



FACULTEIT DIERGENEESKUNDE
approved by EAEVE

**THE ROLE OF THE BOVINE PAPILLOMAVIRUS IN
THE CLINICAL BEHAVIOUR OF EQUINE SARCOIDS
AND THE PRESENCE OF VIRAL LATENCY IN
HORSES**

Lies Bogaert

Thesis submitted in fulfilment of the requirements for the degree of Doctor in Veterinary Science (PhD), Faculty of Veterinary Medicine, Ghent University, December 13th, 2007.

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jezelf een vraag stellen
daarmee begint onderzoek

en dan die vraag aan een ander stellen

(vrij naar Remco Campert)

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ABBREVIATIONS

5-FU	5-fluorouracil
ACTB	actin, beta
AP-1	clathrin adaptor complex
APES	3-aminopropyltriethoxysilane
B2M	beta-2-microglobulin
BCG	Bacillus Calmette-Guérin
bp	base pairs
BPV	bovine papillomavirus
C	control horses
CBP	CREB-binding protein
CC	horses living in contact with papilloma affected cattle
cDNA	copy DNA
CS	horses living in contact with sarcoid-affected horses
Ct	threshold cycle
CV	coefficient of variation
DNA	deoxyribonucleic acid
DNA-PKcs	DNA protein kinase catalytic subunit
dNTP	deoxynucleotides
ELA	equine leucocyte antigens
ERC-55/E6BP	E6 binding protein
EST	expressed sequence tag
GAPDH	glyceradehyde 3-phosphate dehydrogenase
HPRT1	hypoxanthine phosphoribosyltransferase 1
HPV	human papillomavirus
kb	kilobase
LCR	long control region
M	average expression stability value
mAb	monoclonal antibody
MHC	major histocompatibility complex

mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
NF	normalisation factor
NMSC	non-melanoma skin cancer
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PDGF- β	platelet-derived growth factor β
PF	proliferative fraction
pRB	retinoblastoma tumour suppressor protein
qPCR	quantitative PCR
R	Pearson's correlation coefficient
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RPL32	ribosomal protein L32
RT-PCR	reverse transcription PCR
S	sarcoid-affected horses
SNP	short nucleotide polymorphism
Ta	annealing temperature
TBP	TATA box binding protein
TUBA1	tubulin, alpha 1
UBB	ubiquitin B
UDG	uracil DNA glycosylase
UV	ultra violet
V	pairwise variation
VLP	virus like particle

PREFACE

Equine sarcoids are commonly observed skin tumours in horses. Many different clinical presentations exist, ranging from small stable patches to aggressively growing fleshy masses. The bovine papillomavirus (BPV), the causative agent of cattle warts, has an important role in its etiology. Although not life threatening, the presence of equine sarcoids can have important economic repercussions. Treatment may become costly due to frequent recurrences and the breeding value of an affected horse diminishes due to the hereditary character of the disease. Sometimes horses even need to be euthanised because of failure of treatment, functional impairment, multiplicity of tumours or financial burden. Moreover, as an infectious agent is involved in the development of sarcoids, many owners are worried about transmission of the disorder to other horses.

The aims of this PhD thesis were to investigate the correlation between the clinical behaviour of sarcoids and the activity of BPV, as well as to determine the presence of BPV in the normal skin of horses. In particular, differences between control horses and those living in contact with affected horses or cattle were investigated.

Equine sarcoid:

A review of the literature

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Adapted from: *Vlaams Diergeneeskundig Tijdschrift* (in press).

GENERAL INTRODUCTION

Equine sarcoids are the most common skin tumours in horses and other equids like donkeys, mules and zebras (Jackson, 1936; Ragland, 1970; Lazary *et al.*, 1985; Nel *et al.*, 2006). It was described for the first time by Jackson (1936), who considered the tumour as a sarcoma-like mixed (fibro-epithelial) tumour. The equine sarcoid is observed worldwide, independent of breed, coat colour, sex or age (Ragland, 1970; Tarwid *et al.*, 1985). Nevertheless, certain breeds and families are more vulnerable than others (Ragland *et al.*, 1966; Brostrom *et al.*, 1988; Lazary, 1988; Gerber, 1989; Marti *et al.*, 1993) and sarcoids occur most commonly in young adults, between 3 and 6 years (Marti *et al.*, 1993; Torrontegui & Reid, 1994).

Clinically and pathologically, sarcoids present most of the features of a true neoplasm. The predominant cell type is a malignant/transformed fibroblast. Sarcoids generally have a high capacity for local tissue invasion into the dermis and subcutis but true metastatic dissemination does not occur (Pascoe & Knottenbelt, 1999). The clinical presentation of an equine sarcoid can vary a lot between different individuals and even within the same individual. The number of tumours per horse varies from one single sarcoid over a few to more than 100 lesions (Pascoe & Knottenbelt, 1999). A sarcoid can remain stable for years or it can show a rapid and aggressive growth with infiltration of surrounding skin (Ragland, 1970; Marti *et al.*, 1993). The reason for these large differences in clinical presentation is presently not known. The infiltrative behaviour together with the infectious etiology is responsible for the frequent recurrences after treatment. Sarcoids themselves are never lethal, but failure to treat them and the functional impairment they cause may result in euthanasia or slaughtering. The economic repercussion can be great: besides the cosmetic aspect, the normal use of the horse can be hindered, especially when tumours are located at the level of the girth and bridle, distal limbs, corner of the mouth and eyelids. Moreover a genetic component has a role in the development of the disease, diminishing the breeding value of affected animals (Lazary *et al.*, 1985; Meredith *et al.*, 1986; Gerber *et al.*, 1988). Diseased horses are therefore difficult to sell (Gerber, 1989).

In the pathogenesis, the bovine papillomavirus (BPV) plays a major role. Papillomaviruses are normally strictly species-specific, but equine sarcoids result from a natural cross-species infection. Although no intact viral particles have been demonstrated in sarcoids so far, DNA, RNA and proteins of the virus can be found (Amtmann *et al.*, 1980;

Trenfield *et al.*, 1985; Otten *et al.*, 1993; Teifke *et al.*, 1994; Nasir & Reid, 1999; Carr *et al.*, 2001b). Moreover, many similarities can be observed between equine sarcoids and papillomatosis in other species: fast growth, tendency for multiplicity and spreading by contact over different parts of the body (Jackson, 1936). Epizootics have been described in closed herds of horses and zebras, pointing to an infectious agent (Ragland *et al.*, 1966; Nel *et al.*, 2006).

CHAPTER 1.1.

Clinical aspects of equine sarcoids

CLINICAL PRESENTATION

Clinical types

Many clinical manifestations of equine sarcoids are known. They can be single or multiple, small to very large, stable or aggressive growing. At cross-section sarcoids appear as dermal thickening with a pale yellow colour and a firm texture due to fibroblastic proliferation and a small number of capillaries within the tumour (Foy *et al.*, 2002). The epidermis varies from thick, rough and hyperkeratotic to thinned or ulcerated. A classification based on morphological features has been proposed by Pascoe and Knottenbelt (1999) and will be followed throughout this thesis.

Verrucous sarcoids (Fig. 1) have a typical wart-like appearance with a rough, thickened, scabby surface above the fibroblastic part of the tumour (Knottenbelt *et al.*, 1995). They can appear as small exophytic growing masses or as flat, often extended, scaly areas of skin with multiple smaller wart-like lesions on the surface. Common differential diagnoses for this sarcoid type are warts (induced by equine papillomavirus), aural plaque, chronic sweet itch and chronic blistering (Pascoe & Knottenbelt, 1999).

Nodular sarcoids (Fig. 2) are subcutaneous, easily moveable nodules, often but not always spherical, covered by intact, apparently normal skin (Foy *et al.*, 2002). In some cases, however, the overlying skin can be thinner, shiny and adherent to the tumour (Knottenbelt *et al.*, 1995). Differential diagnoses include eosinophilic granuloma, melanoma and other nodular diseases (Knottenbelt *et al.*, 1995). Histologically, nodular sarcoids can mimic schwannomas although their clinical appearance and the presence of BPV demonstrate the sarcoid origin of the tumour (van Heerden *et al.*, in preparation).

Occult sarcoids (Fig. 3) are flat circular to oval areas characterised by alopecia and a roughened or scaly appearance (Knottenbelt *et al.*, 1995). Sometimes small cutaneous nodules can be observed (Pascoe & Knottenbelt, 1999). In some cases, occult sarcoids can be very subtle, displaying no more than a slightly thickened skin with a thin hair coat and slight changes in pigmentation. This relatively benign type can evolve rapidly towards a more aggressive type, either spontaneously or following injury such as biopsy or an erroneous treatment. Common differential diagnoses are dermatophytosis, chronic skin rubbing, alopecia areata and vitiligo (Knottenbelt *et al.*, 1995).

Fibroblastic sarcoids (Fig. 4) are large fibrous masses with an ulcerated surface. This is the most aggressive sarcoid type and can evolve from any other type after accidental or iatrogenic manipulation, including biopsy (Knottenbelt *et al.*, 1995; Foy *et al.*, 2002). In these cases it is observed that the original tumour starts to grow very quickly and begins to ulcerate. Skin wounds, especially at the distal limbs, are also at risk for development of fibroblastic sarcoids. Both dehiscence of the suture line after surgery without an apparent reason as well as the presence of a slow healing hypergranulating wound can be suggestive of sarcoid development (Pascoe & Knottenbelt, 1999). Fibroblastic sarcoids are liable to trauma, haemorrhage and local infection with bacteria or maggots. Fibroblastic sarcoids can be sessile with an invasive character or pedunculated with a small base. Differential diagnoses are exuberant granulation tissue, botryomycosis, squamous cell carcinoma and habronemiasis in warmer climates (Knottenbelt *et al.*, 1995).

Mixed sarcoids (Fig. 5) are a combination of two or more of the above mentioned types. They can represent a progressive or transient state between the verrucous/occult types and the fibroblastic/nodular types (Pascoe & Knottenbelt, 1999).

Malevolent sarcoids are very rare and have been described only by one group (Knottenbelt *et al.*, 1995). These tumours infiltrate in lymphatic vessels resulting in multiple nodular or fibroblastic masses along these vessels. Local lymph nodes might also be involved. This type of sarcoid usually evolves from any of the other types following repeated injury, although spontaneous transformation is also possible.

In our clinic, sarcoids infiltrating the underlying muscles have occasionally been observed. These sarcoids can arise spontaneously or following failure of treatment resulting in deterioration of the tumour.

This classification into different types is also reflecting the clinical behaviour of the tumours: occult types are the most stable tumours, nodular and verrucous sarcoids display a moderate growth, fibroblastic sarcoids are often fast growing and malevolent and infiltrating sarcoids, although rarely seen, are the most aggressive sarcoid types.



Figure 1. Verrucous sarcoid on the right mandible.



Figure 2. Nodular sarcoid on the upper eyelid.



Figure 3. Occult sarcoids in the paragenital region of a mare (ventral abdomen and medial side of the thigh).



Figure 4. Fibroblastic sarcoid in the dorsolateral aspect of the metacarpus.



Figure 5. Mixed sarcoid (verrucous – fibroblastic) on the upper eyelid.

Histopathological properties

Jackson (1936), who described the equine sarcoid for the first time, considered it as a biphasic tumour with an epidermal and dermal component. Typical changes are subepidermal proliferation of spindle-shaped to fusiform fibroblasts showing hyperchromasia with a moderate to high cell density (Tarwid *et al.*, 1985; Goodrich *et al.*, 1998). These immature fibroblasts show a higher density in the superficial part of the tumour compared to the deeper layers (Martens *et al.*, 2000a). The fibroblasts are fusiform to spindle-shaped, forming whorls, interlacing bundles and haphazard arrays with one another (Goodrich *et al.*, 1998). At the dermo-epidermal junction fibroblasts are oriented perpendicular to the basement membrane, which is known as a picket-fence pattern (Ragland, 1970; Tarwid *et al.*, 1985; Scott, 1988; Pulley & Stannard, 1990; Marti *et al.*, 1993). The mitotic rate is invariably low (Goodrich *et al.*, 1998; Martens *et al.*, 2000a). The amount of collagen varies considerably between tumours (Goodrich *et al.*, 1998). In between the fibroblasts of the deeper layers of the dermis, large polygonal cells with large basophilic nuclei can be found. These are infiltrating dendritic cells that have an antigen presenting function suggesting an immune mediated defence against virus infected cells (von Tscharnner *et al.*, 1996; Lepage *et al.*, 1998). Sometimes small epithelial inclusion cysts are found (Ragland, 1970; Tarwid *et al.*, 1985; Pulley & Stannard, 1990). In the most typical cases, pseudoepitheliomatous hyperplasia and hyperkeratosis are observed. There is marked formation of rete pegs, which are broad invaginations (up to more than 20 cells) of epidermal cells into the dermis (Goodrich *et al.*, 1998). However, in less typical sarcoids the epidermal component can be normal, atrophic or even absent (Marti *et al.*, 1993; Lepage *et al.*, 1998; Martens *et al.*, 2000a). In situ hybridisation for BPV DNA of tissue sections from sarcoids reveals viral DNA in the dermal layer within the fibroblasts but not within the epithelial cells (Lory *et al.*, 1993; Teifke *et al.*, 1994; von Tscharnner *et al.*, 1996). Epithelial changes seen in sarcoids are likely the result of growth-promoting factors expressed by the neoplastic fibroblasts that stimulate proliferation of surrounding epithelial cells (Carr *et al.*, 2001a).

The histological properties seem to be mainly dependent of the clinical type (Martens *et al.*, 2000a). Most of the verrucous and mixed sarcoids display the histological features as described above. In verrucous sarcoids, the epithelial component is much more important than the dermal, sometimes only lying as a small band of active fibroblasts against the epidermis (Pulley & Stannard, 1990). In the fibroblastic type there is always partial or total ulceration of the epidermis with infiltration of polymorphonuclear cells (Martens *et al.*, 2000a). The

epidermis just next to the ulcerative lesions is more or less hyperplastic. In nodular sarcoids the epidermis is often thinned (Martens *et al.*, 2000a). If rete pegs are present, they are short. If the dermal proliferation is not making contact with the epidermis, the latter is normal. In occult sarcoids the epidermis is usually normal or only displaying slight changes. The only typical aspect of this sarcoid type is the increased density of subepidermal fibroblasts infiltrating between a reduced number of hair follicles and sweat glands (Martens *et al.*, 2000a). They do not show a typical morphology or specific whorling distribution pattern. The density of dermal fibroblasts is also lower compared to the other types of sarcoids. The only common property for all types of sarcoids is the increased density of dermal fibroblasts compared to normal skin (Martens *et al.*, 2000a).

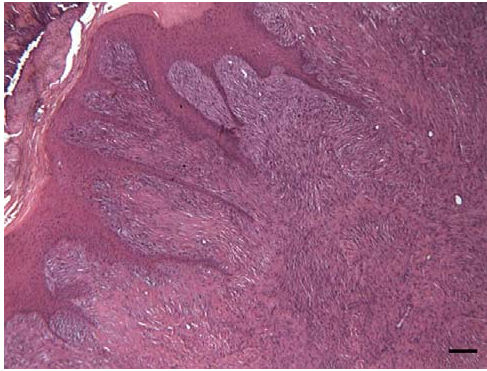


Figure 6. Histological view of a verrucous sarcoid. Note the irregular hyperplasia of the epidermis, that forms deep rete ridges in the superficial dermis. The dermis is replaced by a highly cellular mass, arranged in short interlacing and palisading bundles of small moderately pleiomorphic spindle cells surrounded by a basophilic matrix (bar = 250 μ m).

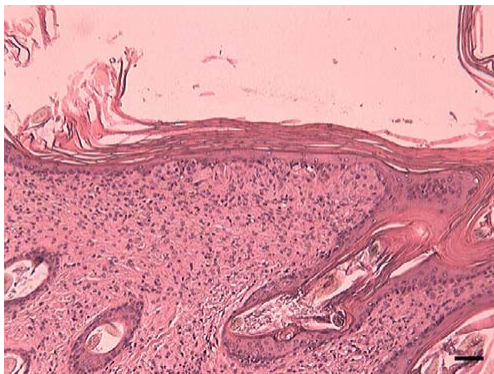


Figure 7. Histological view of an occult sarcoid. Only an increased density of subepidermal fibroblasts can be seen together with diffuse orthokeratotic hyperkeratosis of the overlying epidermis (bar = 250 μ m).

Diagnosis

Diagnosis can be made in three ways: clinical examination, histopathology and detection of BPV DNA. A thorough clinical examination combined with a focused anamnesis (duration of problems, localisation of lesions, age, breed, evolution, multiplicity of lesions...) should be sufficient in the majority of cases. Lack of clinical experience or atypical tumour characteristics may cause confusion and necessitate lab-assisted diagnosis. Histopathological examination is often diagnostic, but it should be remarked that taking a biopsy, in particular of small stable lesions, may induce rapid growth and ulceration. If a non-excisional biopsy must be performed, sites within the mass must be carefully chosen to minimise the confounding factors of surrounding inflammation and granulation and to include intact epidermis (Goodrich *et al.*, 1998). Possible deterioration of the sarcoid following biopsy is the reason why taking a biopsy of verrucous, occult and small nodular lesions is contra-indicated, even if making a definitive diagnosis is not possible in such cases (Pascoe & Knottenbelt, 1999). Another possibility is to perform a full surgical excision as if the lesion was a sarcoid, including excision of wide margins of normal skin and non-touch approach, followed by histological confirmation afterwards. This allows the pathologist to observe the range of morphological characteristics of the tumour allowing a correct diagnosis (Goodrich *et al.*, 1998).

A more recent approach in diagnosing equine sarcoids is the detection of BPV DNA in lesions by means of polymerase chain reaction (PCR). This can be performed on histopathological samples of tissue suspected of equine sarcoid but not displaying the typical histological features (Angelos *et al.*, 1991). A new approach is the detection of BPV DNA by means of PCR in the limited amount of material obtained by swabbing or scraping the lesions. This technique is especially valuable for the diagnosis of equine sarcoid tissue in non-healing wounds and in case of recurrences after former surgery (Martens *et al.*, 2001a). PCR detection of BPV DNA has many advantages: it is not invasive, sampling is easy and the trauma to the tumour is minimal. Disadvantages are the unsuitability for diagnosing occult sarcoids and the lower sensitivity compared to clinical diagnosis (Martens *et al.*, 2001a).

EPIDEMIOLOGY

Prevalence

The equine sarcoid is the most common tumour in horses, donkeys, mules and zebras. Depending on the study, they represent 12 to 67% of all equine tumours and 70% of all skin tumours in horses (Jackson, 1936; Ragland, 1970; Miller & Campbell, 1982; Teifke *et al.*, 1994; Lepage *et al.*, 1998). A prevalence of 0.6 to 2% in clinical populations has been reported (Ragland, 1970; Mohammed *et al.*, 1992; Goodrich *et al.*, 1998). In our clinic, 2.2% of the patients are admitted for treatment of equine sarcoids. This percentage is biased, since it can be higher than the true prevalence because veterinary referral hospitals can be specialised in sarcoid treatment and thus have a high case load. On the other hand, not all horses with sarcoids are referred to the clinic: small tumours are often treated at home by local practitioners or are left untreated. Recent studies conducted on three-year-old horses presented for field tests in Switzerland showed a prevalence of 12% in the Swiss Warmblood Horse and the Freiburger (Mele *et al.*, 2007; Studer *et al.*, 2007), which might be a more accurate estimation of the true population prevalence. In populations of Cape mountain zebras in South Africa the prevalence of equine sarcoids mounted up to 53%, but this high prevalence could be influenced by a high degree of inbreeding (Zimmerman, 2004; Nel *et al.*, 2006).

Localisation of tumours

Sarcoids can appear on any part of the body, but they are mostly localised on the ventral abdomen, the paragenital region, head and limbs (Jackson, 1936; Pulley & Stannard, 1990; Torrontegui & Reid, 1994; Goodrich *et al.*, 1998; Piscopo, 1999). On the head, most tumours develop at the eyelids, ears and commissure of the lips (Foy *et al.*, 2002). Sarcoids are least common on the dorsum of the trunk (Knottenbelt *et al.*, 1995). In northern regions, lesions occur predominantly on the head and abdomen while in warmer climates the limbs are more often involved (Marti *et al.*, 1993). Apart from these predilection sites, sarcoids can develop at any site in injured skin (Torrontegui & Reid, 1994; Foy *et al.*, 2002). The location of sarcoids has a significant relationship to their size: sarcoids on the head are mostly smaller whereas sarcoids on the limbs are larger when compared to all other sites (Brostrom, 1995).

Sarcoids can appear singly or in clusters (Brostrom, 1995; Goodrich *et al.*, 1998). Horses with multiple lesions tend to have larger-sized tumours compared to horses with a single sarcoid (Brostrom, 1995).

Risk factors

AGE

In contrast to most tumours observed in people and animals, the equine sarcoid predominantly develops in young adults. The majority of affected horses are younger than 6 years, with a peak incidence between 3 and 6 years (Miller & Campbell, 1982; Scott, 1988; Marti *et al.*, 1993; Torrontegui & Reid, 1994; Brostrom, 1995; Piscopo, 1999; Foy *et al.*, 2002). The mean age of sarcoid development is 3.5 to 4 years (Brostrom, 1995; Studer *et al.*, 2007). Nevertheless, also younger and older horses can develop sarcoids. According to Mohammed *et al.* (1992) a gradual increase in incidence is observed up to the age of 15 years, followed by a decreasing incidence. A study on a large group of donkeys revealed that mainly males between 0.5 and 3 years were at high risk (Reid & Gettinby, 1996). An obvious reason for this age distribution has not been demonstrated yet, but possibly older horses acquire a certain level of immunity which results in spontaneous regression of existing tumours and prevention of new tumour development. Another hypothesis is that genetically susceptible animals develop tumours early in life (Torrontegui & Reid, 1994).

SEX

Most authors do not assume that there is a gender predisposition for sarcoid development (Ragland, 1970; Miller & Campbell, 1982; Pulley & Stannard, 1990; Torrontegui & Reid, 1994). However, others claim that geldings are more susceptible than stallions and mares. Mohammed *et al.* (1992) observed that the risk for geldings to develop sarcoids was twice as high compared to stallions and mares. In donkeys, a predisposition in males was observed (Reid *et al.*, 1994a; Reid & Gettinby, 1996). A possible explanation is that in castrated males a wound is created which is a possible entrance for BPV (Reid *et al.*, 1994a). On the other hand, in spite of the frequent localisation of sarcoids in the paragenital region, the castration wound itself is only seldom affected. Another hypothesis is that female animals have protective factors, rather than a predisposition in males (Reid & Mohammed, 1997).

BREED

There is a clear breed predisposition for the development of equine sarcoids (Marti *et al.*, 1993). In North-America, Quarter horses are twice as likely to develop sarcoids compared to thoroughbreds (Angelos *et al.*, 1988). Also Appaloosas and Arabian horses are more frequently affected than thoroughbreds (Angelos *et al.*, 1988; Mohammed *et al.*, 1992). Standardbreds on the other hand are rather resistant (Meredith *et al.*, 1986; Mohammed *et al.*, 1992; Brostrom, 1995). A possible explanation is that Quarter horses and Appaloosas are often used on large cattle farms which results in a higher risk to get in contact with BPV (Mohammed *et al.*, 1992). Moreover they often work on rough surfaces resulting in frequent injury to the limbs and the possibility for sarcoid development in these wounds (Brostrom, 1995). Another more plausible explanation for breed predisposition can be found in the different genetic background of these breeds (Lazary *et al.*, 1985; Meredith *et al.*, 1986).

GENETIC BACKGROUND

The most important genes known to play a role in sarcoid development are those of the major histocompatibility complex (MHC), but also other genes are involved. The genes of the MHC code for proteins involved in the immune response and for protein components of the complement system (Piscopo, 1999). In horses these proteins are called equine leucocyte antigens (ELA). Three major classes of MHC genes exist (Lazary *et al.*, 1994): class I genes code for glycoproteins on the cell wall of most nucleated cells. They have a role in recognition and killing of virus infected cells. Seventeen internationally accepted alleles (A1-10, A14-15, A19, W16-18, W20) and 5 regional variants (Be22, Be24-26, Be108) are distinguished serologically in the horse. Class II proteins are expressed on the cell surface of antigen presenting cells. Within this class the DQB gene is highly polymorphic. In the horse 3 internationally accepted alleles (W13, W22-23) and 2 local variants (BeVIII, Be200) can be determined serologically (Lazary, 1988; Hesford *et al.*, 1989). To date, the complete sequence coding the ELA-DQB gene is known (Szalai *et al.*, 1994) and more than 23 DQB sequences have been reported in domestic horses (Gyllensten *et al.*, 1990; Szalai *et al.*, 1993; Horin & Matiasovic, 2002), but not all reported sequences can be assigned to a serological specificity (Villegas-Castagnasso *et al.*, 2003). Recent evidence suggests the presence of at least two copies of the horse DQB gene (Horin & Matiasovic, 2002; Villegas-Castagnasso *et al.*, 2003), one of them probably being a pseudogene. This genome organisation hampers an easy classification of DQB variants with modern molecular techniques such as PCR-RFLP. Class

III genes encode several proteins involved in the complement system, but these are not associated with predisposition for sarcoids.

In most breeds such as the Swiss Warmblood, the Irish Warmblood, the Swedish Halfbred and the Selle Français, the presence of equine sarcoids is strongly correlated with ELA W13 (Meredith *et al.*, 1986; Brostrom *et al.*, 1988; Gerber *et al.*, 1988; Lazary *et al.*, 1994; Brostrom, 1995). A higher percentage of recurrences is also observed in the presence of this allele (Brostrom, 1995). Standardbreeds lack W13, which might be a reason for the low prevalence of equine sarcoids in this breed (Meredith *et al.*, 1986). In the Freiburger, a Swiss draft horse, W13 is also lacking, but a higher sensitivity for sarcoid development is correlated with Be108, a local variant (Lazary *et al.*, 1994). In the Selle Français, A3 is correlated with a higher prevalence of sarcoids, but this could be due to linkage disequilibrium with W13 (Lazary *et al.*, 1994). Indeed, ELA A3W13 is frequently seen as one of the paternal haplotypes in families where equine sarcoids are more commonly observed than in others (Brostrom *et al.*, 1988; Gerber *et al.*, 1988). A5 would be correlated with early onset of sarcoids (Brostrom, 1995). The association of equine sarcoids with certain antigens is however not absolute. A large proportion of horses are carriers of W13, but only a small part of them will develop sarcoids (Goodrich *et al.*, 1998). On the other hand, also horses lacking W13 may be affected by sarcoids. Maybe these alleles are only markers of other susceptibility genes in linkage disequilibrium with the MHC, with a more direct influence on the pathogenesis (Brostrom *et al.*, 1988; Gerber *et al.*, 1988; Lazary *et al.*, 1994). Still it seems plausible that genes coding for proteins regulating the immune system can also influence the susceptibility for certain diseases (Piscopo, 1999). Indeed, in virus-induced tumours the virus-infected cells express new cell wall antigens, both virus specific and virus induced non-viral products. These foreign antigens should normally be recognised by the immune system resulting in destruction of tumour cells. If a failure occurs in the immune system, recognition and destruction of tumoural cells may also fail. In people and rabbits, a linkage between papillomavirus induced cancer and MHC alleles has also been observed (Wank & Thomssen, 1991; Han *et al.*, 1992).

In Arabian horses a significant correlation exists between presence of equine sarcoids and heterozygosity for the defective DNA protein kinase catalytic subunit (DNA-PKcs) allele, which is in homozygous condition responsible for severe combined immunodeficiency. In a population of Arabian horses, 8.7% of the animals were carriers of the defective DNA-PKcs allele, compared to 18.6% of the equine sarcoid-affected animals (Ding *et al.*, 2002).

Transmission

The way horses get infected with BPV is not yet clarified. Possible transmission routes are direct contact with cattle (Jackson, 1936), indirect transmission from cattle to horses (e.g. housing of horses in cattle stables, transmission by caretakers of the animals...) and transmission from horses with sarcoids to other horses (direct or indirect via caretaker, tack, grooming equipment, common rubbing posts,...). In donkeys it is known that animals having close contact with affected animals are at higher risk for development of sarcoids (Reid *et al.*, 1994a). Epidemiological data, multiplicity of tumours on one horse, spontaneous development of sarcoids on intact skin and absence of contact with cattle or affected horses suggest that flies or other insects may play an important role as mechanical vector in BPV infection of the horse (Voss, 1969; Torrontegui & Reid, 1994; Reid *et al.*, 1994a; Knottenbelt *et al.*, 1995; Pascoe & Knottenbelt, 1999). Recently, Kemp-Symonds and Kirk (2007) have demonstrated the presence of BPV-1 and -2 in *Musca autumnalis* face flies infesting sarcoid-affected horses. Moreover, it is observed that in one specific region identical variants of BPV are seen both in horses and in cattle, also pointing into the direction of a flyborn transmission (Otten *et al.*, 1993). In case of a skin wound, BPV coincidentally present in the environment of the horse can immediately come into contact with the subepidermal fibroblasts via insects (Torrontegui & Reid, 1994; Reid *et al.*, 1994a; Knottenbelt *et al.*, 1995; Pascoe & Knottenbelt, 1999). Furthermore, cell growth is already stimulated as part of wound regeneration. This can eventually lead to malignant transformation of these cells (Phelps *et al.*, 1999; Foy *et al.*, 2002).

CURRENT TREATMENT MODALITIES

The treatment of equine sarcoids has always been challenging, due to the variable clinical presentation of lesions and the frequent local recurrences. Therefore, careful selection of the appropriate treatment for each sarcoid and each horse should be made, taking into account the localisation, number and size of the tumours, the treatment history, the financial value of the animal and the owner compliance to fulfil the treatment schedule (Marti *et al.*, 1993; Carstanjen & Lepage, 1998).

Both surgical and non-surgical techniques have been described with variable success rates. Since none of the current techniques has been proven to be 100% successful (Marti *et al.*, 1993), the confidence of owners in conventional treatments may decrease, leading them to use a whole scale of topical ointments (including toothpaste), homeopathy... Despite the lack of any scientific evidence, tumour regression can occasionally be seen after these alternative therapies. Cure, however, may also be a result of spontaneous regression, which is observed in up to 32% of the cases (Brostrom, 1995; Martens *et al.*, 2001c). Therefore, benign neglect can be an option, especially in case of small tumours (Knottenbelt *et al.*, 1995; Pascoe & Knottenbelt, 1999). Careful monitoring is yet very important, since aggravation can happen anytime without an obvious reason. It should be kept in mind that not all horses with equine sarcoids can be cured: in horses with a very large number of sarcoids, or with sarcoids with a very large surface treatment may be impossible. Even in case of a small single sarcoid owners should be warned about possible local recurrence and deterioration of the sarcoid (Knottenbelt *et al.*, 1995; Carstanjen & Lepage, 1998). Moreover, the development of new sarcoids on other locations is always possible (Brostrom, 1995; Carstanjen *et al.*, 1997; Carstanjen & Lepage, 1998). It is generally accepted that the prognosis for treatment is significantly worse if one or more unsuccessful attempts have been made previously (Knottenbelt & Walker, 1994). Therefore, the best available treatment option with the highest chance of success should always be used at the first attempt of treatment (Pascoe & Knottenbelt, 1999).

In this section, only the most commonly applied treatments in equine practice will be presented. Treatments with only anecdotic evidence, such as Sanguinara canadensis (XXTerra®), photodynamic therapy, homeopathy, acupuncture, ligation, hyperthermia and various topical ointments will not be discussed further (Hoffman *et al.*, 1983; Schwierczena, 1993; Brostrom, 1995; Thoresen, 1995; Pascoe & Knottenbelt, 1999; Martens *et al.*, 2000b; von Felbert *et al.*, 2005).

Surgical treatment

CONVENTIONAL EXCISION

Surgical excision of equine sarcoids has been applied for decades with variable success. High recurrence rates of 40 to 72% are reported (Ragland, 1970; Diehl *et al.*, 1987; McConaghy *et al.*, 1994; Brostrom, 1995). This can be attributed to the infiltrative nature of the tumour and auto-transplantation of tumour cells during the surgical procedure (Klein, 1990; Howart, 1990). On histology, fronds of tumour cells infiltrating the surrounding dermis and sometimes the underlying tissues can be observed (Ragland, 1970; Tarwid *et al.*, 1985). The detection of BPV DNA at the surgical margin of an excised sarcoid correlates with an increased risk of recurrence (Martens *et al.*, 2001a). When the excision is performed with wide normal skin margins (at least 12 mm; Fig. 8) and a non-touch approach (i.e. changing of gloves and material after tumour removal and extensive flushing of the wound), auto-inoculation with tumour cells and viral material can be avoided and success rates of more than 80% are achieved (Howart, 1990; Brostrom, 1995; Martens *et al.*, 2001c). Another major factor in the prognosis is the possibility to perform the excision under general anaesthesia (80% success) compared to working on the standing horse (24% success). General anaesthesia provides the surgeon a clear view on the sarcoids, a good ability to remove wide margins and creates good conditions to avoid auto-inoculation (Brostrom, 1995).

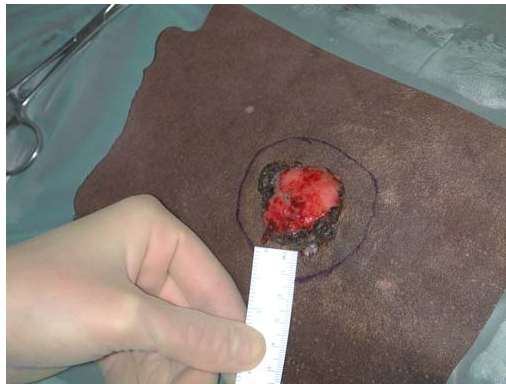


Figure 8. Demarcation of a normal skin margin of 12 mm to perform excision of a fibroblastic sarcoid.

CO₂ LASER SURGERY

Excision with the CO₂ laser is an efficient tool for the treatment of equine sarcoids. Success rates range from 60 to 89% (Diehl *et al.*, 1987; Vingerhoets *et al.*, 1988; Carstanjen *et al.*, 1997; Martens *et al.*, 2001c). Large masses are commonly laser-excised using the same approach as described for the conventional excision. Small or flat lesions can be entirely vaporised with the CO₂ laser (Diehl *et al.*, 1987; Vingerhoets *et al.*, 1988; Carstanjen *et al.*, 1997; Weigand *et al.*, 1997). Laser ablation causes less damage to the surrounding tissues and less spread of malignant cells to healthy regions compared to sharp surgical excision with mechanical tools (Carr, 2006). However, poor cosmetic results including scar tissue formation, alopecia, leucotrichia and transient abnormal hair colour can sometimes be observed (Vingerhoets *et al.*, 1988; Carstanjen *et al.*, 1997). Other drawbacks are the cost of the equipment and the required safety precautions against burns, fire, eye damage and smoke inhalation (Palmer, 1989).

CRYOSURGERY

Both in human and veterinary medicine cryosurgery has become an accepted way of treating malignancies. Cell death is obtained by repeated freeze-thaw cycles, with optimal cryonecrosis achieved by rapid freezing and slow thawing of the tissue (Farris *et al.*, 1976; Fretz & Barber, 1980). Prior to freezing, large sarcoid masses are debulked to the level of the surrounding skin or even lower, followed by freezing of the tumour base and a normal skin margin with liquid nitrogen (Fig. 9). Tissue should be frozen to at least -20 to -25 °C. Temperature control is essential and can be obtained with thermocouple needles (Farris *et al.*, 1976). After the procedure, a black leathery crust develops within a few days, and sloughs gradually over several weeks eventually leading to an open wound which heals by second intention.

Success rates vary from 60 to 100% (Lane, 1977; Fretz & Barber, 1980; Klein *et al.*, 1986; Diehl *et al.*, 1987; Martens *et al.*, 2001c). Occasionally, spontaneous regression of non-treated sarcoids on the same horse can be observed, indicating that destruction of one tumour can result in an immune response against other, distant sarcoids (Lane, 1977). Disadvantages of the technique are the prolonged anaesthesia time required, the cosmetic effects (scarring, leucodermia, leucotrichia) and the possible damaging of underlying structures. The latter makes cryosurgery less suitable for periocular sarcoids or tumours located on large vessels, nerves, ligaments or joints (Lane, 1977; Fretz & Barber, 1980; McConaghy *et al.*, 1994; Knottenbelt *et al.*, 1995).



Figure 9. Continuous circulation contact probes for cryosurgery of debulked sarcoid lesions. Tissue temperature is monitored carefully with thermocouple needles inserted into the tumour mass.

Non-surgical treatment

CHEMOTHERAPY

Administration of cytotoxic drugs results in tumour cell death and can be performed in two ways: systemically and locally. Local chemotherapy can be applied intralesionally or topically. The rationale for local chemotherapy is based on achieving a high drug concentration over time in the tumour while sparing normal tissue. The general circulation is bypassed and a low systemic exposure is obtained, thereby minimising toxicity (Theon, 1998). Due to its cutaneous localisation, sarcoids are most convenient for local chemotherapy.

Intralesional injection of cytotoxic drugs directly into the tumour allows higher intratumoural drug concentrations and permits accurate placement of the drug within the tumour (Theon, 1998; Mair & Couto, 2006). The beneficial effects are enhanced if drug carriers that prolong the persistence of the drug in the tumour are used. Viscous fluid preparations such as sesame seed or almond oil are frequently used to achieve this effect (Theon *et al.*, 1993). The most widely used drug in this manner is cisplatin with a 2-year local control rate of approximately 90% (Theon *et al.*, 1993; Theon *et al.*, 1994; Theon, 1998). Intralesional cisplatin can be effective as the sole treatment for small sarcoids, but for large tumours the combined use with surgery is recommended (Theon *et al.*, 1994; Theon, 1998). Local cisplatin injected at the time of surgical sarcoid excision was shown not to have any adverse effect on wound healing (Theon *et al.*, 1994). Emulsions are made up from 1 ml of an

aqueous solution of 10 mg cisplatin and 2 ml sesame oil and are mixed using 2 syringes and a 3-way valve (Fig. 10). A dose rate of 1 mg cisplatin/cm³ of tumour can be applied. The sarcoid as well as a margin of normal skin of 1-2 cm should be injected using a parallel-row or field-block technique (Theon *et al.*, 1997). A standard treatment protocol includes 4 injections at 2-week intervals. Side effects of treatment are strictly local (inflammation, swelling, focal ulceration) and self-limiting (Theon, 1998). Prophylactic antibiotic therapy and anti-inflammatory drugs are recommended after each treatment session to prevent acute clostridial infection. The cosmetic and functional results are excellent (Theon, 1998). Alternative cisplatin formulations with almond oil or epinephrine as well as local chemotherapy with bleomycin and 5-fluorouracil (5-FU) are described but appear to be less effective (Bouré *et al.*, 1991; Doyle, 1998; Knottenbelt & Kelly, 2000; Stewart *et al.*, 2006).



Figure 10. Preparation of cisplatin emulsion by mixing of an aqueous cisplatin solution and sesame oil through a 3-way valve.

Topical chemotherapy has been used for over 100 years to treat equine sarcoids. However, the effect of only a few of these drugs has been studied scientifically. 5-FU can be applied as a cream for single, small occult or verrucous sarcoids but also for larger areas of occult or verrucous sarcoids that are not amenable to any other form of treatment. Due to poor diffusion and inadequate distribution from the surface to the deep margins, the use of 5-FU should be limited to flat lesions (Theon, 1998). Topical 5-FU needs to be applied daily for 30-90 days. Alternatively, it can be applied less frequently under a bandage (Roberts, 1970). A success rate of 66% was observed (Knottenbelt & Kelly, 2000). To aid the penetration through the epidermis, 5-FU can be mixed with podophylin, an irritant cathartic (McConaghy *et al.*, 1994; Piscopo, 1999). The treated area can show a marked inflammatory reaction, but scarring is usually minimal (Pascoe & Knottenbelt, 1999). Unlicensed topical ointments (AW-3-LUDES and AW-4-LUDES) containing a variety of heavy metals with 5-FU and thiouracil can be applied on successive or alternate days for 3 to 5 treatments, depending on the size, number and nature of the sarcoids (Knottenbelt & Walker, 1994; Knottenbelt & Kelly, 2000). This treatment may cause severe local irritation and scar contracture but a resolution rate of 80% has been reported in sarcoids being treated for the first time (Knottenbelt & Walker, 1994). The success rate decreases by 30 to 40% for each previous unsuccessful treatment.

RADIOTHERAPY

Radiotherapy induces tumour destruction by ionizing radiation. The most frequently used radiotherapeutic method in horses is interstitial brachytherapy with iridium-192 reporting one- and five-year progression-free survival rates of 87 and 74% respectively for non-ocular sarcoids and 98-100% for periocular sarcoids (Wyn-Jones, 1983; Turrel *et al.*, 1985; Theon & Pascoe, 1995; Knottenbelt & Kelly, 2000; Byam-Cook *et al.*, 2006). It involves the implantation of sealed radioactive sources within the tissue to treat the tumour at short source-object distances. Because of the long half-life of iridium-192 (74.2 days), the implants are removed once the required dose has been administered, usually after 5 to 6 days. The primary advantage is that high radiation doses can be given directly into the tumour with minimal damage to the surrounding tissues (Henson & Dobson, 2004). The technique is very suitable for tumours on locations where surgical excision is not possible (eyelids, commissure of lips) and good results have been obtained for recurrent sarcoids. Local complications such as necrosis and infection and minor cosmetic defaults (leucotrichia, alopecia, minimal scarring) have been observed (Turrel *et al.*, 1985; Theon & Pascoe, 1995; Theon, 1998;

Byam-Cook *et al.*, 2006). A major drawback of this otherwise valuable treatment is the safety of the operators and the need for patient isolation (Wyn-Jones, 1979; Walker *et al.*, 1991).

IMMUNOTHERAPY

The purpose of immunotherapy is to enhance the ability of the host to reject the tumour. Antitumoural effects are produced primarily through enhanced immunologic activity. Many immunostimulants have been evaluated in horses, including Bacillus Calmette-Guérin (BCG) vaccine, autovaccination and imiquimod 5% cream.

BCG is an attenuated strain of *Mycobacterium bovis* originally developed as a tuberculosis vaccine in people and is currently still in use for intravesical treatment of superficial bladder cancer (Brake *et al.*, 2000). Live organisms, dead organisms and cell wall extracts have been used in combination with various adjuvants for intratumoural injection of equine sarcoids. Tumour regression involves both non-specific and specific mechanisms and is based on a delayed hypersensitivity reaction (Morton & Barth, 1995). BCG vaccination consists of multiple injections into the tumour mass and tumour bed to ensure complete infiltration of the target volume. Most commonly 4 injections at a 2-3 week interval are performed. The success rate ranges from 60-100% and is mainly dependent on tumour size and localisation (Klein *et al.*, 1986). Periocular sarcoids are most responsive to treatment with local control rates up to 100%. Sarcoids on the limbs respond poorly and vaccination can result in massive oedema formation, lymphangitis or even septic arthritis (Klein *et al.*, 1986; Owen & Jagger, 1987; Theon, 1998). Cytreductive surgery prior to treatment of bulky tumours is recommended (Theon, 1998). General (anaphylactic shock, death, fever) and local (oedema, purulent discharge, ulceration) side effects have been described and therefore administration of non-steroidal or steroidal anti-inflammatory drugs is recommended prior to BCG vaccination (Theon, 1998).

Autogenous vaccination of sarcoids has been performed with variable success rates. Although promising results of autovaccination combined with food supplements have been reported (Kinnunen *et al.*, 1999), other authors have reported worsening of the condition or new sarcoid development after autovaccination (Knottenbelt *et al.*, 1995; Pascoe & Knottenbelt, 1999).

Imiquimod (AldaraTM) is an immune response modifier with potent antiviral and antitumoural activity showing promising results for the treatment of equine sarcoids. Successful treatment of human anogenital warts, actinic keratosis and superficial basal cell carcinoma has been reported (Beutner *et al.*, 1998; Marks *et al.*, 2001; Salasche *et al.*, 2002).

Recent studies have shown good results for the treatment of equine sarcoids (Nogueira *et al.*, 2006; Bogaert & Martens, 2007). Treatment consists of application of a thin layer of cream on the tumour surface 3 times a week during several weeks to months. In one study, 60% of the treated sarcoids resolved entirely and another 20% showed more than 75% reduction, most of them being recurrences after previous treatment (Nogueira *et al.*, 2006). Local side effects, such as ulceration, pain and depigmentation are often observed (Bogaert & Martens, 2007).

CHAPTER 1.2.

Association of bovine papillomavirus with equine sarcoids

CLASSIFICATION AND GENOME ORGANISATION

Bovine papillomaviruses are small non-enveloped DNA viruses (Fig. 11) and are members of the family *Papillomaviridae*, a large family of animal and human viruses that normally infect epithelial cells causing hyperproliferative lesions. To date, 8 BPV types (BPV-1 to -8) have been characterised and classified into 3 genera: the Delta-, Epsilon- and Xi-papillomaviruses (de Villiers *et al.*, 2004). BPV-1 and -2 belong to the Delta-papillomaviruses and induce fibropapillomas (warts). BPV-3, -4 and -6 are members of the Xi-papillomaviruses and infect only the epithelium, inducing true papillomas. BPV-5 and -8 belong to the Epsilon-papillomaviruses and cause both fibropapillomas and epithelial papillomas (Bloch *et al.*, 1994b; Tomita *et al.*, 2007). Phylogenetic analysis based on the complete L1 ORF suggests that BPV-7 should be classified in a novel genus (Ogawa *et al.*, 2007).

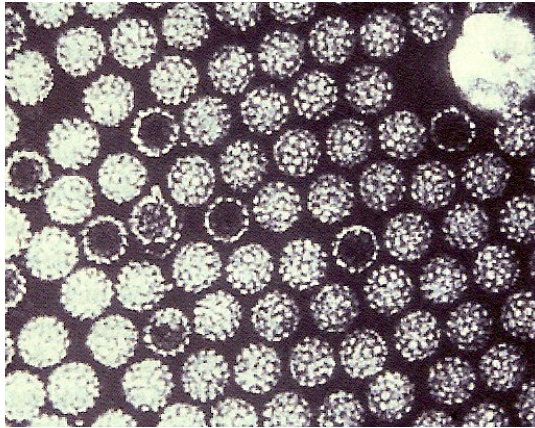


Figure 11. Electron microscopic picture of human papillomavirus particles (Stanley *et al.*, 2006).

BPV has a genome of 7900 bp of double-stranded DNA and contains 6 early and 2 late genes (Chen *et al.*, 1982; Campo, 1988) (Fig. 12). Early genes (E1, E2, E4, E5, E6 and E7) are responsible for replication and transformation, whereas late genes (L1 and L2) encode the structural proteins. The early and late regions are separated by a stretch of non-transcribed DNA, called the long control region (LCR), which contains the transcriptional promoters and enhancers, the origin of DNA replication and binding sites for numerous cellular transcription factors as well as for the virally encoded early gene product E2 (McBride *et al.*, 1991; Phelps *et al.*, 1999; Chambers *et al.*, 2003a).

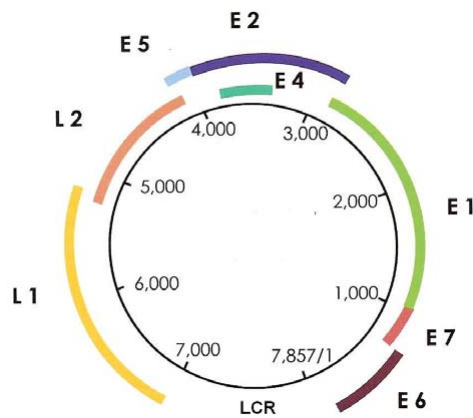


Figure 12. Genome organisation of papillomaviruses (Muñoz *et al.*, 2006).

The most extensively studied BPV gene is E5, which plays a prominent role in sarcoid development. E5 is a small hydrophobic protein, consisting of 44 amino acids, residing in the membranes of the Golgi apparatus and the endoplasmic reticulum. In vitro studies on murine fibroblasts have shown that BPV E5 is one of the most important genes in neoplastic transformation of the cell (Marchetti *et al.*, 2002). As soon as E5 is expressed, a rapid transformation occurs without the help of other proteins. E5 contributes to cellular transformation of fibroblasts in different ways. It has been shown to induce activation of the platelet-derived growth factor β (PDGF- β) receptor by binding to it (Petti & DiMaio, 1992; DiMaio & Mattoon, 2001). Activation of PDGF- β receptors of the dermal fibroblasts by E5 results in mitogenesis in nearby epithelial cells (Carr *et al.*, 2001b). This event may explain the typical pseudoepitheliomatous hyperplasia. Furthermore the vacuolar H^+ -ATPase is

targeted by BPV E5 (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). This proton pump is essential for the function of cellular compartments that process growth factors. By binding of E5, an alteration in this process occurs. The acidification of the endosomes is blocked and degradation of the receptors for growth factors is inhibited resulting in a prolonged stimulation time, besides an increased receptor recycling (Carr *et al.*, 2001b). This hypothesis is objected by the findings of Ashbey *et al.* (2001) who found no alteration in the function of the vacuolar H⁺-ATPase. The latter authors suppose that the binding to the vacuolar H⁺-ATPase has only a transport function in the cell in order to reach other intracellular targets. The BPV E5 also binds to 16K ductin/subunit c, a component of the gap junctions, responsible for the contact between neighbouring cells. Through this binding, the contact is disrupted and the transformed cell can replicate uncontrolled (Campo, 1997). Interaction with 16K leads also to the alkalization of the endosomes and the Golgi apparatus with consequent intracellular retention of MHC class I molecules (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). The absence of MHC class I on the cell surface would help the infected cells to evade host immunosurveillance (Chambers *et al.*, 2003a).

The BPV E6 protein is localised in membrane and nuclear fractions and contains 2 highly conserved zinc finger domains typical of DNA-binding transcription activator proteins. However, cell transformation by E6 seems to be independent of its transcription transactivation function (Ned *et al.*, 1997). Human papillomavirus (HPV) E6, the most important transforming protein of HPV, is known to bind to p53 and subsequently stimulate its degradation (Scheffner *et al.*, 1990). However, BPV E6 does not bind to p53 (Rapp *et al.*, 1999). Instead, the transformational ability of BPV E6 is linked to its ability to bind ERC-55/E6BP, a calcium binding protein (Chen *et al.*, 1995) and in part CBP/p300, a transcriptional co-activator (Zimmermann *et al.*, 2000) which interferes with the normal cell functions. BPV E6 also binds the focal adhesion protein paxillin (Tong *et al.*, 1997; Tong & Howley, 1997; Vande Pol *et al.*, 1998) and the γ subunit of the clathrin adaptor complex AP-1 (Tong *et al.*, 1998) leading to disruption of cytoskeleton and vesicular traffic pathways. The cytoskeleton is vital for the maintenance of cellular morphology, motility, division and cell-cell and cell-matrix interactions and the AP-1 complex plays an important role in the control of cell proliferation and differentiation (Chambers *et al.*, 2003a).

The function of the other early BPV genes is less well studied, but it has been established that E1 is responsible for initiation of replication and maintenance of the genome. E2 is, besides its role in replication, an important regulator of transcription. The exact role of E4 is still unknown, but its functions suggest a role in facilitating and supporting viral genome

amplification, the regulation of late gene expression, control of virus maturation and mediation of virus release (Roberts, 2006). E7 has a minor role in cell transformation and is a regulator of the BPV copy number (Campo, 1988). This genome organisation is somewhat different from the epitheliotropic BPVs (BPV-3, -4 and -6) which lack the E6 gene. Instead they have an E5 (formerly defined as E8) ORF between E7 and the LCR on the genome (Campo, 1992; Morgan & Campo, 2000). Also in HPV a different organisation is seen, where E6 and E7 are the most important transforming genes, and E5 is only active in the early stages of neoplastic transformation (Blair *et al.*, 1998).

Specific BPV-1 variants associated with equine sarcoids have been reported. Most variation in nucleotide sequences of papillomaviruses in general is found in the transforming genes E5, E6 and E7. Chambers *et al.* (2003b) found 7 E5 sequence variants in 34 sarcoids, none of them identical to the prototype sequence found in cattle, which might reflect the specific clinical presentation of equine sarcoids. On the other hand, E1 and E2 have a more conserved sequence, probably due to strict conditions for replication and transcription (Chan *et al.*, 1992; Nasir *et al.*, 2007). LCR variants of sarcoid BPV-1 with a higher activity in equine cells compared to bovine cells have been identified suggesting that these BPV variants have an enhanced function in equine sarcoids (Nasir *et al.*, 2007). These findings suggest that sarcoids are associated with variant BPV-1 genomes that preferentially infect horses and are maintained within equids (Nasir *et al.*, 2007).

PAPILLOMAVIRUS INFECTION IN THE NATURAL HOST

Natural infection with papillomaviruses occurs in epithelial cells and induces benign, self-limiting cutaneous and mucosal proliferations, called “warts”. During acute virus infection, replication of the virus genome is strictly linked to the state of differentiation of the infected cell. The virus initially infects the basal keratinocytes. The early genes are then expressed in the undifferentiated basal and suprabasal layers. Viral DNA is replicated in the differentiating spinous and granular layers and expression of the late structural proteins is limited to the terminally differentiated cells of the squamous layer, where the new virus particles are encapsidated and released into the environment as the cells shed (Chambers *et al.*, 2003a).

In cattle, mainly young animals are affected; later on they acquire immunity so that older animals rarely develop warts (Nicholls & Stanley, 2000). Initially transformation of subepithelial fibroblasts is seen, followed by epithelial acanthosis and the typical papilloma formation. BPV-1 mainly affects the genital region (teat and penile papillomas and the surrounding skin), while BPV-2 can infect the entire skin surface as well as the epithelial layers of the gastro-intestinal tract (Jarrett, 1984; Campo, 1987; Campo, 1997). In Europe, warts in cattle are commonly observed on the abdomen, limbs and dorsum, whereas in the USA mainly the head, neck and shoulders are affected (Phelps *et al.*, 1999). Most animals will spontaneously cure after several weeks to months as a result of a cell-mediated immune response. Reinfection is prevented by neutralising antibodies against capsid proteins. This immunity is type specific, so infection with other BPV types is still possible (Nicholls & Stanley, 2000).

In a small number of cases, no regression or even malignant transformation to squamous cell carcinoma is seen. Initiation of malignant transformation is linked to the deregulated expression of the early virus genes, which results in an uncontrolled proliferation and loss of differentiation of the infected cells (Campo, 1997). The presence of BPV-2 DNA in urinary bladder cancer in cattle is known, both naturally as well as experimentally induced (Campo & Jarrett, 1986; Borzacchiello *et al.*, 2003; Yuan *et al.*, 2007).

HISTORY OF ASSOCIATION BETWEEN BPV AND EQUINE SARCOIDS

Epidemiological data suggest an etiological role of an infectious agent in the development of equine sarcoids (Jackson, 1936; Voss, 1969; Ragland, 1970). Many similarities can be observed between equine sarcoids and papillomatosis in other species: fast growth, tendency for multiplicity and spreading over the body by contact between different parts of the body (Jackson, 1936). Epizootics have been described in closed herds of horses and zebras, pointing to an infectious agent (Ragland *et al.*, 1966; Nel *et al.*, 2006). Inoculation studies conducted in the early fifties suggested a role of BPV in sarcoid development (Olson and Cook 1951). Nevertheless, transmission studies from sarcoids to cattle were not successful (Ragland *et al.*, 1970). Other viruses, such as the equine cutaneous papillomavirus (Gorman, 1985; Obanion *et al.*, 1986) and a retrovirus (England *et al.*, 1973; Cheevers *et al.*, 1982), were supposed to be involved, but later on enough evidence was collected to assign an etiological role in equine sarcoid development only to BPV-1 and -2 (Lancaster *et al.*, 1977; Angelos *et al.*, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Bloch *et al.*, 1994a; Reid *et al.*, 1994b; Nasir *et al.*, 1997; Nasir & Reid, 1999; Carr *et al.*, 2001b).

Inoculation studies

The earliest suggestion that sarcoids may have an infectious origin was made in the early fifties. Horses were experimentally infected with cell-free extracts from cattle papillomas resulting in tumours grossly and histologically mimicking equine sarcoids (Olson & Cook, 1951). Later studies have confirmed these findings (Ragland & Spencer, 1968; Voss, 1969; Ragland & Spencer, 1969; Ragland *et al.*, 1970; Koller & Olson, 1972; Lancaster *et al.*, 1977; Makady *et al.*, 1990). Yet many differences were also observed between the induced tumours and naturally arising sarcoids. Histologically, only the dermal layers were changed without involvement of the epidermis and picked fence formation was not observed. Moreover, all induced tumours regressed spontaneously within 4 to 12 months and neutralising antibodies against BPV were formed resulting in resistance against re-infection. In natural infection, spontaneous regression exists but is less common. Moreover, no neutralising antibodies against BPV are formed and horses remain sensitive to re-infection (Ragland & Spencer, 1969; Lancaster *et al.*, 1977; Brostrom *et al.*, 1979). Furthermore,

attempts to identify BPV particles in natural cases of equine sarcoids by electron microscopy or using anti-BPV antibodies have been unsuccessful (Barthold & Olson, 1978; Sundberg *et al.*, 1984). One reason for the observed differences might be that with experimental inoculation numerous viral particles as well as bovine antigens are inserted in the organism inducing a strong immune reaction. In natural infection, possibly only one or a few virus particles infect the cell causing latent or non-productive infection (Carr *et al.*, 2001a). Moreover, the horse is not the natural host for BPV and cannot support the vegetative portion of the viral life cycle (Gorman, 1985; Sousa *et al.*, 1990). In natural sarcoids BPV DNA is kept in a non-integrated episomal state in the nucleus, only the early genes are transcribed and only a few copies of the genome are produced (Goodrich *et al.*, 1998). This might explain why inoculation of sensitive calves with equine sarcoid material does not induce papillomas and why these animals remain sensitive for BPV infection (Ragland *et al.*, 1970).

Detection of BPV DNA

Since the seventies, several studies have demonstrated the presence of BPV DNA in equine sarcoids with molecular techniques. Measurements of DNA-DNA reassociation kinetics on fresh or frozen material yielded 80-86% of sarcoids positive for BPV DNA (Lancaster *et al.*, 1977). Southern blot hybridisation on fresh or frozen material was positive in 86-100% of the sarcoids (Trenfield *et al.*, 1985; Angelos *et al.*, 1991; Reid *et al.*, 1994b). PCR on paraffin embedded material demonstrated BPV DNA in 74-91% of all cases (Teifke *et al.*, 1994; Bloch *et al.*, 1994a) and PCR on fresh or frozen samples resulted in a 100% detection rate (Otten *et al.*, 1993; Martens *et al.*, 2001a).

An explanation for the fact that BPV DNA can sometimes not be demonstrated in 100% of the examined sarcoid samples might be the insensitivity of the technique used e.g. when only a low copy number of viral genomes per cell is present (Angelos *et al.*, 1991) or after unsuited or too long formalin fixation (Teifke *et al.*, 1994; Bloch *et al.*, 1994a).

Detection of BPV gene expression

Nasir and Reid (1999) demonstrated that in 95% of the sarcoids where BPV DNA can be detected with PCR, expression of viral genes is also present. BPV E2 and E5 mRNA were detected in respectively 90% and 80% of the sarcoids. This was the first evidence of direct involvement of BPV in the pathogenesis of equine sarcoids. In additional studies, BPV E5

and E6 were expressed in respectively 85 and 72% of the sarcoids (Chambers *et al.*, 2003b; Nixon *et al.*, 2005). Carr *et al.* (2001b) could demonstrate the E5 protein in all examined sarcoids. Collectively, these studies clearly provide evidence that the viral genes are expressed and that the presence of BPV DNA in equine sarcoids is not coincidental.

PATHOGENESIS OF BPV INFECTION OF THE HORSE

Although most papillomaviruses are extremely species- and tissue-specific, it has now been established that a minority of them can also infect other hosts resulting in a different pathological outcome. BPV infection of the horse is one of the most extensively studied species-crossing infections. Both BPV-1 and BPV-2 infection occur, independent of each other, but no correlation exists between BPV type and clinical presentation of the equine sarcoid (Reid *et al.*, 1994b). Regional differences in frequency of both types are observed: in Europe, 74 - 93% of all examined samples of sarcoids are infected with BPV-1, compared to only 7 - 26% BPV-2 (Angelos *et al.*, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Martens *et al.*, 2000a; Martens *et al.*, 2001a). In the USA however, both types are seen in equal proportions (Teifke *et al.*, 1994; Carr *et al.*, 2001a). In a single tumour only one BPV type has been found up till now, but in case of multiple tumours on the same horse, both BPV types can be demonstrated (Otten *et al.*, 1993; Carr *et al.*, 2001a; Martens *et al.*, 2001a). These findings point towards lack of immunity to superinfection (Otten *et al.*, 1993). In Australia, a BPV type has been found in a small number of sarcoids different from type 1 and type 2. Maybe this is a third and until now unidentified type (Trenfield *et al.*, 1985).

The number of copies of BPV DNA per diploid equine sarcoid cell varies between 0.1 and 500 (Lancaster *et al.*, 1977; Amtmann *et al.*, 1980; Otten *et al.*, 1993; Goodrich *et al.*, 1998; Yuan *et al.*, 2007). They are present in a non-integrated circular episomal state in the cell (Amtmann *et al.*, 1980). In situ hybridisation studies have only shown BPV DNA in the nuclei of fibroblasts, especially at the dermo-epidermal junction and not in the epidermis (Teifke *et al.*, 1994). This is in contrast with BPV infection in cattle, where the virus is mainly located in the epidermal layers. Only in the first stages of infection in cattle, BPV DNA is found in the fibroblasts (Phelps *et al.*, 1999). Infection of BPV in a non-permissive host results in a non-productive cycle. There is only transcription of early genes, responsible for genome maintenance, regulation of cell growth and cell transformation (Gorman, 1985; Sousa *et al.*, 1990). A productive infection only occurs in bovine skin in well-differentiated keratinocytes: only here considerable replication and formation of complete virus particles takes place (Campo, 1997; Carr *et al.*, 2001b). It has been shown that intra-type sequence variation occurs within papillomavirus types, which can affect the cellular location and function of viral proteins, including E5, which in turn can affect the pathogenesis and

transforming effects of the virus (Reid *et al.*, 1994a; Giannoudis & Herrington, 2001; Chambers *et al.*, 2003b).

When an equine fibroblast gets infected by BPV-1 or -2, a number of cellular changes can happen resulting in cell transformation and uncontrolled growth. First of all, the MHC involved in immune regulation plays a major role (see paragraph 1.2.3.). Other cellular proteins are also involved, such as p53, c-myc and in Arabian horses DNA-PKcs, and probably many more.

The tumour suppressor gene p53 is coding for a nuclear phosphoprotein which will activate, in case of genetic damage, the transcription of genes inhibiting the cell cycle progression, resulting in DNA repair (Nasir *et al.*, 1999). P53 inhibits expression of the basic fibroblast growth factor and inhibits angiogenesis by up-regulation of thrombospondin-1 (Bucher *et al.*, 1996). These events result in conservation of the genetic stability of the cell (Nasir *et al.*, 1999). In human cancers it is observed that somatic mutation of p53 is the most commonly observed genetic alteration (Levine *et al.*, 1994). In horses and donkeys with equine sarcoids this is not observed, although a point mutation cannot be excluded (Bucher *et al.*, 1996; Nasir *et al.*, 1999). Other explanations for p53 inactivation are complex formation with viral or cellular proteins (Nasir *et al.*, 1999). HPV E6 is known to bind to p53 and subsequently stimulate its degradation (Scheffner *et al.*, 1990). However, BPV E6 does not (Rapp *et al.*, 1999). Yet it is supposed that in one way or another function loss of p53 takes place resulting in uncontrolled proliferation of fibroblasts and angiogenesis (Bucher *et al.*, 1996). Another hypothesis is cytoplasmic sequestration of p53, leading to inability to exert its function in the nucleus (Nasir *et al.*, 1999). Immunohistochemical staining has demonstrated that 9-44% of the sarcoids show perinuclear staining for p53 (Martens *et al.*, 2000a; Nixon *et al.*, 2005). These data indicate that nuclear exclusion seems to be a possible explanation for the deficient p53 mechanism.

ACKNOWLEDGMENTS

Prof. Dr. Hilde De Cock (University of Antwerp) is greatly acknowledged to provide us with Figures 6 and 7.

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Aims of the study

The clinical presentation of equine sarcoids can vary a lot between different horses and even within a single individual. A sarcoid can remain stable for years or even regress spontaneously, or it can show a rapid and aggressive growth with infiltration of surrounding skin. The reasons for these large differences in clinical presentation are presently not known.

The first aim of this research project was to investigate the correlation between the clinical behaviour of sarcoids and the activity of BPV using an accurate and reliable gene expression model.

The transmission of BPV to the horse and the pathogenesis of tumour development are not fully understood yet. Hypotheses for BPV infection in horses include direct or indirect contact with infected cattle or horses, as well as transmission by mechanical vectors such as insects. Furthermore, there is discussion about the occurrence of virus latency. In many species, including man and cattle, latency of papillomaviruses in asymptomatic individuals has been demonstrated. In horses, BPV DNA has been detected in biopsies obtained from the normal skin of horses affected with equine sarcoid, suggesting the occurrence of virus latency. However, the distinction between BPV DNA contamination of the skin surface and a true latent infection has never been made. Neither has there been any investigation on the risk of BPV infection in horses depending on their living environment.

The second aim of this thesis was to determine the presence of BPV on and in the normal skin of both sarcoid-affected and healthy horses. In particular, differences were investigated between healthy control horses and those living in contact with affected horses or with cattle presently or recently infected with BPV.

**Role of bovine papillomavirus in the clinical
behaviour of equine sarcoids**

Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids

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Adapted from: *BMC Biotechnology* (2004) 6:24.

SUMMARY

Real-time quantitative PCR can be a very powerful and accurate technique to examine gene transcription patterns in different biological conditions. One of the critical steps in comparing transcription profiles is accurate normalisation. In most of the studies published on real-time PCR in horses, normalisation occurred against only one reference gene, usually *GAPDH* or *ACTB*, without validation of its expression stability. This might result in unreliable conclusions, because it has been demonstrated that the expression levels of so called “housekeeping genes” may vary considerably in different tissues, cell types or disease stages, particularly in clinical samples associated with malignant disease. The goal of this study was to establish a reliable set of reference genes for studies concerning normal equine skin and equine sarcoids, which are the most common skin tumour in horses.

In the present study the gene transcription levels of 6 commonly used reference genes (*ACTB*, *B2M*, *HPRT1*, *UBB*, *TUBA1* and *RPL32*) were determined in normal equine skin and in equine sarcoids. After applying the geNorm applet to this set of genes, *TUBA1*, *ACTB* and *UBB* were found to be most stable in normal skin and *B2M*, *ACTB* and *UBB* in equine sarcoids.

Based on these results, *TUBA1*, *ACTB* and *UBB*, respectively *B2M*, *ACTB* and *UBB* can be proposed as reference gene panels for accurate normalisation of quantitative data for normal equine skin, respectively equine sarcoids. When normal skin and equine sarcoids are compared, the use of the geometric mean of *UBB*, *ACTB* and *B2M* can be recommended as a reliable and accurate normalisation factor.

INTRODUCTION

Gene expression analysis has become increasingly important in biological research where e.g. gene expression profiles from tissues associated with diseases and disorders have to be compared with each other and with those from normal tissues. One of the most powerful tools in this area is real-time quantitative reverse transcription PCR (qRT-PCR). To account for differences in starting material, RNA preparation, RNA quality and cDNA synthesis, adequate normalisation is frequently performed by comparing expression profiles of the genes of interest with those of constitutively expressed genes (= reference genes). Housekeeping genes are most widely used as reference genes, based on the assumption that they are constitutively expressed in most tissues and under certain circumstances, and that they are more or less resistant to cell cycle fluctuations (Zhang *et al.*, 2005; Radonic *et al.*, 2005). However, it has been demonstrated that the expression levels of these genes may vary considerably in different tissues, different cell types and different disease stages, particularly in clinical samples associated with malignant disease (Thellin *et al.*, 2000; Suzuki *et al.*, 2000). Housekeeping genes are not only involved in the basal cell metabolism, but appear to participate in other functions too, and therefore are prone to regulation (Petersen *et al.*, 1990; Singh & Green, 1993; Ishitani *et al.*, 1996). Especially in tumours, the metabolism is generally elevated because of permanent proliferation and expansion. Moreover, some housekeeping genes may have a specific function essential for the tumour metabolism and therefore be up or down regulated (Neuvians *et al.*, 2005). Because of these findings, Vandesompele *et al.* (2002) proposed to identify a set of stable housekeeping genes in the tissue of interest and use them as internal reference genes for accurate normalisation.

Up till now, only a few gene expression studies using real-time qRT-PCR have been performed in horses. *GAPDH* or *ACTB* were commonly used as a single non-validated reference gene (Leutenegger *et al.*, 1999; Giguere & Prescott, 1999; Swiderski *et al.*, 1999; von Rechenberg *et al.*, 2001; Lim *et al.*, 2003; Ainsworth *et al.*, 2003), since at that time not much information was available concerning this issue. Recently, Waguespack *et al.* (2004) compared 4 housekeeping genes (*ACTB*, *B2M*, *GAPDH* and *TBP*) in the lamellae of the hoof in horses.

In this study, 6 commonly used reference genes in both human and animal studies were investigated, both in normal skin and in equine sarcoids of horses. Equine sarcoids are fibroblastic skin tumours and are the most common tumours in horses. The disease not only

induces esthetical defects, but also diminishes the economical value of affected horses (Gerber, 1989). Moreover affected horses show a genetic predisposition for the development of equine sarcoids, through which the breeding value of an animal with sarcoids sharply declines (Lazary *et al.*, 1985; Meredith *et al.*, 1986; Gerber *et al.*, 1988). The bovine papillomavirus (BPV) plays an important role in the etiology of equine sarcoids (Otten *et al.*, 1993; Teifke *et al.*, 1994; Nasir & Reid, 1999). Several clinical types exist, ranging from small, stable patches to large, aggressive and fast growing tumours (Pascoe & Knottenbelt, 1999). To be able to examine the gene expression profile of BPV in these different clinical types and to compare equine sarcoids with normal skin asymptotically infected with BPV, a well suited internal control should first of all be established.

METHODS

Sample collection

Eight equine sarcoid samples were obtained from surgically treated horses. A whole range of tumours were sampled (one occult type, one verrucous type, one nodular type and five fibroblastic types). Three sarcoids were located on the medial part of the thigh, two in the axilla and two on the ventral side of the abdomen. Care was taken to obtain only tumoural tissue, without underlying normal stroma. Normal skin samples were obtained from healthy horses undergoing elective surgery or euthanasia (umbilical hernia, castration, osteochondrosis dissecans, dorsal displacement of the soft palatum). Six of the samples were collected from the ventral abdomen, one from the throat region and one from the shoulder. All samples were freshly collected and stored immediately in RNAlater (Ambion). After overnight incubation at 4 °C, the samples were frozen at -18 °C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated from the samples using TRIR (ABgene) according to the manufacturer's instructions. Subsequently, approximately 4 µg of the total RNA solution, measured with the BioPhotometer (Eppendorf), were treated with 3 units DNase I (Ambion) to remove genomic DNA. This was followed by a spin-column purification (Microcon YM-10, Millipore). A minus RT control with primers for *GAPDH* was performed to check for

successful removal of all the contaminating DNA. These primers were designed by the Primer 3 software (Rozen & Skaletsky, 2000) using publicly available sequences from the Nucleotide Sequence Database from the National Center for Biotechnology Information (NCBI). The initial denaturation was performed at 94 °C for 10 minutes. Thirty-five cycles of amplification were performed. Each cycle involved a denaturation step of 30 seconds at 94 °C, followed by 30 seconds primer annealing at 61 °C and 60 seconds primer extension at 72 °C. After the last cycle, the PCR-mix was heated during 10 minutes at 72 °C to become extension of the partially elongated primers.

First strand cDNA synthesis was carried out on approximately 2.6 µg of the total RNA solution with SuperscriptTM II Reverse Transcriptase (Invitrogen), an engineered version of M-MLV RT, and a combination of random primers (Invitrogen) and oligo(dT)₂₀ primers (Invitrogen) in a total volume of 20 µl, following the manufacturer's instructions. After this step, a PCR was performed with primers for *GAPDH* to check for the presence of cDNA. The reaction conditions used in this PCR were identical to the PCR described for the minus RT control.

Reference gene selection and primer design

Six reference genes (*ACTB*, *B2M*, *HPRT1*, *UBB*, *TUBA1* and *RPL32*) belonging to different functional classes were selected to reduce the chance that these genes might be co-regulated (Table 1).

The primers, based on horse RNA and DNA sequences found in the NCBI database, were designed by the Primer 3 software (Rozen & Skaletsky, 2000). The specificity of the primers was tested using a BLAST analysis against the genomic NCBI database. The complete nucleotide sequences of the genes of interest were characterised using Mfold (Zuker, 2003) to take into account possible secondary structures at the primer binding sites which might influence the PCR efficiency. The PCR products were cloned (pCR 2.1 vector, Invitrogen) and sequenced for verification (Thermo Sequenase Primer Cycle Sequencing Kit, Amersham Bioscience) with an ALF Express sequencer (Amersham Bioscience). Technical information about the primers and amplicons are listed in Table 2.

Table 1. Name and function of genes mentioned in the text.

Symbol*	Gene name*	Function
<i>ACTB</i>	actin, beta	Cytoskeletal structural protein
<i>B2M</i>	beta-2-microglobulin	Beta-chain of major histocompatibility complex class I molecules
<i>HPRT1</i>	hypoxanthine phosphoribosyltransferase I	Purine synthesis in salvage pathway
<i>UBB</i>	ubiquitin B	Protein degradation
<i>TUBA1</i>	tubulin, alpha 1 (testis specific)	Structural protein
<i>RPL32</i>	ribosomal protein L32	Member of the 80 different ribosome proteins
<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme
<i>TBP</i>	TATA box binding protein	General RNA polymerase II transcription factor

* Nomenclature according to Genew (Wain *et al.*, 2002)

Table 2. Information on the primers used in this study.

Gene	Genbank accession number	Primer sequence	Ta (°C)	Product size (bp)	Primer concentration
<i>ACTB</i>	AF035774	CCAGCACGATGAAGATCAAG GTGGACAATGAGGCCAGAAT	59	88	0.5 µl 10 µM
<i>B2M</i>	X69083	GTTCCATCCGCCTGGAGATT GGGGTCTTTGAGAGTAGAGTG	60	182	0.25 µl 10 µM
<i>HPRT1</i>	AY372182	GGCAAAAACAATGCAAAACCTT CAAGGGCATAATCCTACGACAA	60	163	0.5 µl 10 µM
<i>UBB</i>	AF506969	GCAAGACCAATCACCCCTGGA CTAACAGCCACCCCTGAGAC	61	206	0.5 µl 10 µM
<i>TUBA1</i>	AW260995	GCCCTACAACCTCCATCCTGA ATGGCTTCATTGTCCACCA	60	78	0.5 µl 10 µM
<i>RPL32</i>	CX594263	AGCCATCTACTCGGCGTCA TCCAATGCCTCTGGGTTTC	61	149	0.25 µl 10 µM
<i>GAPDH</i>	AF157626	GATGCCCAATGTTTGTGA AAGCAGGGATGATGTTCTGG	61	250	0.5 µl 10 µM

Real-time quantitative PCR

Eight normal skin samples and eight equine sarcoid samples were used for quantification of reference genes.

PCR reactions were performed in a 15 µl reaction volume on the iCycler iQ Real-Time PCR Detection System (Bio-Rad) using the Platinum[®] SYBR[®] Green qPCR SuperMix UDG (Invitrogen) supplemented with 0.02 µM fluorescein and 20 ng of cDNA. Primer concentration varied according to the primers used. A blank was incorporated in each assay.

First, an UDG-treatment was done at 50 °C to prevent cross contamination. The initial denaturation was performed at 95 °C for 2 minutes to activate the *Taq* DNA polymerase, followed by 40 cycles of denaturation at 95 °C for 20 seconds and a combined primer annealing/extension at the specific annealing temperature (Table 3) for 40 seconds during which fluorescence was measured. A melt curve was generated to confirm a single gene-specific peak and to detect primer dimer formation by heating the samples from 70 to 95 °C in 0.5 °C increments with a dwell time at each temperature of 10 seconds while continuously monitoring the fluorescence.

PCR efficiencies were calculated using a relative standard curve derived from a pooled cDNA mixture (a ten-fold dilution series with four measuring points). The pooled cDNA was obtained from normal equine skin and equine sarcoids, using the same RNA isolation and cDNA synthesis protocols as described above.

Each reaction was run in duplicate, whereby a no-template control was included.

During optimisation of the protocol, the PCR products were loaded on a 3% agarose gel after each run to confirm specific gene amplification and the absence of primer dimer formation.

Determination of reference gene expression stability

To determine the stability of the selected reference genes, the geNorm Visual Basic application for Microsoft Excel was used as described by Vandesompele *et al.* (Vandesompele *et al.*, 2002). This approach relies on the principle that the expression ratio of two perfect reference genes should be identical in all samples, independent of the experimental condition or cell type.

RESULTS AND DISCUSSION

Transcription profiling of the candidate genes

cDNA was synthesised from DNA-free RNA (checked with minus RT control) isolated from 8 normal equine skin and 8 equine sarcoid samples. A real-time PCR assay, based on SYBR[®] Green detection, was designed for the transcription profiling of six frequently used reference genes (*ACTB*, *B2M*, *HPRT1*, *UBB*, *TUBA1* and *RPL32*) in these cDNA samples. During optimisation of the protocol, real-time PCR products were visualised by gelelectrophoresis and sequenced for verification. For every assay, a single amplicon with the expected size was generated without primer dimer formation. Indeed, the formation of primer dimers and unspecific amplification, which can falsely increase the gene expression levels, is a major point of attention, particularly when using intercalating dyes such as SYBR[®] Green.

Amplicon sequences of *ACTB*, *B2M*, *HPRT1*, and *TUBA1* were 100% identical with the described sequences on which primer design was based. The sequencing of *UBB* revealed 1 gap and 3 SNPs compared to the original sequence (98% identity). The gap is probably due to sequencing errors; the SNPs did not result in an amino acid variation. When translated to amino acid sequence, the *RPL32* sequence was 100% identical to publicly accessible horse ESTs and human *RPL32* amino acid sequences.

After optimisation, gene-specific amplification was confirmed by a single peak in melt-curve analysis. For each assay, a standard curve was generated by using 10-fold serial dilutions of pooled cDNA, generated of both normal skin and equine sarcoid tissue, characterised by a linear correlation coefficient (R^2) varying from 0.991 to 0.998 and a PCR efficiency between 88.1 and 104.6%. These findings showed that these assays are suitable for quantitative purposes.

In order to select a reliable set of reference genes, each assay was performed in duplicate and included the appropriate control samples. To compare the transcription level of the selected genes across the different samples, the Ct values, ranging from 16.4 to 31.4 were converted into raw data based on the PCR efficiency, gathered by standard curve analyses.

GeNorm analysis

The gene expression stability over the different samples was analysed using the geNorm software (Vandesompele *et al.*, 2002). The ranking of the 6 candidate reference genes according to their M value was not equivalent between the normal skin samples, the equine sarcoid samples and the combination of both kinds of samples. For normal skin *ACTB*, *TUBA1* and *UBB* were the 3 most stable genes (Figure 1A(a)). In equine sarcoids on the other hand, *ACTB*, *B2M* and *UBB* proved to be the most stable genes with *TUBA1* being the least stable gene (Figure 1B(a)). When both sets of samples were analysed together, the results showed that the combination of 3 genes (*ACTB*, *UBB* and *B2M*) is sufficient for adequate normalisation (Figure 1C(a)). The results are listed in Table 3. In another study (Waguespack *et al.*, 2004), where *ACTB*, *B2M*, *GAPDH* and *TBP* were compared as reference genes, *ACTB* and *B2M* were found to be the best endogenous control genes for real-time qPCR of lamella in the hoof of horses.

When calculating a normalisation factor (NF), a careful choice of the number of reference genes should be made. The more genes included, the more accurate the NF is. However, including too many genes may increase the risk of using unsuitable genes, and is also impractical. On the other hand, if the cut off is made too stringent, stably expressed reference genes may be excluded and accuracy might drop. In order to determine how many reference genes should be included, normalisation factors (NF_n), based on the geometric mean of the expression levels of the n best reference genes, were calculated by inclusion of an extra, less stable, reference gene according to Vandesompele *et al.* (2002). Figures 1A(b), 1B(b) and 1C(b) show the pairwise variation V_n/V_{n+1} between 2 sequential normalisation factors NF_n and NF_{n+1} for normal skin, equine sarcoids and the combination of both kinds of samples. In all 3 cases, the inclusion of a 4th gene had no significant contribution (low $V_{3/4}$ value) to the NF. The 3 member sets as described above are a good choice for the calculation of the NF.

Figure 1A(a)

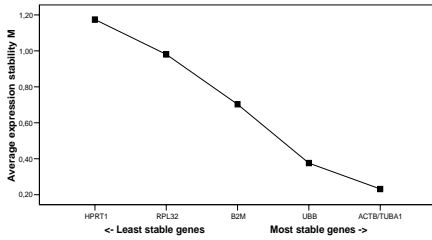


Figure 1A(b)

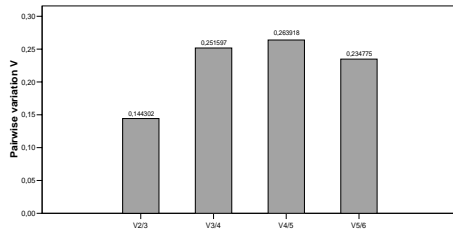


Figure 1B(a)

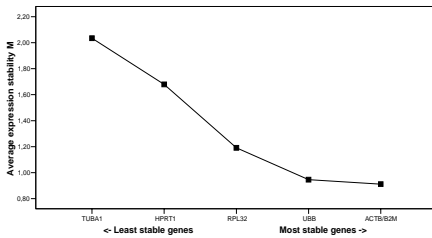


Figure 1B(b)

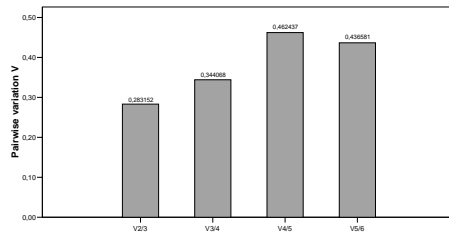


Figure 1C(a)

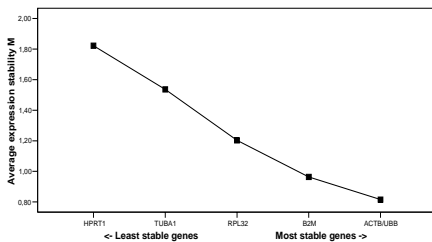


Figure 1C(b)

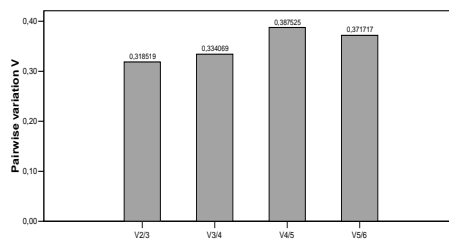


Figure 1. Gene expression stability of the 6 candidate reference genes analysed by the geNorm program (Vandesompele *et al.*, 2002).

- Average expression stability values (M) of the control genes, plotted from least stable (left) to most stable (right).
- Pairwise variation analysis between the normalisation factors NF_n and NF_{n+1} , to determine the optimal number of control genes for normalisation. The higher $V_{3/4}$ value is due to the inclusion of a relative unstable gene and is in accordance with the average expression stability M.

A – Normal skin

B – Equine sarcoids

C – Combination of normal skin and equine sarcoids

Table 3. Ranking of the reference genes.

The reference genes are ranked in order of their expression stability in normal equine skin, in equine sarcoids and in the combination of both kinds of samples, decreasing from top to bottom. The reference genes chosen to calculate the normalisation factor, used for comparing equine sarcoids and normal skin, are printed in bold.

Normal skin	Equine sarcoid	Combination
<i>TUBA1</i>	<i>B2M</i>	<i>UBB</i>
<i>ACTB</i>	<i>ACTB</i>	<i>ACTB</i>
<i>UBB</i>	<i>UBB</i>	<i>B2M</i>
<i>B2M</i>	<i>RPL32</i>	<i>RPL32</i>
<i>RPL32</i>	<i>HPRT1</i>	<i>TUBA1</i>
<i>HPRT1</i>	<i>TUBA1</i>	<i>HPRT1</i>

Implementation of results in clinical research

For the reasons discussed above, we have confidence that our gene expression results are accurate and reliable. The described set of reference genes can be used in gene expression studies both in normal skin, equine sarcoids and the combination of both. One of the points of interest in veterinary medicine is the expression level of BPV in different kinds of equine sarcoids. With the normalisation technique described in this study, reliable results can be obtained. Another research topic in this domain is the study of BPV expression in normal skin, showing latent infection with BPV. Also, the expression level of specific horse genes with a putative role in tumourigenesis, can be investigated.

CONCLUSION

In conclusion, a method for genomic DNA-free RNA extraction from normal equine skin and equine sarcoids was optimised and a reference gene assay for reliable normalisation of real-time qPCR data, obtained from normal skin, equine sarcoids and the combination of both, was designed. The profiling of the gene expression pattern of 6 putative reference genes showed that 3 reference genes should be used. *ACTB*, *TUBA1* and *UBB* can be used in normal skin, while *ACTB*, *B2M* and *UBB* are the best choice in equine sarcoids. If normal skin and equine sarcoids have to be compared, the same member set as proposed for equine sarcoids can be used.

ACKNOWLEDGEMENTS

This research was funded by the Special Research Fund, Ghent University, grant no. 01D01103.

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Bovine papillomavirus load and mRNA expression, cell proliferation and p53 expression in four clinical types of equine sarcoids

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Adapted from *Journal of General Virology* (2007) 88, 2155-2161.

SUMMARY

Equine sarcoids, the most common skin tumours in horses, are induced by the bovine papillomavirus (BPV). Its clinical appearance varies from small stable patches to aggressively growing masses. Differences in BPV load and mRNA expression, Ki67 and p53 immunostaining between 4 clinical types (fibroblastic, occult, nodular and verrucous sarcoids) were evaluated to test the hypothesis that the clinical behaviour of equine sarcoids is correlated with BPV activity. Viral load and expression of BPV E2, E5, E6 and E7 genes were determined with quantitative real-time PCR. The proliferative fraction (PF) of the tumours was determined by Ki67 immunostaining and expression of p53 was analysed by immunohistochemistry. Nodular sarcoids showed a significantly higher viral load than the other types. A significant overall difference between the 4 types was observed for E2, E5, E6 and E7 mRNA expression. Nodular sarcoids showed the highest expression level for each examined BPV gene, followed by verrucous, fibroblastic and occult tumours. Viral DNA and mRNA outcomes were correlated which indicates a similar transcriptional pattern in each type of sarcoids. The PF was significantly higher in the superficial layers of verrucous and fibroblastic sarcoids compared to occult and nodular types. No significant difference was observed for PF in the deep layers and for p53 expression. These results clearly demonstrate the omnipresence and active transcription of BPV in equine sarcoids. However, the hypothesis that the clinical behaviour of an equine sarcoid can be explained on the basis of differences in BPV activity could not be demonstrated.

INTRODUCTION

Equine sarcoid is the most common skin tumour in horses. The clinical aspect can vary remarkably, ranging from small, stable patches to aggressively growing tumours up to more than 10 cm in diameter. A sarcoid can appear as a single tumour, but horses often show multiple lesions. Although equine sarcoids do not metastasise, they recur frequently if not treated properly. Different clinical types have been described (Pascoe & Knottenbelt, 1999). The fibroblastic type, with a typically ulcerated epidermis, usually presents an aggressive clinical behaviour. Occult types are flat, alopecic patches, sometimes with hyperkeratosis or small nodules on their surface. These tumours will often not evolve, even over several years. Nodular sarcoids are round masses with an intact epithelium. The overlying skin can be loose from the stromal tissue or (partially) attached to the fibroblastic portion of the tumour. Verrucous sarcoids have a typically warty appearance. Nodular and verrucous sarcoids display a clinical behaviour in between the fibroblastic and the occult sarcoid, with mostly a moderate growth. Mixed sarcoids, composed of two or more of the described types, are also commonly observed. Occult sarcoids, and even nodular and verrucous sarcoids, can remain stable for many years and then suddenly, without any apparent reason, change dramatically into a rapidly growing tumour, often fibroblastic, that can be difficult to treat.

The most important aetiological factor in the development of equine sarcoids is the bovine papillomavirus type 1 and 2 (BPV-1, - 2). BPV DNA has been found in up to 100% of the examined sarcoids in several studies (Teifke & Weiss, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Carr *et al.*, 2001a; Martens *et al.*, 2001a; Carr *et al.*, 2001b; Martens *et al.*, 2001b; Martens *et al.*, 2001c). Also BPV mRNA and BPV proteins have been detected in equine sarcoid tissue (Nasir & Reid, 1999; Carr *et al.*, 2001b), which provided evidence for a direct involvement of BPV in equine sarcoids.

The BPV genome contains 8 early and 2 late genes (Chen *et al.*, 1982; Campo, 1988). Early genes (E1, E2, E3, E4, E5, E6, E7 and E8) are responsible for replication and transformation, whereas late genes (L1 and L2) encode for capsid proteins. The four most important genes in equine sarcoid development are E2, E5, E6 and E7. E2 is a regulator of transcription; E5 is the most important transforming protein, in cooperation with E6. E7 is, beside its minor role in transformation, a regulator of the BPV copy number. Complete (encapsulated) virions have not been isolated from equine sarcoids so far and therefore a non-productive infection is assumed (Amtmann *et al.*, 1980).

The objective of the present study was to investigate differences in bovine papillomaviral load and mRNA expression of the four most important early genes for equine sarcoid development (E2, E5, E6 and E7) in four different clinical types of equine sarcoids (fibroblastic, occult, nodular and verrucous types). Furthermore, cell proliferation using Ki67 immunostaining and variations in p53 expression were analysed. Our hypothesis was that clinically aggressive sarcoids were characterised by a higher activity of the bovine papillomavirus as well as a higher proliferative fraction and a higher degree of abnormal p53 staining as compared to stable sarcoids.

METHODS

Sample collection

Equine sarcoids included in this study were obtained from patients referred to the clinic for surgery and anaesthesiology of domestic animals of Ghent University for surgical treatment between February 2004 and November 2005. Fibroblastic, occult, nodular and verrucous sarcoids were identified and classified based on their gross morphology according to the criteria of Pascoe & Knottenbelt (1999). If a sarcoid could not clearly be assigned to one specific clinical type, it was excluded from the study. From each tumour, three representative samples of at least 100 mg were taken. The first sample was stored at -18 °C for BPV DNA analysis. Another sample was stored in RNAlater (Ambion) for RNA analysis. A third part of the tumour was snap frozen in liquid nitrogen after embedding in KP-CryoBlock (Klinipath) and was stored at -80 °C for immunohistochemical staining. The following parameters were recorded for each tumour: localisation, size and time of onset of the sarcoid and breed and sex of the horse. It was also noted whether the tumour was a recurrence after former surgery or not.

Quantitative bovine papillomavirus DNA and mRNA analysis

DNA extraction of the first sample of each sarcoid and subsequent PCR analysis for BPV DNA detection, with a threshold of 20 copies per sample, was performed as described by Bogaert *et al.* (2005). Positive controls were sarcoid samples formerly confirmed by sequencing. H₂O served as no-template control. To identify the BPV type, a restriction

fragment length polymorphism assay was applied as described by Martens *et al.* (2000). Next, viral load of BPV-1 and -2 was determined by quantitative real time PCR using Taqman probes.

Total RNA isolation and subsequent first strand cDNA synthesis were performed on the second sample of each sarcoid as described earlier (Bogaert *et al.*, 2006). Briefly, RNA was isolated using TRIR (Abgene), followed by DNase treatment and a minus RT control with primers for *GAPDH* to check for successful removal of all contaminating DNA. Next, first strand cDNA synthesis was carried out with SuperscriptTM II Reverse Transcriptase (Invitrogen) and a combination of random primers (Invitrogen) and oligo(dT)₂₀ primers (Invitrogen) in a total volume of 20 µl. After this step, a PCR was performed with primers for *GAPDH* to check for the presence of cDNA.

Primers and probes were designed using Primer 3 software (Rozen & Skaletsky, 2000) for E1 (BPV load analysis) and for E2, E5, E6 and E7 (BPV mRNA analysis) of both BPV-1 and BPV-2. Specificity was tested using a BLAST analysis against the genomic NCBI database. The amplicon and the surrounding sequences were characterised using Mfold (Zuker, 2003) to take into account possible secondary structures at the primer and probe binding sites which might influence the PCR efficiency. The PCR products were cloned (pCR 2.1 vector, Invitrogen) and sequenced for verification (Thermo Sequenase Primer Cycle Sequencing Kit, Amersham Bioscience) with an ALF Express sequencer (Amersham Bioscience). Information about primers, probes and amplicons is listed in Table 1.

Reactions were performed in a 15 µl reaction volume on the iCycler iQ Real-Time PCR Detection System (Bio-Rad) using the iQ Supermix (Bio-Rad) for DNA analysis and the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) supplemented with 0.02 µM Fluorescein (Bio-Rad) for mRNA analysis. Samples were 1:1000 (DNA) or 1:100 (mRNA) diluted. First, an UDG treatment was done at 50 °C to prevent cross contamination. The initial denaturation was performed at 95 °C for 3 minutes to activate the *iTaq* DNA polymerase (DNA) or for 2 minutes to activate the Platinum *Taq* DNA polymerase (mRNA), followed by 40 cycles of denaturation at 95 °C for 20 seconds and a combined primer annealing/elongation at the specific annealing temperature for 40 seconds during which fluorescence was measured. During analysis of mRNA samples, a melt curve was generated to confirm a single gene-specific peak and to detect primer dimer formation by heating the samples from 70 to 95 °C in 0.5 °C increments with a dwell time at each temperature of 10 seconds while continuously monitoring the fluorescence. Each reaction was run in duplicate, whereby two no-template controls and dilution series for BPV-1 and BPV-2 were included in

each run. Additionally, two normal skin samples from sarcoïd-free horses, two samples of equine melanomas and two samples of exuberant granulation tissue were included as controls. During optimisation of the protocol, the PCR products were loaded on a 3% agarose gel after each run to confirm specific gene amplification and the absence of primer dimer formation. PCR efficiencies were calculated using a relative standard curve (a ten-fold dilution series with five measuring points) derived from pooled DNA obtained from a mixture of 13 equine sarcoïds for BPV DNA analysis and pooled cDNA from a mixture of 10 sarcoïds for BPV mRNA analysis, both for BPV-1 and BPV-2. Ct values (threshold cycle) were obtained at the time the fluorescence exceeded the threshold value. Ct values were subsequently transformed to “raw data”, taking into account PCR efficiency and all values measured during the same run.

Data obtained from viral load analysis were normalised against ubiquitin B, which was quantified using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Obtained data from the mRNA analysis were normalised against a set of 3 reliable reference genes (beta-actin, beta-2-microglobulin and ubiquitin B). The choice of the employed reference genes has been elaborated by Bogaert *et al.* (2006). Briefly, a normalisation factor was computed for each single sample by calculating the geometric average of the obtained results from the 3 reference genes, followed by dividing the value of the gene of interest of the same sample by this normalisation factor, thus allowing reliable comparison of different samples.

Table 1. Primer and amplicon properties.

Primer/Probe	Primer/Probe sequence (5'-3')	Primer concentration (nM)	Annealing temperature (°C)	Accession number	Position
BPV qualitative	CAAAGGCAAGACTTTCGAAACAT AGACCTGTACAGGACCTCAA	1000	60	X02346 M20219	3759-4002 3760-4006
BPV quantitative	AAATCGGTGAGCAACCTTT TGCTGTCTCCATCCTCTTCA	333	60	X02346 M20219	2500-2619 2497-2616
BPV-1 probe	FAM-CGTCAAATCAGGTCTAAACGCCCC-BHQ1	100	60	X02346	2565-2586
BPV-2 probe	TexasRed-TCAACCCAGGTCTAAGCGCCC-BHQ2	100	60	M20219	2562-2581
BPV-1 E2	TGCAGTTGCTTTTGCAGGAG AGCACCGTTTAGTTCTGACAT	333	60	X02346	2831-2935
BPV-1 E5	GGTGTACTATGCCAAATCTATGGTT AGCACTCAAATGATCCAGT	333	60	X02346	3870-3989
BPV-1 E6	ATGGACCTGAAACCTTTTGC CAGCCTTCCCAGATTACAAC	333	60	X02346	4200-4333
BPV-1 E7	CACAACGTGGACACAAGAGG CTGTCTGCTGTGGAAACTGC	333	60	X02346	714-833
BPV-2 E2	TGCAGTTGCTTTTGCAGGAG AGCACCGTTTAGTTCTGACAT	333	60	M20219	2828-2932
BPV-2 E5	TGCTGCTTTACTGTTCTGCT GAGCACTCAAATGATCCAGT	167	60	M20219	3934-3994
BPV-2 E6	GGGGAAAACCTAACCAAAAAC AGTCGTAGCAGCTCCTCTT	167	60	M20219	370-469
BPV-2 E7	TCAAGACTGGCTCTACGACCT GCAACGGGACACAACA	167	60	M20219	756-832
GAPDH	GATGCCCAATGTTTGTA AAGCAGGATGATGTTCTGG	333	61	AF157626	248-497
ubiquitin B	GCAAGACCATCACCCCTGGA CTAACAGCCACCCCTGAGAC	333	61	AF506969	831-1036
beta-2-microglobulin	GTTCATCCGCCCTGGAGATT GGGGTCTTTGAGAGTAFAGAGTG	167	60	X69083	155-336
beta-actin	CCAGCACGATGAAGATCAAG GTGGACAATGAGGCCAGAAT	333	59	AF035774	963-1052

Immunohistochemical staining procedures

Cryosections of the third sample of each sarcoid were cut at 6 μm and mounted on APES coated slides (StarFrostPlus). Sections were allowed to dry at 37 °C for 30 minutes and then fixed in acetone for 5 minutes at room temperature. After drying, slides were stored at -20 °C until staining. Slides were mounted in disposable immunostaining chambers (Shandon) and endogenous peroxidase activity was blocked using a 3% H_2O_2 in methanol solution. After rinsing, sections were pre-incubated for 30 minutes with 30% goat serum (DAKO) at 25 °C. Next, the appropriate primary monoclonal antibody was applied. The 297M monoclonal antibody (BioGenex) was employed to detect Ki67 at 1:20 dilution (2 hours at 37 °C) and the mouse DO-7 monoclonal antibody (Novocastra) against p53 at 1:50 dilution (2 hours at 25 °C). After rinsing, biotin conjugated goat-anti-mouse Ig (DAKO) served as a second antibody reagent (30 minutes at 21 °C). Signal amplification was obtained by incubating the slides for 30 minutes at 21 °C with the horseradish peroxidase conjugated streptavidin-biotin complex (DAKO). Diaminobenzidine tetrahydrochloride (Sigma) was used as a chromogen and H_2O_2 as a substrate, and the slides were counterstained with haematoxylin. Positive control tissues were equine papilloma for Ki67 and equine squamous cell carcinoma for p53. Normal equine skin served as negative control. On the Ki67 stained slides eight high power fields (640x) of the superficial and eight of the deep layers of each tumour were randomly chosen to count all nuclei and the number of Ki67 positive nuclei. The proliferative fraction (PF) was calculated separately for the superficial and the deep tumour portion, as well as globally. The p53 stained slides were evaluated for presence or absence of p53 expression.

Statistical analysis

All numerical data were log transformed to achieve a normal distribution. Correlations between outcomes were investigated with Pearson's correlation coefficient. For all different outcome variables (viral load and mRNA of E2, E5, E6 and E7), differences between the sarcoid types, BPV type, localisation, size, time of onset, breed, sex and recurrence after former surgery were analysed using a linear mixed effect model, with "horse" as random effect. Differences in the PF between the superficial and deep layers of the sarcoids was analysed using a paired sample t-test. Differences in p53 expression in relation to the sarcoid type were analysed by means of logistic regression. For all tests, differences were considered

statistically significant if p-values were below 0.05. All statistical analyses were performed in S-plus (S-plus 7.0 for Windows, Insightful Corp.).

RESULTS

Sample population

In this study, 99 equine sarcoids originating from 60 horses of different breeds (48 warmbloods, 3 Arabian horses, 3 draft horses, 2 thoroughbreds, 2 standardbreds and 2 ponies) and 1 donkey were included. Three of them were stallions, 25 were geldings and 33 were mares. The median age of the animals was 8 years (range from 2 to 19 years). Tumours were classified as fibroblastic (n=39), occult (n=20), nodular (n=21) or verrucous (n=19) based on their clinical appearance. The median size of the sarcoids was 29 mm (ranging from 4 to 100 mm). Forty-two percent of them were localised on the ventral side of the body, 29% on the limbs, 15% in the genital region, 10% on the dorsal side and 3% on the head. Thirty-three percent of the sarcoids were observed less than 6 months before the time of presentation at the clinic, 48% were present since more than 6 months and for 19% of the tumours the time of onset was not known. Twenty-one percent of the sarcoids were recurrences after previous treatment; the others had not been treated before.

Quantitative bovine papillomavirus DNA and mRNA analysis

All tumours showed presence of BPV DNA. The majority of the sarcoids (88%) harboured BPV-1, the remaining 12% BPV-2.

Figure 1 illustrates that nodular sarcoids had a more than fourfold higher viral load than fibroblastic, verrucous and occult sarcoids. The BPV load of the three last named sarcoid types was not significantly different. No-template controls, samples of normal skin, melanomas and exuberant granulation tissue were all negative for BPV DNA.

Figure 2 represents the mean and the 95% confidence interval of the log transformed values of all samples quantitatively analysed for the 4 BPV early genes (E2, E5, E6, E7) in each group of equine sarcoids. For each examined BPV gene, nodular sarcoids showed the highest expression, followed by verrucous, fibroblastic and occult sarcoids. Differences between nodular and fibroblastic and between nodular and occult sarcoids were always

statistically significant. On the other hand, the distinction between nodular and verrucous sarcoids was too small to be significant. Verrucous and occult tumours varied significantly for each examined BPV gene, except for E6. Verrucous and fibroblastic sarcoids only showed a statistical significant difference for E7 expression but not for the other BPV genes. Finally, fibroblastic and occult sarcoids showed a significant difference for E2 and E5 but not for E6 and E7. All negative controls did not contain BPV mRNA. No significant correlation was found between outcome of any examined BPV gene and BPV type, localisation, size, time of onset, breed, sex and recurrence after former surgery (data not shown). Tumours originating from the same horse showed related outcomes, despite a sometimes different clinical appearance.

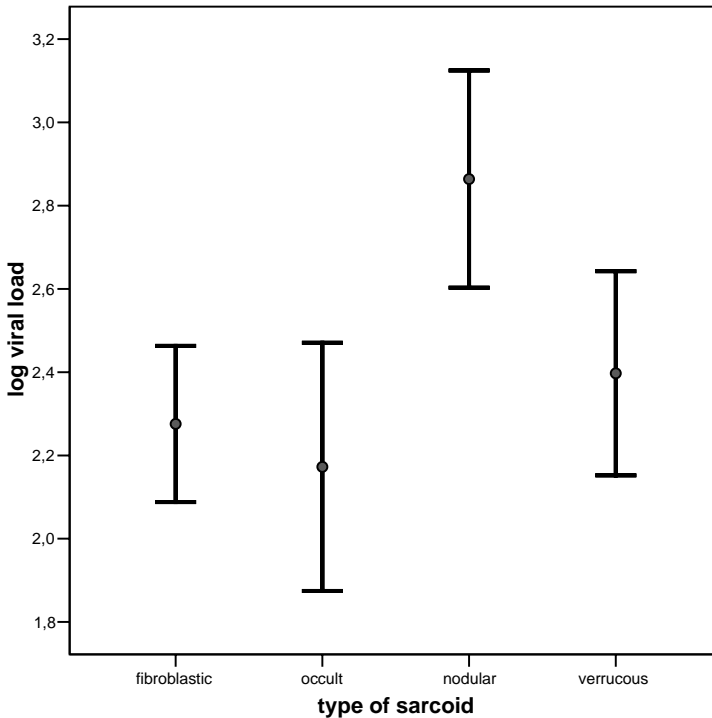


Figure 1. Graphic representation of BPV load in a logarithmic scale in the four different types of equine sarcoids. Dots represent the mean and bars the 95% confidence interval.

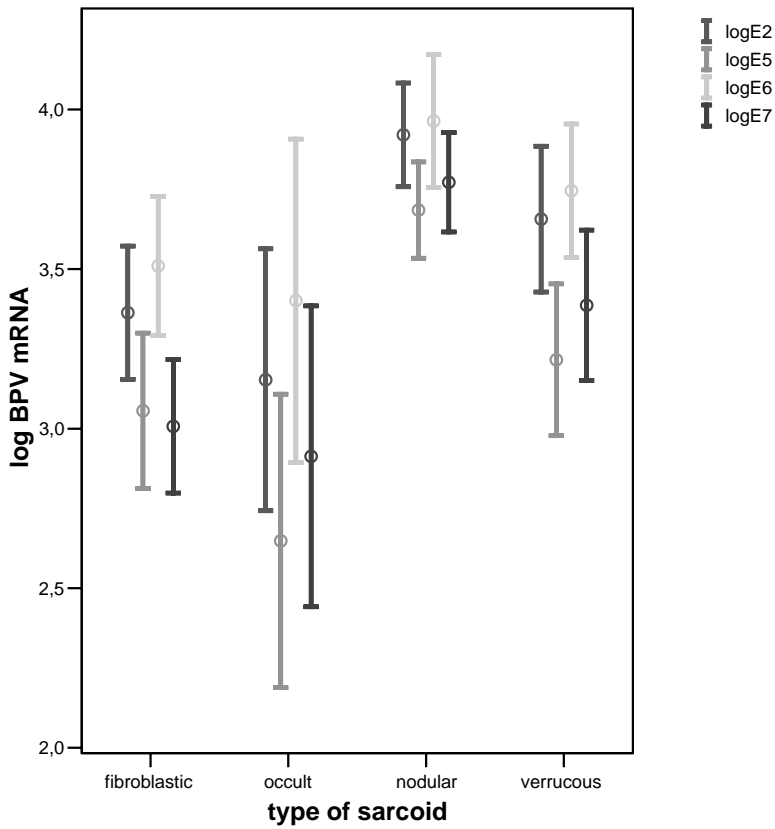


Figure 2. Graphic representation of BPV mRNA expression in a logarithmic scale in the four different types of equine sarcoids. Dots represent the mean and bars the 95% confidence interval.

Immunohistochemical staining

The mean PF for all equine sarcoids was 2.41%. The log transformed PF for the superficial portion of each sarcoid was significantly higher ($p < 0.001$) than the log transformed PF for the deep portion. Verrucous and fibroblastic sarcoids showed a significantly higher PF in the superficial layers compared to occult and nodular tumours. No significant difference for PF in the deep layers could be observed. Data are graphically represented in Figure 3.

Perinuclear p53 expression was present in 36% of the tumours. A relatively high proportion of the verrucous and fibroblastic sarcoids showed p53 expression (respectively 47% and 41%), in occult sarcoids the expression was present in 35% of the tumours and only 19% of the nodular sarcoids showed p53 expression. However, these differences were not statistically significant.

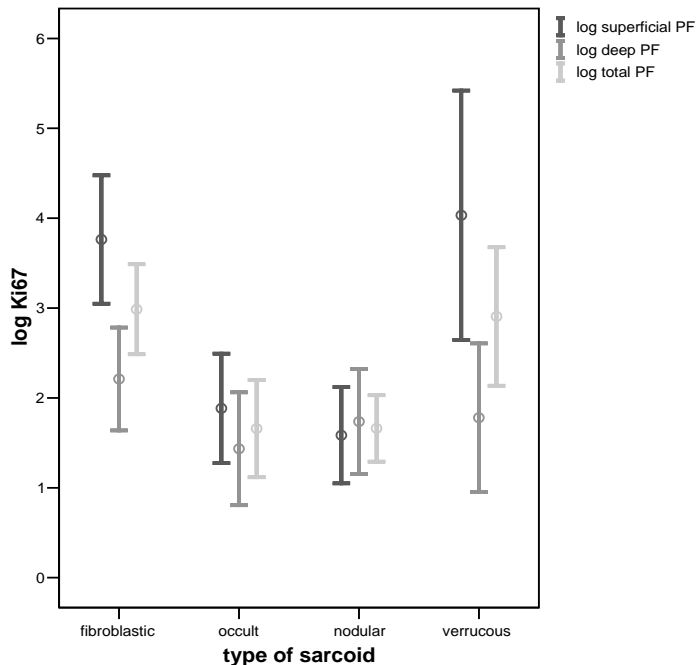


Figure 3. Graphic representation of log transformed proliferative fraction (PF) in the four different types of equine sarcoids. Dots represent the mean and bars the 95% confidence interval.

Correlation between results

Expression of different BPV genes was highly correlated (Pearson's correlation coefficient "r" between 0.675 and 0.923). A good correlation between viral load and BPV mRNA expression was observed (r between 0.518 and 0.633). On the other hand, neither viral load nor BPV mRNA expression were significantly correlated with PF ($r < 0.135$) except for E6 mRNA and PF in the deep layers, which showed a rather weak correlation ($r = 0.205$).

DISCUSSION

The present study clearly confirms the causative role of BPV in equine sarcoids. In agreement with other studies, BPV DNA was present in 100% of the sarcoids (Teifke & Weiss, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Carr *et al.*, 2001a; Martens *et al.*, 2001a; Carr *et al.*, 2001b; Martens *et al.*, 2001b; Martens *et al.*, 2001c). Moreover, all examined early BPV genes were expressed in every sarcoid, whereas all samples of normal skin from sarcoid-free horses and non-sarcoid tumours were negative for BPV DNA and mRNA. Some sarcoids however showed a very low BPV expression. Following our hypothesis which postulates that clinical behaviour is correlated with BPV activity, these findings might reflect a very stable character of the tumour or even regression.

As expected, fibroblastic tumours, which show an aggressive character, have a high proliferative fraction. This is in contrast with the observed low BPV mRNA expression in these tumours. In contrast, HPV mRNA expression increases in human cervical cancer when lesions progress from low grade dysplasia to invasive carcinoma (Wang-Johanning *et al.*, 2002; Kraus *et al.*, 2004; Scheurer *et al.*, 2005). Also viral load was not in the expected concordance with the clinical behaviour, with nodular sarcoids showing a much higher viral load than the three other types. In human medicine, it has been shown that women with low grade cervical intraepithelial lesions accompanied by a high HPV load are at higher risk for progression to high grade lesions (Ho *et al.*, 2006) and that a higher HPV load is seen in high grade lesions than in low grade lesions (Carcopino *et al.*, 2006; Kovacic *et al.*, 2006; Lai *et al.*, 2006). On the other hand, Dahlgren *et al.* (2006) could not determine any influence of HPV load (except for HPV-16) on survival in patients with early stage cervix carcinoma. Possibly the role of BPV becomes less important in tumour progression once certain cellular abnormalities have been established, which might indicate a "hit and run" role of BPV.

Another possible explanation for lower BPV mRNA expression in fibroblastic sarcoids is that these tumours are composed of not only sarcoid cells, but also a relative high amount of endothelial cells due to high vascularisation, and inflammatory cells as a result of superficial bacterial infection. This can reduce the relative amount of BPV DNA and mRNA in those tumours. Verrucous sarcoids have a relative greater proportion of epithelial cells which may also lower the proportion of BPV infected fibroblastic cells. In occult sarcoids, there is usually a mixture of transformed and normal fibroblasts, since these tumours represent the initial stage of transformation. Nodular sarcoids on the other hand are very dense tumours with a high cellularity, which might explain the relatively higher amount of virally infected cells and therefore a higher quantity of BPV DNA and mRNA. Since BPV DNA and mRNA levels were correlated, it can be expected that in each equine sarcoid, independent of its clinical type, every BPV DNA molecule displays a similar transcriptional pattern. Only very small tumours or tumours with clinical signs of regression seem to have a lower BPV activity.

Carr *et al.* (2001b) found expression of BPV E5 protein in all examined sarcoids with the highest expression observed in biologically aggressive fibroblastic variants. However, this expression was not quantified. Another explanation of the discrepancy between their and our results is that there is no linear correlation between mRNA expression and protein expression due to possible post-transcriptional regulation under varying cellular conditions.

The proliferative fraction of the equine sarcoids examined in this study was low. This is in accordance with other studies, where the observed PF varied between 0 and 11% (Martens *et al.*, 2000; Nixon *et al.*, 2005). This might reflect the sometimes very stable character of sarcoids. However, even fast growing and clinically aggressive tumours still showed a rather low PF. Possibly only highly malignant tumours, such as squamous cell carcinomas, show a high proliferative fraction.

P53 expression was comparable with other studies on equine sarcoids (Johnston *et al.*, 1996; Martens *et al.*, 2000; Nixon *et al.*, 2005). A subset of equine sarcoids displays perinuclear expression of p53, by which the normal function of this nuclear tumour suppressor protein is abrogated resulting in perturbation of the balance of cell growth and cell death (Nixon *et al.*, 2005). A significant difference between the different clinical types could not be observed and therefore abnormal p53 expression might not be the reason for aggressive behaviour of an equine sarcoid.

CONCLUSION

In conclusion, these results confirm that BPV is omnipresent and they show that it is actively transcribed in equine sarcoids irrespective of the clinical type. However, our hypothesis that the clinical behaviour of an equine sarcoid could be explained by differences in BPV activity could not be demonstrated. Further research is required to elaborate the rationale for differences in the behaviour of equine sarcoids.

ACKNOWLEDGEMENTS

This research was funded by the Special Research Fund, Ghent University, grant no. 01D01103.

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**Presence of bovine papillomavirus in the
normal skin and in the surroundings of horses
with and without equine sarcoids**

**Detection of bovine papillomavirus DNA on
the normal skin and in the habitual
surroundings of horses with and without
equine sarcoids**

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Adapted from: *Research in Veterinary Science* (2005) 79, 253-258.

SUMMARY

The purpose of the present study was to examine whether bovine papillomavirus (BPV) DNA can be detected on the normal skin and in the habitual surroundings of horses with and without equine sarcoids by means of superficially taken swabs. In affected horses, no significant difference in presence of BPV DNA could be observed between samples obtained from the equine sarcoid surface, from normal skin close to the tumour and from a normal skin site in direct contact with the tumour. From the group of healthy horses living in contact with affected horses, 44% were BPV DNA positive. The surroundings of affected and non-affected horses are probably not a major source of BPV DNA contamination. It can be concluded that BPV DNA is present on the normal skin of horses affected by equine sarcoid and to a lesser degree, on the normal skin of horses living in contact with affected horses.

INTRODUCTION

The bovine papillomavirus (BPV) plays an important role in the etiology and pathogenesis of equine sarcoids. With a number of molecular techniques, including Southern blot hybridisation and PCR, BPV DNA can be detected in 86 to 100% of equine sarcoids (Trenfield *et al.*, 1985; Angelos *et al.*, 1991; Otten *et al.*, 1993; Bloch *et al.*, 1994; Reid *et al.*, 1994b; Martens *et al.*, 2001b). Nasir and Reid (1999) demonstrated that in 95% of the sarcoids where BPV DNA could be detected by means of PCR, and expression of viral genes was also present. Carr *et al.* (2001b) demonstrated the presence of the BPV E5 protein in all examined sarcoids.

The exact manner of BPV infection of horses and the development of equine sarcoids is not yet fully understood. In contrast to the cattle BPV infection, where complete virus particles are formed, a non-productive infection occurs in equines. Hypotheses for infection of the horse include direct or indirect contact with other infected horses and cattle, and transmission by insects may also play a role (Reid *et al.*, 1994a; Knottenbelt *et al.*, 1995; Chambers *et al.*, 2003). However, contact with the virus alone is insufficient for tumoural proliferation: skin trauma, the immunological status and the genetic constitution of the individual horse also play an important role (Lazary *et al.*, 1985; Meredith *et al.*, 1986; Brostrom *et al.*, 1988; Gerber *et al.*, 1988; Phelps *et al.*, 1999; Chambers *et al.*, 2003). Furthermore, there is discussion about the occurrence of viral latency. In humans and cattle, latency of papillomaviruses in asymptomatic individuals has clearly been demonstrated (Jarrett, 1985; Campo *et al.*, 1994). In horses, BPV DNA has been demonstrated in biopsies obtained from the normal skin of horses affected with equine sarcoid, possibly demonstrating the occurrence of viral latency (Trenfield *et al.*, 1985; Carr *et al.*, 2001a; Martens *et al.*, 2001a). However the virus has not yet been demonstrated in the normal skin of horses without equine sarcoid (Otten *et al.*, 1993; Teifke *et al.*, 1994; Carr *et al.*, 2001a). There is not much known about the prevalence and survival of BPV or its DNA in the surroundings of horses with equine sarcoids.

The purpose of the present study was to determine the presence of BPV DNA on the normal skin and in the habitual surroundings of horses with and without equine sarcoid by means of swabs, in order to better understand the contamination rate of the environment with bovine papillomavirus.

MATERIAL AND METHODS

Population

In the present study, samples were taken from 39 horses divided into four groups (Table 1). The horses in group 1 were admitted to our clinic with at least one ulcerated equine sarcoid. In group 2, the horses were unaffected but lived in direct contact with affected horses (e.g. stabled in neighbouring boxes, grazing on the same pasture...). Group 3 included non-affected horses living in direct contact with cattle. Both the horses and the cattle were housed on the same farm and were tended by the same person. One of the horses of group 3 was sampled twice: at the time of first sampling, the cattle that were held together with the horse showed no clinical signs of papillomatosis. Five months later, the horse was sampled again because a two-year-old cow suffered from warts for a few weeks. The eight other horses from this group were kept on farms where none of the cows had developed papillomatosis for at least six months. In group 4, the group of control horses, 9 of the horses had no skin problems. One horse had a squamous cell carcinoma of the penis, confirmed by histopathological examination.

Table 1. Characterization of the 4 different groups of horses.

	<i>Group 1</i>	<i>Group 2</i>	<i>Group 3</i>	<i>Group 4</i>
Equine sarcoid-affected	yes	no	no	no
Contact with affected horses	no	yes	no	no
Contact with cattle	no	no	yes	no
Number of horses	11	9	9	10

Sampling and storage of the samples

To determine the presence of BPV DNA, samples were taken by means of swabs from the surface to be examined (3 x 3 cm for skin samples, 5 x 5 cm for samples from the surroundings of the horse). The swabs were rubbed firmly during at least 30 seconds until the tip of the swab was clearly covered with material present on the skin. When the surface was dry, the swabs were moistened with a sterile NaCl 0.9% solution in order to pick up as much material as possible. During the sampling, care was taken not to cause cross contamination between the samples. Swabs were stored in empty polypropylene vials (1.5 ml, Eppendorf®) at -18 °C.

The locations to be sampled depended on the group the horse belonged to. In group 1 (horses with equine sarcoids) samples were taken from the sarcoid surface, the normal skin close to (< 4 cm) and further distant from (> 20 cm) from the tumour and at a site of the normal skin in direct contact with the tumour (e.g. medial side of the carpus for tumours on the side of the head, prepuce for tumours on the medial side of the thigh, dorsal side of the hind leg for tumours on the ventral abdomen...). Furthermore, samples were obtained from the normal skin on the medial corner of the eye and the genitals (prepuce for males or tip of the teat for females). In the horses from groups 2, 3 and 4, samples were taken from the lateral side of the neck, the dorsal side of the carpus, the axilla, the ventral abdomen, the medial corner of the eye and the genitals. Samples were also taken from the surroundings of every horse included in this study: the stable wall and the feed tub were examined for this purpose. If possible, flies from the stable were caught with methomyl granules (Staflex®).

Processing of the samples

DNA extraction was performed by means of a commercial protocol (Puregene® DNA Isolation Kit, Gentra Systems) and resulted in a pellet of DNA which was dissolved in a final volume of 100 µl. Samples were stored at -18 °C. For detection of the presence of BPV DNA, a polymerase chain reaction (PCR) was used. The primer set used is complementary to a common sequence of the E5L2 open reading frame of both BPV-1 and BPV-2 (upper primer: 5'-CAAAGGCAAGACTTTCTGAAACAT-3'; lower primer: 5'-AGACCTGTACAGGAGCACTCAA-3'). The initial denaturation was performed at 94 °C for 10 minutes. Thirty cycles of amplification were performed. Each cycle involved a denaturation step of 30 seconds at 94 °C, followed by 30 seconds primer annealing at 58 °C

and 1 minute primer extension at 72 °C. After the last cycle, the PCR-mix was heated during 10 minutes at 72 °C to become extension of the partially elongated primers. As a positive control, cloned BPV-1 and BPV-2 were used. H₂O served as negative control. When a specific fluorescent band at 250 bp was visible, the sample was considered positive for BPV DNA. In case of a smear of DNA on the gel, the PCR was repeated with a 1/10 dilution of the DNA. The sensitivity of this PCR was tested via 1/10 dilution series. The threshold could be determined at 20 copies of BPV DNA per sample in vitro.

Statistical analysis

The results were processed with SPSS 11.0. In the group of horses with equine sarcoids, a X²-test for an RxC contingency table was used to determine the positivity of the swab in relation to the location of sampling. For the individual comparisons, Fisher's exact tests (2x2 tables) were used. The X²-test was also used to check for any significant difference in the presence of BPV DNA on the normal skin of horses without sarcoids, depending on the surroundings they were held in. Differences were considered significant at p<0.05.

RESULTS

The results of the PCR analysis of the horses from group 1 are presented in Table 2. Table 3 summarises the results of all examined samples and horses per group. When results are grouped per horse, a horse is considered to be positive for BPV DNA as soon as one of the normal skin samples is positive. All together, the normal skin of 39 horses was sampled and one of them was sampled twice.

In group 1, 10 of the 11 samples obtained from the sarcoids were positive for BPV DNA (91%). Of the "normal skin" samples, 47% were positive and all horses had at least one normal skin sample positive for BPV DNA. No positive BPV DNA samples were obtained from the surroundings of this group of sarcoid-affected horses. Statistical analysis revealed that the different sampling locations on the normal skin did not form a homogenous group (p≤0.0001). Individual comparisons between the sarcoid surface and the different normal skin locations, showed that samples taken close to the tumour ("< 4 cm") and on a site of the normal skin in direct contact with the tumour ("contact") were as likely to be positive for BPV DNA compared to samples from the equine sarcoid surface. There was a significantly

lower probability of obtaining a positive sample on all other normal skin locations ($p < 0.05$ for “> 20 cm”, $p < 0.01$ for “eye” and “genitals”).

In group 2, 4 of the 9 horses (44%) had at least one positive normal skin sample. In total, 6 out of 54 normal skin samples (11%) were positive for BPV DNA. All of the examined samples of the surroundings were negative. In group 3, all samples of the normal skin and the surroundings obtained from the papillomatosis-free farms were negative. However, 4 of the 6 skin samples from the horse living in contact with a papillomatosis-infected cow were positive whereas the samples taken before the papillomatosis infection were all negative. The sample of the stable wall from that horse was also positive after the papillomatosis infection occurred. In group 4, there was only one positive normal skin sample: it was obtained from the horse with the squamous cell carcinoma. All samples from the other horses as well as all samples of the surroundings were negative. Although some variation could be observed between the 3 groups of horses without equine sarcoid, a statistical significant difference for the presence of BPV DNA depending on the surroundings in which the horses were held (in contact with affected horses or not, in contact with cattle or not) could not be proven ($p = 0.114$). In addition, grouping of the samples per horse could not reveal a statistical difference in positivity for BPV DNA depending on the surroundings of the horses ($p = 0.106$).

Table 2. Results of the individual samples in horses from group 1.

Horse	sarcoid	< 4 cm	> 20 cm	contact	eye	genitals	stable	feed tub	flies
S1	+	+	+	-	-	-	-	-	-
S2	+	+	-	-	-	-	/	/	/
S3	+	+	-	+	-	-	/	/	/
S4	-	+	-	+	-	+	/	/	/
S5	+	+	-	+	-	-	-	-	-
S6	+	+	-	-	-	-	-	-	/
S7	+	+	-	-	-	-	-	-	-
S8	+	+	-	-	-	-	/	/	/
S9	+	+	+	+	+	-	/	/	/
S10	+	+	+	+	+	-	-	-	/
S11	+	+	-	+	+	+	/	/	/
Total +	10	11	4	6	3	2	0	0	0
% +	91%	100%	36%	55%	27%	18%	0%	0%	0%

+: positive for BPV DNA

-: negative for BPV DNA

/: no sample available

Total +: total number of positive samples

% +: percentage of positive samples

Table 3. Results of the examined samples and horses (number and percentage of positive samples and horses). A horse is considered positive as soon as one of the normal skin samples is positive for BPV DNA. The surroundings of a horse are considered positive as soon as at least one of the samples of the surroundings is positive for BPV DNA.

	Sarcoid		Normal skin		Surroundings			
	N_s	n_s (%) positive	N_h	n_h (%) positive	N_s	n_s (%) positive	N_h	n_h (%) positive
Group 1	11	10 (91%)	11	10 (91%)	13	0 (0%)	5	0 (0%)
Group 2			55	26 (47%)	21	0 (0%)	9	0 (0%)
Group 3			54	6 (11%)	22	1 (5%)	10	1 (10%)
Group 4			60	4 (7%)	20	0 (0%)	9	0 (0%)
Total	11	10 (91%)	228	37 (16%)	76	1 (1.3%)	33	1 (3%)

N_s : number of samples examined

n_s (%) positive: number (percentage) of positive samples

N_h : number of horses examined

n_h (%) positive: number (percentage) of positive horses

In group 2, 4 of the 9 horses (44%) had at least one positive normal skin sample. In total, 6 out of 54 normal skin samples (11%) were positive for BPV DNA. All of the examined samples of the surroundings were negative. In group 3, all samples of the normal skin and the surroundings obtained from the papillomatosis-free farms were negative. However, 4 of the 6 skin samples from the horse living in contact with a papillomatosis-infected cow were positive whereas the samples taken before the papillomatosis infection were all negative. The sample of the stable wall from that horse was also positive after the papillomatosis infection occurred. In group 4, there was only one positive normal skin sample: it was obtained from the horse with the squamous cell carcinoma. All samples from the other horses as well as all samples of the surroundings were negative. Although some variation could be observed between the 3 groups of horses without equine sarcoid, a statistical significant difference for the presence of BPV DNA depending on the surroundings in which the horses were held (in contact with affected horses or not, in contact with cattle or not) could not be proven ($p=0.114$). In addition, grouping of the samples per horse could not reveal a statistical difference in positivity for BPV DNA depending on the surroundings of the horses ($p=0.106$).

DISCUSSION

The detection of BPV DNA on the normal skin of horses with and without equine sarcoid can be explained in several ways: it can be the result of a superficial contamination of BPV DNA originating from a sarcoid or from a cattle wart, via direct or indirect contact. The detection of BPV DNA can also be caused by the presence of viral material in the normal skin, referred to as latency. On the basis of the results of this study it could not be determined whether the presence of BPV DNA on the normal skin of horses with and without sarcoid is the result of superficial contamination with the viral DNA or BPV latency in the normal equine skin. In situ hybridisation showed that BPV DNA could only be detected in fibroblasts from equine sarcoids and not in their epidermal cells (Teifke *et al.*, 1994). This would mean that it is unlikely that, even in the presence of viral latency, BPV DNA would be detected on the normal intact skin by means of swabs. However, firmly rubbing on the skin during sampling could result in the collection of surface cells from the dermo-epidermal margin without visual damage of the skin. Indeed, BPV DNA has also been detected on swabs obtained from sarcoids with an intact surface (Martens *et al.*, 2001b). In situ PCR on

histological slides could possibly reveal the difference between contamination and infection, and could therefore offer the solution for this problem.

The hypothesis about superficial contamination of the normal skin is very likely, certainly in horses with ulcerated sarcoids where the fibroblasts are exposed. The present study shows that there is a significant difference in positivity for BPV DNA in normal skin samples depending on the location of normal skin sampling. No significant difference could be observed between samples obtained from the equine sarcoid surface, from normal skin close to the tumour and from a normal skin site in direct contact with the tumour whereas there is a significantly lower chance of obtaining a positive sample on other normal skin locations. These results would indicate that smearing of the viral DNA on the normal skin next to the tumour and on contact sites is very likely. However, the study of Carr *et al.* (2001a) showed that viral latency is also possible: BPV DNA could be detected in biopsies of normal skin from horses with sarcoids, whereas swabs obtained from the same site just before taking the biopsy were all negative. This would mean that superficial contamination was not present. The sampling procedure as done by Carr *et al.* (2001a) was performed in a similar way to this study. Their negative results could be due to the storage of the samples or to the method for isolation of DNA. A preliminary investigation done before this study showed that storage of swabs under dry conditions at -18 °C resulted in a higher yield of DNA compared to storage in formalin as performed by Carr *et al.* (2001a) or in NaCl 0.9% as done by Martens *et al.* (2001b).

In our study only swabs and no biopsies were taken, and BPV DNA could be detected in a high percentage of normal skin samples of horses with equine sarcoid. Moreover, BPV DNA could also be detected on the normal skin of 4 out of 9 non-affected horses living in contact with sarcoid-affected horses, as well as on the normal skin of one horse living in contact with a cow with warts and on one horse not having any contact with cattle or other affected horses. Statistical analysis failed to demonstrate a significant difference between these last 3 groups, although a tendency to a higher yield of positive results was observed in horses living in contact with horses with equine sarcoid compared to horses living in contact with cattle and control horses.

Latency of the bovine papillomavirus has been demonstrated in the normal skin and in circulating lymphocytes of cattle both in animals with and without clinical signs of papillomatosis (Campo *et al.*, 1994). In horses however, presence of viral material has only been shown in skin fibroblasts but no virus has yet been retrieved in lymphocytes, liver, spleen or lymph nodes (Otten *et al.*, 1993; Teifke *et al.*, 1994; Nasir *et al.*, 1997). Also, BPV

DNA has not yet been detected in the normal skin of healthy horses or horses with other skin diseases (Otten *et al.*, 1993; Teifke *et al.*, 1994; Carr *et al.*, 2001a). For groups 2, 3 and 4 (non-affected animals), only horses at least 7 years old were selected, because equine sarcoids rarely develop afterwards (Miller & Campbell, 1982; Torrontegui & Reid, 1994; Brostrom, 1995; Foy *et al.*, 2002). In this way, horses that were still in a susceptible phase for the development of equine sarcoids and possible asymptomatic carriers of bovine papillomavirus were excluded as much as possible. They could indeed falsely increase the number of BPV DNA positive samples.

Literature is not conclusive on whether contact with cattle is a risk factor for the development of equine sarcoids. This question has often been raised but never been answered (Jackson, 1936; Mohammed *et al.*, 1992). We have sampled horses living in contact with cattle, without distinguishing between farms with and without bovine papillomatosis. The main conclusion for this group of animals is that contact with cattle does not lead to an increased contact with BPV DNA, unless there has been a recent papillomavirus infection on the farm. Actually it is not yet known how long horses on such a farm stay BPV DNA positive after the cattle are cured of the papillomavirus infection. It has been observed that donkeys living in close contact with sarcoid-affected donkeys (“donkey-friends”) are more likely to develop sarcoids compared to an average donkey (Reid *et al.*, 1994a). A similar transmission from affected to non-affected animals has not yet been shown in horses, and isolation of affected horses is therefore not considered to be necessary.

The bovine papillomavirus is very resistant to physical and chemical reagents. It is not inactivated after 30 minutes at 67 °C, is stable at a pH between 4 and 8, is stable in ether and survives easily in 50% glycerol when frozen or lyophilised (Buxton & Frazer, 1977). Due to these *in vitro* properties of the virus, BPV is supposed to have a long survival time in its natural environment. Nevertheless no hard evidence can be found in the literature about the degree of contamination of the natural environment with BPV or BPV DNA and its rate of survival. In the present study, samples from the surroundings of affected and healthy horses were all negative for BPV DNA, with one exception for the horse living in contact with a cow affected by warts. This can indicate that the surroundings of a horse do not form a major source of BPV DNA contamination. However, since only a limited area was sampled, the persistence of point sources of BPV DNA in the environment cannot be excluded. The present data give us an indication that the initial BPV infection probably occurs a long time before any lesion becomes visible, possibly resulting in viral latency. At the moment of tumoural proliferation, the virus had already disappeared from the surroundings. It would definitely be

interesting to take samples from a larger group of horses living in contact with cattle affected by warts and with healthy cattle where it is known how long ago a BPV infection took place. In this way, the spread and the survival of BPV can be observed and the chance for tumour development in these horses could be compared to other horses. Due to the enhanced hygienic circumstances on cattle farms, papillomaviral infections occur less frequently than in the past. It remains an open question whether this will result in a decreased incidence of equine sarcoids in horses.

Contact with BPV alone is probably not enough to induce a clinical tumoural transformation (Carr *et al.*, 2001a). The virus might remain in a latent phase in the fibroblasts until other factors trigger cell transformation. In women it has been proven that infection of cervical epithelial cells by papillomaviruses results in a higher risk for the development of cervical neoplasia, but that viral infection does not directly lead to tumoural transformation (Jarrett, 1985). Tumoural transformation of the cervix is a multifactorial process with papillomavirus infection as one of the initiating factors. It is tempting to make such a comparison with equine sarcoids where other factors could also play a role, such as trauma and genetic constitution of the animal. Indeed, except for the results in this study, BPV DNA has not been demonstrated yet in the normal skin of horses without equine sarcoid (Otten *et al.*, 1993; Teifke *et al.*, 1994; Carr *et al.*, 2001a).

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High prevalence of bovine papillomaviral DNA in the normal skin of equine sarcoid-affected and healthy horses

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Adapted from: *Veterinary Microbiology*, in press.

SUMMARY

Bovine papillomavirus (BPV), the causative agent of papillomas in cattle, has been shown to play a major role in the pathogenesis of equine sarcoids in horses. BPV has also been detected occasionally in normal equine skin. In this study, presence and activity of BPV in normal skin and peripheral blood of 4 groups of horses was evaluated: sarcoid-affected horses, horses living in contact with sarcoid-affected horses, horses living in contact with papilloma affected cattle and control horses. From each horse, 3 samples on 4 locations were collected: a swab of the intact skin surface and both a swab and a biopsy after decontamination. BPV DNA was found in the normal skin of 24 of 42 horses (57%). Mainly sarcoid-affected horses and horses living in contact with cattle were carriers (73%), but BPV DNA was also detected in 50% of the horses living in contact with sarcoid-affected horses and in 30% of the control population. BPV mRNA was detected in 58% of the samples positive for BPV DNA, although in a much lower quantity compared to sarcoids. In most of the BPV DNA positive samples mild acanthosis, slight basophilic cytoplasmic swelling of the epidermal layers and/or thickening of the basement membrane were noticed, but these observations were also present in several BPV DNA negative normal skin samples. BPV DNA could not be detected in peripheral blood. These findings suggest latent infection and a wide-spread occurrence of BPV in the horse population.

INTRODUCTION

Equine sarcoids are commonly observed fibroblastic skin tumours in horses. They do not metastasise but treatment is often challenging due to frequent recurrences. The bovine papillomavirus type 1 and 2 (BPV-1, -2) is recognised as the most important etiological factor in its development. BPV DNA has been found in up to 100% of the examined sarcoids in several studies (Teifke & Weiss, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Carr *et al.*, 2001a, b; Martens *et al.*, 2001a, b, c). BPV mRNA and BPV proteins have also been identified in equine sarcoid tissue, providing evidence for a direct involvement of BPV in the pathogenesis (Nasir & Reid, 1999; Carr *et al.*, 2001b; Bogaert *et al.*, 2007). There are no differences in the clinical presentation between BPV-1 and BPV-2 induced sarcoids (Reid *et al.*, 1994b).

The transmission of BPV to the horse and the pathogenesis of tumour development are not yet fully understood. In contrast to BPV infection in cattle, the natural host where complete virus particles are formed, a non-productive infection occurs in horses. Hypotheses for BPV infection in horses include direct or indirect contact with infected cattle or horses, as well as transmission by insects (Reid *et al.*, 1994a; Knottenbelt *et al.*, 1995; Chambers *et al.*, 2003). Contact with the virus alone is probably not sufficient for tumour development: skin trauma, the immunological status and the genetic constitution of the individual horse also play an important role (Lazary *et al.*, 1985; Meredith *et al.*, 1986; Brostrom *et al.*, 1988; Gerber *et al.*, 1988; Chambers *et al.*, 2003). Furthermore, there is discussion about the occurrence of virus latency. In many species, including man and cattle, latency of papillomaviruses in asymptomatic individuals has clearly been demonstrated (Jarrett, 1985; Pfister, 1987; Campo *et al.*, 1994; Maran *et al.*, 1995; Antonsson *et al.*, 2000; Broker *et al.*, 2001; Abramson *et al.*, 2004; Burkhart, 2004; Antonsson & McMillan, 2006; Van Doorslaer *et al.*, 2006). In horses, BPV DNA has been demonstrated in swabs and biopsies obtained from the normal skin of horses affected with equine sarcoid, suggesting the occurrence of virus latency (Trenfield *et al.*, 1985; Carr *et al.*, 2001a; Martens *et al.*, 2001a; Bogaert *et al.*, 2005). BPV DNA has also been found occasionally on the normal skin of healthy horses and in non-sarcoid inflammatory skin conditions (Angelos *et al.*, 1991; Chambers *et al.*, 2003; Bogaert *et al.*, 2005; Yuan *et al.*, 2007). However, the distinction between BPV DNA contamination of the skin surface and a true latent infection has never been made.

The purpose of the present study was to determine the presence of BPV DNA and mRNA in the normal skin of horses in combination with a histopathological evaluation, in order to describe the occurrence of latent BPV infection in horses. Not only sarcoid-affected horses but also horses “at risk”, such as horses living in contact with sarcoid-affected horses or with papilloma affected cattle, as well as healthy control horses were examined.

METHODS

Study population and sample collection

Four groups of horses were included in the study. Group S (“sarcoid”) consisted of 11 horses (S1-11) admitted for surgery of one or more equine sarcoids. Group CS (“contact sarcoid”) included 10 unaffected horses (CS1-10) living in direct contact with horses with sarcoids (neighbouring boxes, same pasture...). Group CC (“contact cattle”) consisted of 11 unaffected horses (CC1-11) living in direct contact with cattle having suffered from papillomas in the last 6 months. Group C (“control”) included 10 control horses (C1-10), not having any contact with horses with sarcoids nor with cattle.

From each horse, 3 samples were collected from normal skin on 4 locations: chest, axilla, ventral abdomen and medial side of the thigh. First a pre-wetted (0.9% NaCl solution) cotton-tipped swab was rubbed on an intact skin surface of 5 x 5 cm. Next, this skin area was decontaminated using the following protocol. Hairs were clipped using a trimmer with disposable blades followed by scrubbing with povidonum iodatum 7.5% soap and disinfection with 76% ethanol. Afterwards, the skin was tape-stripped with 3M™ Transpore™ surgical tape as described by Forslund *et al.* (2004). Finally, another swab was rubbed over the skin surface and a full-thickness punch biopsy (8 mm diameter) was obtained. Horses from groups CS, CC and C were sedated with romifidine (0.08 mg kg⁻¹) and/or morphine (0.10 mg kg⁻¹) when needed and a local ring block (100 mg mepivacaine) was performed for the skin biopsies. Horses from group S were sampled during general anaesthesia required for the surgical treatment of the sarcoid. In this group a tissue sample of the sarcoid was also taken. Normal skin samples from horses in group S were removed at a distance of at least 10 cm away from the tumour(s). Skin biopsies and sarcoid samples were divided into 3 pieces: one was stored dry at -18 °C for DNA analysis, one was stored in RNeasy Lysis Buffer (Qiagen) overnight at 4 °C and thereafter at -18 °C for mRNA analysis and one was formalin fixed

during 24h and routinely processed for histopathological examination (HE-staining). From each horse, 10 ml of heparinised peripheral blood was collected for BPV DNA analysis. At the end of the study the owners of the horses were telephonically questioned about possible sarcoid development.

BPV DNA and mRNA analysis

All swabs, skin biopsies and sarcoid samples were routinely DNA extracted using the Puregene DNA extraction kit as described elsewhere (Bogaert *et al.*, 2005). Two hundred microliter of each blood sample was DNA extracted by adding 500 μ l Tris-HCl-EDTA, followed by 30 seconds of centrifuging and removal of the supernatant fluid. The pellet was subsequently washed 3 times with 500 μ l Tris-HCl-EDTA. Resuspension of the pellet was obtained by adding 100 μ l lysis buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.5% Tween 20) and proteinase K (100 μ g ml⁻¹). Next, the samples were incubated for 45 minutes at 56 °C and for 10 minutes at 95 °C. Finally, they were centrifuged for 1 minute, and stored at -18 °C.

Multiplex real time PCR was performed using Taqman probes specific for BPV-1 and BPV-2 as described in Bogaert *et al.* (2007), but performing 50 cycles in stead of 40 to enhance the detection limit. For each sample, 2 to 4 repeated measurements were performed. When BPV DNA was detected in at least one of the samples (swabs and/or biopsy) of a given location, RNA extraction was performed followed by real time RT-PCR of the E2 and E5 mRNA of BPV. When mRNA of one or both genes was retrieved, the same analysis was also performed for BPV E6, E7 and 3 reference genes (beta actin, ubiquitin B, beta-2-microglobulin) for quantitative analysis. All equine sarcoids were analysed for the 4 above mentioned BPV genes and the 3 reference genes. Details on the methods used have been described previously (Bogaert *et al.*, 2006; Bogaert *et al.*, 2007). In short, Ct values were transformed to “raw data”, taking into account PCR efficiency and all values measured during the same run. Next, obtained values were divided by a normalisation factor calculated as the geometric mean of the values of the 3 reference genes, allowing reliable comparison of different samples (both normal skin and sarcoid samples). In order to compare the two groups of sarcoid samples and normal skin samples, a ratio of the average values of both groups for each examined gene was calculated.

Histopathological evaluation

The equine sarcoid samples from group S horses were evaluated histopathologically to confirm the diagnosis. All BPV DNA positive and 10 BPV DNA negative normal skin samples were submitted for histopathological evaluation by 2 independent pathologists. All abnormal findings in epidermal and dermal layers were recorded.

Statistical analysis

The frequency of positive BPV DNA samples in the different groups was compared using logistic regression. In all analyses, location of the sampling was taken into account as co variable. Statistical analyses were performed in SPSS 15.0.

RESULTS

Study population

In group S, 6 horses (S1, S2, S6, S9, S10, S11) had only one sarcoid and 5 horses had multiple sarcoids (S3: 3, S4: 7, S5: 5, S7: 3, S8: 13). The median age was 9 years (range 5-15y) and there were 7 warmbloods, 1 thoroughbred, 1 Frisian, 1 pony and 1 Arabian horse. Seven of them were geldings, the other 4 were mares. In group CS, the median age was 8.5 years (range 3-16y) and there were 6 warmbloods, 1 pony, 1 tinker, 1 standardbred and 1 Andalusian horse. Five were geldings and 5 were mares. In group CC, the median age was 6 years (range 3-27y), 10 were mares and 1 was a gelding. Six were warmbloods, 2 were Belgian draft horses, 1 crossbred standardbred x warmblood, 1 Anglo Arabian and 1 pony. In group C the median age was 6.5 years (range 2-15y); this group consisted of 6 mares, 3 geldings and 1 stallion. Seven horses were warmbloods, next to 1 thoroughbred, 1 Quarter horse and 1 crossbred Appaloosa x Quarter horse.

BPV DNA analysis

Eight out of 9 examined sarcoid samples were positive for BPV DNA. Two sarcoid samples (S1 and S9) as well as the biopsy of the thigh of horse CS4 were not available for analysis. BPV DNA was detected in the normal skin of 24 out of 42 horses (57%). There were clear differences between the groups: in 73% of the horses in group S and CC BPV DNA was detected, compared to 50% in group CS and 30% in group C (Table 1). The positivity for BPV DNA was independent of the type of sample obtained: globally, 17% of the swabs before decontamination were positive, 17% of those after decontamination and 15% of the biopsies. BPV DNA was not detected in any of the peripheral blood samples. Tables 2-5 represent the normal skin locations positive for BPV-1 and BPV-2 DNA in the 4 groups. Thirty-six percent of all 42 horses were carrying BPV-1, 12% BPV-2 and 9% both BPV types in their normal skin samples. All of the horses where both BPV-1 and BPV-2 were detected were living in contact with cattle having suffered recently from papillomas. The BPV type detected in the normal skin samples in group S always corresponded to the BPV type of the respective sarcoid. Significantly more normal skin swabs (both before and after decontamination, $p=0,02$ and $p<0,01$ respectively) were positive for BPV DNA in group S (12 and 41% respectively) compared to the control group (0 and 1%). Group CC differed significantly from the control group for the swabs before decontamination and the biopsies (10 and 13% vs 0 and 4%; $p=0,01$ and $p=0,04$ respectively). Group CS was not significantly different from the control group for any of the obtained samples. In horses with at least one positive sample, the mean number of BPV DNA positive locations was also remarkably different: horses in group S and CC had a higher number of positive locations (respectively 2.6 and 2.5 out of 4) compared to group CS and C (respectively 1.6 and 1.3 out of 4) (Table 1). No significant effect of location of the sampling on BPV DNA presence was observed. From all normal skin locations examined ($n=168$), 11 showed BPV DNA positivity only in the swab taken before decontamination. Eight locations were positive in both swabs but not in the biopsy. In 11 locations BPV DNA was demonstrated only in the biopsy and not in the swabs. In 2 locations, both the swab before decontamination and the biopsy were positive for BPV DNA. In 4 locations, the swab before decontamination was negative, but the swab after decontamination as well as the biopsy were positive. Eight locations were positive in the 3 samples. Eight locations were only positive in the swab taken after decontamination but not in the two other samples.

Interestingly, one of the horses of group CS (CS6) developed a sarcoid on the abdomen 8 months after sampling. That horse had the highest number of positive locations in this group. None of the other horses of groups CS, CC or C developed equine sarcoids after a mean follow up time of 13 months (range of 4 – 19 months).

Table 1. Proportion of horses positive for BPV DNA and mRNA in the four groups and mean number of positive locations in horses carrying BPV DNA.

Horses	BPV DNA + horses	Mean № of BPV DNA + locations*	BPV mRNA + horses	Mean № of BPV mRNA + locations*
Group S	8/11 (73%)	2.6	7/11 (64%)	1.5
Group CS	5/10 (50%)	1.6	3/10 (30%)	0.8
Group CC	8/11 (73%)	2.5	7/11 (64%)	1.6
Group C	3/10 (30%)	1.3	2/10 (20%)	0.6
Total	24/42 (57%)	2.2	19/42 (45%)	1.3

Group S: horses with equine sarcoids

Group CS: healthy horses having contact with sarcoid-affected horses

Group CC: healthy horses having contact with cattle having recently suffered a papillomavirus infection

Group C: healthy control horses

* in horses with at least one positive sample

BPV mRNA analysis

Seventy-nine percent of the horses where BPV DNA was detected in at least one of the samples (swabs or biopsy) were positive for the presence of BPV mRNA. This corresponds to 45% of all horses. This was mainly attributed to the horses in groups S and CC, where BPV mRNA was detected in 88% of the BPV DNA positive horses, compared to only 60% in group CS and 67% of group C (Table 1). Globally, sixty percent of the samples positive for BPV DNA also harboured BPV mRNA (68% in group CC, 57% in group S,

50% in groups CS and C). The mean number of BPV mRNA positive locations in BPV DNA positive horses was 1.6 in group CC, 1.5 in group S, 0.8 in group CS and 0.6 in group C. These results are shown separately per group of horses in Tables 2-5.

The quantity of BPV mRNA detected in the normal skin samples was much lower compared to the equine sarcoid samples. Quantitative mRNA analysis demonstrated that BPV mRNA expression of the examined BPV genes was on average 3439 times lower in the normal skin samples compared to the equine sarcoids (data not shown). Particularly the equine sarcoid to normal skin ratio of E2 expression was low (10387:1) in comparison with E5 (659:1), E6 (1915:1) and E7 (796:1). Not all examined BPV genes were expressed in all samples: E5 mRNA was most often present (93% of samples with any BPV mRNA), followed by E7 (56%) and E2 (52%). E6 mRNA was only expressed in 33% of the samples with BPV activity. The mean and the 95% confidence interval of the BPV mRNA expression levels of the 4 examined BPV genes in the 2 groups of samples are shown in Fig. 1.

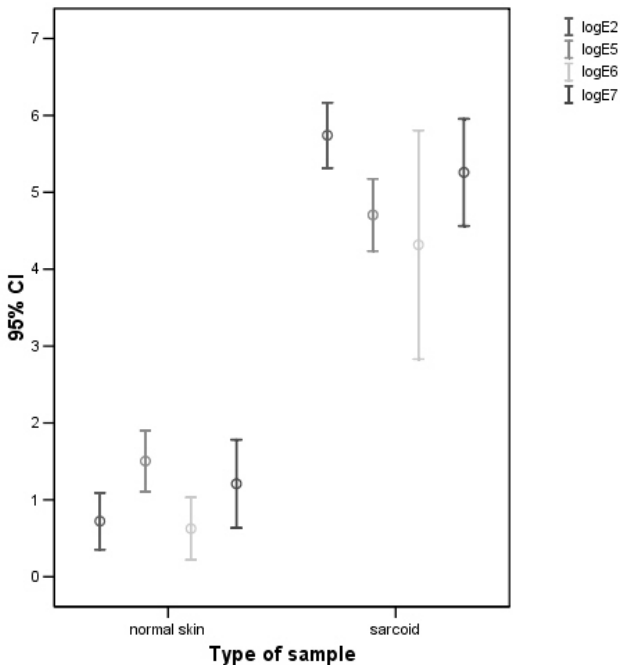


Figure 1. Graphic representation of BPV mRNA expression in a logarithmic scale for the normal skin samples and the sarcoid samples. Dots represent the mean and bars the 95% confidence interval.

Table 2. Normal skin locations positive for BPV DNA and mRNA in horses with equine sarcoids (group S).

Horse	BPV-1				BPV-2			
	DNA swab ¹	DNA swab ²	DNA biopsy	mRNA biopsy*	DNA swab ¹	DNA swab ²	DNA biopsy	mRNA biopsy*
S1	chest, axilla	thigh	-	thigh	-	-	-	-
S2	-	axilla, thigh	-	axilla	-	-	-	-
S3	-	-	-	-	-	-	-	-
S4	chest	chest	-	chest	-	-	-	-
S5	chest, axilla	chest, axilla	-	-	-	-	-	-
S6	-	chest, axilla, thigh	chest	chest	-	-	-	-
S7	-	-	-	-	chest, axilla, abdomen	chest, axilla, abdomen, thigh	chest, axilla, thigh	chest, axilla, abdomen
S8	-	thigh	chest, thigh	chest	-	-	-	-
S9	-	-	-	-	-	-	-	-
S10	-	-	-	-	-	-	-	-
S11	chest, axilla, thigh	chest, axilla, abdomen, thigh	chest, abdomen	chest, axilla, abdomen, thigh	-	-	-	-

¹ before decontamination² after decontamination

* only locations positive for BPV DNA were analysed for BPV mRNA

- : no BPV DNA detected in any of the 4 locations (chest, axilla, ventral abdomen, medial side of the thigh)

Table 3. Normal skin locations positive for BPV DNA and mRNA in healthy horses living in contact with sarcoid-affected horses (group CS).

Horse	BPV-1				BPV-2			
	DNA swab ¹	DNA swab ²	DNA biopsy	mRNA biopsy*	DNA swab ¹	DNA swab ²	DNA biopsy	mRNA biopsy*
CS1	-	-	thigh	thigh	-	-	-	-
CS2	-	-	-	-	-	-	-	-
CS3	-	abdomen	-	-	-	-	-	-
CS4	-	-	†	†	-	-	-	†
CS5	-	-	-	-	-	-	-	-
CS6	chest, axilla, abdomen, thigh	chest, axilla, abdomen, thigh	abdomen, thigh	axilla, thigh	-	-	-	-
CS7	-	thigh	-	-	-	-	-	-
CS8	-	-	-	-	-	-	-	-
CS9	axilla	-	-	axilla	-	-	-	-
CS10	-	-	-	-	-	-	-	-

¹ before decontamination

² after decontamination

* only locations positive for BPV DNA were analysed for BPV mRNA

- : no BPV DNA detected in any of the 4 locations (chest, axilla, ventral abdomen, medial side of the thigh)

† biopsy of the thigh was missing

Table 4. Normal skin locations positive for BPV DNA and mRNA in healthy horses living in contact with cattle having suffered a recent papillomavirus infection (group CC).

Horse	BPV-1				BPV-2			
	DNA swab ¹	DNA swab ²	DNA biopsy	mRNA biopsy*	DNA swab ¹	DNA swab ²	DNA biopsy	mRNA biopsy*
CC1	chest, abdomen	-	-	abdomen	-	-	thigh	-
CC2	abdomen, thigh	-	abdomen	thigh	-	-	chest	chest
CC3	-	-	-	-	-	-	-	-
CC4	-	-	abdomen	abdomen	chest	-	-	chest
CC5	-	-	abdomen	-	-	-	axilla	axilla
CC6	-	-	thigh	thigh	-	-	-	-
CC7	chest, axilla, abdomen, thigh	axilla, abdomen, thigh	chest, axilla, abdomen, thigh	chest, axilla, abdomen, thigh	-	-	-	-
CC8	-	-	-	-	-	-	-	-
CC9	-	-	-	-	-	-	-	-
CC10	-	-	-	-	chest, axilla	-	-	-
CC11	-	-	-	-	axilla, abdomen	-	-	axilla, abdomen

¹ before decontamination

² after decontamination

* only locations positive for BPV DNA were analysed for BPV mRNA

- : no BPV DNA detected in any of the 4 locations (chest, axilla, ventral abdomen, medial side of the thigh)

Table 5. Normal skin locations positive for BPV DNA and mRNA in healthy control horses (group C).

Horse	BPV-1				BPV-2			
	DNA swab ¹	DNA swab ²	DNA biopsy	mRNA biopsy*	DNA swab ¹	DNA swab ²	DNA biopsy	mRNA biopsy*
C1	-	-	-	-	-	-	thigh	thigh
C2	-	-	-	-	-	-	abdomen	abdomen
C3	-	-	-	-	-	-	-	-
C4	-	-	-	-	-	-	-	-
C5	-	-	-	-	-	-	-	-
C6	-	abdomen	chest	-	-	-	-	-
C7	-	-	-	-	-	-	-	-
C8	-	-	-	-	-	-	-	-
C9	-	-	-	-	-	-	-	-
C10	-	-	-	-	-	-	-	-

¹ before decontamination² after decontamination

* only locations positive for BPV DNA were analysed for BPV mRNA

- : no BPV DNA detected in any of the 4 locations (chest, axilla, ventral abdomen, medial side of the thigh)

Histopathological examination

All examined tumour samples were confirmed to be equine sarcoids. Two samples (S1 and S9) were not available for analysis. Forty-nine BPV DNA positive normal skin samples originating from 23 horses and 10 BPV DNA negative samples from 5 horses were examined histopathologically by 2 independent pathologists. The majority of the BPV DNA positive normal skin samples showed discrete abnormalities: minimal to mild focal epidermal hyperplasia of the stratum spinosum (acanthosis) together with slight basophilic cytoplasmic swelling were frequently observed (Fig. 2). Diffuse or focal hyperpigmentation was also commonly noticed. Several samples displayed a thickened basement membrane, with or without acanthosis. Mild perivascular dermatitis was sometimes noticed. Yet, some of these changes were also seen in 6 out of 10 normal skin samples negative for BPV DNA. No correlation between histopathological abnormalities and the presence of BPV mRNA was observed.

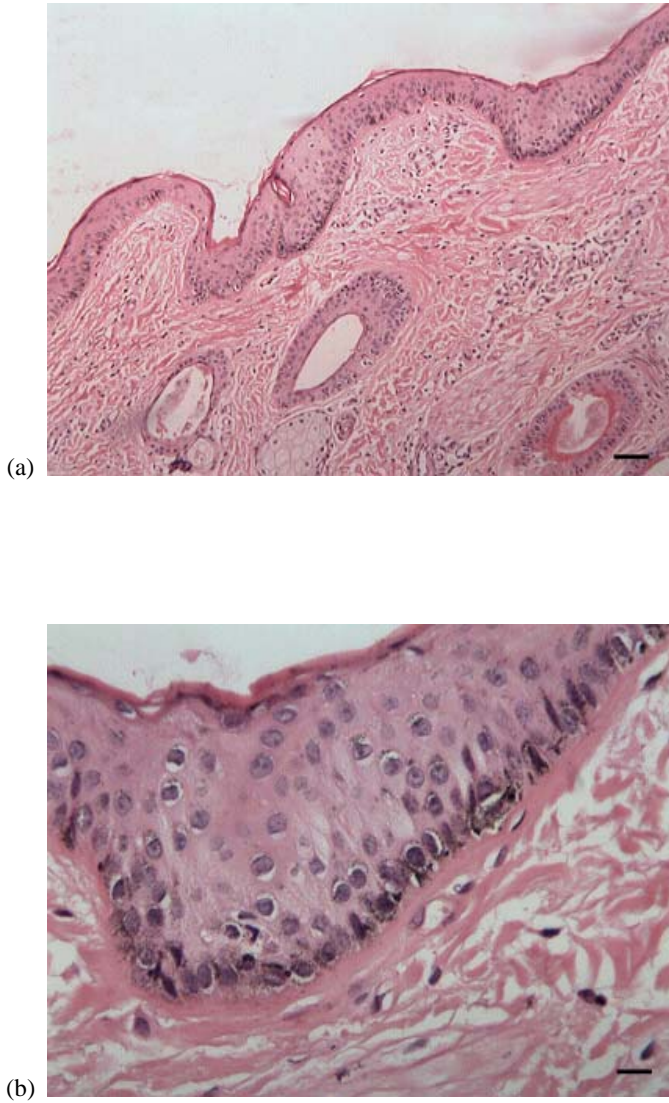


Figure 2. Mild focal acanthosis, slight basophilic cytoplasmic swelling and parakeratosis of a normal equine skin sample positive for BPV DNA. (a) Overview of epidermal and dermal layers (bar = 50 μ m). (b) Detail of the epidermal layers (bar = 25 μ m).

DISCUSSION

The present study systematically investigated the presence of bovine papillomavirus in the normal skin of horses. It was found that horses were often carriers of BPV DNA, not only horses with equine sarcoids, but also healthy horses, without any evidence of dermatological disease. In people, latency of HPV is defined as the presence of papillomaviral DNA without any clinical or histological evidence of infection, and extremely low levels of HPV mRNA compared to papillomas (Maran *et al.*, 1995). Similarly, we found BPV DNA together with very low levels of BPV mRNA in the clinically normal equine skin. Although no macroscopic changes were present on the normal skin samples, microscopic changes such as mild acanthosis and slight basophilic cytoplasmic swelling were observed in some cases. However, these changes were also observed in normal skin without the presence of BPV DNA, showing that these features might be inherent to normal equine skin.

Sarcoids can appear on any part of the body, but they are mostly localised on the ventral abdomen, the paragenital region, head and limbs (Jackson, 1936; Pulley & Stannard, 1990; Torrontegui & Reid, 1994; Goodrich *et al.*, 1998; Piscopo, 1999). In the present study, samples were obtained from these predilection sites to enhance the chance of finding latent carriers. On the other hand, sampling only 4 locations might be insufficient to detect all horses carrying BPV in their normal skin. Therefore, sampling on more locations might increase the percentage of horses with latent BPV infection.

One of the aims of the present study was to distinguish between the presence of BPV DNA on the skin surface (contamination) and in the epidermal and/or dermal layers (latency). In order to differentiate the two conditions, the skin surface was decontaminated before taking a biopsy. However, the interpretation of the results is not straightforward. BPV DNA positivity only in the swab taken before decontamination can be considered as superficial skin contamination with BPV DNA from shed sarcoid cells or with BPV produced in cattle papillomas. Locations positive in both swabs but not in the biopsy might be the result of superficial skin contamination combined with unsuccessful decontamination. BPV DNA demonstrated only in the biopsy and not in the swabs can be regarded as true latent infection of the dermis or of the basal layers of the epidermis. If both the swab before decontamination and the biopsy were positive for BPV DNA, this can be seen as a combination of superficial skin contamination and latent dermal infection. If the swab before decontamination was negative, but the swab after decontamination as well as the biopsy were positive, this may be

the result of latent infection of the epidermis and possibly also the dermis. Locations with all 3 samples positive for BPV DNA may be the result of a combined superficial contamination and latent epidermal infection or of a combined superficial contamination, unsuccessful decontamination and dermal latent infection. Locations only positive in the swab taken after decontamination but not in the two other samples might have got re-contaminated because the horse moved with consequent contact with other parts of the body or whirling of hairs or shed epidermal cells on the decontaminated area. It is also possible that the decontamination protocol was not efficient enough. A study on HPV prevalence on the top of skin tumours compared to stripped biopsies demonstrated that the same decontamination protocol resulted in a rather good but not 100% effective removal of HPV DNA from superficial skin layers (Forslund *et al.*, 2004). These findings can be explained by the rather resistant character of papillomaviruses against conventional disinfection protocols. The presence of BPV DNA in the swab obtained after decontamination as well as in the biopsy combined with a negative swab before decontamination can be the result of epidermal BPV infection. Equine sarcoids are fibroblastic tumours but they often have an important epithelial component. Thus far, BPV DNA has only been demonstrated in the fibroblasts and not in the epidermal cells (Lory *et al.*, 1993; Teifke *et al.*, 1994). However, the presence of BPV DNA in swabs of decontaminated skin together with the observed epidermal changes could generate the hypothesis that BPV infection of equine skin might start in the epidermis, followed by shifting of the viral material to the subepidermal fibroblasts where the BPV genome can fully exert its transformational activity. Another remarkable finding is that BPV mRNA was demonstrated in sample locations, positive in the swab (either before or after decontamination) but negative in the biopsy. Theoretically it is possible that the BPV mRNA in BPV DNA negative biopsies originates from the source of superficial contamination although mRNA production is not expected when intact BPV particles contaminate the skin. Another possibility is that the quantity of BPV DNA in the normal skin samples is close to the detection limit of the applied technique, which may result in a positive outcome in some samples and a negative in others. Since BPV load and mRNA expression are correlated (Bogaert *et al.*, 2007), it can be supposed that very low mRNA expression levels reflect very low levels of viral DNA in these samples.

Among the healthy horses, mainly those having contact with cattle with papillomas were positive for BPV DNA. These were often affected by 2 different BPV types. Even if the BPV infection in cattle had already disappeared for up to 6 months, contact horses still got a higher chance of harbouring BPV in their skin. Horses having contact with sarcoid horses did

not have a significantly higher chance of harbouring BPV compared to the control group. This could be explained by the non-productive BPV infection in equine sarcoids: no capsid proteins are synthesised and thus no infectious virus particles are formed (Sousa *et al.*, 1990). Nevertheless, outbreaks of equine sarcoids in closed herds of equids have been reported (Ragland *et al.*, 1966; Reid *et al.*, 1994a; Nel *et al.*, 2006; personal observations) and BPV DNA was detected in half of the horses living in contact with sarcoid-affected horses in our study. This could indicate that BPV DNA alone can be sufficient for transmission of infection between horses, although to a lower degree compared to transmission by cattle where infectious viral particles are formed. This stresses the need for hygienic measures when horses with sarcoids are present in the close surroundings of other horses.

In the control group, 30% of the horses harboured BPV DNA in the skin which indicates that a substantial part of the horse population is infected even without recent contact with diseased animals. In many sarcoid cases, owners do not report any contact with cattle or sarcoid-affected horses. It is however possible that horses got infected by neighbouring cattle a long time ago, maybe with previous owners, and that BPV infection was latent during months or years, ultimately resulting in the development of sarcoids. In people, acquisition of HPV infection of the skin probably occurs in early infancy through close skin contact with infected individuals (Antonsson *et al.*, 2003). Multiple studies in people have shown that a substantial proportion of the healthy population is infected with HPV on the normal skin (Boxman *et al.*, 1997; Astori *et al.*, 1998; Antonsson *et al.*, 2000; Forslund *et al.*, 2004; de Koning *et al.*, 2007). It is hypothesized that in immunocompetent individuals an intact immune system inhibits the development of clinical disease (Astori *et al.*, 1998). In horses, sarcoid susceptibility is associated with certain equine leukocyte antigen (ELA) haplotypes of the major histocompatibility complex. In the Swiss Warmblood Horse, the Irish Sporthorse and the Selle Français, horses with the ELA W13 haplotype are more susceptible to equine sarcoid development (Meredith *et al.*, 1986; Brostrom *et al.*, 1988; Lazary *et al.*, 1994; Brostrom, 1995), which might indicate a role of the antigen presenting system in the host defence against BPV infection. In Arabian horses a significant correlation exists between sarcoid development and heterozygosity for the defective DNA-PKcs allele responsible for severe combined immunodeficiency (Ding *et al.*, 2002). The present study shows that the proportion of latent BPV carriers in the horse population (52% of the healthy horses) is much higher compared to the prevalence of equine sarcoids, which mounts up no higher than 12% (Gerber, 1989; Mele *et al.*, 2007; Studer *et al.*, 2007). A high clearance rate and a low progression rate towards tumour development can therefore be assumed.

In this study, 38% of the horses positive for BPV DNA harboured BPV-2. This is remarkably higher than the proportion of BPV-2 induced sarcoids in Europe, which amounts not more than 20% (Angelos *et al.*, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Martens *et al.*, 2001a; Bogaert *et al.*, 2007). However, this high percentage can be attributed to the horses living in contact with cattle having suffered a recent papillomavirus infection. When this group is excluded from the calculation, the expected 80/20 ratio of BPV-1 to BPV-2 is seen. These results might indicate that BPV-2 infection in horses is more easily cleared and less likely to induce sarcoids.

In people, HPV DNA is very often shown in normal epithelial tissues, which raises the hypothesis that HPV might be part of the normal microflora (Broker *et al.*, 2001; Forslund *et al.*, 2004), causing disease in some, but staying dormant for life in others (Burkhart, 2004). Particularly in an environment which is sub-optimal for completion of the productive life cycle of papillomaviruses, resulting in an abortive infection, progression to cancer is more likely to happen (Doorbar, 2006). The same could be true for the formation of equine sarcoids in horses compared to cattle, the natural host of BPV, where papillomas only rarely progress to cancer and most often regress spontaneously. A cottontail rabbit papillomavirus (CRPV) model showed that in latent infection E1 and E2 are expressed, although at very low levels, but transcripts of E6 and E7 are not detected (Zhang *et al.*, 1999). In our study, similar low levels of E2 were present, but also transcripts of genes playing a role in cell transformation (E5, E6 and E7) were detected. This discrepancy might be due to the more sensitive detection methods used in the present study or to a different pathogenesis of equine sarcoids. Latent BPV infections have been demonstrated in normal skin, blood lymphocytes and normal bladder mucosa of both healthy and immunosuppressed cows (Campo *et al.*, 1994; Borzacchiello *et al.*, 2003). Our results confirm the absence of BPV DNA in peripheral blood of sarcoid-affected and non-affected animals (Otten *et al.*, 1993; Nasir *et al.*, 1997).

It remains unclear how equine sarcoids eventually develop after BPV infection. Skin trauma can certainly play an important role because it allows direct access of BPV to the subepidermal fibroblasts which may lead, together with formation of different growth factors necessary for skin regeneration, to proliferation of the BPV transformed cells. In people it is observed that not only patients with psoriasis, which is strongly correlated with HPV-5, but also patients with epidermal repair following extensive second degree burns or autoimmune bullous diseases, generate antibodies to HPV-5 (Favre *et al.*, 2000). Healthy cattle housed in isolation can develop papillomas harbouring BPV-1 and/or -2 following immunosuppression or skin trauma (Campo *et al.*, 1994). Also in horses, sarcoid development in wounds is

frequently observed (Torrontegui & Reid, 1994; Knottenbelt *et al.*, 1995; Foy *et al.*, 2002). Nevertheless, many sarcoids appear without any clinical evidence of skin trauma, although microtrauma (e.g. insect bites) cannot be excluded.

CONCLUSION

In conclusion, BPV can be detected in a large proportion of normal skin samples in sarcoid-affected and healthy horses, but not in their blood. Among the healthy horses, those living in contact with cattle with papillomas were at equal high risk for BPV positivity compared to sarcoid-affected horses. Both viral DNA and mRNA were detected in low quantities, which points in the direction of true latent infection and not superficial contamination. A 30% BPV infection rate of healthy horses not living in contact with cattle or sarcoid-affected horses demonstrates the wide-spread occurrence of BPV in the horse population.

ACKNOWLEDGEMENTS

This research was funded by the Special Research Fund, Ghent University, grant no. 01D01103.

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General discussion and perspectives

At the onset of this research project, not much information was available on the activity of BPV in relation to the clinical behaviour of equine sarcoids. BPV mRNA and proteins had already been demonstrated in sarcoids (Nasir & Reid, 1999; Carr *et al.*, 2001b; Chambers *et al.*, 2003b), but these studies were performed non-quantitatively, on rather small numbers of tumours and irrespective of the clinical behaviour. Furthermore, only anecdotal evidence regarding the presence of BPV on normal skin of horses was available whereby BPV DNA was shown on the normal skin of horses with equine sarcoids, but not on healthy horses (Trenfield *et al.*, 1985; Carr *et al.*, 2001a; Martens *et al.*, 2001a). Moreover, no differentiation between true latent infection and superficial contamination of shed tumoural cells was made.

Before performing quantitative gene expression analysis, a good normalisation process needs to be established. Differences in starting material, RNA preparation, RNA quality and cDNA synthesis may cause important fluctuations between measurements and therefore expression profiles of the genes of interest should be compared with those of constitutively expressed reference genes (Zhang *et al.*, 2005; Radonic *et al.*, 2005). However, it has been demonstrated that the expression level of these genes may vary considerably particularly in clinical samples associated with malignant disease (Thellin *et al.*, 2000; Suzuki *et al.*, 2000; Neuvians *et al.*, 2005). Because of these findings, not only one, but a whole set of stable reference genes should be determined in each tissue of interest to use them as internal reference genes for accurate normalisation (Vandesompele *et al.*, 2002). When calculating a normalisation factor, a careful choice of the number of reference genes should be made. The more genes included, the more accurate the normalisation factor is. However, including too many genes may increase the risk of using unsuitable genes, and is also impractical. On the other hand, if the cut off is made too stringent, stably expressed reference genes may be excluded and accuracy might drop. The results of our study (Chapter 3.1.) demonstrate that the use of 3 reference genes (ubiquitin B, beta-2-microglobulin and beta actin) is appropriate for gene expression analysis of equine sarcoids, normal equine skin and the combination of both.

The study results from Chapter 3.2. clearly confirm the omnipresence and activity of BPV in equine sarcoids. Although numerous studies have demonstrated the presence of BPV DNA in equine sarcoids (Lancaster *et al.*, 1977; Amtmann *et al.*, 1980; Trenfield *et al.*, 1985; Otten *et al.*, 1993; Teifke *et al.*, 1994; Carr *et al.*, 2001a; Martens *et al.*, 2001b; Yuan *et al.*,

2007a), its importance has sometimes been questioned, since BPV DNA was also observed occasionally in normal equine skin and in inflammatory skin conditions (Trenfield *et al.*, 1985; Angelos *et al.*, 1991; Carr *et al.*, 2001a; Martens *et al.*, 2001a; Yuan *et al.*, 2007b). Previous studies had already shown the presence of BPV mRNA in 95% of sarcoids (Nasir & Reid, 1999) and BPV E5 protein in all sarcoids (Carr *et al.*, 2001b). The demonstration of large amounts of BPV mRNA in equine sarcoids shows that the viral genome is actively transcribed allowing the oncogenes E5 and E6 to exert their transformational activity in the fibroblasts. Binding of E5 to cellular proton pumps results in inhibition of the degradation of growth factor receptors (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). Furthermore, the PDGF- β receptor itself is targeted by E5 leading to activation of this receptor (Petti & DiMaio, 1992; DiMaio & Mattoon, 2001). E5 also binds to 16K ductin/subunit c, a component of the gap junctions. Through this binding, the contact with neighbouring cells gets lost and the transformed fibroblast can replicate uncontrolled (Campo, 1997). It has been suggested that the activation of the dermal fibroblasts results in mitogenesis in the nearby epithelial cells, which might explain the typical pseudoepitheliomatous hyperplasia (Carr *et al.*, 2001b). BPV E6 on its side is able to bind the focal adhesion protein paxillin (Tong *et al.*, 1997; Tong & Howley, 1997; Vande Pol *et al.*, 1998) and the γ subunit of the clathrin adaptor complex AP-1 (Tong *et al.*, 1998) leading to disruption of cytoskeleton and vesicular traffic pathways. The cytoskeleton is vital for the maintenance of cellular morphology, motility, division and cell-cell and cell-matrix interactions and the AP-1 complex plays an important role in the control of cell proliferation and differentiation (Chambers *et al.*, 2003a).

Although BPV gene expression was observed in every sarcoid in our study, no correlation with clinical behaviour could be demonstrated (Chapter 3.2.). Significant differences in expression were observed between the clinical types, but nodular and verrucous sarcoids, displaying an intermediate growth rate, showed a higher BPV expression than the more aggressive fibroblastic sarcoids. Also the viral load was not in the expected concordance with the clinical behaviour, with nodular sarcoids showing a much higher viral load than the verrucous, fibroblastic and occult sarcoids. This might be explained by a higher tumour cell density in the first types compared to a higher amount of endothelial and inflammatory cells in the latter, which might decrease the relative amount of BPV DNA and mRNA in fibroblastic sarcoids. Another explanation for this unexpected outcome is that the role of BPV becomes less important once certain cellular abnormalities have been established. This is in contrast with the situation in human medicine, where HPV mRNA expression increases in

cervical cancer when lesions progress from low-grade dysplasia to invasive carcinoma (Wang-Johanning *et al.*, 2002; Kraus *et al.*, 2004; Scheurer *et al.*, 2005). It has also been shown that women with low-grade cervical intraepithelial lesions accompanied by a high HPV load are at higher risk for progression to high-grade lesions (Ho *et al.*, 2006) and that a higher HPV load is seen in high-grade lesions compared with low-grade lesion (Carcopino *et al.*, 2006; Kovacic *et al.*, 2006; Lai *et al.*, 2006). On the other hand, Dahlgren *et al.* (2006) could not determine any influence of HPV load (except for HPV-16) on survival in patients with early-stage cervical carcinoma. Our findings support a “hit-and-run” hypothesis of BPV in horses, which assigns an essential role of BPV to the onset of equine sarcoids, but a less important role in its progression to a more malignant state. One particular case in our clinic supports this hypothesis: treatment of a horse for a “classic” fibroblastic sarcoid, with a normal amount of BPV DNA and mRNA, resulted for unknown reasons into a strong deterioration of the tumour, with massive growth and infiltration into underlying muscles. In this last tumour, BPV DNA was still detected but no BPV mRNA was present. Although this is only a single observation, it can be suggested that in such cases of severe worsening of the clinical condition, cellular transformation has reached a level where transformed cells can replicate independently without assistance from the viral genome. Nevertheless, it should be clearly stated that in the vast majority of sarcoids, BPV is still present and active, demonstrating that it remains one of the determining factors in the multifactorial process of sarcoid pathogenesis.

In Chapter 4 the presence of bovine papillomavirus in the normal skin of horses was investigated. In the first study (Chapter 4.1.), BPV DNA was demonstrated in swabs of normal equine skin, both of sarcoid-affected and healthy horses. In sarcoid-affected horses, particularly the skin near the sarcoid and the contact locations (e.g. head – front leg, ventral abdomen – hind leg) had a significantly higher chance of being positive for BPV DNA than more distant skin locations. This can be seen as superficial contamination of the skin by smearing of tumoural cells, especially when an ulcerated fibroblastic sarcoid is present. On the other hand, BPV DNA was also detected on other skin locations, not in direct contact with the sarcoid, as well as in horses without sarcoids. Other studies had already demonstrated BPV DNA in biopsies of normal skin but not in swabs, therefore proposing the hypothesis of viral latency (Carr *et al.*, 2001a). These confusing observations have prompted us to start a more extensive study allowing us both to differentiate between superficial skin contamination and latency and to determine the prevalence of latency.

In people, latency of HPV is defined as the presence of papillomaviral DNA without any clinical or histological evidence of infection, and extremely low levels of HPV mRNA compared to papillomas (Maran *et al.*, 1995). Our findings (Chapter 4.2.) correspond to this definition, although slight histological changes were seen in most of the BPV DNA positive samples. However, these changes were also observed in some of the BPV DNA negative samples, which might indicate that these changes could be inherent to normal equine skin. The demonstration of BPV DNA and mRNA in normal equine skin may explain why horses already affected by equine sarcoids often develop additional tumours, even after successful treatment of the original tumours. It can also explain why horses develop sarcoids without any known contact with cattle, the natural host of BPV. A possible explanation might be that horses got infected by neighbouring cattle a long time ago, maybe with previous owners, and that BPV infection was latent during months or years, ultimately resulting in the development of sarcoids.

One of the aims of this study was to distinguish between the presence of BPV DNA on the skin surface (contamination) and in the epidermal and/or dermal layers (latency). In order to differentiate the two conditions, the skin surface was decontaminated before taking a biopsy. However, the interpretation of the results was not straightforward. In some samples, BPV DNA was only present in the swab taken before decontamination, which can be seen as superficial skin contamination with shed sarcoid cells or cattle papilloma cells. BPV DNA which was demonstrated only in the biopsy and not in the swabs can be regarded as true latent infection of the dermis or of the basal layers of the epidermis. In some cases, both the swab before decontamination and the biopsy were positive for BPV DNA. This can be seen as a combination of superficial skin contamination and latent dermal infection. Locations only positive in the swab taken after decontamination but not in the two other samples might have got re-contaminated because the horse moved with consequent contact with other parts of the body or whirling of hairs or shed epidermal cells on the decontaminated area. It is also possible that the decontamination protocol was not efficient enough. A study on HPV prevalence on the top of skin tumours compared to stripped biopsies demonstrated that the same decontamination protocol resulted in a rather good but not 100% effective removal of HPV DNA from superficial skin layers (Forslund *et al.*, 2004). These findings can be explained by the fact that conventional disinfection protocols are rather ineffective against papillomaviruses. If the swab before decontamination was negative, but the swab after decontamination as well as the biopsy were positive, this may be the result of latent infection

of the epidermis and possibly also the dermis. Equine sarcoids are known as fibroblastic tumours but they often have an important epithelial component. Thus far, BPV DNA has only been demonstrated with in situ hybridisation in the fibroblasts and not in the epidermal cells (Lory *et al.*, 1993; Teifke *et al.*, 1994). However, the presence of BPV DNA in swabs of decontaminated skin together with the observed epidermal changes could generate the hypothesis that BPV infection of equine skin might start in the epidermis, followed by shifting of the viral material to the subepidermal fibroblasts where the BPV genome can fully exert its transformational activity. Selective isolation of the epidermal and dermal components using laser dissection microscopy, followed by PCR analysis of the obtained cellular material could help to confirm this hypothesis. Indeed, PCR analysis is much more sensitive than in situ hybridisation, thus allowing determining whether the isolated epidermal layers would also harbour a limited amount of BPV DNA.

Among the healthy horses, especially those having contact with cattle with a current or recent BPV infection often showed the presence of BPV DNA. The results from the swab analysis for the horses living in contact with cattle (Chapters 4.1. and 4.2.) show a noticeable difference in BPV DNA positivity: when no recent history of BPV infection in cattle was present, horses did not have a higher chance for presence of BPV DNA compared to the control group. In case of a current infection or even when the BPV infection in cattle had already disappeared for up till 6 months, horses got a significantly higher chance of harbouring BPV in their skin. Although latency of BPV in the skin and peripheral blood of cattle exists (Campo *et al.*, 1994), these animals do not seem to be a major source of BPV infection for horses in view of the low infection rate in the horses living in contact with unaffected cattle. The presence of cattle with a current or recent BPV infection at the farm significantly increased the proportion of BPV infected horses to a level comparable to sarcoid-affected horses. These results show that this specific group of horses is at high risk to get infected, possibly resulting in development of sarcoids after a certain period of time.

Horses having contact with sarcoid-affected horses did not have a significantly higher chance of harbouring BPV compared to the control group. This can be explained by the non-productive BPV infection in horses. In equine sarcoids, no capsid proteins are synthesised so no infectious viral particles are formed (Gorman, 1985; Sousa *et al.*, 1990). Still, half of the horses were latently infected in their normal skin. Together with the existence of occasionally reported outbreaks of equine sarcoids in closed herds of equids (Ragland *et al.*, 1966;

Gorman, 1985; Reid *et al.*, 1994; Nel *et al.*, 2006; personal observations) this can indicate that BPV DNA alone can be sufficient for transmission between horses, although to a lower degree compared to transmission by cattle actively producing infectious viral particles. Moreover, sarcoid specific BPV-1 sequence variants have been reported, suggesting that these variants preferentially infect horses and are maintained in equids (Chambers *et al.*, 2003b; Nasir *et al.*, 2007). Interestingly, one of the horses in our study living in contact with a sarcoid-affected horse (stabled in neighbouring boxes) developed itself an equine sarcoid close to one of the sites of sampling where BPV DNA and mRNA were detected. This horse was kept in a riding school of 135 horses, which were regularly let out in large groups on the same pasture. In this riding school, equine sarcoids are regularly seen among the horses. The same observation has been made in several other riding schools and stud farms. On the other hand, other riding schools with similar hygienic conditions are less frequently affected. The results from the present study cannot explain these differences, but it can be proposed that once the virus is present on a farm, eradication is very difficult with custom hygienic measures. The bovine papillomavirus is known for its very resistant character against physical and chemical agents. It is not inactivated after 30 minutes at 67 °C, is stable at a pH between 4 and 8, is stable in ether and survives easily in 50% glycerol when frozen or lyophilised (Buxton & Frazer, 1977). As a result of these *in vitro* properties, BPV is supposed to have a long survival time in its natural environment. Nevertheless no hard evidence can be found in literature about the degree of contamination of the natural environment with BPV or BPV DNA and about its survival rate. In our study (Chapter 4.1.), samples from the surroundings of affected and healthy horses were all negative for BPV DNA, with one exception for the horse living in contact with a cow affected with papillomas. This can indicate that the surroundings of a horse do not form a major source of BPV DNA contamination. However, since only a limited area was sampled, the persistence of point sources of BPV DNA in the environment cannot be excluded. Furthermore, latently infected horses remain a source of infection, and affected animals (both horses and cattle) living at a distance from the farm might re-infect the premises via vector transmission.

Thirty percent of the healthy horses without contact neither with cattle nor with sarcoid-affected horses harboured BPV DNA in their skin, indicating that a substantial part of the horse population is infected even without recent contact with diseased animals. Since the proportion of latent BPV carriers in horses (52% of the healthy horses) is much higher than the prevalence of equine sarcoids in the population, which mounts up no higher than 12%

(Gerber, 1989; Mele *et al.*, 2007; Studer *et al.*, 2007), a high clearance rate and a low progression rate towards tumour development can be assumed.

Currently it is not known what the incubation time is for BPV infection in the horse. Experimental BPV infection of horses and donkeys results in sarcoid development after 6 days to 11 months (Olson & Cook, 1951; Ragland & Spencer, 1968; Voss, 1969; Ragland & Spencer, 1969; Ragland *et al.*, 1970). However, in natural cases of equine sarcoids, contact with cattle is often non-existent or already years ago. These observations suggest that the time span between BPV infection and sarcoid development might be very long, probably months or years. In people, the most determining factors in the development of non-melanoma skin cancer (NMSC) are UV radiation and immune suppression, but HPV-5 and -8 are believed to be co-carcinogens in a certain number of tumours, particularly in epidermodysplasia verruciformis patients (Muñoz *et al.*, 2006). However, cutaneous HPV infection is thought to occur early in life whereas NMSC is mainly seen in elderly (Antonsson *et al.*, 2003; de Koning *et al.*, 2007). Multiple studies in people have shown that a substantial proportion of the healthy population is infected with HPV on the normal skin (Boxman *et al.*, 1997; Astori *et al.*, 1998; Antonsson *et al.*, 2000; Forslund *et al.*, 2004; de Koning *et al.*, 2007). This rises the hypothesis that HPV might be part of the normal microflora of man (Broker *et al.*, 2001; Forslund *et al.*, 2004) causing disease in some, but stay dormant for life in others (Burkhart, 2004). It is hypothesized that in immunocompetent individuals an intact immune system inhibits the development of clinical disease (Astori *et al.*, 1998). Anogenital HPV infections are usually caught in young adults short after the first sexual contact. Most people will spontaneously clear the infection after several months and only a limited proportion of infected individuals will develop cancer. Persistent HPV infection is one of the prerequisites for neoplastic transformation (O'Brien & Campo, 2002). This can result in a slow development of subtle intraepithelial neoplasia towards in situ and eventually frank carcinomas, a process which is sometimes taking decades (Moscicki *et al.*, 2006). It would be interesting to follow the horses included in our study (Chapter 4.2.) to determine whether the observed BPV infection was transient or persistent, as well as to quantify the proportion of horses developing sarcoids following latent BPV infections.

In horses, sarcoid susceptibility is associated with certain equine leukocyte antigen (ELA) haplotypes of the major histocompatibility complex. In the Swiss Warmblood Horse, the Irish Sporthorse and the Selle Français, horses with the ELA W13 haplotype are more

susceptible to equine sarcoid development (Meredith *et al.*, 1986; Brostrom *et al.*, 1988; Lazary *et al.*, 1994; Brostrom, 1995), which might indicate a role of the antigen presenting system in the host defence against BPV infection. It would be interesting to determine the ELA haplotype of the horses in our study (Chapter 4.2.) and to correlate it with the prevalence of BPV latency. However, preliminary experiments with PCR-RFLP in our laboratory showed that the determination of the ELA haplotype is not straightforward due to the existence of multiple loci and many different alleles. In Arabian horses a significant correlation exists between sarcoid development and heterozygosity for the defective DNA-PKcs allele responsible for severe combined immunodeficiency (Ding *et al.*, 2002). In a sub-optimal environment for completion of the productive life cycle of papillomaviruses, resulting in an abortive infection, progression to cancer is more likely to happen (Doorbar, 2006). This can explain the formation of equine sarcoids in horses compared to cattle, the natural host of BPV, where papillomas only rarely progress to cancer and most often regress spontaneously.

It remains unclear how equine sarcoids eventually develop after BPV infection. Skin trauma may play a role, because it allows direct access of BPV to the subepidermal fibroblasts which may lead, together with the formation of different growth factors necessary for skin regeneration, to proliferation of the BPV transformed cells. In people it is observed that not only in patients with psoriasis, which is strongly correlated to HPV-5, but also in patients with epidermal repair following extensive second degree burns or autoimmune bullous diseases, antibodies to HPV-5 are generated (Favre *et al.*, 2000). In experimental conditions, healthy cattle housed in isolation can develop papillomas harbouring BPV-1 and/or -2 following immunosuppression or skin trauma (Campo *et al.*, 1994). Also in horses, sarcoid development in wounds is frequently observed (Torrontegui & Reid, 1994; Knottenbelt *et al.*, 1995; Foy *et al.*, 2002). Nevertheless, many sarcoids appear without any clinical evidence of skin trauma, although microtrauma (e.g. insect bites) cannot be excluded.

The presence of BPV DNA on the normal skin of horses without any clinical evidence of sarcoid development supports the multistep pathogenesis of equine sarcoids. As demonstrated in numerous studies, BPV DNA is a prerequisite for transformation of fibroblasts, but it is not sufficient on its own to induce malignancy. Host factors, such as the genetic constitution and the immunological status, are also playing a major role. Papillomavirus infections are only maintained when the virus can remain hidden from the immune system. A genetically predisposed insufficient immune response against virally

infected tumour cells can give papillomaviruses all opportunities to transform host cells, together with a loss of cell cycle regulation or an increase in cellular proliferation (Carr *et al.*, 2001a).

The distribution of BPV-1 and BPV-2 in the horses with latent BPV infection was remarkably different from the distribution in sarcoid-affected horses. In Europe, the proportion of BPV-1 in sarcoids is between 74 and 93%, compared to 7 to 26% of BPV-2 (Angelos *et al.*, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Martens *et al.*, 2000; Martens *et al.*, 2001a). The results from Chapter 3.2. confirm this proportion. However, among the healthy horses examined in Chapter 4.2., 63% of the BPV DNA positive horses were carrier of BPV-1, 21% of BPV-2 and 17% of both types. This distorted distribution was mainly due to the horses living in contact with papilloma affected cattle. In this group, 75% of the BPV DNA positive horses harboured BPV-2, a number as high as the BPV-1 positive horses. Fifty percent of the horses in this group carried both types. When this group is excluded, the expected 80/20 ratio is observed. These results suggest that BPV-2 might be more easily cleared after infection than BPV-1 or that BPV-2 is less likely to transform equine skin.

Due to improved hygiene on cattle farms, papillomavirus infections are not as common anymore nowadays. It could therefore be expected that the prevalence of sarcoids in horses would also decrease. Nevertheless, sarcoids are more and more observed in practice. One possible reason for this apparently increased incidence is that owners as well as veterinarians are more aware of the disorder and that sarcoids are more easily diagnosed compared to some decades ago. Another possibility is that BPV is circulating between horses, a hypothesis which is supported by the existence of sarcoid-specific genetic variants (Chambers *et al.*, 2003b; Nasir *et al.*, 2007). Although this transmission seems to be not as effective as transmission from cattle to horses, passage of the virus exists and can even lead to outbreaks of sarcoids in closed herds of equids (Ragland *et al.*, 1966; Nel *et al.*, 2006; personal observations). This possibility of “horse to horse” transmission stresses the need for hygienic measures when a horse with equine sarcoids is held together with other horses. Examples of such hygienic measures are the use of separate grooming equipment, bridle and tack as well as avoiding contact with the sarcoids and washing the hands after touching them. Especially wounds are vulnerable to BPV infection and care should be taken to prevent transmission, both in the affected and in contact horses. Eradication of BPV by hygienic measures only is utopian. Many animals, both horses and cattle, are asymptotically

infected which will cause the regular appearance of clinically affected animals. Moreover, since no 100% effective treatment exists for equine sarcoids, some horses will always remain affected thus being a source of infection to others.

Prophylactic vaccination could possibly be a valuable tool in the prevention of equine sarcoids. In human medicine, a prophylactic vaccine against HPV-6, -11, -16 and -18 is commercially available since 2006 and another one against HPV-16 and -18 is available since 2007. HPV-6 and -11 are responsible for over 90% of anogenital warts and HPV-16 and -18 for approximately 70% of cervical cancers. Less common diseases, such as vulval, vaginal, penile and anal cancer, recurrent respiratory papillomatosis, head and neck cancer etc. are in a substantial number of cases also induced by one of those 4 HPV types. This prophylactic vaccine consists of virus like particles (VLP) composed of the L1 capsid antigen. These VLPs resemble the authentic virions, but they do not contain the viral genome and are therefore not infectious. The vaccine induces high and persisting titres of virus-specific neutralising antibodies and absolute protection against persistent infection and virus-associated premalignant cervical lesions (Koutsky *et al.*, 2002). However, this mechanism of prophylaxis will probably not be effective in horses to protect them against BPV infection. Indeed, horses can get infected by BPV DNA alone as sarcoid transmission between horses has been observed. Antibodies against the viral capsid will therefore not protect the horse from infection and subsequent sarcoid development. Moreover, the prevalence of equine sarcoids is not high enough to justify vaccination of the entire horse population.

A more eligible line of thought is the development of a therapeutic vaccine. Therapeutic vaccines are aimed at eliminating or reducing already existing lesions. However, development of such a vaccine is not straightforward. While the main purpose of prophylactic vaccination is to generate neutralising antibodies, therapeutic papillomavirus vaccines attempt to elicit cellular immune responses targeting non-structural early viral antigens. T-cell responses are of uppermost importance in clearing papillomavirus infections and papillomavirus induced lesions (Stern, 2005; Inglis *et al.*, 2006; Stanley, 2006). A basic principle of therapeutic vaccines is to achieve upregulation of MHC class I alleles and to stimulate cytotoxic T lymphocytes as effector cells that will recognise and target infected cells (Leggatt & Frazer, 2007). In people, numerous therapeutic HPV vaccines are under investigation, including peptide, viral-vector, DNA and dendritic cell vaccines (Giles & Garland, 2006). Although empirical clinical trials have been successful in inducing cell-

mediated immunity, cure of HPV-associated epithelial disease is rare and immunogenicity is not correlated with clinical outcome (Frazer, 2004; Stern, 2005). Overcoming local immunoregulation in the skin will be a key determinant of success (Leggatt & Frazer, 2007). Possible targets of a therapeutic vaccine against equine sarcoids are BPV E5 and E6 because of their major role in transformation and the constitutive expression in all sarcoid types. But that will be a long and winding road...

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SUMMARY

The present work focuses on the role of bovine papillomavirus (BPV) in the pathogenesis of equine sarcoids and on its prevalence in the normal skin of sarcoid-affected and healthy horses. Equine sarcoids are the most common skin tumours in equids. Although not life threatening, the presence of sarcoids can have important economic repercussions due to expensive treatments and the hereditary character of the disorder.

In Chapter 1, both clinical and virological aspects of equine sarcoids are reviewed. Many clinical manifestations of sarcoids have been described, ranging from single small lesions to multiple aggressively growing masses. Histopathologically, it is considered as a biphasic tumour with epidermal hyperplasia and subepidermal proliferation of transformed fibroblasts. The diagnosis can be made clinically, histopathologically and/or by detection of BPV DNA. Sarcoids can appear on any part of the body, but they are mostly localised on the ventral abdomen, the paragenital region, head and limbs. Sarcoids develop independent of breed, coat colour, sex or age, but they are mostly observed in young adults and certain families and breeds are more vulnerable than others. Transmission of BPV is supposed to happen from cattle to horse or from horse to horse, possibly via insects.

Treatment of sarcoids is often challenging, due to the variable clinical presentation of lesions and the frequent local recurrences. In this thesis, both the surgical and the non-surgical treatment of equine sarcoids are reviewed. It is generally accepted that the prognosis is worse if unsuccessful attempts have been made previously. Therefore, the best available treatment option should always be used at the first attempt of treatment. Different surgical approaches have been reported, including conventional excision, cryosurgery and CO₂ laser surgery. Success rates are high if a non-touch approach, wide surgical margins and general anaesthesia can be applied. Local chemotherapy is a valuable addition in the treatment of sarcoids and can be combined with surgery. Radiotherapy is another successful treatment, but safety precautions prevent routine application. Local immunotherapy including Bacillus Calmette-Guérin vaccination and imiquimod cream are commonly applied treatments which induce quite effective tumour regression.

Genetic material of the bovine papillomavirus (BPV), a small non-enveloped DNA virus, can be demonstrated in virtually all sarcoids. Eight different BPV types have been

described, all inducing benign proliferation of epithelium in cattle, but only BPV-1 and -2 are less strictly species-specific and can induce sarcoids in horses. Historically, association between BPV and equine sarcoids has been demonstrated using inoculation studies and detection of BPV DNA and BPV gene expression. The BPV genome is composed of 6 early and 2 late genes, with E5 and E6 being the most important transforming genes. Specific BPV-1 variants associated with equine sarcoids have been reported, suggesting circulation of the virus between horses. In horses, a non-productive BPV infection occurs, whereby only transcription of early genes, responsible for genome maintenance, regulation of cell growth and cell transformation, occurs. There is no formation of new infectious virus particles as seen in the natural host.

In Chapter 2, the scientific aims of this work are formulated. The first aim of this research project was to investigate the correlation between the clinical behaviour of sarcoids and the activity of BPV using an accurate and reliable gene expression model. The second aim of this thesis was to determine the presence of BPV on and in the normal skin of both sarcoid-affected and healthy horses. In particular, differences were investigated between healthy control horses and those living in contact with affected horses or with cattle presently or recently infected with BPV.

In Chapter 3.1., a reliable set of reference genes was determined to allow accurate normalisation of quantitative real-time RT-PCR for studies concerning normal equine skin and equine sarcoids. In most of the studies published on real-time PCR in horses, normalisation occurred against only one reference gene without validation of its expression stability. This might result in unreliable conclusions, because it has been demonstrated that the expression levels of so called “housekeeping genes” may vary considerably in different tissues, cell types or disease stages, particularly in clinical samples associated with malignant disease. The gene transcription levels of 6 commonly used reference genes (*ACTB*, *B2M*, *HPRT1*, *UBB*, *TUBA1* and *RPL32*) were determined in normal equine skin and in equine sarcoids. After applying the geNorm applet to this set of genes, *TUBA1*, *ACTB* and *UBB* were found to be most stable in normal skin and *B2M*, *ACTB* and *UBB* in equine sarcoids. When normal skin and equine sarcoids are compared, the geometric mean of *UBB*, *ACTB* and *B2M* can be recommended as a reliable and accurate normalisation factor.

In Chapter 3.2., 99 sarcoid samples of 4 different clinical types (fibroblastic, occult, nodular and verrucous) were analysed for differences in BPV load and mRNA expression, proliferative fraction (PF) and p53 expression to test the hypothesis that the clinical behaviour of equine sarcoids is correlated with BPV activity. Viral load and expression of BPV E2, E5, E6 and E7 genes were determined with quantitative real-time PCR. The PF of the tumours was determined by Ki67 immunostaining and expression of p53 was analysed by immunohistochemistry. Nodular sarcoids showed a significantly higher viral load than the other types. A significant overall difference between the 4 types was observed for E2, E5, E6 and E7 mRNA expression. Nodular sarcoids showed the highest expression level for each examined BPV gene, followed by verrucous, fibroblastic and occult tumours. Viral DNA and mRNA outcomes were correlated which indicates a similar transcriptional pattern in each type of sarcoids. The PF was significantly higher in the superficial layers of verrucous and fibroblastic sarcoids compared to occult and nodular types. No significant difference was observed for PF in the deep layers and for p53 expression. These results clearly demonstrate the omnipresence and active transcription of BPV in equine sarcoids. However, the hypothesis that the clinical behaviour of an equine sarcoid can be explained on the basis of differences in BPV activity could not be demonstrated.

In Chapter 4.1. it was examined whether BPV DNA could be detected on the normal skin and in the habitual surroundings of horses with and without equine sarcoids by means of superficially taken swabs. All sarcoid-affected horses had at least one normal skin sample positive for BPV DNA. Moreover, there was no significant difference in BPV DNA positivity between samples obtained from the equine sarcoid surface, from normal skin close to the tumour and from a normal skin site in direct contact with the tumour. From the group of healthy horses living in contact with affected horses, 44% were BPV DNA positive. All horses living in contact with clinically unaffected cattle and the control horses were negative for BPV DNA, with one exception for a horse with a squamous cell carcinoma. The samples of the surroundings were all negative except for those obtained from the surroundings of a horse living in contact with a papilloma affected cow. Therefore, the surroundings of affected and non-affected horses are probably not a major source of BPV DNA contamination.

In Chapter 4.2., presence and activity of BPV in normal skin and peripheral blood of 4 groups of horses was evaluated: sarcoid-affected horses, horses living in contact with sarcoid-affected horses, horses living in contact with papilloma affected cattle and control horses.

From each horse, 3 samples on 4 locations were collected: a swab of the intact skin surface and both a swab and a biopsy obtained after skin decontamination. BPV DNA was found in the normal skin of 24 of 42 horses (57%). Mainly sarcoid-affected horses and horses living in contact with presently or recently infected cattle were carriers (73%), but BPV DNA was also detected in 50% of the horses living in contact with sarcoid-affected horses and in 30% of the control population. BPV mRNA was detected in 58% of the samples positive for BPV DNA, although in a much lower quantity compared to sarcoids. In most of the BPV DNA positive samples mild acanthosis, slight basophilic cytoplasmic swelling of the epidermal layers and/or thickening of the basement membrane were noticed, but these observations were also present in several BPV DNA negative normal skin samples. BPV DNA could never be detected in peripheral blood. These findings suggest latent infection and a wide-spread occurrence of BPV in the horse population.

The final chapter (Chapter 5) presents the general discussion of the obtained results and the future perspectives of research in the field of equine sarcoids. One of the most exiting challenges is the development of a therapeutic vaccine against sarcoids. However, development of such a vaccine is not straightforward due to the need to generate a potent cellular immune response in the skin.

SAMENVATTING

Deze thesis richt zich op de rol van het boviene papillomavirus (BPV) in de pathogenese van equine sarcoïden en op de prevalentie ervan in de normale huid van paarden met en zonder sarcoïden. Equine sarcoïden zijn de meest voorkomende huidtumoren bij paarden en andere paardachtigen. Hoewel de aandoening niet levensbedreigend is, kan de aanwezigheid van sarcoïden een belangrijke financiële weerslag hebben omwille van de dure behandelingen en het erfelijke karakter van de aandoening.

In Hoofdstuk 1 wordt een overzicht gegeven van zowel de klinische als de virologische aspecten van equine sarcoïden. Verschillende klinische vormen zijn beschreven, gaande van enkelvoudige kleine laesies tot multipele agressief groeiende gezwellen. Histopathologisch wordt de tumor beschouwd als een bifasisch gezwel met epidermale hyperplasie en subepidermale proliferatie van getransformeerde fibroblasten. De diagnose wordt gesteld aan de hand van het klinisch onderzoek, histopathologie en/of de detectie van BPV DNA. Sarcoïden kunnen over het hele lichaam voorkomen, maar meestal zijn ze terug te vinden onderaan de buik, in de paragenitale regio, op het hoofd en op de ledematen. Sarcoïden ontwikkelen zich onafhankelijk van ras, haarkleur, geslacht of leeftijd, maar worden toch meestal waargenomen bij jong volwassen dieren. Bepaalde lijnen en rassen zijn eveneens meer gevoelig dan andere. Transmissie van BPV gebeurt vermoedelijk zowel van rund naar paard als tussen paarden onderling, mogelijks via insecten.

De behandeling van sarcoïden is vaak een uitdaging omwille van het variabel klinisch beeld en het frequent optreden van lokale recidieven. In deze thesis wordt een overzicht gegeven van zowel de chirurgische als niet-chirurgische behandeling. Algemeen wordt aangenomen dat de prognose daalt wanneer eerder een niet-succesvolle behandeling werd ingesteld. Daarom is het van het grootste belang om bij de behandeling onmiddellijk de best mogelijke optie aan te wenden. Verschillende chirurgische methodes worden beschreven, o.a. conventionele excisie, cryochirurgie en CO₂ laser behandeling. Hoge slaagpercentages worden bereikt indien een “non-touch” benadering, wegname van brede randen normale huid en een algemene anesthesie worden toegepast. Lokale chemotherapie is een waardevolle aanvulling in de behandeling van sarcoïden en kan eventueel gecombineerd worden met chirurgie. Bestraling is een andere succesvolle behandeling, maar de strenge

veiligheidsmaatregelen verhinderen een routinematige toepassing hiervan. Lokale immunotherapie, zoals vaccinatie met Bacillus Calmette-Guérin en imiquimod crème, wordt regelmatig toegepast en veroorzaakt behoorlijk goede tumorregressie.

Het genetisch materiaal van het boviene papillomavirus (BPV), een klein DNA virus zonder envelope, kan worden aangetoond in virtueel alle sarcoïden. Er worden 8 verschillende BPV types beschreven die allen goedaardige epitheliale proliferaties veroorzaken bij runderen, maar enkel BPV-1 en -2 zijn minder strikt diersoortspecifiek en kunnen bij paarden equine sarcoïden induceren. Historisch gezien werd de associatie tussen BPV en equine sarcoïden aangetoond door middel van inoculatiestudies en detectie van BPV DNA en BPV genexpressie. Het BPV genoom is samengesteld uit 6 vroege en 2 late genen, waarbij E5 en E6 de meest belangrijke transformerende genen zijn. Specifieke BPV-1 varianten geassocieerd met sarcoïden worden beschreven, wat suggestief is voor circulatie van het virus tussen paarden onderling. Bij paarden vindt een niet-productieve infectie plaats, waarbij er enkel transcriptie is van vroege genen die verantwoordelijk zijn voor onderhoud van het genoom, celgroeiregulatie en celtransformatie. Er is geen vorming van nieuwe infectieuze viruspartikels zoals dit het geval is in de natuurlijke gastheer.

In Hoofdstuk 2 worden de wetenschappelijke doelstellingen van dit onderzoek geformuleerd. Het eerste doel van dit onderzoeksproject was om het verband na te gaan tussen het klinisch gedrag van sarcoïden en de activiteit van BPV met behulp van een accuraat en betrouwbaar genexpressie model. Het tweede doel van deze thesis was om de aanwezigheid van BPV op en in de normale huid van paarden met en zonder sarcoïden te bepalen. Meer in het bijzonder werden de verschillen tussen gezonde controlepaarden en paarden die in contact leven met aangetaste paarden of met runderen (met een recente of een huidige BPV infectie) onderzocht.

In Hoofdstuk 3.1. werd een betrouwbare set referentiegenen bepaald die accurate normalisatie van kwantitatieve real-time RT-PCR toelaten in studies met normale paardenhuid en equine sarcoïden. In de meeste studies over real-time PCR bij paarden gebeurde normalisatie vaak slechts tegenover één enkel referentiegen zonder validatie van zijn expressiestabiliteit. Zoiets kan leiden tot onbetrouwbare conclusies aangezien het is aangetoond dat het expressieniveau van dergelijke “huishoudgenen” aanzienlijk kan fluctueren in verschillende weefsels, celtypes of ziektestadia, vooral in klinische stalen geassocieerd met kwaadaardige processen. Het gentranscriptieniveau van 6 vaak gebruikte

referentiegenen (ACTB, B2M, HPRT1, UBB, TUBA1 en RPL32) werd bepaald in normale paardenhuid en in equine sarcoïden. Na het toepassen van de geNorm technologie op deze set van genen werd gezien dat TUBA1, ACTB en UBB de meest stabiele genen waren in normale huid en B2M, ACTB en UBB in equine sarcoïden. Wanneer normale huid en sarcoïden vergeleken moeten worden, kan het geometrisch gemiddelde van UBB, ACTB en B2M aanbevolen worden als een betrouwbare en accurate normalisatiefactor.

In Hoofdstuk 3.2. werden 99 sarcoïd stalen van 4 verschillende klinische types (fibroblastisch, occult, nodulair en verruceus) geanalyseerd voor verschillen in BPV lading en mRNA expressie, proliferatieve fractie (PF) en p53 expressie om de hypothese te testen dat het klinisch gedrag van equine sarcoïden gecorreleerd is met BPV activiteit. De virale lading en de expressie van BPV E2, E5, E6 en E7 werd bepaald met kwantitatieve real-time PCR. De PF van de tumoren werd bepaald door middel van Ki67 immunohistochemie en de expressie van p53 werd eveneens met immunohistochemie beoordeeld. Nodulaire sarcoïden vertoonden een significant hogere virale lading dan de 3 andere types. Een significant globaal verschil tussen de 4 types werd geobserveerd voor E2, E5, E6 en E7 mRNA expressie. Nodulaire sarcoïden vertoonden het hoogste expressieniveau voor elk onderzocht gen, gevolgd door verruceuze, fibroblastische en occulte sarcoïden. De resultaten van de DNA en mRNA analyses waren gecorreleerd wat indicatief is voor een gelijkaardig transcriptiepatroon in elke type sarcoïd. De PF was significant hoger in de oppervlakkige lagen van de verruceuze en fibroblastische sarcoïden vergeleken met de nodulaire en occulte types. Er was geen significant verschil voor de PF in de diepe delen van de tumor en evenmin voor de p53 expressie. Deze resultaten tonen duidelijk de alomtegenwoordigheid en de actieve transcriptie van BPV aan in sarcoïden. De hypothese dat het klinisch gedrag van equine sarcoïden verklaard kan worden aan de hand van verschillen in BPV activiteit kon echter niet worden bevestigd.

In Hoofdstuk 4.1. werd onderzocht of BPV DNA kan gedetecteerd worden op de normale huid en in de leefomgeving van paarden met en zonder sarcoïden door middel van oppervlakkig genomen swabs. Alle aangetaste paarden hadden minstens één staal van de normale huid positief voor BPV DNA. Bovendien was er geen significant verschil in BPV DNA positiviteit tussen stalen genomen ter hoogte van het sarcoïd oppervlak, de normale huid dicht bij de tumor en een zone normale huid in direct contact met de tumor. Binnen de groep gezonde paarden die in contact leefden met aangetaste paarden, was 44% positief voor BPV

DNA. Alle paarden die in contact leefden met klinisch niet-aangetaste runderen en alle controlepaarden waren negatief voor BPV DNA, met uitzondering van één paard met een squameus cel carcinoma. De stalen van de omgeving waren alle negatief behalve deze van de omgeving van een paard dat in contact leefde met een rund met papiloma's. Hieruit kunnen we concluderen dat de omgeving geen belangrijke bron van contaminatie met BPV DNA vormt.

In Hoofdstuk 4.2. werd de aanwezigheid en activiteit van BPV in de normale huid en in het perifere bloed van 4 groepen paarden geëvalueerd: door sarcoïd aangetaste paarden, paarden die in contact leven met door sarcoïd aangetaste paarden, paarden die in contact leven met runderen met papiloma's en controlepaarden. Van elk paard werden 3 stalen op 4 locaties genomen: een swab van het intacte huidoppervlak alsook een swab en een biopsie na decontaminatie van de huid. BPV DNA werd teruggevonden in de normale huid van 24 van de 42 paarden (57%). Vooral paarden met equine sarcoïden en paarden die in contact leven met huidig of recent geïnfecteerde runderen waren dragers (73%), maar BPV DNA werd ook teruggevonden bij 50% van de paarden die in contact leven met door sarcoïd aangetaste paarden en bij 30% van de controlepopulatie. BPV mRNA werd gedetecteerd in 58% van de stalen positief voor BPV DNA, hoewel in een veel lagere hoeveelheid vergeleken met sarcoïden. Bij de meeste BPV DNA positieve stalen werd milde acanthose, geringe basofiele cytoplasmatische zwelling van de epidermis en/of verdikking van de basaalmembraan vastgesteld. Deze veranderingen werden echter ook in verschillende BPV DNA negatieve normale huidstalen teruggevonden. BPV DNA kon in geen enkel perifeer bloedstaal worden gedetecteerd. Deze bevindingen suggereren het bestaan van latente infectie met BPV bij paarden evenals het wijdverspreide voorkomen van BPV in de paardenpopulatie.

Het laatste hoofdstuk (Hoofdstuk 5) is de algemene discussie van de bekomen resultaten en de toekomstperspectieven van het onderzoek over equine sarcoïd. Eén van de grootste uitdagingen is de ontwikkeling van een therapeutisch vaccin tegen equine sarcoïden. Dit is echter niet evident gezien de noodzaak om een krachtige cellulaire immuunrespons op te wekken ter hoogte van de huid.

CURRICULUM VITAE

Lies Bogaert werd geboren op 20 september 1979 te Dendermonde. Na het beëindigen van het secundair onderwijs, richting Wetenschappen-Wiskunde, aan het Sint-Vincentiusinstituut te Dendermonde, begon ze in 1997 met de studies Diergeneeskunde aan de Universiteit Gent. In 2003 behaalde ze haar diploma met grote onderscheiding en ontving voor haar scriptie over “Detectie van BPV DNA op de normale huid en in de omgeving van paarden met en zonder equine sarcoïden” de prijs van de Belgian Equine Practitioners Society.

Onmiddellijk daarna trad zij in dienst bij de vakgroep Heelkunde en Anesthesie bij de Huisdieren, aanvankelijk als assistent en nadien als doctoraatsbursaal ten laste van het Bijzonder Onderzoeksfonds van de Universiteit Gent. Ze verrichtte een onderzoeksstudie getiteld: “De rol van het boviene papillomavirus in het ontstaan en het klinisch gedrag van equine sarcoïden”. Daarnaast stond zij ook in voor het onderzoek en de behandeling van huidtumoren bij het paard. In 2006 voltooide zij de doctoraatsopleiding in de Diergeneeskundige Wetenschappen.

Lies Bogaert is auteur of mede-auteur van meerdere publicaties in internationale tijdschriften en nam actief deel aan verschillende nationale en internationale congressen. Zij is ook referee voor meerdere internationale tijdschriften.

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DANKWOORD

WORD OF THANKS

Tot slot nog een woordje van dank aan al degenen die op één of andere manier hebben bijgedragen aan het voltooien van dit proefschrift. In de eerste plaats mijn promotor, Prof. Dr. Martens, Ann, bedankt voor jouw verhelderend inzicht in deze soms complexe materie en om jouw steun in dit onderzoek. Vanaf de eerste dag, toen nog als laatstejaarsstudent, heb je me het volste vertrouwen gegeven en me laten experimenteren met de papillomavirusjes. Je hebt me de “liefde” voor de sarcoïden bijgebracht en me ook de kans gegeven om het papillomavirus-team verder uit te bouwen en er voor te zorgen dat ik dit onderzoek ook na mijn doctoraat kan verderzetten! Ongeloofelijk bedankt daarvoor! Verder wil ik ook mijn medepromotor Prof. Dr. F. Gasthuys bedanken voor de wetenschappelijke vrijheid die je me hebt gegeven om mijn onderzoek verder uit te diepen en voor de mogelijkheid die je me telkens boodt om op congres te gaan, zelfs al was het vaak niet echt bij de deur... Deze congressen hebben me telkens een enorme boost aan ideeën gegeven!

I would like to thank Prof. Dr. M.S. Campo and Dr. L. Nasir from the University of Glasgow for the fruitful collaboration already from the very beginning of my PhD. Saveria and Lubna, you have always been so kind to give me any information I needed, you gave me the opportunity to visit your lab and you supported us with the E5 antiserum. I'm also very honoured with your presence here today; your lab can be considered as the most experienced BPV lab in the world! Also many thanks to Dr. Georges Chambers for all the information you gave us.

Verder wil ik ook Prof. Dr. Marc Van Ranst en Dr. Annabel Rector van het Rega instituut van de KULeuven bedanken voor de immer enthousiaste samenwerking op het gebied van de detectie van nieuwe papillomavirussen. Marc, bedankt voor de interessante babbels en de vernieuwende inzichten die je me gaf in de papillomavirussen. Annabel, ik sta nog steeds versteld van jouw encyclopedische kennis van de PV-stamboom en alle mogelijke combinaties van primers die je kan gebruiken om een nieuw virus aan te tonen. Ook een dikke merci voor de uitleg op jullie labo over de rolling circle amplification!

Prof. Dr. R. Ducatelle, bedankt voor jouw constructieve opmerkingen en voor de vaak pittige discussies over mijn onderzoek. Het was zeer zeker nuttig om mijn resultaten soms eens van een andere hoek te bekijken en op die manier vaak mooie besluiten te trekken! Ook bedankt aan alle mensen van het labo pathologie voor het gebruik van de faciliteiten en de deskundige uitleg. Christian en Delphine, jullie hebben me zo vaak geholpen bij het inbedden van de weefselstaaltjes. Isabelle en Sarah, zonder jullie zou de immunohistochemie niet zijn wat ze is! Griet, bedankt voor de toffe samenwerking i.v.m. de PIN laesies en voor de vele tijd die je erin gestoken hebt tussen het blokken voor jouw examen door. Ik duim voor jou! Ook een woord van dank aan Koen, Filip, Marjolein, Sarah, Valerie en Iris voor het aflezen van coupes en het helpen met het nemen van foto's.

Prof. Dr. Nauwynck, Hans, enorm bedankt voor de vele tijd die je voor mij hebt vrijgemaakt om te discussiëren over mijn viruskes! Ik weet nog dat je in het begin van mijn doctoraat zei: "Maar kind toch, wat voor een ambetant virus hebt gij nu toch uitgekozen!" maar gaandeweg denk ik toch dat ook dit virus je gebeten heeft en jouw suggesties zijn heel vaak waardevol geweest! Wij zijn je ook heel dankbaar voor het gebruik van jouw labo voor het werk met de celculturen. Ook bedankt aan alle mensen van het labo virologie voor de steeds enthousiaste en bereidwillige hulp bij het opstarten van de cellijnen. Jullie stonden steeds klaar om ons met raad en daad bij te staan als ze niet wilden groeien en jullie waren mee blij als ze dan eindelijk een mooie cultuur werden! Bedankt Lieve, Carine, Sarah C., Sarah G., Filip, Annick, Karen, Chris, Gerald, Nele, David, Mathias, Els, Peter, Wander, Nick, Kalina, Celine...

Prof. Dr. Peelman, Luc, bedankt voor jouw hulp aan de "genetische" kant van de zaak en voor het ter beschikking stellen van het labo, reeds zovele jaren. Het is lovenswaardig dat wij steeds bij jullie terecht kunnen zowel voor wetenschappelijke uitleg als voor het gebruik van de (vaak dure) toestellen. Een héél groot woord van dank voor Dr. Mario Van Poucke: jij hebt me keer op keer een super-deskundige uitleg gegeven en me ingewijd in de wereld van het DNA en mRNA. Dank zij jou heeft real-time PCR niet te veel geheimen meer voor mij (alhoewel?? zo'n crack als jou zal ik nooit worden!). Ook bedankt Karen, Tim, Evelyne, Ruben, Linda, Nicolas en Domi voor de vele woordjes van uitleg en voor de aangename koffiepauzes! Jullie hebben me meer dan eens "ont-strest"!

Veel dank ook aan Prof. Dr. Hilde De Cock voor de vele aangename en leerrijke babbels en voor de toffe samenwerking! Ik ben razend benieuwd naar de resultaten van de laser capture microscopie en ik hoop dat we jouw hypothese over het epitheel kunnen bevestigen! Ook bedankt voor de bemoedigende woorden en om mijn enthousiasme over de USA aan te wakkeren!

Verder mag ik ook mijn collega's van de verloskunde, Jan en Maarten niet vergeten te bedanken voor het gebruik van jullie proefpaarden toen jullie een stier met wratten hadden gehad en voor de namen die jullie me hebben doorgespeeld van klanten met paarden met sarcoïden. Hopelijk kunnen we in de toekomst nog wat proefpaarden delen voor onze experimenten. Ook dank aan Prof. Dr. Jeroen Dewulf voor het oplossen van heel wat statistische vraagstukken! Je zei me steeds: "Zeg het als je niet meer kan volgen hé" en meestal kon ik na 2 woorden al niet meer volgen maar toch had je steeds het geduld en het vermogen om de statistische analyses haarfijn uit te leggen.

Wie ik zeker niet mag vergeten zijn de collega's van de heekunde!! Hoewel ik niet zo vaak in de kliniek heb gestaan als jullie, hebben jullie me toch steeds als een echte collega aanschouwd en vergaten jullie me niet te roepen wanneer er een paard binnenkwam met een "bolleke" of een "plekske". En de vraag die dan steevast volgde: "En collega, ruikt dit naar sarcoïd?". Steeds hilariteit alom, maar die tumoren hebben echt wel hun geur hoor! Bedankt ook voor de leuke middagpauzes waarin steeds stoom afgeblazen kon worden, voor de vele practical jokes (Borat!) en voor de "stakingen" tussendoor om eens efkes je gedachten te verzetten. Bedankt aan de mensen die de operatiezaal draaiende houden: Prof. Steenhaut, Lieven, Jeroen, Tamara, Geert, Stijn, Lindsey, Hannah, Miguel, Caroline, Valérie, Caroline, Cindy, Nadine en vroeger ook Stefan, Els, Linda en Véronique. Ook de mensen van de receptie: Frederik, Michèle, Maarten, Tim en vroeger Prof. Desmet; de hospitalisatie met Eva en Annelies; de hoefsmeden Jan, Didier en Remi; de mensen uit de stallen: Erwin, Hubert, Nadine, Didier, Wilfried; het secretariaat met Heidi, Veerle, Cindy en Johan en ook Annemie voor het elke avond piekfijn achterlaten van de onderzoeksruimten; verder ook veel dank aan de internships om bijna onzichtbaar er voor te zorgen dat alles steeds in orde was en dat elk fiche van een etiket voorzien was! En ook een héél groot woord van dank aan onze laborante Cindy De Baere! Cindy, zonder jou had mijn doctoraat nooit zo snel klaar geweest! Jij hebt zo ongelooflijk veel verstand van PCR en aanverwanten dat ik jou deze delicate taak met mijn ogen dicht kon toevertrouwen! Je was altijd even nieuwsgierig naar mijn resultaten als ikzelf

en je was nooit te beroerd om wat langer door te werken als het moest. Ook extra opleidingen waren voor jou nooit een probleem om je verder te bekwamen in je functie. Prachtig werk! Ook de leuke babbels in ons bureau hielden de sfeer er steeds in en hielpen om deze laatste dagen relatief stress-vrij door te komen! Verder wens ik ook nog veel succes toe te wensen aan Pieter, die het sarcoïd-team is komen versterken. Ik hoop dat je gauw mooie resultaten mag bereiken! Prof. Dr. A. De Moor, jij hebt jaren terug het sarcoïd-onderzoek op gang getrokken als promotor van Ann en nu nog steeds blijf je hierin geïnteresseerd. Bedankt voor de vele aanmoedigingen en voor jouw interesse in dit onderwerp!

Ik zou ook nog mijn scriptiestudenten willen bedanken voor het vuur waarmee jullie aan jullie onderwerp gewerkt hebben of werken! Femke, je hebt vorig jaar een zeer mooi overzicht gemaakt over chemotherapie en onze ervaringen hiermee eens mooi op een rijtje gezet. Eva, jij bent supergemotiveerd om jouw onderzoek over EcPV-2, zelfs zonder de beloofde primers ;-), tot een goed einde te brengen. Jij zult het nog ver schoppen in het onderzoek!

Furthermore, I would like to thank all the people from the HPV community who I met on the conferences in Vancouver, Prague and Beijing. Meeting you and listening to your experiences was of such a high importance for my research. I picked up a lot of ideas and I managed to find solutions for quite some difficulties I had, only by talking with you! Thanks a lot to Marc A., Marc B., Dennis, Hilde, Inez, Johannes (we'll keep in touch!), Sabien, Peter, Martin, Shreya, Diane, Laura, Annabel, Koenraad, Yuan, Mark, Lubna, Saveria, Tim, Véronique and all the others not only for the scientific discussions but also for the most enjoyable chats during dinner and the spare free moments! I hope to see you back on the next meetings! I would like to address a special word of thank to Prof. Dr. Martin Kast for offering me the most exciting opportunity to spend a year in your lab to allow me to gain experience in the wonderful world of the immunobiology of papillomaviruses! Also many thanks to Peter Emtage for introducing me to Martin and to help me so much with the working out of my post-doc despite of your very busy days! Thank you so much for helping me with this!

Onderzoek is duur, en dankzij de firma Intervet, met name Peter van Woensel en Mirjam van der Sluijs, zijn we in staat om ons equine sarcoïd onderzoek verder te zetten. Van harte bedankt voor de financiering en het geloof in onze know-how! Een therapeutisch vaccin ontwikkelen zal niet gemakkelijk zijn en het zal nog niet voor morgen zijn, maar geloof me,

we komen er wel! Ooit zullen we vele paarden kunnen genezen met een simpel prikje in de hals!

Over geld gesproken: ook de Universiteit van Gent wil ik zeker niet vergeten. Dank zij het Bijzonder Onderzoeks Fonds (BOF beurs nr. 01D01103) heb ik de mogelijkheid gekregen om gedurende 4 jaar onderzoek te verrichten aan deze instelling. Dank ook aan de Facultaire Onderzoekscommissie voor het toekennen van een krediet voor het bijwonen van mijn allereerste papillomavirus congres in Vancouver. En eveneens dank aan Prof. Gasthuys voor de mogelijkheid om meer geld te kunnen spenderen aan het onderzoek dan strikt genomen voorzien via het werkingskrediet. Ook dank aan het Equine Research and Welfare Fund van de Stichting Prins Laurent voor het toekennen van een krediet.

Voor veel van mijn studies ben ik ook afhankelijk geweest van de goodwill van heel wat paardeneigenaars. Ontzettend bedankt voor het ter beschikking stellen van jullie paarden om me swabjes en biopten te laten nemen! Ook bedankt aan alle dierenartsen die me heel wat adressen hebben gegeven van eigenaars met sarcoïdpaarden waar ik mijn ding kon gaan doen. Het is altijd tof om met practici te kunnen samenwerken en om ervaringen uit te wisselen!

Naast de hulp van al deze collega's mag ik zeker niet vergeten om ook mijn vrienden en familie te bedanken! Jullie hebben dan wel niet rechtstreeks bijgedragen aan mijn onderzoek en waarschijnlijk meer dan eens jullie wenkbrauwen gefronst wanneer ik over mijn "huidkanker" en mijn "wrattenvirus" begon, maar jullie waren steeds daar om mijn zinnen te verzetten en me te doen beseffen dat er ook nog andere dingen in de wereld bestaan dan doctoreren! De laatste tijd heb ik veel te veel zaken moeten afzeggen (of viel ik zelfs in slaap wegens te vermoeid!) maar ik beloof het, dat haal ik zeker in! Lynn, jij was er steeds voor mij, zowel om over de kleinste onnozelheden te babbelen als om grote levensvragen te beantwoorden (alhoewel, sommige vragen lijken geen antwoord te vinden!). Wij zijn twee handen op één buik! Proximus wordt rijk aan ons soms oneindig lijkende gesprekken, maar toch doet het enorm deugd te weten dat er steeds iemand voor je klaar staat! Thanks!! Verder ook veel dank aan de mensen van "den action". 't Is allemaal heel toevallig begonnen op de Gentse Feesten een dikke 2 jaar geleden met een paar onnozelaars (forgive me the word) die overgebleven VIP-hapjes stonden uit te delen, maar intussen heb ik met jullie al heel veel fijne momenten mogen beleven. Loofie, Sofia, Lexie, Lieven, Peter, Gino, Robyne, Tom, Christian, Pluis, Franske, Claudine, Griet en al de anderen: merci voor alles! Ook mijn oude

studiegenoten mag ik niet vergeten: Sofie en Kris, Liesbeth en Bart, Tamara en Danny, Véronique, bedankt voor de vele aangename momenten en ook al zien we elkaar niet zo veel meer, toch is het steeds leuk om mekaar af en toe nog eens te zien en bij te kletsen over de honden of de paarden!

Deze laatste woorden van mijn doctoraat zijn helemaal gewijd aan de allerbelangrijkste mensen in mijn leven: mama en papa, Katrien en Pieter, Laura en Jurriaan, Maarten en Lien. Jullie hebben me steeds gesteund in alles wat ik deed in mijn leven, nooit hebben jullie me laten vallen, zelfs al ging het soms wat moeilijk. Ik ga dat nooit vergeten, van mijn leven niet! Bedankt ook voor de vele hulp in de verbouwingen van mijn huis, zonder jullie was dat niet gelukt. Mama, Katmandu en Brammeke kunnen zich geen betere “pleegmoeder” voorstellen, ik zie dat ze heel gelukkig zijn daar in Berlare. Thanks, folks!

Lies, 13 december 2007

