INVESTIGATIONS OF EQUINE SARCOIDS AND BOVINE PAPILLOMAVIRUS IN WESTERN CANADA

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By

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ABSTRACT

Equine sarcoids are the most common skin tumors of horses. Despite being such a common entity, relatively little is known about many features of sarcoid epidemiology or growth. In addition, due to the detection of Bovine Papillomavirus (BPV) DNA of 2 different types, BPV type 1 (BPV1) and BPV type 2 (BPV2), in equine sarcoids BPV has been suggested as the causative agent of sarcoid development. Recently, however, BPV DNA has also been detected in other skin conditions of horses; the significance of this is unclear. Multiple studies to learn more about sarcoids were undertaken.

To investigate the epidemiology of sarcoids in horses in Western Canada the records of five veterinary diagnostic laboratories were searched to identify submissions of sarcoids from horses. The submission record and diagnostic reports of 802 separate submissions of equine sarcoids were reviewed for age, breed, and gender of the horse and the number, location and clinical type of sarcoid. Based on these submissions, horses of a wide variety of ages and 23 different equine breeds were affected, within these breeds, Donkeys were overrepresented.

The presence of BPV was determined by Polymerase Chain Reaction (PCR). BPV was found in 74 of 96 (77.1%) samples, and using Restriction Fragment Length Polymorphism, BPV1 and BPV2 were identified in these samples. BPV2 was present in 59 (79.7%) of these. Unlike other areas in the world, in Western Canada, equine sarcoids are most commonly associated with BPV2.

A second study examined different clinical types of sarcoids to determine if there was differential expression of immunohistochemical markers associated with apoptosis, Cleaved Caspase 3(ClC3), and antiapoptotic factors, B-Cell Lymphoma 2 (Bcl-2) and Survivin. No differences in the expression of any of these markers regardless of BPV type were noted. Survivin was expressed in equine sarcoids of all types and increased levels of expression are associated with more aggressive clinical behaviour.

Finally, the location of BPV DNA was determined in both sarcoids and a variety of non-sarcoid inflammatory skin conditions of horses, as well as, normal skin. PCR for BPV DNA was performed on 86 skin biopsies from horses with non-sarcoid skin conditions, as well as, normal skin. BPV DNA was present in 41 of 86 biopsies. These positive samples, in addition to BPV positive sarcoid samples from the earlier study, were dissected into tissue compartments using laser microdissection followed by 2 forms of BPV DNA amplification, PCR and isothermal loop

mediated amplification. BPV DNA was more often located in the epidermis of non-sarcoid skin conditions than in sarcoids. In addition, areas of inflammation within the dermis and epidermis were more likely to contain BPV DNA than non-inflamed areas. These results suggest that while BPV is commonly found in equine skin, the location where it is found differs between sarcoids and non-sarcoid samples. When BPV DNA was found in non-sarcoid samples, it was commonly associated with inflammation suggesting that microscopic damage to the epidermal barrier of the skin maybe an adequate predisposing factor to the development of sarcoids.

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LIST OF ABBREVIATIONS

Bcl-2	B-Cell Lymphoma 2
BLAST	Basic Local Alignment Search Tool
bp	base pair
BPV	Bovine papillomavirus
BPV1	Bovine papillomavirus type 1
BPV2	Bovine Papillomavirus type 2
ClC3	Cleaved caspase 3
DNA	deoxyribonucleic acid
DNA-PKcs	deoxyribonucleic acid protein kinase subunit
dNTP	deoxynucleoside triphosphate
E5	early protein 5
ELA	Equine leukocyte antigen
EPV	Equine papillomavirus
FFPE	formalin-fixed paraffin embedded
HPV	Human papillomavirus
ISH	*in situ* hybridization
IS-PCR	*in situ* polymerase chain reaction
LAMP	isothermal loop mediated amplification
LCM	laser microdissection
MHC	major histocompatability complex
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDS	Prairie Diagnostic Services, Inc.
RFLP	restriction fragment length polymorphism

1. INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Introduction

1.1.1 Context

Sarcoids are neoplasms of dermal fibroblasts in ungulates, especially horses and other equids, including donkeys, mules and zebras (Jackson, 1936;Kidney and others, 2001;Lohr and others, 2005;Martens and DeMoor, 1996;Ragland, Keown, and Spencer, 1970;Reid and others, 1994). They are the most common neoplasm reported in these species (Marais and others, 2007;Reid and others, 1994;Valentine, 2006). The term sarcoid was first used to describe these neoplasms in 1936, in South Africa, and to distinguish them from other fibroblastic skin tumours such as fibromas, fibrosarcomas and papillomas (Jackson, 1936). Since that time, sarcoids have been reported worldwide in wild and domesticated equids and a large variety of horse breeds (Bloch, Breen, and Spradbrow, 1994;Borzacchiello and others, 2008;Chambers and others, 2003;Valentine, 2006).

The initial description depicted a sarcoid as "a unique locally invasive, benign neoplastic like tumour of the skin with a variable epidermal component which has a high propensity for recurrence" (Jackson, 1936). The invasive nature of sarcoids and their tendency to recur following treatment remain defining features of sarcoids today (Bogaert and others, 2008;Knottenbelt, 2005;Ragland, Keown, and Spencer, 1970). An individual horse may have a single sarcoid but, more frequently, horses have multiple lesions and hundreds of lesions per animal have been reported (Bogaert and others, 2007;Nasir and Campo, 2008). Sarcoids can occur over any area of the body, but have a predilection for the region around the genitalia, the chest, abdomen and around the head (Angelos and others, 1988;Voss, 1969). Sarcoids generally do not metastasize, but their proliferative and invasive growth can lead to disfigurement, pain, and functional impairment (Bogaert and others, 2008).

Currently there is no universally effective treatment for sarcoids and recurrence following treatment is common (Bogaert and others, 2008;Knottenbelt, 2005). As a result, the presence of sarcoids may lead to the "failure" of the animal during a pre-purchase exam and may result in exclusionary clauses for loss of use or veterinary fee limitations on insurance policies (Knottenbelt, 2005;Nasir and Campo, 2008).

1.1.2 Clinical types of sarcoids

The growth pattern and clinical appearance of sarcoids can vary greatly amoung horses and amoung individual lesions (Knottenbelt, 2005). Based on their clinical appearance, six different types of sarcoids have been described (Table 1.1) (Knottenbelt, 2005). While five types of sarcoids are commonly reported, the malevolent type has only been reported by one group (Knottenbelt and Kelly, 1995). The typical histologic appearance of sarcoids is a proliferation of whorls and interlacing bundles of spindle shaped fibroblasts in the dermis, with epidermal hyperkeratosis, hyperplasia, and rete peg formation. Frequently, these fibroblasts grow in a perpendicular "picket fence" arrangement to the epidermal basement membrane along the dermal epidermal junction (Pascoe and Knottenbelt, 1999). The mitotic rate is typically low (Goodrich and others, 1998). While increased numbers of dermal fibroblasts are common to all clinical types, the other histologic changes are not consistently seen. The different clinical types cannot be distinguished based on their histologic appearance (Martens and others, 2000).

Sarcoid diagnosis is most commonly made on the basis of clinical appearance, or histopathologic examination of biopsy specimens (Martens and others, 2000). However, variability of clinical appearance and the common presence of ulceration, inflammation and secondary infection may hinder making a definitive diagnosis. As an additional diagnostic test, the presence of Bovine papillomavirus (BPV) as demonstrated by *in situ* hybridization (ISH) is sometimes used (Angelos and others, 1991;Martens and others, 2000), but this, as will be described later, may be problematic.

1.1.3 Sarcoid prevalence

Although, sarcoids are the most common skin tumour of horses representing over 50% of all skin tumours (Marti and others, 1993;Pascoe and Summers, 1981;Valentine, 2006), it is unclear what the true prevalence of sarcoids is in the horse population. The reported prevalence varies widely depending on the study population examined. For example, the reported prevalence in horses admitted to veterinary hospitals varies from 0.6% to 2% (Angelos and others, 1988;Mohammed, Rebhun, and Antczak, 1992), while prevalence based on samples of equine origin submitted to diagnostic laboratories was reported to be 15.18% in the Pacific northwest of the United States (Valentine, 2006). Estimation of the relative frequency of the

various clinical types of sarcoid is even more difficult to determine. This estimation is hampered in large part by the variability in the severity of disease caused by sarcoids, and the tendency for sarcoids to transform to a more aggressive type following surgical intervention. This may mean that small, inoffensive lesions are treated with benign neglect and may not be reported to veterinarians (Scott D.W. and Miller W.H., 2003). Occult and small verrucose sarcoids often do not cause clinically significant disease, and as such, they are often overlooked or not treated (Pascoe and Knottenbelt, 1999). Conversely, horses with large, aggressive fibroblastic sarcoids are much more likely to receive treatment or biopsy. As a result, the larger and more aggressive types are likely overrepresented in clinical or diagnostic centre populations, while the smaller, more benign lesions are likely underrepresented (Scott D.W. and Miller W.H., 2003).

1.2 Risk factors for sarcoid development

1.2.1 Age

The risk factors for development of sarcoids are not fully understood. Horses of almost any age may develop sarcoids, but most reports suggest that sarcoids are most commonly seen in young horses with a peak incidence somewhere between three and six years (Brostrom, 1995;Scott D.W. and Miller W.H., 2003). However, one study stated that the incidence of sarcoids increases in horses until 15 years of age and then decreases thereafter (Mohammed, Rebhun, and Antczak, 1992). Why younger horses are at increased risk of developing these tumours is unclear, but it has been suggested that sarcoids may be caused by infection with a virus and that as horses age they are likely to develop immunity to this virus.

1.2.2 Gender

Most studies do not identify any gender predilection for sarcoid development. Where a gender predisposition has been reported, geldings have been at an increased risk compared to stallions and mares (Mohammed, Rebhun, and Antczak, 1992;Reid and others, 1994). Although it has been suggested that castration may place geldings at an increased risk for sarcoid development (Reid and Mohammed, 1997), this remains unproven and seems unlikely as the actual surgical site is rarely affected.

1.2.3 Breed

Breed predispositions to sarcoid development have been shown in several studies. Studies in the United States have demonstrated that Quarter Horses, Arabians and Appaloosas are roughly twice as likely to develop sarcoids as compared to Thoroughbreds (Angelos and others, 1988;Mohammed, Rebhun, and Antczak, 1992). Further, Thoroughbreds are roughly twice as likely to develop sarcoids as Standardbreds (Brostrom, 1995;Meredith and others, 1986;Mohammed, Rebhun, and Antczak, 1992). The reason for this predisposition is not known, but it has been suggested that Quarter Horses and Appaloosas are more likely to be in contact with cattle and thus more likely to be in contact with BPV (Mohammed, Rebhun, and Antczak, 1992). Alternatively, it may be that these breeds are more likely to work on rough surfaces and thus more likely to develop wounds to the legs (Brostrom, 1995). In addition to lifestyle factors, differences in the genetic makeup of these various breeds may contribute to the differences in susceptibility to development of sarcoids.

1.2.4 Immune system and equine leukocyte antigens

The immune system may play a role in the development of sarcoids. In Arabian horses, a defective DNA protein kinase subunit (DNA-PKcs) that is associated with severe combined immunodeficiency is also associated with an increased risk of sarcoid development (Ding and others, 2002). Genetic variation based differences in susceptibility to sarcoid formation may also be due to expression of various equine leukocyte antigens (ELA) (Lazary and others, 1985). ELA are serologically identifiable gene products of the Major Histocompatability Complex (MHC) which are involved in the immune response. Of these, ELA in two different classes have been associated with sarcoid formation (Brostrom and others, 1988;Brostrom, 1995;Gerber, Dubath, and Lazary, 1988;Lazary and others, 1985;Meredith and others, 1986). Class 1 ELA genes encode proteins that are present on the surface of all nucleated cells. ELA of this class that are associated with an increased risk of sarcoid formation include: W11 in Irish Warmbloods (Lazary and others, 1985), W5 in Swiss and French Warmbloods (Lazary and others, 1985), A5, W20 in Swiss Warmbloods (Gerber, Dubath, and Lazary, 1988). A5 has also been associated with the early onset of sarcoid formation (Brostrom, 1995). Class 2 ELA genes encode proteins that are expressed on the surface of antigen presenting cells and the expression of W13 (formerly called W3,B1) is strongly associated with increased risk of sarcoid formation in Swiss and Irish Warmbloods, Thoroughbreds, Swedish Halfbreds and the Selle Francais (Brostrom and others,

1988;Brostrom, 1995;Gerber, Dubath, and Lazary, 1988;Meredith and others, 1986). Standardbred horses do not express W13 and this has been suggested as a possible explanation for the lower frequency of sarcoids in this breed (Meredith and others, 1986). However, the expression of W13 alone is not sufficient to explain sarcoid formation, as large numbers of horses express W13, but most do not develop sarcoids, and horses that do not express W13 may develop sarcoids.

1.3 Etiologic agents in sarcoid development

1.3.1 Early studies

In early studies, the pattern of horses that developed sarcoids suggested the possibility of an infectious agent being the cause of sarcoids (Jackson, 1936). Voss et al. were able to transmit sarcoids between naturally affected horses and non-affected horses by transmission of whole sarcoid material or cell-free supernatant fluid (Voss, 1969). Viral agents were deemed to be the most likely etiology for sarcoid formation. Several viruses have been proposed as the causative agent, including a retrovirus and two papillomaviruses, one equine and one bovine.

1.3.2 Attempts to transmit sarcoids

In 1951, Olsen and Cook inoculated biopsy material from the affected skin of cattle with active papillomaviral infections into the skin of horses (Olson and Cook, 1951). These horses developed "sarcoma-like" tumours that did not involve the epithelium, often recurred after resection and tended to resolve spontaneously. Replication of these findings was attempted in 1969 and although lesions that grossly resembled sarcoids were produced, they were histologically distinct (Ragland and Spencer, 1969). These histologic differences included: no change in the overlying epidermis, and the arrangement of fibroblasts to the epidermal basement membrane did not show the "picket fence" arrangement commonly seen in sarcoids. In addition, unlike true sarcoids which have a low spontaneous regression rate, all of the induced lesions regressed within four to 12 months. This was attributed to the production of a strong immune response to the BPV which is not seen in naturally occurring sarcoids. Attempts to transmit sarcoids by inoculation of sarcoid extracts into horses, donkeys and cattle by this group were unsuccessful (Ragland, McLaughlin, and Spencer, 1970).

1.4 Bovine papillomavirus

1.4.1 Detection of BPV

Three viruses have been considered as possible causative agents of sarcoid formation. These included BPV, Equine papillomavirus (EPV) and a retrovirus isolated from sarcoid cell culture lines (Cheevers and others, 1982). However, further research demonstrated that EPV DNA could not be found in sarcoids (Postey, Appleyard, and Kidney, 2007), and that the retrovirus was an endogenous retrovirus and did not demonstrate transforming activity (Cheevers, Fatemi-Nainie, and Anderson, 1986) and thus that neither were involved in sarcoid transformation. Southern blotting had facilitated detection of BPV in some sarcoids (Lancaster, Olson, and Meinke, 1977), but results were inconsistent. Inconsistencies may have resulted from the very low levels of virus in sarcoids which may have been undetectable using the Southern blotting technique. In the early 1990s, multiple groups detected BPV DNA in sarcoids by the use of the more sensitive polymerase chain reaction (PCR) (Otten and others, 1993;Teifke, Hardt M., and Weiss, 1994;Teifke and Weiss, 1991).

1.4.2 BPV types

Papillomaviruses are doubled-stranded DNA viruses and are divided into genera and types based on the DNA sequences of their L1 open reading frames (de Villiers and others, 2004). Two different BPV have been found in sarcoids – BPV type 1 (BPV1) and BPV type 2 (BPV2) (Otten and others, 1993;Teifke, Hardt M., and Weiss, 1994;Teifke and Weiss, 1991). Recently, a third, a putative new BPV has been recovered from a sarcoid in Brazil (Anjos and others, 2010). Papillomaviral particles have not been found in sarcoids and the papillomaviral infection is considered to be non-productive with the papillomavirus existing episomally (Goodrich and others, 1998). Using PCR, BPV DNA has been consistently detected in sarcoids worldwide with detection rates varying from 73% to 100% (Bloch, Breen, and Spradbrow, 1994;Borzacchiello and others, 2008;Carr and others, 2001;Carr and others, 2001;Teifke, Hardt M., and Weiss, 1994). Both BPV1 and BPV2 are δ papillomaviruses causing fibropapillomas in their natural host – cattle (Nasir and Campo, 2008). A recent study using quantitative PCR demonstrated a positive correlation between increasing BPV viral load and increasing speed of tumour growth (Haralambus, Klukowskarutzler, and Brandt, 2010). Papillomaviruses are typically species specific, with the exception of BPV1, BPV2 (Nasir and Campo, 2008), and

possibly the feline sarcoid-associated papillomavirus (Munday, Knight, and Howe, 2010). BPV1 has been the papillomavirus most consistently found in sarcoids, present in over 90% of sarcoids from Europe (Angelos and others, 1991;Otten and others, 1993;Teifke, Hardt M., and Weiss, 1994), as compared to the Western United States where BPV2 is the dominant type, being identified in 63% of sarcoids (Carr and others, 2001).

1.4.3 BPV transforming genes

1.4.3.1 E5 gene

Papillomaviruses encode three transforming oncoproteins called early protein 5 (E5), E6 and E7 (Nasir and Campo, 2008). E5 and E6 are the major transforming proteins, with E7 having a complementary role to enhance the transformative abilities of E5 and E6 (DeMasi and others, 2007). The mechanism by which E5 transformation of cells occurs appears to be primarily through activation of the platelet derived growth factor (PDGF) β receptor (Petti and DiMaio, 1994;Petti, Nilson, and DiMaio, 1991). In addition, E5 prevents acidification of endosomes and Golgi apparatus through binding of the 16kDa subunit of vacuolar H^+ ATPase. The resultant lack of acidification leads to failure to degrade PDGF receptors. This in turn makes recycling of these undegraded receptors possible, indirectly reducing receptor down-regulation (Schapiro and others, 2000). E5 also causes retention of MHC class 1 molecules within the Golgi apparatus and prevents their expression on the cell surface, which may allow BPV infected cells to evade normal detection and removal by the immune system (Ashrafi and others, 2002;Marchetti and others, 2002).

1.4.3.2 E6 gene

E6 exerts its oncogenic effect in other papillomaviruses by binding to the tumour suppressor gene p53 and stimulating its degradation (Scheffner and others, 1990). However in BPV, E6 does not have this binding property (Werness, Levine, and Howley, 1990), and may instead exert its effect by sequestering p53 in the cytoplasm of the cell and preventing it from functioning by denying p53's entrance into the nucleus (Nasir and Reid, 1999). Immunohistochemical studies on sarcoids have supported this theory by demonstrating p53 in an abnormal perinuclear location in 9 to 44% of the sarcoids examined (Martens and others,

2000;Nixon and others, 2005). In addition to its effects on p53, E6 in BPV1 has been shown to interact with a cellular adhesion protein called paxillin (Tong and Howley, 1997;Wade, Brimer, and Vande, 2008). This interaction between E6 and paxillin leads to the disruption of the actin cytoskeleton of cells, a change characteristic of transformed cells (Tong and Howley, 1997).

1.4.3.3 E7 gene

The mechanism by which E7 contributes to sarcoid formation is not as clear as those of E5 and E6. E7 alone is not able to transform cells. Rather, its presence seems to be required for full expression of the transforming abilities of E5 and E6 (DeMasi and others, 2007). More specifically, E7 appears to enhance the survivability of transformed cells. It does this through inhibition of anoikis, an apoptosis pathway associated with cell detachment. BPV transformed cells expressing E7 show increased numbers and sizes of anchorage independent colonies in cell cultures when compared to non-transformed cells (DeMasi and others, 2007). The mechanism by which this occurs is uncertain, but may be related to E7 binding of a cellular protein known as p600 (DeMasi and others, 2007).

1.4.3.4 Sequence variants

A DNA sequence region encompassing portions of the transforming gene E5 and a second early gene, E2, is a common target for PCR amplification in sarcoids (Teifke and Weiss, 1991). Numerous sequence variants in E5 have been found in sarcoids. Chambers et al. found six different E5 sequences in sarcoids, including three which resulted in sarcoid-specific amino acid sequences (Chambers and others, 2003). Thus far, the significance of these variants is unclear. More recently, sequence variants in the long control region and E2 open reading frame of BPV1 in sarcoids, as opposed to these same regions in BPV1 in cattle, have been discovered (Nasir and others, 2007). Comparisons of activity of these sarcoid specific variants in equine and bovine cells suggest that these variants have increased activity in equine cells compared to bovine cells and, therefore, may represent an adaptation for function in equine cells (Nasir and others, 2007).

1.4.4 Diagnosis based on the presence of BPV

1.4.4.1 Presence of BPV on equine skin

The basis of BPV detection as a tool for use in diagnosing sarcoids is founded upon the presence of BPV DNA in sarcoids and the lack of BPV DNA in other equine tumours including melanomas, papillomas and squamous cell carcinomas (Carr and others, 2001;Carr and others, 2001;Chambers and others, 2003;Otten and others, 1993). However, two recent studies have shown that BPV DNA can be detected by PCR in locations on the skin of horses apart from within the sarcoid itself (Bogaert and others, 2005;Bogaert and others, 2008). One study found that in sarcoid affected horses, BPV could be detected on apparently normal skin, the sarcoid surface and from normal skin in contact with the sarcoid (Bogaert and others, 2005). There was no significant difference in detection rates between these sites. As well, 44% of horses in contact with these affected horses were also BPV positive on their skin. A second study revealed the presence of BPV DNA on the normal skin of 57% of tested horses (Bogaert and others, 2008). Using PCR, BPV DNA was detected on the apparently normal skin of horses with sarcoids (73% of which were positive), horses in contact with horses with sarcoids (30% positive), horses in contact with cattle with recent papillomaviral infections (73% positive), and in control horses (30%) (Bogaert and others, 2008).

The risk of aggressive transformation of sarcoids following diagnostic biopsying (Knottenbelt and Kelly, 1995) has led to attempts to find alternate methods for the initial diagnosis of sarcoids and to determine the prognosis for recurrence following surgery. These attempts include the use of PCR for the detection of BPV on superficial swabs and skin scrapings of suspected sarcoids (Martens, De Moor, and Ducatelle, 2001). Overall, this method had a diagnostic sensitivity rate for the detection of BPV of 88% in swabs and 91% in skin scrapings. This sensitivity rate was felt to be less than that of clinical diagnosis and the sensitivity was even less in occult or nodular sarcoids where the overlying epithelium was intact. PCR for BPV DNA has also been used in an attempt to determine the probability of recurrence of sarcoids following their surgical resection (Martens and others, 2001). Samples from the surgical margins following resection were examined using PCR for the detection of BPV DNA. Increased risk for the recurrence of a sarcoid was associated with detection of BPV in the surgical margins.

1.4.4.2 Presence of BPV in other equine skin conditions

In addition to normal skin, BPV DNA has also been found in inflammatory skin conditions of horses without sarcoids (Angelos and others, 1991;Chambers and others,

2003;Yuan and others, 2007). It is unclear where in these biopsies the BPV DNA was located. Perhaps based on these findings, BPV is simply a common skin contaminant of horses and the presence of its DNA in sarcoids is coincidental rather than causal. This, in turn, would mean that the simple detection of BPV in suspect skin lesions is not sufficient to confirm that these lesions are sarcoids.

1.5 Transmission of sarcoids

Whether sarcoids are a transmissible tumour from one horse to another is unknown. It has been suggested that direct contact between sarcoid affected horses and unaffected horses may allow for transmission of sarcoids (Bogaert and others, 2005;Nasir and Campo, 2008). If sarcoids are caused by BPV, perhaps BPV may be transmitted from one horse to another, but the method by which BPV is transmitted is currently unknown (Nasir and Campo, 2008). There is no epidemiologic evidence that BPV is transmitted from cattle to horses (Nasir and Campo, 2008). It is possible that BPV may be transmitted from one horse to another by direct contact between sarcoid affected horses or by fomites (Pascoe and Knottenbelt, 1999). In addition, one study found that face flies (*Musca autumnalis*) feeding on sarcoid affected horses contained BPV DNA identical to that in the sarcoids and may act as vectors for transmission (Kemp-Symond, 2000). As yet, these studies are preliminary and no definitive evidence for transmission has been published.

Alternatively, it had been suggested that like some other tumours, sarcoids may be transmitted from one animal to another as a result of the direct inoculation of a transformed cell line from one horse to the next. The genome of the transformed cells would be unique to inoculated cell lines and distinct from other cells within the horse. However, this has been recently refuted by showing that transformed cells in sarcoids contain DNA of an identical genotype to that of other non-sarcoid circulating cells in the blood (Gobeil and others, 2007).

1.6 Treatment

Surgical excision is the most common treatment for sarcoids, but trauma, including surgical intervention, may induce sarcoids to undergo a rapid and invasive growth phase (Knottenbelt, 2005;Pascoe and Knottenbelt, 1999), making development of other treatment modalities desirable.

With the recent widespread use of vaccines to prevent cervical cancer caused by Human papillomaviruses (Oaknin and Barretina, 2008), there is increasing interest in the development of an analogous vaccine for prevention of sarcoids in horses. Small scale trials vaccinating sarcoid-bearing donkeys and horses with chimeric viral particles containing the BPV L1 (late protein) and E7 proteins have recently been reported (Ashrafi and others, 2008;Mattil-Fritz and others, 2008). Both trials showed trends to increased regression and reduced progression, but the numbers were too small in either case to show any statistical significance.

1.7 Conclusion

Despite sarcoids being the most common tumour of horses with no consistently effective treatment and causing significant distress owners, there remains a surprising lack of basic information about this neoplasm. True understandings of the pathogenesis of sarcoids, risk factors for their development, prognostic factors for recurrence and if and how transmission occurs, are still lacking. Further research into these and other areas is needed to guide both the development of new treatment protocols and new diagnostic methods.

Table 1.1 Clinical classification of equine sarcoids based on appearance as described by Knottenbelt (Knottenbelt, 2005).

Clinical Type of Sarcoid	**Appearance**
Occult	Variable from a coat colour change to alopecia
Verrucose	Hyperkeratotic, scaly, thickened skin
Nodular	Firm, distinct subcutaneous nodules with no epidermal change
Fibroblastic	Ulceration with marked exophytic, proliferative growth
Mixed	Combination of 2 or more of the above types
Malevolent	Extensive infiltration of lymphatics and extension to local lymph nodes

2. EPIDEMIOLOGY OF EQUINE SARCOIDS IN HORSES IN WESTERN CANADA

Although sarcoids are a very common tumour of horses their descriptive epidemiologic features have not been described in Western Canadian horses. To do this, we collected the diagnostic records of sarcoids from multiple veterinary diagnostic centres across Western Canada. The paraffin histologic blocks of a subsample of these sarcoids were selected for further study. In addition to providing information on sarcoids in Western Canada, this study[1] allowed for the collection of the samples of sarcoids that would be used for study throughout the rest of the thesis.

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2.1 Abstract

Sarcoids are the most common tumour of the equine skin. Although much research has been performed on sarcoids in the rest of the world, only one study describing the epidemiologic features of sarcoids in Canadian horses has been published. The records of five veterinary diagnostic laboratories in Western Canada were searched to identify submissions of sarcoids from horses. The submission record and diagnostic reports of 802 separate submissions of equine sarcoids were reviewed for age, breed, and gender of the horse and the number, location and clinical type of sarcoid. From these records, the 307 submissions that were submitted to laboratories in Saskatchewan were compared to a reference group to test for breed and gender predisposition. Based on clinical history and lesion descriptions, five clinical types of sarcoids were identified. Horses of a wide variety of ages and 23 different equine breeds were affected and, within these breeds Donkeys were overrepresented. A second stratified subset of 96 sarcoids representing each of five clinical types was selected, the associated formalin-fixed paraffin-embedded tissues were collected and polymerase chain reaction for a segment of Bovine papillomavirus (BPV) DNA was performed on these tissues. BPV was found in 74 of 96 (77.1%) samples, and using restriction fragment length polymorphism, BPV Type 1 (BPV1) and BPV Type 2 (BPV2) were differentiated in these samples. BPV2 was present in 59 (79.7%) of these. Unlike other areas in the world, in Western Canada, equine sarcoids are most commonly associated with BPV2.

Key Words: Equine Sarcoid; Bovine Papillomavirus 2; Western Canada.

2.2. Introduction

Equine sarcoids are the most common skin tumour of horses and have been found in horses worldwide. A wide variety of other equids including zebras, donkeys and mules have been affected (Jackson, 1936;Kidney and Berrocal, 2008;Lohr and others, 2005;Martens and DeMoor, 1996;Reid and others, 1994). The term "sarcoid" was first used to describe these tumours in 1936, in South Africa, to distinguish them from other fibroblastic skin tumours such as fibromas, fibrosarcomas and papillomas (Jackson, 1936). Although these tumours do not metastasize, they can be invasive, are regarded as cosmetic defects, can become ulcerated and infected and, when occurring near the eyes or on the eyelids, can impair vision. The treatment of horses for equine sarcoids can represent a considerable expense to horse owners (Nasir and Campo, 2008).

Descriptive epidemiologic features of equine sarcoids (i.e., age, breed, clinical type, location of tumour, presence of multiple tumours) have not been well defined in Canadian horses and, as best can be determined, only a single study has been published (Fretz P.B. and Barber S.M., 1980). Regional estimates of sarcoid prevalence based on records from diagnostic centres vary considerably depending on the source of samples, from a high of 15.18% of submissions to a diagnostic centre (Valentine, 2006) to a low of 0.74% of horses admitted to a veterinary hospital (Angelos and others, 1988). The age at diagnosis in published studies varies with one study suggesting that sarcoids rarely develop in animals older than seven years (Bogaert and others, 2005), while a second study, from the Pacific Northwest of the United States determined that the mean age at diagnosis was nine years (Valentine, 2006).

Equine sarcoids are associated with the presence of two types of Bovine papillomavirus (BPV); BPV-type 1 (BPV1) and BPV-type 2 (BPV2) (Teifke, Hardt M., and Weiss, 1994). Much of the work on the molecular pathogenesis of sarcoids has been conducted in Europe and performed exclusively on animals from which BPV1 was detected in lesions. BPV2 is reportedly much more common in sarcoids from horses in the Western United States (Carr and others, 2001). It is unknown if the previous results of research on BPV1 associated sarcoids applies to those associated with BPV2 (Carr and others, 2001).

To address the knowledge gap in the descriptive epidemiology of equine sarcoids in horses in Western Canada the following studies were performed. Evaluation of submission records and diagnostic reports from multiple veterinary diagnostic laboratories in Western

Canada were used to describe the epidemiologic features of equine sarcoids. From these records, a subset of cases that had been submitted to laboratories in Saskatchewan were compared to a reference group to test for breed and gender predisposition.

A second subset of the most recently submitted cases, stratified by province of origin, and representing different clinical types of sarcoids, was selected and the associated formalin-fixed paraffin-embedded tissues utilized. Polymerase chain reaction (PCR) was performed on this subset to detect the presence of BPV, and the type of BPV was identified by restriction fragment length polymorphism (RFLP) on PCR positive samples. Confirmation that the PCR product was either BPV1 or BPV2 was conducted by nucleotide sequencing on four samples.

2.3 Materials and Methods

2.3.1 Cases

Surgical biopsies of equine sarcoids diagnosed during the 12 year period between January 1, 1996, and December 31, 2007, inclusive, were identified by using computer-based record searches at five veterinary diagnostic laboratories in Western Canada: IDEXX Reference Laboratories Ltd., located in Langley, British Columbia (formerly Central Laboratory for Veterinarians), and their associated laboratories in Edmonton and Calgary, Alberta; the Department of Veterinary Pathology at the University of Saskatchewan; and Prairie Diagnostic Services, Inc. (PDS) in Regina and Saskatoon, Saskatchewan. For each case, the submission records and diagnostic reports were obtained. Formalin-fixed paraffin embedded (FFPE) tissues of the most recently submitted samples stratified by region of each the five clinical types of sarcoids were retrieved for molecular based testing.

Submission records and diagnostic reports for these biopsies were used to determine breed, gender, age at the time of submission, number of sarcoids on the animal, location of the sarcoids on the animal and in which province the animal was located. The submitted history, gross appearance and information in the biopsy description were used to determine the clinical type of sarcoid. Sarcoids were classified using the clinical classification scheme proposed by Knottenbelt (Knottenbelt, 2005) and these included: fibroblastic, nodular, occult, verrucose, mixed and malevolent.

2.3.2 Reference population

Data on all equine skin biopsies not diagnosed as sarcoids within the same time period were collected from the records of the Department of Veterinary Pathology at the University of Saskatchewan, and PDS in Regina and Saskatoon, Saskatchewan. These data served as the reference population for identifying potential risk factors associated with the development of sarcoids. Only those sarcoids identified at these same diagnostic laboratories located in Saskatchewan were used for these comparisons.

2.3.3 Data Analysis

Risk factors considered for associations with the development of sarcoids were as follows: gender (gelding, mare, and stallion), age (in years), and breed. Comparisons of median age of horses with different clinical classifications of sarcoids in this study were performed using Kruskal-Wallis Equality of Populations rank test and Dunn's Multiple Comparison Test. Gender and breed information on horses diagnosed with one or more sarcoids was compared to this same information on horses with other skin conditions diagnosed by Saskatchewan veterinary diagnostic laboratories and the reference population were evaluated for these risk factors using a χ^2 goodness of fit test. Relative risk of the occurrence of sarcoid in different breeds and genders was made based on comparisons with the occurrence in the breed or gender with the lowest risk of disease. Significance was set at $p<0.05$. Analysis was performed with the aid of a statistical software package.[a]

2.3.4 Determination of the presence and type of Bovine papillomavirus

For each clinical type of sarcoid, the 20 most recently submitted biopsies from horses, stratified by province of origin, were selected from the submissions to the various veterinary diagnostic centres. The FFPE tissue blocks from these samples were utilized for PCR as previously described (Teifke, Hardt M., and Weiss, 1994). Briefly, the single primer set that amplifies a 244 base pair (bp) sequence spanning portions of the E2 gene, the intergenic spacer region and a small portion of the E5 gene of BPV1, or a similarly located 248 bp sequence of BPV2, was utilized (Figure 2.1). Previous studies using this set of primers have identified the amplified sequence as the E5- open reading frame (Carr and others, 2001;Kidney and others, 2001;Teifke and others, 2003), but comparisons to BPV sequence data in GenBank[b] revealed that the above description of the sequence is more correct.

Following amplification, typing of the BPV in the PCR product was performed. RFLP was utilized to distinguish between the two potential virus types (Teifke, Hardt M., and Weiss, 1994). The BPV1 amplicon was cleaved using *Bst*XI which cleaves the 244 bp sequence between nucleotides 3888 and 3889 into a 130 bp fragment and a 114 bp fragment. The BPV2 amplicon was cleaved using *Hinf*I which cleaves the 248 bp sequence between nucleotides 3887 and 3888 to create 2 fragments; 129 bp and 119 bp long. RFLP was performed in parallel with the two different restriction endonucleases. To confirm that PCR was amplifying BPV, four samples of PCR product, two of each, identified as BPV1 and BPV2 by RFLP, were sequenced.

2.4 Results

Eight hundred two equine sarcoids were submitted to the veterinary diagnostic laboratories during the 12 year time period. Two hundred thirty one, 378 and 193 samples were from horses resident in Alberta, British Columbia and Saskatchewan, respectively. Four hundred ninety five samples were seen by the IDEXX laboratories and 307 were seen by diagnostic laboratories in Saskatchewan. All laboratories received sarcoids from horses in each of the three provinces. Where the number of sarcoids on the animal could be determined, 204 of 722 (28.7%) animals had multiple sarcoids present at the time of submission.

Five different clinical types of sarcoids were present in the submitted biopsy samples; the malevolent type of sarcoid was not present in any samples. The most common type of sarcoid was the fibroblastic type (Table 2.1).

Data on the age of horses with each of the five clinical types of sarcoids present in this sample are summarized in Table 2.1. Median ages ranged from five years for fibroblastic sarcoids to seven years for occult, nodular and mixed sarcoids. The median age of horses with fibroblastic sarcoids was significantly younger than for horses with mixed, nodular and occult types of sarcoids.

Horses of a wide range of ages were diagnosed with sarcoids. Comparisons of reference groups of horses revealed that the mean age of affected horses was 6.8 years and the range was from 0.5 years to 31 years. Reference population horses that did not have sarcoids had a mean age of 8.9 years and the age range was one day to 30 years. Horses with sarcoids were significantly younger than horses whose skin was biopsied for other reasons ($p<0.01$).

The location of 746 sarcoids on the bodies of 686 horses was determined (Table 2.2). The most common location from which sarcoids were biopsied was the head with 307, followed by the limbs (149), neck and shoulder (116), abdomen (81), axilla and chest (59) and the paragenital region (34). Thirty-eight horses had sarcoids in more than one of these locations.

The reference group comprised 443 reports from horses with non-sarcoid skin conditions diagnosed by Saskatchewan veterinary diagnostic laboratories over the same time period. The 307 sarcoids diagnosed in these laboratories in this 12 year period were used as a study group. Sarcoids represented 41.9% of all equine skin submissions seen in these laboratories for this period of time.

Twenty-three breeds of horses were affected. The most commonly identified breeds were Quarter Horses and Thoroughbreds (Table 2.3). Breed relative risks for the presence of sarcoids in horses diagnosed in Saskatchewan veterinary laboratories as compared to reference population horses were determined (Table 2.3). The breed with the lowest risk of development of sarcoids was Warmbloods. Compared to the risk of development of sarcoids in Warmbloods, one breed had a significantly elevated risk, Donkeys (3.1 times more likely to develop, $p = 0.01$).

Geldings, mares and stallions were all affected with the most commonly affected horses being geldings (Table 2.3). No significant difference in the risk for the development of sarcoids was identified with any of these groups.

PCR for amplification of BPV was performed on 96 biopsies, selected from veterinary diagnostic laboratories across Western Canada, representing each of the five clinical types seen (Table 2.4). BPV DNA was amplified from 74 of 96 biopsies (77%). RFLP was performed on the PCR product from all BPV positive cases and, using this method, 14 of 74 tested BPV1 positive (19%), 59 of 74 tested BPV2 positive (80%) and one biopsy tested positive for both BPV1 and BPV2 (1%).

Nucleotide sequencing was performed on two of the BPV1 positive samples and two of the BPV2 positive samples. These sequences were compared to sequences from GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul and others, 1997). Multiple sequence alignments of the entire amplified region were performed using a computer program.[c] The two BPV1 positive samples (GenBank accession numbers FJ895875, FJ895876) showed greater than 98% identity with the previously described British II BPV1 sequence recovered from equine sarcoid in the United Kingdom (accession number AY232263.1) (Chambers and

others, 2003) (Figure 2.2). While BPV2 PCR products from one sarcoid (accession number FJ895874) showed 100% identity with the previously described Swiss V sample found in equine sarcoids from Switzerland (accession number AY232264.1) (Chambers and others, 2003) (Figure 2.3). PCR product from the other Western Canadian sarcoid (accession number FJ895877) had over 98% identity with three nucleotide differences from the Swiss V sample.

2.5 Discussion

Sarcoids on horses have been described since historical times (Erk, 1976) and have been recognized as a clinical entity since 1936 (Jackson, 1936). Although there has been abundant research on sarcoids in Europe and the United States there has not been, until now, an attempt to describe the epidemiologic features of sarcoids in horses in Western Canada. In this study, 802 horses with sarcoids were identified based on biopsy submission records to multiple veterinary diagnostic laboratories in Western Canada.

As sarcoids are the most common skin tumour of horses (Scott D.W. and Miller W.H., 2003;Valentine, 2006), it is not surprising that horses with sarcoids were present in large numbers in each of the three provinces that were examined. Submissions from the largest number of horses were from British Columbia (378) followed by Alberta (231) and Saskatchewan (193). According to a 2003 report on horse numbers in Canada, Alberta had the largest number of horses with 299,753, British Columbia had 141, 410 and Saskatchewan had 126, 252 (Evans, 2003). While results from this study may suggest that sarcoids are more common on a per capita basis in British Columbia, the data for this study was derived from veterinary practitioner submitted biopsies from horses and the results may be biased based on the willingness of owners to spend money on veterinary diagnostic services. With this in mind, the same report states that owners in British Columbia spend an average of $379 annually on veterinary care per horse as compared to $255 in Alberta which may explain the apparent difference in frequency of diagnosis per capita (Evans, 2003).

As noted in other studies on sarcoids, affected horses frequently have multiple sarcoids at the time of biopsy (Martens and others, 2001;Torrontegui B.O. and Reid, 1994). In this study, horses with multiple sarcoids present at the time of biopsy represented 28.7% of the submissions. In addition, sarcoids composed 41.9% of all biopsies involving equine skin over this same period

at Saskatchewan veterinary diagnostic laboratories making them the most frequently diagnosed skin condition.

Of the six different clinical classifications of sarcoids suggested by Knottenbelt (Knottenbelt, 2005), five were found within this study (Table 2.1). No malevolent sarcoids were found in this study and this clinical type has only been reported by one group (Knottenbelt and Kelly, 1995). All other clinical types were present in relatively equal proportions with the exception of the fibroblastic type which was present at approximately twice the frequency of other types. While this may represent the actual clinical distribution of cases, it may also represent the greater frequency with which these larger and more aggressive tumours are likely to receive diagnostic attention (Scott D.W. and Miller W.H., 2003).

Sarcoids may occur on any part of the body and in this study sarcoids were present in many different locations (Table 2.2). The majority of sarcoids occurred on the head, followed by the limbs and the neck and shoulder. This distribution differs from other studies, which found the lower abdomen to be the most common region for sarcoids to develop (Brostrom, 1995;Head, 1965;Torrontegui B.O. and Reid, 1994), but is similar to that of another study (Ragland, Keown, and Spencer, 1970). Interestingly, all of the studies where the abdomen was the most frequent location of sarcoid development were from Europe, while the study with findings similar to the current study was from the Pacific Northwest of the United States. It has been postulated that the reason for the variability in sites is due to differences in the use of horses in different geographic areas with horses in North America being more likely to injure their legs on rough ground than are those in Europe (Brostrom, 1995), but this remains speculative and has not been scientifically examined. One possible reason for the large number of sarcoids on the head in this study may be a result of sampling bias. The sarcoids included in this study were submitted by veterinary practitioners removing these sarcoids from client-owned horses. It is possible that lesions on the head of horses may be more likely to be removed for aesthetic reasons or may interfere with the use of the horse more than a similar lesion located on the flank.

A large number of equine breeds, 23 in total, were diagnosed with sarcoids in this study. Several studies have examined relative risk for the development of sarcoids among breeds (Angelos and others, 1988;Meredith and others, 1986;Mohammed, Rebhun, and Antczak, 1992). Two of these studies found that among horses diagnosed with sarcoids in New York State, Quarter Horses were more than twice as likely to develop sarcoids as compared to

Thoroughbreds which, in turn, were twice as likely to develop sarcoids as Standardbred horses (Angelos and others, 1988;Mohammed, Rebhun, and Antczak, 1992). In one of these studies, Arabians were identified as the breed with the highest risk of sarcoid development, more than three times higher than Thoroughbreds (Mohammed, Rebhun, and Antczak, 1992). This breed disposition for development of sarcoids was not present in the current study. Only Donkeys were at a statistically significantly higher risk to be diagnosed with a sarcoid (Table 2.3). The increased risk of Donkeys developing sarcoids has not been previously reported. Warmbloods, Standardbreds and Arabians were among breeds at the lowest risk of developing sarcoids in the current study. However, in none of these breeds was the risk reduction statistically significant. This difference in results may represent a variation based on the geographic location and genetic heritage of the horses involved, but a more likely explanation maybe the reference populations used to make these comparisons. The reference population of the earlier study was comprised of horses with corneal ulcers, indigestion, carpal bone chip fractures and tendonitis (Angelos and others, 1988), while the current study's reference population was comprised of horses with skin conditions other than sarcoids.

Similarly, the apparent predilection of donkeys for the development of sarcoids may also be related to the reference population chosen in the current study. It may be that donkeys are less frequently biopsied for skin disease than are other breeds of horses thus lowering the number of donkeys in the reference population and artificially elevating the risk of donkey sarcoid development.

Although more geldings than mares or stallions were diagnosed with a sarcoid, in this study, no differences in relative risk were noted (Table 2.3). In previous studies where a gender predilection has been shown, geldings have been found to be at an increased risk when compared to stallions and mares (Mohammed, Rebhun, and Antczak, 1992;Reid and others, 1994). In these studies, it was suggested that castration surgery is a risk factor for the development of sarcoids, but the lack of a gender predilection and the relative rarity of paragenital location of sarcoids in this study does not support this conclusion.

Sarcoids have been reported to be a disease of young horses (Brostrom, 1995;Scott D.W. and Miller W.H., 2003), and in this study, the mean age of horses with sarcoids in the study group was significantly younger than those of the reference population, but there was a wide range of affected animals from six months to 31 years old. Of particular note, is that unlike

previous reports which suggest that sarcoids rarely develop in horses older than seven years (Brostrom, 1995;Foy, Rashmir-Raven, and Brashier, 2002;Miller R.I. and Campbell R.S.F., 1982;Torrontegui B.O. and Reid, 1994), the median age of all horses in the current study with several clinical types of sarcoids (nodular, occult and mixed) was seven years, suggesting that half of the horses with this diagnosis were this age or older at the time of diagnosis. Of interest, is that the fibroblastic type classification, the most aggressive clinical type, was also the type associated with the youngest median age, statistically significantly younger than horses with other clinical types (Table 2.1). It has been suggested that sarcoids develop initially as one of the less aggressive types such as occult or verrucose sarcoids and then transform into the more aggressive fibroblastic form (Brostrom, 1995;Knottenbelt and Kelly, 1995;Marti and others, 1993). If this were the case, it seems counterintuitive that the most aggressive form of sarcoids is seen more often in younger horses than are the less aggressive forms from which it may derive.

BPV has been reported as the cause of equine sarcoids (Otten and others, 1993;Teifke, Hardt M., and Weiss, 1994;Teifke and Weiss, 1991) and in this study BPV DNA was amplified from 74 of 96 samples tested (77%). The amplification rates of BPV DNA from sarcoids in various studies has ranged from 73 to 100%, with studies with lower identification rates using formalin fixed paraffin-embedded samples as in the current study (Bloch, Breen, and Spradbrow, 1994;Borzacchiello and others, 2008;Carr and others, 2001;Carr and others, 2001;Teifke, Hardt M., and Weiss, 1994). Confirmation that BPV DNA was in fact being detected was conducted by sequencing of a small number of randomly chosen DNA products from amplified sarcoid samples. A high degree of identity with BPV previously recovered from equine sarcoids was present. One BPV2 DNA amplicon contained three previously unidentified nucleotide variations. The significance of these changes is uncertain, but nucleotide variation in other regions within these genes has been previously found in equine sarcoids (Chambers and others, 2003).

BPV was detected in all clinical types of sarcoids examined (Table 2.4). The lowest detection rates were in occult sarcoids with 12 of 19 positive (63%). Whether this represents lower levels of BPV in these types of sarcoids or if some of these biopsies were not truly sarcoids is unclear. Occult sarcoids have the most subtle changes histologically of any of the sarcoid types and also have the smallest lesions by volume, often confined to a small

proliferation of fibroblasts beneath the skin surface. It is possible that some of these lesions were misdiagnosed as sarcoids or that the smaller volume of neoplastic tissue in occult sarcoid as compared to other types of sarcoids reduces the success of DNA amplification resulting in a lower BPV positivity rates in this classification type of sarcoid.

The majority of sarcoids with amplified BPV DNA in this study contained solely BPV2 DNA based on RFLP (59 of 74 samples, 80% of the total). BPV1 was found as the only amplified BPV type in 14 of 74 samples (19%) while a single sample had a superinfection with both BPV1 and BPV2 detected. This is dramatically different from the results of most studies of sarcoids. In Europe, BPV1 accounts for a vast majority of DNA detected with only a small number of sarcoids containing BPV2 (Angelos and others, 1991;Otten and others, 1993;Teifke, Hardt M., and Weiss, 1994). Studies in the United States have found BPV1 and BPV2 in roughly equal proportions in the Eastern United States (Teifke, Hardt M., and Weiss, 1994), while BPV2 predominates in the Western United States representing 63% of the amplified DNA (Carr and others, 2001). No previous studies have found as high a rate of BPV2 infection as this current study. Most of the work conducted on equine sarcoids was performed on horses infected with BPV1 rather than BPV2. Although no clinical or pathogenetic differences between infection with BPV1 and BPV2 have been reported, no specific research has been published to confirm this. As such, the relevance of studies performed on horses with sarcoids containing BPV1 to horses with sarcoids containing BPV2 is not entirely certain (Carr and others, 2001).

In summary, this study is the largest study of equine sarcoids specifically focused on Western Canadian horses. Sarcoids appear to be a common lesion of the skin of horses in Western Canada. Although sarcoids in horses in Western Canada may seem similar to those reported elsewhere in the world, there are several differences in the details. Specifically, donkeys appear to be at an increased risk of sarcoid development, older horses are more commonly affected by sarcoids than some previous reports suggest and, perhaps most importantly, BPV2, not BPV1 is the predominant viral type detected in sarcoids in these horses. More research is needed to determine the importance of BPV2 as a cause of sarcoids and to determine if there are different clinical implications in sarcoids caused by BPV2 versus those caused by BPV1 or differences in the pathogenesis of these lesions.

2.6 Sources and Manufacturers

[a] Stata/IC 10.1 for Windows, StataCorp LP, College Station Texas

[b] GenBank, National Center for Biotechnology Information, National Institutes of Health, USA

[c]EMMA multiple alignment program, Emboss Version 5.0.0 (http://haruspex.usask.ca/emboss/).

Table 2.1. Clinical type of sarcoid and age of sarcoid affected horses based upon biopsy submissions to multiple veterinary diagnostic laboratories in Western Canada (n=467).

	Number	**Median Age (years)**	**Minimum Age (years)**	**Maximum Age (years)**
Fibroblastic	145	5[a]	0.6	25
Nodular	104	7	1.0	23
Occult	72	7	1.1	21
Verrucose	72	6	0.5	19
Mixed	74	7	0.8	31
Malevolent	0	N/A	N/A	N/A

[a]Significantly lower than nodular, occult and mixed types of sarcoids. ($p<0.05$)
N/A = Not applicable

Table 2.2. Known locations of 1 or more sarcoids on the body of horses in Western Canada.*

Location on Body	Number (%) of horses with at least 1 sarcoid at a given location
Head	307 (41.1%)
Limb	149 (20.0%)
Neck / Shoulder	116 (15.5%)
Abdomen	81 (10.9%)
Axilla / Chest	59 (7.9 %)
Paragenital	34 (4.6%)
Total	746 (100%)

*Some horses had sarcoids in more than one location on the body

Table 2.3. Breed and gender of horses with at least one equine sarcoid diagnosed at a veterinary diagnostic laboratory in Saskatchewan between January 1, 1996, and December 31, 2007. Relative risk of development of a sarcoid is based on comparisons to horses with skin conditions other than sarcoids submitted to these laboratories during the same time period.

	Number (row %) of horses with sarcoids	Number (row %) of horses with skin conditions other than a sarcoid	Risk	Relative Risk	P-Value	95% Confidence Interval
Breed						
Quarterhorse	147 (46.8%)	167 (53.2%)	0.47	1.9	0.07	0.87 - 4.04
Thoroughbred	22 (46.8%)	25 (53.2%)	0.47	1.9	0.11	0.91 - 4.39
Arabian	20 (31.7%)	43 (68.3%)	0.32	1.3	0.59	0.60 – 3.05
Donkey	10 (76.9%)	3 (23.1%)	0.77	3.1[a]	0.01	1.46 – 5.56
Draft Horse	9 (27.3%)	24 (72.7%)	0.27	1.1	1.00	0.45 - 2.84
Appaloosa	7 (31.8%)	15 (68.2%)	0.32	1.3	0.74	0.48 - 3.37
Warmblood	5 (25.0%)	15 (75.0%)	0.25	Reference		
Standardbred	3 (37.5%)	5 (62.5%)	0.38	1.5	0.65	0.45 - 4.24
Other/unknown	84	146				
Total	307	443				
Gender						
Gelding	146 (42.3%)	199 (57.7%)	0.42	1.2	0.37	0.76 – 2.14
Mare	111 (41.3%)	158 (58.7%)	0.41	1.1	0.51	0.83 - 1.60
Stallion	27 (36.5%)	47 (63.5%)	0.36	Reference		
Unknown	23	39				
Total	307	443				

[a]Significantly higher relative risk for the development of sarcoids

Table 2.4. Type of Bovine Papillomavirus identified in various clinical types of sarcoids submitted to multiple veterinary diagnostic laboratories in Western Canada.

Clinical type of sarcoid	Number tested by PCR	Number BPV1 positive (row %)	Number BPV2 positive (row %)	Total number BPV positive
Fibroblastic	20	3 (15.0%)	11 (55.0%)	14 (70.0%)
Nodular	19	0 (0%)	15 (78.9%)	15 (78.9%)
Occult	19	4 (21.1%)	8 (42.1%)	12 (63.2%)
Verrucose	19	4* (21.1%)	15* (78.9%)	18* (94.7%)
Mixed	19	4 (21.1%)	11 (57.9%)	15 (78.9%)
Total	96	15* (15.6%)	60* (62.5%)	74* (77.1%)

*One biopsy tested positive for both BPV1 and BPV2.

Figure 2.1 Diagrammatic representation of the genome of Bovine papillomavirus type 1 (BPV1). Open reading frames of Early genes (E1 to E8) and Late genes (L1 and L2).

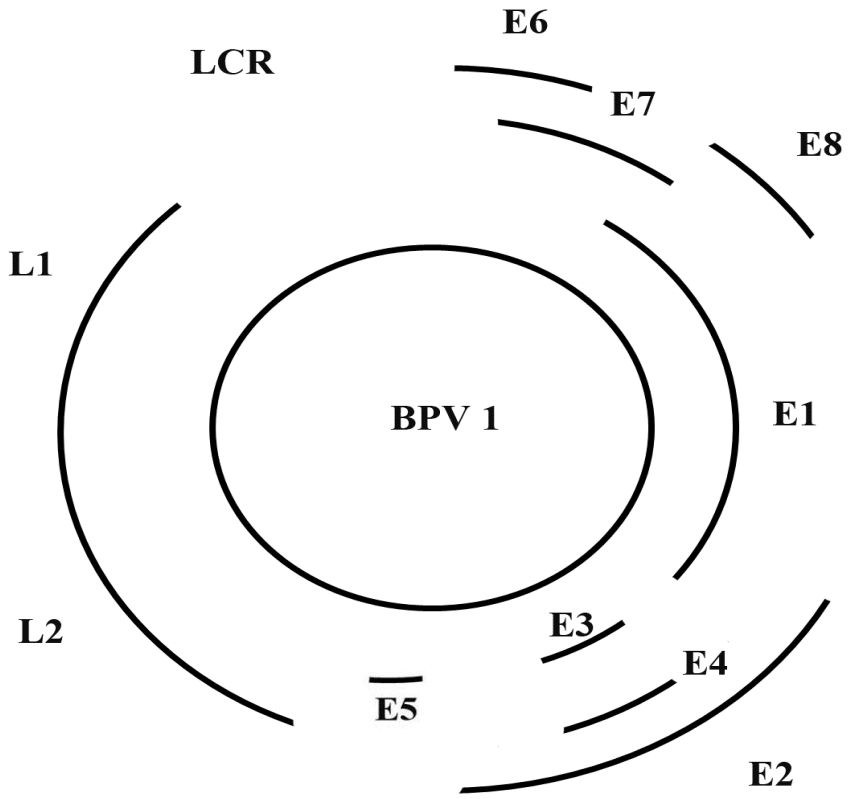

Figure 2.2 BPV1 PCR product sequences, primers and restriction enzyme cleavage sites. Comparison of recovered BPV DNA from equine sarcoids to reference strain of BPV1 previously recovered from equine sarcoids.

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                    *        20         *        40         *        60         *        80
1           : CCAAAGGCAAAGACTTTCTGAAACATGTACCACTACCTCCTGGAATGAACATTTCCGGCTTTACAGCCAGCTTGGACTTCTGATCAC :
2           : CCAAAGGCAAAGA-TTTCTGAAACATGTACCACTACCTCCTGGAATGAACATTTCCGGCTTTACAGCCAGCTTGGACTTCTGATCAC :
AY232263.1 : CCAAAGGCAA-GACTTTCTGAAACATGTACCACTACCTCCTGGAATGAACATTTCCGGCTTTACAGCCAGCTTGGACTTCTGATCAC :
Consensus    CCAAAGGCAAaGAcTTTCTGAAACATGTACCACTACCTCCTGGAATGAACATTTCCGGCTTTACAGCCAGCTTGGACTTCTGATCAC

                  *       100         *       120         *       140         *       160         *
1           : TGCCATTGCTTTTTCTTCATCTGACTGGTGTACTATGCCAAATCT/ATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAACT :
2           : TGCCATTGCCTTTTCTTCATCTGACTGGTGTACTATGCCAAATCT/ATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAACT :
AY232263.1 : TGCCATTGCTTTTTCTTCATCTGACTGGTGTACTATGCCAAATCT/ATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAACT :
Consensus    TGCCATTGCtTTTTCTTCATCTGACTGGTGTACTATGCCAAATCTATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAACT

                   180         *       200         *       220         *       240
1           : GCTGCTGTTACTGTTCTTACTCTTGTTTTTTCTTGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCT : 246
2           : GCTGCTATTACTGTTCTTACTCTTGTTTTTTCTTGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCT : 245
AY232263.1 : GCTGCTGTTACTGTTCTTACTCTTGTTTTTTCTTGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCT : 245
Consensus    GCTGCTgTTACTGTTCTTACTCTTGTTTTTTCTTGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCT
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Primers are highlighted in light grey, restriction enzyme cleavage site in dark grey and nucleotide variations in black.

Figure 2.3 BPV2 PCR product sequences, primers and restriction enzyme cleavage sites. Comparison of recovered BPV DNA from equine sarcoids to reference strain of BPV2 previously recovered from equine sarcoids.

```
                    *        20         *        40         *        60         *        80
1           : CAAAGGCAA-GACTTTCTGAAACATGTCCCACTACCTCCTGGAATGAACATTTCCGGCTTTACGGCCAGCTTGGACTTTTAATCACT :
2           : CAAAGGCAAAGA-TTTCTGAAACATGTCCCACTACCTCCTGGAATGAACATTTCCGGCTTTACGGCCAGCTTGGACTTTTAATCACT :
AY232264.1  : CAAAGGCAA-GACTTTCTGAAACATGTCCCACTACCTCCTGGAATGAACATTTCCGGCTTTACGGCCAGCTTGGACTTTTAATCACT :
              CAAAGGCAA GAcTTTCTGAAACATGTCCCACTACCTCCTGGAATGAACATTTCCGGCTTTACGGCCAGCTTGGACTTTTAATCACT

                  *       100         *       120         *       140         *       160         *
1           : GCCATTTGTTTTTTTCATATCTCGTCTAGGCATACTATGCCG/AATCTATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAA :
2           : GCCATTTGTTATTTTCATATCTCGTCTAGGCATACTATGCCG/AATCTATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAA :
AY232264.1  : GCCATTTGTTTTTTTTCATATCTCGTCTAGGCATACTATGCCG/AATCTATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAA :
              GCCATTTGTTtTTTTCATATCTCGTCTAGGCATACTATGCCGAATCTATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAA

                 180         *       200         *       220         *       240
1           : CTGCTGCTGTTACTGTTTCTGCTGCTATTTTTCTTGGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCT : 247
2           : CTGCTGCTGTTACTGTTTCTGCTGCTATTTTTCTTGGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCT : 247
AY232264.1  : CTGCTGCTGTTACTGTTTCTGCTGCTATTTTTCTTGGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCT : 247
              CTGCTGCTGTTACTGTTTCTGCTGCTATTTTTCTTGGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCT
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Primers are highlighted in light grey, restriction enzyme cleavage site in dark grey and nucleotide variations in black.

3. IMMUNOHISTOCHEMICAL EXPRESSION OF CLEAVED CASPASE-3, B- CELL LYMPHOMA 2, AND SURVIVIN IN SARCOIDS FROM HORSES

This study used the paraffin histologic blocks acquired for use in the studies described in Chapter two. These blocks contained tissue from sarcoids of different clinical types. BPV was detected using PCR and, when present, BPV type was determined by RFLP. The method by which sarcoid tumours actually grow is unknown. Immunohistochemistry detection of three different antigens related to apoptosis was used to test the hypothesis that sarcoids grow by evading apoptosis. We were able to determine that sarcoids express Survivin and that there are differences amoung clinical types of sarcoids in the level of Survivin expression.

3.1 Abstract

Sarcoids are the most common tumour of the skin of horses. Despite being common, much of the detail of sarcoid transformation and growth is not understood. Whether sarcoid growth is associated with evasion of apoptosis by neoplastic fibroblasts is currently unknown. The aims of this study were to examine different clinical types of sarcoids and sarcoids associated with different Bovine papillomavirus (BPV) types to determine if there was differential expression of immunohistochemical markers associated with apoptosis, Cleaved Caspase-3 (ClC3), and antiapoptotic factors, B-Cell Lymphoma 2 (Bcl-2) and Survivin. Seventy-six sarcoids that included tumours of five clinical types collected from multiple diagnostic centres in Western Canada were used. All 76 of these tumours were immunohistochemically stained for the identification of ClC3 and Survivin, and 66 were stained for Bcl-2. ClC3 staining was present in only small numbers of neoplastic cells in all types of sarcoids. However, a significantly higher proportion of cells in fibroblastic type sarcoids had positive staining compared to occult type sarcoids. No differences in the expression of Bcl-2 between clinical types were noted. All five types of sarcoids had Survivin staining of some of the neoplastic cells. Nodular and fibroblastic sarcoids had significantly higher proportions of Survivin staining cells than occult sarcoids. There was no relationship between BPV type and the staining characteristics of any of these markers. Survivin expression was detected in equine sarcoids and increased levels of expression were associated with more aggressive clinical behaviour.

3.2 Introduction

Sarcoids are fibroblastic tumours of the equine skin and are the most common tumours seen in horses (Scott D.W. and Miller W.H., 2003;Valentine, 2006). Sarcoids have been reported in many countries around the world (Bloch, Breen, and Spradbrow, 1994;Borzacchiello and others, 2008;Brostrom and others, 1988;Carr and others, 2001;Fretz P.B. and Barber S.M., 1980;Jackson, 1936) and their existence has been described since ancient times (Erk, 1976). Although these tumours typically do not metastasize, they are regarded as cosmetic defects, can become ulcerated and infected and, when occurring near the eyes or on the eyelids, can impair vision.

Six types of sarcoids have been identified based on their clinical appearance: occult, verrucose, nodular, fibroblastic, mixed and malevolent (Knottenbelt, 2005). The first five of these are relatively commonly seen (Martens and others, 2000) while the sixth, the malevolent type, has only been described by one group (Knottenbelt and Kelly, 1995). Occult sarcoids are the most clinically benign and often appear as an area of hair loss. The verrucose type, as the name implies, is a wart-like growth. Nodular sarcoids are protuberances beneath an intact epithelium. Fibroblastic sarcoids are the most clinically aggressive, with exophytic, sometimes rapid growth, and frequent ulceration. The final common type of sarcoid is the mixed type which exhibits characteristics of two of the previously described types within a single growth or contiguous series of growths.

Despite being such a common tumour, much of the detail of how dermal fibroblasts transform to produce sarcoids is not understood. The use of molecular diagnostic techniques, most particularly polymerase chain reaction (PCR), has identified that Bovine papillomavirus (BPV) is commonly associated with equine sarcoids (Carr and others, 2001;Teifke, Hardt M., and Weiss, 1994;Teifke and Weiss, 1991;Wobeser and others, 2010). Two types of BPV are associated with the development of equine sarcoids, BPV types 1 (BPV1) and 2 (BPV2). Currently, no differences in the sarcoids associated with these two types have been published.

Growth of a tumour depends upon an increase in the size of neoplastic cells, an increase in the number of neoplastic cells or a reduction in the rate at which neoplastic cells die and are removed, as individual processes or combined processes. Sarcoids are tumours of equine dermal fibroblasts which are spindle-shaped cells and significant tumour growth due to increase in cell size seems unlikely. More likely, tumour growth depends on increases in replicative rate,

reduction in cell death rates, or both. Several studies have examined the replicative rate of equine sarcoids as defined by expression of Ki-67, an immunohistochemical marker of cell proliferation (Borzacchiello and others, 2008;Martens and others, 2000;Nixon and others, 2005). All of these studies found that the replicative rate of sarcoids was low, and in some cases not dissimilar to normal equine skin.

Evasion of apoptosis is thought to be an important event in Human papillomavirus (HPV) induced malignant transformation of cervical epithelial cells (Branca and others, 2005). Studies to examine whether neoplastic fibroblasts in equine sarcoids evade apoptosis by some method have not been published.

Apoptosis is a well-defined chain of reactions that leads to the orderly death, dismantling and removal of senescent or damaged cells. The ability of neoplastic cells to evade apoptosis allows for their continued growth and survival. Numerous cell markers have been used in both human and veterinary medicine to assess apoptosis in tumours. However, compared to studies in cats and dogs, little work has been done on the use of molecular markers in equine neoplasms, including sarcoids.

There are a variety of immunohistochemical antibodies available to detect markers associated with apoptosis. Cleaved caspase-3 (ClC3) is expressed in cells undergoing apoptosis and its frequency can be evaluated in tumours and compared to appropriate normal tissues to determine if different levels of apoptosis are present in the tumour. Other genes, such as B-Cell Lymphoma 2 (Bcl-2) and Survivin, are not expressed by normal dermal fibroblasts, but are expressed in the cells of some tumours; expression of these genes alters normal cellular processes allowing these cells to evade the normal apoptotic mechanisms. In addition, expression of Bcl-2 and Survivin, as detected by immunohistochemistry, has been found within HPV virally induced dysplastic and cancerous cervical lesions of women. The expression of these markers was associated with worsened prognosis and more aggressive clinical tumour type in these tumours (Frost and others, 2002;Yaqin, Runhua, and Fuxi, 2007).

The aims of this study were to use immunohistochemistry to detect expression of ClC3, Bcl-2 and Survivin in equine sarcoids and to determine if expression differs with clinical type of sarcoid and BPV type within the sarcoid.

3.3 Materials and Methods

3.3.1 Cases

Formalin-fixed paraffin-embedded (FFPE) biopsy samples of equine sarcoids representing each of the five clinical types (occult, verrucose, nodular, fibroblastic and mixed) were collected from multiple veterinary diagnostic centres in Western Canada. These samples had been previously diagnosed as being PCR positive or negative for BPV and, where positive, the type of BPV had been determined using restriction fragment length polymorphism (RFLP) (Wobeser and others, 2010). From biopsies in this collection, the FFPE tissue samples of sarcoids of each clinical type were included in this study. Each of the samples originated from a different horse. All samples had been reviewed and had a histologic appearance consistent with an equine sarcoid. Of the 76 sarcoids examined in this study, 13 were BPV1 positive, 52 were BPV2 positive and eleven were BPV negative.

3.3.2 Immunohistochemical staining

Expression of ClC3, Bcl-2 and Survivin was evaluated by immunohistochemistry. From each FFPE sarcoid sample three 4 μm serial sections were cut; one for each antibody used. Staining was performed using a Benchmark staining platform[a], with BMK iVIEW DAB Paraffin detection kits[b] using a streptavidin-biotin amplification system. Heat-induced epitope retrieval consisted of applying cell conditioner #1 to sections. Slides used for antibody staining were counterstained with hematoxylin and bluing reagent. For the detection of Bcl-2, a commercial mouse monoclonal antibody[c] was utilized at a dilution rate of 1:10. Staining for ClC3 was performed with a commercial rabbit polyclonal antibody[d] at a dilution rate of 1:50. The presence of Survivin was determined using a commercial rabbit polyclonal antibody[e] at a dilution rate of 1:1000. Only 66 of the 76 samples were stained with antibody to Bcl-2 because of a limited quantity of antibody available.

3.3.3 Control Tissues

These immunohistochemical markers have not been previously used in FFPE equine tissue so positive and negative equine control tissues were chosen based on reported staining characteristics in other species. FFPE control tissues of equine origin including adult haired skin, and fetal tissue including thymus, stomach, pancreas and lymph node, were used for each staining run of the three antibodies. Basal epithelial cells of adult haired skin in other species

have been shown to express Bcl-2 immunohistochemically (Madewell and others, 1999). This being the case, adult haired equine skin was used as a positive control for this antibody. Also, in the same samples of adult haired skin, dermal fibroblasts are present and these do not stain immunohistochemically with Survivin or Bcl-2 in other species (Johnson and Howerth, 2004;Madewell and others, 1999). Consequently, equine dermal fibroblasts were used as negative controls for both Bcl-2 and Survivin. Based on immunohistochemical staining in other species, equine thymus and lymph node were also included as positive controls for Bcl-2 (Korsmeyer, 1992;Madewell and others, 1999) and Clc3 (Resendes and others, 2004). Equine stomach and thymus were included as positive controls for Survivin based on their reported staining in other species (Johnson and Howerth, 2004).

3.3.4 Assessment of staining

Sections of each sarcoid sample were examined in the same manner for antigen expression. Stained sections were examined by a single pathologist without knowledge of sarcoid classification type or BPV status. Sections were considered positive if specific brown intracytoplasmic or intranuclear immunohistochemical staining was observed in neoplastic fibroblasts. For each staining batch, the control slide was carefully examined to determine staining characteristics. Then each sarcoid sample in that batch was examined at 200x magnification to subjectively identify the area within the neoplasm that exhibited the largest concentration of positively staining neoplastic cells, if present. When positive staining was present, five microscopic fields at 400x magnification were selected within the area chosen as most intensely stained and 100 neoplastic cells were counted in each field. The number of positively staining neoplastic cells within these 500 cells was recorded and the mean number of positively staining neoplastic cells in each sample was determined and recorded as a percentage of positively staining cells per field. If no staining was present in the sample, the sample was considered to be negative for staining for that marker and the percentage of positively staining cells per field was recorded as zero. To better define the character of the immunohistochemical staining in addition to the number of cells with positive staining, the location within the cell (nuclear or cytoplasmic) and staining pattern (diffuse, granular or vesicular) was recorded.

3.3.5 Data Analysis

For each of the antibodies, comparisons of the median percentage of positive staining among clinical classifications of all sarcoids and among BPV status groups (BPV1, BPV2 and BPV negative) were performed using Kruskal-Wallis Equality of Populations rank test and Dunn's Multiple Comparison Test. Significance was set at $p<0.05$. Analysis was performed with the aid of a statistical software package[f].

3.4 Results

Sarcoids of five clinical types were selected from a collection of equine sarcoid biopsies from horses in Western Canada. Immunohistochemical staining results for Clc3, Survivin, Bcl-2 are shown in Tables 3.1, 3.2, and 3.3. All three of the antibodies produced immunohistochemical staining of control tissues consistent with the staining patterns described in other species.

3.4.1 ClC3

Not surprisingly, ClC3 was expressed in control samples of thymus and lymph node (Figure 3.1C) and was not expressed in control skin samples (Figure 3.1A). ClC3 staining was present in 60 of 76 sarcoid samples, including all types of sarcoids (Table 3.1). Only small numbers of neoplastic cells stained positively in all types of sarcoids. When staining was present, cells had prominent cytoplasmic granular staining (Figure 3.1B). The highest proportion of staining was in fibroblastic sarcoids where 15 of 15 sarcoids stained positively, with a median of 1.2% of neoplastic fibroblasts in this type stained positively. The lowest proportion of staining was present in occult sarcoids where eight of 15 samples had positive staining of any neoplastic fibroblasts and a median of 0.40% of cells stained positively. These two medians are significantly different ($p<0.05$). Complete results are summarized in Table 3.1.

3.4.2 Bcl-2

As expected, there was positive staining for Bcl-2 in the basal cells of the epithelium of control and sarcoid samples (Figure 3.2A), cells in lymph node (Figure 3.2C), and in pancreas. Fibroblasts in control skin samples did not stain with Bcl-2 antibody (Figure 2A). Weak diffuse cytoplasmic staining for Bcl-2 was present only in small numbers of sarcoid samples (19 of 66 sarcoids) and a small number of neoplastic cells in these samples, (Table 3.2, Figure 3.2B).

Again, the highest proportion of staining in neoplastic cells was within fibroblastic sarcoids, but the magnitude of difference amoung the clinical types of sarcoids was not statistically significant.

3.4.3 Survivin

Control tissues stained as expected in that, Survivin was detected in fetal thymus (Figure 3.3C) and gastric epithelium and no staining was present in control adult equine dermal fibroblasts (Figure 3.3A). All types of sarcoids had neoplastic cells that stained for Survivin and 59 of the 76 sarcoids stained positively (Table 3.3). Higher proportions of nodular and fibroblastic sarcoids stained positively (14 of 15 samples and 13 of 15 samples respectively), as compared to occult sarcoids where seven of 15 sarcoids had positive staining. Median percentage of positively staining cells in the area of greatest staining intensity varied from 0% in occult sarcoids to 6.6% in fibroblastic sarcoids which is statistically significant ($p<0.05$). Positive staining was nuclear and granular in character (Figure 3.3B).

3.4.4 Immunohistochemical staining and BPV type

No statistically significant difference was found in staining characteristics amoung any of the antibodies (Clc3, Survivin, Bcl-2) and BPV type (BPV1, BPV2 or BPV negative) in the 76 sarcoids (data not shown).

3.5 Discussion

Apoptosis, the programmed death and removal of cells, is a common occurrence in neoplastic processes as a result of irreparable DNA damage. However, evasion of apoptosis by neoplastic cells is also an adaptation seen in many different tumours and allows for neoplastic cell survival and replication. As far as can be determined, examination of equine sarcoids to detect evasion of apoptosis by neoplastic cells has not been previously reported. In this study, equine sarcoids of different clinical types were examined immunohistochemically to determine their expression of three markers: one associated with apoptosis (ClC3) and two associated with the evasion of apoptosis (Bcl-2 and Survivin).

No previous reports describing the use of these markers in FFPE equine tissues of any sort have been published. All of these proteins are highly conserved among species and have

been studied in other domestic animal species. Based on staining characteristics reported in other species, numerous normal FFPE equine tissues, including adult haired skin, fetal tissues including thymus, stomach, pancreas and lymph node, were examined and found to exhibit the expected staining characteristics for these antibodies (Johnson and Howerth, 2004;Korsmeyer, 1992;Madewell and others, 1999;Resendes and others, 2004), as shown in Figures 3.1, 3.2 and 3.3.

Immunohistochemistry has been previously used to help identify apoptotic cells in formalin-fixed tissue sections. Two methods are commonly used, immunohistochemistry with antibodies to ClC3 and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). ClC3 was chosen for use in this study as it has been reported to be more specific in the identification of apoptotic cells than is TUNEL (Duan and others, 2003;Gown and Willingham, 2002). The number of apoptotic cells present in sarcoids in the current study was generally low (Table 3.1). However, differences in positive staining of neoplastic fibroblasts were present between the types of sarcoids examined; all fibroblastic sarcoids had some positive staining neoplastic cells, while seven of 15 occult sarcoids had no positive staining whatsoever. A significantly higher proportion of neoplastic cells in fibroblastic sarcoids were positive for ClC3 than in occult sarcoids. This may reflect a more unstable population of neoplastic cells in fibroblastic sarcoids, as compared to occult sarcoids. By definition, fibroblastic sarcoids are more likely to be ulcerated and secondarily inflamed than occult sarcoids, (Knottenbelt, 2006) and, as ulceration and inflammation are positively associated with apoptosis (Myers and McGavin, 2007), the observed differences in apoptotic levels may be attributable to this.

In the current study Bcl-2, a marker of decreased apoptosis, was present in a relatively few sarcoids (19 of 66 sarcoids) of any clinical type and a low percentage of neoplastic cells in these 19 sarcoids (Table 3.2). Having few neoplastic fibroblasts evading apoptosis, as evidenced by Bcl-2 staining, and few of these cells in apoptosis as demonstrated by Clc3 staining is not contradictory, as Bcl-2 is not normally expressed at all in the fibroblasts of adult skin, yet this lack of expression is not associated with increased levels of apoptosis (Hockenbery and others, 1991;Pablos and others, 1997). No significant difference in expression of Bcl-2 was present among the various types of sarcoids examined. This would suggest that the expression of Bcl-2 has a limited role in the development of the different clinical classifications of equine sarcoids.

Survivin is a member of a group of proteins, collectively known as inhibitor of apoptosis proteins, that are found in a wide variety of life forms from yeasts to vertebrates (Johnson and Howerth, 2004). Survivin has a role in two important cell functions: regulation of normal cell division (Li and others, 1998) and evasion of apoptosis (Altieri and Marchisio, 1999). Normally, Survivin is not expressed in differentiated adult tissues (Ambrosini, Adida, and Altieri, 1997), but it is widely expressed in fetal tissues (Adida and others, 1998). The expression of Survivin in many different neoplastic processes has been described and its expression is frequently associated with more clinically aggressive tumour types (Frost and others, 2002;Murakami and others, 2008;Rebhun and others, 2008;Takai and others, 2002;Yaqin, Runhua, and Fuxi, 2007).

In the current study, expression of Survivin was commonly detected (59 of 76 samples) in the neoplastic fibroblasts of equine sarcoids (Table 3.3). Positive staining for Survivin was present in most nodular (14 of 15 sarcoids) and fibroblastic sarcoids (13 of 15 sarcoids), but in only eight of 15 occult sarcoids. The proportion of positively staining neoplastic cells varied among samples and significantly higher proportions were present in the areas of greatest staining intensity of fibroblastic and nodular type sarcoids than in occult type sarcoids (Table 3.3). The occult type of sarcoid is the most clinically benign, while the fibroblastic type is the most clinically aggressive (Knottenbelt, 2005). The increased likelihood of Survivin expression with increasing clinical aggressiveness has been described in other tumours caused by papillomaviruses, most notably in cervical cancers in women caused by HPV (Yaqin, Runhua, and Fuxi, 2007). Increased Survivin expression may represent an explanation for the increased clinical aggressiveness of fibroblastic sarcoids as compared to less aggressive types like occult sarcoids.

Expression of E6, an oncogene of papillomaviruses, in HPV infections has been positively correlated with Survivin expression (Borbely and others, 2006). This correlation was shown to be related to effects of E6 on p53, an important tumour suppressor gene. Specifically, in HPV, E6 causes degradation of p53 and removal of p53 is correlated to increased Survivin expression in these infected cells. In contrast to HPV, BPV E6 does not cause degradation of p53 (Werness, Levine, and Howley, 1990), but instead has been shown to sequester p53 in an abnormal perinuclear location which may prevent its normal function (Martens and others,

2000;Nixon and others, 2005). Whether the prevention of normal p53 functioning is the cause of the expression of Survivin in sarcoids seen in the current study is unknown.

Expression of Survivin in sarcoids may have implications for the recurrence of sarcoids following treatment. However, further research would be needed to determine this. In addition, this expression may be useful for the development of new treatment modalities for equine sarcoids. As Survivin is not normally expressed in adult tissues, it may represent a novel therapeutic target. Several studies have been recently published using therapies targeted at Survivin expression in neoplastic cells as treatment methods (Chen and others, 2009;Lu and others, 2008;Wu and others, 2008). Whether equine sarcoids could be treated in a similar manner is currently unknown.

The BPV present in the sarcoid samples in this study had been previously typed as BPV types 1 or 2 using PCR and RFLP. No difference in the expression of any of the three markers used in this study could be seen according to BPV type associated with these sarcoids.

In summary, in this study immunohistochemistry was used to detect the presence of three markers associated with apoptosis or evasion of apoptosis (ClC3, Bcl-2 and Survivin) in different clinical types of equine sarcoids. The use of these immunohistochemical markers on FFPE equine tissues has not been previously reported. Levels of apoptosis as measured by immunoreactivity for ClC3 were significantly higher in fibroblastic sarcoids than in the less clinically aggressive occult sarcoids. No differences in Bcl-2 expression were present among sarcoid types. Survivin was expressed in all types of sarcoids and in more clinically aggressive types of sarcoids the expression of Survivin was significantly higher. This suggests that evasion of apoptosis is a clinically important factor in sarcoid development. No differences in the expression of any of the three markers were present among sarcoids associated with different BPV types. This study provides further insight into the pathogenesis of sarcoid progression and suggests that Survivin may be a useful therapeutic target for the development of new therapies for treatment of sarcoids.

3.6 Sources and Manufacturers

[a]Benchmark staining platform, Ventana Medical Systems Inc., Tucson, AZ

[b]BMK iVIEW DAB Paraffin detection kits, Ventana Medical Systems Inc., Tucson, AZ

[c]Bcl2 Oncoprotein VP-B201, Vector Laboratories, Burlingame, CA

[d]Cleaved Caspase-3 (Asp175) Antibody #9661, Cell Signaling Technologies, Danvers, MA

[e]Survivin Antibody NB500-201, Novus Biologicals LLC, Littleton, CO

[f]Prism 5 for Windows, Graphpad Software, La Jolla, CA

Table 3.1. Immunohistochemical expression of Cleaved Caspase-3 in 5 clinical types of equine sarcoids.

Clinical Type of Sarcoid	Proportion of Samples positive	% of Positively Staining Cells		
		Minimum	Median	Maximum
Occult	8/15	0.00	0.40	1.00
Verrucose	13/16	0.00	0.70	2.20
Nodular	13/15	0.00	0.60	2.00
Fibroblastic	15/15	0.60	1.20	4.00
Mixed	11/15	0.00	0.60	4.00

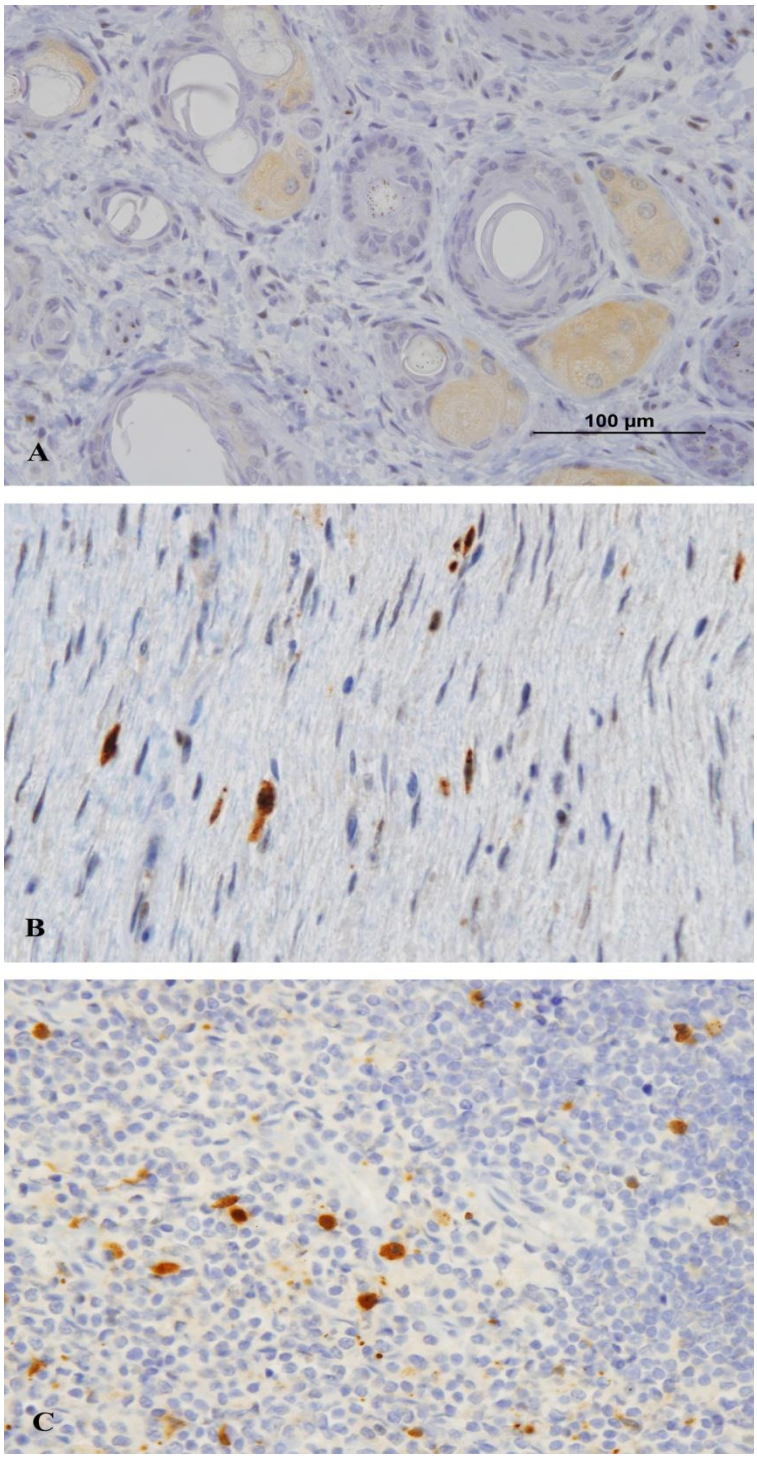
Table 3.2. Immunohistochemical expression of B-Cell Lymphoma 2 in 5 clinical types of equine sarcoids.

Clinical Type of Sarcoid	Proportion of Samples positive	% of Positively Staining Cells		
		Minimum	Median	Maximum
Occult	2/13	0.00	0.00	2.80
Verrucose	3/14	0.00	0.00	5.00
Nodular	4/14	0.00	0.00	2.80
Fibroblastic	6/12	0.00	1.20	9.80
Mixed	4/13	0.00	0.00	4.80

Table 3.3. Immunohistochemical expression of Survivin in five clinical types of equine sarcoids.

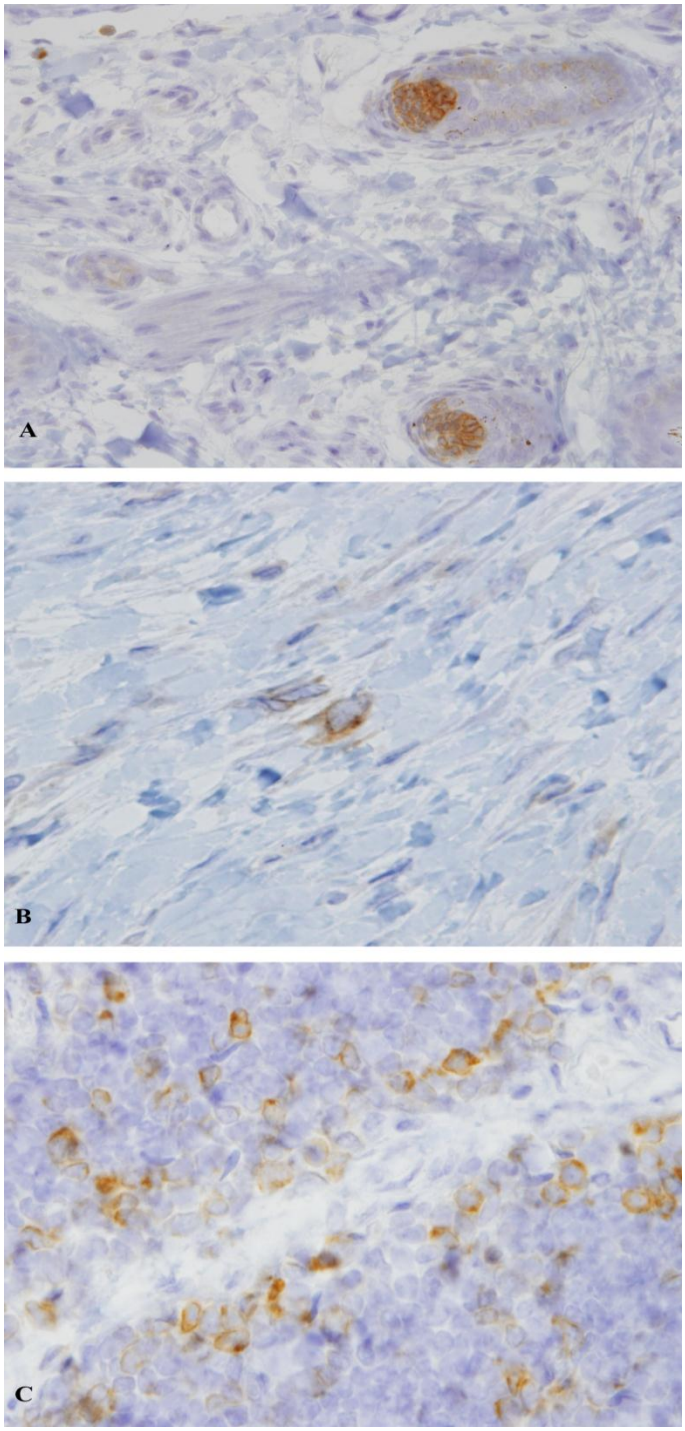
Clinical Type of Sarcoid	Proportion of Samples positive	% of Positively Staining Cells		
		Minimum	Median	Maximum
Occult	7/15	0.00	0.00	10.20
Verrucose	13/16	0.00	3.00	17.60
Nodular	14/15	0.00	5.20	18.40
Fibroblastic	13/15	0.00	6.60	21.40
Mixed	12/15	0.00	4.00	15.00

Figure 3.1. Immunohistochemical staining using antibody to a marker of apoptosis in sections of normal and sarcoid bearing equine tissue. Antibody staining for Cleaved Caspase 3.



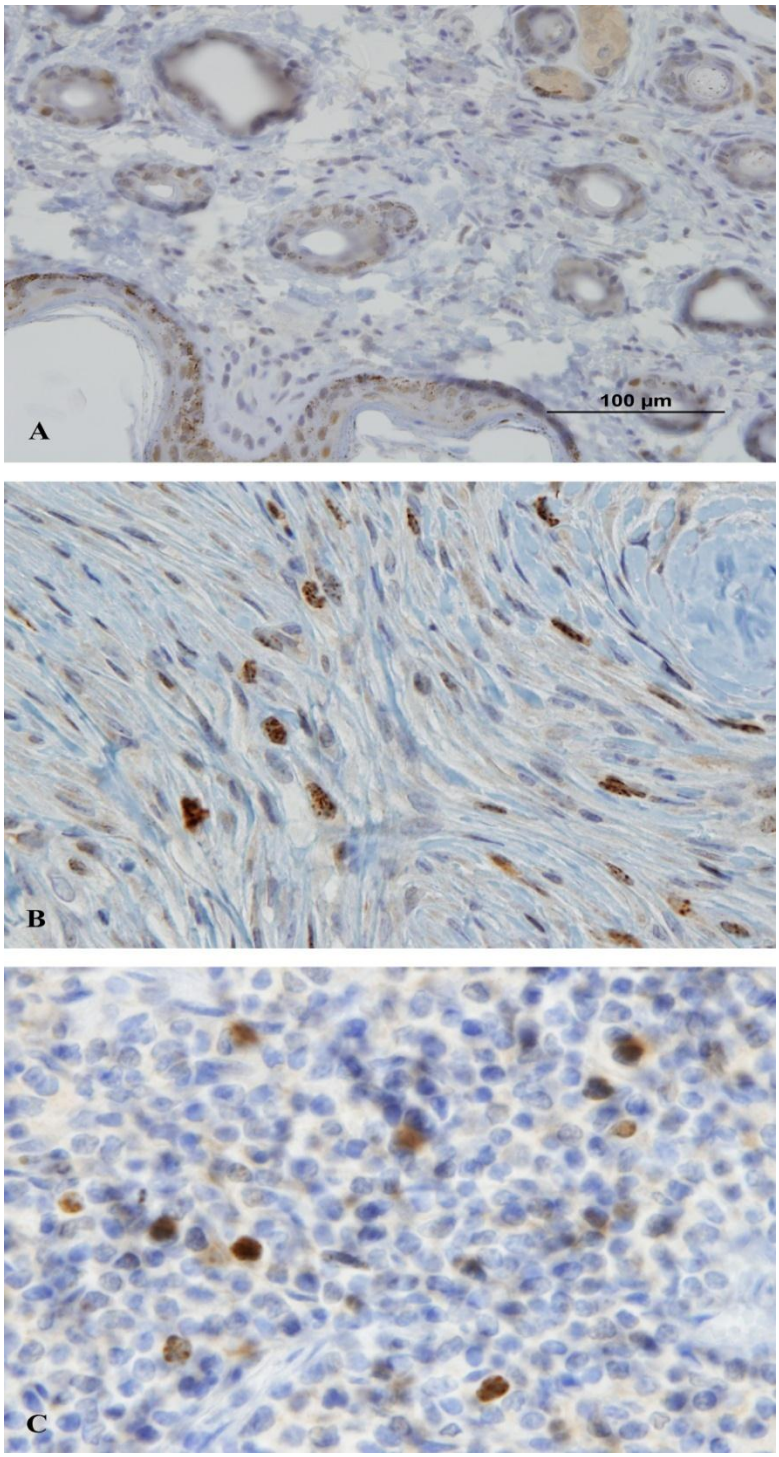
A. Negative Control - Normal equine skin: note lack of staining of dermal fibroblasts. B. Fibroblastic sarcoid: note strong positive cytoplasmic staining of neoplastic fibroblasts. C - Positive control lymph node: note positive cytoplasmic staining.

Figure 3. 2. Immunohistochemical staining using antibody to a marker of evasion of apoptosis in sections of normal and sarcoid bearing equine tissue. Antibody staining for B-Cell Lymphoma 2.



A. Negative Control- Skin: note positive staining of basal epithelial cells and lack of staining of dermal fibroblasts. B. Fibroblastic sarcoid: note positive cytoplasmic staining of neoplastic fibroblasts. C. Positive control - Lymph Node: note positive cytoplasmic staining.

Figure 3.3. Immunohistochemical staining using antibody to a marker of evasion of apoptosis in sections of normal and sarcoid bearing equine tissue. Antibody staining for Survivin.



A. Negative Control- Skin: note lack of staining of dermal fibroblasts. B. Fibroblastic sarcoid: note strong positive granular cytoplasmic staining of neoplastic fibroblasts. C. Positive control – Thymus: note positive cytoplasmic staining.

4. LOCALIZATION OF BOVINE PAPILLOMAVIRUS IN EQUINE SARCOIDS AND INFLAMMATORY SKIN CONDITIONS OF HORSES USING LASER MICRODISSECTION AND TWO FORMS OF DNA AMPLIFICATION

In addition to sarcoids, BPV has been found in non-sarcoid skin conditions. How often this occurs and whether the location of BPV is different in sarcoids than in these other skin conditions is unclear. To investigate this, PCR was used to detect BPV in sample paraffin histologic blocks of a variety of inflammatory skin conditions of horses and normal equine skin. Those samples in which BPV was detected were used for further study. In addition to these, the BPV positive paraffin histologic blocks from sarcoids in the studies described in Chapter Two were used. Using laser microdissection, all of the samples were divided into various tissue compartments and each of these compartments was then individually tested using PCR and a new amplification technique, LAMP, for the presence of BPV DNA. BPV DNA was detected in nearly half of all non-sarcoid samples tested. In sarcoids, BPV DNA was more likely to be found in morphologically neoplastic fibroblasts than in other tissues from the same histologic section. BPV DNA was more often present in the epithelium of non-sarcoid samples than in the epithelium of sarcoid samples. In non-sarcoid samples areas of inflammation were significantly more likely to contain BPV DNA than were non-inflamed areas.

4.1 Abstract

Equine sarcoids are the most common skin tumour of horses. Bovine papillomavirus (BPV) has been suggested as the causative agent of sarcoid formation. Yet, several studies have shown that BPV is present in swabs or biopsies from non-sarcoid bearing equine skin. The frequency of BPV detection in or on skin biopsies from non-sarcoid inflammatory conditions has never been examined, nor has the location of the BPV DNA in these samples been determined. BPV DNA has recently been demonstrated in keratinocytes of a small number of equine sarcoids and its presence in these cells may depend on the clinical type of sarcoid examined. In the current study, equine skin biopsies from a variety of different non-sarcoid skin conditions and histologically normal skin from horses with no reported history of sarcoids were examined by PCR for the presence of BPV DNA. BPV DNA was found in all different types of skin conditions, as well as normal skin. Overall, 41/86 skin biopsies from horses without sarcoids were found to contain BPV DNA. Laser microdissection followed by DNA amplification by both PCR and isothermal loop mediated amplification was performed on these biopsies and upon, 70 BPV positive sarcoid biopsies in order to localize BPV DNA within these biopsies. Sarcoid samples were more likely to have BPV DNA in morphologically neoplastic fibroblasts than in other tissues on the same histologic slide ($p=0.0005$). The location of BPV DNA was different between sarcoid and non-sarcoid groups. Non-sarcoid skin biopsies were significantly more likely to have BPV DNA within intact or inflamed epidermis than sarcoids ($p=0.016$ and $p=0.007$, respectively). Sarcoids were more likely to have BPV DNA in the non-inflamed dermis than non-sarcoid samples, but not significantly so. In addition, areas of inflammation within the dermis and epidermis were more likely to contain BPV DNA than non-inflamed areas ($p=0.008$ and $p=0.009$, respectively). BPV DNA was also found in the epidermis of all clinical types of equine sarcoids examined, but more frequently in occult sarcoids than in fibroblastic and nodular types ($p=0.03$ and $p=0.01$, respectively). These results suggest that BPV is commonly found in equine skin. In non-sarcoid samples, BPV is more often located in the epidermis than in sarcoids. Wherever it is located, it is more likely to be associated with inflammation suggesting that microscopic damage and inflammation within the skin may be a sufficient predisposing factor to the development of sarcoids.

Key Words: equine sarcoid; Bovine papillomavirus; laser microdissection, LAMP

4.2 Introduction

Bovine papillomavirus (BPV) has been associated with the development of the most common skin tumour of horses, the equine sarcoid (Chambers and others, 2003). This association has been made based on the detection of BPV DNA in equine sarcoids and its absence in other equine skin tumours (Nasir and Campo, 2008). Given this association, BPV has been suggested as the cause of equine sarcoids. However, some studies have found BPV DNA in the skin of a small number of horses with non-sarcoid related inflammatory skin conditions, but the significance of this is unknown (Angelos and others, 1991;Chambers and others, 2003;Yuan and others, 2007).

The frequency with which BPV can be found in non-sarcoid skin conditions has not been determined in a larger sample of equine tissues. In addition, it is unclear where BPV is located histologically in these biopsies from non-sarcoid bearing horses. If the same BPV can be commonly found in non-sarcoid skin conditions of horses and is present in the same histologic locations in these conditions as in equine sarcoids, the theory that BPV causes equine sarcoids may be called into question.

Previous studies have utilized polymerase chain reaction (PCR) to amplify the BPV DNA present in samples to allow for its identification, but this technique does not identify where within the sample BPV is located (Bloch, Breen, and Spradbrow, 1994;Carr and others, 2001;Martens and others, 2001;Otten and others, 1993;Teifke, Hardt M., and Weiss, 1994). Studies have demonstrated the location of BPV in sarcoids using *in situ* hybridization (ISH) (Kidney and Berrocal, 2008;Teifke, Hardt M., and Weiss, 1994). This technique allows for the visualization of BPV, but does not amplify the BPV to any significant degree when compared to PCR and is, therefore, a much less sensitive technique for identification of DNA when it is present in small quantities (Martens, De Moor, and Ducatelle, 2001). Laser microdissection (LCM) allows for the very precise excision of a cell or cells from a sample of interest. These excised cells become the template for DNA amplification allowing an accurate determination of where in a tissue sample the DNA of interest is located.

Numerous techniques exist to amplify DNA. PCR has frequently been used in the study of equine sarcoids. More recently, another technique, isothermal loop mediated amplification (LAMP), has been developed (Notomi and others, 2000). It has been purported to be as sensitive as PCR for the detection of DNA and is potentially more rapid and less expensive (Tomita and

others, 2008). The use of LAMP in equine tissue or in combination with laser microdissection has not, to the authors' knowledge, been previously reported.

Historically, BPV DNA had only been found within the neoplastic fibroblasts of equine sarcoids and not within the epidermis. However, a recent study examined a small number of sarcoids using LCM and found BPV DNA in the keratinocytes of the epidermis (Bogaert and others, 2010). The presence of BPV DNA varied depending on the type of sarcoid examined. The authors' suggested that BPV DNA location may change as sarcoids progress to different clinical types. Only one clinical type of sarcoid was identified and examined in this 2010 study, so further research to see if this result is repeatable and applies to different clinical types of sarcoids is required.

This study consisted of three parts. Part A was conducted to determine the frequency with which BPV DNA could be detected in non-sarcoid skin conditions of horses and whether it was the same type and has the same partial gene sequence as that found in sarcoids. Part B used BPV positive sarcoid biopsies and BPV positive non-sarcoid skin condition biopsies from part A and utilized LCM in combination with PCR and LAMP to determine if differences existed in the location of BPV in BPV positive sarcoids and non-sarcoid skin conditions of horses. In addition, the viability of LAMP as a DNA amplification technique in formalin-fixed paraffin embedded (FFPE) equine tissue dissected by LCM was examined. The information gleaned from part A and B, helped further clarify the role of BPV in the development of equine sarcoids. In part C of this study, the location of BPV DNA in a variety of different clinical types of sarcoids was determined to discover if BPV location varies between types and between more and less clinically aggressive types.

4.3 Materials and Methods

4.3.1 Detection of BPV in non-sarcoid conditions of equine skin

Surgical equine skin biopsies of non-neoplastic conditions diagnosed during the 12 year period between January 1, 1986, and December 31, 2007, inclusive, were identified by using computer-based record searches at the Department of Veterinary Pathology at the University of Saskatchewan and Prairie Diagnostic Services, Inc., (PDS) in Regina and Saskatoon, Saskatchewan. From this series of collected records, samples of inflammatory skin conditions of a variety of different types were selected. These conditions were chosen based on their

frequency of occurrence or clinical similarity to equine sarcoids (Table 4.1). Normal skin samples were selected from horses with no reported history of sarcoids and which had morphologically and histologically normal skin biopsies taken as control samples in cases of non-sarcoid inflammatory skin conditions. In total, the 86 most recent cases were chosen (Table 4.2). From these cases, the formalin-fixed paraffin embedded (FFPE) tissues were retrieved for PCR. The FFPE tissue blocks from these samples were utilized for PCR as previously described (Teifke, Hardt M., and Weiss, 1994). Briefly, 100 µm thick sections were cut from the FFPE block using a microtome. The microtome was cleaned using acetone between samples and blank paraffin blocks were cut every five samples for use as negative controls. The single primer set that amplifies a 244 base pair (bp) sequence spanning portions of the E2 gene, the intergenic spacer region and a small portion of the E5 gene of BPV type 1 (BPV1), or a similarly located 248 bp sequence of BPV type 2 (BPV2), was utilized (Table 4.3). To confirm that amplified product was BPV, the 17 most strongly positive PCR samples were nucleotide sequenced.

4.3.2 Laser microdissection, PCR and LAMP amplification of BPV positive equine sarcoids and non-sarcoid equine skin conditions

The 41 tissue biopsies from Part A that were positive by PCR for BPV DNA and all BPV positive equine sarcoid biopsies with sufficient remaining tissue (70 samples) collected for a previous study (Wobeser and others, 2010) were used for this study. Sections 5 µm thick were cut from the FFPE blocks, affixed to specialized membrane covered glass slides designed for use with laser microdissection (MembraneSlide 1.0 PEN; Carl Zeiss Canada Ltd., Toronto, Canada), and stained with hematoxylin and eosin. No coverslips were affixed.

Using a laser microscope dissection system (PALM MicroBeam; Carl Zeiss Canada Ltd., Toronto, Canada) each slide was examined under 200x magnification by a Diplomate of the American College of Veterinary Pathologists (BKW). When present on the slide, portions consisting of various tissue compartments (Table 4.4) were excised with the laser and catapulted into individual 500 µL microcapillary tubes (AdhesiveCap 500; Carl Zeiss Canada Ltd., Toronto, Canada). A minimum of 5×10^6 μm^3 of tissue was collected into each tube. DNA from the tissue within these tubes was extracted as previously described (Teifke, Hardt M., and Weiss, 1994). DNA extracted and precipitated was resuspended in 10 µL of Tris EDTA buffer.

To confirm that amplifiable DNA was present in each sample following DNA extraction, PCR using primers that amplify a 247 bp sequence of the equine actin gene was utilized (Table 4.3). The reaction mixture was as follows : 5 mM KCl and 1 mM Tris HCl, 3 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of each primer, 1.25 units of *Taq* DNA polymerase, 3 µL of template DNA and sufficient water for a final reaction volume of 50 µL. Amplification conditions were 94º C for 3 minutes, followed by 40 cycles of 94º C for 30 seconds, 59º C for 60 seconds, 72º C for 60 seconds followed by a final extension period of 72º C for 10 minutes. Confirmation that the PCR product was equine actin was conducted by nucleotide sequencing.

After confirmation that amplifiable DNA was present in the sample, PCR for BPV DNA using the same method used in the part A was performed on each sample with the exception that the number of cycles was increased from 40 to 45 to account for the smaller sample volume acquired by laser microdissection. In addition to PCR, LAMP was also performed on each sample. Positive and negative controls for both PCR and LAMP included sequenced, BPV-positive bovine papilloma, tissue-free sections of slide membrane to act as negative controls and standard no template controls. The LAMP primer set used amplifies a portion of the same sequence of the E2 and E5 genes used for PCR (Table 4.3). Using Primer Explorer V4 (Eiken Chemical Co LTD, Japan, http://primerexplorer.jp/e/) and the 244 base pair sequence of E2 and E5 that the BPV PCR amplifies, four sets of primers were designed. All were optimized using a cloned BPV E2 and E5 amplicon originally derived from an equine sarcoid. The primer set used in this experiment was chosen from the four designed sets based on consistent and strong amplification. LAMP reaction solution was composed of 2 mM Tris-HCl, 1 mM $(NH_4)_2SO_4$, 1 mM KCl, 0.2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8, 2 mM $MgCl_2$, 200 µM of each dNTP, 1.6 µM of FIP and BIP primers, 0.2 µM of F3 and B3 primers, 32 units of Bst DNA polymerase large fragment, 3 µL of template DNA and sufficient water for a final reaction volume of 50 µL. The reaction was heated at 65º C for 60 minutes. Following isothermal amplification, positive products were identified using electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

4.3.3 Presence of BPV DNA in the keratinocytes of different clinical types of sarcoids

The clinical types of sarcoids used in Part B of this study had been previously identified (Wobeser and others, 2010). Sarcoids from four different clinical types (occult, verrucous,

nodular and fibroblastic) were considered in this portion of the study. A fifth clinical type of sarcoid (mixed) used in part B was not utilized for this portion of the study as mixed sarcoids exhibit features from one or more of the other four sarcoid types and are, therefore, not a homogenous clinical type. BPV DNA positivity in the epidermal compartments from each of these four different clinical types of sarcoids was compared.

4.3.4 Statistical analysis

Comparisons of the recovery rates of BPV DNA between sarcoid and non-sarcoid groups were made using Fisher's exact tests. Comparisons of recovery rates of BPV DNA between matched samples of sarcoid or non-sarcoid groups were made using McNemar's test. Relative risk of BPV positivity in the epidermis amongst different clinical types of sarcoids was made based on comparison with the clinical types with the lowest risk of BPV positivity. The comparison of agreement between LAMP and PCR was made using an unweighted Kappa test. P values of <0.05 were considered significant.

4.4 Results

4.4.1 Presence of BPV in non-sarcoid conditions of equine skin

A total of 86 cases of non-sarcoid, non-neoplastic skin conditions including histologically normal skin from horses with no history of sarcoids were examined. Results are summarized in Table 4.2. BPV DNA positive samples were found in all conditions examined including normal skin. Overall, 41 of 86 (47.6%) samples were positive for BPV DNA. This is significantly lower than the proportion of sarcoid samples found to contain BPV DNA by the same methodology ($p=0.0001$). No significant difference in the proportion of positive samples was present amoung any skin conditions or normal skin. All negative controls including blank paraffin blocks cut during the microtome cutting procedure were negative.

A total of 17 of the 41 positive samples were sequenced. These sequences were compared to sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul and others, 1997). Of the 17 samples sequenced, eight were identical to BPV1 obtained from equine sarcoids in Western Canada (GenBank accession number FJ895875) and nine of the 17 showed 100% identity to BPV2 found in sarcoids in Western Canada (GenBank accession number FJ895874).

4.4.2 Laser microdissection, PCR and LAMP amplification of BPV positive equine sarcoids and non-sarcoid equine skin conditions

Sufficient material remained within the BPV DNA positive FFPE blocks to perform LCM, PCR and LAMP in 70 equine sarcoid samples and 37 non-sarcoid samples. These 107 samples were cut into a total of 334 tissue compartments (Table 4.4) for examination for BPV by DNA amplification.

PCR for equine actin was positive in all 334 tissue compartments showing that amplifiable DNA was present in all samples. BPV was detected by PCR, LAMP, or both in 157 of these compartments (47%). Results are summarized in Table 4.5 and examples of corresponding PCR and LAMP gels are given in Figure 4.1. PCR detected BPV DNA in more samples than did LAMP, detecting BPV DNA in 155 compartments, including 19 not detected by LAMP. LAMP detected BPV DNA in 138 compartments including two not detected by PCR. Overall, results from PCR and LAMP agreed on 313 of 334 samples (93.7%). This corresponds to an unweighted Kappa score of 0.87 (95% confidence interval 0.76-0.98), which is considered "almost perfect" agreement (Landis and Koch, 1977). Given this, tissue compartments from samples that were positive by either PCR or LAMP were considered positive for BPV DNA for the other parts of this study.

BPV DNA was found in 47% of tissue compartments in both sarcoid and non-sarcoid samples (Table 4.6). Not all tissue compartments were present in any given sample. For example, in normal skin, areas of inflamed epidermis or dermis do not exist. Similarly, some samples of sarcoid no longer contained areas of morphologically neoplastic fibroblasts. These areas were likely contained within sections of the FFPE block that were consumed during other testing. In sarcoid samples, BPV DNA was most commonly detected within areas of morphologically neoplastic fibroblasts in the dermis. BPV DNA was detected within 57 of the 65 samples where this tissue compartment existed. Other areas of the superficial and deep dermis that were morphologically normal also contained BPV DNA (4/14 and 10/21, respectively, Table 4.7). Overall, in sarcoid samples, BPV DNA was significantly more likely to be recovered from morphologically neoplastic fibroblasts than from other tissues on the same histologic slides ($p=0.0005$, Table 4.8).

Non-sarcoid samples were more likely to have BPV DNA in intact or inflamed epithelium than were sarcoid samples (p=0.016 and p=0.007 respectively, Table 4.7). Sarcoid samples were more likely to have BPV DNA in non-inflamed dermis than non-sarcoid samples, but the differences were not significant. Non-sarcoid samples were more likely to contain BPV in inflamed dermis and epidermis as compared to normal dermis and epidermis (p=0.008 and p=0.009, respectively, Table 4.8).

4.4.3 Presence of BPV DNA within the epidermis of different clinical types of sarcoids

A total of 49 sarcoids of four clinical types contained sufficient epidermis for analysis. When these 49 sarcoids were subdivided based on clinical type into occult, verrucous, nodular, and fibroblastic, BPV DNA was present within the epidermis of all types (Table 4.9). There was a significantly higher risk of BPV DNA being present in the epidermis of occult sarcoids compared to fibroblastic or nodular sarcoids. There were no significant differences between the detection rate of BPV between verrucous sarcoids and occult ones..

4.5 Discussion

Equine sarcoids are the most common skin tumour of horses (Scott D.W. and Miller W.H., 2003;Valentine, 2006). Their appearance in horses has been described for centuries. Currently, the development of sarcoids is believed to be associated with infection by BPV (Nasir and Campo, 2008). This association has been made based upon the presence of BPV DNA in sarcoid lesions and its absence in other equine skin lesions. The association of BPV with equine sarcoids is unlike other papillomaviral diseases for several reasons. First, other papillomaviruses are typically species specific and do not cause disease outside of their host species (Nasir and Campo, 2008). Second, unlike other papillomaviral diseases, papillomavirus has not been isolated from sarcoids. Third, using electron microscopy, viral particles have not been visualized in equine sarcoids. Finally, atypical of other papillomaviral diseases, attempts to recreate the disease by inoculation of BPV into the skin of horses have been unsuccessful (Olson and Cook, 1951;Ragland and Spencer, 1969;Voss, 1969). The causal association between the presence of BPV and the development of sarcoids is strengthened by the presence of BPV DNA in sarcoids and a lack of BPV DNA in other skin conditions of horses notably other skin tumours of horses. However, several studies have found BPV on the skin of normal horses (Bogaert and others,

2005;Bogaert and others, 2008) and one small study found BPV in all four cases of inflammatory skin conditions of horses that the researchers examined (Yuan and others, 2007). If BPV is commonly found in non-sarcoid skin lesions the association between BPV infection and sarcoid development is weakened. The purpose of this study was to examine a larger group of non-sarcoid skin conditions of horses for the presence of BPV DNA and, if present, to determine if the distribution of BPV DNA in the skin is different in these non-sarcoid lesions than in sarcoids.

A total of 86 skin biopsies including a variety of non-sarcoid skin lesions and morphologically normal equine skin from horses with no reported history of sarcoids were examined using PCR on the FFPE tissues to determine if BPV DNA was present in these samples. Nine categories of non-sarcoid equine skin samples were chosen based on the frequency of diagnosis over a ten year period at PDS and their similar clinical appearance to sarcoids (Table 4.1).

BPV DNA was present in all categories of skin lesions examined, as well as normal skin (Table 4.2). The overall prevalence varied from a low of 4/17 cases in eosinophilic granulomas to a high of 4/5 cases in photoactivated dermatitis. The overall rate of 41/86 BPV DNA positive cases was surprisingly high. In a study by the current authors examining the epidemiology of equine sarcoids in Western Canada, identical methods to determine the presence of BPV DNA in sarcoid biopsies was used (Wobeser and others, 2010). In that study, 74/96 sarcoid samples were positive for BPV DNA. While the recovery rate of BPV DNA in the current study was lower than that found in sarcoids ($p=0.0001$), BPV DNA was found with considerable frequency.

Two types of BPV are commonly associated with equine sarcoids, BPV1 and BPV2. Sequencing of the BPV recovered from these non-sarcoid skin samples confirmed that one or the other of these types of BPV was present in the samples described here and that their partial gene sequences were identical to those found in equine sarcoids from the same geographic region. A previous study on the presence of BPV DNA in sarcoids and the skin of non-sarcoid bearing horses found that the proportion with BPV2 positivity was higher in non-sarcoid bearing horses than in sarcoid bearing horses suggesting that BPV2 may be a more easily cleared infection than BPV1 (Bogaert and others, 2008). However, in the current study BPV1 and BPV2 DNA were recovered in roughly equal proportions in non-sarcoid bearing horses. This result was the opposite of that found in the 2008 study and the level of BPV1 recovery was actually higher than

the recovery rate from sarcoid bearing horses in Western Canada. This suggests that there may be no difference in the duration of infection between BPV1 and BPV2.

If the belief that BPV causes equine sarcoids is largely based on the presence of BPV DNA in sarcoids and absence from other equine skin samples, the fact that identical BPV DNA can be recovered from over 40% of other skin conditions in horses is problematic. Direct visualization of the presence of BPV DNA in sarcoids in the past has relied on i*n situ* hybridization. While specific, this methodology does not have the required sensitivity to detect the small amount of BPV DNA in some components of tissue biopsies (Martens, De Moor, and Ducatelle, 2001).

To enhance the sensitivity of detection, researchers have used other sample collection methodologies in combination with PCR for DNA amplification. These include skin swabbing and scraping techniques to determine the presence of BPV on the surface of sarcoid bearing and non-sarcoid bearing horses. In one such study, BPV DNA was detected in most of the sarcoid samples, but none of the non-sarcoid control samples (Martens, De Moor, and Ducatelle, 2001) which is in contrast to the frequency with which some studies, including the current one, were able to find BPV DNA in non-sarcoid equine skin samples (Bogaert and others, 2005). In this 2005 study, BPV DNA was detected on the skin surface of both non-sarcoid and sarcoid bearing horses. Perhaps, it is not simply the presence of BPV DNA alone that is important in sarcoid development; but also its location within the skin.

In one previous study the use of swabbing, cleaning and biopsying equine sarcoids, followed by PCR on the collected samples, was attempted to determine where within the lesion BPV was located (Bogaert and others, 2008). The results were somewhat difficult to interpret as the methodology lacks the ability to precisely determine what is being sampled. For example, in one horse BPV DNA was not present on a swab of the uncleaned skin surface, but was present after cleaning the skin surface. To avoid this problem LCM was used in the current study. The advantages of LCM are the ability to precisely choose specific areas or individual cells from a histologic slide of a lesion, and the ability to excise and capture these target areas without contamination from surrounding areas. The PALM laser microdissection scope uses a UV laser to excise the cells of interest and then uses a diffuse blast of laser light to propel the excised cells into a collection chamber so that DNA contamination from surrounding cells or instruments does

not occur. Very recently a study has used LCM on equine sarcoids following the same reasoning (Bogaert and others, 2010).

Using this methodology, in the current study it was found that there were significant differences in the anatomic location from which BPV can be recovered from non-sarcoid and sarcoid skin samples (Table 4.7). Non-sarcoid skin samples were more likely to have BPV DNA on or in the epidermis, than were sarcoid samples.

In addition, the presence of BPV DNA was significantly associated with the presence of inflammation. BPV DNA was more likely to be found in inflamed epidermis or dermis than in non-inflamed areas on the same slide (Table 4.8). Skin trauma has been suggested as an important mechanism to allow BPV access to the dermal fibroblasts and to provide an environment conducive to the proliferation of BPV transformed cells (Bogaert and others, 2008). The current results suggest that macroscopic trauma is not required and that inflammation alone may be sufficient to allow BPV access to the dermal fibroblasts. This may explain sarcoid development in locations on horses with no previous history of injury at that site. In addition, inflamed areas may represent potential sites for transmission of BPV from one horse to another by either contact or potentially via fomites. In support of this theory is a report that the same BPV DNA found within equine sarcoids could also be found in face flies (*Musca autumnalis*) in the horses' surroundings (Kemp-Symond, 2000).

A 2008 study demonstrated the presence of BPV DNA in circulating peripheral blood mononuclear cells of sarcoid bearing horses (Brandt and others, 2008). This suggested a possible alternate explanation for the presence of BPV DNA in inflamed areas. Rather than the environment being the source of BPV DNA infection at these sites, circulating blood mononuclear cells within the horse are the source and are concentrated at these sites of inflammation. However, although areas of inflammation were more likely to have BPV DNA within them, BPV DNA was also found in tissue compartments without inflammation. So a strictly internal source of infection seems unlikely.

A third possible explanation for the frequent presence of BPV in inflamed areas of the skin is that BPV infection itself may be a contributing cause of the inflammation. Epidermal changes associated with BPV infection have been described (Bogaert and others, 2010;Bogaert and others, 2008). Whether these changes predispose horses to the development of other inflammatory skin conditions in these areas is unclear. It may be that most horses are able to

clear the inflammatory processes associated with these BPV infections, but others are unable to avert infection and progression to sarcoid development occurs.

Recently, BPV DNA has been recovered from keratinocytes within the epidermis of sarcoids (Bogaert and others, 2010). In that study, BPV DNA was amplified from keratinocytes in occult sarcoids but not from "advanced" sarcoids. The clinical classification of the sarcoids described as "advanced" was not identified. The current study demonstrated that BPV DNA could be found within the epidermis of all examined clinical types of sarcoids. However, BPV DNA in the epidermis was significantly more likely to be identified in occult sarcoids than in fibroblastic or nodular ones (Table 4.9). Presuming that fibroblastic and nodular sarcoids are types of the "advanced" sarcoids described in the other study, these current studies results would agree that occult sarcoids are more likely to contain BPV DNA in the epidermis than are fibroblastic or nodular ones. This may represent a shift in the location of BPV DNA as sarcoids change from less to more clinically aggressive.

Two methods of DNA amplification were utilized in this study, PCR and LAMP. PCR has been extensively used to detect BPV in sarcoids in FFPE tissue (Bloch, Breen, and Spradbrow, 1994;Brandt and others, 2008;Carr and others, 2001;Carr and others, 2001;Martens and others, 2001;Otten and others, 1993;Teifke, Hardt M., and Weiss, 1994;Wobeser and others, 2010), while LAMP has not. LAMP is a relatively new DNA amplification technique first reported in 2000 (Notomi and others, 2000). Some studies have shown it to be more sensitive for detection of low copy numbers than PCR (Chen, Chu, and Lu, 2010). While LAMP has been used in a number of applications its use in FFPE equine tissue or with laser microdissection has not been described. In the current study, agreement, as measured by unweighted Kappa, between PCR and LAMP was almost perfect. PCR was more sensitive than LAMP, detecting 19 positive samples that LAMP did not, while LAMP detected two positive samples that PCR did not. This level of agreement suggests that LAMP is a viable DNA amplification technique for use with laser microdissection.

In summary, BPV DNA was present in over 40% of all samples of non-sarcoid equine skin biopsies tested. It was found in a variety of inflammatory skin conditions as well as normal equine skin. The distribution of BPV within the skin was different in these samples than in equine sarcoids, as it was more likely to be found on or in the epidermis. BPV was more likely to be found in microscopically inflamed areas than in microscopically normal areas of non-

sarcoid samples. This inflammation may cause defects in the skin barrier which allows BPV access through the skin surface to the underlying dermis of the skin where it may contribute to equine sarcoid formation. Alternately, the inflammation itself may be partially a result of BPV infection and the inability of some horses to resolve the inflammation in these areas may lead to sarcoid formation. These results may provide new insight into the pathogenesis of sarcoid formation and also imply that diagnostic techniques for equine sarcoids which rely upon detection of BPV DNA from skin swabs, scrapings or biopsies are unlikely to be adequately specific.

Table 4.1. Non-sarcoid equine skin condition categories assessed for the presence of Bovine papillomavirus, brief descriptions and rationale for their inclusion.

Skin condition type	**Description and inclusion rationale**
Dermatophytosis	Infection by dermatophytes leading to hyperkeratosis and alopecia; clinically similar to occult sarcoids (Knottenbelt, 2006)
Eosinophilic dermatitis	Common skin condition characterized by perivascular eosinophilic inflammation; BPV positive in another study (Yuan and others, 2007)
Eosinophilic granuloma	Nodular dermal eosinophilic inflammation; clinically similar to nodular sarcoid; BPV positive in another study (Yuan and others, 2007)
Exuberant granulation tissue	Ulcerative, dermatitis with proliferative granulation tissue, also known as "proud flesh"; clinically similar to neoplastic sarcoid (Knottenbelt, 2006) and BPV positive in another study (Yuan and others, 2007)
Normal skin	Selected from horses with no reported history of sarcoids which had morphologically and histologically normal biopsies of skin taken by clinicians as control samples in cases of non-sarcoid inflammatory skin conditions
Pemphigus foliaceous	Most common auto-immune skin disease of horses, clinically similar to occult sarcoid (Knottenbelt, 2006)
Photoactivated dermatitis	Sunlight induced vasculopathy; relatively common lesion in horses (Scott D.W. and Miller W.H., 2003)
Urticaria	Clinically distinct variant of hypersensitivity characterized by dermal edema, serum exudation and alopecia; common skin lesion of horses (Scott D.W. and Miller W.H., 2003);
Vasculitis	Type III hypersensitivity induced vasculitis; pathogenetically distinct from the more common photoactivated dermatitis

Table 4.2. The number and percentage of biopsies that were polymerase chain reaction positive for Bovine papillomavirus DNA grouped by inflammatory skin condition type as well as normal skin.

Skin Condition Type	**Number of cases**	**Number of cases BPV positive (%)**
Photoactivated dermatitis	5	4 (80.0%)
Eosinophilic dermatitis	5	3 (60.0%)
Vasculitis	5	3 (60.0%)
Dermatophytosis	9	5 (55.6%)
Pemphigus foliaceous	9	5 (55.6%)
Exuberant Granulation Tissue	14	7 (50.0%)
Normal Skin	12	6 (50.0%)
Urticaria	10	4 (40.0%)
Eosinophilic Granuloma	17	4 (23.5%

Table 4.3. Polymerase chain reaction (PCR) and isothermal loop mediated amplification (LAMP) DNA amplification primers used in formalin fixed paraffin embedded equine sarcoid and non-sarcoid skin biopsies.

Primer Name	**Nucleotide Sequence (5'-3')**
PCR	
Actin	
Forward	CACACTGTGCCCATCTACGA
Reverse	GCAGCTCGTAGCTCTTCTCC
Bovine Papillomavirus	
Forward	CAAAGGCAAGACTTTCTGAAACAT
Reverse	AGACCTGTACAGGAGCACTCAA
LAMP	
Bovine Papillomavirus	
F3	CAAAGGCAAGACTTTCTGAA
B3	CAAGAAAAAACAAGAGTAAGAACAG
FIP	CAGAAGTCCAAGCTGGCTGTACATGTACCACTACCTCCT
BIP	ATCACTGCCATTGCTTTTTCTTCGCAGCAACTAGTCCCAAG

Table 4.4. Tissue compartment descriptions for use in laser microdissection for the detection of Bovine papillomavirus in sarcoid and non-sarcoid skin samples

Name of tissue compartment	Description of tissue compartment
Intact epidermis	Epidermis extending from the stratum basale to the stratum corneum
Inflamed epidermis	As per intact epidermis and including any of: increased inflammatory cell numbers, hemorrhagic or serum exudates, dysplasia, erosion
Superficial dermis	Dermis extending from the level of the dermal/epidermal junction to the level of adnexal structures of the skin
Deep dermis	Dermis extending deep to the level of adnexal structures of the skin
Inflamed dermis	As per either superficial or deep dermis and including any of: increased inflammatory cell numbers, granulation tissue, hemorrhage, reactive fibroblasts and hypertrophic endothelial cells
Neoplastic dermis	Superficial or deep dermis, inflamed or not, and composed largely of morphologically neoplastic fibroblasts

Table 4.5. Comparison of number of samples positive and negative for the presence of Bovine papillomavirus (BPV) DNA using 2 different amplification techniques, polymerase chain reaction (PCR) and isothermal loop mediated amplification (LAMP), in 334 laser microdissected tissue compartments of equine skin.

		PCR detection of BPV DNA		**LAMP Total**
		Number of samples BPV Positive	Number of samples BPV Negative	
LAMP detection of BPV DNA	Number of samples BPV Positive	136	2	138
	Number of samples BPV Negative	19	177	196
	PCR Total	155	179	334

Table 4.6. Sarcoids and non-sarcoid equine skin samples dissected into tissue compartments using laser microdissection and examined for the presence of Bovine papillomavirus (BPV) DNA using polymerase chain reaction and isothermal loop mediated amplification

Skin Condition Sample Type (number of samples)	Name of Tissue compartment	Number of times present	Number of times BPV positive (%)
Sarcoid (70)	Inflamed Epidermis	57	22 (38.6)
	Intact Epidermis	52	6 (11.5)
	Superficial Dermis	14	4 (28.6)
	Deep Dermis	21	10 (47.6)
	Inflamed Dermis	4	1 (25.0)
	Neoplastic Dermis	65	57 (87.7)
Exuberant Granulation Tissue (6)	Inflamed Epidermis	6	6 (100)
	Intact Epidermis	3	2 (66.7)
	Superficial Dermis	2	1 (50.0)
	Deep Dermis	4	2 (50.0)
	Inflamed Dermis	6	6 (100.0)
	Neoplastic Dermis	0	0
Dermatophytosis (5)	Inflamed Epidermis	5	3 (60.0)
	Intact Epidermis	4	0 (0)
	Superficial Dermis	2	0 (0)
	Deep Dermis	0	0
	Inflamed Dermis	4	1 (25.0)
	Neoplastic Dermis	0	0
Normal Skin (5)	Inflamed Epidermis	0	0
	Intact Epidermis	5	4 (80.0)
	Superficial Dermis	5	1 (20.0)
	Deep Dermis	3	1 (33.3)
	Inflamed Dermis	0	0
	Neoplastic Dermis	0	0
Eosinophilic Granuloma (4)	Inflamed Epidermis	4	2 (50.0)
	Intact Epidermis	2	0 (0)
	Superficial Dermis	3	0 (0)
	Deep Dermis	0	0
	Inflamed Dermis	4	2 (50.0)
	Neoplastic Dermis	0	0
Pemphigus foliaceous (4)	Inflamed Epidermis	3	2 (66.7)
	Intact Epidermis	4	3 (75.0)
	Superficial Dermis	4	2 (50.0)
	Deep Dermis	3	1 (33.3)

	Inflamed Dermis	0	0
	Neoplastic Dermis	0	0
Urticaria (4)	Inflamed Epidermis	4	3 (75.0)
	Intact Epidermis	4	2 (50.0)
	Superficial Dermis	0	0
	Deep Dermis	2	0 (0)
	Inflamed Dermis	4	2 (50.0)
	Neoplastic Dermis	0	0
Hypersensitivity (3)	Inflamed Epidermis	3	2 (66.7)
	Intact Epidermis	3	2 (66.7)
	Superficial Dermis	0	0
	Deep Dermis	3	1 (33.3)
	Inflamed Dermis	3	2 (66.7)
	Neoplastic Dermis	0	0
Photoactivated Dermatitis (3)	Inflamed Epidermis	3	1 (33.3)
	Intact Epidermis	3	0(0)
	Superficial Dermis	1	0(0)
	Deep Dermis	0	0
	Inflamed Dermis	2	1(50.0)
	Neoplastic Dermis	0	0
Vasculitis (3)	Inflamed Epidermis	2	2(100.0)
	Intact Epidermis	2	0(0)
	Superficial Dermis	3	0(0)
	Deep Dermis	0	0
	Inflamed Dermis	3	0(0)
	Neoplastic Dermis	0	0

Table 4.7. Number and percentage of sarcoid and non-sarcoid equine skin samples dissected into tissue compartments using laser microdissection and examined for the presence of Bovine papillomavirus (BPV) DNA using polymerase chain reaction and isothermal loop mediated amplification.

Tissue Compartment	**Skin Sample classification**	**Number of samples BPV DNA positive**	**Number of samples BPV DNA negative**	**% positive**	**P value[a]**
Intact epidermis	Sarcoid	6	46	12	0.016
	Non-sarcoid	9	16	36	
Inflamed epidermis	Sarcoid	22	35	39	0.007
	Non-sarcoid	21	9	70	
Superficial dermis	Sarcoid	4	10	29	0.689
	Non-sarcoid	4	16	20	
Deep dermis	Sarcoid	10	11	48	0.501
	Non-sarcoid	5	10	33	
Inflamed dermis	Sarcoid	1	3	25	0.598
	Non-sarcoid	14	12	54	

[a] P value refers to the difference between percentages of BPV positive non-sarcoid skin samples to the percentage of BPV positive sarcoid skin samples within the same tissue compartment type as measured by Fisher's exact test

Table 4.8. Comparison of BPV DNA detection rates from laser microdissected equine skin samples where both compared tissue compartments were present within the same histologic sample.

Skin Sample Classification	Compartment Type	Number of matched samples	Number of samples BPV DNA positive	Number of samples BPV DNA negative	% positive	P value[a]
Sarcoid	Neoplastic dermis	65	57	8	88	0.0005
	Non-neoplastic Tissue		43	22	66	
Non-sarcoid	Inflamed Epithelium	25	19	6	76	0.009
	Non-inflamed Epithelium		9	16	36	
Non-sarcoid	Inflamed Dermis	26	14	12	54	0.0077
	Non-inflamed dermis		5	21	19	

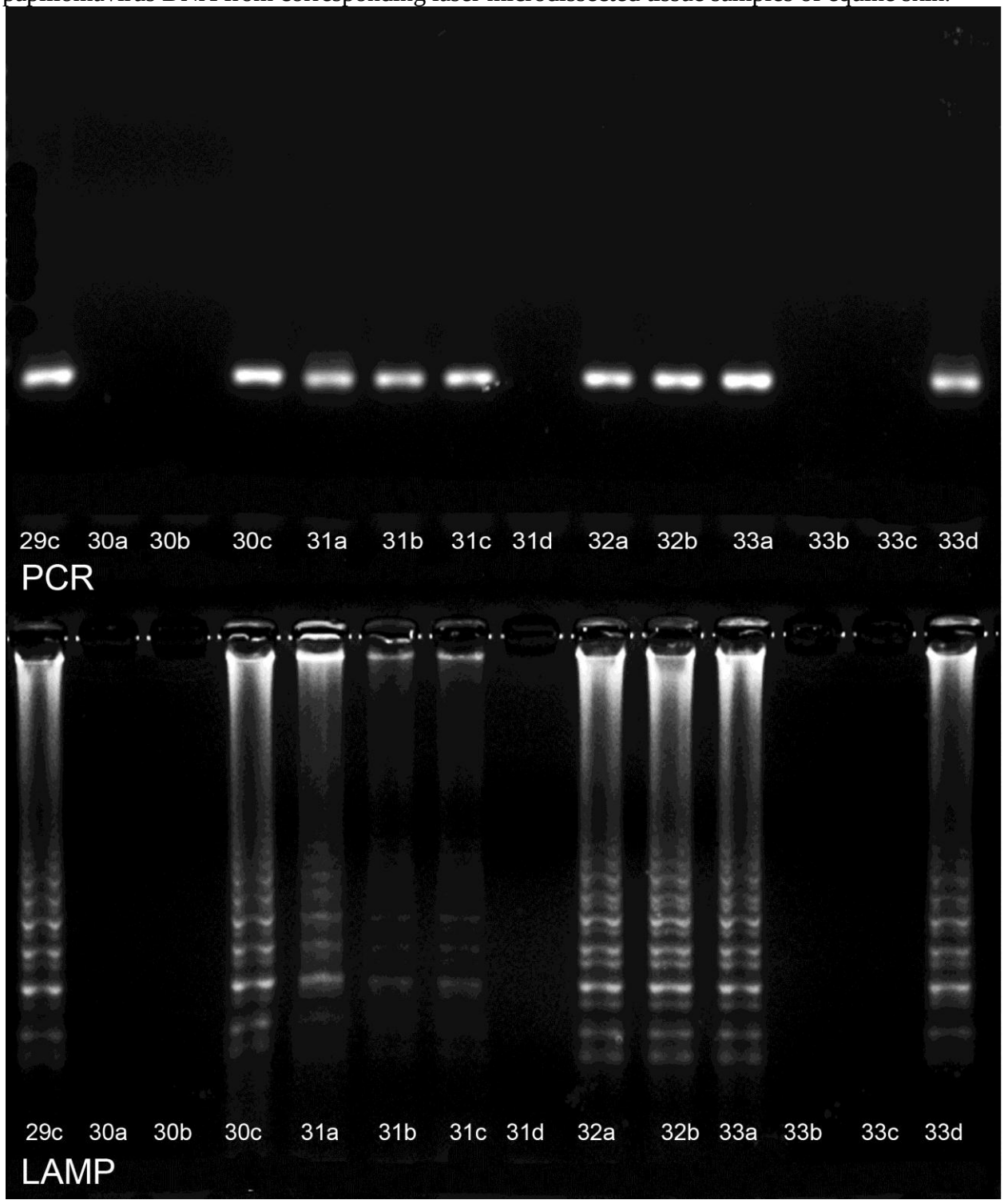
[a] P value refers to the association of presence of BPV DNA from matched equine skin samples within the compared compartment types as measured by McNemar's test.

Table 4.9. Presence of Bovine papillomavirus (BPV) within the epidermis of laser microdissected equine sarcoids of different clinical types and the relative risk of BPV positivity based on comparison to BPV positivity in occult sarcoids.

Clinical Type of Sarcoid	Number of samples with epidermis present on slide	Number with BPV DNA in the epidermis	Risk of BPV presence	Relative Risk of BPV presence	P value
Occult	11	7	0.64	5.8	Reference
Verrucous	14	5	0.36	3.2	0.23
Nodular	15	2	0.13	1.2	0.01
Fibroblastic	9	1	0.11	1.0	0.03

[a] significantly higher relative risk of being BPV positive

Figure 4.1. Ethidium bromide stained agarose gels of PCR and LAMP amplified Bovine papillomavirus DNA from corresponding laser microdissected tissue samples of equine skin.



5. GENERAL DISCUSSION

5.1 Introduction

This thesis represents a series of investigations into various aspects of equine sarcoid epidemiology and etiopathogenesis. Although equine sarcoids are very common neoplasms of horses, relatively little is known about several characteristics of sarcoid occurrence in Western Canada. In addition to basic epidemiologic features of affected horses, how the actual neoplasms grow or the role of Bovine papillomavirus (BPV) in sarcoid development is unknown. The studies contained within this thesis were designed to investigate these characteristics. Summaries of these studies and their role in better understanding equine sarcoids along with possible future studies follow.

5.2 Epidemiology of sarcoids in Western Canada

5.2.1 Summary of Results

As was introduced in the first chapter, although equine sarcoids are a common neoplasm, relatively little is known about basic epidemiologic features, particularly these features in Western Canada. In particular, breed, gender, age, and BPV type features associated with equine sarcoids, were examined at some depth in this thesis. To investigate these, the largest collection of sarcoid records (802) in the reported literature was evaluated.

Various previous studies had suggested that some breeds of horses are more likely to develop sarcoids than are others (Angelos and others, 1988;Meredith and others, 1986;Mohammed, Rebhun, and Antczak, 1992). However, this thesis demonstrated that in Western Canada, Donkeys, but no specific breed of horse, were more likely to have sarcoids. As discussed in Chapter 2, it is possible that this variance in reported breed predilection may be a result of different populations that were used to make these comparisons or may represent differences in the horses that inhabit these different geographic regions.

Horses with sarcoids were statistically younger than horses that had been biopsied for other reasons, which is not surprising as sarcoids are generally considered a disease of young horses. Interestingly, the most aggressive clinical type of sarcoid, the fibroblastic type, was present in younger horses more often than were other clinical types of sarcoids. This is curious because the general theory of sarcoid development is that sarcoids begin as a relatively benign

lesion of the occult or verrucose types and then these may transform into the more aggressive clinical types. If this were the case, then the most aggressive clinical types would be more common in older horses than would the more benign clinical types. A possible reason that this was not the case in this thesis may be that the sarcoid samples on which this thesis was based were derived from biopsy samples submitted by practicing veterinarians. It may be that these veterinarians or the owners of the horses involved were more likely to biopsy these aggressive neoplasms early in the course of the disease, and were willing to wait a longer period of time before biopsying the more clinically benign lesions.

In this thesis, a large majority of the sarcoids contained BPV2 while a small minority contained BPV1. Even though most studies done elsewhere in the world have found BPV1 to be the more common type found in sarcoids, the predominance of BPV2 was not entirely unexpected as one study found that BPV2 is more common in sarcoids than BPV1 in the Western United States (Carr and others, 2001). Whether this variation in type prevalence is a result of differences in the virus or the horses present in different geographic regions is unknown, as no studies on the prevalence of the various types of BPV in different geographic regions have been done.

5.2.2 Possible future studies based on this research

Sequences of the BPV DNA amplified from the sarcoids in this thesis were similar to sequences amplified from other sarcoids. BPV1 and BPV2 are interesting viruses as the vast majority of other papillomaviruses are species specific, but these types of BPV seemingly are not. This suggests a possible new research opportunity. The typing and identification of BPV present in sarcoids is based on relatively short DNA sequences, commonly a section of the E2 and E5 early genes, representing only a small portion of the total BPV genome. It is possible that there are substantial differences in other portions of the genome of the BPV present in sarcoids. The virus present in sarcoids may not be identical to the BPV1 and BPV2 viruses found in cattle, but may be novel viruses that simply share the previously sequenced portions. Subtypes of papillomaviruses, may only vary by 2% from one another based on sequence of a single gene, and the complete genome of very few non-human papillomaviruses are known (de Villiers and others, 2004). It is expected that perhaps hundreds of currently unknown papillomaviruses exist (de Villiers and others, 2004). To determine if BPV DNA recovered from

sarcoids is identical to BPV1 from cattle, amplification and sequencing of the whole genome of BPV from equine sarcoids would be needed. This thesis used formalin-fixed paraffin embedded tissue (FFPE) which is very useful for retrospective studies, but cannot be used for total genome amplification as the fixation process prevents amplification of long DNA sequences. As such, new studies attempting to sequence the entire genome would require the use of fresh sarcoid tissue in addition to other amplification and sequencing techniques, such as rolling circle amplification (Rector and others, 2005;Reimar and others, 2009).

5.3 Immunohistochemistry of markers of apoptosis and evasion of apoptosis in equine sarcoids

This portion of the thesis was stimulated by the finding that the mitotic rate of neoplastic cells in sarcoids is not dramatically higher than in normal tissue (Borzacchiello and others, 2008;Martens and others, 2000;Nixon and others, 2005). If sarcoids do not grow by increased cell division then some other process such as increased cell longevity must result in tumour growth. Evasion of apoptosis as a means of tumour growth as detected by immunohistochemical staining for Survivin and B-Cell Lymphoma 2 (Bcl-2) was, therefore, chosen to study in this thesis. In addition to tumour growth, cervical neoplasms in women caused by papillomavirus have immunohistochemically detectable production of Survivin (Frost and others, 2002;Yaqin, Runhua, and Fuxi, 2007). The production of this protein in these cervical tumours is associated with poorer prognosis and increased clinical aggressiveness. In addition to providing information on how sarcoid tumours might grow, it was hoped that, based on immunohistochemical detection of production of Survivin and Bcl-2, prognostic information useful to veterinarians and horse owners might be provided. One of the frustrations with the diagnosis of sarcoids is that beyond identifying the lesion as a sarcoid more specific information on the prognosis of that particular sarcoid cannot be given. Sarcoids may never progress to clinical aggressiveness, may spontaneously regress or may, as is often the case, become more clinically aggressive. If production of Survivin within a sarcoid could provide useful clinical information this would potentially be a useful diagnostic tool.

The results of this thesis support evasion of apoptosis as a method of sarcoid growth. Survivin was immunohistochemically detected in all types of sarcoids and was more often expressed in more clinically aggressive types of sarcoids than in less clinically aggressive types.

Statistically, this was significant, but for prognostic purposes the difference in Survivin protein production may not be large enough to provide accurate prognostic information on an individual sarcoid biopsy basis. If expression of the Survivin gene is an important factor in transformation of sarcoids from less aggressive to more aggressive clinical types, it may be that immunohistochemistry is not a sufficiently sensitive test to detect small, but clinically important differences in Survivin expression. Measurement of mRNA activity of the Survivin gene may be a more sensitive technique. While mRNA can be detected from FFPE blocks, it can be technically difficult to do so. Again, as with whole genome amplification, fresh sarcoid tissue may be the ideal substrate for this type of research. Using fresh tissue from different clinical types of sarcoids and quantitatively measuring mRNA levels of Survivin may allow for better differentiation of different clinical types of sarcoids and possibly provide better prognostic information.

5. 4 Localization of BPV in equine sarcoids and non-sarcoid equine skin samples

The presence of BPV DNA in almost half of non-sarcoid skin samples was surprising. This level of detection suggests that any method of sarcoid diagnosis based on simple detection of BPV DNA is unlikely to be useful. However, this thesis was able to show that although BPV DNA was present in these non-sarcoid samples, its location was different than in the sarcoid samples. Laser microdissection and subsequent DNA amplification was an effective way of determining where the BPV DNA was located, but it was difficult and time consuming. *In situ* methods, such as *in situ* PCR (IS-PCR) or *in situ* LAMP offer appealing alternative methods for localization of BPV DNA. Other groups have had success demonstrating DNA with IS-PCR in other conditions. Indeed, a considerable amount of time was spent during this PhD research trying to develop these techniques. After some early success, the development of these was abandoned as consistent or predictable results could not be obtained. The reasons for the failure of these tests in the hands of this researcher are not clear. Troubleshooting of all parts of the processes was attempted, but without success.

The initial finding of the frequency of BPV DNA detection in non-sarcoid skin samples initially suggested that perhaps BPV was not a likely causative agent of sarcoid development. As was demonstrated in the later parts of the research the detection rate was significantly lower

in non-sarcoid skin conditions than in sarcoids and the location of BPV DNA within the sample differed between sarcoids and non-sarcoid skin samples. These suggest that although simple detection of BPV DNA alone is insufficient to diagnose a sarcoid, there is ample evidence that BPV is a necessary factor in sarcoid development.

The presence of BPV DNA in normal dermal fibroblast in both sarcoid and non-sarcoid samples may provide an explanation of both the frequency of recurrence of sarcoids and the tendency of sarcoids to develop in multiple areas on the body. If BPV DNA is present in these fibroblasts, when these fibroblasts are induced to more activated forms such as those present in areas of wound healing or inflammation then the BPV DNA within them may be stimulated to express oncogenic early genes and transform these morphologically normal cells into the neoplastic fibroblasts of a sarcoid.

It is interesting to speculate on why BPV DNA, which seems to be relatively ubiquitous on horses' skin, causes development of sarcoids in some horses, but not in others. This thesis suggests that the location of the BPV DNA may be one important factor in sarcoid development and that the presence of BPV DNA within the skin in non-sarcoid skin samples is associated with inflammation. If there is inflammation present in these areas, why doesn't the horse's immune system react to and remove the BPV DNA before sarcoids are formed? Tempting hints as to why this may occur can be found in earlier research which reported that sarcoids are found more frequently in horses with some equine leukocyte antigen (ELA) types (Brostrom, H. et al., 1988; Brostrom, H., 1995; Gerber, H. et al., 1988; Lazary, S. et al., 1985; Meredith, D. et al., 1986). As ELAs are associated with the immune response, it may be that horses with these ELA types have impaired clearance of BPV. If this were to be the case then rather than BPV being efficiently removed, it may persist and lead to sarcoid development. Research into this could provide valuable insight into the pathogenesis of sarcoid development. In addition, if an impaired immune response is important in the development of sarcoids this may have implications for treatment or prevention modalities that rely on the immune system such as vaccination.

5.5 Final discussion

This thesis has provided valuable insight into sarcoid development and answered several questions about the role of BPV in sarcoids. However, like all research, by providing answers to

some questions it raises several new questions and new possible avenues of research. As sarcoids remain the most common equine neoplasm and their clinical behavior remains frustratingly difficult to predict considerable research remains to be performed.

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APPENDIX A.

Data tables for matched equine skin samples for comparison of detection rate of BPV DNA from laser microdissected equine skin samples of sarcoid and non-sarcoid equine skin (Table 4.8).

1. BPV DNA in neoplastic and non-neoplastic tissues

This table shows the data used to generate the p value for comparison of BPV DNA detection rate from neoplastic tissue to the BPV DNA detection rate from non-neoplastic tissue (Table 4.8) in equine sarcoid skin samples. Only skin samples where both neoplastic and non-neoplastic tissues were present were used for this comparison (n=65).

		Neoplastic dermis		**Non-neoplastic dermis totals**
		Number of samples BPV Positive	Number of samples BPV Negative	
Non-neoplastic dermis	Number of samples BPV Positive	43	0	43
	Number of samples BPV Negative	14	8	22
	Neoplastic dermis totals	57	8	65

2. BPV DNA in inflamed and non-inflamed epidermis

This table shows the data used to generate the p value for comparison of BPV DNA detection rate from inflamed epithelium to the BPV DNA detection rate from non-inflamed epithelium in non-sarcoid equine skin samples (Table 4.8). Only skin samples where both inflamed and non-inflamed epitheliums were present were used for this comparison (n=25).

		Inflamed epidermis		**Non-inflamed epidermis totals**
		Number of samples BPV Positive	Number of samples BPV Negative	
Non-inflamed epidermis	Number of samples BPV Positive	8	1	9
	Number of samples BPV Negative	11	5	16
	Inflamed epidermis totals	19	6	25

3. BPV DNA in inflamed and non-inflamed dermis

This table shows the data used to generate the p value for comparison of BPV DNA detection rate from inflamed dermis to the BPV DNA detection rate from non-inflamed dermis in non-sarcoid equine skin samples (Table 4.8). Only skin samples where both inflamed and non-inflamed dermis were present were used for this comparison (n=26).

		Inflamed dermis		**Non-inflamed dermis totals**
		Number of samples BPV Positive	Number of samples BPV Negative	
Non-inflamed dermis	Number of samples BPV Positive	5	0	5
	Number of samples BPV Negative	9	12	21
	Inflamed dermis totals	14	12	26