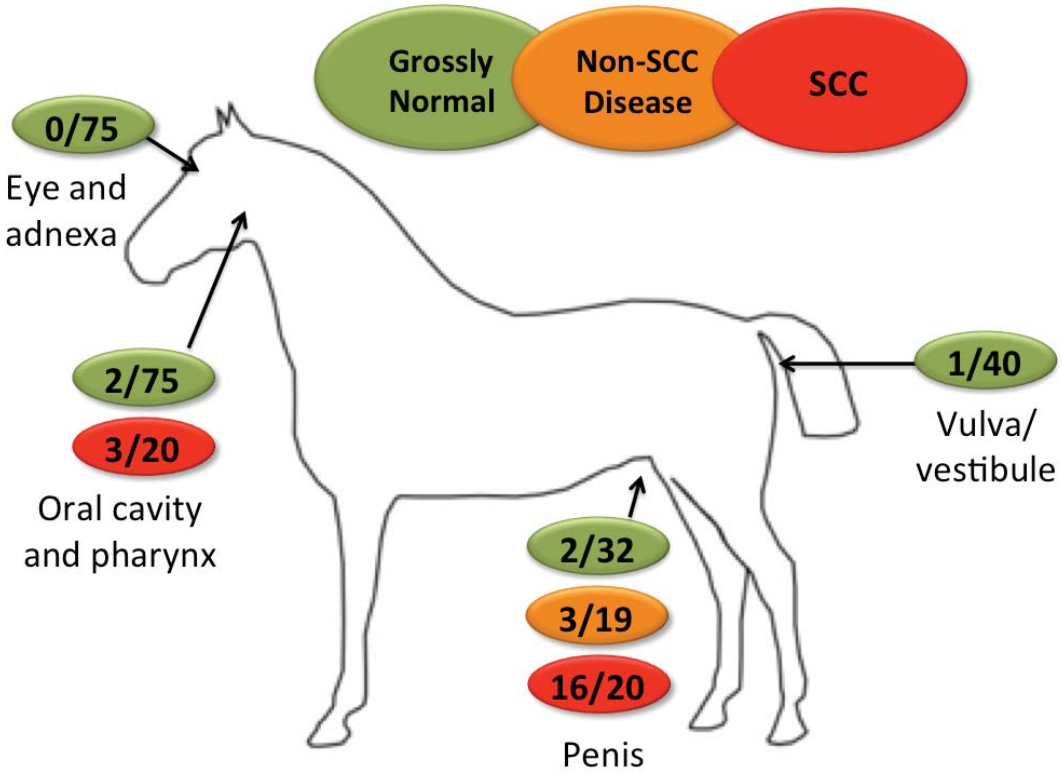


*Figure 5.1. EcPV-2 infection in different body sites as determined by quantitative PCR (number positive/number of samples tested). Results from both fresh and fixed equine tissues are included in this figure. EcPV-2: equine papillomavirus type 2. SCC: squamous cell carcinoma*

## 5.4 Discussion

The work described in this chapter is the first quantitative investigation of EcPV-2 infection in equine neoplastic and non-neoplastic diseased tissues. In addition, this was the first investigation into EcPV-2 infection in tissues from a large number of healthy horses. In the following paragraphs the results pertaining to each of the three aims for this chapter are discussed in turn.

### 5.4.1 Investigation of EcPV-2 Viral DNA Load in Penile Samples

We chose to investigate EcPV-2 in penile tissue for the reasons given in chapter 2. Briefly, EPSCCs are common[49] and a convincing cause has not been established. Given that approximately 50% of PSCCs in men are associated with high-risk HPV infection[1,38] it is plausible that a PV could be associated with PSCC development in horses. Several previous studies have shown the presence of EcPV-2 in EPSCCs.[26,29,39]

Our results showed that EcPV-2 was present significantly more frequently and in significantly higher copy numbers in EPSCC lesions than in either grossly normal penile mucosa or non-SCC penile lesions. Put another way, penile mucosa rarely harbored EcPV-2 unless affected by EPSCC and, when it did, the viral load was low. This is similar to the findings of two previous studies, in which EcPV-2 DNA sequences were detected by conventional PCR significantly more frequently in EPSCC lesions than in non-SCC penile lesions.[26,29] Our findings extend the results of those previous studies by providing information about viral DNA load in SCC-affected and healthy tissues.

While higher viral load in EPSCCs than in non-SCC tissues does not prove that EcPV-2 causes these neoplasms, it is one of the criteria currently considered necessary for establishing microbial disease causation,[17] and provides additional support for a link between EcPV-2 infection and development of EPSCCs. However, an alternative explanation for the higher EcPV-2 load seen in EPSCCs than in non-SCC tissues should also be considered. It is possible that the greater EcPV-2 copy numbers seen in EPSCCs may simply be a byproduct of the increased epithelial cell replication in a developing neoplasm, with dividing epithelial cells copying viral DNA sequences each time they replicate. If this were true then a similar viral load could be expected in many non-SCC penile lesions because there is also increased epithelial cell replication due to ulceration and repair. Our results demonstrated that this was not the case, and that the mean viral load in EPSCCs was fifty times higher than in non-SCC lesions. This implies that EcPV-2 has a different biologic behavior in EPSCCs than in non-SCC lesions, possibly driving epithelial cell replication rather than being a simple passenger. This finding also supports EcPV-2 having a causal role in the development of neoplasia.

There was no significant difference between EcPV-2 viral DNA loads in EcPV-2-positive grossly normal penile mucosa and EcPV-2-positive non-SCC penile lesions. The viral load for both was significantly lower than in EcPV-2-positive EPSCCs. This suggests that EcPV-2 is not involved in the development of non-SCC penile disease.

EcPV-2 was detected in a proportion (2 of 32) of grossly normal penile mucosal samples from clinically healthy horses. This is a novel finding and suggests that EcPV-2-

infected but lesion-free horses may either (a) have not yet developed lesions of PV infection, including papillomas and possibly EPSCCs, or (b) have not yet cleared EcPV-2 infection. These scenarios are analogous to those for high-risk HPV infection in women; 5-15% of women are infected with high-risk HPVs at some stage in their life yet the majority go on to clear the infection.[5,6] Only a fraction of women remain infected and go on to develop cervical neoplasia, and the interval between infection and development of cancer is typically long.[5,6] Detection of high-risk HPV infection in the cervix of a clinically normal woman, therefore, is not predictive of outcome and only suggests that regular monitoring is necessary. At this stage too little is known about the clinical course of EcPV-2 infection to predict outcome, but our findings add the important information that grossly normal mucosa may carry this virus.

### 5.4.2 Investigation of EcPV-2 Viral DNA Load in Samples from Other SCC-Prone Body Sites

#### *5.4.2.1 Oral and Pharyngeal Samples*

Equine oral and pharyngeal mucosa was chosen for investigation because SCCs are the most common neoplasms in this region[14,40] and no cause has been established. Unlike the external genitalia and nictitating membrane, exposure to UV radiation cannot be a factor in SCC development in the throat. Up to one third of human throat cancers are associated with PV infection[27] and a similar papillomaviral association with throat cancer in horses is plausible.

To date, no primary equine oral or pharyngeal SCCs have been reported to contain PV DNA. However, EcPV-2 DNA sequences were detected by PCR in mucosal samples from a New Zealand horse euthanized for laryngeal SCC (fig. 5.2; Knight, C.G., unpublished data). Additionally, EcPV-2 detection by PCR has been reported in two cases of equine SCC that metastasized to the mouth or pharynx from a primary SCC of the periocular tissues.[4,21]

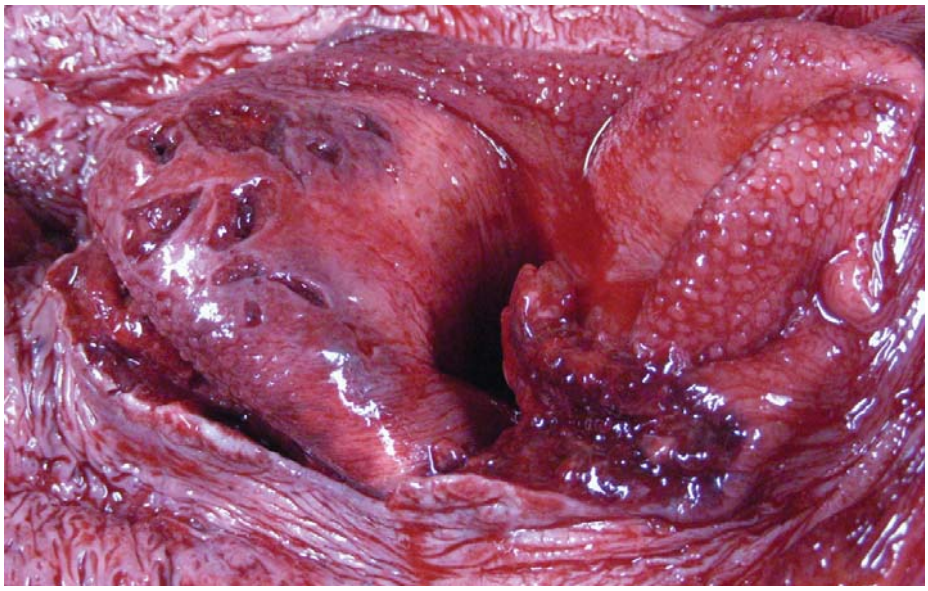

*Figure 5.2. Squamous cell carcinoma of the left aryepiglottic fold in a New Zealand horse; left lateral view. EcPV-2 DNA sequences were detected by PCR in lesional mucosa. The lacerations in the epiglottic mucosa were due to the epiglottis being grasped by forceps during surgery.*

In the work described in this chapter, EcPV-2 DNA sequences were detected within 5 of 95 samples of oral or pharyngeal mucosa. This is a novel finding and shows that the virus can infect these epithelial surfaces, even in horses without gross lesions. However, EcPV-2 sequences were not detected significantly more frequently in SCCs than in grossly normal pharyngeal mucosa. In addition, the mean EcPV-2 viral DNA load within the three SCCs was not significantly higher than the viral load detected in the two normal pharyngeal mucosal samples. These data, therefore, do not support a causal link between EcPV-2 infection and development of SCC in the mouth and pharynx. It should be emphasized, however, that these results are based on a small number of EcPV-2 positive horses. In addition, SCC samples had undergone formalin fixation, paraffin embedding, and prolonged storage, whereas the grossly normal tissues had not. It is possible that this reduced the amount of intact viral DNA available for amplification within SCC lesions.[13] Thus, a potential role of EcPV-2 in the development of oral or pharyngeal SCCs should not be fully excluded without further investigation.

#### *5.4.2.2 Vulvar Samples*

A proportion of vulvar SCCs in women is associated with PV infection,[16] which is typically contracted venereally.[47] In mares a similar association is plausible, particularly

given the association between genital SCCs and EcPV-2 infection in male horses.[4,25,26,29,36,39,46]

In the experiments described in this chapter, low levels of EcPV-2 DNA were detected in just one of 40 samples of vulvovestibular mucosa from grossly normal mares. This is similar to the findings of two earlier studies that reported EcPV-2 DNA in 2 of 51 vulvovestibular swabs[46] and in 0 of 20 vulvovestibular swabs[4] from normal mares. These results all suggest that EcPV-2 is not a common, commensal inhabitant of grossly normal equine female genitalia. This implies that if EcPV-2 were detected significantly more frequently in vulvovestibular SCCs than in grossly normal mucosa there could be a causal association between infection and neoplasm development.

There have been just two reports describing the detection of EcPV-2 in vulvovestibular SCCs. EcPV-2 DNA sequences were detected in 4 of 5 vulvar SCCs in one study,[39] and in 2 of 2 vulvovestibular neoplasms (one SCC and one intraepithelial neoplasia) in another.[4] An additional report described development of an *in situ* vulvar SCC at the site of prior surgical excision of vulvar papillomas, but the PV type was not specified.[43] While the results of these three studies suggest an association between PV infection and vulvovestibular SCCs, further studies screening a large number of vulvovestibular SCCs are needed.

#### *5.4.2.3 Nictitating Membrane Samples*

SCCs of the eye and its adnexa (referred to as periocular SCCs) are common in horses[30,41,44] and believed to be associated with ultraviolet light exposure.[15,19] Periocular SCCs also occur in humans and, as in horses, are strongly associated with UV exposure.[20,35] There is currently limited and contradictory evidence suggesting that human periocular SCCs are associated with PV infection, although this may change as further studies are conducted.[2,20,42] Similarly, there is little evidence suggesting that equine periocular SCCs are associated with EcPV-2 infection. However, equine penile SCCs were also attributed to UV exposure until very recently, when investigations revealed a papillomaviral association. Therefore, based on the premise that a similar

association between PV infection and periocular SCCs should not be ruled out without investigation, we included periocular samples in this study.

In the experiments described in this chapter, EcPV-2 DNA was not detected in any of 75 nictitating membranes from grossly normal horses. This result is similar to that of the only other study of PVs in grossly normal periocular tissues, in which EcPV-2 DNA was detected in just 1 of 30 ocular swabs from healthy horses.[46] In combination, these results suggest that periocular infection by EcPV-2 is rare in grossly normal tissues.

The presence of EcPV-2 has been reported just once in an equine periocular SCC.[21] Two other studies reported detection of EcPV-2 in 0 of 12 and 0 of 42 equine periocular SCCs.[36,39] However, the lack of association between EcPV-2 infection and equine periocular SCCs should be confirmed by additional studies with larger sample sizes. The possibility that equine periocular SCCs are associated with a PV other than EcPV-2 should also be considered. A single conference abstract described detection of a novel, non-EcPV-2 PV in 79% of equine periocular SCCs and in 15% of normal periocular tissues.[22] This abstract has not been followed up by the original authors and no other studies have been conducted to reproduce their findings.

EcPV-2 DNA sequences were reportedly detected in 9 of 9 equine ocular SCCs in one study.[51] This paper was later retracted at the authors' request due to contamination of the original experiment. Regrettably, the article's retraction did not receive the same attention as the original article, which has been cited several times since and continues to be cited.[29,48] Although this has been pointed out,[24] it is possible that the erroneous belief will persist that EcPV-2 is strongly associated with equine periocular SCCs.

In conclusion, it is not yet clear whether EcPV-2 is linked to development of periocular SCCs, and further investigation should be carried out.

### 5.4.3 Overall Interpretation of qPCR Results

#### *5.4.3.1 Assay Design and Performance*

This is the first quantitative investigation of EcPV-2 in neoplastic and non-neoplastic tissues from a large number of horses. Quantitative analysis using qPCR allowed us to compare the amount of PV DNA within different tissues, providing some indication of the behaviour of the virus within lesions.

During development of the qPCR assay (chapter 4) we demonstrated that the assay had acceptable efficiency and $R^2$ values, as well as high analytical sensitivity, repeatability, and reproducibility. However, the test samples that were used to optimize the assay consisted of pure solutions of the target EcPV-2 sequence. Pure samples do not necessarily predict assay performance with clinical samples that contain equine genomic DNA. This is because the presence of genomic DNA in a sample may reduce assay efficiency by competitive inhibition of primer-template hybridization or by decreasing the rate of diffusion of reaction components in the presence of numerous long strands of host DNA.[7,37]

In the work described in this chapter, the qPCR assay retained acceptable efficiency and $R^2$ values when using template DNA extracted from equine tissues. In samples where the viral DNA content was lower than one copy per µL after 45 cycles our results were obtained by extrapolation. Values this low may lead to concerns about false positives due to sample contamination. However, we believe that the extrapolated low values reflect true low levels of viral DNA detected in our samples rather than contamination. This is based on the fact that the melting peaks in these samples were within the expected range and that the negative controls included in the test were consistently negative.

Once viral copy number is calculated, the final result must be reported relative to a defined unit of interest. In our experiments viral copy number values were normalized to the amount of equine template DNA added to each reaction volume and expressed as viral copies per nanogram of template DNA. This is appropriate for correlating viral copy number with a disease state, but assumes that (1) the majority of template DNA

is host genomic (i.e., the contribution of viral DNA to total template DNA is not significant) and (2) that cells from different body sites contain a relatively constant amount of DNA.

Another way to express viral load would have been viral copies per equine cell. The number of cells present in each PCR reaction could have been estimated based on the number of copies of a selected house-keeping gene such as beta actin (ACTB). Although ACTB PCR, in theory, could have been used for this purpose, the performance of the assay we used did not allow for reliable quantification of the ACTB target. Frequently, two different products were amplified, one of the expected size and a smaller one similar in size to the expected mRNA transcript. The latter most likely represented amplification of a pseudogene.[45] Thus, while the assay was suitable to demonstrate the presence of amplifiable DNA in the sample, it could not reliably quantify the number of cells that DNA came from.

#### *5.4.3.2 Interpretation of EcPV-2 Viral DNA Load*

It is important to distinguish between EcPV-2 *infection*, as detected by conventional PCR, versus EcPV-2 *activity* within lesions; the latter may be inferred from viral loads measured by qPCR. Since viral DNA load may be regarded as a surrogate marker for viral replication and possibly for increased concentration of viral oncoproteins,[52,53] our qPCR assay may provide prognostic information for EcPV-2 infected horses. This is analogous to humans, in whom HPV load is reported to be an important prognostic indicator in patients with tonsillar,[8] cervical,[12,23] and oropharyngeal[28] cancer, with a higher viral load correlating with improved survival and response to therapy. Conversely, in humans with epidermodysplasia verruciformis a larger number of HPV-positive cells and/or higher viral copy numbers per cell correlate with an increased risk for skin cancer development.[52] However, even calculating viral load may not always be prognostically useful. A human-based study has shown that it is expression of the HPV oncogenes E6 and E7, rather than viral load, that is predictive of outcome in patients with cervical cancer.[11] Another study has shown that a single measurement of HPV viral load made at an indeterminate point during infection does not reliably predict the risk of a woman developing cervical neoplasia.[9] Therefore, a single measure of HPV

viral load cannot be considered a clinically useful biomarker in women, and EcPV-2 viral load may prove to be similarly unhelpful in horses. The potential prognostic significance of EcPV-2 viral load needs further investigation.

To date there have been no other studies of EcPV-2-infected horses that are comparable to ours. Therefore, the data presented in this chapter should be considered a starting point for further investigation into the prognostic relevance of measuring EcPV-2 viral load. In humans, the implications of HPV detection for patient management and prognosis are still debated, and a positive result for the presence of HPV is interpreted only in light of documented persistence through repeated tests, the HPV type(s) present, viral load, and cytology and biopsy results.[33] The same is likely to be true for EcPV-2 infection in horses.

## 5.5 Chapter Summary

The work in this chapter showed that EcPV-2 was present significantly more frequently and in higher copy numbers in EPSCC lesions than in either healthy penile mucosa or in non-SCC penile lesions. Healthy penile mucosa, or mucosa from penile tissues affected by diseases other than SCC, rarely harbored EcPV-2. These findings support the hypothesis that EcPV-2 is etiologically involved in the development of equine penile SCCs.

In addition, we provided the first evidence that EcPV-2 can infect clinically normal pharyngeal mucosa and showed that EcPV-2 may be detected in a proportion of oral or pharyngeal SCCs. Our data do not support a causal link between EcPV-2 infection and development of SCCs in this region; however, the sample size was small, and the potential role of EcPV-2 in development of oral or pharyngeal SCCs needs further investigation.

EcPV-2 DNA was not detected in any of a large number of grossly normal nictitating membranes, and was detected only rarely in grossly normal vulvar mucosa, suggesting that asymptomatic EcPV-2 infection is uncommon at these sites. If, in future studies,

EcPV-2 were detected in periocular or vulvovestibular SCCs this would support a causal association between infection and development of neoplasia.

This is the first time that large numbers of healthy equine tissues have been screened for EcPV-2 or that qPCR has been used to assess and compare viral loads in neoplastic and non-neoplastic tissues. As evidence accumulates that PVs are associated with neoplasia in domestic animals,[34] the results from this chapter should provide useful information for future investigation into the role of EcPV-2 in the development of equine neoplasia.

The results and conclusions from this chapter have been published in the article:

> *Knight CG, Dunowska M, Munday JS, Peters-Kennedy J, Rosa BV:* ***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****. Veterinary Microbiology 166: 257-262, 2013*

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# CHAPTER SIX

# Immunohistochemical Investigation of Oncogenic Mechanisms of EcPV-2

## 6.1 Introduction

It is now accepted that human cervical and a proportion of human oral squamous cell carcinomas (SCCs) are caused by infection with high-risk human PVs.[26] For obvious ethical reasons, these PVs were not proven to cause cancer by prospective studies of people experimentally infected with HPVs. Instead, a causal association was proven using evidence from two types of study: epidemiological and biological.[6] Epidemiological studies included case series, case-control studies, cohort studies, and intervention studies. Biological studies included in vitro experiments, animal

experiments, and molecular studies that demonstrated a mechanism for induction of cancer.

The experiments reported in previous chapters showed that EcPV-2 DNA sequences can be detected at a significantly higher rate in SCCs of the equine penis (EPSCCs) than in non-SCC penile samples.[15,16] This result suggests that EcPV-2 infection may cause SCC development. However, since the majority of PV infections in most species are ubiquitous and asymptomatic,[3] the possibility that PV DNA present within a SCC is there simply due to the SCC providing a more permissive environment for the virus cannot be excluded.

Many of the studies investigating the association between HPV infection and cancer have sought to establish a plausible mechanism for PV-induced oncogenesis. From these, two predominant pathways have emerged as the most significant means by which HPV infection causes cancer. As discussed in Chapter 1, one pathway involved in the carcinogenic effect of high risk HPV is the E6 oncoprotein-mediated degradation of transformation-related protein 53 (TRP53, p53).[32] The other is mediated by the E7 oncoprotein and results in inactivation and degradation of the retinoblastoma protein (pRb).[7,23] This reduction in the amount of active pRb results in a compensatory intracellular increase in the cyclin-dependent kinase inhibitor protein $p16^{CDKN2A}$ (p16).[27] Since p53, pRb, and p16 all have functions in controlling the cell cycle, their dysregulation by the PV virus impairs cell cycle arrest and promotes neoplastic transformation of the infected cell.

Changes in the intracellular amounts of pRb, p16, and p53 can be investigated using immunohistochemistry (IHC).[20] When a PV induces neoplastic transformation of a cell, these proteins are altered in a consistent pattern. This results in an immunohistochemical 'fingerprint,' which can be used to confirm a PV etiology for certain neoplasms.[20] For example, a human oral SCC that has reduced pRb, increased p16, and no p53 immunostaining is considered to have been caused by PV infection.[2] In contrast, an oral SCC that has normal pRb, normal p16, and visible p53 immunostaining is unlikely to have been caused by PV infection.

We hypothesized that if equine penile and throat SCCs are caused by EcPV-2 then these cancers may contain similar immunohistochemical features to those observed in human PV-induced neoplasms. Therefore, the aim of the work described in this chapter was to compare the immunostaining of p16, pRb, and p53 in SCCs with, and without, evidence of EcPV-2 infection. If significant differences in immunostaining were detected between SCCs that contained EcPV-2 DNA and SCCs that did not contain detectible PV DNA, this would support the hypothesis that EcPV-2 influenced the development of cancer by altering these regulatory proteins within the infected cells.

## 6.2 Materials and Methods

### 6.2.1 Sample Collection

The same formalin-fixed, paraffin-embedded (FFPE) samples of EPSCCs and throat SCCs that were used in Chapter 5 were used for the IHC investigations. All samples were located by searching the archives of the Department of Biomedical Sciences, Cornell University, College of Veterinary Medicine, Ithaca, New York and the diagnosis was confirmed by examination of the original hematoxylin and eosin-stained sections.

### 6.2.2 Immunohistochemistry

Four 5 µm thick sections were cut from each FFPE tissue block and mounted on charged glass slides. The mounted sections were then deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water.

#### *6.2.2.1 p16*

To detect p16, endogenous peroxidases were first neutralized with 1% hydrogen peroxide in methanol for 15 minutes. This was followed by rinsing for 5 minutes in distilled water. Antigen retrieval was achieved by incubating slides in epitope retrieval solution 2 (Leica Microsystems GmBH, Wetzlar, Germany) for 30 minutes at 100°C with a 20-minute cool-down. The primary antibody was applied by incubating slides for 15 minutes with a mouse anti-human p16 monoclonal antibody (BD Biosciences, San Jose, CA) at a dilution of 1:50. The immunoreaction was visualized using a Bond Refine

Detection staining kit (Leica Microsystems GmBH) using 3,30 -diaminobenzidine substrate with hematoxylin counterstaining. Basal keratinocytes in adjacent non-neoplastic tissue were used as an internal positive control while a sample of feline Bowenoid *in situ* carcinoma was included as an external positive control. The primary antibody was omitted from the negative controls.

#### *6.2.2.2 pRb*

Immunohistochemistry to detect pRb was performed by heating slides in a pressure cooker for 15 minutes in 0.01M citrate buffer, pH 6.0, with a 10-minute cool-down. Endogenous peroxidases were blocked by incubating slides for 5 minutes in 0.3% hydrogen peroxide in 0.05M Tris-buffered saline, pH 7.6. Non-specific staining was blocked by incubating slides with equine serum (Vector Laboratories, Burlingame, CA) for 20 minutes. The primary antibody was applied by incubating slides overnight with mouse anti-human pRb antibodies (BD Biosciences) at a dilution of 1:50. To visualize the immunoreaction, the slides were incubated for 30 minutes with biotinylated anti-mouse/rabbit secondary antibody (Vector Laboratories) and then incubated for 30 minutes with a biotin-avidin complex (Vector Laboratories), followed by 3,3-diaminobenzidine substrate (Liquid DAB Substrate Chromagen System, Dako, Carpinteria, CA) with hematoxylin counterstain. Cells in adjacent non-neoplastic tissue were used as an internal positive control. The primary antibody was omitted from the negative controls.

#### *6.2.2.3 p53*

Endogenous peroxidases were first neutralized with 1% hydrogen peroxide in methanol for 15 minutes followed by rinsing for 5 minutes in distilled water. Antigen retrieval was achieved by incubating slides in epitope retrieval solution 2 (Leica Microsystems GmBH) for 30 minutes at 100°C with a 20-minute cool-down. Slides were then incubated for 15 minutes with a mouse anti-human p53 clone pAb 240 antibody (BD Biosciences) at a dilution of 1:100. The immunoreaction was visualized using a Bond Refine Detection staining kit (Leica Microsystems GmBH) using 3,30 -diaminobenzidine substrate and sections were counterstained with hematoxylin. A

human neoplasm known to contain p53 mutations was used as a positive control. The primary antibody was omitted from the negative controls.

### 6.2.3 Interpretation and analysis of IHC slides

Each slide was assessed without knowledge of the viral status of the sample. Evaluation of pRb and p53 immunostaining revealed faint cytoplasmic immunostaining in many of the cells. However, this was considered background reactivity, and only neoplastic cells with nuclear immunostaining for pRb or p53 were considered positive. For penile SCCs, the total sample score for each protein was determined by examining five randomly chosen 400 X fields within each lesion. The total number of tumor cells with and without immunostaining in each field was counted manually using CellSens software (Olympus Corporation, Shinjuku, Tokyo, Japan). The total score was the average percent of cells within the five fields exhibiting immunostaining.

Immunostaining in the samples of penile SCCs that contained EcPV-2 DNA was compared to the immunostaining in samples of penile SCCs that did not contain EcPV-2 using analysis of variance for p53 and, as the percentage of immunostained cells was not normally distributed, using a Wilcoxon-Mann-Whitney test for pRb. For the samples of penile SCCs that contained EcPV-2 DNA, the relationship between EcPV-2 viral load and the percentage of tumor cells with immunostaining in each sample was assessed for both pRb and p53 by simple linear regression. For both models the residuals were normally distributed. All statistical analyses were performed using SAS statistical software version 9.4 (SAS Institute, Cary, NC, USA).

## 6.3 Results

### 6.3.1 Sample Collection

Twenty EPSCCs were included in this experiment. This included 16 samples that had previously been found to contain EcPV-2 and four samples in which no EcPV-2 DNA was detected. As determined in the experiments described in Chapter 5, within the samples that contained EcPV-2 DNA the viral copy number ranged from 0.55 to 308 EcPV-2 copies/ng template DNA. Three SCCs from the throat that had been found to

contain EcPV-2 DNA were also included; their EcPV-2 viral copy numbers ranged from 1.51 to 24.1 copies/ng template DNA.

### 6.3.2 Immunohistochemistry

#### *6.3.2.1 p16*

Immunostaining for p16 was not observed in any equine penile or throat SCC. However, immunostaining was also not visible within the adjacent non-neoplastic basal keratinocytes that were used as the internal positive control. In contrast, the feline positive control sample had moderate nuclear immunostaining within non-neoplastic basal keratinocytes surrounding the Bowenoid *in situ* carcinoma and intense cytoplasmic and nuclear immunostaining throughout the neoplastic cells.

#### *6.3.2.2 pRb*

All 23 slides that were immunostained for pRb showed nuclear immunostaining within a proportion of the neoplastic cells (Figure 6.1 and Table 6.1). Additionally, the nuclei of non-neoplastic cells adjacent to the neoplasms showed immunostaining within all sections. The negative controls did not demonstrate any immunostaining.

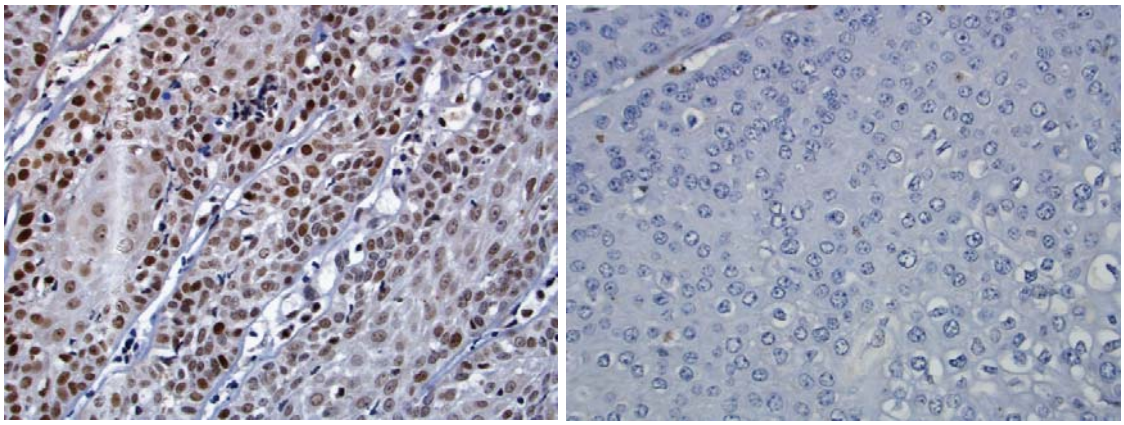

*Figure 6.1. Immunostaining against tumor suppressor protein pRb from two representative horses with penile squamous cell carcinomas. The left hand image shows a high percentage tumor cells with nuclear immunostaining. The right hand image shows a low percentage of tumor cells with nuclear immunostaining. Both images are taken using the 40 X microscope objective.*

On average, 72.85% of tumor cells within the 20 EPSCCs had moderate to intense nuclear immunostaining for pRb (range 7.0% to 96.2%). There was only one slide in which less than 50% of the cells showed pRb nuclear immunostaining. EPSCCs that

contained EcPV-2 DNA by PCR had an average pRb immunostaining of 76.75%. This was not significantly different from the pRb immunostaining in the EPSCCs that did not contain detectible EcPV-2 DNA by PCR (57.25%, p=0.16). The presence of pRb immunostaining was not significantly correlated with the EcPV-2 viral load within the EPSCCs that contained EcPV-2 DNA (p=0.48).

The three throat SCCs that contained EcPV-2 DNA by PCR had pRb immunostaining present within the nuclei of nearly all neoplastic cells. There was no evidence of loss of pRb immunostaining within any of the throat SCCs. (Figure 6.2)

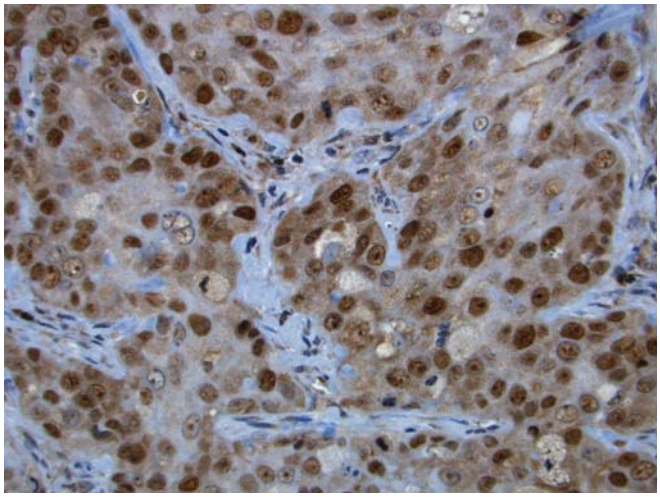

*Figure 6.2. Immunostaining against tumor suppressor protein pRb from a representative horse with pharyngeal squamous cell carcinoma. The image shows a high percentage tumor cells with nuclear immunostaining. The image is taken using the 40 X microscope objective.*

#### *6.3.2.3 p53*

Nuclear immunostaining for p53 was present in a proportion of neoplastic cells in 19 of the 20 samples of EPSCCs (Figure 6.3). One EPSCC sample (horse 2) had no immunostaining visible within any cell on the slide. The absence of any immunostaining on this slide was likely due to a failure of the immunostaining process. Unfortunately, as insufficient tissue remained in the FFPE block, immunostaining could not be repeated in this case and the cause of this isolated immunostaining issue could not be investigated.

Within the EPSCCs, p53 immunostaining was visible in an average of 48.7% of the neoplastic cells, with immunostaining ranging from 1.2% to 96.3% of the neoplastic

cells. EPSCCs that contained PV DNA by PCR had an average p53 immunostaining of 44.75%. This was not significantly different from the immunostaining in the EPSCCs that did not contain detectible EcPV-2 DNA by PCR (63.5%%, p=0.24). The presence of p53 immunostaining was not significantly correlated to the EcPV-2 viral load within the EPSCCs that contained EcPV-2 DNA (p=0.26).

The three throat SCCs that contained EcPV-2 DNA by PCR had p53 immunostaining present within the nuclei of a majority of neoplastic cells. There was no evidence of any reduction in p53 within the neoplastic cells.

| Horse Number | Tumor cells with pRb immunostaining (%) | Tumor cells with p53 immunostaining (%) | EcPV-2 viral load (copies/ng template DNA) |
|---|---|---|---|
| 1 | 68.0 | 50.6 | 3.39 |
| 2 | 80.0 | -* | 61.43 |
| 3 | 54.0 | 63.8 | 12.23 |
| 4 | 76.0 | 16.0 | 62.41 |
| 5 | 60.0 | 1.2 | Negative by PCR |
| 6 | 84.0 | 59.4 | 0.55 |
| 7 | 70.0 | 37.8 | 20.43 |
| 8 | 50.0 | 72.0 | 4.73 |
| 9 | 84.0 | 21.8 | 60.75 |
| 10 | 92.8 | 70.3 | 7.34 |
| 11 | 50.4 | 44.2 | 8.27 |
| 12 | 72.0 | 96.3 | Negative by PCR |
| 13 | 96.2 | 22.6 | 21.31 |
| 14 | 90.0 | 62.9 | Negative by PCR |
| 15 | 7.0 | 93.5 | Negative by PCR |
| 16 | 87.8 | 31.9 | 308.01 |
| 17 | 73.8 | 43.6 | 223.58 |
| 18 | 89.2 | 21.5 | 2.69 |
| 19 | 81.6 | 25.4 | 50.18 |
| 20 | 90.2 | 90.1 | 45.40 |

*Table 6.1. Immunostaining and viral load within 20 equine penile squamous cell carcinomas. * indicates absence of immunostaining consistent with a failure of the immunostaining process.*

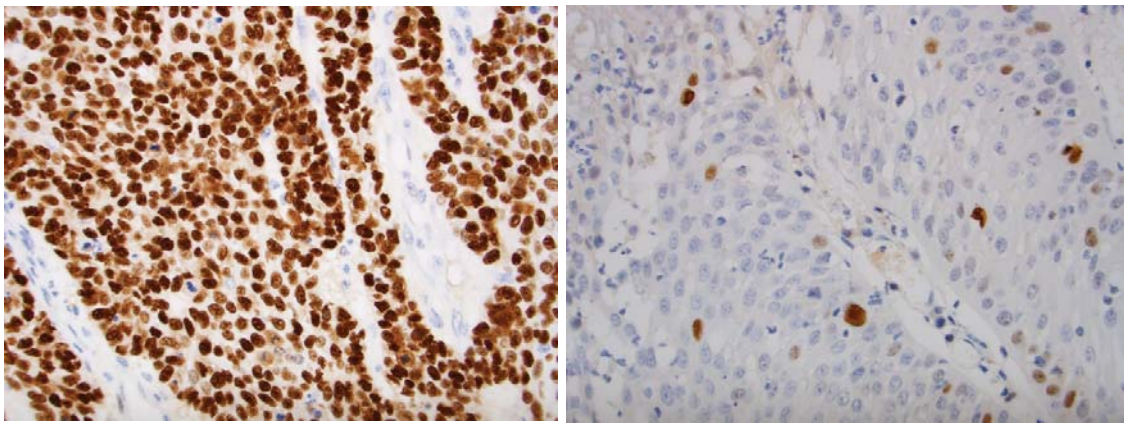

*Figure 6.3 Immunostaining against tumor suppressor protein p53 from two representative horses with penile squamous cell carcinomas. The left hand image shows a high percentage tumor cells with nuclear immunostaining. The right hand image shows a low percentage of tumor cells with nuclear immunostaining. Both images are taken using the 40 X microscope objective.*

## 6.4 Discussion

A key feature of the PV life cycle is the ability of these viruses to alter the host cell cycle to maximize viral replication.[12] Some PV types are so effective at promoting host cell growth and replication that they result in the development of a visible papilloma.[9,11,19] In horses, EcPV-2 has been reported to cause penile papillomas, suggesting that it has the ability to significantly promote cell division and growth;[4,17,18,31,36] however, the mechanism by which EcPV-2 achieves this is currently unknown.

Human high-risk alphaPVs consistently promote neoplastic transformation of cells by reducing levels of the tumor suppressor proteins pRb and p53.[7,12,32] In contrast, we found that EcPV-2-positive EPSCCs did not have significantly less pRb immunostaining in neoplastic cells than did EcPV-2-negative EPSCCs. Likewise, EcPV-2-positive EPSCCs did not have reduced p53 immunostaining when compared to EcPV-2-negative EPSCCs. The absence of a reduction in p53 or pRb immunostaining in EcPV-2-positive SCCs suggests two possibilities. The first is that EcPV-2 does not play a causative role in the development of SCCs. The second is that EcPV-2 may have a role in SCC development, but through different molecular mechanisms than those used by high-risk HPVs.

The following sections discuss our IHC results in more detail.

### 6.4.1 pRb

In contrast to most of the PV types that have been previously studied,[7] our results show that EcPV-2 does not appear to influence the cell cycle by E7-mediated degradation of pRb. This was shown in our experiment by the lack of a reduction in pRb immunostaining in EcPV-2-positive SCCs relative to EcPV-2 negative SCCs.

While there was no evidence that EcPV-2 degraded pRb within our SCC samples, it is important to note that IHC only detects the presence of a protein and cannot be used determine whether or not the protein is functional. In studies in humans and cats, evidence suggests that the PV E7 protein initially inhibits pRb function prior to later protein degradation.[20,24] If the E7 oncoprotein of EcPV-2 inhibited, but did not subsequently degrade pRb, then cell growth and division would be promoted without any detectable reduction in pRb immunostaining. One means of circumventing this possible confounder would have been immunostaining for p16; as cellular p16 production rises following either inhibition or degradation of pRb,[20,27] p16 immunostaining of the EPSCCs would have allowed estimation of pRb function within the neoplasms. Unfortunately, p16 immunostaining was not possible in this experiment. Thus, it remains unknown whether or not EcPV-2 E7 can alter the function of pRb in an EcPV-2 infected cell.

Most of the 20 EPSCC samples contained pRb immunostaining within a high percentage of their neoplastic cells. While this could suggest cross-reactivity with other nuclear proteins, the specificity of the anti-pRb antibody used in this study for equine pRb had been previously confirmed.[13]

One EPSCC contained pRb immunostaining in just 7% of the neoplastic cells. Since no EcPV-2 DNA was detected in this sample, PV-induced degradation of pRb can be excluded. We hypothesize that the low level of immunostaining in the neoplastic cells in this case may have been caused by spontaneous mutations in the pRb gene.

### 6.4.2 p53

Interpretation of p53 immunostaining is more difficult than interpretation of pRb. This is because a low level of p53 immunostaining in a section can indicate either loss of p53 protein or, because wild-type p53 has a short-half life in a cell, the normal production of p53 protein.[30] Furthermore, the presence of abundant p53 immunostaining does not indicate that increased functional p53 protein is present in the cell. Instead, abundant p53 immunostaining suggests that a mutation in the p53 gene has resulted in the accumulation of a non-functional, but abnormally stable, form of p53 within the cell.[8] Additional information on this can be found in Chapter 1 (Section 1.6.2.3 *p53*).

In the 19 EPSCCs for which p53 immunostaining was determined in our experiment, all contained neoplastic cells with p53 immunostaining. In nine of the 19 EPSCCs more than half of the neoplastic cells immunostained for p53. These results are consistent with those of an earlier study that detected p53 immunostaining in seven of nine EPSCCs.[35] While this could cause concern about cross-reactivity with other nuclear proteins, the specificity of the anti-p53 antibody used in this study for equine p53 had been previously confirmed.[1] Therefore, we believe that this result indicates that many of the cells in the EPSCCs in our experiment contained p53 mutations that led to abnormal accumulation in the cells. However, sequencing of the p53 gene would be required to confirm the presence of mutations in these SCCs.[22]

Half of all human neoplasms contain p53 mutations, but these mutations are reported to be particularly frequent in UV-induced cutaneous SCCs.[8,10,29] Likewise, 6 of 6 equine ocular neoplasms, which are thought to be UV-induced, were reported to contain p53 immunostaining in one study.[35] Therefore, the high rate of p53 immunostaining observed in our experiment could be evidence that the EPSCCs are caused by sun exposure. However, p53 immunostaining was also frequent in the equine throat SCCs, which were definitely not caused by UV exposure. Since unpigmented skin may be more susceptible to UV damage than pigmented skin, a comparison of the p53 immunostaining in EPSCCs from unpigmented versus heavily pigmented penises would provide additional information on the role of UV light in the development of p53

mutations. Unfortunately, the color of the penis was not recorded for the 20 EPSCCs in our experiment.

Our results do not support the hypothesis that EcPV-2 influences neoplasm development by degrading the p53 protein. Therefore, the prevalence of p53 immunostaining within the EPSCCs may be due to spontaneous mutation of the p53 gene rather than indicative of any specific etiology. p53 gene mutations have previously been reported in neoplasms from horses,[28] cats[21,25] and dogs.[14,34,37] While this study did not reveal evidence to support an interaction between EcPV-2 and p53, it remains possible that EcPV-2 could degrade the p53 protein early in oncogenesis, but that mutations in the p53 gene that develop subsequently as the cancer progresses result in inactive p53 protein accumulating in the cell.

In one EPSCC, only 1.2% of the neoplastic cells immunostained for p53. This EPSCC did not contain EcPV-2 DNA, which excludes EcPV-2-induced degradation as the cause of the relative lack of p53 in this neoplasm compared to the other EPSCCs. It remains possible that an unidentified PV type was present and had degraded the p53 within the neoplasm. However, it is also possible that the p53 gene had not mutated within the majority of the tumor cells and these cells contained normal p53 protein with its typical short half-life.

### 6.4.3 p16

A significant finding in our experiment was that we were unable to immunostain p16 in equine tissues. While this failure may have resulted from suboptimal conditions in any of the steps involved in the immunostaining process, intense p16 immunostaining within the expected cells was visible in feline tissue that was processed in parallel within the horse samples. The contrast between the immunostaining in the feline and equine tissues suggests insufficient cross-reactivity between the equine p16 protein and the anti-human p16 antibody used. Although it was recently reported that the anti-p16 antibody used in our experiment was able to bind equine p16 protein in a Western blot assay,[33] to our knowledge p16 immunostaining has never been successfully performed using equine tissue. Therefore, it is most likely that the

commercially available anti-p16 antibodies have a low affinity for the equine p16 protein. The affinity between the antibody and protein appears to be sufficient for binding to native proteins in a Western blot, but insufficient for this antibody to be used for IHC. Therefore, in order to investigate p16 in horses it would be necessary to create an equine-specific anti-p16 antibody. We did not do this because of time constraints and expense, but this is a potential direction for future research.

### 6.4.4 Throat SCC Samples

Three equine throat SCCs that contained EcPV-2 DNA were included in this experiment. We initially intended to perform IHC for p16, pRb, and p53 on all 20 throat SCCs that we had obtained during the work described in Chapter 5. However, immunostaining of the three SCCs that contained PV DNA revealed widespread pRb and p53 immunostaining and a lack of p16 immunostaining in both neoplastic cells and positive controls. As these results provided no evidence of a viral etiology and did not allow pRb function to be assessed, we decided that immunostaining the additional 17 throat SCCs was unlikely to provide further useful information.

## 6.5 Chapter Summary

Immunohistochemistry was performed to detect p16, pRb, and p53 in 20 EPSCCs and three equine throat SCCs. These three proteins were investigated because they are important in cell regulation and all are altered during PV-induced oncogenesis by high-risk HPV types.[7,12,27,32]

Immunostaining to detect p16 in our samples was not possible, presumably due to insufficient affinity of the human anti-p16 antibody for the equine p16 protein. In contrast to PV-induced neoplasms in people, the EPSCCs and the equine throat SCCs frequently contained immunostaining for pRb and p53. EPSCCs that contained EcPV-2 DNA did not have reduced pRb or p53 immunostaining within the neoplastic cells. These results provide no evidence that EcPV-2 causes SCCs through either E7-mediated pRb degradation or E6-mediated p53 degradation. However, it remains possible that

EcPV-2 could inhibit pRb function without resulting in degradation of the protein or that EcPV-2 could degrade p53 as an early event in viral oncogenesis.

In summary, the mechanisms by which EcPV-2 dysregulates cell growth and division during its normal life-cycle remain unknown but appear not to involve *E6* or *E7*-mediated inactivation of p53 or pRb. Additionally, since EcPV-2 does not express an E5 protein,[31] an interaction with the PDGF-βR receptor similar to that of the delta bovine PV during oncogenesis,[5] can be excluded. Further studies are required to determine how EcPV-2 stimulates the development of a papilloma and, therefore, to determine the likelihood that this PV has the ability to cause neoplastic transformation of infected cells.

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# CHAPTER SEVEN

# General Discussion and Conclusions

## 7.1 Introduction

When I began the research described in this thesis only one equine papillomavirus (PV) was known (EcPV-1) and there was no evidence that it was associated with the development of squamous cell carcinomas (SCCs). In 2010, I found an association between a novel PV and equine penile SCCs (EPSCCs). Other research groups around the world were also investigating this, and three articles describing the newly characterized *Equus caballus* papillomavirus type 2 (EcPV-2) and its association with EPSCCs were published in close succession.[23,34,41]

During the course of my subsequent research several other groups also investigated EcPV-2 and published their findings. If these findings were available at the time my thesis chapters were written, then I have referred to them in the relevant chapter. However, some findings by other groups were published after my own experimental

work was completed. I have introduced and discussed these results in this chapter (section 7.3), where I review my own research findings in the context of others' work and summarize what is now known about EcPV-2.

In section 7.4 I conclude by considering whether or not my overall hypothesis that some equine SCCs are caused by PV infection is supported, and briefly discuss potential directions for future research into EcPV-2 and its role in equine cancer.

## 7.2 Brief Summary of the Results Presented in this Thesis

The results of the research presented in this thesis showed for the first time that:

- EcPV-2 is present in New Zealand;
- EcPV-2 viral loads are significantly higher in penile SCCs than in non-SCC penile tissues;
- Immunostaining localizes EcPV-2 antigen to the nuclei of neoplastic cells but not to those of normal, adjacent cells. It also shows that viral replication in EPSCCs is rare;
- Equine penile papillomatosis, which had not been previously described, is associated with EcPV-2 infection;
- Biopsy, cytobrushing, or swabbing are equally effective methods of detecting EcPV-2 infection;
- EcPV-2 DNA is detectible in a proportion of equine throat cancers;
- EcPV-2 is able to infect grossly normal equine pharyngeal mucosa;
- EcPV-2 infection is not common in grossly normal nictitating membranes;
- Unlike high-risk human PVs, EcPV-2 likely does not cause SCC development through degradation of either of the tumor suppressor proteins p53 or pRb.

The results of the research presented in this thesis corroborate other reports that:

- The presence of EcPV-2 is significantly correlated with the presence of EPSCCs;
- EcPV-2 is the PV found in penile papillomas;
- Grossly normal vulvovestibular mucosa rarely contains EcPV-2.

## 7.3 Cumulative Knowledge about EcPV-2 to Date

### 7.3.1 EcPV-2 and EPSCCs

In chapter 2 we reported finding EcPV-2 DNA sequences significantly more frequently in EPSCC lesions than in non-SCC penile disease lesions. This suggests, but does not prove, that EcPV-2 may be involved in the development of penile neoplasia. Since 2010, similar findings have been reported in nine publications by other researchers.[2,26,27,29,33,34,37,40,43] Reported detection rates of EcPV-2 DNA sequences in EPSCCs or carcinomas *in situ* ranged from 45%[29] to 100%,[27] with an average detection rate of 88%; the sample sizes in those studies ranged from 1 horse[26] to 87 horses[40] (average 22). There are many possible reasons for these differences in detection rate, including variations in horse age, penile pigmentation, UV exposure, choice of PCR technique, choice of PCR primers, and sample preservation or age. However, it is clear that EcPV-2 is a common inhabitant of the majority of EPSCC lesions and that its presence must be considered when investigating the cause of this disease.

### 7.3.2 EcPV-2 and Penile Papillomas

In chapter 3 we reported finding EcPV-2 in multiple penile papillomas in a horse with penile papillomatosis. We used the term 'penile papillomatosis' to describe the massive number of papillomas present; this novel clinical manifestation of EcPV-2 infection[24] has not subsequently been described in any additional horses.

That PVs cause equine penile papillomas was first proposed in 1984, but the specific PV type was unknown.[19,30,31,36] Five other research groups have now also demonstrated the presence of EcPV-2 in equine penile papillomas, which strengthens the case that EcPV-2 is the virus responsible for their development.[2,11,27,34,40]

No PV other than EcPV-2 has ever been detected in equine penile papillomas. While this further supports EcPV-2 as the cause of equine penile papillomas, the possibility of an additional, undetected PV present in these lesions cannot be excluded. Equine penile papillomas are unusual among papillomas in that they frequently progress to SCCs.[5,12,18] Since EcPV-2 is the only PV that has ever been detected in equine penile

papillomas, it is, therefore, plausible that this virus not only induces papilloma development but also influences the progression from papilloma to SCC. Evidence that EcPV-2 could promote neoplastic progression was presented in a 2015 study, in which the authors demonstrate the presence of EcPV-2 using *in situ* hybridization (ISH) in both an EPSCC that arose within a focus of epithelial hyperplasia (likely PV-induced) and within the hyperplastic epithelium.[43] Again, this may support the hypothesis that EcPV-2 infection drives progression of penile papillomas to EPSCCs.

### 7.3.3 EcPV-2 and Healthy or Non-SCC Genital Mucosa

In chapters 2 and 5 we tested grossly normal or non-SCC-diseased male and female genital tissues for EcPV-2. The results confirmed our hypothesis that EcPV-2 would be present less frequently and at a lower viral load in non-SCC tissues than in SCCs. A shortcoming of our work was that, although normal vulvovestibular mucosa was tested, no vulvovestibular SCC samples were tested, and no comparison of viral presence or load in diseased versus healthy mucosa was possible.

Our finding that EcPV-2 was present infrequently in grossly normal penile mucosa (2 of 32; 6.3%)[22] is now in agreement with four other studies. These reported EcPV-2 in 1 of 27 (3.7%),[34] 4 of 39 (10.3%),[2] 2 of 43 (4.7%),[37] and 1 of 12 (8.3%)[40] penile swabs or mucosal samples. Our finding that EcPV-2 was present infrequently in grossly normal vulvovestibular mucosa (1 of 40; 2.5%)[22] is also in agreement with four other studies. These reported EcPV-2 in 2/52 (3.8%),[33] 0 of 20 (0%),[2] 0 of 1 (0%),[20] and 2 of 51 (3.9%)[37] vulvovestibular swabs or mucosal samples. Overall, evidence suggests that EcPV-2 infection is infrequent, but possible, in grossly normal male and female genital mucosa. However, it is not known whether horses with EcPV-2-infected but grossly normal genital mucosa will clear the infection, remain latently infected carriers, or go on to develop papillomas, precancerous plaques, or SCCs. For this reason, future work should include long-term monitoring of EcPV-2-infected horses.

The finding in our experiments that EcPV-2 was present infrequently in non-SCC penile lesions (3 of 20; 15%)[22] has been investigated in just one subsequent study, which reported EcPV-2 DNA in 6 of 11 (54.5%) non-SCC diseased penile biopsy samples.[27]

There are several possible explanations for the large difference between our results and those of the later study. These differences in results may reflect a true difference in the infection rate between two separate populations. They may also be caused by a difference in the types of non-SCC lesions tested in each study, with some lesions being more or less favorable to EcPV-2 infection than others. However, the types of non-SCC penile lesions in which EcPV-2 was detected in these two studies were diverse, and there were no obvious connections between them. They included neoplasia (melanoma), inflammatory disease (balanoposthitis), and non-inflammatory disease (amyloidosis and follicular cyst). Another possible reason for the large difference between the results of these two studies is that our study used formalin-fixed, paraffin-embedded tissue that had been stored for years, while the other study used freshly collected biopsy material. It is, therefore, possible that fragmentation of the DNA in our samples reduced the amount of intact EcPV-2 sequences available for amplification relative to the DNA in the samples used by the second group. The difference between the results of these two studies may also reflect differences in the sensitivities of the EcPV-2-specific primers used by each group. However, both groups used primer sets designed to amplify segments of the EcPV-2 L1 gene and our own set had very high analytical sensitivity, as discussed in chapter 4, so this possibility seems less likely.

While the rates of EcPV-2 DNA detection in non-SCC penile lesions differed between our study and the later experiment, the results of both suggest that EcPV-2 may be present in diseased, non-SCC penile tissues more commonly than in grossly normal penile mucosa, but less commonly than in EPSCCs. However, it is not possible to reach a definitive conclusion based on just two studies, especially given the differences in their results. There is currently insufficient evidence to draw any conclusion about the influence of EcPV-2 in non-SCC penile lesions. While EcPV-2 does appear to be present with reasonable frequency in non-SCC penile lesions, it may be that non-SCC penile lesions simply provide a more permissive environment for EcPV-2 infection than healthy mucosa but otherwise has no effect. An obvious question now is whether EcPV-2-infected non-SCC lesions behave differently than do their non-infected counterparts, and whether any of them progress to SCCs.

### 7.3.4 EcPV-2 and Non-Genital Tissues

In chapter 5 we investigated the presence of EcPV-2 in SCCs and grossly normal mucosa from two other sites at which SCCs commonly develop in horses. These were the throat (oral cavity and pharynx) and third eyelid (nictitating membrane). We were especially interested to study the throat because, unlike SCCs of the genitals, a role of UV light in the development of cancer can be definitively excluded at this site.

We showed that EcPV-2 is able to infect the equine throat, although infrequently (2 of 75; 2.7%). This was a novel finding and no other study since has investigated EcPV-2 in grossly normal equine throat tissues. We also reported EcPV-2 infection in 3 of 20 (15%) throat SCCs and have detected it in a single additional aryepiglottic SCC (unpublished). EcPV-2 detection in an equine throat SCC has only been described once since, in a case report.[20] Overall, therefore, there is currently insufficient information to draw any conclusion about the role of EcPV-2 in equine throat cancer. However, as it has been proven that this PV is able to inhabit the throat and no other potential cause of equine throat SCCs has been identified, EcPV-2 remains a potential significant cause of these invariably fatal cancers of horses.

We did not detect EcPV-2 in any of 75 grossly normal third eyelids. Only one group since has investigated EcPV-2 in grossly normal periocular tissues; they detected EcPV-2 in 1 of 30 (3.3%) periocular swabs.[37] We did not test third eyelid SCCs because of a lack of availability, which is a shortcoming of our work. This has been addressed to some extent by two other studies that reported EcPV-2 detection in 0 of 12,[34] and 0 of 42[29] periocular SCCs. In contrast, EcPV-2 was detected in 1 of 2[37] and 1 of 1[20] periocular SCCs in two other reports. Therefore, as for equine throat tissues, there is insufficient information to draw any conclusion about the role of EcPV-2 in periocular SCCs. However, since EcPV-2 can infect a small proportion of grossly healthy periocular tissues and has been detected in two periocular SCCs, it appears that EcPV-2 could potentially play a role in SCC development at this site.

### 7.3.5 Summary of Conditions Found to Contain EcPV-2 DNA

EcPV-2 DNA has been detected in lesions of the following conditions:

1. penile SCC, carcinoma *in situ*, or precancerous plaque,[2,22,23,26,27,29,33,34,37,41,43]
2. penile papilloma,[2,11,27,34,40]
3. vulvovestibular SCC, carcinoma *in situ* or precancerous plaque,[2,34]
4. vulvovestibular papilloma,[34]
5. periocular SCC,[20,37]
6. pharyngeal SCC,[20,22]
7. perianal SCC.[2]

It is important to recognize that simply detecting EcPV-2 in a limited number of lesions does not prove that the PV had any influence on the development of those lesions. Additionally, the number of publications that report finding EcPV-2 in a specific disease does not influence the likelihood that EcPV-2 causes that disease. For example, the higher number of publications reporting EcPV-2-positive penile lesions (fourteen) than EcPV-2-positive vulvovestibular lesions (two) may simply reflect greater interest in penile research based on the findings from earlier studies. Nevertheless, higher rates of EcPV-2 have been consistently reported in studies of large numbers of penile cancerous and precancerous lesions and there is currently more evidence that EcPV-2 plays a significant role in the development of these lesions than in lesions at other body sites.

### 7.3.6 Progression of EcPV-2 Infection

In chapter 3 we reported observing a horse with EcPV-2-induced penile papillomas over a period of 16 months. This is the only report to date of EcPV-2 infection being investigated in the same horse at more than one time point. In women infected by high-risk HPVs, lesions may take years to decades to progress to neoplasia, with only a fraction of the infected women ever going on to develop cancer.[35] It is currently unknown what proportion of EcPV-2-infected horses will clear infection, become latent carriers, or develop neoplasia. This knowledge would help us understand the clinical

importance of EcPV-2 infection; thus, future studies should focus on long-term monitoring of EcPV-2-infected horses.

### 7.3.7 Laboratory Techniques Used to Investigate EcPV-2

#### *7.3.7.1 Swabbing or Cytobrushes*

In chapter 3 we developed and tested non-invasive methods for diagnosing EcPV-2 infection in horses. We initially utilized cytobrushes, and subsequently used cotton swabs because of their lower cost and equal effectiveness. These same methods have since been used in five other studies,[2,11,28,33,37] and the use of swabs or cytobrushes is now an accepted technique for PCR-based diagnosis of EcPV-2 infection. Compared to biopsy based diagnostic techniques, swab or brush-based diagnostic methods are both less-invasive and cheaper, and therefore allow greater numbers of samples to be collected.

#### *7.3.7.2 Quantitative PCR*

In chapter 4 we used quantitative PCR (qPCR) to quantify the EcPV-2 viral load within healthy and diseased equine tissues. Of the fifteen reported investigations into EcPV-2 to date,[2,11,20,22-24,26,27,29,33,34,37,40,41,43] only two others have used qPCR,[20,40] while the remainder have used conventional PCR. This could suggest that the information on viral load provided by qPCR is not considered useful by other groups investigating EcPV-2. However, as discussed in chapter 5 (section 5.4.1 *Investigation of EcPV-2 Viral DNA Load in Penile Samples*), there is some evidence that comparing the EcPV-2 viral loads of cancerous and non-cancerous tissues may help to differentiate between the virus causing neoplasia and the virus being present as a harmless commensal. In human HPV-associated cancers, some studies suggest that viral load may be an important prognostic indicator.[6,7,10,21,25,42] While the evidence is sometimes contradictory, I consider information on EcPV-2 viral load to be relevant as it has the potential to help predict lesion progression and prognosis in horses with EPSCCs. The two other EcPV-2 studies that used qPCR both measured EcPV-2 mRNA transcripts as an indication of gene expression, rather than measuring viral load.[20,40] This may provide more information on the oncogenic activity of EcPV-2 within a neoplasm than

does viral load,[9] but both measurements contribute to a fuller understanding of the biologic behavior of this PV.

#### *7.3.7.3 In Situ Hybridization*

In chapter 2 we used ISH with a generic consensus PV probe to show that PV DNA was present within neoplastic cells in 6 of 9 (70%) EcPV-2 PCR-positive SCC lesions but not in adjacent, normal epithelium. Since that work was done, ISH has been used to investigate EcPV-2 in three studies by other researchers.[27,34,43] The three studies used different EcPV-2-specific probes and detected viral DNA in 5 of 6 (83%)[34], 2 of 2 (100%)[27] and 6 of 13 (46%)[43] samples that had been confirmed by PCR to contain EcPV-2. With regard to the localization of hybridization, two groups reported similar findings to our own: nuclear staining in neoplastic cells but not in those of the adjacent normal epithelium.[27,34] The third group reported that, in addition to nuclear staining in neoplastic cells, there was an intensely stained band of basal epithelial cells in the hyperplastic mucosa adjacent to the neoplasm.[43] This likely represents the epithelial hyperplasia induced as part of the normal life cycle of any PV, but at whose center a neoplasm developed from a mutated cell. This could suggest that EcPV-2 positive EPSCCs begin as hyperplastic or papillomatous lesions. Overall, other groups' ISH findings agree with our own and strengthen the evidence for an association between EcPV-2 infection and development of EPSCCs. In addition, the positive results obtained by other groups when using EcPV-2 specific probes suggest that the unspecified PVs detected by our own consensus probes were, in fact, EcPV-2 and not some other type of PV.

One factor that has not been considered by any research group, including our own, when using ISH to investigate EcPV-2 in PCR-positive tissues is the influence of viral load on hybridization. As shown in chapter 4 of this thesis, viral loads that are extremely low may still be detected using qPCR and specific primers because the viral DNA is amplified to higher levels over 30–40 cycles. However, ISH does not include an amplification step and a viral load of only a few copies per ng of template DNA may not be visually evident using this technique.

#### *7.3.7.4 Immunohistochemistry for p53, pRb, p16 and Ki67*

In chapter 6 we used IHC to detect p53 as part of our investigation of possible mechanisms of EcPV-2-induced oncogenesis. Our results did not support the hypothesis that EcPV-2, like the high risk human PVs, promotes neoplasia by degrading p53. Only one other study has subsequently investigated p53 in relation to EcPV-2. Using 101 samples from 87 horses, that study found that penile lesions that were negative for EcPV-2 by PCR had significantly higher nuclear p53 immunostaining than EcPV-2-positive lesions.[40] While this result was interpreted as possible evidence that the E6 protein of EcPV-2 may degrade p53, there was no significant correlation between the quantity of EcPV-2 E6 mRNA and the reduction in p53 immunostaining. Thus the role of EcPV-2 in the loss of p53 in neoplasms remained uncertain. That same study also found significantly more p53 immunostaining in less differentiated EPSCCs than in well-differentiated EPSCCs, carcinomas *in situ*, or normal skin. The authors suggested that measuring p53 could be useful for "predicting disease severity." However, histologic evaluation and grading of lesions is already an established method for predicting the future behaviour of penile neoplasms in horses[39] and humans.[8] In my opinion, therefore, measuring p53 may not provide any useful information beyond that already provided by histologic grading.

We also used IHC for pRb and p16 to investigate whether EcPV-2 influences neoplasia development through degradation of pRb. We did not detect any evidence to suggest that pRb was lost in those SCCs that contained EcPV-2 DNA. In humans and cats, loss of pRb results in a readily-detectible increase in p16 within the neoplasms. Unfortunately, we were unable to detect immunostaining for p16 in any of our samples; this was likely due to a lack of cross-reactivity between the equine p16 protein and the human anti-p16 antibody we used. No other studies have used pRb or p16 IHC to investigate EcPV-2.

Finally, a single study has investigated immunostaining of the cellular proliferation marker Ki67 in equine penile SCCs.[40] The expression of Ki67 is correlated to lymph node metastasis in human penile cancer, although not to overall survival.[15] When investigating Ki67 in equine penile cancer the authors found no difference in Ki67

immunostaining between well- and poorly differentiated neoplasms, and no prognostic value in its measurement.[40]

Overall, the immunohistochemical markers p53, pRb, p16, and Ki67 do not appear to have the same value when investigating EcPV-2 infection in horses as when investigating high-risk HPV infections in humans.

### 7.3.8 Other Known Equine Papillomaviruses

#### *7.3.8.1 In Genital Lesions*

In chapters 2 and 3 we used two consensus PV primer sets and sequenced their PCR products to show that EcPV-2 was present in a subset of EPSCC lesions and in penile papillomas. As discussed in these chapters, because EcPV-2 was the only viral type amplified from these lesions it is likely that no other PV was present. However, we cannot rule out the possibility that additional PV types were present but were either not amplified, or were amplified less efficiently, by the MY09/MY11 consensus primers used. Since our experiments there have been numerous PCR-based investigations into EcPV-2 by our own and other groups, but all have used EcPV-2-specific primers rather than consensus primers.[2,11,20,22,27,29,33,34,37,40,43] None of those studies would, therefore, have detected either known or novel non-EcPV-2 PVs.

Since the work described in chapters 2 and 3, only one other study has used PV consensus primers to investigate equine lesions.[28] In that study, three sets of consensus primers were used; one was the FAP59/FAP64 set that we used in chapter 2, and the other two were different from ours. Using the FAP consensus primer set, EcPV-2 was amplified from a penile plaque and a vulvar plaque. In addition, a novel sequence was also amplified from the vulvar plaque using the FAP primer set. This was sequenced and named EcPV-4. When using the CP4/CP5 consensus primer set, a novel sequence was also amplified from a "penile mass." This was sequenced and named EcPV-7. Therefore, since two additional EcPVs (4 and 7) have now been demonstrated in equine genital lesions, it is worth considering whether these PVs may have been present, but not detected, in the samples we tested in chapter 2. Since we tested our 40 equine penile lesions with the FAP consensus primers, which detected EcPV-4 in the

2013 study,[28] it is likely that EcPV-4 would have been detected in our samples if it were present. However, in our experiments, the FAP primer set did not amplify PV DNA from any equine tissue tested, even those 10 in which EcPV-2 was detected using the MY09/MY11 primer set. Because of this we did not use the FAP primer set in any later work. It is, therefore, unexpected that the FAP primer set amplified EcPV-2 in the 2013 study but not in our own. One possible reason for this is that the DNA in our samples may have been degraded by prolonged formalin fixation and paraffin embedding, while the samples from the 2013 study were obtained fresh from living horses. The results of the 2013 study suggest that the FAP primer set may still be useful for amplifying EcPV-2, and that a variety of consensus primer sets should be used in future studies to detect novel PVs. While EcPV-2 is the PV most likely to have a causal role in equine genital cancer, it must be acknowledged that other PVs may also have a role.

#### *7.3.8.2 In Non-Genital Lesions*

Since the start of our experimental work, when just one equine PV was known (EcPV-1), six more equine PVs have been discovered (EcPV-2 through 7). Equine PVs 2, 4, and 7 have been detected in genital lesions, as discussed above.[28] Equine PVs 3, 4, 5, and 6 have been detected in equine aural plaques.[14,28,38] These findings are not surprising, given that there are now nearly 200 known human PV types, but they do emphasize the ubiquity and site-specificity of PVs. These findings also show that investigating other equine body sites for novel PVs could be worthwhile. It is worth noting that the throat samples tested in chapter 5 were only investigated using primers specific for EcPV-2, and that novel PV types may have been present but undetected in these samples.

## 7.4 Conclusions

### 7.4.1 Evidence for Causality

The central hypothesis of this thesis is that, as in humans, some equine SCCs are caused by PV infection. In the preceding sections of this chapter I summarized the findings from my own and others' research into SCCs and PVs in horses over the past

five years. In the remainder of this chapter I re-examine my hypothesis in light of what is now known about EcPV-2, and discuss future directions for research.

Overall, the most frequent and important finding since 2010 has been the consistent and significant association of EcPV-2 DNA with EPSCCs or precursor lesions. However, a simple association is insufficient to establish causality. A similar problem exists in human medicine, where a statistical association between the presence of a suspected carcinogen and development of cancer may be evident, but cannot be tested experimentally for ethical reasons. Despite this, it is now accepted beyond reasonable doubt that certain HPV types cause cervical cancer, that cigarette smoking causes lung cancer, and that chronic hepatitis B virus infection causes liver cancer.

This acceptance has been achieved through collection of a large amount of epidemiologic and animal experimental data. From this body of data, certain criteria are commonly assessed in order to determine whether an association is causal or not.[17] These criteria are widely accepted and have been adopted by the International Agency for Research on Cancer (IARC), which is the agency of the World Health Organization that decides on the carcinogenicity of different agents.[1] They include (1) strength, (2) consistency, (3) specificity, (4) temporality, (5) biological gradient, (6) plausibility, (7) experimental evidence, and (8) analogy. In the following paragraphs, each criterion is discussed as it pertains to HPV infection and cervical cancer, and then compared to the current data on the association between EcPV-2 infection and equine SCCs. In this way, I will assess how the evidence supporting my hypothesis "weighs up" against the evidence showing irrefutably that HPV causes human cervical cancer.

#### *7.4.1.1 Strength of Association*

Strong associations between exposure to/presence of an agent and development of disease are more likely to be causal than are weak associations. This is based on the assumption that if a strong association were to arise by chance because of some confounding variable or bias, then the biasing influence would have to be even stronger and would therefore be easy to detect. In women, there is a very strong association between the presence of cervical cancer and the PCR-based detection of

HPV DNA, with DNA detectible in over 90% of cancers. In horses, the average detection rate of EcPV-2 in EPSCCs and precursor lesions over all 11 reported studies is approximately 83%.[2,22,23,26,27,29,33,34,37,40,43] Therefore, just as for human cervical cancer, nearly all EPSCCS contain PV DNA, and very few EPSCCs do not contain PV DNA. This supports my hypothesis that EcPV-2 causes penile cancer in horses.

In human studies, data on the association between cervical cancer and HPV infection are usually discussed in terms of relative risk (RR), odds ratio (OR), and attributable fractions (AF). Although different studies report different values, the typical reported increase in the OR for cervical cancer is in the range of 50 to 100 fold for HPV DNA, with an AF of more than 95%.[4] Due to the small number of investigations into the prevalence of EcPV-2 infection in horses without EPSCCs, it is not yet possible to calculate these values for horses. Knowing if an increased risk for EPSCC development is caused by EcPV-2 infection would be helpful when deciding whether to test horses for this PV and whether the development of preventative strategies, such as vaccination, is worthwhile.

#### *7.4.1.2 Consistency*

Consistency refers to the repeated observation of an association in different studies of different populations under different circumstances. There is marked consistency in the results of epidemiologic studies of human cervical cancer and its association with HPV infection, regardless of the country in which the study was conducted, the population investigated, or the protocol used. The number of studies investigating EcPV-2 infection and EPSCCs is relatively small (11) but the results are fairly consistent, although less so than in the human cervical cancer studies.[2,22,23,26,27,29,33,34,37,40,43]
Equine studies have investigated horse populations from eight countries (USA, England, Austria, the Netherlands, Switzerland, New Zealand, Belgium and Australia) using a variety of PCR protocols and primer sets, and in all but two[23,29] EcPV-2 DNA was found in the majority of EPSCCs or *in situ* SCCs. All studies to date have consistently found a significant association between EcPV-2 and EPSCCs or *in situ* SCCs, regardless of country or PCR protocol, and no published study has challenged this finding. These observations, therefore, support my hypothesis.

In more detail, when data from the 11 studies investigating the association between EcPV-2 infection and EPSCCs are pooled, 191 of 236 (80.9%) samples were positive for EcPV-2. The average percentage of samples containing EcPV-2 was similar over the eleven studies (83.1%; range 45% to 100%). It should be noted that these numbers are not completely accurate since several research groups, including our own, retested the same samples using new primers or PCR protocols and published these results in subsequent studies. However, this has had the benefit of explaining at least some of the variability in detection: our first study using consensus primers and conventional PCR detected EcPV-2 in 9 of 20 samples, while our second study using specific primers and qPCR detected EcPV-2 in 16 of 20 samples. The same 20 samples were used in both studies; thus, our results demonstrate the variability in detection that occurs with different detection protocols. This highlights the fact that EcPV-2 detection methods are not yet standardized and that variation in primer choice, PCR conditions, and sample processing may reduce the detection rate in some studies and lower the cumulative average for all studies, especially given the small number of studies that have been conducted to date. The average EcPV-2 detection rate may increase in future studies as PCR techniques and primers are optimized.

Interestingly, if the average EcPV-2 detection rate rises in future studies, then the prevalence of EcPV-2 in EPSCCS will more closely resemble that of HPV in human cervical cancers than in human penile or throat cancers. Whereas cervical cancer essentially does not occur without HPV infection, only a proportion of human penile or throat cancers are associated with HPV infection, and these have a better prognosis than HPV-negative tumors. If EcPV-2 is ultimately found to be present in every EPSCC, then the potential prognostic value of testing for EcPV-2 in EPSCCs will be lost. This is summarized well in a 2015 study of metastasis and EPSCC outcome, which states, “In man, HPV presence in penile SCC is seen as a positive prognosticator for survival, but we could not confirm this for horses. The most likely explanation is that EcPV-2 presence in equine urogenital SCC is so common that this parameter is not discriminative enough.”[40]

#### *7.4.1.3 Specificity*

Specificity ("one agent/one disease") is less relevant to assessment of causality for cancer than for microbial disease. This is because carcinogenesis is typically complex, and a particular carcinogen may affect different organs and cause different types of neoplasia. Since more than 15 HPV types are linked to cervical cancer in women, and many of these same types are also associated with cancer at other body sites, specificity of cause and effect for HPV and SCCs cannot be shown based on the narrow definition typically used for non-neoplastic microbial disease. In contrast, EcPV-2 is the only PV type that has ever been found in EPSCCs. EcPV-2 is not significantly associated with SCCs from any non-penile body sites tested (throat and periocular tissue) and has never been associated with any disease other than SCC or its precursor lesions. These observations appear to support a causal role of EcPV-2 in penile SCCs.

However, it is important to recognize the limitations of my claim of specificity, most of which are to do with the limited number of studies conducted. First, EcPV-2 may be the only PV detected in EPSCC lesions to date simply because EcPV-2-specific primers have been used in all but two studies, [23,28] and in one of the two studies in which consensus primers were used, a second PV (designated EcPV-7) was detected. However, that study described only a "penile mass" so it is unclear whether or not this was an EPSCC. Given the number of different PV types found in human cervical cancers, or even equine aural plaques, it is plausible that EcPV-2 may be just one of several types of EcPV eventually associated with EPSCCs. Second, the claim that EcPV-2 is not significantly associated with SCCs from any non-penile body sites tested may reflect nothing more than the low number of samples tested; EcPV-2 has in fact been detected in five equine throat cancers, although this this was only a small proportion of the throat SCCs evaluated. Too few vulvovestibular, periocular, or other body site samples have been tested to state that EcPV-2 is not associated with any disease other than penile SCC or its precursor lesions.

#### *7.4.1.4 Temporality*

This refers to the necessity that cause should precede effect. If disease arises before exposure to a particular agent, then that agent did not cause the disease, even if it is

later shown to be present within lesions. In humans, it has been proven that HPV infection precedes development of cervical cancer, typically by decades. In horses, there is no information about the timing of PV infection relative to development of EcPV-2 positive EPSCCs. Every EPSCC in which EcPV-2 has been detected represents only a single time point, with no information available about the presence of EcPV-2 before diagnosis. The possibility that these EPSCCS developed due to a non-PV cause and were later infected by EcPV-2 cannot be ruled out. Therefore, there is no information on temporality that supports or refutes my hypothesis.

#### *7.4.1.5 Biological Gradient*

This indicates that an increasing magnitude of exposure to an agent increases the risk of disease, following a dose-response curve. While this is applicable to chemical agents of carcinogenesis, it is less important in viral carcinogenesis. This is because viruses are able to replicate or, alternatively, be cleared by the immune system, which makes the initial infective dose (i.e., magnitude of exposure) less relevant. For example, nearly all women who are exposed to high-risk HPVs and become infected will clear the infection completely, regardless of the initial infective dose.

As a substitute for initial exposure, various studies have measured HPV viral loads in cervical samples and calculated the risk of HPV-positive women developing cervical cancer relative to HPV-negative women. Some studies have found higher viral load to be a determinant of future cancer risk, while others have not.[3] No similar data are available for horses with EcPV-2 infection, and viral load has been measured in just one study.[22] Although EcPV-2 viral loads were significantly higher in SCC-affected penile samples than non-SCC penile samples, no horses in the study were available for long-term observation. The recorded viral loads represented a single time point, and it was not possible to correlate viral load with progression of disease. Therefore, there are no data on biologic gradient that support or refute my hypothesis.

#### *7.4.1.6 Biological Plausibility*

This asks whether it is plausible that the presence of an agent could, in fact, result in the disease. In the case of HPV and induction of cervical carcinoma there is an

enormous body of evidence that clearly demonstrates several mechanisms by which high risk HPVs can cause neoplasia. These include: degradation of p53 and pRb by E6 and E7 oncogene products; expression of E6 and E7 oncogenes in tumor cells but not stromal cells; the ability to immortalize human keratinocytes in cell culture; induction of morphologic changes in raft cultures that resemble preinvasive cervical lesions; maintenance of a transcriptionally active viral genome in every lesional cell; consistent higher levels of transcription of E6 and E7 oncogenes in high-grade lesions than low-grade lesions; and consistent integration of the virus into the host cell genome. Thus, an association between the presence of HPV DNA in cervical specimens and development of cervical cancer is plausible. In comparison to HPV work, investigation into the biological plausibility of EcPV-2 as a cause of SCCs is only beginning. In the work reported in this thesis, I chose two possible oncogenic mechanisms (p53 and pRb degradation by PV oncogenes) for investigation and my results provided no evidence that EcPV-2 degrades either tumor suppressor protein. The only other study to investigate p53 found that penile lesions that were negative for EcPV-2 by PCR had significantly more nuclear p53 immunostaining than EcPV-2-positive lesions. While this could suggest that EcPV-2 degrades p53, there was no correlation between P53 immunostaining and EcPV-2 E6 mRNA expression; such a correlation would have been expected had EcPV-2 infection promoted neoplasia by the same mechanism as high-risk HPVs. Overall, the results of investigations into potential oncogenic mechanisms EcPV-2 are scarce and somewhat inconsistent. There is no mechanistic evidence to support my hypothesis that EcPV-2 causes cancer.

#### *7.4.1.7 Experimental Evidence*

This refers to animal model experiments or human experiments. However, there is no suitable animal model for HPV-induced cervical cancer, and humans may not be experimentally infected with HPVs for ethical reasons. Therefore, carcinogenicity investigations using humans are typically preventive intervention studies that explore whether a reduction in exposure leads to a reduction in cancer incidence. In terms of HPV-induced cervical cancer, one of the most significant cancer prevention studies in history is currently underway in the form of prophylactic vaccination against specific high-risk HPV types. These vaccines have been widely used for just over a decade.

While their efficacy is currently unproven, these vaccines are predicted to cause a marked decline in the incidence of cervical cancer. If so, this will be the strongest and least refutable evidence for a causal relationship between HPV infection and cancer. In horses, there is currently no experimental evidence that EcPV-2 either does or does not cause cancer. Horses have never been deliberately infected with EcPV-2 and observed for development of cancer. Horses known to be EcPV-2-negative have not been vaccinated against the virus and then infected in order to compare incidence of EPSCCs with the unvaccinated population. While such an experiment would be extremely useful, the mechanism of transmission of EcPV-2 is currently unknown and no vaccine against EcPV-2 exists, making such a study impossible at this time. This is discussed further in sections 7.4.2.2 and .3. Therefore, my hypothesis is currently neither supported nor refuted by any experimental data.

#### *7.4.1.8 Analogy*

This criterion is the least essential, but can suggest that an association is causal rather than incidental. In simple terms, analogy means that if an agent can cause cancer in one species then perhaps (a) another similar agent could also cause cancer in that species or (b) the same agent could cause cancer in a different species. In terms of EcPV-2 and equine SCCs, I used human cervical cancer as an analogy to justify my research, and I consider this analogy even more valid after my experiments. The proven role of HPVs in cervical cancer, taken together with the results of the studies presented in this thesis, supports my hypothesis that EcPV-2 plays a role in the development of EPSCCs.

### 7.4.2 Summary

#### *7.4.2.1 Does EcPV-2 Cause Cancer?*

Possibly; this is still unknown.

According to the IARC guidelines on carcinogenicity[1] discussed in the previous section, the information about EcPV-2 and EPSCCs accumulated over the past 5 years meets certain criteria that suggest causality. These are: strength of association between viral presence and EPSCCs; consistency in results over several studies, locations and

populations; specificity of the association between the virus type and the cancer type; and analogy to a similar disease (in this case human cervical cancer). There is currently no evidence refuting the hypothesis that EcPV-2 causes EPSCCs.

On the other hand, several IARC criteria have not been met sufficiently to show that the association between EcPV-2 and EPSCCs is causal. These are temporality, biological gradient, biological plausibility, and experimental evidence. However, it should be noted that these criteria have not been met because appropriate or sufficient equine studies have not yet been conducted, rather than because contradictory evidence has been found.

#### *7.4.2.2 Is it Possible to Prove that EcPV-2 Causes Cancer?*

In theory, if every study type on HPV and cervical cancer were repeated for EcPV-2 and EPSCCs, it would be possible to prove whether or not this virus causes cancer. However, even if the constraints of money, research time, and animal ethics were removed, this would be complex for numerous reasons, including the following:

- The large-scale, international collection of epidemiologic data used for human studies is not possible in horses, whose birth, health, movements, and death are not recorded as they are for humans. These human data have been crucial to understanding the significance of HPV infection over long periods.
- If EcPV-2 infection is analogous to high-risk HPV infection then the interval between natural or experimental infection and lesion development could be decades. Keeping track of a large number of horses and obtaining regular biopsy samples from them over such a long period is impractical.
- The observation that the majority of people clear high-risk HPV infection over time has allowed research to focus on women with persistent HPV infection as being at greater risk for cervical cancer. We don't yet know if horses can clear EcPV-2 infection in the same way.
- Given that there are nearly 200 known human PVs, it is very likely that many more equine PVs than the seven currently known exist, and that EcPV-2 will not be the only "high-risk" equine PV. It is possible that EPSCCs and SCCs at other body sites will be associated with novel PVs. These may have overlapping or

synergistic effects with EcPV-2, similar to HPVs-16 and -18 in humans. Untangling these effects would likely require a larger number of infected horses than would ever be available.

- We don't know how horses are infected with EcPV-2 or at what age. In humans, high-risk HPV infection is acquired venereally after sexual maturity. This gives a long, pre-infection window of time during childhood in which prophylactic HPV vaccination will be effective. If horses acquire EcPV-2 infection transplacentally, perinatally, via fomites, or via insect vectors then the window for vaccination is reduced or lost. If so, it would not be possible to prove that EcPV-2 causes EPSCCs by demonstrating a post-vaccination reduction in the incidence of cancer.

Many of the techniques used to investigate HPV and cervical cancer are impractical or unavailable for use in horses. Therefore, I believe that it cannot be proven unequivocally that EcPV-2 causes EPSCCs without widespread prevention of new EcPV-2 infections (for example by vaccinating) and observation of a subsequent decline in cancer rates over the following decades. However, I feel that the available evidence points to EcPV-2 being carcinogenic and that work should proceed under this assumption.

Because EPSCCs have a significant welfare and economic cost, a possible cause, and are potentially preventable, I agree with A. B. Hill, who says, "*All scientific work is incomplete—whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have, or to postpone the action that it appears to demand at a given time.*"[17]

#### *7.4.2.3 What Directions should EcPV-2 Research Take in the Future?*

Future equine PV research could take many directions, but some are more likely to yield clinically useful results than others. I have reservations about experimental infection of horses with EcPV-2, for the reasons listed in section 7.4.2.2 and because of ethical considerations.

I believe that *in vitro* cell culture work, where cultured equine keratinocytes or raft cultures are infected with EcPV-2, has the potential to provide very important information about the capabilities of the EcPV-2 virus. If cells can be immortalized or induced to develop neoplastic lesions after infection, this would provide strong evidence that this PV is able to cause cancer in horses. This would answer the question of *whether* EcPV-2 causes cancer, but not *how*, which could be considered a shortcoming. However, even without understanding the mechanism, I believe that research showing that EcPV-2 can cause cancer will lead more quickly to research into therapy or intervention. Equine keratinocyte cell culture work is currently underway in Switzerland, although results have not yet been reported.[32]

I also think that development of EcPV-2 virus-like particles (VLPs) is an important next step in EcPV-2 research. This would provide proof of principle that development of a prophylactic vaccine against EcPV-2 is theoretically possible. While there is currently insufficient evidence to justify development of an EcPV-2 vaccine, I believe that there will be in the future. Virus-like particles have already been produced for EcPV-1 with the aim of developing a vaccine that could prevent cutaneous warts in horses.[13] Similarly, research into production of BPV-1 VLPs is underway with the eventual aim of vaccinating to prevent development of equine sarcoids.[16] Production and use of either of these vaccines is still some time off, but the possibility of combining these three VLPs to produce a multivalent vaccine that protects horses against EPSCCs, sarcoids, and warts should be considered.

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# APPENDIX A

## List of Publications

- (2011) **Equine penile squamous cell carcinomas are associated with the presence of equine papillomavirus type 2 (EcPV-2) DNA sequences**. CG Knight, JS Munday, J Peters-Kennedy, M Dunowska. *Veterinary Pathology 2011, 48(6):1190-1194.*

- (2011) **Persistent, widespread papilloma formation on the penis of a horse: a novel presentation of equine papillomavirus type 2 (EcPV-2) infection**. CG Knight, JS Munday, BV Rosa, M Kiupel. *Veterinary Dermatology 2011, 22(6):570-574.*

- (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**. CG Knight, M Dunowska, JS Munday, J Peters-Kennedy, BV Rosa. *Veterinary Microbiology 2013, 166 (1-2): 257–262*

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# Equine Penile Squamous Cell Carcinomas Are Associated With the Presence of Equine Papillomavirus Type 2 DNA Sequences

**C. G. Knight[1], J. S. Munday[1], J. Peters[2], and M. Dunowska[1]**

**Abstract**

Forty cases of equine penile disease were screened with polymerase chain reaction for the presence of papillomaviral DNA. Cases consisted of 20 squamous cell carcinomas (average age of horse, 23.9 years) and 20 non–squamous cell carcinoma diseases (average age of horse, 13.3 years). All horses but one originated from the Northeastern United States. Breeds were not recorded. As based on MY09/MY11 consensus primers, DNA sequences from equine papillomavirus type 2 were amplified from 9 of 20 horses (45%) with penile squamous cell carcinoma and only 1 of 20 horses (5%) with non–squamous cell carcinoma penile disease. Equine papillomavirus type 2 DNA was the only papillomaviral DNA amplified from any of the 40 horses. Tissues from the 10 horses in which papillomaviral DNA was detected by polymerase chain reaction were also screened with in situ hybridization and immunohistochemistry. The presence of papillomavirus was demonstrated in a subset of these by in situ hybridization (6 of 10) and immunohistochemistry (1 of 10). This report describes a possible association between equine penile squamous cell carcinomas and equine papillomavirus type 2. This study is also the first report of equine papillomavirus type 2 infection in North American horses.

**Keywords**

genital, horse, equine papillomavirus type 2, polymerase chain reaction, in situ hybridization, immunohistochemistry, squamous cell carcinoma

Squamous cell carcinomas (SCCs) constitute approximately 18% of all equine cutaneous neoplasms.[23] Of these, 45% are reported to involve the male external genitalia,[22] and SCCs are the most common neoplasm in this location.[24] Equine penile SCCs (PSCCs) commonly recur after treatment, and the long-term prognosis is poor.[5,12] Whereas ultraviolet light is an accepted cause of SCC in sun-exposed skin in some species, ultraviolet overexposure may be a less likely cause of equine PSCCs because of the ventral location of the male genitalia. Although equine PSCCs have been associated with chronic inflammation and smegma accumulation, the role of these factors in neoplasm development is unclear.[8,25]

Approximately 5% of all human cancers and 50% of human PSCCs are caused by papillomavirus (PV) infection.[2,16] The aim of this study is to investigate whether equine PSCCs are also associated with PV infection. The rate of detection of PVs in equine PSCCs is compared with the rate of detection in non-SCC equine penile lesions. If PVs are detected more frequently in PSCC than non-SCC lesions, this will provide evidence of a possible association between equine PSCC and PV infection.

## Materials and Methods

Cases of equine penile disease were located by searching the archives of the Department of Biomedical Sciences, Cornell University, College of Veterinary Medicine, Ithaca, New York. Histologic diagnoses were confirmed for this study by one of the authors (J.P.). Ages were recorded, where available. Standard tests for normality were conducted using SAS 9.1. Differentiation between samples from geldings and stallions was not possible, because this information was not recorded.

For polymerase chain reaction (PCR), DNA was extracted from formalin-fixed paraffin-embedded tissues. Briefly, 10-μm sections were deparaffinized with xylene and then washed twice with ethanol. DNA was extracted with a DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. Two primer sets were used to amplify PV DNA from the samples by PCR. Both primer sets amplify conserved regions of the PV *L1* gene.[6,9] The primer set MY09/MY11 detects mucosal-adapted PVs, whereas

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the primer set FAP59/FAP64 detects cutaneous-adapted PVs.[6,9] Both primer sets have been shown to detect PVs from a variety of animal species.[1] Amplification conditions for both primer sets were as previously published.[14] All PCR reactions were carried out in duplicate. DNA extracted from a bovine cutaneous fibropapilloma was used as a positive control, whereas template DNA was not included in the negative controls. Differences in PV detection rates were analyzed with Fisher exact test on a $\chi^2$ model, as performed with SPSS 15.0.

Amplicons were purified (Qiaex II 150 Gel Extraction Kit, Qiagen GmbH) and subjected to automatic dye-terminator cycle sequencing with BigDye Terminator 3.1 Ready Reaction Cycle Sequencing Kit and with an ABI3730 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA). Results were compared with known sequences from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) using the basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In situ hybridization (ISH) for PV was performed using the Discovery automated slide-processing system (Ventana Medical Systems Inc, Tucson, AZ). The slides received pretreatment through mild cell conditioning using the citrate buffer–based RiboCC reagent (Ventana Medical Systems Inc) and enzyme pretreatment with Protease 3 for 12 minutes (Ventana Medical Systems Inc) as programmed. The sections were then incubated for hybridization with a generic antisense probe for PVs at 200 ng per slide for 1 hour at 37°C after a denaturing step for 4 minutes at 95°C (technique courtesy of Dr Matti Kiupel, of Michigan State University's Department of Pathobiology and Diagnostic Investigation). Three stringency wash steps using 0.5× Ribo-Wash (Ventana Medical Systems Inc; equivalent to 0.5× saline sodium citrate) for 4 minutes at 42°C were followed by incubation of anti-rabbit anti-digoxigenin antibody (Sigma, St Louis, MO) for 32 minutes at 42°C. After streptavidin–alkaline phosphatase conjugate UMap anti-Rb AP (Ventana Medical Systems Inc) incubation for 16 minutes at 42°C, the signal was automatically detected with the BlueMap NBT/BCIP substrate kit (Ventana Medical Systems Inc) for 2 hours at 42°C. Finally, the sections were counterstained with the nuclear fast red–equivalent reagent Red Stain II (Ventana Medical Systems Inc) for 4 minutes before coverslipping.

Immunohistochemical (IHC) labeling was performed with an antibody that detects human PVs 1, 6, 11, 16, 18, and 31 (Lifespan Biosciences, Seattle, WA ). Briefly, IHC staining was performed with an automated staining system (Benchmark, Ventana Medical Systems Inc) that incorporated a commercial detection system (Enhanced V-Red Detection Kit, Ventana Medical Systems Inc). Antigen retrieval was achieved by incubating slides in a high-pH antigen retrieval solution (CC2 Std, Ventana Medical Systems Inc, Tucson, AZ) for 60 minutes. The primary antibody was applied at a concentration of 1:100 for 32 minutes. Sections were counterstained with hematoxylin. Positive control specimens included tissues known to be infected with canine PV. For negative control specimens, the primary antibody was replaced with Tris-buffer saline (Dako, Carpinteria, CA).

## Results

Forty cases of equine penile disease were identified. These included 20 PSCCs and 20 cases of non-SCC penile disease (Table 1). The ages were available for 18 of 20 horses from the PSCC group and 14 of 20 horses from the non-SCC group. The average age of horses in the SCC group (23.9 years) was significantly higher than the average age of horses in the non-SCC group (13.3 years; $P < .001$). Age data for both groups followed a normal distribution. All horses in the study originated from the Northeastern United States with the exception of one, which was from Virginia.

As based on MY09/MY11 primers, PV DNA was amplified from 9 of 20 PSCCs. The PV DNA sequenced from all 9 was 98% to 100% similar to equine PV type 2 (EcPV-2; GenBank accession No. EU503122). The mismatching of small numbers of bases within the sequences is likely the result of errors in the sequencing process rather than evidence of multiple PV types. Based on MY09/MY11 primers, PV DNA was amplified from only 1 of 20 horses with non-SCC penile disease. The PV DNA amplified from this single case, a lesion of chronic ulcerative and granulomatous balanoposthitis, was 99% similar to EcPV-2 (GenBank accession No. EU503122). PV DNA was also amplified from the bovine fibropapilloma control according to MY09/MY11 primers. As based on the FAP59/FAP64 primer set, PV DNA was amplified from only the bovine cutaneous fibropapilloma control, suggesting that these primers have low affinity for EcPV-2.

PV DNA was detected significantly more frequently in lesions of equine PSCC than in lesions of non-SCC penile disease ($P < .05$). Because horses with PSCC in this study were significantly older than those with non-SCC disease, it was considered possible that older horses are more frequently infected by PVs. However, the average age of horses in which PV was detected (22.4 years) was compared with the average age of horses in which PV was not detected (17.9 years), and there was no significant difference ($P = .1470$).

As based on ISH, PV DNA was detected in 6 of the 10 samples in which PV DNA had been detected by PCR. In these cases, PV DNA was detected in the nuclei of scattered neoplastic epithelial cells within lesions but not in adjacent, histologically normal tissue (Fig. 1). As based on IHC, PV antigen was detected in only 1 of the 10 samples in which PV DNA had been detected by PCR. In this case, antigen was detected in the nucleus of scattered neoplastic epithelial cells but not in adjacent, histologically normal tissue (Fig. 2).

## Discussion

In this study PV DNA was detected by PCR significantly more frequently in equine PSCCs than in non-SCC penile diseases. In all 9 cases of PSCC in which PV DNA was detected, sequences from EcPV-2 were amplified. This report describes a possible association between PSCCs and EcPV-2, and is the first report of EcPV-2 infection in North American horses.

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**Table 1.** Study Horses: Ages, Original Diagnoses, and EcPV-2 Amplification

| Horse[a] | Age, Years | Diagnosis |
|---|---|---|
| 1 | 19 | PSCC |
| 2 | 30 | PSCC |
| 3 | 22 | PSCC |
| 4 | 38 | PSCC |
| 5 | 20 | PSCC |
| 6 | 20 | PSCC |
| 7 | 21 | PSCC |
| 8 | 20 | PSCC |
| 9 | 20 | PSCC |
| 10 | 29 | PSCC |
| 11 | 20 | PSCC |
| 12 | 25 | PSCC |
| 13 | 30 | PSCC |
| 14 | 10 | PSCC |
| 15 | 28 | PSCC |
| 16 | Unknown | PSCC |
| 17 | 22 | PSCC |
| 18 | 27 | PSCC |
| 19 | 30 | PSCC |
| 20 | Unknown | PSCC |
| 21 | 14 | Chronic ulcerative and granulomatous balanoposthitis |
| 22 | 4 | Granulation / ulceration |
| 23 | Unknown | Chronic balanitis |
| 24 | 0.0274 (10 days) | Lymphocytic and granulomatous posthitis |
| 25 | Unknown | Inflammatory polyp |
| 26 | 16 | Inflammatory polyp |
| 27 | 17 | Eosinophilic granuloma |
| 28 | Unknown | Eosinophilic granuloma |
| 29 | 13 | Eosinophilic dermatitis with lymphoid hyperplasia |
| 30 | 17 | Ductal apocrine adenoma |
| 31 | 14 | Focal neutrophilic vasculitis |
| 32 | "Old" | Melanoma |
| 33 | 14 | Melanoma |
| 34 | Unknown | Amelanotic melanoma |
| 35 | 25 | Undifferentiated sarcoma / amelanotic melanoma |
| 36 | 18 | Lymphosarcoma |
| 37 | 12 | Lymphosarcoma |
| 38 | 14 | Mastocytoma |
| 39 | "Mature" | Tricholemmoma |
| 40[b] | 8 | Habronemiasis |

EcPV-2 = Equine papillomavirus type 2; PSCC = penile squamous cell carcinoma.
[a] Horse Nos. 1–9 and 21: EcPV-2 DNA amplified.
[b] Horse originating from Virginia. All others originated from the Northeastern United States.

EcPV-2 is distinct from EcPV-1, which causes self-limiting cutaneous papillomas in young horses.[19] EcPV-1 has never been demonstrated in equine genital lesions, even those in which PV was visible immunohistochemically.[15] EcPV-2, the complete genome of which was characterized in 2008 (GenBank accession No. EU503122), is classified as a *Dyoiotapapillomavirus*, the only member of this genus.[3] In contrast, EcPV-1 belongs in the genus *Zetapapillomavirus*, and human PV 16, strongly associated with human penile cancer, is an *Alphapapillomavirus*.[2,7]

In humans, approximately half of PSCCs are associated with PV infection. For certain histologic subtypes, PV DNA can be identified in close to 100% of PSCCs.[2,4,20] In horses, reports of an association between PVs and PSCCs are limited to the present study and a single conference presentation.[21]

The significantly higher rate of detection of EcPV-2 in PSCCs in the current study suggests an association between this PV and equine genital neoplasia. However, given that PVs can asymptomatically infect skin, it is not possible to determine whether the PV is the cause of neoplasia in this study or simply an "innocent bystander."[13] To prove that EcPV-2 causes equine PSCCs, further work is needed to investigate an oncogenic mechanism of the PV. Additionally, determining the incidence of EcPV-2 in horses and the mechanism of transmission may provide additional information on the relationship of this PV to equine PSCCs.

In addition to papillomas and invasive SCCs, a well-recognized lesion on the genitalia of old horses is the "precancerous plaque." Precancerous plaques cannot be grossly distinguished from papillomas or SCCs and, because they frequently progress to invasive SCC, they are considered premalignant.[5,10,12] Histologically, they consist of a dysplastic and hyperplastic epithelium, with or without koilocytes, overlying an intact basement membrane.[26] In a recent study, DNA of an unspecified PV was amplified by PCR from 10 of 10 penile and vulvar plaques.[26] These findings, along with the frequent detection of EcPV-2 in PSCCs in the current study, raise the possibility that the unspecified PV in precancerous plaques may be EcPV-2. In the current study, EcPV-2 DNA was amplified from 1 of 20 lesions of non-SCC disease. It is possible that this lesion, from a 14-year-old horse diagnosed with "chronic ulcerative and granulomatous balanoposthitis," may have represented an early lesion of SCC, suggesting that PCR detection of EcPV-2 is warranted in cases of equine penile disease lacking a definitive diagnosis.

Interestingly, a 2007 study amplified a novel PV DNA sequence from 3 equine genital papillomas.[19] It is possible that the 2007 study actually amplified EcPV-2 DNA but could not classify it, because the EcPV-2 sequence was not added to GenBank until 2008. EcPV-2 sequences have recently been amplified from equine penile papillomas (C. G. Knight et al, unpublished data).

Several nonviral causes of equine PSCCs have been proposed. Because of the reported higher incidence of PSCCs in horses with lightly pigmented genital skin, ultraviolet radiation exposure has been suggested to promote neoplasm development.[5,11] However, darkly pigmented horses also develop PSCCs.[5,11] The accumulation of preputial smegma has been suggested as promoting equine PSCCs. While early studies reported that smegma was carcinogenic,[18] this finding is not universally accepted.[25] A possible role of smegma in development of equine PSCC was supported by the observation that geldings

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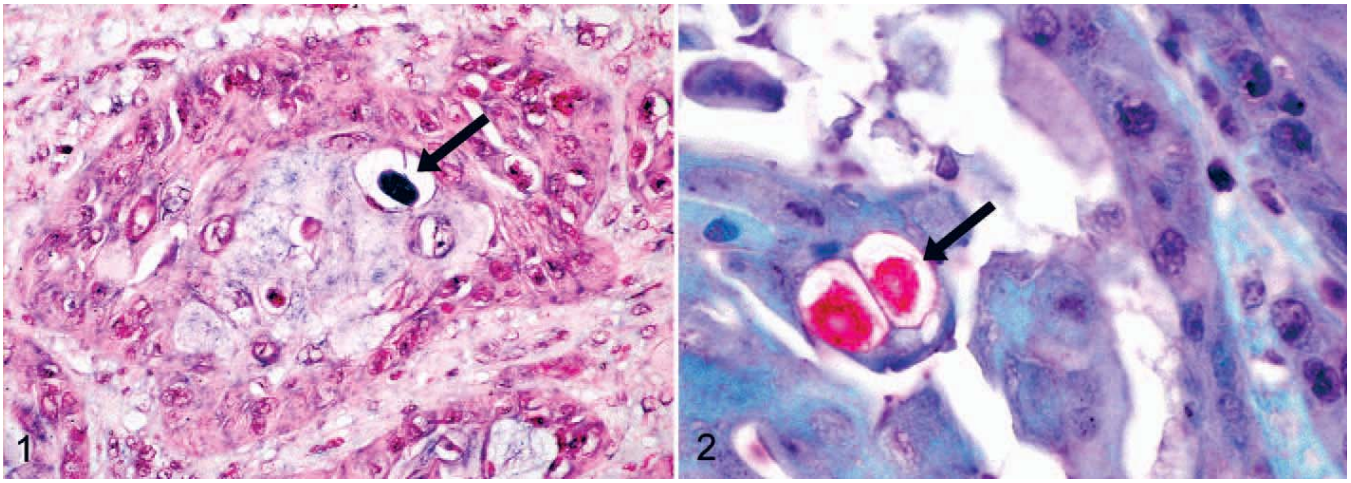


**Figure 1.** Penile squamous cell carcinoma; horse No. 5. In situ hybridization using a generic antisense probe for papillomaviruses shows papillomaviral DNA in the nucleus of a neoplastic epithelial cell (arrow). Counterstained with the nuclear fast red equivalent reagent Red Stain II. **Figure 2.** Penile squamous cell carcinoma; horse No. 5. Immunohistochemistry shows papillomaviral antigen in the nuclei of 2 neoplastic epithelial cells (arrow). Immunohistochemistry for human papillomavirus with hematoxylin counterstain.

develop PSCC more frequently than do stallions, which tend to accumulate less smegma.[5,8] This observation is not supported by all studies.[10,12] In humans, chronic inflammation is associated with PSCC development.[4] Histologically, equine PSCCs often contain infiltrates of chronic inflammatory cells, but these are thought to be a response to the tumor, rather than a cause.[17]

In the current study, tissues from the 10 horses in which PV DNA was detected by PCR were also screened using ISH and IHC. The presence of PV was demonstrated in a subset of these by ISH (6 of 10) and IHC (1 of 10), which suggests that PCR is a more sensitive test for the presence of EcPV-2 than either ISH or IHC; it also suggests that the virus is present in low quantities. In contrast to PCR or ISH, IHC detects structural capsid antigens and demonstrates these when replicating PV is present. Lack of detection of viral antigen by IHC in 9 of 10 horses in which PV DNA was amplified by PCR may indicate that there was no replicating PV or that the amount of antigen was reduced by prolonged formalin fixation.

In conclusion, the current study detected PV DNA by PCR significantly more frequently in equine PSCCs than in non-SCC lesions of the penis. The only PV amplified was EcPV-2. Although this study suggests that EcPV-2 is associated with a proportion of equine PSCCs, additional experiments are required to determine whether EcPV-2 is a cause of neoplasia.

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## Declaration of Conflicting Interests

The authors declared that they had no conflicts of interest with respect to their authorship or the publication of this article.

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Veterinary Dermatology

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# Persistent, widespread papilloma formation on the penis of a horse: a novel presentation of equine papillomavirus type 2 infection

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**Conflict of Interest**
No conflicts of interest have been declared.

## Abstract

**A 9-year-old gelding presented with approximately 100 papillomas that covered about 75% of the distal penis. Biopsy was performed, and histology showed evidence of viral cytopathic change and koilocytosis. Polymerase chain reaction using DNA extracted from biopsied tissue amplified equine papillomavirus type 2 (EcPV-2) DNA sequences. Sixteen months later, the horse was re-examined and the appearance of the papillomas was unchanged. Equine papillomavirus type 2 DNA sequences were again amplified from both biopsied tissue and swabs of the penis. Papillomavirus was localized to the lesions by immunohistochemistry and *in situ* hybridization. An examination 2 years after the initial presentation revealed no detectable change in the appearance of the penis. The large number of papillomas and their failure to regress over an extended period support a clinical classification of papillomatosis. To the authors' knowledge, this is the first report of papillomatosis of the equine penis. This novel clinical manifestation suggests that persistent EcPV-2 infection is possible in horses. As there is evidence that EcPV-2 may promote development of equine penile squamous cell carcinoma, understanding the natural history of EcPV-2 infections may be important in preventing equine penile neoplasia.**

Accepted 8 April 2011

## Introduction

A gelding from New Zealand presented with numerous nonresolving papillomas that covered the majority of the distal penis. Polymerase chain reaction (PCR), immunohistochemistry (IHC) and *in situ* hybridization (ISH) were used to demonstrate equine papillomavirus type 2 (EcPV-2) DNA and antigen in lesions from this horse. While recent studies have demonstrated an association between EcPV-2 and squamous cell carcinomas (SCCs)[1–3] and equine genital papillomas,[2,3] to the authors' knowledge, this clinical manifestation of EcPV-2 infection has not been previously reported. Additionally, this is the first time that repeated sampling has shown that horses can remain persistently infected with EcPV-2. Owing to the extensive involvement of the penis in this case, the term equine penile papillomatosis is proposed.

## Case report

A 9-year-old standardbred cross gelding presented with a several-month history of excessive preputial debris and infrequent protrusion of the penis from the sheath. The horse was sedated routinely and the penis exteriorized. Extending distally from the preputial ring, both the free part of the penis and the glans penis were covered by approximately 100 discrete to confluent, raised, smooth-surfaced, broad-based masses up to 2 cm in diameter (Figure 1). These were largest and most numerous proximally, leaving no normal skin between masses, but were discrete and smaller distally. Ulceration was not present. Multifocally, masses produced hard, keratinized horns up to 1.5 cm in length.

Biopsy of three representative masses on the penis was performed under local anaesthesia. Samples were processed routinely for histological examination. All three masses consisted of well-demarcated areas of epidermal hyperplasia and hyperkeratosis, with retention of orderly keratinocyte maturation and no penetration of the underlying basement membrane (Figure 2a). Keratinocytes of the stratum granulosum frequently contained giant keratohyalin granules and increased amounts of wispy grey-blue cytoplasm, consistent with viral cytopathic change. The nuclei of these cells were pale and vesicular and occasionally contained eosinophilic intranuclear bodies consistent with viral inclusions (Figure 2b). Deeper within the epidermis, numerous keratinocytes had dark, condensed nuclei surrounded by a clear cytoplasmic halo (koilocytosis; Figure 2b).

Polymerase chain reaction was used to amplify papillomavirus (PV) DNA from formalin-fixed, paraffin-embedded penile tissues. Papillomavirus DNA sequences were amplified from all penile samples using the MY09/MY11 consensus primer set, which detects mucosal-adapted PVs in a variety of animal species.[4] No PV DNA sequences

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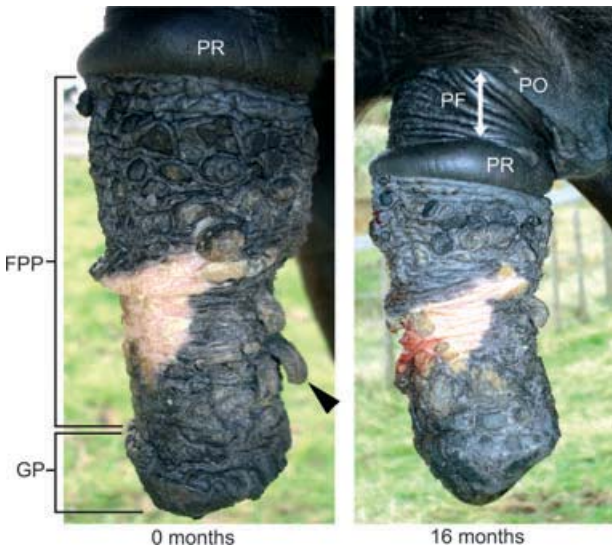


**Figure 1.** Penis of horse with penile papillomatosis. Photographs are taken 16 months apart and demonstrate lack of either ulceration or lesion progression. The haemorrhage in the right-hand photograph is due to biopsy. Multifocally, keratinized horns protrude up to 1.5 cm from the skin surface (arrowhead). Abbreviations: FPP, free part of the penis; GP, glans penis; PF, preputial fold; PO, preputial orifice; and PR, preputial ring.

were amplified from penile biopsy samples using the FAP59/FAP64 consensus primer set, which detects cutaneous-adapted PVs.[5] Both DNA extraction and PCR were performed according to previously published methods.[1,6]

Amplified PV DNA was sequenced and results were compared with known sequences from GenBank.[1] Papillomavirus DNA from the penile biopsy samples was identical to EcPV-2 (GenBank accession number EU50312).

The horse was re-examined 16 months later. The penis was examined as before. No evidence of ulceration was observed, and comparison with photographs taken 16 months earlier showed neither progression nor regression of lesions (Figure 1).

Prior to biopsy, swabs from the surface of the affected penis were collected to assess a less invasive method than biopsy for diagnosing EcPV-2 infection. The tip of a sterile cotton-tipped swab was moistened in sterile saline and drawn back and forth 15 times over an affected area of approximately 5 cm × 5 cm according to a previously published method.[7] The swab tip was then placed into 500 μL of sterile saline in an Eppendorf tube and frozen at –20°C until used. To extract DNA from frozen swabs, the Eppendorf tube containing 500 μL thawed saline and the swab tip was vortexed for 15 s. The swab tip was then discarded. The tube and fluid were centrifuged at 5900 *g* for 3 min and the supernatant was aspirated and discarded. The residual tissue pellet was resuspended in 200 μL of sterile saline. DNA was extracted from the swab suspension using a DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

After swabbing, three representative papillomas were chosen for biopsy. Biopsy samples were then taken in the same way as 16 months earlier, with one exception: prior to each biopsy, scotch tape was used to remove surface debris, as previously described.[7]

Histologically, all three masses were similar to those taken 16 months earlier. Polymerase chain reaction using the MY09/MY11 primer set amplified PV DNA sequences from DNA extracted from biopsy tissues and swabs. The PV DNA from all samples was between 98 and 100% similar to EcPV-2 (GenBank accession number EU50312).

Immunohistochemistry was performed as previously described[1] and demonstrated PV antigen within the nuclei of several hypertrophied keratinocytes, predominantly those of the stratum granulosum (Figure 2c). Papillomaviral DNA was also visualized within hypertrophied keratinocytes using ISH. The PV DNA appeared confined to the enlarged cells and was not detected within keratinocytes in the deeper layers of the epidermis (Figure 2d). Papillomaviral ISH was performed by Matti Kiupel.

Examination of the horse 8 months after the second set of samples was taken did not reveal regression or progression of penile papillomas.

## Discussion

In the horse described in this report, numerous papillomas covered more than three-quarters of the free part of the penis and glans. This was considered consistent with the clinical classification of penile papillomatosis.[8] To the authors' knowledge, genital papillomatosis has not been described previously in horses. Equine papillomavirus type 2 DNA was consistently amplified from penile biopsy samples at different time points. This is the first time that EcPV-2 has been detected within repeated samples from the same horse taken over an extended time period, suggesting that this PV is able to infect some horses persistently.

Equine papillomavirus type 2 DNA has previously been associated with equine genital papillomas;[2,3] however, the horse described in this report appears to have developed a novel clinical manifestation of EcPV-2 infection. The large number and lack of either resolution or progression of lesions over more than 2 years is unusual. Equine genital papillomas are typically described as solitary and progressive,[9,10] although one text mentions that some remain static for many years.[9]

In the present case, IHC and ISH were used to demonstrate PV antigen and DNA, respectively, within the nuclei of epithelial cells in papillomas but not in adjacent, histologically normal epithelium. The localization of PV antigen and DNA to the lesion is important, as EcPV-2 DNA has previously been amplified by PCR from samples of clinically normal equine epithelium,[3] suggesting that asymptomatic infection by this virus is possible. To the authors' knowledge, ISH has not previously been used to localize the presence of PV DNA within an equine papilloma.

Equine papillomavirus type 2 is classified as a Dyoiotapapillomavirus, the only member of this genus.[11] Little is known about the transmission, prevalence or behaviour of EcPV-2. It is unknown why some EcPV-2 infections remain asymptomatic, while others are associated with the development of genital lesions.

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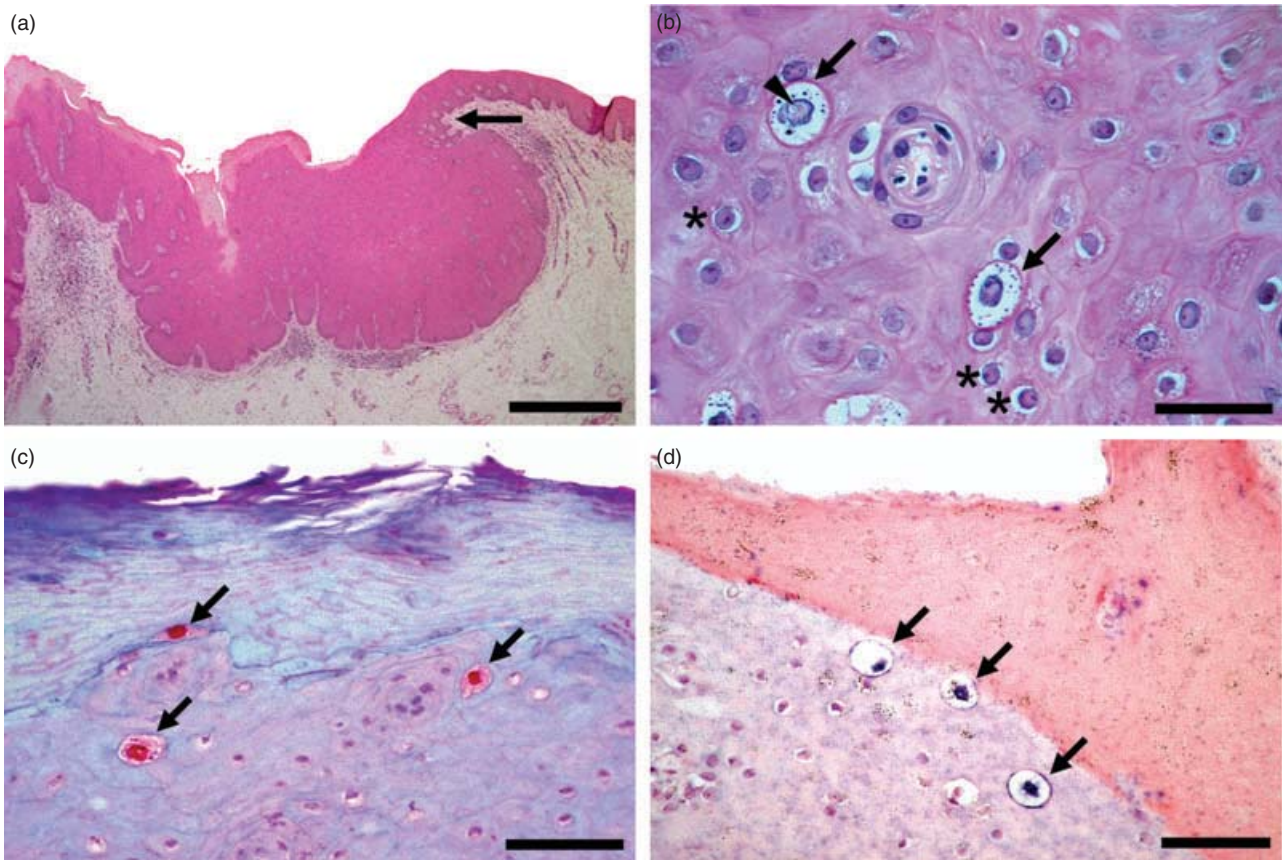


**Figure 2.** (a) Photomicrograph of equine penile papilloma. Well-demarcated focus of epidermal hyperplasia. The junction of normal and hyperplastic epidermis is sharply defined (arrow). The basement membrane is intact, and keratinocyte maturation is orderly. Haematoxylin & eosin. Scale bar represents 200 μm. (b) Photomicrograph of equine penile papilloma. Keratinocytes within the stratum spinosum and stratum granulosum contain evidence of viral effects, including expanded, pale cytoplasm with giant keratohyalin granules (arrows), koilocytosis (asterisks) and occasional intranuclear inclusions (arrowhead). Haematoxylin & eosin. Scale bar represents 30 μm. (c) Photomicrograph of equine penile papilloma. Immunohistochemistry demonstrates papillomaviral antigen within the nuclei of several hypertrophic keratinocytes (arrows). Mouse monoclonal antibody raised against sodium dodecyl sulfate-disrupted bovine PV type 1 that detects multiple papillomavirus types. Haematoxylin counterstain. Scale bar represents 60 μm. (d) Photomicrograph of equine penile papilloma. *In situ* hybridization demonstrates papillomaviral DNA within the nuclei of several hypertrophic keratinocytes (arrows). Hybridization with a generic antisense probe for papillomaviruses. Red Stain II counterstain. Scale bar represents 60 μm.

Evidence suggests that PVs may be a significant cause of SCCs in humans and domestic mammals.[12] Equine genital papillomas have been reported to progress to SCCs,[10,13] and EcPV-2 has recently been associated with the development of equine penile SCCs.[1–3] In the present case, despite persistent PV infection and the presence of numerous papillomas, there was no evidence of progression to SCC within a 2 year period. This suggests that factors other than PV infection and the presence of papillomas are needed for genital SCC development in horses. This may be important when considering treatment options for nonresolving equine genital papillomas.

The proximal extent of the papillomatosis in the present horse was sharply demarcated by the preputial ring. The preputial ring is unique to equids and is distinct from the preputial orifice, which is present in all male domestic mammals (Figure 3). In the retracted equine penis, the skin proximal to the preputial ring is in constant, intimate contact with the skin distal to the ring and, presumably, each is exposed to any infectious agent carried by the other. Therefore, the restriction of the papillomatosis to the skin distal to the ring suggests that EcPV-2 has a specific tropism for the free part and glans of the penis. It also suggests that skin proximal to the preputial ring may have different properties to more distal skin of the penis, despite there being no gross or histological difference. On a practical level, it suggests that swabbing the preputial orifice of a retracted equine penis may be of limited value in the diagnosis of EcPV-2 infection, and that the glans and free part of the penis may need to be exteriorized for swabbing if false negatives are to be avoided.

The differential diagnosis for proliferative diseases of the equine penis resembling papillomatosis includes SCC, fibropapilloma and sarcoids. These diseases were ruled out by gross and histological appearance and by the absence of disease progression. Squamous cell carcinoma would be expected to progress and become invasive over a 2-year period, causing ulceration and desmoplastic thickening of the penis.[14] These were not present in the present case, and lesions remained static. Neither fibropapillomas of the penis, which may grossly resemble the present case,[15] nor sarcoids have histological features consistent with those of the present horse. In addition, the presence of PV was demonstrated by IHC and

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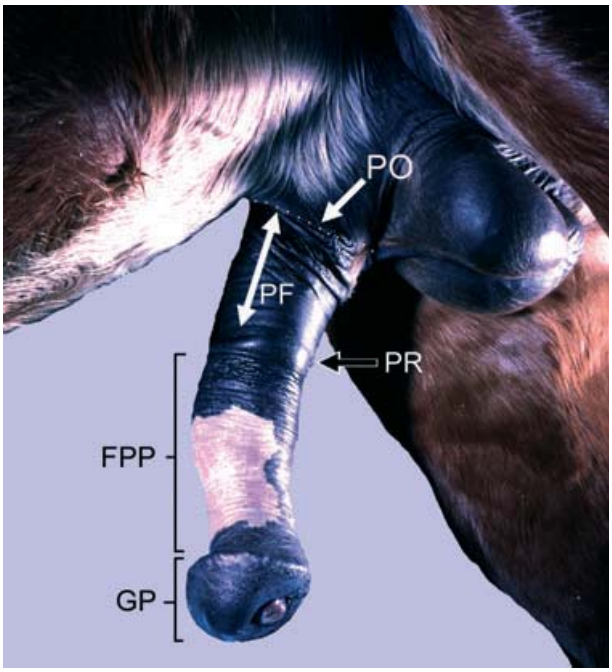


**Figure 3.** Anatomy of the normal equine penis for comparison with Figure 1. Note that the preputial ring is the junction between the preputial fold and the free part of the penis. Abbreviations: FPP, free part of the penis; GP, glans penis; PF, preputial fold; PO, preputial orifice; and PR, preputial ring. Image courtesy of Cornell University College of Veterinary Medicine.

ISH in the epithelium in the present case, whereas PVs are typically located in subepithelial fibroblasts in sarcoids.

In conclusion, this report describes a case of penile papillomatosis in a gelding. Unusual features include the extent and sharply demarcated anatomical distribution of lesions, the lack of progression or regression of lesions, and the consistent amplification of EcPV-2 DNA sequences from biopsy samples and swabs over a 16 month period. A PV aetiology was supported by visualization of PV antigen and DNA within lesions, but not in surrounding skin. Therefore, penile papillomatosis appears to represent a new clinical manifestation in the disease spectrum associated with EcPV-2 infection. The results suggest that persistent infection with EcPV-2 is possible. While EcPV-2 has been associated with the development of equine penile SCCs, observations from the present case suggest that factors in addition to the presence of papillomas and EcPV-2 are needed for neoplastic transformation. As more is learned about this virus, and its role in equine genital lesions becomes clearer, improvements in therapy or prognosis may become possible.

## Acknowledgements

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**Résumé** Un hongre de 9 ans est présenté avec approximativement 100 papillomes qui couvrent près de 75% de l'extrémité distale du pénis. Des biopsies ont été réalisées et l'examen histopathologique a révélé la présence de modifications cytopathiques virales et de la koïlocytose. Une PCR sur ADN tissulaire des biopsies a permis l'amplification de séquences d'ADN du papillomavirus équin de type 2 (EcPV-2). Seize mois plus tard, le cheval était réexaminé et l'apparence des papillomes était inchangée. Les séquences d'ADN de EcPV-2 ont été de nouveau amplifiées à partir des deux biopsies tissulaires et des écouvillons péniens. Un examen immunohistochimique et une hybridation *in situ* ont localisé le papillomavirus dans les lésions. Un examen deux ans après la présentation initiale n'a révélé aucun changement dans l'apparence du pénis. Le grand nombre de papillomes et l'absence de régression sur une longue période supporte la

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classification clinique de papillomatose. A la connaissance des auteurs, ceci est le premier rapport de papillomatose pénienne chez le cheval. Cette nouvelle manifestation clinique suggère qu'une infection à EcPV-2 persistante est possible chez le cheval. Il existe des éléments pour prouver que le EcPV-2 peut entrainer le développement de carcinomes épidermoïdes péniens chez le cheval. Ainsi, comprendre l'évolution naturelle des infections à EcPV-2 pourrait être important dans la prévention des tumeurs péniennes du cheval.

**Resumen** Un semental de 9 años de edad se presentó con aproximadamente 100 papilomas que cubrían alrededor del 75% del pene distal. Se obtuvo una biopsia y la histología demostró coilocitosis y otros cambios asociados con infección viral. La reacción de PCR utilizando DNA extraído de tejido lesional amplificó secuencias del virus papiloma equino tipo 2 ( EcPV-2). Dieciséis meses después el caballo fue reexaminado y el aspecto de los papilomas permaneció sin cambios. Las secuencias de DNA EcPV-2 fueron nuevamente amplificadas en el tejido de biopsia y de hisopos del pene. Se localizó papilomavirus en las lesiones mediante inmunohistoquímica e hibridación *in situ*. Un examen 2 años después de la presentación inicial no reveló ningún cambio detectable en el aspecto del pene. El número tan grande de papilomas y la ausencia de regresión espontanea en un período tan largo de tiempo sugiere una denominación clinica de papilomatosis. A nuestro entender este es el primer informe del papilomatosis del pene equino. Esta novedosa manifestación clínica sugiere que la infección persistente con EcPV-2 es posible en caballos. Como hay evidencia de que EcPV-2 puede promover el desarrollo de carcinoma de escamosas del pene en equinos, entender la historia natural de las infecciones por EcPV-2 puede ser importante en la prevención de neoplasia del pene en equinos.

**Zusammenfassung** Ein 9-Jahre alter Wallach wurde mit ungefähr 100 Papillomen, die etwa 75% des distalen Penis bedeckten, vorgestellt. Eine Biopsie wurde durchgeführt und die histologische Untersuchung zeigte das Auftreten einer zytopathischen Veränderung sowie Koilozytose. Eine Polymerasekettenreaktion mit DNA aus biopsiertem Gewebe amplifizierte DNA Sequenzen des equinen Papillomavirus Typ 2 (EcPV-2). Sechzehn Monate später wurde das Pferd wieder untersucht und das Erscheinungsbild der Papillome war unverändert. EcPV-2 DNA Sequenzen wurden wiederum aus Biopsien wie auch aus Tupfern, die vom Penis entnommen worden waren, amplifiziert. Papillomavirus wurde in den Lokalisationen mittels Immunhistochemie und *in situ* Hybridisierung nachgewiesen. Zwei Jahre nach der Erstpräsentation zeigte eine neuerliche Untersuchung keine erkennbare Veränderung im Aussehen des Penis. Die große Anzahl an Papillomen und die Tatsache, dass sie sich über einen langen Zeitraum nicht zurückbildeten, bekräftigt die klinische Klassifizierung als Papillomatose. Nach dem besten Wissen der Autoren handelt es sich hierbei um den ersten Bericht einer Papillomatose des Pferdepenis. Diese neue klinische Manifestation weist auf die Möglichkeit persistierender EcPV-2 Infektionen bei Pferden hin. Da es Beweise gibt, dass EcPV-2 die Entstehung von Plattenepithelkarzinomen am Penis des Pferdes begünstigt, könnte das Wissen über den natürlichen Krankheitsverlauf von EcPV-2 Infektionen bei der Verhinderung von Neoplasien am Penis des Pferdes wichtig sein.

**要約** 9 歳齢の去勢馬が約 100 個の乳頭腫が陰茎の遠位約 75%を覆っているという主訴で来院した。生検を行い組織学的にウイルス性細胞変性変化と空胞細胞症の証拠が見られた。生検した組織から抽出した DNA を使用したポリメラーゼ連鎖反応で 2 型ウマパピローマウイルス（EcPV-2）の DNA 配列が増幅された。6 カ月後、馬を再検査したが、パピローマの存在には変化がなかった。再度、生検組織と陰茎のぬぐい液から EcPV-2DNA 配列を増幅した。免疫組織化学染色と *in situ* ハイブリダイゼーションではパピローマウイルスは病変部に限局していた。初診から 2 年後の検査で陰茎の外観には特に変化がないことが明らかになった。乳頭腫の数の多いことと長期間にわたって退行しない病変であることは乳頭腫症の臨床分類を支持している。筆者の知る限りでは、これは馬の陰茎の乳頭腫症の最初の報告である。EcPV-2 が馬のペニスの扁平上皮癌の発生を促進するかもしれないという報告があるために、EcPV-2 感染の自然経過はおそらく馬の陰茎の腫瘍の予防において重要である。

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Short communication

# 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

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ABSTRACT

*Equus caballus* papillomavirus type 2 (EcPV-2) infection has been associated with equine genital squamous cell carcinomas (SCCs). However, quantitative PCR (qPCR) has not been performed to determine viral copy numbers within these lesions. Additionally, the frequency with which EcPV-2 can be detected in other common sites of equine SCC development remains uncertain. The aim of this study was to develop a qPCR assay to estimate the viral load in a variety of equine tissue samples. These included 40 SCC lesions, 19 penile non-SCC or precursor disease lesions, and 222 tissues without observable lesions from SCC-prone sites on clinically normal horses. EcPV-2 DNA was present significantly more frequently, and in higher copy numbers, in equine penile SCC lesions than in either healthy penile mucosa or non-SCC penile lesions. This supports the hypothesis that EcPV-2 is involved in development of penile SCCs and suggests that penile EcPV-2 infection is rare in the absence of SCCs. Samples of normal vulval mucosa rarely contained EcPV-2 DNA and none of the nictitating membrane samples contained EcPV-2 DNA, indicating that asymptomatic EcPV-2 infection is uncommon at these sites. EcPV-2 DNA was detected in a proportion of both SCCs and normal samples from the oral cavity or pharynx, although there were no significant differences in the rate of infection or viral copy number between the SCCs and the normal mucosal samples. As such, the role of EcPV-2 in development of SCCs in this location remains to be established

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## 1. Introduction

Squamous cell carcinomas (SCCs) are the most common neoplasm of the equine penis (van den Top et al., 2008). Penile SCCs may recur after treatment and the long-term prognosis can be poor (Howarth et al., 1991). Although equine penile SCCs have been previously associated with ultraviolet light exposure, chronic inflammation and smegma accumulation, the role of these factors in neoplasm development remains unclear (Elce, 2009).

An association between *Equus caballus* papillomavirus type 2 (EcPV-2) infection and equine penile SCCs has been recently suggested by several authors based on detection of EcPV-2 in SCC tissues (Bogaert et al., 2012; Knight et al., 2011; Lange et al., 2012; Scase et al., 2010). Two of these studies, in addition, investigated the presence of EcPV-2 DNA in tissues from penile lesions other than SCC or its precursor lesions (Knight et al., 2011; Lange et al., 2012). To date, however, there have been just two investigations into

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the prevalence of EcPV-2 infection in clinically normal horses (Bogaert et al., 2012; Sykora et al., 2012). Furthermore, all studies to date have used conventional PCR to detect EcPV-2. As this does not allow quantification of PV DNA, it is currently unknown whether SCCs and non-SCC tissues contain different copy numbers of EcPV-2.

The first aim of this study was to screen a large number of penile samples to compare the EcPV-2 infection rates and levels of EcPV-2 DNA in clinically normal and diseased horses in order to further investigate a causative association between EcPV-2 infection and development of penile SCCs. The second aim of this study was to screen a large number of normal and neoplastic equine oral or pharyngeal tissues for EcPV-2 DNA. In humans, up to one third of oropharyngeal SCCs are thought to be caused by PV infection (Kreimer et al., 2005). The final aim of this study was to screen a large number of vulval mucosal samples and nictitating membranes from healthy horses for EcPV-2. Horses frequently develop SCCs of the vulva and eye or its adnexa. Although these SCCs are thought to be associated with ultraviolet (UV) light exposure (Giuliano, 2010; McCue, 1998), a non-EcPV-2 PV has been detected in a proportion of ocular SCCs (Kim et al., 2009). In the current study, qPCR was used to determine the levels of EcPV-2 DNA in a series of clinically normal vulval mucosal samples and nictitating membranes.

## 2. Materials and methods

### 2.1. Samples

Samples for this study were collected from two sources. Firstly, 222 fresh tissue samples were collected using sterile technique from 75 clinically normal horses that were slaughtered on one day at an abattoir in southern New Zealand. Samples were collected as follows: penile mucosa ($n = 32$) was sampled at the junction of the glans penis and the free part of the penis; vulval mucosa ($n = 40$) was sampled from the vulvovestibular floor lateral to the clitoral fossa; pharyngeal mucosa ($n = 75$) was sampled at the aryepiglottic fold; nictitating membranes ($n = 75$) were exteriorized and excised. Samples were placed into individual, labeled plastic bags on ice. Bags were shipped to the laboratory overnight on ice and transferred to −80 °C freezer for storage before DNA extraction. The ages of horses were estimated by examining eruption and wear of incisor teeth according to a previously published method (Davis, 1988). The age of one horse could not be estimated. Of the 74 horses whose ages were estimated, 32 were less than 5 years of age, 16 were 6–10 years old, 14 were 11–15 years old, 10 were 16–20 years old and 2 were over 21 years old.

Secondly, 60 formalin-fixed, paraffin-embedded (FFPE) tissue samples from diseased horses were obtained by searching the archives of the Department of Biomedical Sciences at the College of Veterinary Medicine, Cornell University, Ithaca, NY, USA. The following samples were collected: penile SCCs ($n = 20$), oral or pharyngeal SCCs ($n = 20$), and penile non-SCC lesions ($n = 20$). Oral or pharyngeal SCC samples did not include any samples from anatomic locations that could have been exposed to UV light (i.e., haired skin and mucocutaneous junctions were excluded). Penile non-SCC lesions included 4 samples of granulomatous balanoposthitis, 4 melanomas, 3 eosinophilic granulomas, 2 inflammatory polyps, 2 lymphomas, and single samples of habronemiasis, vasculitis, mast cell tumor, tricholemmoma, and apocrine adenoma. Penile non-SCC lesions did not include sarcoids or precursor lesions of SCC, such as papillomas and precancerous plaques. The age of each horse was determined from patient records. The horses with penile SCCs had a mean age of 23.9 years, while the horses with oral or pharyngeal SCCs had a mean age of 19.2 years. The mean age of horses with non-SCC penile lesions was 13.3 years.

### 2.2. DNA extraction and quantification

Total DNA was extracted from fresh and FFPE tissues using a commercially available extraction kit according to the manufacturer's instructions (Roche High Pure PCR Template Preparation Kit). For fresh tissues the "Isolation of Nucleic Acids from Mammalian Tissues" protocol was followed and 1 $mm^3$ of tissue used. For FFPE tissues the "Isolation of Nucleic Acids from Formalin-Fixed Paraffin-Embedded Tissues" protocol was followed and two 10 μm thick block sections used. The quantity and quality of extracted DNA were assessed using a Nanodrop spectrophotometer (Thermo Scientific). The presence of amplifiable DNA was confirmed in each sample by qPCR with equine beta actin primers, using a previously described method (Bogaert et al., 2006). Samples testing negative for beta actin were excluded from the analysis.

### 2.3. Generation of template for standard curve experiments

Forward MD.L1(B).F (GCGGTGTCGAGGTGTCACGG) and reverse MD.L1(A).R (AGCGATCCACTTGGCGTGGC) primers were designed to amplify a larger (591 bp) fragment of the EcPV-2 L1 gene containing the sequence targeted by qPCR. The sample from which this 591 bp fragment was amplified was an equine SCC lesion shown previously to be EcPV-2 positive. The PCR was performed using HOT FIREPol Blend Master Mix (Solis BioDyne), a final concentration of 0.4 μM of each primer and 0.5 μL of template DNA in a total volume of 10 μL. The cycling conditions consisted of a 10 min denaturation step followed by 40 cycles of denaturation (95 °C for 20 s), annealing (65 °C for 20 s) and elongation (72 °C for 60 s), with a final extension step (72 °C for 10 min). The identity of the PCR products was confirmed by sequencing of a gel-purified (Qiaex II 150 Gel Extraction Kit, Qiagen) amplicon. A stock solution containing $10^9$ copies/μL of the gel-purified amplicon was prepared, which was used for all subsequent standard curve experiments.

### 2.4. Quantitative PCR assay

Quantitative PCR assays were run using an Eco real time instrument (Illumina Inc., San Diego, CA, USA). The qPCR was designed to amplify a 118 bp fragment of the EcPV-2 L1 gene. Each qPCR reaction was performed using

AccuMelt™ HRM SuperMix (Quanta Biosciences, Gaithersburg, Maryland, USA), a final concentration of 0.5 μM of each primer (MD.L1(B).F: GCGGTGTCGAGGTGTCACGG and MD.L1(B).R): CGCTCCCCTGCAGCTTCCTG) and 2 μL of template DNA in a total volume of 10 μL. The cycling conditions consisted of a 15 min denaturation step followed by 45 cycles of denaturation (95 °C for 2 s), annealing (65 °C for 5 s) and elongation (72 °C for 25 s). The cycling was followed by a melting step from 55 °C to 95 °C. The viral copies per μL of template DNA were calculated from quantification cycle (Cq) values using either Eco (single run) or EcoStudy (multiple runs) software (Illumina Inc.) based on the standard curve generated from serial dilutions ($10^8$–$10^0$ copies/μL) of a stock solution containing a known concentration of a 591 bp fragment of the EcPV-2 L1 gene. Values lower than 1 copy/μL were extrapolated. Automatic threshold detection settings were used. Samples were considered positive if the amplification curve crossed the threshold, and there was a corresponding melting peak between 85.3 °C and 85.6 °C. The copy number values were then normalized to the amount of template DNA added to each reaction volume and expressed as viral copies per nanogram of template DNA.

All standards and samples were run in duplicate, unless specified otherwise. A non-template control (water) was included in each run. For analysis of samples from multiple runs, the master standard curve was used within the EcoStudy software. One standard ($10^6$ copies/μL) was designated as a plate control and included on each contributing plate. Plates that showed a Cq variation between a master standard and a control standard of ≤0.5 were considered valid.

### 2.5. Quantitative PCR assay validation

Validation was performed by determination of the analytical sensitivity, linearity, precision and reproducibility of the assay. The sensitivity of the assay was determined by testing serial dilutions of a stock solution containing a known concentration of a 591 bp sequence of the EcPV-2 L1 gene. Linearity was assessed based on the analysis of the standard curves (calculated reaction efficiency and $R^2$ values) generated from the same standards. Precision was evaluated by calculating intra-assay variability based on distribution of Cq values for 5 replicates of each standard in a single PCR run. Reproducibility was evaluated by calculating inter-assay variability by comparison of the Cq values obtained for the same standards in 3 separate PCR runs. The intra- and inter-assay variability was expressed as coefficient of variation (CV), which was calculated by dividing the Cq value of each replicate of the standard by the mean Cq value for that standard.

### 2.6. Statistical analysis

Data were analyzed in SAS 9.2. Fisher's exact test was used to compare the frequency of detection of EcPV-2 in tissues with and without SCC. Differences in mean viral load were compared using the Wilcoxon–Mann–Whitney test. Differences were considered statistically significant if $p < 0.05$.

## 3. Results and discussion

### 3.1. Performance of the qPCR assay

The qPCR assay was able to detect the equivalent of 1 copy of EcPV-2 genomic DNA, indicating good analytical sensitivity. A single melting peak between 85.3 °C and 85.6 °C was observed in all runs (Fig. 1). The assay was linear within the tested range from 1 to $10^8$ target copies/μL, with an efficiency of 99.6% and $R^2$ of 0.994, calculated based on 3 separate runs. The intra-assay CV values ranged from 0.4% to 3.3%, indicating high precision of the assay. The inter-assay CV values ranged from 0.4% to 2.9%, indicating high reproducibility.

This study describes a sensitive qPCR assay for the detection and quantification of EcPV-2 DNA in tissues. To the authors' knowledge this is the first quantitative, rather than qualitative, description of EcPV-2 in neoplastic and non-neoplastic tissues from horses. Quantitative PCR was considered superior to conventional PCR in this study for

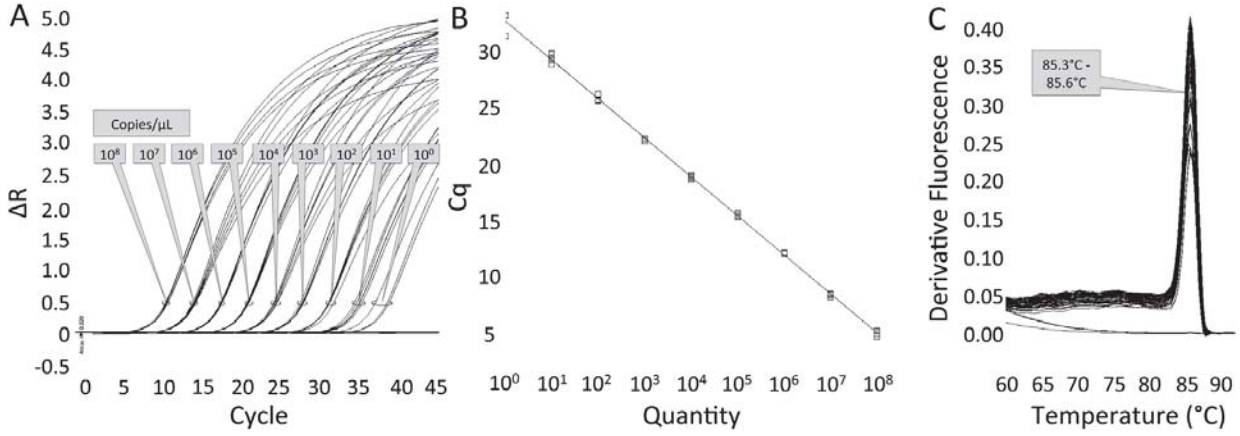


**Fig. 1.** Amplification curves (A), the corresponding standard curve (B) and melting peaks obtained (C) in a run to determine intra-assay variability of a qPCR test to detect EcPV-2. Standards consisted of a serially diluted stock solution containing a known concentration of a 591 bp sequence of the EcPV-2 L1 gene from an equine SCC lesion shown previously to be EcPV-2 positive. The assay shown in this figure had an efficiency of 94.45% and an $R^2$ of 0.999.

two reasons. Firstly, because viral DNA load may be regarded as a surrogate marker of viral replication (Weissenborn et al., 2010), qPCR provides an indication of the biologic behavior of EcPV-2 within a lesion. Secondly, contamination of samples can be problematic when using highly sensitive PCR assays. Such contamination is likely to result in consistent, low levels of viral DNA present within the samples. While conventional PCR will record all such samples as positive, the use of qPCR allows the amount of PV DNA within each group to be compared.

### 3.2. Testing of the samples

The results of EcPV-2 qPCR are shown in Table 1. One sample, a penile non-SCC lesion, tested negative by beta-actin qPCR and was excluded from the analysis. Overall, EcPV-2 DNA sequences were detected in 27 of 281 (9.6%) samples including 19 of 40 (47.5%) SCC lesions, 3 of 19 (15.8%) non-SCC lesions and 5 of 222 (2.3%) mucosal samples taken from clinically normal horses. The copy number of EcPV-2 ranged from 0.01 to 308.01 per ng template DNA. The mean viral load within the 19 SCCs that contained EcPV-2 DNA was 49.18 copies/ng template DNA. This was significantly higher than the mean viral load detected in the 8 EcPV-2-positive non-SCC samples (2.29 copies/ng template DNA; $p = 0.0006$).

EcPV-2 DNA sequences were amplified from 21 of 71 (29.6%) penile samples. Sequences were detected significantly more frequently in penile SCCs than in penile tissues without SCC ($p < 0.0001$). EcPV-2 DNA was not detected significantly more frequently in penile non-SCC disease lesions than in clinically normal penile mucosa ($p = 0.36$). This is in agreement with two earlier studies, in which EcPV-2 DNA sequences were detected by PCR significantly more frequently in penile SCC lesions than in non-SCC lesions (Knight et al., 2011; Lange et al., 2012). A novel finding in the current study was the viral load was significantly higher (mean 55.79 copies/ng template DNA) in penile SCC lesions than in the 5 samples of normal or non-SCC penile lesions that contained EcPV-2 DNA (mean 3.66 copies/ng template DNA; $p = 0.01$). While this does not prove that EcPV-2 causes SCC, it is one of the criteria currently considered necessary for establishing microbial disease causation (Fredericks and Relman, 1996), and provides additional support for a link between EcPV-2 infection and development of equine penile SCCs.

When penile SCCs were excluded, there was no significant difference in viral copy number between EcPV-2-positive diseased penises and EcPV-2-positive clinically normal penises. This suggests that equine penile diseases other than SCC are not associated with an increased EcPV-2 load. Since many non-SCC penile diseases result in increased epithelial cell replication due to ulceration and repair, this suggests that the higher viral loads seen in SCCs are not simply a result of increased epithelial cell division, but that EcPV-2 has a different biologic behavior in SCCs.

Up to one third of human oropharyngeal cancers are associated with human PV infection (Kreimer et al., 2005). To date, no primary equine oral or pharyngeal SCCs have been investigated for the presence of PV DNA, although EcPV-2 DNA has been reported in two cases of SCC that metastasized to the mouth or pharynx (Bogaert et al., 2012; Kainzbauer et al., 2012). In the current study, EcPV-2 was detected within 5 of 95 samples of oral or pharyngeal mucosa, confirming that the virus can infect these epithelial surfaces. However, EcPV-2 was not detected significantly more frequently in SCCs from the oral cavity or pharynx (3/20 samples) than in non-SCC controls (2/75 samples). In addition, the mean viral load within the 3 SCCs (13.92 EcPV-2 copies/ng template DNA) was not significantly higher than the viral load detected in the normal pharyngeal mucosal samples (0.02 EcPV-2 copies/ng template DNA; $p = 0.2$). These data, therefore, do not support the hypothesis of a causative link between EcPV-2 infection and development of SCC in oral and pharyngeal regions. However, our results are based on a very small sample size. Hence, a potential role of EcPV-2 in development of oral or pharyngeal SCC cannot be fully excluded and needs to be further investigated.

To the authors' knowledge this study is the first to investigate EcPV-2 infection rates in tissues from a large number of healthy horses. Overall, EcPV-2 was detected in only 5 of 222 tissue samples from 75 clinically healthy horses, suggesting that EcPV-2 only rarely infects horses. These results are similar to those of an earlier study, in which EcPV-2 DNA was detected in just 5 of 124 swabs from the genitalia and conjunctiva of healthy horses

**Table 1**
Summary of results of qPCR evaluation of equine tissues for the presence of *Equus caballus* papillomavirus type 2 (EcPV-2) DNA sequences.

| Tissue | Total samples | Samples containing EcPV-2 DNA (%) | Mean EcPV-2 copy number/ng template DNA (range) |
|---|---|---|---|
| Penis | | | |
| Non-lesional mucosa | 32 | 2 (6.3) | 7.40 (0.32–14.65) |
| Non-SCC lesion[a] | 19 | 3 (15.8) | 1.10 (0.03–3.17) |
| SCC[a] | 20 | 16 (80.0)[b] | 55.79 (0.55–308.01)[b] |
| Oral cavity or pharynx | | | |
| Non-lesional mucosa | 75 | 2 (2.7) | 0.02 (0.01–0.02) |
| SCC[a] | 20 | 3 (15.0) | 13.92 (1.51–24.08) |
| Vulva | | | |
| Non-lesional mucosa | 40 | 1 (2.5) | 0.006 |
| Nictitating membrane | | | |
| Non-lesional mucosa | 75 | 0 (0) | NA |

[a] DNA was extracted from formalin-fixed paraffin-embedded tissues.
[b] Penile SCCs contained EcPV-2 DNA significantly more frequently and at significantly higher copy numbers than non-SCC penile samples ($p < 0.05$).

(Sykora et al., 2012). In other species, many PVs are ubiquitous and detectable in a high proportion of clinically normal animals, making it difficult to determine their role in disease development (Lange et al., 2011; Munday and Witham, 2010). The infrequent detection of EcPV-2 in healthy horses in this study, in conjunction with its frequent detection in SCCs, suggests that EcPV-2 is a pathogenic virus rather than one of the many types of commensal PVs that infect human and animal epithelia (Antonsson and Hansson, 2002). It should be noted, however, that the clinically normal horses sampled in this study were from a restricted geographic location (the South Island of New Zealand) and many of these horses were younger than horses that typically develop SCCs.

In humans, vulval SCCs are also associated with PV infection (Fox and Wells, 2003). In horses, a similar association is plausible, given the association between equine penile SCC and EcPV-2 infection. To date, the number of equine vulvovaginal SCCs or precursor lesions studied is small, but EcPV-2 DNA was detected in 4 of 5 vulval SCCs in one study (Scase et al., 2010), and in 2 of 2 vulvovaginal neoplasms (one SCC and one vaginal intraepithelial neoplasia) in another (Bogaert et al., 2012). In the current study, very low levels of EcPV-2 DNA were detected in only 1 of 40 samples of vulval/vestibular mucosa from healthy mares, indicating that this virus is not a common, commensal inhabitant of healthy female genitalia. This is in agreement with two earlier studies that reported EcPV-2 DNA in 2 of 51 vulval swabs (Sykora et al., 2012) and in 0 of 20 vulval swabs (Bogaert et al., 2012) from normal mares. Since EcPV-2 infection is uncommon in the vulva of normal mares, its detection in vulval SCCs would make a causative association more likely.

SCCs of the eye and its adnexa are well recognized in horses and believed to be associated with ultraviolet light exposure (Giuliano, 2010). While a novel, non-EcPV-2 PV was detected in these tumors (Kim et al., 2009), there is little evidence to date that EcPV-2 is involved in development of SCCs in this location. EcPV-2 has only been reported once in an equine ocular SCC (Kainzbauer et al., 2012; Sykora et al., 2012). Another study reported detection of EcPV-2 in 0 of 12 ocular SCCs (Scase et al., 2010). It should be noted that EcPV-2 DNA sequences were initially reported in 9 of 9 equine ocular SCCs in another study, but this was later retracted (Vanderstraeten et al., 2011). In the current study EcPV-2 DNA was not detected in any of 75 nictitating membranes from clinically normal horses, suggesting that infection by EcPV-2 is rare at this location. This is in agreement with another study, in which EcPV-2 DNA was detected in just 1 of 30 ocular swabs from healthy horses (Sykora et al., 2012). The role, if any, of EcPV-2 in SCC of the nictitating membrane should be further investigated using a large number of SCC lesions from this site.

## 4. Conclusions

In conclusion, the results of this study showed that EcPV-2 was present significantly more frequently, and in higher copy numbers, in equine penile SCC lesions than in either healthy penile mucosa or in non-SCC penile lesions. Healthy penile mucosa, or mucosa from penile tissues affected by diseases other than SCC, rarely harbored EcPV-2. These findings support the hypothesis that EcPV-2 is etiologically involved in the development of equine penile SCCs. In addition, the results of this study provide the first evidence that EcPV-2 can infect clinically normal pharyngeal mucosa and show that EcPV-2 may be detected in a proportion of oral or pharyngeal SCCs. EcPV-2 DNA was not detected in any of a large number of healthy nictitating membranes, and was detected only rarely in normal equine vulval mucosa, suggesting that asymptomatic EcPV-2 infection is uncommon at these sites. This study is the first to screen large numbers of healthy equine tissues for EcPV-2 or to use quantitative PCR to assess and to compare viral load in neoplastic and non-neoplastic tissues. As evidence accumulates that PVs are associated with neoplasia in domestic animals (Munday and Kiupel, 2010), the results of this study should provide useful information for future investigation into the role of EcPV-2 in the development of equine neoplasia.

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