New insights in transmission, diagnosis and treatment of equine sarcoids

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Don't you remember when you first went to school? You went to kindergarten. And in kindergarten, the idea was to push along so that you could get into first grade, and then push along so that you could get into second grade, third grade, and so on, going up and up.

And then you went to secondary school and now the pressure was being put on. You must *get ahead. You* must *go up the grades and finally be good enough to get to university. And then when you got to university, you were still going step by step, step by step, up to the great moment in which you were ready to go out into the world.*

And when you finally got out into this famous world, then came the struggle for success in profession. And again, there seemed to be a ladder before you, something for which you were reaching all the time.

And then, suddenly, in the middle of your life, you wake up one day and say "huh, I've arrived?". And you feel pretty much the same as you've always felt. In fact you're not so sure that you don't feel a little bit cheated.

Because, you see, you were fooled. You were always living for somewhere where you aren't. And while it is of use for people to be able to look ahead and to plan, there is no use in planning for a future, for which when you get to and it becomes the present, you won't be there. You'll be living in some other future which hasn't yet arrived.

And so in this way, one would never be able to actually inherit and enjoy the fruits of ones actions.

You can't live at all,

unless you can live fully now*.*

Adapted from Alan Watts (1915 – 1973)

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ABBREVIATIONS

BPV	Bovine papillomavirus
PCR	Polymerase chain reaction
LCR	Long control region
ELA	Equine leucocyte antigen
PBMC	Peripheral blood mononuclear cell
BCG	Bacillus Calmette-Guérin
VLP	Virus like particle
GEE	Generalized estimating equations
CI	Confidence interval
DP	Diagnostic protocol
HSV	Herpes simplex virus
PBS	Phosphate buffered saline
UPLC	ultra-performance liquid chromatography
DD	Deep dermis
SD	Superficial dermis
E	Epidermis
LOD	Limit of Detection
LOQ	Limit of quantification
Jss	Steady state flux
Kp,v	Permeability coefficient
Cv	Concentration in the donor solution
Q48h	Cumulative percentage after 48 hours
GLM	General linear model
SE	Standard error
VAS	Visual analog scale
IFNb	Interferon beta
SiRNA	Small interfering RNA

An introduction to equine sarcoids

In the earliest report of an equine sarcoid, the lesion was described as a “locally invasive, fibroblastic tumour of skin found in horses, donkeys, and mules” (Jackson, 1936). While at first this term was used along with other terms to address a broad range of tumours of fibroblastic origin, equine sarcoids were gradually acknowledged as a separate tumoural entity (Tarwid et al., 1985) and defined as benign, but locally aggressive fibroblastic tumours of the equine skin (Ragland, 1970). The occurrence of equine sarcoids is limited to the skin and they do not metastasize. They rarely show infiltrative growth, and if they do, this is limited to draining lymphoid tissues (Knottenbelt et al., 1995). Nevertheless, spread to other body sites due to contact of healthy skin with sarcoid tissue is common (Jackson, 1936) and affected horses are likely to have multiple tumours.

The fact that equine sarcoids are considered benign tumours does not mean they don’t affect an animals welfare or value. Small sarcoids located at body sites where they don’t interfere with movement or riding gear do not cause any problems, but when they are larger, ulcerated or ill-located, they can cause serious discomfort or even prevent the use of the horse (Taylor and Haldorson, 2013). Because initial sarcoid stages are mostly harmless, they are often underdiagnosed or underestimated and are only being treated after they have started to grow. As larger tumours have a less favourable prognosis (Bergvall, 2013), it is important to gain more insight in the origin, development and treatment modalities of equine sarcoids.

Below, the current knowledge on equine sarcoids is summarized, with an emphasis on clinical management.

Etiopathogenesis

To date, insights in how and why equine sarcoids develop are limited. Already from their first description, it was suggested that a virus could play a role in the development of equine sarcoids (Jackson, 1936), and early inoculation experiments in which lesions resembling equine sarcoids could be induced in horses by exposing them to cell-free extracts from bovine warts (Olson and Cook, 1951) seemed to confirm this suspicion. Similar inoculation experiments with more controlled inocula were carried out and evidence emerged that the bovine papillomavirus (BPV), which is known to cause warts in cattle, could play a causative role in equine sarcoid formation (Ragland and Spencer, 1969). Nevertheless, the lesions that were induced in these experiments

were not *exactly* equine sarcoids: while they histologically resembled equine sarcoids, they differed from naturally occurring tumours in that they remained limited in size and that they regressed spontaneously. Even in the most recent inoculation experiments, it was not possible to induce *real* equine sarcoids by inoculating horses with BPV (Hainisch et al., 2009; Hartl et al., 2011).

After these inoculation experiments had made a strong case for a causative relationship between BPV and equine sarcoids, advances in biotechnology helped to crystallize this hypothesis. BPV DNA was now being detected in most equine sarcoid tissues by DNA-DNA hybridization (Lancaster et al., 1977) and southern blotting (Trenfield et al., 1985; Angelos et al., 1991). Polymerase chain reaction (PCR) techniques provided even more evidence (Bloch et al., 1994; Teifke et al., 1994) and further specified that only viruses of the genus *Deltapapilomavirus* (BPV type 1 and 2 (Otten et al., 1993) and to a lesser extent type 13 (Lunardi et al., 2013)) are associated with equine sarcoids.

Of course, the mere presence of BPV DNA in equine sarcoids is no proof of the virus actually causing these lesions. Nevertheless, there is evidence of expression of the main oncogenes in the majority of equine sarcoids (Nasir and Reid, 1999; Chambers et al., 2003b; Bogaert et al., 2007). Further, viral DNA load (ranging from 0.001 to 568.5 copies per cell) seems to be associated with lesion severity (Haralambus et al., 2010) and m-RNA loads are highly correlated with DNA loads, which is an indication for stable gene expression in equine sarcoids (Bogaert et al., 2007).

The non-enveloped BPV consists of nothing more than a capsid containing a circular dual strand DNA genome. The genome has a length of a little less than 8000 base pairs and consists of 8 open reading frames, containing 6 early genes (E1-E2; E4-E7) and 2 late genes (L1 and L2), and a long control region (LCR) which assists in replication and transcription (Campo, 2006) (Figure 1).

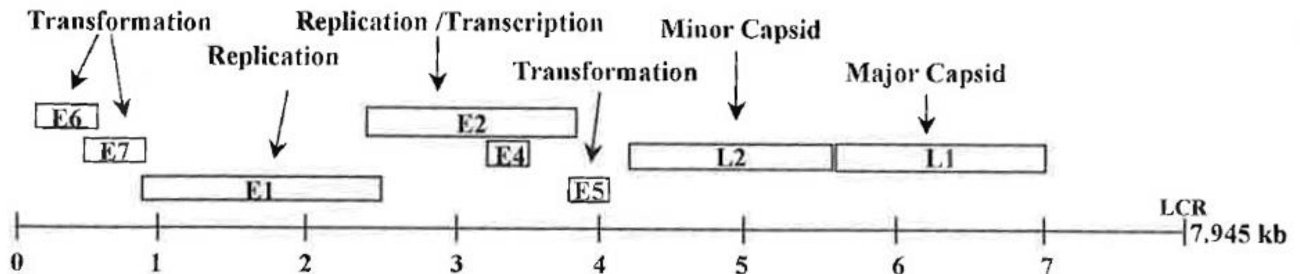

Figure 1 - Linear representation of the BPV-1 and BPV-2 genome. Rectangles indicate open reading frames. (Campo, 2006)

In cattle, after the virus has infected keratinocytes close to the basal layer through skin lacerations, the early genes come to expression first. E1, E2 and E4 regulate replication and transcription of the viral DNA, but their exact roles are not well known. The LCR is not being transcribed, but offers several binding sites for the E2 protein and binding of this protein to the LCR inhibits or promotes transcription of genes. E5, E6 and E7 induce transformation of the host cell and are better studied. The E5 protein is the main oncoprotein of the virus. When it comes to expression, it interferes with intercellular communication by inhibiting gap junctions between cells. By doing this, the host cell becomes isolated and cellular growth is no longer inhibited by the normal homeostatic processes. Concurrently, the E5 protein inhibits acidification of endosomes and by doing so, it causes the host cell to retain and recycle activated growth factors. It also directly activates the platelet derived growth factor receptor. These processes, combined with the inhibition of receptors for downregulation, results in uncontrolled cellular growth and mitosis (Campo, 2006; Venuti et al., 2011). The E6 protein adds to the cellular transformation process by indirectly inhibiting the function of p53, which in normal cells is responsible for cell cycle arrest and apoptosis (Campo, 2006). Both E5 and E6 are assisted by E7 in exerting their effect, but the exact role of E7 is not known (Campo, 2006). The combined effect of E5, E6 and E7 results in a fast growing and constantly dividing cell, which on a tissue scale leads to the formation of warts. Once an infected cell reaches the more superficial epidermal layers, the late genes L1 and L2 come to expression, leading to the formation of a major and minor viral capsid protein, respectively. Several copies of these proteins are then joined together to form a capsid and infectious viral particles are assembled and released. In

most cases, warts are benign and regress after the immune system has cleared the infection.

Contrary to what is known for cattle, the exact mechanism of how BPV leads to the formation of equine sarcoids remains to be elucidated. In horses, the virus is mainly located in fibroblasts of the dermis (Teifke et al., 1994), although its DNA has also been detected in epidermal skin cells (Bogaert et al., 2010; Brandt et al., 2011b). How exactly the BPV reaches the dermal layers is unknown, but it seems to be common sense that skin lacerations up to the level of the dermis are needed. After infection, BPV leads to transformation of fibroblasts (Yuan et al., 2011a) through a largely unknown mechanism in which suppression of p53 functionality (Bucher et al., 1996; Martens et al., 2001b) and overexpression of p38 seem to play an important role (Yuan et al., 2011b). In horses, the viral infection is not cleared by the immune system, which is presumably the result of immune-evasion through major histocompatibility complex class 1 (called equine leucocyte antigen (ELA) in the horse) inhibition (Marchetti et al., 2009), downregulation of toll like receptor 4 (Yuan et al., 2010) and an immune-suppressed cytokine micro-environment (Mählmann et al., 2014; Wilson and Hicks, 2016). Most researchers agree that equine sarcoid formation is the result of a localized infection, where BPV DNA is being confined in intracellular episomes (non-integrated DNA that can replicate independently of chromosomal DNA) (Lancaster, 1981), but the detection of BPV DNA in equine peripheral blood mononuclear cells (PBMCs) (Brandt et al., 2008a) has led to the suggestion that hosts might go through a viraemic phase before lesions occur (Brandt et al., 2009). This could be one possible explication for the observation that horses tend to have multiple sarcoids on different body sites. While expression of L1 and L2 DNA has been observed in some sarcoids (Nasir and Reid, 1999; Wilson et al., 2013), inoculation of cattle with sarcoid extract failed to induce wart formation (Ragland and Spencer, 1969) and BPV infection in equine sarcoids is believed to be non-productive.

Epidemiology

Equine sarcoids occur in horses, donkeys and even zebra. They are the most common of all equine skin tumours, representing up to 90% of them (Scott and Miller, 2011). Their true population incidence is hard to estimate, because most reports are based on a referral clinic population and therefore biased. Reported incidences range from

approximately 0.6% (Ragland, 1970; Mohammed et al., 1992) to about 12% (Studer et al., 2007). There is no strong evidence for equine sarcoid development being associated with demographic parameters, but several studies reveal certain tendencies, which are discussed below.

The reported ages at which sarcoid incidence is the highest vary from 5.8 years to 15 years (Mohammed et al., 1992; Reid et al., 1994; Torrontegui and Reid, 1994; Schaffer et al., 2013; Knowles et al., 2015). Researchers seem to agree that where other tumours are more likely to affect primarily older animals, equine sarcoids can occur at all ages. Nevertheless, the risk of developing other tumours than equine sarcoids increases with age (Knowles et al., 2015) and the risk of equine sarcoid diagnosis decreases above the age of 15 years (Mohammed et al., 1992), indicating that sarcoids are more likely to develop in relatively young horses.

Some researchers observed the incidence of equine sarcoids to be significantly higher in geldings compared to stallions (Mohammed et al., 1992) and in geldings compared to mares (Reid et al., 1994). Others did not find a significant difference in incidence between sexes (Torrontegui and Reid, 1994; Schaffer et al., 2013) and this seems to be in agreement with what most experts believe (Scott and Miller, 2011).

Equids of all breeds are susceptible to the development of equine sarcoids (Scott and Miller, 2011), but some breed predilections have been observed. Standardbreds were found to be less likely to have equine sarcoids than Thoroughbreds, while they in turn were less likely to have sarcoids compared to all other breeds (including Appaloosa, Quarter and Arabian breeds) (Mohammed et al., 1992). These observations are confirmed in another report where Quarter horses were at higher risk of equine sarcoid development and Standardbreds at lower risk, compared to Thoroughbreds (Angelos et al., 1988). There is no confirmed explanation for these observations, but as there is evidence of a certain genetic predisposition for equine sarcoid development, certain breeds might be genetically more vulnerable.

After early observations that equine sarcoids occurred more frequently in certain families (Ragland et al., 1966; James, 1968), more specific evidence was discovered in support of the existence of a genetic predisposition. The occurrence of sarcoids has been associated with different alleles of the ELA gene (Lazary et al., 1985, 1994; Meredith et al., 1986; Brostrom, 1995), of which the product is responsible for antigen

presentation, an important step in initiating the immune response to (a viral) infection. More recently, a whole genome scan of a population of Swiss Warmblood horses presented evidence in favour of a polygenic inheritance for equine sarcoid susceptibility (Jandova et al., 2012). This suspicion was confirmed on a phenotypic level in a heritability study of a population of Franches-Montagnes, in which the heritability of equine sarcoids was estimated to be 8% to 21%, depending on the model (Christen et al., 2014).

When all evidence is combined, it becomes clear that equine sarcoids can be considered a multifactorial disease in which the BPV is the etiological agent, but for which other factors also add to the developmental process.

Transmission

While it is now widely accepted that BPV infection is the main cause for equine sarcoid development, the question of how this virus, originating from cattle, is being transmitted to and possibly between horses, remains to be elucidated. Papillomaviruses are usually very species-specific and the BPV is the only member of the family that is known to spread and cause disease in other species than its natural host. The most evident way for the virus to spread would be by direct contact, but not all horses that develop equine sarcoids live together with or have been around cattle. BPV DNA has been identified in the surroundings of horses with and without sarcoids (Bogaert et al., 2005), indicating that indirect transmission could be possible as well. It has been proposed by several authors that insects could act as a vector for BPV transmission (Knottenbelt and Kelly, 2000; Chambers et al., 2003a). This suspicion became even stronger when BPV DNA was detected by PCR in *Musca autumnalis* (Kemp-Symonds and Kirk, 2007) and several other fly species (Finlay et al., 2009) in proximity of sarcoid-bearing horses. It remains however unclear whether these findings were coincidental or whether flies actually transmit BPV.

Because no certain proof of infective virus has ever been found in equine sarcoids, most authors believe BPV infection to be abortive in equids. Nevertheless, some evidence points in the direction of the production of infectious BPV virions in tumoural tissue. In an early transmission experiment, researchers were able to induce equine sarcoids by inoculating scarified skin of unaffected horses with a sarcoid suspension (Voss, 1969). Sarcoid outbreaks in populations of donkeys (Reid et al., 1994; Abel-

Reichwald et al., 2016) and zebra (Nel et al., 2006) also suggest that infection could spread between equids. Of course, if it is assumed that insects act as a vector, these outbreaks could possibly also be *de novo* infections. Moreover, one study could not detect intact virion in any of the animals affected by the outbreak (Abel-Reichwald et al., 2016). On the other hand, in another study, donkeys were kept in pairs of one sarcoid-affected animal and one healthy animal. If healthy animals developed sarcoids, the BPV present in the lesions was of the same genotypic variant as the sarcoid affected animal in the same stable, but differed from other animal pairs (Nasir and Campo, 2008). Further, as mentioned earlier, L1 and L2 DNA expression have been detected in equine sarcoids (Nasir and Reid, 1999; Wilson et al., 2013) and immunocapture PCR has demonstrated the presence of BPV DNA in association with L1 major capsid protein in equine sarcoids (Brandt et al., 2008b). In addition to this, one researcher published an electron microscopic image of a structure that resembles an intact BPV virion in an equine sarcoid (Wilson et al., 2013).

The detection of DNA mutations specific to BPV originating from equine sarcoids (Chambers et al., 2003b; Nasir et al., 2007; Wilson et al., 2013; Trewby et al., 2014; Savini et al., 2015) is pointing towards another possible theory, in which separate strains of BPV exist. On the one hand there would be the wild type BPV originating from cattle, and on the other hand there would be an equine adapted strain able to produce infectious virions in sarcoids. The effect of these mutant gene sequences on viral replication were also tested. Transcriptional activity of virus containing an LCR variant was twice as high compared to virus containing the reference LCR in equine cells, but not in bovine cells (Nasir et al., 2007). Nevertheless, combined with the finding that variant E2 increased transcriptional activity more in bovine samples compared to equine samples, the authors suspected that the mutant E2 and LCR combination did not contribute to better replication and viral maintenance of variants in equine cells (Nasir et al., 2007). Despite these findings, BPV sequencing was mainly aimed at the LCR and E5 regions, whereas the complete BPV genome has rarely been sequenced. As it is not known at what location most mutations occur, it is very likely that when the genome would be sequenced at another location, more mutations will be discovered both in BPV originating from cattle wart tissue and from sarcoids and thus the importance of these mutations remains unclear.

Clinical presentation

Equine sarcoids can occur anywhere on the skin, but are observed more often at the level of the head, ventral thorax and abdomen and in the paragenital region (Torrontegui and Reid, 1994). They are also observed quite frequently on distal limbs (Taylor and Haldorson, 2013), most likely because this region is more prone to skin lacerations. Indeed, sarcoid development is often associated with a history of non-healing wounds (Torrontegui and Reid, 1994). Although some rare cases of mucosal involvement have been described, these were always complementary to a primary dermal equine sarcoid with local invasion of the mucosa (Knottenbelt, 2005; Knottenbelt and Kelly, 2011).

Morphologically, equine sarcoids can have very different appearances ranging from small hairless hyperkeratotic spots on the skin with little clinical importance, to large ulcerated masses that impede movement and cause discomfort to the horse. Because of this variety in clinical presentation, a clinical classification system has been developed to better describe these tumours (Pascoe and Knottenbelt, 1999). Despite this system, equine sarcoids can change morphology and horses often have multiple tumours of the same or different type at different body locations.

Occult equine sarcoids (Figure 2) appear as hairless spots on intact skin, with a rough or mildly hyperkeratotic aspect, and with or without slight skin thickening (Pascoe and Knottenbelt, 1999). They often are very small and because the changes to the skin can be very subtle (Knottenbelt, 2005), they are easy to miss if one does not know what to look for. They can range in size from very small circular lesions to large irregularly shaped patches and are often mistaken for dermatophytosis (Pascoe and Knottenbelt, 1999). Although they are slow growing or even stable most of the time, in some cases they can change to verrucous sarcoids (see further) or even show quick and aggressive growth towards fibroblastic sarcoids (see further), usually following trauma (Knottenbelt, 2005).

Verrucous equine sarcoids have a more wart-like appearance (Figure 3). The skin is dry, thickened and hyperkeratotic to scaly (Pascoe and Knottenbelt, 1999) in a more pronounced way compared to occult sarcoids. Lesions can be pedunculated or stalkless (Knottenbelt, 2005) and as with occult sarcoids, sizes range from almost imperceptibly small to dozens of square centimetres of affected skin. If small they are

easily mistaken for papillomas or “warts” (Knottenbelt, 2005) and are mostly harmless and stable. They can however grow larger and change into tumours of the fibroblastic type (see further), often starting from small tissue nodules, located within the area of verrucous sarcoid (Knottenbelt, 2005).

Nodular equine sarcoids (Figure 4) are the only type of sarcoid where no changes to the epidermis can be seen. They consist of spherically shaped nodules which are very hard on palpation and lie directly under the skin (Pascoe and Knottenbelt, 1999). This category of sarcoids is further divided into type A nodular sarcoids, where the skin is not attached to the mass and freely moveable, and type B nodular sarcoids, where the skin is attached to the mass and cannot be moved independently (Knottenbelt, 2005). Nodule sizes range from 0.5 to over 20 cm in diameter (Knottenbelt, 2005) and these masses can easily be misdiagnosed as other nodular tumours like (neuro)fibroma, melanoma (Pascoe and Knottenbelt, 1999) and mastocytoma. Nodular sarcoids often show slow but steady growth, but can ulcerate and quickly develop into fibroblastic tumour types (see further). They can occur at any body site but are often seen in the upper eyelid (Pascoe and Knottenbelt, 1999). While they are harmless at first, they can grow into larger nodules, interfering with eyelid movement or even preventing the eye from opening, with obvious implications for the animal’s sight.

Fibroblastic equine sarcoids (Figure 5) have a somewhat misleading name, because all sarcoids actually are of fibroblast cell origin. They appear as ulcerated, proliferative masses (Pascoe and Knottenbelt, 1999) and have been divided further into 3 categories (Knottenbelt, 2005). Type 1A fibroblastic sarcoids are pedunculated and their stalk is thin and consists of macroscopically normal skin. Type 1B fibroblastic sarcoids are also pedunculated, but the stalk is thicker and a substantial part of the tumour is present in the skin at the base of the stalk. Type 2 fibroblastic sarcoids are sessile. The fibroblastic sarcoid is associated in particular with a history of chronic wounds or trauma to or unsuccessful treatment of other sarcoid types (Knottenbelt, 2005), but can also develop spontaneously. This type of sarcoid rarely changes to other sarcoid types and often causes discomfort, because it bleeds, is mechanically hindering and attracts flies, which in turn can cause myiasis. Fibroblastic sarcoids are often mistaken for hypergranulation tissue if occurring at wound sites, and it is therefore important to check if non-healing wounds do not consist of sarcoid tissue (Knottenbelt,

2005) (see diagnosis). Other common misdiagnoses are fibrosarcoma and squamous cell carcinoma (Pascoe and Knottenbelt, 1999).

Two or more (or even all) of the previously mentioned sarcoid types can co-occur in the same lesion. When this is the case, the tumour is called a **mixed equine sarcoid** (Figure 6) (Pascoe and Knottenbelt, 1999). They probably represent a transient state between the abovementioned types (Knottenbelt, 2005).

In one paper, an additional type of sarcoid is reported, called the **malevolent equine sarcoid** (Knottenbelt et al., 1995). This form distinguishes itself from the other forms in that it infiltrates in lymphoid vessels and lymph nodes, resulting in a strand of tumours along those tracts. There have not been other reports of this type of tumour and the question has been raised whether these tumours are actually sarcoids (Wobeser, 2017).

Figure 2 - An occult equine sarcoid at the inner side of the thigh.

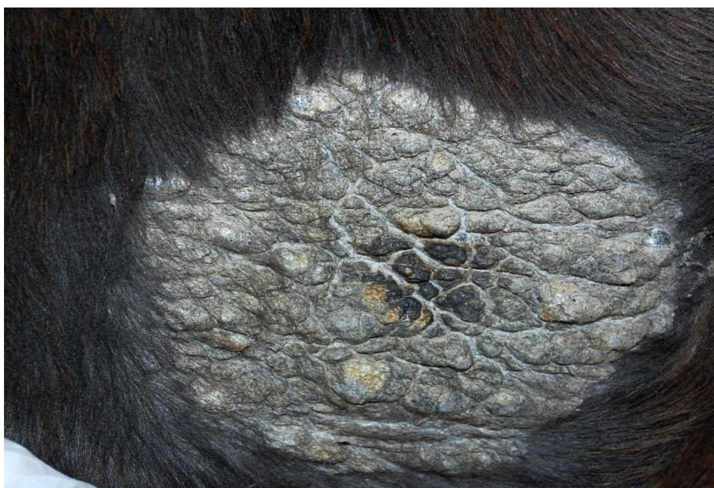

Figure 3 - A verrucous equine sarcoid at the level of the left shoulder.

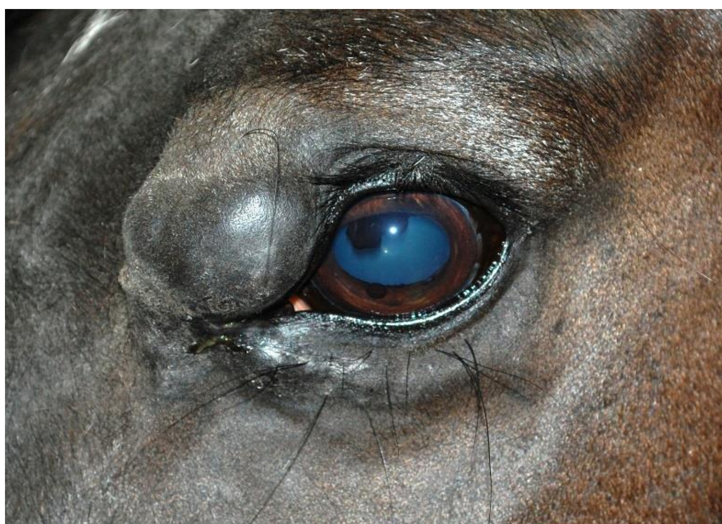

Figure 4 - Type B nodular equine sarcoid at the level of the left upper eyelid.

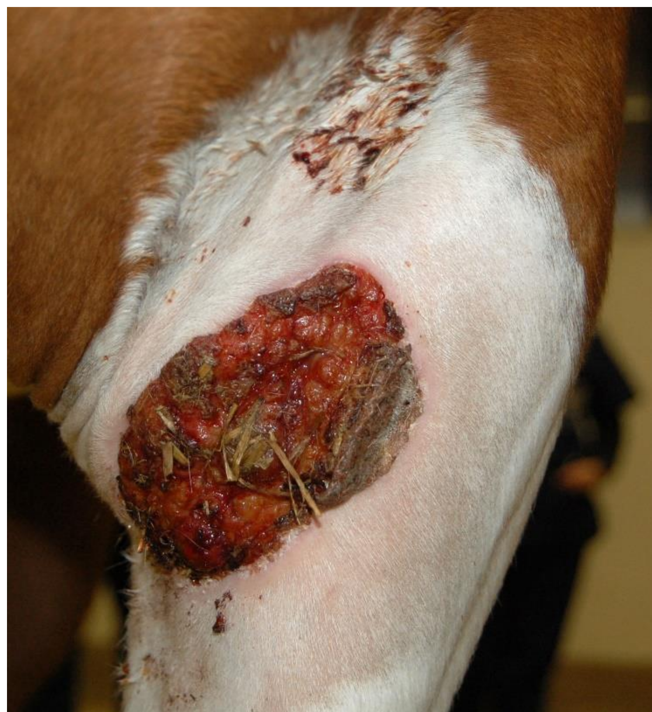

Figure 5 - Type 2 fibroblastic equine sarcoid at the level of the left axilla.

Figure 6 - Mixed (occult-verrucous-fibroblastic) equine sarcoid at the level of the right shoulder.

Diagnosis

In a recent guest editorial, the author states that in theory, the diagnosis of equine sarcoids should be easy: “Similar to how US Supreme Court Justice Potter Stewart described pornography: ‘I know it when I see it.’” (Wobeser, 2017). Indeed, the clinical image of a relatively young horse with multiple skin tumours of different types at typical body sites is pathognomonic to the experienced clinician. However things become more complicated if one or more of these typical characteristics are absent or if the clinician does not have adequate experience with sarcoids. There are currently no scientifically confirmed guidelines for making a clinical diagnosis, and research for the reliability of such diagnosis is lacking.

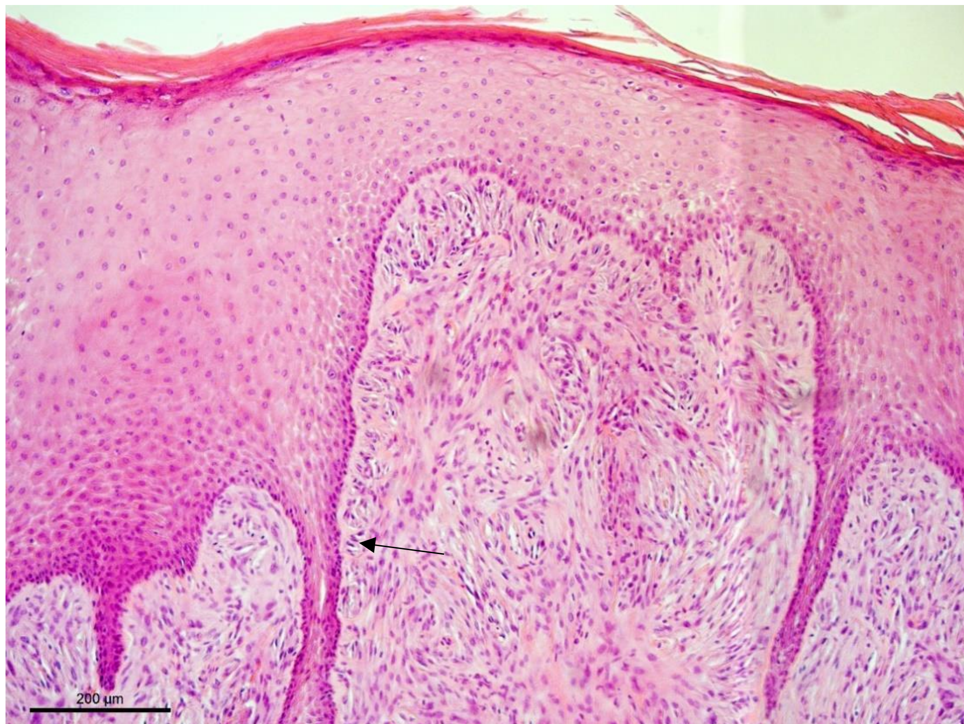

Figure 7 - Histological preparation of an equine sarcoid (H&E). At the dermo-epidermal junction fibroblasts are arranged perpendicular to the basement membrane (picket-fence formation) (arrow) and dermal fibroblasts in the tumour mass are organized in whorls and bundles.

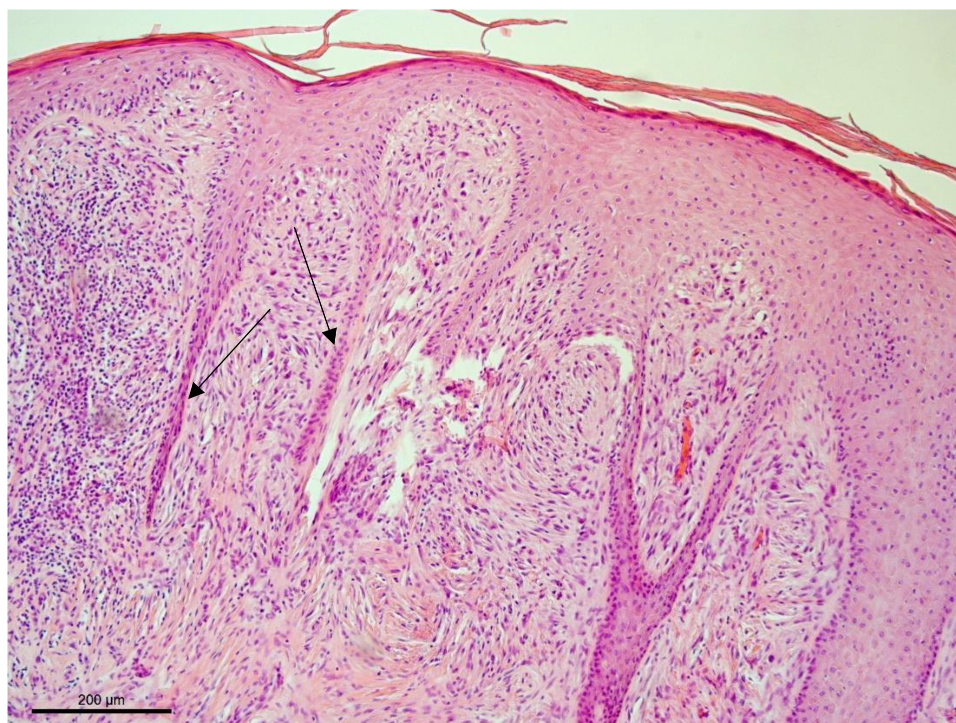

Figure 8 - Histological preparation of an equine sarcoid (H&E). There are long, sharp rete ridges (arrows) and the dermal fibroblasts are organized in bundles and whorls. At the left of the image, dermal infiltration by lymphocytes and plasma cells is visible.

Histopathological examination of tumoural tissue is still the gold standard for equine sarcoid diagnosis (Scott and Miller, 2011; Taylor and Haldorson, 2013). Equine sarcoid tissue typically shows a thickened dermis with increased fibroblast density and spindle-shaped, hyperchromatic neoplastic cells embedded in a matrix of collagen fibers, which can be arranged in whorls, tangles or fishbone configurations (Figure 7 and 8). At the dermo-epidermal junction, fibroblasts often arrange perpendicularly to the basement membrane in a “picket fence” configuration (Figure 7) (Scott and Miller, 2011). Epidermal changes can consist of hyperkeratosis, hyperplasia and the formation of long, sharp rete ridges (Figure 8) (Scott and Miller, 2011). Although epidermal hyperplasia and rete ridges are most often seen in verrucous sarcoids, and ulceration in fibroblastic lesions, all these characteristics can be present in any given sarcoid (Martens et al., 2011). The only constant between different equine sarcoids is an increased fibroblast density (Martens et al., 2000). This makes it particularly difficult to histologically differentiate equine sarcoids from other fibromatous tumours such as schwannomas (Scott and Miller, 2011). Immunostaining for the S-100 protein which should be present in schwannomas has been suggested to differentiate said tumours

from equine sarcoids (Bogaert et al., 2011), although this is debated by others (Epperson and Castleman, 2017).

Because BPV DNA has been shown to be present in up to 100% of tested equine sarcoids (Chambers et al., 2003a) and because PCR techniques are becoming more widely available and cheap, PCR detection of BPV DNA in tumoural tissue is gaining popularity as a diagnostic method for equine sarcoid. Nevertheless, BPV DNA has also been detected in normal equine skin (Bogaert et al., 2005, 2008), hoof canker lesions (Brandt et al., 2011a) and other inflammatory skin conditions (Wobeser et al., 2012), which makes PCR detection somewhat less specific as a diagnostic method. That being said, when a lesion has the clinical appearance of a sarcoid and is positive for the presence of BPV DNA, chances are high that it is indeed an equine sarcoid.

Taking a biopsy can cause a previously stable equine sarcoid to start growing more aggressively or change into a fibroblastic lesion (Scott and Miller, 2011), making it necessary that owners are committed to treatment before biopsying. When technically feasible, it is also possible to do a full excision first, taking into account some precautions (see treatment), and perform histopathology on the excised tumour afterwards. Another possible approach is to perform PCR for BPV DNA on a swab taken from the suspected lesion (Martens et al., 2001b), which does not damage the tissues and limits the risk of activation of the tumour. While BPV DNA could be detected in the vast majority of swabs taken from fibroblastic and verrucous sarcoids, sensitivity was lower in occult and nodular sarcoids where the skin surface is completely intact (Martens et al., 2001b). Nevertheless, as the PCR protocol yields quick results, a good approach might be to analyse a swab sample first and only take a biopsy when the result of the PCR is negative. By doing so, the necessity for biopsy and therefore the risk of tumour activation are reduced to a minimum.

Treatment

Spontaneous regression of equine sarcoids is traditionally believed to be rare (Scott and Miller, 2011), although it has been reported in up to 48% of cases (Brostrom, 1995; Martens et al., 2001c; Berruex et al., 2016). When spontaneous regression occurred, this was mainly in young horses (age 3 or younger) with small, occult or verrucous sarcoids (Berruex et al., 2016). Equine sarcoids are prone to recurrence after any treatment and tend to become more aggressive after treatment failure (Scott and Miller,

2011). Therefore, for small, stable sarcoids, benign neglect with careful monitoring of tumoural behaviour is defendable. Nevertheless, sarcoids are notorious for their unpredictable behaviour (Wobeser, 2017) and can, even without external stimulus, quickly turn into large, ulcerated masses that are hard to treat. Several treatments with varying success rates have been described, but to date, no treatment exists that can cure and/or prevent all equine sarcoids. It is therefore important that in the prognosis, it is made clear to owners that a successful treatment can never be guaranteed and that tumours can recur or new lesions can appear at other body locations.

Sharp surgical excision is perhaps the most commonly performed equine sarcoid treatment in clinical practice. Because this treatment technique has been associated with a recurrence rate of up to 82% (McConaghy et al., 1994; Brostrom, 1995; Knottenbelt and Kelly, 2000), some authors discourage it (Pilsworth and Knottenbelt, 2007; Tupper, 2017). Nevertheless, with a careful tumour selection and the application of an adequate surgical technique, success rates of up to 82% can be achieved (Martens et al., 2001c). Precautions which can be taken during surgery to prevent recurrence include a strict non-touch surgical protocol (Martens et al., 2001c), isolating the tumour from the surgical field by adequate draping (Tupper, 2017), performing the procedure under general anaesthesia (Brostrom, 1995) and the excision of wide margins of apparently normal skin (McConaghy et al., 1994; Martens et al., 2001c). The suggested width of these margins is variable and ranges from 0.5 to 2 centimetres (McConaghy et al., 1994; Martens et al., 2001c; Scott and Miller, 2011; Tupper, 2017). Recurrence of equine sarcoids after excision is suggested to be significantly higher when BPV DNA is present in the surgical margins (Martens et al., 2001c), although other evidence contradicts this finding (Taylor et al., 2014). A surgical margin of 12 mm seems to be the optimum between surgical practicality and detection rate of BPV DNA (Martens et al., 2001a). After sharp excision, wounds are sutured whenever possible and are otherwise left open to heal by second intention.

As an alternative for sharp surgical excision, **laser surgery** has been used to treat equine sarcoids. Carbon dioxide (Carstanjen et al., 1997; Martens et al., 2001c; McCauley et al., 2002), diode (Compston and Payne, 2013; Compston et al., 2013, 2016) or Nd:YAG (Compston et al., 2016) lasers can be used. As with conventional excision, more or less wide margins are being treated, but the wound bed is usually vaporized and the wound left open to heal by second intention. In a recent review,

success rates are reported to be “significantly better” compared to conventional excision (Tupper, 2017), but there is no research supporting this statement. Reported success rates are similar to conventional excision (62% - 71%) (Carstanjen et al., 1997; Martens et al., 2001c; Hawkins and McCauley, 2005; Compston et al., 2016) and one author even formally tested the above hypothesis, finding that success rates between conventional and laser excision were not significantly different (Martens et al., 2001c).

For **cryosurgery**, equine sarcoids are first debulked if necessary and then frozen. Multiple freeze-thaw cycles until at least -20 °C are used to efficiently induce cell death in tumour cells (Diehl et al., 1987; McConaghy et al., 1994; Martens et al., 2001c). Monitoring of tissue temperatures by thermocouple needles is advisable (Carr, 2012) to confirm adequate freezing of tumoural tissue and at the same time avoid unintended damage to other tissues. To obtain these low tissue temperatures, liquid nitrogen is mostly used, either by direct application or in the form of a contact probe (Diehl et al., 1987; McConaghy et al., 1994; Martens et al., 2001c). Because the freezing process is difficult to control, cryosurgery can have serious consequences when carried out in proximity of vulnerable structures such as the eye, nerves, blood vessels or synovial structures (Knottenbelt et al., 1995). After the procedure, a cryogenic crust gradually forms and falls off as the cells become necrotic and are being sloughed by the body. The remaining wound is left to heal by second intention. Success rates are said to range from 60% to 100% (Carr, 2012), although reports were published with lower success rates of 42% or even 9% in specific cases (McConaghy et al., 1994; Knottenbelt and Kelly, 2000). One study reports the use of **hyperthermia** for treating equine sarcoids (Hoffman et al., 1983), but there are too few reported cases to allow evaluation of the effectiveness of this treatment.

Local chemotherapy can be used as a stand-alone treatment, or as an adjuvant therapy after excision or cryosurgery. A cytotoxic agent is applied topically in the form of an ointment or is injected intralesionally, resulting in cell death of exposed cells. Because the cytotoxic agent is applied locally, systemic side-effects are avoided and high local concentrations can be achieved at the level of the tumour cells (Théon, 1998). Because the cytotoxic agents pose a hazard for the operator, appropriate precautions need to be taken to avoid any contact with skin or eyes. Measures can include, but are not limited to, wearing a face mask, long impermeable sleeves, special gloves and the preparation and administration through a specialised no-spill system.

For intralesional chemotherapy, cisplatin is the most frequently used agent. Because the product is easily absorbed and eliminated by the body, it is injected in the form of an emulsion with sterile sesame oil, which helps to keep it in place as a controlled-release (Théon et al., 1993). Injections aiming at saturating tumoural tissues are repeated at 2 week intervals for a total of 4 injections (Théon et al., 1993) or until the desired effect is reached. The dosage was estimated at 1mg of cisplatin / cm^3 of tissue (Théon et al., 1994). There are no systemic side effects and local side effects are limited (Théon et al., 2007). Adjuvant cisplatin treatment can be initiated during surgical excision, does not interfere with primary wound closure (Théon et al., 1994, 2007) and cisplatin can even be administered in the form of intralesionally implanted biodegradable beads (Hewes and Sullins, 2006). Nevertheless, there are no benefits compared to starting the adjuvant treatment postoperatively (Théon et al., 1999). For intralesional chemotherapy with cisplatin, success rates of up to 93% are reported (Théon et al., 2007). Other cytotoxic drugs that can be injected intralesionally include bleomycin (Scott and Miller, 2011), mitomycin-C (McKane and Coomer, 2013) and carboplatin, but there is limited information available on the use of these agents and their success rates. Intralesional chemotherapy can be combined with electroporation to increase intracellular drug concentrations, and beneficial effects of electrochemotherapy have been described *in vitro* (Souza et al., 2016) and *in vivo* (Tamzali et al., 2012; Tozon et al., 2016). Nevertheless, administering a high voltage electrical shock requires the horse to be in general anaesthesia, which is not always practical or wanted.

Small occult or verrucous sarcoids can be treated topically by applying a 5% 5-fluorouracil ointment twice daily (Knottenbelt et al., 1995; Tupper, 2017). Another cytotoxic topical treatment which has been described for equine sarcoids is the so called “AW-3-lude” or “AW-4-lude”. The ointment consists of a mixture of “a number of heavy metal salts and antimitotic compounds” (Knottenbelt and Walker, 1994), but the exact formulation remains undisclosed and the treatment is only available through one equine clinic. Nevertheless, an 80% success rate has been described (Knottenbelt and Walker, 1994).

Because the horse’s immune system does not adequately react to BPV infection and sarcoid formation, **immunotherapy** is since long considered as a logical treatment. Bacillus Calmette-Guérin (BCG) is an attenuated strain of *Mycobacterium bovis* which

was used as a vaccine for tuberculosis in humans. Later, it was discovered that the vaccine has antitumoural properties and it is nowadays in use as an intravesical treatment for bladder cancer. Equine sarcoids are saturated by intralesional injection of a solution containing either BCG cell wall extract or inactivated or attenuated BCG bacteria. Injections are repeated every 2-3 weeks for a total of 4 injections or until tumoural regression occurs (Klein et al., 1986; Knottenbelt et al., 1995; Martens et al., 2001c; Tupper, 2017). Severe side effects, including anaphylactic shock and sudden death, have been described (Knottenbelt et al., 1995; Théon, 1998), but these are rare. Common side effects are limited to local swelling, abscess formation and a slight fever (Théon, 1998; Martens et al., 2001c). The exact mechanism of action of BCG is not known, but it induces a local unspecific primarily cellular immune response which kills tumour cells as bystanders in the inflammatory process. When this happens, tumoural antigens are probably being exposed, which can result in a specific immune response against tumoural cells (Théon, 1998). Success rates up to 100% are reported (Théon, 1998), but vary heavily between locations and tumour types (Knottenbelt et al., 1995; Martens et al., 2001c). Best results are obtained for periocular nodular or fibroblastic sarcoids and success rates are drastically lower for other sarcoid types and at other locations (Knottenbelt et al., 1995; Théon, 1998; Tupper, 2017).

For smaller equine sarcoids, topical treatment has been described with a cream containing 5% imiquimod (Nogueira et al., 2006). Imiquimod is being used in humans to treat genital warts and superficial basal cell carcinoma. While the exact mechanism of action is not entirely understood, it is known that imiquimod stimulates the production of cytokines which in turn initiates a nonspecific cellular immune response and activates natural killer cells and macrophages (Sauder, 2000). For the treatment of equine sarcoids in horses, there is only one study available, reporting complete tumour regression in 9/15 (60%) of cases and 75% reduction in tumour size for an additional 3 lesions (Nogueira et al., 2006). The cream is applied three times a week for a total duration of up to 32 weeks, but all tumours that completely disappeared did so within 16 weeks (Nogueira et al., 2006). Side effects are very common and include exudation, erythema, erosions, depigmentation and alopecia (Nogueira et al., 2006). Some topical creams commercialised to treat equine sarcoids contain bloodroot, which is also said to have immunostimulating qualities, but apart from a study based on owner perception (Wilford et al., 2014), scientific proof of its effectiveness is lacking.

In cattle, both prophylactic and curative vaccination can be used to prevent or treat BPV-2 induced papillomas (Campo, 1997) and in several species, papillomavirus-induced warts are often treated by autogenous vaccination (Nicholls and Stanley, 2000). Similarly, autogenous vaccination can be used to treat equine sarcoids with studies reporting full tumoural regression in 16 out of 21 (Kinnunen et al., 1999), 12 out of 15 cases (Espy, 2008) and 11 out of 16 cases (Rothacker et al., 2015). Others report little effect or even worsening of the condition (Knottenbelt et al., 1995). Recent research has focussed on developing a prophylactic vaccine for equine sarcoids, based on virus-like particles (VLP's) containing L1 and L2 capsid proteins. These vaccines were well tolerated, induced high neutralizing antibody titres (Hainisch et al., 2012) and prevented horses from developing sarcoid-like lesions which are normally induced upon intradermal BPV injection of non-vaccinated horses (Hainisch et al., 2016). Nevertheless, these pseudo-sarcoids are known to remain small and spontaneously disappear over time in non-vaccinated horses (Hartl et al., 2011). They are therefore not representative for true equine sarcoids. Curative treatment of equine sarcoids with BPV VLP-based vaccines has been unsuccessful to date (Ashrafi et al., 2008; Mattil-Fritz et al., 2008).

During **radiotherapy**, ionising radiation is used to cause fatal DNA damage to tumoural cells, resulting in cell death and tumour remission. While teletherapy (radiation from a distant source) has been used to treat equine sarcoids (Henson and Dobson, 2004), interstitial brachytherapy is far more common. Interstitial brachytherapy consists of intratumoural implantation of radioactive iridium-192 with the obvious advantage that radiation doses can be maintained for a prolonged time in tumoural tissues. The technique is very successful with reported success rates consistently high (87% to 98%), especially for periocular sarcoids (Théon and Pascoe, 1994; Knottenbelt and Kelly, 2000; Byam-Cook et al., 2006). Nevertheless, radiotherapy is not widely available, due to the need for a specialised infrastructure and the inherent danger for operators and caretakers.

Other treatments for equine sarcoids have been described. Mistletoe extracts were able to inhibit equine sarcoid cell proliferation *in vitro* (Felenda et al., 2015), but *in vivo* trials revealed a success rate of only 38% (Christen-Clottu et al., 2010). Topical application of acyclovir yields a success rate of 68% (Stadler et al., 2011), but raises questions as to how it can affect replication of the BPV, which is not a member of the

herpesviridae and therefore does not stimulate infected cells to produce the enzyme necessary to activate the drug.

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Scientific aims

Equine sarcoids are common and their treatment is difficult. Since it became widely accepted that the BPV plays an important role in disease onset, scientific interest has increased because the BPV, which causes harmless warts in cattle but cancerous lesions in horses, is one of the few papillomaviruses to cross the species-barrier. The pathogenesis and mode of transmission of equine sarcoids are not fully understood and further insights are needed to find better ways to prevent and cure the disease. The **general aim** of this research project was therefore to fill some of these gaps in the current knowledge which will eventually lead to a better clinical management of the disease and to a better understanding of disease transmission.

Because equine sarcoids are causally linked to the BPV, one way to prevent the disease would be to prevent exposure to the virus. While the mode of viral transmission remains unclear, recent research indicates that flies could play a role in this process and a biting fly would be an excellent vector. The **first aim** was to establish if and for how long a biting fly can become positive for BPV DNA after exposure to cattle warts or equine sarcoids under controlled experimental conditions.

Currently, a paradox exists in equine sarcoid diagnosis: histopathological examination stands as the gold standard, but a biopsy is needed, which can lead to quick exponential tumour growth. While PCR techniques can solve part of this issue, they are not widely available to practitioners. Clinical diagnosis of equine sarcoids is cheap and requires no additional tests, but is said to be non-specific, although substantial research on this subject is lacking. Therefore, the **second aim** was to validate clinical diagnosis of equine sarcoids against histopathological examination.

Many treatments for equine sarcoids have been described, but there is no agreement on how to decide which treatment to use in order to optimise the outcome. The **third aim** was to establish a standardized treatment selection protocol and evaluate its use. Because many of these treatments are expensive, time-consuming or invasive, topical treatments are gaining popularity. Topical treatment with acyclovir has been described, but did not prove to be very successful in our hands and controlled studies were missing. The **fourth aim** was to find out if topically administered acyclovir reaches the dermal layers of the skin, where it should exert its effect, and if acyclovir is better at treating equine sarcoids compared to a placebo.

The possible role of *Stomoxys calcitrans* in equine sarcoid transmission

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Summary

The association between the bovine papillomavirus (BPV) and equine sarcoids is well established, but it is unclear how the virus spreads. Although evidence in support of viral spread through direct animal contact exists, this does not explain sarcoid development in isolated equids. BPV DNA has been detected in flies, which could indicate that these insects serve as a vector. This study aimed to investigate whether BPV-negative stable flies (*Stomoxys calcitrans*) become positive for BPV DNA after exposure to equine sarcoid or bovine papilloma tissue under experimental conditions and if so, for how long.
A total of 420 stable flies were caught alive and exposed to BPV positive equine sarcoid or bovine papilloma tissue. During the following week, dead flies were collected daily and BPV loads were determined by quantitative PCR.

There was a significant rise in BPV load after tissue exposure both in sarcoid and papilloma exposed flies, but the viral load was higher and remained high for a longer time after exposure to papilloma tissue compared to sarcoid tissue. Within days, viral loads decreased again and became indifferent from loads before exposure.

The results of these experiments indicate that BPV transmission by *Stomoxys calcitrans* seems possible and is more likely to occur after contact with bovine papillomas than with equine sarcoids. Transmission seems only possible shortly after tissue exposure. Further research could include experimental induction of sarcoids with BPV positive stable flies, or a repeat of the experiment with micro-dissection prior to PCR.

Introduction

Equine sarcoids are common tumours of the equine skin. While they do not affect the general health of equids, they can be mechanically, hygienically and cosmetically compromising and treatment is difficult, costly and often unrewarding (Martens et al., 2001; Haspeslagh et al., 2016). An association between equine sarcoids and the bovine papillomavirus (BPV) is well established (Chambers et al., 2003a; Nasir and Campo, 2008) and BPV DNA is present in most (if not all) equine sarcoids. Inoculation experiments demonstrated that sarcoid-like lesions can be induced in young, healthy horses by injecting cattle-derived BPV-1 virion intradermally (Hartl et al., 2011). The lesions histologically resembled equine sarcoids and expressed the E5 oncoprotein, but spontaneous regression occurred in all cases, probably due to a cellular immune response (Hartl et al., 2011). Interestingly, intradermal inoculation of sarcoid-derived fibroblasts or naked BPV-1 DNA failed to induce such lesions, confirming that an intact virus is necessary for sarcoid formation (Hartl et al., 2011).

The mechanism of transmission of the virus from cattle to equids is not currently understood. Furthermore it has not been established whether an infective virus production occurs in equine sarcoids, that is capable of spreading from horse to horse. BPV DNA has been found on the skin and in the surroundings of healthy horses living in contact with sarcoid-affected equids or cattle suffering from papillomatosis (Bogaert et al., 2005). Early transmission experiments demonstrated that equine sarcoid formation could be triggered in healthy horses by inoculating scarified skin with minced sarcoid suspension (Voss, 1969) or bovine papilloma extract (Olson and Cook, 1951). These findings suggest that viral transmission through direct animal contact could be possible, but imply that, except for horses living in close contact with cattle, infective virus has to be produced in equine sarcoids. BPV DNA was also found in several fly species surrounding horses with and without equine sarcoids (Kemp-Symonds and Kirk, 2007; Finlay et al., 2009). This could indicate that insects may act as a vector for the spread of BPV from cattle to horses or between equids.

Some observations point to the possibility that equine sarcoids are a source of infective BPV. Epidemic outbreaks of equine sarcoids have been observed in horses (Ragland, 1970), donkeys (Reid et al., 1994) and an isolated group of zebra (Nel et al., 2006). In BPV DNA originating from equine sarcoids, several sequence variants have been

reported to differ from the reference sequence originating from cattle (Chambers et al., 2003b; Nasir et al., 2007; Wilson et al., 2013; Trewby et al., 2014; Savini et al., 2015). This could indicate that an equine specific BPV subtype exists. Further findings include the detection of BPV DNA in a complex with L1 major capsid proteins in about half of investigated sarcoids (Brandt et al., 2008) and an electron microscopic image of putative intact BPV virions in an equine sarcoid section (Wilson et al., 2013).

The present study investigates whether BPV-negative stable flies (*Stomoxys calcitrans*) become positive for the presence of BPV DNA after exposure to equine sarcoid or bovine papilloma tissue under experimental conditions and if so, for how long.

Materials and methods

Fly collection and maintenance

Preliminary experiments (results not included) revealed that *Stomoxys calcitrans* was by far the most common fly at the stables used in this experiment (96% of all caught flies) and that none of the wild caught flies were positive for the presence of BPV DNA. At the time of fly collection, no horses with sarcoids or papilloma-bearing cattle were present.

Flies used for the experiment were caught alive between June and September 2015 in the stables of the Faculty of Veterinary Medicine, Ghent University, under the same conditions as during the preliminary experiment. This was done using a handheld vacuum cleaner with filter and transparent container (FC6093, Philips). *Stomoxys calcitrans* were positively identified by examination of their proboscis. Twenty-seven batches of around 40 flies in each batch were captured. After capturing, the container with the flies was detached from the motor unit of the vacuum cleaner and put into a dark freezer at -20°C for seven minutes to immobilize the flies temporarily. Three flies from each batch were transferred into separate Eppendorf containers to serve as controls before the remaining flies were exposed to sarcoid or papilloma tissue. They were kept at -20°C until further processing. The rest of the flies were moved to specially made plastic containers which allowed for easy handling, feeding and sampling. A new, disinfected container was used for every new batch of flies. Immediately after transferring the flies, a sterile polystyrene petri dish (SPL Life Sciences) with a surface

area of 21.50 cm², containing either equine sarcoid tissue from a fibroblastic equine sarcoid or bovine papilloma tissue at room temperature, was placed in the container with the flies. The tissue was defrosted and sliced approximately 2 mm thick, and covered the complete bottom of the petri dish. The petri dish was left inside the container for 24 h and then removed. Equine sarcoid or bovine papilloma tissue was alternated between batches. During the entire experiment, flies were fed daily through an adapted disposable plastic pipette with citrated BPV negative equine blood.

Every 24 h, dead flies were collected from the containers, transferred into separate labeled Eppendorf tubes and kept at -20°C until further processing. If after 7 days flies were still alive, the experiment was stopped and flies were killed by freezing them at -20°C during 24 h before transferring them into separate labeled Eppendorf tubes kept at -20°C until further processing. The whole procedure was repeated until 30 flies were collected for each sampling point (24, 48, 72, 96, 120, 144 and 168 h after capture), both for batches exposed to equine sarcoid tissue and bovine papilloma tissue.

PCR procedures

DNA from all samples (flies, sarcoid, equine blood and papilloma tissue) was extracted using a commercial kit (DNeasy blood and tissue kit, Kiagen) following the tissue protocol supplied by the manufacturer.

Details on PCR procedures can be found in Table 1. Quantitative real-time PCR for the presence of BPV DNA was performed on all samples. With each run, previously established quantified dilution series of BPV-1 and BPV-2 DNA, harvested from a mix of several equine sarcoids were included. They served as positive controls and as a reference for quantification of the viral load of the sample. To allow for absolute quantification of the viral load and to confirm successful DNA extraction, all samples were also analysed for the presence of a single copy housekeeping gene (equine, bovine or fly) (primers listed in Table 1). For equine sarcoid tissue samples, this was a unique sequence in the equine interferon beta gene (GenBank M14546) (Haralambus et al., 2010). Bovine tissue samples were analyzed for the presence of a unique sequence in the 8th bovine chromosome and for flies, a unique sequence in the ORCO gene (GenBank JX996042.1) was used. Quantified dilution series with known cell quantities were included in all runs, which allowed to determine the number of cells present in the samples. The viral load per cell could then be calculated as the number

of BPV copies in a sample divided by the number of equine, bovine or fly cells in the sample, respectively. All samples were analyzed in duplicate on the same plate and when the difference between the threshold cycle of both replicates was greater than one, the analysis of that sample was repeated in duplicate. For further calculations, the mean of the 2 measurements was used.

Table 1 - Primers and cycling programs used for real time quantitative PCR detection of the targets. (BPV = bovine papillomavirus; IFNb = interferon beta; ORCO = odorant receptor coreceptor; CHROM8 = chromosome 8; BHQ = black hole quencher; FAM = 6-carboxyfluorescein)

Target (Dye)	**Primer**	**Cycling program**
BPV	**f**-AATCGGGTGAGCAACCTTT **r**-TGCTGTCTCCATCCTCTTCA	95°C – 3 min 45 cycles: 95°C – 20 s ; 60°C – 40 s
BPV-1 probe	**FAM**-CGTCAAtCAGGTCTAAaCGCCC-**BHQ1**	
BPV-2 probe	**TexasRed**-TCAAcCAGGTCTAAgCGCCC-**BHQ2**	
Equine IFNb	**f**-AGGTGGATCCTCCCAATGG **r**-CGAAGCAAGTCATAGTTCACAGAAA	95°C – 3 min 45 cycles:
IFNb probe	**FAM**-CCTGCTGTGTTTCTCCACCACGGC-**BHQ**	95°C – 20 s ; 60°C – 40 s
Fly ORCO (SYBR Green)	**f**-TGACAAGGAGACAAACTCAACCATT **r**-CGAAAATCAGCCAGGAGCAG	95°C – 3 min 40 cycles: 95°C – 20 s ; 60°C – 40 s
BosTau CHROM8 (SYBR Green)	**f**-ACTCCCTGATTCTATTACCCATGT **r**-TTTGGTGCTTGTTCCTCTCA	95°C – 3 min 40 cycles: 95°C – 20 s ; 65°C – 60 s

Statistical analysis

All analyses were performed using commercial statistical software (SPSS 23, IBM). To allow for a clear interpretation, abstraction was made from the BPV type (1 or 2) and viral load was defined as number of BPV copies per cell. Extreme values were identified in each group (sarcoid or papilloma tissue) and at each time point (0, 24, 48, 72, 96, 120, 144 and 168 h), by drawing boxplots and calculating the means and 5% trimmed means. If an explanation could be found for the observed extreme values, they were eliminated from the database. When no explanation could be found, they were considered genuine data and included in all further analyses. At each time point, a generalized linear mixed model with a gamma distribution and a logarithmic link function was used to determine if BPV loads were higher in flies exposed to papilloma or to sarcoid tissue. BPV load was used as the dependent variable, while the tissue type (papilloma or sarcoid) was used as a fixed effect in the model. To correct the model for grouping by batch, a random effect of batch number was also included. In order to determine if BPV loads differed significantly between different sample times within exposure groups, for both tissue types (papilloma and sarcoid), a generalized linear mixed model with gamma distribution and logarithmic link function was used. BPV load was the dependent variable and sample time (0, 24, 48, 72, 96, 120, 144 or 168 h) the fixed effect. Batch number was also included as a random effect to correct the model for the effect of grouping. If multiple comparisons were made, a Bonferroni correction was applied. Significance was set at $P \leq 0.05$.

Results

In total, 210 flies in 13 batches were collected after exposure to bovine papilloma tissue and 210 flies in 14 batches after exposure to equine sarcoid tissue. Nine fly samples were eliminated from the analysis, six because no fly DNA was detected in the samples, which indicates a problem with extraction, and three because they were considered extreme values (because the detected fly DNA was unusually low, extremely high BPV loads were obtained). Mean BPV loads of bovine papilloma tissue and equine sarcoid tissue were 227921,27 copies/cell and 18,49 copies/cell, respectively. Table 2 summarizes the number of samples and the mean BPV load in each group at the different sample times.

Table 2 - The total and included number of samples and the mean bovine papillomavirus (BPV) load (copies/cell) for each exposure group at all sampling times (T0, T24, T48, T72, T96, T120, T144, T168). An asterisk indicates a BPV load significantly higher compared to the load at T0 within the exposure group (papilloma or sarcoid).

		T0	T24	T48	T72	T96	T120	T144	T168
Papilloma	**Samples total**	39	30	30	30	30	30	30	30
	Samples Included	38	30	30	28	29	30	30	30
	Mean BPV Load	0.00	21.33*	4.92*	2.01	1.99	6.24*	1.00	0.62
	(± 95% CI)	(± 0.00)	(± 16.2)	(± 2.57)	(± 1.25)	(± 0.88)	(± 7.26)	(± 0.59)	(± 0.25)
Sarcoid	**Samples Total**	42	30	30	30	30	30	30	30
	Samples Included	41	30	29	29	29	30	30	29
	Mean BPV Load	0.00	0.51*	0.03	0.04	0.05	0.02	0.01	0.00
	(± 95% CI)	(± 0.00)	(± 0.54)	(± 0.02)	(± 0.04)	(± 0.06)	(± 0.02)	(± 0.01)	(± 0.01)

Before exposure to sarcoid or papilloma tissue (at T0), no significant difference was present in BPV load ($P > 0.05$) between both groups. Statistical analysis revealed that fly BPV load was significantly higher after exposure to bovine papilloma tissue compared to exposure to sarcoid tissue at all sample times ($P < 0.05$).

Flies that were exposed to bovine papilloma tissues had a significant increase in BPV load between T0 and T24 ($P < 0.01$). At T48, the BPV load had decreased, but not significantly ($P > 0.05$) and was still significantly higher compared to T0 ($P < 0.05$). At T72, the load had become significantly smaller compared to T24 ($P < 0.05$) and was no longer significantly different from the load at T0 ($P > 0.1$). From T72 until T168, the fly BPV load was not significantly different from the load at T0 ($P > 0.1$), except for at T120, where a peak in fly BPV load was observed which was significantly different from the load at T0 ($P < 0.05$).

Flies exposed to equine sarcoid tissue also showed a significant increase in BPV load between T0 and T24 ($P < 0.01$). At T48, the BPV load decreased again significantly compared to T24 and was no longer significantly different compared to the BPV load at T0. At all further sample points, the BPV load remained more or less stable and there was no significant difference compared to the load at T0.

Discussion

If it is assumed that BPV infection is not productive in equine sarcoids, there has to be a vector involved in the spread of BPV from cattle to isolated equids or groups of equids. Equine sarcoids most often occur in the inguinal region, in the axilla and on the head (Taylor and Haldorson, 2013; Haspeslagh et al., 2016). The skin is less thick in these regions (Nel et al., 2006; Scott and Miller, 2011), which makes them more preferred and vulnerable for stable fly bites. The detection of BPV DNA in flies surrounding horses with and without equine sarcoids (Kemp-Symonds and Kirk, 2007; Finlay et al., 2009) further incriminates these insects.

In this study, *Stomoxys calcitrans* was selected because it was found to be by far the most common fly species around horses and cattle and because of its ability to pierce the skin. Nevertheless, BPV DNA has also been identified in *Musca autumnalis* (Kemp-Symonds and Kirk, 2007), *Fannia carnicularis* (Finlay et al., 2009) and *Musca domestica* (Finlay et al., 2009). While none of these species are biting flies, they are known to feed on already existing wounds. Other possible biting vectors could be *Haematobia irritans*, which lives almost exclusively around cattle but is known to feed on horses living in close proximity to cattle as well, or different tabanid species, in which BPV DNA has been identified previously (Abel-Reichwald et al., 2016).

The results of this research show that stable flies can become positive for the presence of BPV DNA both after exposure to equine sarcoid tissue and bovine papilloma tissue. The mean viral load was over 10 times higher immediately after papilloma exposure compared to after sarcoid exposure and remained significantly higher during the entire experiment. This difference could be explained by the fact that the viral load of the papilloma tissue was many times higher compared to the sarcoid tissue. Nevertheless, this would also be the case in real life and the results from this study indicate that viral spread from cattle to equids may be more likely than between equids. Only fibroblastic sarcoid tissue was used here, but there does not seem to be a relationship between sarcoid type and viral load (Haralambus et al., 2010) although BPV DNA in a complex with L1 major capsid proteins was predominantly detected in fibroblastic sarcoids (Brandt et al., 2008). Nevertheless it would be interesting to see if flies become positive for BPV DNA after contact with sarcoid types with intact skin, as BPV DNA has been detected in the epidermis of such lesions (Bogaert et al., 2010).

Since fly BPV load rapidly decreased after initial exposure, and if BPV DNA measured in this study would originate from intact virion, the window of opportunity for BPV transmission by flies is limited in time and therefore distance. If flies are responsible for BPV transmission and transmission only occurs from cattle to equids, horses could be protected by keeping them at sufficient distance from cattle. For other viruses that have blood feeding arthropods as a vector, a separation of 200 m between affected and healthy horses is sufficient to prevent disease spread (Issel and Foil, 2015).

At T120 after papilloma exposure, there was an apparent peak in fly BPV load. There is no biological explanation for this, but the peak in mean BPV load was caused by three out of 30 samples with extremely high BPV loads. These samples were not eliminated as extreme values because the high load was not related to an unusually low amount of fly DNA in the sample, but was due to truly elevated BPV loads.

Currently, it is not known what viral concentration is required for infective transmission. One research group succeeded in producing sarcoid-like lesions by inoculating up to 10^7 viral particles intradermally, but these lesions all regressed spontaneously due to a cellular immune response (Brandt et al., 2009; Hainisch et al., 2009; Hartl et al., 2011). In the present study, detected fly viral loads per cell are relatively low, but the total amount of viral particles in a fly could be in the same range as what was used in the inoculation study. It is also likely that the virus is concentrated at the proboscis or legs of the fly, where tissue contact was intensive. If this is the case, the proboscis would make an excellent tool for transmission, as the virus is inserted directly at its target location when a stable fly pierces the equine skin.

Many questions in the transmission and pathogenesis of equine sarcoids are still to be solved. While the evidence that flies may have a role in BPV transmission and sarcoid onset is increasing, the exact mechanisms remain to be elucidated.

In conclusion, BPV transmission by *Stomoxys calcitrans* seems possible and is more likely to occur after contact with bovine papillomas than with equine sarcoids. Transmission seems only possible for a short time after tissue exposure. Further research could include analysis of flies for the presence and amount of intact virions using quantitative immunocapture PCR and transmission electron microscopy for screening of microdissected fly segments such as the proboscis and intestine, and experimental induction of sarcoids in equids with BPV positive stable flies.

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The clinical diagnosis of equine sarcoids – Part I: assessment of sensitivity and specificity using a multicentre case-based online examination

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Summary

Equine sarcoids are common tumours of the equine skin that are difficult to treat. Correct diagnosis of these tumours is paramount for treatment selection and for scientific research. The gold standard for equine sarcoid diagnosis is histopathological examination, but this requires a tissue biopsy, which can cause lesion exacerbation and can interfere with the outcome of clinical trials. Because equine sarcoids have a typical clinical appearance for the experienced eye, clinical diagnosis can be a good alternative, but has not been validated. To remediate this, 40 clinical cases of histologically confirmed equine skin lesions were compiled and put into an online examination. Care was taken that the selected lesions were a good representation of clinical occurrence of equine sarcoids. Fourteen equine sarcoid experts, 39 board certified equine specialists, 103 equine practitioners and 25 novices categorized the cases as equine sarcoid or not. The overall success rate was 82% while sensitivity and specificity were 83.3% and 79.6% respectively. Equine sarcoid experts were significantly better at diagnosing the lesions and felt more confident doing so, compared to all other expertise levels. Thanks to this research, clinical diagnosis of equine sarcoids can now be accepted as valid. There is room for improvement of clinical diagnosis by less experienced veterinarians and guidelines for making a correct clinical diagnosis could help to achieve this.

Introduction

Equine sarcoids are the most common skin tumours found in equids (Marti et al., 1993; Scott and Miller, 2011). The exact aetiology of equine sarcoids is still not understood, but occurrence and clinical course of the disease are likely influenced by viral, host, and environmental factors (Marti et al., 1993; Taylor and Haldorson, 2013; Wilson and Hicks, 2016). Clinically, equine sarcoids are responsible for a wide spectrum of disease manifestations ranging from small, quiescent solitary tumours to voluminous, multiple, rapidly growing tumours that affect large surfaces of the integument. Lesion morphology is highly variable and comprises four principal forms, i.e. occult, verrucous, nodular, fibroblastic, or mixtures thereof (Knottenbelt et al., 1995). Sites of predilection include the face, neck, axilla, ventral abdomen, paragenital region and distal extremity, as well as areas of previous injury or scarring (Marti et al., 1993). Given this heterogeneity of clinical features and their impact on the use and welfare of the affected equid, many forms of treatment for equine sarcoids have been described. However, no specific treatment is universally effective (Haspeslagh et al., 2016b) and these tumours remain notorious for a high rate of recurrence.

As with any form of neoplasia, the definitive diagnosis is based on histological examination. However, in equine practice histology is often not performed to confirm the clinical suspicion of equine sarcoid. This is explained by the high index of clinical suspicion in cases with typical clinical and morphological features of equine sarcoid, and by the fear of lesion exacerbation (Knottenbelt and Kelly, 2000). Especially for milder manifestations of the disease, including occult and verrucous lesions, the surgical stimulus of taking biopsies is believed to carry a significant risk of transforming quiescent lesions into more aggressively growing tumours (Ragland, 1970; Pascoe and Summers, 1981; Howarth, 1990; Knottenbelt, 2009). Furthermore, in many clinical situations, treatment or a definitive diagnosis are deemed unnecessary and are thus not pursued by the owner or attending veterinarian.

Nonetheless, without confirmation of the clinically suspected diagnosis, it is difficult to draw reliable conclusions from experiments or interpret findings of clinical studies on equine sarcoid. Therefore, representative analyses that assess the agreement between clinical diagnosis and histology of cutaneous tumours thought to be equine sarcoids, will improve our understanding of different epidemiological and clinical

aspects of the disease. The objective of this study was to assess the sensitivity, specificity, positive and negative predictive values and the influence of the level of expertise of the observer when diagnosing equine sarcoid lesions clinically.

Materials and Methods

Online examination

In order to assess the ability to correctly identify equine sarcoids in as many equine veterinarians as possible, an online test was compiled, including 40 clinical cases with skin lesions for which equine sarcoid was considered a possible differential diagnosis and for which an unequivocal histological diagnosis was available. Each case was carefully processed to provide all relevant information as concise as possible. This information always included patient data of the affected subject and, whenever available, when the lesion had first been observed, its growth behaviour, any previous treatments and response to treatment, and the presence and location of other lesions on the affected individual. No information relevant for the assessment of the lesion was intentionally withheld. However, the case information was filtered to some extent in order to present cases as uniformly as possible.

For each case, respondents were asked if they thought the lesion in question was an equine sarcoid ("yes" or "no"), and how confident they were of their clinical diagnosis on a scale from 1 (not confident at all) to 6 (very confident). Furthermore, each respondent was asked to identify himself, specify how long he or she had been working in equine practice, and provide his or her email address. At the end of the exam, each respondent was also asked what 3 features they found to be most reassuring that a skin lesion was actually an equine sarcoid, with the following answers to choose from: (1) Lesion localization, (2) Lesion morphology (occult/ verrucous/ nodular/ fibroblastic/ mixed), (3) Multiple lesions in the same horse with typical sarcoid morphology, (4) Age of the animal, (5) Changing lesion morphology (e.g.: changing from occult to verrucous), (6) (Aggressive) recurrence after treatment.

The online examination was made available using Google Forms and can be accessed under the following link: https://goo.gl/forms/I0iGWYq0mDbOrrZ83. Potential candidates were directly contacted by the first authors (C.K. and M.H.) via email and kindly requested to take part in the online examination. Furthermore, the study

objectives were clearly disclosed and all candidates were informed that results of the exam will be subjected to peer-review and publication in an anonymised format.

Candidates were categorized as (1) “equine sarcoid expert”, if they had previously published on the topic of this tumour in peer-reviewed journals and had at least 2 years of clinical experience, (2) “board certified equine veterinarian”, if they were qualified specialists with Diplomate status of the American or European Colleges of Veterinary/Equine Surgery, Internal Medicine, or Dermatology, (3) “equine practitioner”, if they had at least 1 year of clinical experience in equine practice, or (4) “novice”, if they had less clinical experience, including veterinary medicine students (2 final years of studies) and recent graduates.

Once all required fields of the online examination had been filled in by a respondent, the responses were automatically recorded in an Excel-spreadsheet.

Case-composition for the online examination

To determine the proportion of equine sarcoid and non-equine sarcoid skin lesions to be included in the online examination, a preliminary analysis was carried out on the histological diagnoses of all equine skin lesions (including external submissions, and not just restricted to neoplastic lesions) to the histopathology diagnostic laboratory at the University of Berne between January 2011 and September 2016. To assess a representative proportion of diagnostically challenging cases, this data set was augmented with the histological diagnosis of skin lesions submitted to the histology diagnostic laboratories at the veterinary teaching hospital at the Universities of Ghent and Vienna (not including external submissions) between June 2011 and June 2016. For this analysis, the determining inclusion criterion was that the suspected clinical diagnosis had to be listed on the histology request form or could conclusively be deducted from the medical records.

Cases of cutaneous masses and skin alterations that were seen by the first authors (C.K. at the ISME Equine Clinic Berne and M.H. at the Equine Hospital of Ghent University) between 2011 and 2016 were screened for completeness in documentation, including histopathologic examination and comprehensive written medical and digital photo documentation. All cases included in the pool for the online exam were clinical cases and an informed consent that case data may be used for

teaching and research purposes had been signed by the owners for each case. Cases for which photographic documentation of the relevant skin lesion was of inadequate quality or unclear and cases with an equivocal histological diagnosis were excluded.

Clinical cases with adequate documentation to be included in the online examination were categorized as “equine sarcoid” or “non-equine sarcoid” cases, based on the results from histology, and furthermore as “typical” or “diagnostically challenging” cases. To differentiate between typical and diagnostically challenging cases, the authors adhered to a list of criteria summarized in Table 1. This resulted in a final case pool, in which cases were assigned to one of the following four categories: 1. “typical equine sarcoid”, with case features typical of equine sarcoid disease; 2. “diagnostically challenging equine sarcoid”, with case features that are less typical, or unspecific of equine sarcoid; 3. “typical non-equine sarcoid”, with case features that are highly suggestive and typical of another differential diagnosis; and 4. “diagnostically challenging non-equine sarcoid”, with case features that are also compatible with equine sarcoid. According to pre-determined proportions (equine sarcoid, non-equine sarcoid, diagnostically challenging or typical), the appropriate number of cases from each category was selected at random from this case pool to assemble the online examination consisting of 40 clinical cases (Figure 1), at the same time ensuring that lesions of all 4 basic morphologies and different localisations were included in the final version of the online examination.

Table 1 - Discriminatory criteria to differentiate “typical” and “diagnostically challenging” equine sarcoid (ES) cases.

	typical ES case features	features of **diagnostically challenging** cases and/or case features not typical of ES
age	2 years and older	< 2 years and > 15 years of age
history of…	observed changes from occult to verrucous, or verrucous to fibroblastic morphological appearance	recent wounding
	tumour recurrence within 2 months after excision	signs of pruritus
lesion morphology and number	several lesions clearly compatible with one or more typically described ES tumour morphologies (occult, verrucous, nodular, fibroblastic)	solitary nodular mass
		hairless lesions with symmetrical distribution over the two body halves, or over surfaces exposed to rubbing or in direct contact with tack and blankets
lesion localisation	typical ES localisation (eyes, ears, axilla, chest, abdomen, medial proximal legs)	lesion localised at (oral, ocular or genital) muco-cutaneous junction or at the base of the tail

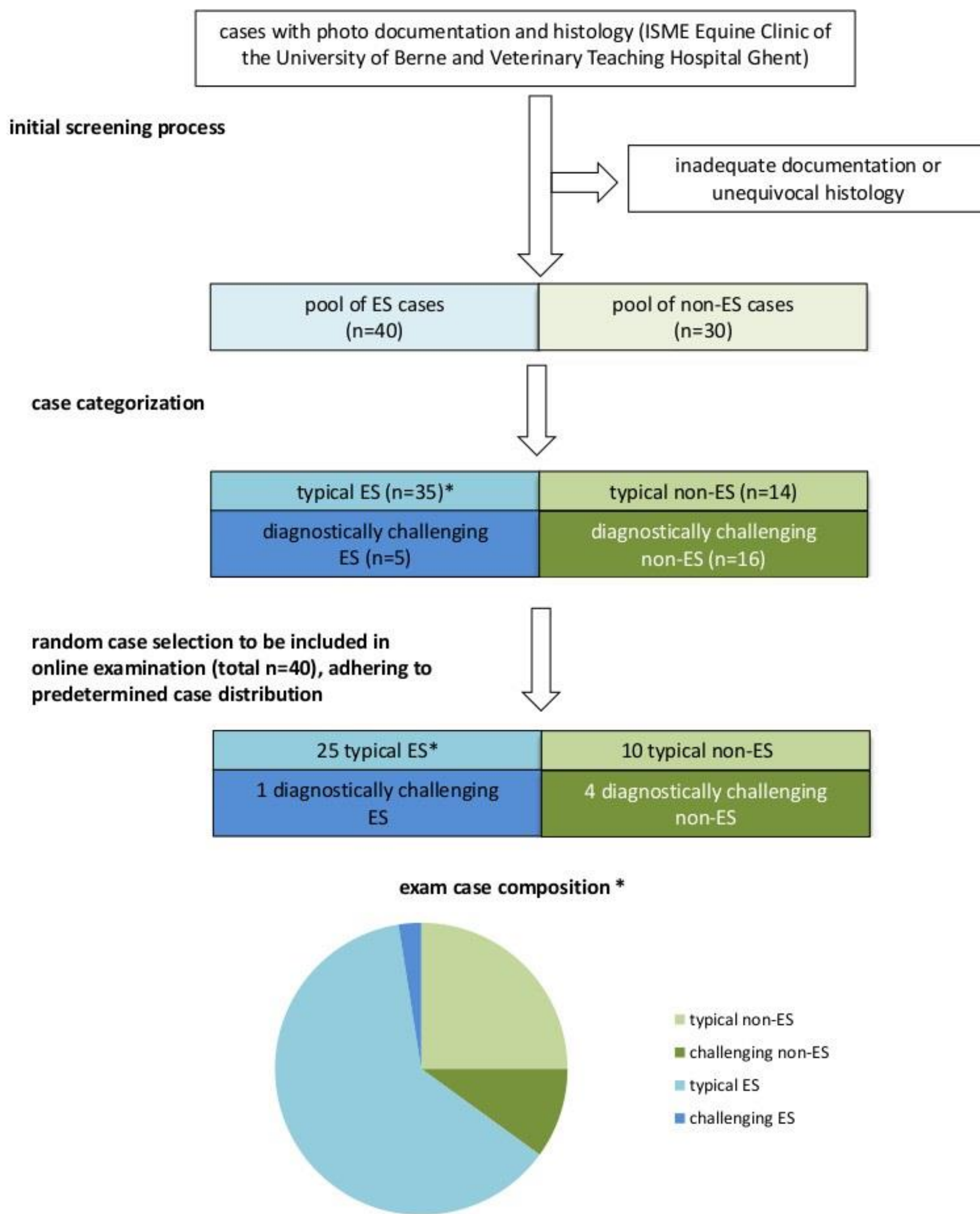

Figure 1 - Flow diagram of case selection and the resulting case composition of the online exam.

Statistical Analyses

Descriptive statistics and calculations of success rate, sensitivity, specificity, positive predictive value and negative predictive value were carried out in a spreadsheet (Excel 2013). Formal statistical analyses were carried out using commercially available software (SPSS 23).

To check whether there was a difference in ability to differentiate between equine sarcoid and non-equine sarcoid lesions between different expertise levels of respondents or between typical and diagnostically challenging lesions, a generalized estimating equations (GEE) model with a binomial error distribution and a logit link function was fitted. The ability to correctly differentiate equine sarcoids from other lesions (Yes/No) was used as dependent variable and the expertise level (equine sarcoid Expert/Board certified veterinarian/Practitioner/Novice) and lesion difficulty (typical/diagnostically challenging) were used as independent variables. Because there were more than 2 levels of expertise, the estimated marginal means for this variable were calculated and pairwise comparisons were carried out, applying a Bonferroni correction. A second GEE model with a multinomial error distribution and a cumulative logit link function was fitted to test if there was a difference in confidence between different expertise levels of the respondents or between typical and diagnostically challenging cases. Confidence level (1 to 6) was used as the dependent variable and expertise level (equine sarcoid Expert/Board certified veterinarian/Practitioner/Novice) and lesion difficulty (typical/diagnostically challenging) as the independent variables. To test if confidence levels were higher if a lesion was correctly diagnosed, a third GEE with a multinomial error distribution and a cumulative logit link function was fitted, using confidence level (1 to 6) as the dependent variable and the ability to correctly differentiate equine sarcoid from other lesions (Yes/No) as the independent variable. All models were corrected for the fact that the same respondents evaluated multiple cases and significance was set at $P \leq 0.05$.

Results

Case composition for the online examination

A total of 234 accessions to the histopathology diagnostic laboratory at the University of Berne were available for the preliminary analysis regarding the proportions of equine sarcoid and non-equine sarcoid cases to be included in the online examination (Figure 2). Based on this proportion analysis, a case composition of 66% (26/40) of equine sarcoid cases, and 34% (14/40) other cases was aimed for.

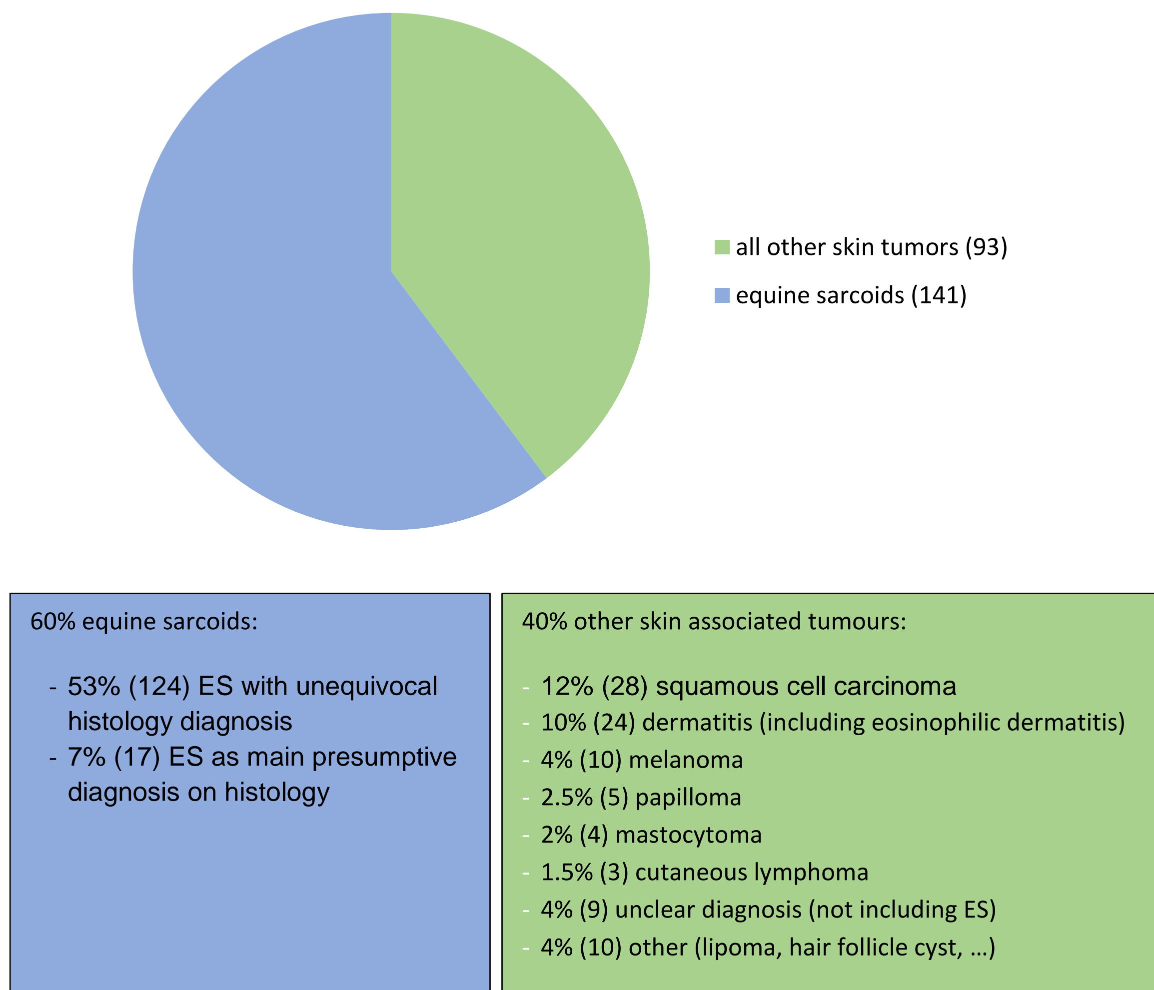

Figure 2 - Distribution of histology diagnoses from 234 accessions to the histopathology diagnostic laboratory at the University of Berne between January 2011 and September 2016

The preliminary analysis of histopathology submissions regarding the prevalence of diagnostically challenging cases, revealed a larger proportion of diagnostically challenging non-equine sarcoid cases (skin lesions that were clinically suspected as equine sarcoid, but histologically diagnosed as other lesions) compared to diagnostically challenging equine sarcoid cases (skin lesions that were not suspected to be equine sarcoid, but histologically diagnosed as equine sarcoid). Overall, 50/235 (21.3%) diagnostically challenging non-equine sarcoid cases were identified in all available data sets (5.6% (7/124) Berne; 28.6% (10/35) Vienna; 43.4% (33/76) Ghent). Here, it needs to be pointed out that the data from Ghent and Vienna is skewed by a strong selection bias, as only excised skin lesions with a doubtful clinical diagnosis (in Ghent 76 out of 600 clinical cases (12.7%)) were submitted for histology. The proportion of diagnostically challenging non-equine sarcoid cases identified at the University of Berne is likely the most representative, as it refers to a time period during which all excised and biopsied cutaneous masses were submitted for histology. Since February 2015, tumour tissue and case details including digital photographs of every case presented to the ISME Equine Clinic Berne for evaluation of a cutaneous mass are systematically archived in a tumour tissue bank. Therefore, it was deemed appropriate to include a proportion of 10% diagnostically challenging non-equine sarcoid cases in the online examination. Diagnostically challenging equine sarcoid cases, on the other hand, were identified in only 2/366 instances in 2 referral centres for which data was available over a 5-year period (0.8% (1/141) Berne; 0.4% (1/225) Ghent). Thus, only 1 diagnostically challenging equine sarcoid case was included in the online examination. The case selection process and proportions of case categories included in the online examination are shown in Figure 1.

Examination respondents

In total, 181 respondents completed the online examination between February 1st and April 15th, 2017. This included 156 veterinarians with more than one year of experience and active in equine practice in 17 different countries from the European, North American, and Australian continents. Furthermore, 4 recent graduates with less than one year of working experience and 21 veterinary students enrolled in the equine emphasis track and studying in their 2 final years at the University of Berne or Ghent. The overall response rate was 44.4%. Respondents from Switzerland and Belgium were overrepresented contributing 33.1% and 17.1% of respondents, respectively.

Fourteen respondents met the criteria of equine sarcoid experts, 39 respondents were board certified equine veterinarians, 103 were categorized as equine practitioners, and 25 as novices.

Examination results

Table 2 summarizes the success rates, sensitivity, specificity, positive and negative predictive value and mean level of confidence for clinical diagnosis of equine sarcoid for all respondents and separately for the different levels of expertise.

Table 2 - Success rate, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), and mean confidence levels for all respondents and for the different levels of expertise (SD = standard deviation; ES = equine sarcoid).

	Success rate (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Mean confidence (± SD)
Overall	82.0	83.3	79.6	88.4	72.0	4.24 (± 1.30)
ES experts	87.9	88.7	86.2	92.3	80.5	4.77 (± 1.19)
Board certified	81.7	82.5	80.2	88.6	71.2	4.25 (± 1.24)
Practitioners	82.0	82.9	80.4	88.7	71.7	4.25 (±1.28)
Novices	79.2	83.2	71.7	84.5	69.7	3.49 (± 1.31)

Statistical analysis revealed that equine sarcoid experts were significantly better at distinguishing between equine sarcoid and other lesions compared to board certified veterinarians (OR = 1.67; $P < 0.01$), equine practitioners (OR = 1.64; $P < 0.01$) and novices (OR = 1.99; $P < 0.001$). Other differences between different levels of expertise were not significant. Equine sarcoid experts were also more confident compared to board certified equine veterinarians (OR = 2.25; $P < 0.001$), equine practitioners (OR = 2.21; $P < 0.001$) and novices (OR = 6.30; $P < 0.001$). The cases with a typical morphology were significantly more likely to be assessed correctly (OR = 4.92; $P < 0.001$) then challenging cases, and respondents of all expertise levels also felt significantly more confident (OR = 1.78; $P < 0.001$) when assessing typical cases compared to challenging ones.

Equine sarcoid experts who correctly diagnosed a case were significantly more confident of their diagnosis compared to equine sarcoid experts who did not correctly diagnose the case (OR = 6.42; $P < 0.001$), but this was not the case for other levels of expertise and overall, there was no significant correlation between the ability to correctly differentiate equine sarcoid from other lesions and the level of confidence (OR = 4.79; $P = 1.00$).

Both overall and within each expertise level, the 3 features that were most reassuring that a skin lesions was actually an equine sarcoid were (1) multiple lesions in the same horse with typical equine sarcoid morphology (84.0% of respondents), (2) typical lesion morphology (80.1% of respondents), and (3) lesion localization (71.8% of respondents).

Discussion

The present study describes the development of an online examination to critically assess the diagnostic accuracy of the clinical diagnosis of equine sarcoid. The conceptual design aimed to elaborate an examination with a representative proportion of skin lesions that are possible differential diagnoses for equine sarcoid and special consideration was given to the diverse clinical aspects of the disease that may influence the reliability of the clinical diagnosis. Based on the examination results from 181 respondents, the clinical diagnosis of equine sarcoid is fairly reliable regardless of the level of expertise of the respondents, with an overall sensitivity and specificity of about 80% and positive predictive value of nearly 90% (Table 2).

Previous publications have mentioned that equine sarcoid diagnosis based on clinical assessment of the lesions should be easy (Wobeser et al., 2010) and that a strong agreement between clinical diagnosis and histopathology of equine sarcoid exists (Lazary et al., 1994; Jandova et al., 2012). Nevertheless, there is no record of specific research focusing on this topic and future research would benefit from insights in the accuracy of the clinical diagnosis (Compston et al., 2016). While histopathology still remains the gold standard for the diagnosis of sarcoids and other skin tumours and should remain the mainstay for diagnosis whenever possible, the high specificity, sensitivity and positive and negative predictive values found in this study indicate that the clinical diagnosis of equine sarcoid may indeed serve as a reliable alternative with

a predictive margin of error. This knowledge is useful in many clinical situations where biopsies are often avoided for practical or economic reasons as well as the fear of possible lesion aggravation (Knottenbelt, 2009). In research settings, taking biopsies and performing histopathology are often incompatible with practical, economic and ethical considerations (Berruex et al., 2016) or deliberately avoided for the risk of interference with the outcome of clinical or experimental trials (Haspeslagh et al., 2016a).

Although widely accepted as the gold standard of diagnosis, it has to be pointed out that histopathology also has its limitations in sarcoid diagnosis. Histopathological characteristics are neither consistent (Martens et al., 2000) nor absolutely specific (Epperson and Castleman, 2017) in all equine sarcoids and the diagnosis depends heavily on the expertise and experience of the pathologist (Taylor and Haldorson, 2013). A polymerase chain reaction (PCR) based screening for bovine papilloma virus (BPV) DNA may complement the diagnostic workup in some clinical settings (Martens et al., 2001).

One of the possible pitfalls of designing a case-based study to investigate the diagnostic accuracy of a clinical assessment is that the selected cases need to be representative of the clinical population. For this study, careful preliminary analyses of histopathological and clinical caseloads of 3 equine hospitals in 3 different countries ensured a representative ratio of equine sarcoid and other cases and of typical and diagnostically challenging cases. The determined proportion of equine sarcoid cases versus non-equine sarcoid cases (66% and 34%, respectively) was within the range of published equine sarcoid prevalence (35% - 90% of all cutaneous neoplasms) (Scott and Miller, 2011). For the classification of typical versus diagnostically challenging cases, a unanimous agreement had to be reached between the first authors, who carefully assessed each case, guided by a list of objective criteria. The analysis of the test results clearly shows that cases thereby classified as ‘typical’ yielded a significantly higher respondent confidence score then cases classified as diagnostically challenging. This finding confirms that the case classification was representative and suggests that the objective discriminatory criteria (Table 1) may be of use for clinical applications.

The ability to correctly discriminate equine sarcoids from other lesions was high for all respondents, but was significantly higher for respondents fulfilling the criteria of equine sarcoid experts compared to all other levels of expertise (board certified veterinarians, practitioners and novices). Equine sarcoid experts were also significantly more confident of their diagnosis compared to all other respondents. Moreover, only for the group of equine sarcoid experts, the level of confidence correlated with the correct differentiation of an equine sarcoid versus a non-equine sarcoid case. Essentially, this means that clinicians with less expertise are more likely to be erroneous in their judgment, despite a deceivingly high level of confidence. Therefore, we argue that it is feasible and potentially useful to develop a diagnostic tool to guide (less experienced) veterinarians with their clinical diagnosis and provide a directive feedback of which skin lesions are better subjected to a biopsy. Ideally, this diagnostic tool would bring the diagnostic abilities of inexperienced veterinarians to the level of equine sarcoid experts. The case features that were identified to discriminate typical equine sarcoids from clinically challenging lesions (Table 1) could serve as starting point to develop such a tool.

Several limitations of this study need to be pointed out. The initial case pool consists of cases seen at equine referral centres in Switzerland and Belgium. Thus, a potential selection bias for clinically challenging case material, skewed towards a higher proportion of aggressive skin tumours compared to first opinion practice needs to be considered. Furthermore, possible geographical differences in the characteristics of equine sarcoids that may exist between continental Europe and other regions of the world were not taken into account. The format of the examination only provides the test respondent with incomplete, filtered case information and impedes any interaction with the subjects, such as palpation of the lesions, or the possibility of asking the owner specific questions, for instance regarding pruritus or details about previous treatments. This may have negatively influenced the respondents' ability to correctly discriminate between equine sarcoids and other lesions.

In conclusion, the results of this study show that clinical diagnosis of equine sarcoids is reliable, especially when carried out by experienced observers. The clinical diagnosis of these tumours has an overall sensitivity and specificity of about 80% with a predictable margin of error. Less experienced veterinarians could benefit from a tool

to guide them in the correct diagnosis of skin lesions and help them decide for which cases diagnostic testing is highly recommended.

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The clinical diagnosis of equine sarcoids – Part II: validation of a decision protocol to guide equine clinicians in the diagnosis of equine sarcoids

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Summary

While histopathology remains the gold standard for equine sarcoid diagnosis, previous research has demonstrated that clinical diagnosis could be a valuable alternative. Nevertheless, less experienced veterinarians were not as good as sarcoid experts in discriminating sarcoids from non-sarcoid lesions. Therefore, and because of the lack of standardized clinical parameters for clinical sarcoid diagnosis, the aim of this study was to develop and test a diagnostic protocol (DP) which could be used to guide veterinarians in the diagnostic process. The DP was designed based on clinical parameters typical of equine sarcoids and then refined by repeated testing on different cases. To assess the functionality of the DP, it was given to experienced and inexperienced equine veterinarians and veterinary students to use on 40 standardized clinical sarcoid and non-sarcoid cases. The respondents were asked to clinically diagnose these cases as sarcoid or non-sarcoid lesions. The scores of respondents using the DP were then compared to those of respondents not using the DP. Overall, respondents using the DP were significantly more likely to correctly diagnose a case compared to respondents not using the DP and felt significantly more confident of their diagnosis. This was mostly because novice respondents performed better when using the DP. For more experienced practitioners, there was no significant effect of the DP. In conclusion, the DP proved to be a reliable tool to increase clinical diagnostic performance and user confidence, although some alterations can be made to improve its functionality even more. By systematically applying the DP, less lesions will need biopsies to be taken, diminishing the risk of lesion exacerbation.

Introduction

Histopathology remains the gold standard for the diagnosis of equine sarcoids. Nevertheless, taking biopsies of sarcoids may aggravate these lesions and is therefore better avoided (Knottenbelt and Kelly, 2000). Earlier findings indicate that the clinical diagnosis of equine sarcoid has an overall high sensitivity and specificity (chapter 4). Given the high index of suspicion and the supposed risk of lesion aggravation, many clinicians elect not to take biopsies to confirm the suspected diagnosis prior to initiating treatment. Arguably, this may represent the most reasonable approach in many cases where lesion morphology and other clinical features of equine sarcoid disease are typical. Nonetheless, the previous analysis revealed that even experienced equine practitioners still misdiagnosed 18.0% of clinical cases. Unsurprisingly, sarcoid experts were significantly more likely to correctly differentiate equine sarcoids from other skin lesions compared to less experienced respondents. Furthermore, novices and practitioners were significantly less confident of their diagnosis compared to experts in the field. This indicates that a diagnostic tool to aid in the differentiation between sarcoid and non-sarcoid lesions and guide less experienced veterinarians with their clinical diagnosis is desirable. Ideally, such a tool will increase the diagnostic accuracy of a clinician assessing a skin lesion and/or cutaneous mass in an equine subject, and guide the selection of lesions with less typical clinical features, for which a biopsy and histology is indicated.

In order to design such a diagnostic tool, unmistakably identifiable case features that are characteristic and at the same time exclusive to a particular condition need to be defined. Unfortunately, such pathognomonic clinical signs are not readily identified for equine sarcoids, a condition with a highly heterogeneous clinical presentation (Knottenbelt, 2005). However, although none are exclusive to equine sarcoids, certain case features are typical for sarcoid cases and very commonly associated with this complex condition. These features include lesion morphology (Knottenbelt, 2005), the presence of multiple, similar lesions in different locations in the same horse, the notorious recurrence after treatment (Scott and Miller, 2011), growth spurts (Wobeser, 2017) and being present in typical localizations (Torrontegui and Reid, 1994). All of these criteria were previously used for the triage of cases in typical and diagnostically challenging cases and some were identified as being important for clinical sarcoid diagnosis by a panel of respondents (chapter 4).

The working hypothesis was that the systematic review and weighting of these criteria, along with the animal's demographic information would increase the discriminatory capacity of less experienced practitioners to correctly differentiate between sarcoid and non-sarcoid lesions. Furthermore, it was hypothesized that the use of a clinically applicable diagnostic tool would improve the level of confidence with which observers make their decision. The objective of the present study was to develop and validate a diagnostic protocol (DP) to guide clinicians in diagnosing equine sarcoids and to select potentially challenging cases for which taking a biopsy is highly advisable to confirm or rule out the differential diagnosis of equine sarcoid.

Materials and Methods

Development of the diagnostic protocol

Case features typical for sarcoids regarding the subject's particulars, medical history, number of lesions compatible with typical sarcoid morphologies, lesion localization and morphology were listed. A weighting coefficient was subjectively assigned to each of these case features to reflect the importance of the given feature in the overall diagnostic decision. This resulted in a preliminary DP that would produce a high score for lesions that were likely equine sarcoids and a low score for lesions that probably were not. This preliminary DP was then tested on all of the 73 histologically confirmed sarcoid and non-sarcoid cases that were pooled for the previous study (chapter 4) and clinical cases presented to the equine referral hospitals of the Universities of Berne and Ghent during the months of December 2016 – February 2017. Based on the resulting total scores, cut-off values were determined indicating either a high probability of equine sarcoid, a low probability of equine sarcoid or a grey area where further diagnostic techniques (biopsy or PCR) are recommended. The DP was then generated in an active Excel spreadsheet to automatically calculate the total score and advise the user of the diagnostic decision (Table 1).

Table 1 - The diagnostic protocol used in this study, filled out for a fictional case. High final scores indicate a high likelihood of equine sarcoid while low final scores indicate a low likelihood of equine sarcoid. In this example, the final score of 10 indicates that further diagnostics (histopathology or PCR) are recommended.

		Score if positive	**Case score**
1. age			
< 1 year old		-4	0
2 - 7 years old		2	**2**
7 - 17 years old		1	0
18 years or older		0	0
weighted subscore (weighting coefficient = 2)			**4**
2. history			
no information	(also: slow continous growth)	1	0
recurrence	after therapy that was (at least temporarly) successful in reducing the lesion	3	0
growth behaviour	rapid growth (spurts) observed	2	**2**
	growth triggered by wounding	2	0
	changes in morphology over time	4	**4**
weighted subscore (weighting coefficient = 1)			**6**
3. number of lesions			
solitary	no other lesions described/found	1	**1**
multiple	2 - 10 lesions with similar (sarcoid-typical) morphology	2	0
	> 10 lesions with similar (sarcoid-typical) morphology	3	0
weighted subscore (weighting coefficient = 2)			**2**

Table 1 (continued) – The diagnostic protocol used in this study

4. localisation			
typical localisation of sarcoids	periocular or auricular	2	0
	lip or cheek	1	0
	neck: atlas/parotid region, jugular groove, lower neck	1	0
	chest, axilla, antebrachium, shoulder	2	0
	girth and ventrum near midline	2	0
	inguinal region and inside of the thigh, fold of the knee	3	0
	prepuce or teats/scrotum (but not shaft of the penis or glans)	3	0
	fetlock or pastern	1	0
atypical localisation	back, saddle region, perineum, muco-cutaneous junctions, penile shaft, glans or clitoris, tail, crest of the neck,…	-2	**-2**
weighted subscore (weighting coefficient = 2)			**-4**
5. lesion morphology			
occult	flat ("thin-skinned"), circular, hairless lesion with hyperkeratotic surface and small (2mm) granules	2	0
verrucose	wart-like, raised hairless cutaneous mass with variable hyperkeratosis	2	0
nodular	spherical, firm subcutaneous or cutaneous mass	1	**1**
fibroblastic	fleshy (pedunculated or sessile) proliferation with an ulcerated surface	1	0
mixed	more than one morphological type (occult, verrucose, nodular, fibroblastic) is present, without one type clearly predominating	3	0
atypical ulcerated	predominantly destructive/ulcerative process or chronic wound healing by second intention with finely granulated surface	-2	0
weighted score (weighting coefficient = 2)			**2**
		total score	**10**
Biopsy highly recommended (5 > score < 15)			
very likely equine sarcoid--> total score = or > 15			
very likely not equine sarcoid --> total score = or < 5			

Online examination to assess inter-observer agreement

To ensure that the DP can be applied correctly and consistently, an online examination was conducted to test the inter-observer agreement. Ten cases were selected from a pool of 40 sarcoid and 30 non-sarcoid cases (chapter 4). It was ensured that both for sarcoid and non-sarcoid lesions, the entire spectrum of scores as determined for each case by the developers of the DP (M.H. and C.K.) was represented in the exam. For each case, photographs of the lesion and a brief case description in a standardized format were provided and made accessible online through the following link: https://goo.gl/forms/2j6JD4npXi2IAVPx2.

Potential respondents had never been exposed to the DP before and were contacted by email and kindly asked to participate in the online examination. A copy of the DP in form of an automated Excel spreadsheet and supplementary illustrations depicting typical sarcoid morphologies and localisations (supplementary items 1 and 2) were attached to the invitation email. Furthermore, guidelines on how to correctly use the DP were provided and the study objectives were clearly disclosed so that all candidates were informed that results of the exam would be subjected to peer-review and publication in an anonymised format. For each case, respondents had to fill in the exact total score obtained by using the DP.

Online examination to assess the clinical value of the decision protocol

To assess the potential clinical value of the DP, an online examination designed to evaluate the clinical diagnosis of equine sarcoid was conducted as previously described in detail (chapter 4). Briefly, this online examination consisted of 40 sarcoid and non-sarcoid cases out of a case pool of well-documented skin lesions with histologically confirmed diagnoses. The proportion of typical and diagnostically challenging cases (both sarcoid and non-sarcoid) aimed to reflect the distribution of a clinical population. Again, photographs of the lesion and a brief case description in a standardized format were provided for each case. The online examination was made accessible through the following link: https://goo.gl/forms/I0iGWYq0mDbOrrZ83.

Candidates were grouped as (1) “sarcoid expert”, if they had previously published on the topic of equine sarcoid in peer-reviewed journals and had at least 2 years of clinical experience, as (2) “equine practitioners”, if they had at least 1 year of clinical

experience in equine practice, or as (3) "novices", if they had less clinical experience, including veterinary medicine students (in their two final years of studies and tracking with an equine emphasis) and recent graduates.

Potential respondents were contacted by email and kindly asked to participate in the online examination. Every other potential respondent in the randomly generated list of candidates for the group of "novices" and the group of "equine practitioners" was provided with the DP, including the appertaining illustrations of typical sarcoid morphologies and localizations. The invitational email also included the guidelines on how to use the DP and it was explained that the DP was intended to help complete the online examination successfully and that it only served as a guideline, not overruling their personal decision. Furthermore, the study objectives were clearly disclosed and all candidates were informed that results of the exam would be subjected to peer-review and publication in an anonymised format.

For each case, respondents had to select whether they thought the lesion was an equine sarcoid or not and indicate how confident they were of this clinical diagnosis (on a scale of 1 to 6).

Once a respondent had filled out all required fields of the online examination, the responses were automatically recorded in an Excel-spreadsheet. Results of respondents using the DP were then compared to results of their peers not using the DP and to results of a group of sarcoid experts, which were obtained in the previous study (chapter 4).

Statistical Analyses

Descriptive statistics were performed in a spreadsheet (Excel 2013). Formal statistical tests were carried out using commercially available software (SPSS 23). To assess the inter-observer agreement, the intraclass correlation coefficient (ICC) was calculated using a two way random model testing for absolute agreement. Normality of the data was assessed using probability-probability plots. To assess whether the use of the DP had a significant effect on the ability to correctly diagnose a case, a generalized estimating equations (GEE) procedure was used with a binomial error distribution and a logit link function. The ability to correctly diagnose the lesion (Yes/No) was used as the dependent variable and whether the respondent used the DP

(Yes/No) as the independent variable. Estimated marginal means were calculated and pairwise comparisons between groups (Expert, practitioner with and without DP, novice with and without DP) were carried out. To find out whether the use of the DP had a significant effect on the confidence of the respondents, a GEE with a multinomial error distribution and a cumulative logit link function was carried out, using the confidence level (1 to 6) as the dependent variable and whether the respondent used the DP (Yes/No) as the independent variable. All models were corrected for the fact that the same respondent scored multiple cases and when multiple comparisons were carried out, a Bonferroni correction was applied. Statistical significance was set at $P \leq 0.05$.

Results

The test to assess the inter-observer agreement was completed by 55 respondents (response rate 42.0%). The single measures ICC was 0.78 (95% confidence interval: 0.62-0.92).

The online examination to assess the clinical value of the DP was completed by 195 respondents. Of the 195, 53 respondents (27.2%) used the DP as a practical tool to help in the decision making process of the online examination (response rate = 17.5%). Of these 53 respondents, 22 were practitioners with at least 1 year of working experience and 31 were novices. The results of these respondents were compared to those of the 142 respondents who completed the online examination without the help of a DP as described in chapter 4 (response rate 41.2 %). Of these 142 respondents, 103 were equine practitioners, 25 were categorized as novices and 14 respondents met the criteria of sarcoid experts (chapter 4). The success rate, sensitivity, specificity, positive and negative predictive value, and mean confidence level for the different groups of respondents with and without DP are listed in Table 2.

Table 2 - Success rate, mean confidence, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the different groups of respondents (SD = standard deviation; DP = diagnostic protocol).

	Novices		**Practitioners**		**ES experts**
	Without DP (n=25)	With DP (n=31)	Without DP (n=103)	With DP (n=22)	Without DP (n=14)
Success rate (%)	79.2	85.7	82.0	83.0	87.9
Mean confidence (± SD)	3.49 (± 1.31)	4.37 (± 1.27)	4.25 (± 1.28)	4.45 (± 1.30)	4.77 (± 1.19)
Sensitivity (%)	83.2	90.4	82.9	87.2	88.7
Specificity (%)	71.7	77.0	80.4	75.0	86.2
PPV (%)	84.5	87.9	88.7	86.6	92.3
NPV (%)	69.7	81.3	71.7	76.0	80.5

Overall, the odds for respondents using the DP to correctly diagnose a case were 1.2 times higher compared to those for their peers not using the DP (OR=1.25; P=0.002). Novices using the DP were significantly more likely to correctly diagnose a case compared to novices not using the DP (OR=1.58; P=0.001) and practitioners not using the DP (OR=1.32; P=0.04), and there was no significant difference compared to sarcoid experts. Practitioners using the DP were not significantly more likely to correctly diagnose a case compared to practitioners not using the DP (OR=1.07; P=1.0) or sarcoid experts (OR=0.67; P=0.18).

Respondents using the DP were 1.5 times more confident of their diagnosis compared to their peers who did not have access to the DP (OR=1.53; P<0.001). Novices using the DP were significantly more confident of their diagnosis compared to novices without DP (OR=3.34; P<0.001) and practitioners without DP (OR=1.19; P=0.002), but were significantly less confident compared to sarcoid experts (OR=0.54; P<0.001). Similarly, practitioners using the DP were significantly more confident compared to practitioners without DP (OR=1.37; P<0.001) and novices without DP (OR=3.85; P<0.001), but significantly less confident compared to sarcoid experts (OR=0.62; P<0.001). There was no significant difference in confidence between novices who used the DP and practitioners who used the DP.

Discussion

This is the first study to establish and test a DP to aid veterinarians and veterinarians in training with clinical sarcoid diagnosis. Similar score systems, as for example the modified sepsis score for equine neonates (Brewer et al., 1988) , are routinely being used in veterinary medicine.

The ICC was good to excellent, indicating that the scores that were obtained when using the DP were consistent between respondents. While there is certainly room for improvement, the results indicate that by using the DP, all respondents became more confident of their diagnosis. Novices using the DP were better at discriminating sarcoids from non-sarcoid lesions compared to peers without DP and the success rates of their diagnostic decisions were statistically indistinguishable from those of sarcoid experts. In contrast, using the DP did not lead to better diagnostic decision in more experienced equine practitioners. A possible explanation is that these respondents may have adhered or referred less frequently to the DP for making their decision, but this suspicion could not be verified.

Interestingly, using the DP mainly increased the sensitivity (Table 2). The specificity decreased when equine practitioners used the DP, but remained above 75% for all respondents using the DP (Table 2). Potentially, this can be adjusted by altering the weighting coefficients or the importance of certain characteristics in the total score. Furthermore, it was considered to include additional parameters to further improve the discriminatory accuracy of the DP. For example, special breeds and/or coat colors could be taken into consideration, as it has been shown that Haflingers (Lassaline et al., 2015) or pale-skinned horses like Appaloosa or Paint horses (Schaffer et al., 2013) are more likely to develop squamous cell carcinomas, and grey horses are more likely to develop melanomas (Phillips and Lembcke, 2013). On the other hand, none of these horses are less prone to ES compared to other breeds or coat colors, so breed and coat color may not be very good discriminatory factors, and it was elected not to use these in the DP.

Respondents using the DP did not only receive the DP, but also additional illustrations of typical sarcoid morphologies and locations. Therefore, the increased ability of respondents using the DP to correctly differentiate equine sarcoids from other lesions could also be due to a learning effect, induced by providing them with a correct

theoretical background on sarcoid characteristics of which they possibly were unaware before.

The DP, as used here, suggests that taking a biopsy of the lesion is highly recommended if the final score falls within a certain range. PCR techniques on swabs are a reliable non-invasive alternative for histopathology, especially on lesions with ulcerated skin (Martens et al., 2001). This could be implemented in the DP by suggesting PCR tests to be carried out on cases that yield a final score within a high range, but not high enough to pass the threshold of 15 points. By doing so, suspected sarcoid cases will be further examined by a non-invasive technique first, avoiding biopsies and possible lesion aggravation.

In conclusion, the routine use of the suggested DP by less experienced veterinarians will lead to a better and more confident clinical diagnosis, which in turn will result in less biopsies and thus decrease the risk of lesion exacerbation. The DP may still be improved by adjusting the weighting coefficients or adding new useful parameters, as more experience is gained with its application in equine practice.

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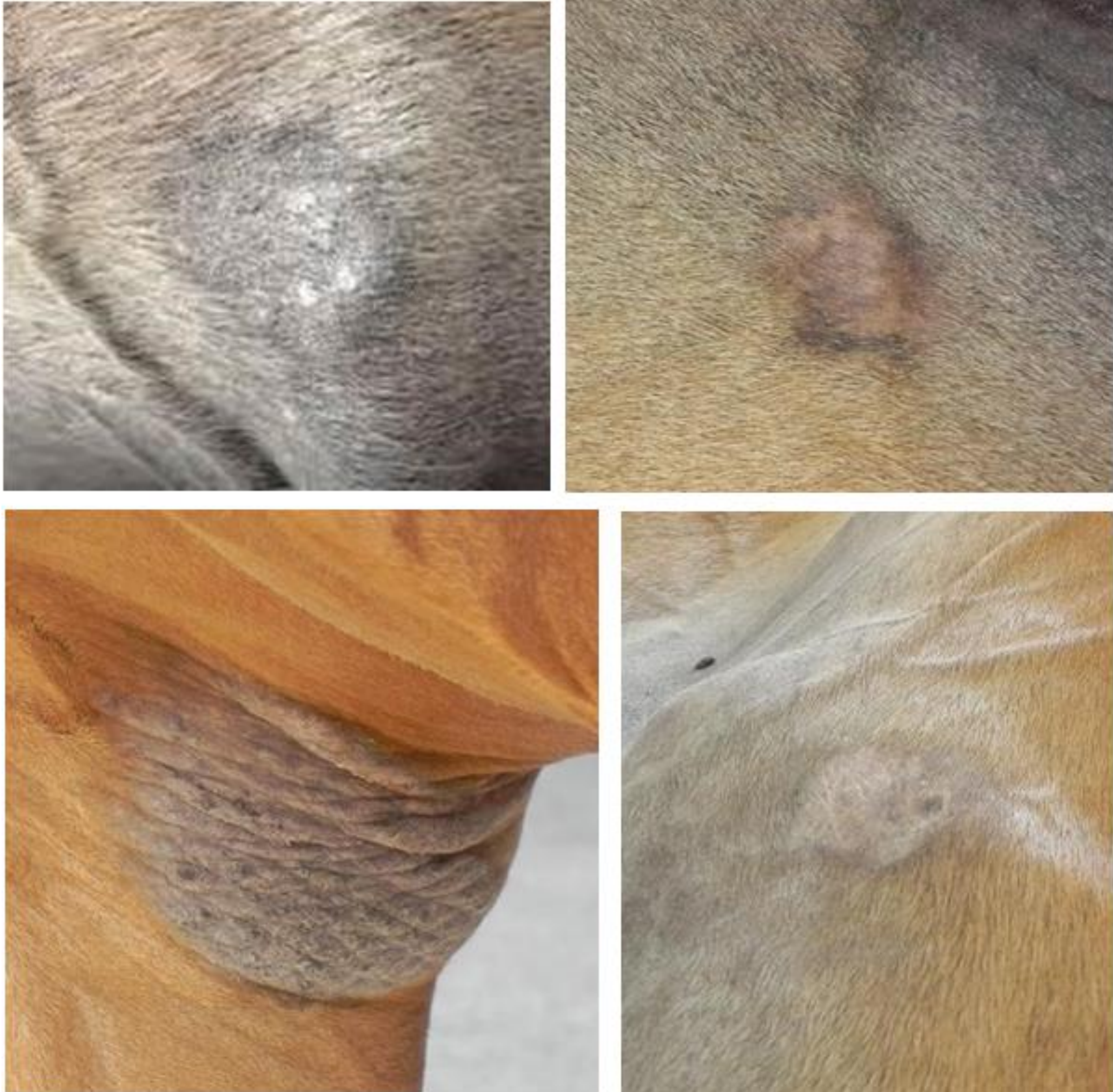
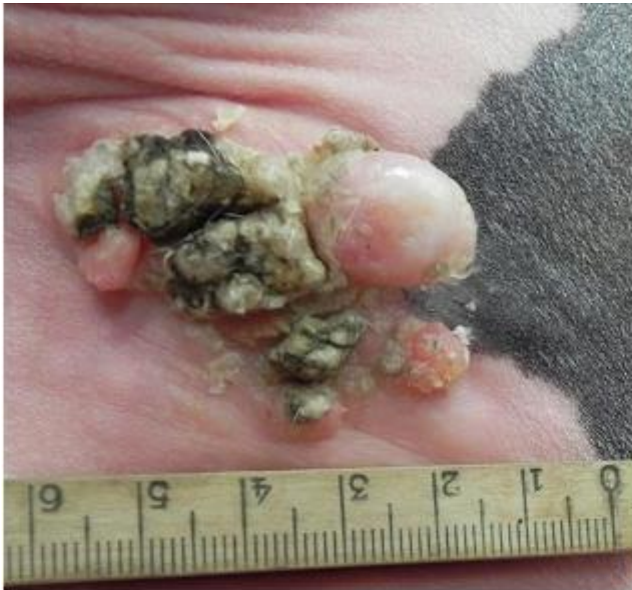
Suplementary item 1 - Examples of typical equine sarcoid morphologies which were included with the diagnostic protocol.

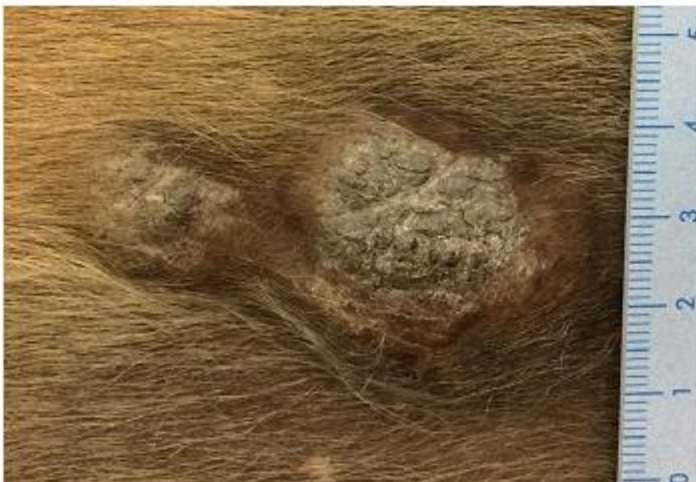
Occult equine sarcoids

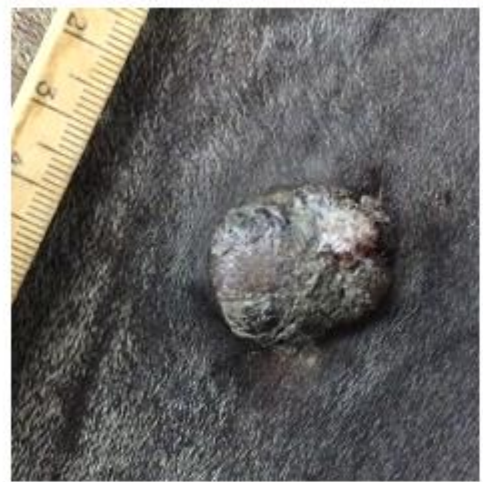


Suplementary item 1 (continued)

Verrucous equine sarcoids

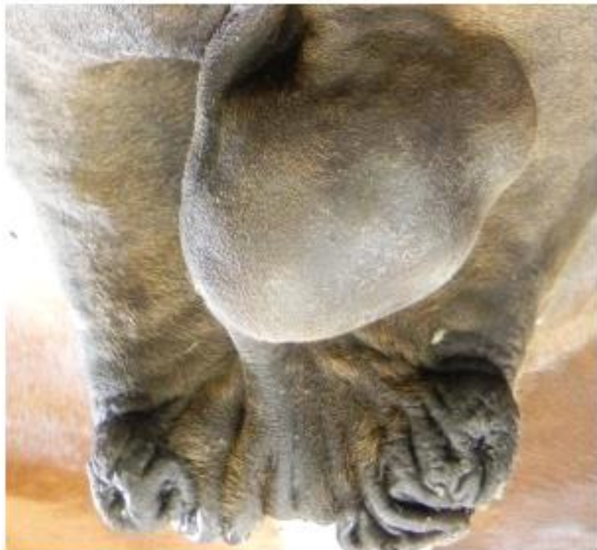
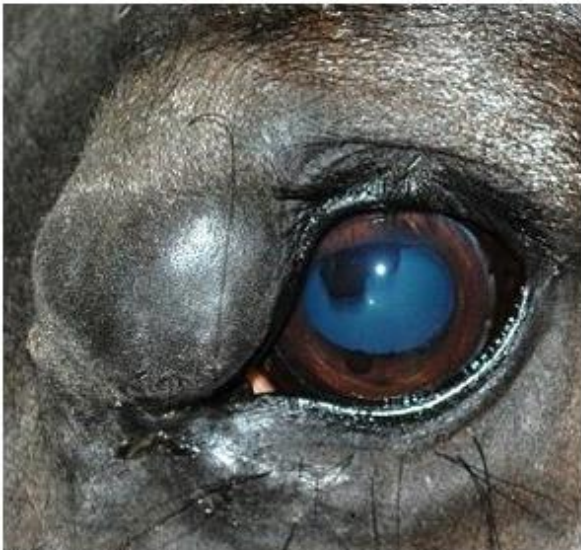
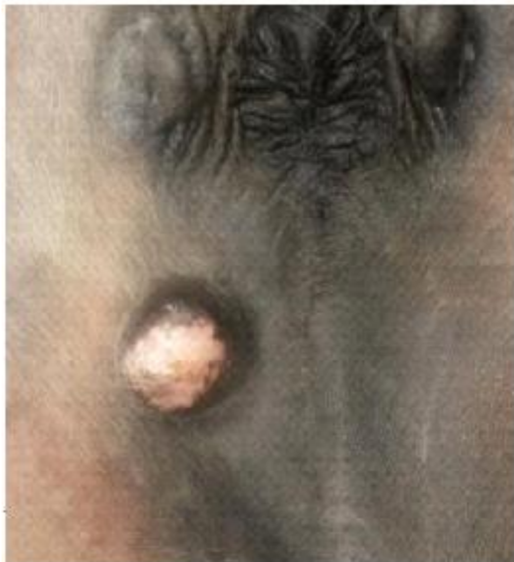
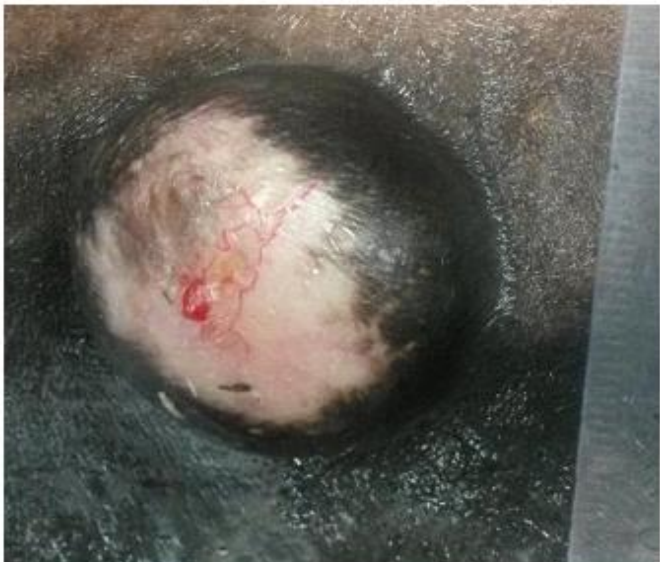






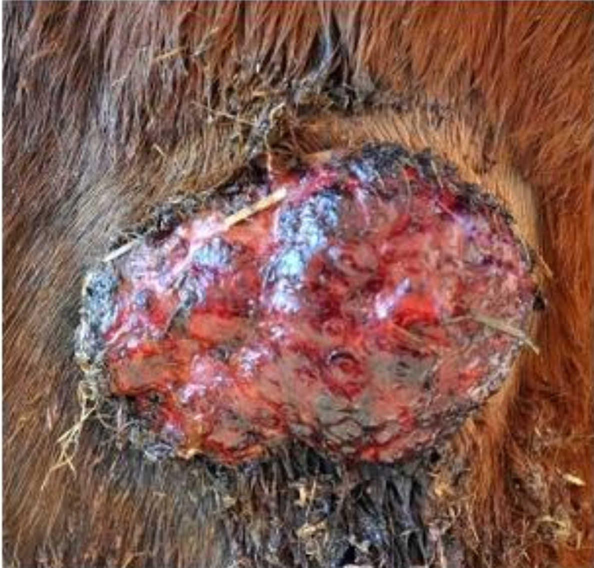
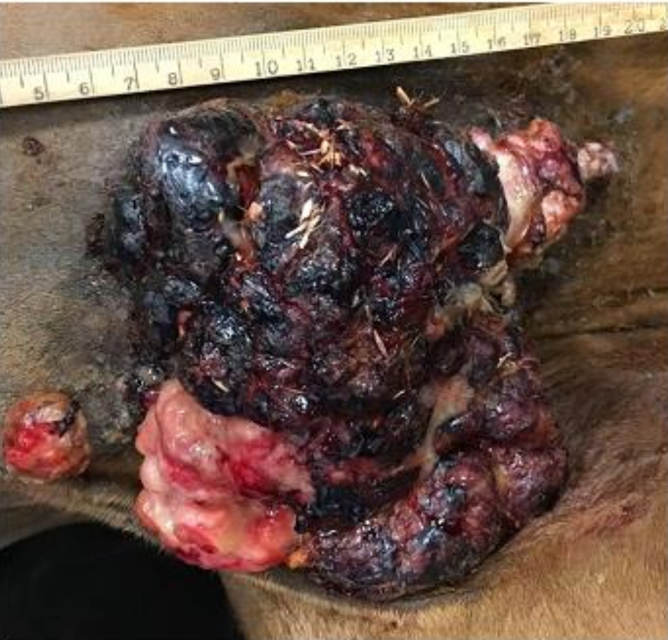
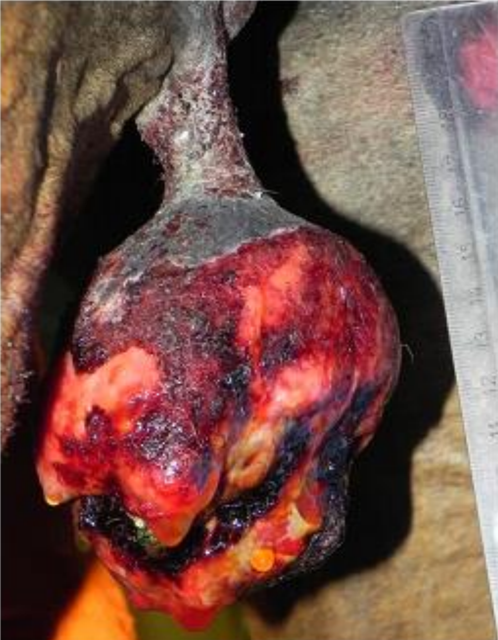
Suplementary item 1 (continued)

Nodular equine sarcoids

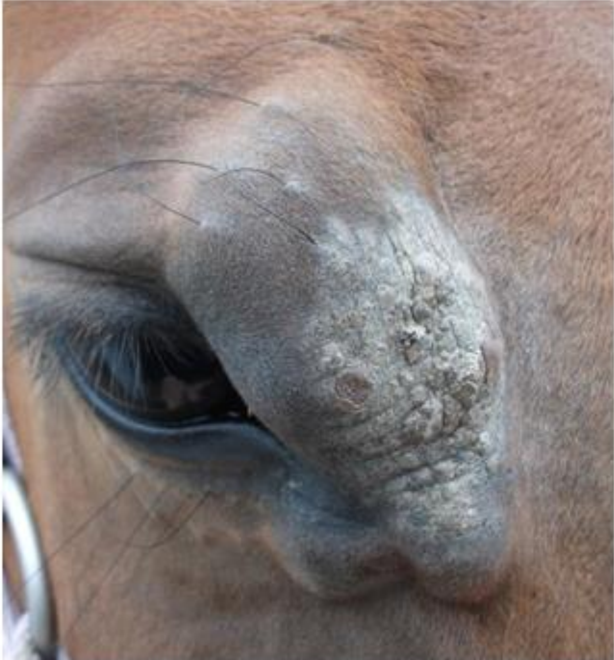
Suplementary item 1 (continued)

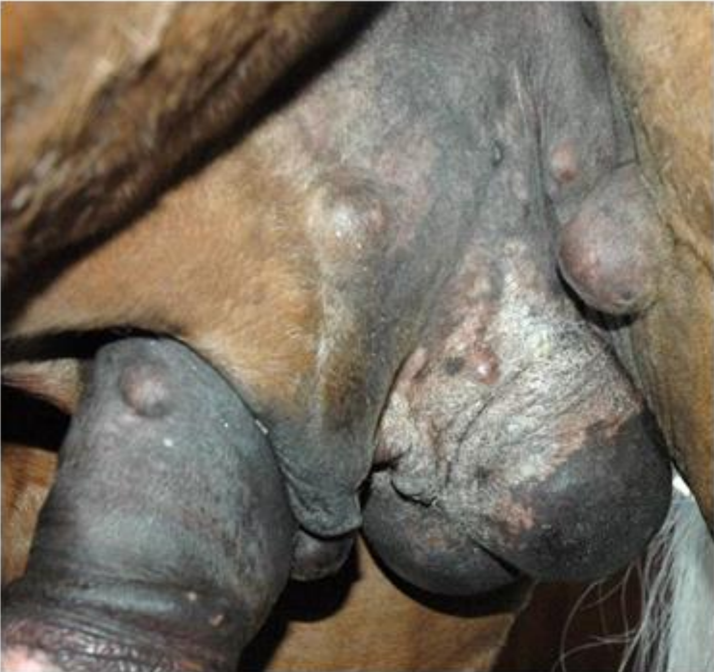
Fibroblastic equine sarcoids

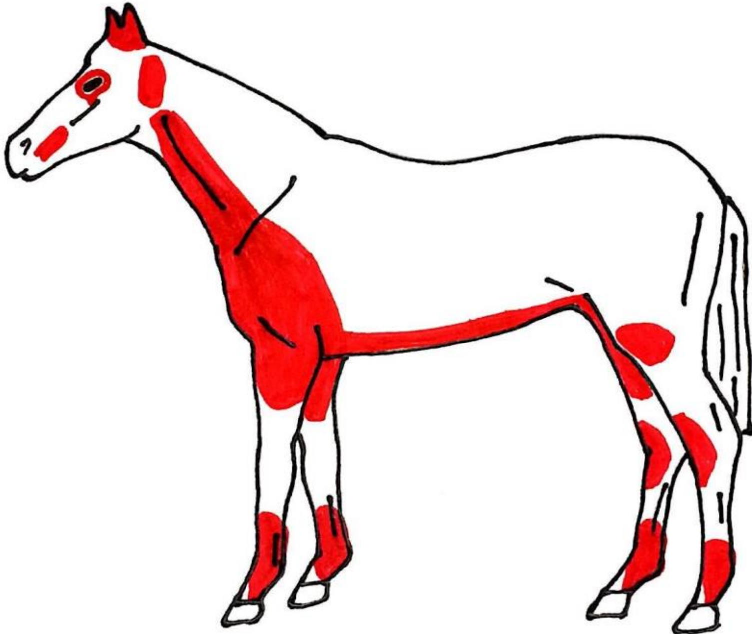
Suplementary item 1 (continued)

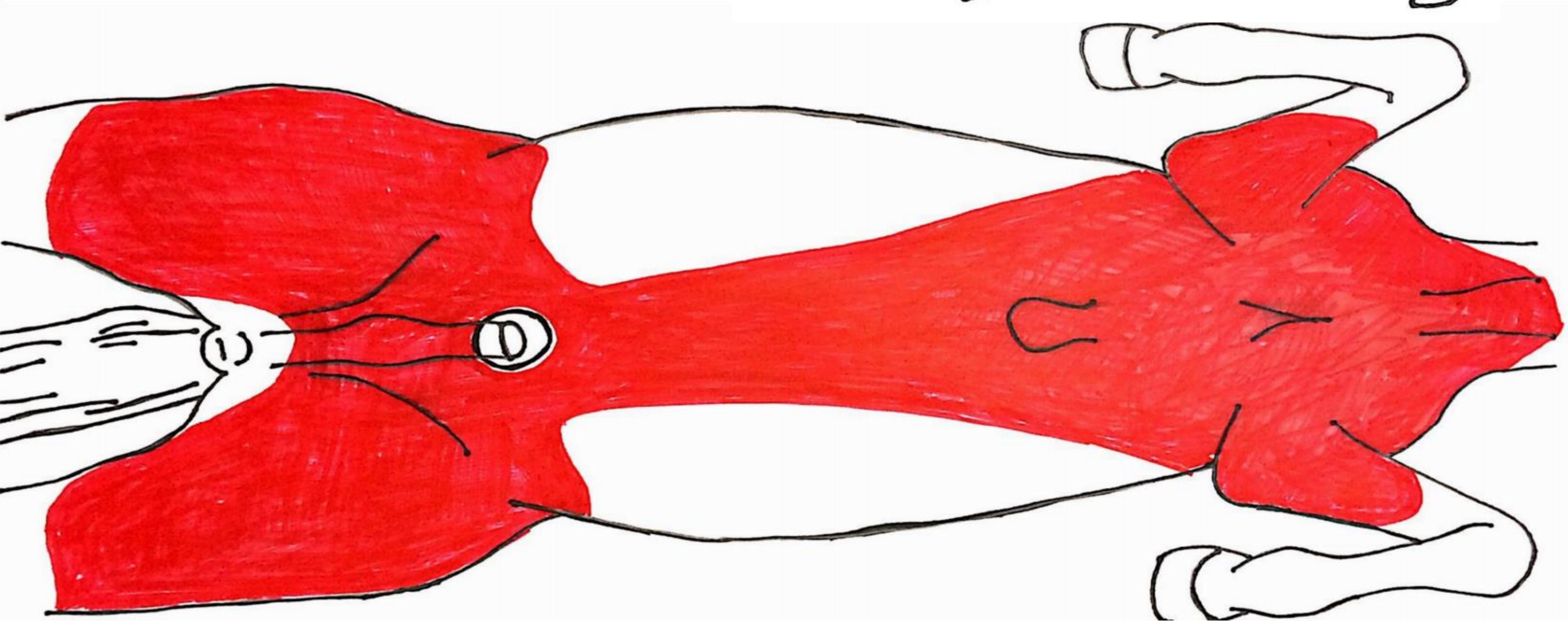
Mixed equine sarcoids





Supplementary item 2 – typical equine sarcoid localisations indicated in red, as included with the diagnostic protocol.





Treatment of sarcoids in equids: 230 cases (2008–2013)

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Adapted from:

Haspeslagh M., Vlaminck L. and Martens A. Treatment of sarcoids in equids: 230 cases (2008-2013) (2016). Journal of the American Veterinary Medical Association 249, 311-318.

Results of this study were presented at the 24th ECVS annual scientific meeting, Berlin, July 2-4 2015.

Summary

The objective of this study was to evaluate outcomes following treatment of sarcoids in equids and to identify risk factors for treatment failure in these patients. A retrospective case series was performed on 230 equids with 614 sarcoids that had been treated according to a standardized treatment decision protocol (electrosurgery, electrosurgery with intralesional placement of cisplatin-containing beads, topical administration of imiquimod or acyclovir, cryosurgery, Bacillus Calmette-Guerin vaccine injection, or intralesional injection of platinum-containing drugs) between 2008 and 2013. Data regarding animal, tumour, treatment, and outcome variables were collected. Complete tumour regression without recurrence for ≥ 6 months was considered a successful outcome. Success rates were calculated; binary logistic regression analysis was used to identify risk factors for treatment failure and to compare effects of the 2 topical treatments. A $\chi 2$ test was used to compare effects of the number of Bacillus Calmette-Guerin vaccine or cisplatin-containing drug injections on outcome. The overall success rate was 460 of 614 (74.9%). Electrosurgical excision resulted in the highest treatment success rate (277/319 [86.8%]); odds of treatment failure were significantly greater for intralesional injections of platinum-containing drugs, cryosurgery, and topical acyclovir treatment. Odds of treatment failure were also significantly greater for sarcoids on equids with multiple tumours than for solitary lesions and significantly lower for sarcoids on equids that received concurrent immunostimulating treatment for another sarcoid than for patients that did not receive such treatment. While selection bias for treatments was inherent to the study design, results may assist clinicians in selecting treatments and in determining prognosis for equids with sarcoids treated according to the described methods.

Introduction

Sarcoids are locally aggressive, nonmetastatic tumours of the skin in equids (Pascoe and Knottenbelt, 1999). Bovine papillomavirus types 1 and 2 are causally associated with sarcoid development in equids (Chambers et al., 2003) but the environment and genetics of animals also have important roles. Sarcoids are the most common skin tumours in horses (Ragland, 1970), and many treatments have been developed (Taylor and Haldorson, 2013). However, no specific treatment is currently considered the standard of care, and therapy must be tailored according to characteristics of the patient and tumour, treatment availability, and owner preference. Because of the heterogeneity in tumour features and treatments used, determining an accurate prognosis is difficult.

Most studies on sarcoid treatments in equids have focused on 1 or 2 selected treatments for specific sarcoid types, locations, or both, which makes it hard to estimate overall success rates for treatment of sarcoids. Reports describing a systematic approach to treatment selection are scarce. Diehl et al. (Diehl et al., 1987) published a report of 117 horses that were treated for sarcoids by means of CO_2 laser surgery, cryosurgery, conventional excision, radiotherapy, or a combination of laser surgery and radiotherapy. The overall success rate was 86 of 117 (73.5%) horses, and the highest success rate was for laser surgery (48/59 horses [81.4%]). However, the number of horses was very low (< 15) for 3 treatment groups, and no specific information was included on how treatment was selected. Our research group previously described the use of 4 different treatments (conventional excision, CO_2 laser excision, BCG vaccination, and cryosurgery) for 197 sarcoids in 95 horses (Martens et al., 2001b). In that study, the overall success rate was 154 of 186 (82.8%) and the best results were also obtained with laser surgery. In that report (Martens et al., 2001b), a defined, systematic approach to treatment selection on the basis of tumour characteristics was described, but some treatment options (eg, use of topically applied medications) were not included.

The objective of the study reported here was to retrospectively evaluate outcomes following treatment of sarcoids in equids at the authors' institution, where treatments

were selected according to a standardized protocol. We also aimed to identify risk factors for treatment failure in these patients.

Materials and Methods

Case selection

Electronic medical records of the surgery department at Ghent University were searched to identify equids treated for clinically diagnosed sarcoids between the first of January, 2008, and June 30, 2013. The minimal follow-up period for inclusion in the study was 6 months after the date of the last treatment. No equid that met these criteria and had treatment information available was excluded.

Medical records review

Data collected included breed or breed type, age at treatment, and sex of the patient; anatomic location, size and type of sarcoids (occult, nodular, verrucous, fibroblastic, or mixed)(Knottenbelt, 2005); presence and number of multiple tumours if applicable, whether sarcoids had been previously diagnosed and had recurred at the same site; and whether ulceration was present. The treatment selected, number of treatments, complications (when reported), and outcomes were recorded. When an equid underwent ≥ 1 treatment type for different tumours, this information was also collected, and the treatment types were assessed for potential immunostimulatory effects. Major complications were defined as complications being substantially hindering or painful to the horse or delaying healing after treatment. Full regression was defined as complete disappearance of the tumour with formation of normal skin, and partial regression was defined as a decrease in size, without complete disappearance; lack of change in tumour size or an increase in size was deemed a nonresponse to treatment. Only full regression without evidence of recurrence at follow-up evaluation ≥ 6 months after treatment was considered to be a success; partial regression and nonresponse to treatment were considered treatment failure. Most information was collected from the medical records. Owners were contacted by phone for a long-term follow-up.

Treatments

During the study period, the following protocol was used to determine treatment choice.

Sarcoids were excised by use of an electrosurgical scalpel (with or without placement of cisplatin-containing beads) whenever well-defined edges were present and the size and location allowed removal of a wide margin of normal skin and primary wound closure. Occult or slightly verrucous sarcoids that were not excised were treated topically with acyclovir cream or imiquimod cream; after explaining the differences in price, possible side effects, and application frequency for each product, treatment choice was at the owner's discretion. Tumours that were ill-defined, inconveniently located for excision, or strongly attached to deeper tissues were treated by cryosurgery when the surgeon anticipated no risk of damaging underlying vital structures. Those that were unamenable for excision or cryosurgery owing to size or location were treated by means of local (intralesional) chemotherapy with cisplatin- or carboplatin-containing sterile sesame oil, except that periocular tumours were treated by intralesional BCG vaccine administration. Some patients underwent treatment by excision, intralesional chemotherapy, or topical acyclovir administration for 1 lesion, whereas another tumour on the same animal was treated by BCG vaccine injection, cryosurgery, or topical imiquimod application. The latter 3 treatment types were considered to have some degree of general immunostimulating effects that could influence results for the concurrently treated tumour. Protocols for sedation, general anaesthesia, and analgesic and antimicrobial administration were at the discretion of the attending surgeon and anaesthesiologist.

Electrosurgical excision—All excisions were performed under general anaesthesia. Direct tumour contact was avoided during the skin preparation protocol and a perimeter of ≥ 12 mm of apparently normal skin (Martens et al., 2001a) around the tumour edge was marked with a sterile pen. Nodules or paler skin detected around the tumour were included within the excision margins. The monopolar electroscalpel (ICC 300, ERBE) was used at a cutting setting of 120 Watts. A non-touch excision technique was used to avoid iatrogenic spread of tumour cells. Following tumour excision, gloves and instruments were changed, and the wound was rinsed with a 0.05% chlorhexidine solution and closed in 2 layers with size 1 polyglactin 910. Excessive tension on wound

edges was relieved by placement of additional stented sutures in a horizontal mattress pattern. Until May, 2012, cisplatin-containing beads (Matrix III cisplatin beads, Royer Biomedical) (1.6 mg/bead at 2 cm intervals) were placed in the wound bed prior to closure for patients with recurrent tumours or for those in which tumour margins were subjectively difficult to identify (Hewes and Sullins, 2006). After this time, the beads were no longer available, and the adjunctive chemotherapeutic treatment of these patients was discontinued. Sarcoids treated by the combination of excision and cisplatin-containing bead placement were analysed as a separate treatment group.

Topical treatments—When selected, a 5% acyclovir cetomacrogol cream (generic preparation) was applied every 12 hours (Stadler et al., 2011) until full tumour regression occurred or until no changes could be seen after 2 months of treatment. The alternate treatment of 5% imiquimod cream (Aldara, 3m) was applied 3 times/week after washing the skin with a mild soap solution. The imiquimod cream was left on for 8 hours and then washed off to avoid excessive skin irritation, and the treatment was performed until full tumour regression or until no changes could be seen after ≥ 3 weeks of treatment (Nogueira et al., 2006).

Cryosurgery—Sarcoids were surgically reduced to the level of the surrounding skin with an electrosurgical instrument in patients under general anaesthesia. One thermocouple probe needle (BAT-10, Physitemp) was then inserted in the tumour base and another was inserted into tissue underneath the base of the tumour to monitor temperature during the freeze-thaw cycles. The tumour base and approximately 5 mm of surrounding skin were frozen (≥ 1 minute at –25 ^{o}C) with liquid nitrogen. The nitrogen was applied by use of continuous circulation contact tips (DFS30, Spembly Medical) or by placement of a plastic ring fitted around the tumour into which liquid nitrogen was directly administered in small amounts at a time to allow for temperature control. The temperature measured by the deep tissue probe was kept > 0°C to avoid damage to underlying tissues. Two freeze-thaw cycles were applied. Between cycles, tissues were allowed to thaw spontaneously until the probes measured temperatures of ≥ 20 ^{o}C.

BCG vaccine treatment—Periocular tumours were treated with BCG (Onco-Tice, Organon) (0.5 X 10^8 to 2 X 10^8 colony forming units/dose) vaccine administration in standing sedated equids. When necessary, ulcerated masses were first debulked to

the level of the surrounding skin as previously described. Sarcoid tissue was saturated with the vaccine by injecting approximately 0.3 ml/cm³ in different planes and directions via a Luer-lock syringe with a 23-gauge needle. Four local injections were scheduled to be given at 2- to 3- week intervals (Martens et al., 2001b). Two months after the 4th injection, patients were re-evaluated; in patients with partial tumour regression, a new cycle of injections was started.

Intralesional injection of chemotherapeutic drugs—A cisplatin (Cisplatine Sandoz, Sandoz) containing emulsion was used for intralesional injections until February, 2012, and a carboplatin (Carbosin, Teva) containing emulsion was used afterward. Fibroblastic tumours were debulked prior to the treatment in general anaesthesia using an electroscalpel as previously described. The chemotherapeutic agent was mixed with sterile sesame oil to a final concentration of 3 mg/mL (cisplatin) (Théon et al., 1993) or 5 mg/mL (carboplatin). The technique of saturation was comparable to that used for BCG vaccine administration but a 21-gauge needle was used for delivery. Injections were repeated every 4 weeks until full tumour regression unless the owner declined continuation of the treatment or no progress was made after 3 consecutive injections.

Statistical analysis

Data were explored with commercially available software (Excel, Microsoft) and subsequently analyzed with a statistical program (SPSS 20, IBM). All model assumptions were met. Outcome after treatment (success vs failure) was modeled as a dependent variable. Nine independent variables (treatment type; presence of multiple sarcoids [yes or no]; whether treatments with immunostimulating effects were used on another sarcoid in the same animal; tumour location, tumour type, and whether ulceration was present; age and sex of the animal; and whether sarcoids had been previously diagnosed and had recurred) were assessed for associations with outcome.

For each categorical independent variable (ie, all except age), the variable with the highest success rate was used as referent. Age was treated as a continuous independent variable in all models. First, univariable binary logistic regressions were carried out to estimate the general effects of each independent variable on the

outcome after treatment. A multivariable binary logistic regression model was then built by selecting independent variables with a univariable *P* value ≤ 0.20 for inclusion, except for tumour type and location, which were considered confounding variables for treatment and therefore forced into all models. Values of $P \leq 0.05$ were considered significant in all further models. The multivariable model was corrected for clustering by animal, and equids with missing data for any of the independent variables were excluded. The model was then refined by repeatedly eliminating the independent variable with the highest nonsignificant *P* value (except that tumour type and location were retained in the model as described). Odds ratios for treatment failure (as compared with that for the referent categories [those with the highest success rate for a given comparison]) were calculated together with Wald 95% confidence intervals (CIs) for significant independent variables in the final multivariable model.

The choice between acyclovir and imiquimod for topically treated tumours was a result of owner preference, and the number of sarcoids in each group was almost equal. Because some patients had multiple lesions, a binary logistic regression with correction for clustering by animal was performed to compare effects of the 2 topical treatments on outcome. For BCG and intralesional chemotherapy (cisplatin or carboplatin emulsion) treatments, the effect of the number of injections on the outcome was tested by means of a χ^2 test.

Results

Medical records review identified 317 equids with 879 sarcoids. Eighty-seven horses (with 265 sarcoids) were lost to follow-up, resulting in 230 horses with 614 sarcoids that were included in the analyses. There were 96 sexually intact females, 22 sexually intact males, and 112 castrated males in the study, including 10 Thoroughbreds, 3 French Trotters, 149 warmblood type horses, 5 half-breed horses, 4 draft type horses, 24 ponies and 6 donkeys. Breed identification was missing for 29 horses. Median age at treatment was 7.9 years (range, 0.5 to 26.9 years) and the median follow-up period was 815 days (range, 167 to 2,256 days).

Overall, 460 of 614 (74.9%; 95% CI: 71.3% - 78.2%) sarcoids were recorded as having a successful outcome after treatment. Although no reliable quantitative data were available regarding complications following treatment, reported major complications

included wound dehiscence after excision by electrosurgery, severe skin irritation after imiquimod application, and abscess formation following BCG vaccine injection. No major complications were reported after cryosurgery, acyclovir application, or intralesional chemotherapy with cisplatin- or carboplatin-containing emulsions. Treatment success rates for sarcoids, categorized by biological variables of interest and treatment type, were tabulated (Table 1).

Sarcoids were most frequently located in the inguinal region (228/614 [37.1%]) or on the head (120 [19.5%]); the least common site was at the distal aspect of the limbs (18 [2.9%]). Tumours of the inguinal area and head were most often treated by electrosurgery (147/228 [64.5%]) and by BCG vaccine injection (47/120 [39.2%]), respectively, whereas those in distal limb regions most commonly underwent topical imiquimod treatment (6/18 [33.3%]). A summary of lesions treated by each method and grouped according to anatomic location and sarcoid type is provided (Table 2).

Measurements were available for 286 sarcoids in 105 equids. The median sarcoid surface measurement was 6.6 cm^2 (range, 0.04 to 600 cm^2). Because the size was not recorded for most sarcoids, this variable was not included in the statistical analysis.

Sarcoids treated with BCG vaccine injection were injected a median of 4 times (range 1 to 7]). There was no significant (P = 0.35) difference in the number of injections between tumours with a successful outcome and those that were nonresponsive to BCG vaccination. Sarcoids treated by intralesional chemotherapy with cisplatin- or carboplatin-containing emulsions underwent a median of 4 injections (range, 1 to 7). There was also no significant difference (P = 0.25) in the number of injections between tumours with a successful outcome and those that were nonresponsive to chemotherapy in this category.

Table 1 - Treatment success rates for 614 sarcoids in 230 equids (200 horses, 24 ponies, and 6 donkeys) in a retrospective study to evaluate outcomes following treatments selected according to a standardized protocol and to identify risk factors for sarcoid treatment failure in these patients.

Variable	No. of sarcoids (% of total for category)	No. of successful treatments	Success rate (95% CI)
Treatment			
Acyclovir	62 (10.1)	33	53.2 (41.0–65.1)
Imiquimod	61 (9.9)	44	72.1 (59.2–82.9)
BCG	48 (7.8)	28	58.3 (43.2–72.4)
ICS	23 (3.7)	12	52.2 (30.6–73.2)
Cryosurgery	67 (10.9)	43	64.1 (51.5–75.5)
Electrosurgery with ICB	34 (5.5)	23	67.7 (49.5–82.6)
Electrosurgery	319 (52.0)	277	86.8 (82.6–90.3)
Multiple sarcoids present			
Yes	386 (62.9)	269	69.7 (64.8–74.2)
No	228 (37.1)	191	83.8 (78.3–88.3)
≥ 1 other sarcoid treated by an immunostimulating method*†			
Yes	135 (22.0)	116	85.9 (78.9–91.3)
No	474 (77.2)	339	71.5 (67.2–75.5)
Tumour location			
Head	120 (19.5)	72	60.0 (50.7–68.8)
Neck	36 (5.9)	31	86.1 (70.5–95.3)
Axilla	81 (13.2)	67	82.7 (72.7–90.2)
Thorax	53 (8.6)	47	88.7 (77.0–95.7)
Abdomen	78 (12.7)	65	83.3 (73.2–90.8)
Inguinal region	228 (37.1)	168	73.7 (67.5–79.3)
Distal aspect of limb	18 (2.9)	10	55.6 (30.8–78.5)
Sarcoid type†			
Occult	83 (13.5)	60	72.3 (61.4–81.6)
Nodular	66 (10.7)	51	77.3 (65.3–86.7)
Verrucous	141 (23.0)	114	80.9 (73.4–87.0)
Fibroblastic	63 (10.3)	47	74.6 (62.1–84.7)
Mixed	125 (20.4)	82	65.6 (56.6–73.9)
Patient sex			
Sexually intact male	48 (7.8)	35	72.9 (58.2–84.7)
Castrated male	283 (46.1)	212	74.9 (69.4–79.9)
Sexually intact female	283 (46.1)	213	75.3 (69.8–80.2)
Ulceration			
Yes	142 (23.1)	102	71.8 (63.7–79.1)
No	472 (76.9)	358	75.9 (71.7–79.6)
Recurrent tumour†			
Yes	89 (14.5)	60	67.4 (56.7–77.0)
No	365 (59.4)	277	75.9 (71.2–80.2)

Success was defined as full regression of the treated tumour without evidence of recurrence on follow-up ≥ 6 months after treatment. Treatment success rates and 95% CIs are reported as percentages. Some patients underwent concurrent treatment of > 1 sarcoid by different methods. *Intratumoural BCG vaccine injection, cryosurgery, and topical imiquimod application were considered to have general immunostimulating effects. †Some animals with missing information were excluded from these analyses.

ICB = Intralesional cisplatin-containing bead placement. ICS= Intralesional injection of cisplatin- or carboplatin-containing emulsion.

Table 2 - Treatment types selected by use of a standardized protocol for 614 sarcoids in 230 equids (200 horses, 24 ponies, and 6 donkeys), grouped according to anatomic location and type of sarcoid.

Variable	Treatment							Total
	Acyclovir	Imiquimod	BCG	ICS	Cryosurgery	Electrosurgery with ICB	Electrosurgery	
Location								
Head	17	24	47	2	16	4	10	120
Neck	1	10	1	0	4	0	20	36
Axilla	7	5	0	4	8	6	51	81
Thorax	5	4	0	0	8	1	35	53
Abdomen	7	4	0	2	8	5	52	78
Inguinal region	24	8	0	12	21	16	147	228
Distal limb	1	6	0	3	2	2	4	18
Type								
Occult	25	12	1	0	0	1	44	83
Nodular	2	1	12	3	5	7	36	66
Verrucous	10	21	5	1	14	3	87	141
Fibroblastic	2	2	3	7	14	3	32	63
Mixed	16	15	16	6	5	11	56	125
Not recorded	7	10	11	6	29	9	64	136

See Table 1 for key.

In the univariable analysis, treatment type ($P < 0.001$), presence or absence of multiple sarcoids ($P < 0.001$), whether another tumour on the same patient received any sarcoid treatment considered to have immunostimulatory effects (BCG vaccine injection, cryosurgery, or topical imiquimod application; $P = 0.001$), patient age at the time of treatment ($P = 0.021$), and anatomic location of the tumour ($P = 0.10$) met the criterion for inclusion in multivariable analysis, whereas patient sex, whether the treated tumours represented a recurrence of previous sarcoids, and whether ulceration was present did not (tumour type, although not meeting the criterion, was retained in the model as described).

One hundred and forty-one sarcoids on 64 horses with missing data were excluded from the multivariable analysis. The quasi-likelihood under independence model criterion for the initial multivariable model was 508.72, and that for the final multivariable model (after removal of animal age, which was nonsignificant in the initial model) was 505.58, indicating that the final model fitted the data slightly better. In the final model, sarcoids that underwent topical acyclovir treatment, cryosurgery, and intralesional treatment with cisplatin- or carboplatin-containing emulsions had significantly higher odds of treatment failure, compared with those treated by electrosurgery (Table 3). Odds of treatment failure for tumours treated by topical application of imiquimod, BCG vaccine injection, or electrosurgery with intralesional placement of cisplatin-containing beads did not differ significantly from the odds for those treated by electrosurgery. The odds of treatment failure were significantly higher for tumours located in an equid with multiple sarcoids than for those in an equid with solitary lesions. These odds were also significantly higher for sarcoids where another tumour in the same equid did not receive immunostimulatory treatments than for those where another tumour did receive such treatment. Patient age at the time of treatment, sarcoid type, and anatomic location of the tumour were nonsignificant ($P > 0.05$ for each overall comparison) in the multivariable models.

Separate analysis of occult and slightly verrucous sarcoids that succesfully underwent topical treatments revealed that a median treatment duration of 50 weeks (range, 3 to 97 weeks) was required to obtain a successful result with acyclovir, compared with a median of 6 weeks (range, 2 to 40 weeks) for imiquimod. The observed difference in success rates between acyclovir-treated sarcoids and those treated with imiquimod

(33/62 [53.2%] in 32 equids vs 44/61 [72.1%] in 43 equids, respectively) was not significant (*P* = 0.105).

Table 3 - Results of multivariable analysis for risk factors associated with treatment failure for 473 of the 614 sarcoids (in 166 of 230 equids) in Table 1.

Variable	Initial model		Final model	
	OR (95% CI)	***P* value**	**OR (95% CI)**	***P* value**
Treatment	—	0.031	—	0.025
Acyclovir	3.89 (1.32–11.46)	0.014	4.05 (1.38–11.88)	0.011
Imiquimod	1.95 (0.68–5.59)	0.22	2.04 (0.72–5.79)	0.18
BCG	2.99 (0.81–11.03)	0.10	3.08 (0.84–11.31)	0.090
ICS	5.65 (1.37–23.30)	0.017	5.86 (1.43–24.09)	0.014
Cryosurgery	5.28 (1.66–16.77)	0.005	5.51 (1.70–17.89)	0.004
Electrosurgery with ICB	2.83 (0.93– 8.54)	0.066	2.92 (0.99–8.65)	0.052
Electrosurgery	Referent	Referent	Referent	Referent
Multiple sarcoids present	—	<0.001	—	< 0.001
Yes	4.16 (2.44 – 7.09)	<0.001	4.13 (2.42–7.05)	< 0.001
No	Referent	Referent	Referent	Referent
≥ 1 other sarcoid treated by an immunostimulating method*	—	0.030	—	0.041
Yes	Referent	Referent	Referent	Referent
No	2.58 (1.10–6.07)	0.030	2.51 (1.04–6.08)	0.041
Patient age	—	0.59	—	—

Independent variables with a *P* value ≤ 0.20 in univariable analysis were included in the multivariable analysis, except that anatomic location and sarcoid type were considered confounding variables for treatment and retained in the models. In the multivariable analysis, values of *P* ≤ 0.05 were considered significant. A significant effect indicates significantly greater odds of treatment failure, compared with that of the referent category. 141 sarcoids (64 equids) were excluded from the analysis because of missing data.

— = Not applicable. See Table 1 for remainder of key.

Discussion

The overall success rate (460/614 [74.9%] sarcoids) found in the present study was comparable to those reported in the few earlier studies in which results for multiple sarcoid treatments in equids were evaluated (86/117 [73.5%] horses by Diehl et al. (Diehl et al., 1987) and 154/186 [82.8%] sarcoids by Martens et al. (Martens et al., 2001b)). For the present study, the overall success rate was generally attributable to the high success rate obtained with electrosurgical excision (277/319 [86.8%]), which was the most commonly performed treatment. In previous reports (Diehl et al., 1987;

Scott and Miller, 2011), substantially lower success rates for surgical excision (ranging from 5/18 [28%] sarcoids to 9/14 [64%] horses) were reported, whereas the data from the present study were in agreement with findings in another retrospective study (Martens et al., 2001b) of sarcoid treatment in equids performed at our institution for the period 1995 through 1999. The high success rate of electrosurgical excision in the present study can be attributed to a careful tumour selection, the fact that all excisions were performed under general anaesthesia, and meticulous use of the electrosurgical instrument with a nontouching technique followed by wound flushing and primary wound closure. Although treatment of all equids under general anaesthesia may not seem necessary, it allows for positioning of patients in a way that provides optimal access to tumours and facilitates excision. Logically, the selection of cases for surgery inherently biased the sample population in our study, and it is clear that surgical excision is not a treatment that can or should be universally used for all sarcoids in all anatomic locations. At the distal aspect of the limbs, for example, there often is less skin available for closure, and electrosurgery would not be an ideal choice for some of these tumours.

The prognosis for sarcoid treatment is often reported to be worse when the tumour has previously undergone unsuccessful treatment (Bogaert et al., 2008; Scott and Miller, 2011; Bergvall, 2013), but to the authors' knowledge, there is no evidence-based research on this topic. In the present study, whether or not the tumour was recurrent had no significant effect on treatment outcome. Nevertheless, information on previous treatment was unavailable for 160 of 614 (26.1%]) sarcoids (60/230 equids), which may have influenced the results. Further research may provide more information on the possible influence of previous treatment failures on prognosis for equids with sarcoids undergoing specific treatment protocols.

Use of a carbon dioxide laser has been suggested to be a promising treatment for sarcoids in horses (Hawkins and McCauley, 2005). Success rates for laser surgery range from 37 of 60 (61.7%) to 48 of 59 (81.4%) of horses (Diehl et al., 1987; Carstanjen et al., 1997; Martens et al., 2001b; McCauley et al., 2002). Owing to the high cost of acquiring a CO_2 laser system and the fact that it is cumbersome to use, some surgeons have used a diode laser instead, with similar results (successful in 21/25 [84.0%] horses) (Compston and Payne, 2013). Considering that these reported success rates for diode or carbon dioxide laser surgery were comparable with the

results of the present study, albeit that success rates were calculated on the sarcoid level in the present study, an advantage for more expensive laser surgery has yet to be proven. Laser surgery was not used for the patients in the present study because it was not available in the clinic at that time.

Earlier reported success rates for cryosurgery range from 21 of 35 (60%) to 10 of 10 (100%) horses (Lane, 1977; Fretz and Barber, 1980; Klein et al., 1986; Diehl et al., 1987; Martens et al., 2001b). The success rate for treatment of sarcoids in the present study with this modality was 43 of 67 (64.1%) sarcoids, and sarcoids treated with cryosurgery had significantly greater odds of treatment failure, including recurrence (OR = 5.51), compared with those treated by electrosurgery. This was partly attributable to the fact that this treatment was primarily used for more complicated sarcoids that were ill-defined, inconveniently located, or strongly attached to underlying tissues. Similarly, the high odds of treatment failure for sarcoids that underwent intralesional injection of platinum-containing chemotherapeutic agents, relative to those for tumours treated by electrosurgery (OR = 5.86) in the present study were likely associated with the fact that these tumours were unamenable to surgical treatments owing to size, anatomic location, or both.

Presently, there is only limited scientific information available on the topical treatment of sarcoids with acyclovir (Stadler et al., 2011) or imiquimod (Nogueira et al., 2006) in equids. The previously reported success rate for treatment of sarcoids with topical acyclovir administration (32/47 [68%] sarcoids) (Stadler et al., 2011) was higher than that found in the present study, whereas our results with imiquimod were more comparable to the previously reported success rate (9 of 15 [60%] sarcoids) (Nogueira et al., 2006). One factor that could have influenced the success rates found in the present study was that owners were responsible for applying the topical treatments; therefore, treatment compliance could not be verified. The success rate for imiquimod-treated tumours in this study was apparently higher, compared with that for acyclovir-treated tumours, but the difference was not significant. Whereas the number of sarcoids in each treatment group was almost identical, there were more animals in the imiquimod group than in the acyclovir group, which influenced the statistical outcome. The authors speculate that if the study design had been more balanced, as in a prospective study, significance might have been reached. Many questions still remain about the working mechanisms of acyclovir and the distribution of acyclovir and

imiquimod in the skin of equids, but on the basis of the present information, the authors believe that imiquimod application might still be a better choice for treatment of occult and slightly verrucous sarcoids in equids that tolerate the skin irritation, even though the observed difference between treatment results was nonsignificant in this study.

In the present study, periocular sarcoids were all treated by BCG vaccine injection; given that the success rate for BCG treatment was low (28/48 [58.3%]), this may explain the low overall treatment success rate for tumours located on the head (72/120 [60.0%]). Previously reported success rates for BCG vaccination for periocular sarcoids range from 36 of 61 (59%) to 31of 31 (100%) of equids (Lavach et al., 1985; Klein et al., 1986; Owen and Jagger, 1987; Vanselow et al., 1988; Knottenbelt and Kelly, 2000; Martens et al., 2001b). A possible explanation for the lower success rate of BCG vaccination in our study, compared with that in an earlier study at the same clinic (21/30 [70%] sarcoids) (Martens et al., 2001b), is that different commercially available BCG strains were used in the 2 studies. In earlier reports, authors warned that nonfatal or fatal anaphylactic shock could occur after BCG vaccination (strain not specified) (Landsheft and Anderson, 1984; Vanselow et al., 1988). Although equids were not premedicated with anti-inflammatory drugs, no such events were identified in the study reported here. Possible treatment alternatives for periocular sarcoids include interstitial brachytherapy and local chemotherapy with cisplatin, carboplatin, or mitomycin (Théon and Pascoe, 1994; Knottenbelt and Kelly, 2000; Byam-Cook et al., 2006). However, specific instruments, skills, and infrastructure are needed to protect the operator and the environment for most of these treatments, and the results of mitomycin treatment have not yet been thoroughly described.

Although the specific role of the immune system in the development and resolution of sarcoids in equids is not yet fully understood, it is likely to play an important role in this bovine papillomavirus–induced tumour. Autovaccination has been used to treat sarcoids (Kinnunen et al., 1999; Espy, 2008), and regression of untreated tumours in horses that had other sarcoids treated with cryosurgery has been previously described (Lane, 1977; Martens et al., 2001b). Spontaneous regression of sarcoids in untreated equids is generally considered to be rare, but it has been reported (Brostrom, 1995; Studer et al., 1997; Martens et al., 2001b). Results of the present study showed that the odds of treatment failure were significantly lower for a sarcoid on a patient that received concurrent immune-stimulating treatment (cryosurgery, BCG vaccine

injection, or imiquimod application) for antoher tumour, compared with those for a sarcoid on a horse that did not, regardless of the primary treatment. This could suggest that the immune system has a role in resolution of these tumours and should not be neglected in future treatment development.

To our knowledge, the present study was the largest published to date in which multiple treatments for different sarcoid types and locations in equids were compared. Although the minimum follow-up time of 6 months can be considered relatively short, compared with that used in other retrospective studies on the subject, the median follow-up period was > 2 years, which facilitates making conclusions for a long-term prognosis. Owing to the large sample size in this study and the fact that the analysis was not focused on a specific treatment or location, these results have a practical relevance for treatment of sarcoids in equids under clinical conditions. It should be noted, however, that the current study focused on a referral population and the results may not directly reflect the situation in ambulatory practice. Other weaknesses of the study included the fact that complications were not always recorded and that the decision regarding whether a treatment was successful was left to the owner in some cases. Nevertheless, during the telephone interview, very specific questions were asked to guide the owner in this process.

With the treatment selection protocol used in the present study, most sarcoids in equids were treated successfully by electrosurgical excision under general anaesthesia; our results also suggested that use of immunostimulating treatments may improve the posttreatment outcome in some equids.

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Topical distribution of acyclovir in normal equine skin and equine sarcoids: an *in vitro* study

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Summary

Topical acyclovir application is an owner-friendly treatment for occult equine sarcoids, without the caustic side-effects other topical treatments have. Variable clinical success rates have been described, but it is not known to what rate and extent acyclovir penetrates in and through equine skin from a topical formulation.
In the current study, an *in vitro* Franz diffusion model was used to determine the permeation parameters for a generic 5% acyclovir cetomacrogol cream for both healthy and sarcoid equine skin. The distribution of acyclovir between different layers of both skin types was also evaluated.

While acyclovir penetrated through both skin types, significantly less acyclovir permeated to the deep dermis of sarcoid skin (197.62 ng/mm^3) compared to normal skin (459.41 ng/mm^3). Within sarcoid skin samples, significantly higher acyclovir concentrations were found in the epidermis (983.59 ng/mm^3) compared to the superficial dermis (450.02 ng/mm^3) and the deep dermis. At each sample point, significantly more acyclovir permeated to the receptor fluid through normal skin compared to sarcoid skin, which is reflected in the significantly higher permeation parameters of normal skin.

Normal skin was found to be more permissive for acyclovir, but even in sarcoid skin, enough acyclovir reached the deep dermis to treat a Herpes simplex virus infection. In the case of equine sarcoids, the treatment is aimed at the Bovine papillomavirus and no information is available on the susceptibility of the DNA polymerase of this virus for acyclovir. Therefore, further research is needed to determine the efficacy of acyclovir to treat equine sarcoids.

Introduction

Equine sarcoids are common Bovine papillomavirus (BPV) induced tumours of the skin of equids. BPV DNA is most often found in the superficial and deep dermis (Wobeser et al., 2012). Various invasive treatments have been described with relatively good results (Taylor and Haldorson, 2013), but they are often expensive, require a high level of commitment of the owner and can lead to patient discomfort. Therefore, a tendency towards topical treatment of the early stages of equine sarcoids exists, using different products ranging from bloodroot (Wilford et al., 2014) or mistletoe extracts (Christen-Clottu et al., 2010) to AW-ludes (Knottenbelt and Kelly, 2000), imiquimod (Nogueira et al., 2006) and acyclovir-based creams (Stadler et al., 2011). In a retrospective study comparing the latter two, higher success rates for complete tumour regression were obtained with imiquimod (Haspeslagh et al., 2016). However, repeated imiquimod application can cause severe local adverse effects including erythema, oedema, scaling, ulceration and exudation (Nogueira et al., 2006), and owners therefore often prefer treatment with the non-irritating 5% acyclovir-based cream. This cream is applied once or twice daily until full tumour regression (Stadler et al., 2011; Haspeslagh et al., 2016).

Acyclovir is an antiviral drug that has been developed for human medicine to treat lesions caused by the Herpes simplex virus (HSV) (Elion et al., 1977). The mechanism of action relies on metabolisation of the drug to its monophosphate form by a thymidine kinase specific to HSV (Elion et al., 1977). The monophosphate form is then further metabolized by cellular enzymes to a bi- and triphosphate form (Miller and Miller, 1980). In its final triphosphate form, acyclovir inhibits the HSV DNA polymerase, thus preventing further virus replication. While BPV lacks the presence of this thymidine kinase and thus the ability to phosphorylate acyclovir (Baker and Howley, 1987), it has been shown that small amounts of acyclovir triphosphate are also formed in the absence of HSV thymidine kinase and certain viral DNA polymerases (for example from the Epstein-Barr virus) are highly sensitive to acyclovir triphosphate, hence an antiviral effect can still be obtained (Datta et al., 1980).

To understand if and how topical acyclovir treatment can help to cure occult equine sarcoids, it is important to know the disposition of the drug in equine skin and if it

reaches the dermis, where viral replication takes place. Topical delivery of acyclovir has been well-described in humans (Freeman et al., 1986; Parry et al., 1992), mice (Gonsho et al., 1990) and pigs (Wei et al., 2012), but nothing is known on topical delivery of acyclovir in horse skin, let alone equine sarcoids.

The present study focusses on (trans)dermal delivery of acyclovir after topical application of a 5% acyclovir cetomacrogol cream in both sarcoids and normal equine skin using an *in vitro* design in Franz diffusion cells.

Materials and methods

Skin sample collection and storage

Twelve occult equine sarcoids with intact epidermis were excised from the skin of ten different healthy adult warmblood horses that were presented for treatment to the Faculty of Veterinary Medicine of Ghent University. After owner consent, surgery was performed under general anaesthesia as described earlier (Haspeslagh et al., 2016). Care was taken not to include the surface of the sarcoids in the surgical preparation protocol. Twelve pieces of approximately five by ten centimeters of normal skin were collected from 12 different adult warmblood horses after euthanasia for unrelated reasons. The location of each piece of normal skin was matched to the location of a corresponding equine sarcoid. The skin was clipped prior to removal, avoiding damage to the epidermis. Immediately after excision all samples were pinned at their edges to a Styrofoam surface and all subcutaneous tissues were carefully removed using a scalpel blade, taking care not to damage the dermis. Skin samples were then rinsed with a sterile isotonic solution (0.9% NaCl, Baxter) and stored separately at -20 °C.

Diffusion experiment

Immediately before the start of the experiment, skin and sarcoid samples were thawed, visually inspected for damage and cut to circular pieces with a radius of approximately three centimeters for further use in the experiment. Leftover samples of the edges of sarcoid pieces were stored in 4% formalin, subsequently colored with hematoxylin – eosin stain and examined histologically to confirm sarcoid diagnosis. Twelve sarcoid and 12 matched normal skin pieces were mounted into static Franz diffusion cells (FDC-6, Logan) with an exposed area of 0.64 cm², with 5.0 ml 0.01 M PBS receptor

fluid (RF) at 32 ± 0.5 °C (Baert et al., 2010). Three-hundred µl of a generic 5% acyclovir cetomacrogol cream was carefully applied to the epidermal side of the skin, covering its complete surface. The donor compartment was covered with a paraffin film.

At specific time intervals (2h, 6h, 12h, 18h, 24h, 31h, 36h and 48h), 200 µl of the receptor fluid of all cells was obtained and stored at -35 °C. Immediately after sample collection, all receptor chambers were refilled with 200 µl of fresh 0.01 M PBS. Immediately after taking the last sample, the remaining donor cream was removed from the skin by using cotton swabs slightly soaked in PBS, followed by dry swabs. The remaining receptor fluid was discarded and the exposed skin area (0.64 cm^2) was cut out with a scalpel blade. All exposed skin samples were placed on microscope slides to ensure they were positioned completely flat and stored at -35 °C.

Tissue collection and acyclovir quantification

Frozen skin samples were embedded in cryotome gel (Cryocompound Frozen Tissue Medium, Klinipath), placed in a cryotome (CM1900, Leica Microsystems) and cut parallel to the skin surface in slices of 50 µm starting from the dermal side. The blade was cleaned between every skin sample. Slices were counted and kept in order while cutting. When the epidermis was reached, which could be visually determined by the operator by a change in color and texture of the sample, the slices already cut were equally divided into two subsets: the deep dermis (DD) and the superficial dermis (SD). The third subset, the epidermis (E), consisted of the remaining part of the sample. The sample was then cut further until slices became incomplete, indicating there was no more epidermis. These last incomplete slices were discarded to avoid any leftover donor solution influencing the results. Slices from the same subset of the same skin sample were combined for analysis. The number of slices in each subset was noted and the thickness of each subset was calculated by multiplying the number of slices by the slice thickness (50 µm). The tissue volume of each subset was calculated by multiplying the subset thickness with slice area (0.64 cm^2). All subset samples were stored at -35 °C.

The amount of acyclovir in skin sample subsets and RF samples was determined by validated ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Matrix-matched calibration curves were prepared in untreated skin

subsets and RF by spiking with an appropriate amount of a working solution of acyclovir. All samples were also spiked with internal standard (ganciclovir) prior to analysis. Skin samples were left to equilibrate for 1 h at room temperature. Extraction from the skin subsets was performed by incubating with 200 µl of UPLC water for 15 min at 60 °C in a warm water bath. Every 5 min the samples were vortexed for 10 sec. Skin samples were cooled down to room temperature and 200 µl of 1 N aqueous perchloric acid was added. The skin samples were vortexed and thereafter centrifuged for 10 min at 13225 g. Finally, the supernatant was filtered through a 0.2 µm Millex nylon filter (Merck-Millipore) and 5 µl was injected onto the UPLC-MS/MS instrument. RF samples were diluted with 1 ml of 0.1 N aqueous hydrochloric acid whereupon they were brought onto a mixed cation exchange - solid phase extraction column previously conditioned with 3 ml of methanol, 3 ml of water and 3 ml of 0.1 N hydrochloric acid. After the sample passed through the column, it was subsequently washed with 3 ml of 0.1N aqueous hydrochloric acid, 3 ml of water and 3 ml of methanol. Acyclovir was eluted with 3 ml of 5% ammonia in methanol. The eluate was evaporated to dryness under a gentle nitrogen stream at 40 °C. The dry residue was dissolved in 150 µl of a 0.1% aqueous acetic acid solution and 5 µl was injected onto the UPLC-MS/MS system.

Chromatographic separation was performed on a UPLC HSS-T3 column (2.1 x 100 mm, 1.8 µm, Waters). A gradient elution was applied with methanol and 0.1% acetic acid in water as mobile phases. The UPLC-MS/MS system consisted of an Acquity UPLC sample manager and autosampler in combination with a Quattro Premier XE triple quadrupole mass spectrometer operating in positive electrospray ionization mode (Waters). For quantification Masslynx software v. 4.1 was used at m/z = 226.02 > 151.92 for acyclovir and m/z = 256.02 >151.98 for ganciclovir.

The method was validated according to EU guidelines (EMEA/CVMP/573/00-FINAL; VICH GL49 (R)(MRK), 2015) and as described previously (Maes et al., 2009) for following parameters: linearity, accuracy, within-day and between-day precision, limit of detection (LOD), limit of quantification (LOQ) and stability in matrix; The LOD and LOQ were respectively determined at 0.16 ng/ml and 1 ng/ml for RF. For the skin subsets the LOD and LOQ were 0.20 ng/subset and 1 ng/subset, respectively.

The concentration of acyclovir in each skin subset was calculated by dividing the absolute amount of acyclovir detected by the subset tissue volume. The concentration of acyclovir in complete skin was calculated for each skin sample by first adding the absolute acyclovir amounts of DD, SD and E and dividing this total by the complete sample tissue volume

Data analysis

Permeation parameters were calculated from the curves of the cumulative amount permeated as a function of time. Steady state flux (J_{ss}) was derived from the slope of the linear portion of the curve, divided by the exposed skin surface (0.64 cm^2). Lag time was estimated by extrapolating the linear part of the curve to the time axis. The permeability coefficient ($k_{p,v}$) was calculated as $k_{p,v} = J_{ss} / C_v$ with C_v being the acyclovir concentration in the donor cream. Q_{48h} was calculated as the cumulative percentage of the applied acyclovir dose that was found in the receptor fluid at 48 h.

Statistical analysis was performed in SPSS 20 (IBM). Table 1 shows an overview of the different fitted models. A general linear model (GLM) approach was used. When multiple pairwise comparisons were performed post-hoc, a Bonferroni correction was applied. Significance was set at $P \leq 0.05$. Normality of unstandardized residuals was tested for every model by a Kolmogorov-Smirnov test. When residuals did not show a normal distribution ($P < 0.05$) or when a large amount of heteroscedasticity was detected, a logarithmic transformation of the data was performed and variances and normality of the residuals was rechecked.

Table 1 - Overview of the fitted general linear models. N = normal skin; S = sarcoid skin; DD = deep dermis; SD = superficial dermis; E = epidermis; AC = acyclovir; RF = receptor fluid.

Model number	Independent variable(s)	Covariate	Dependent variable
1	Type (N, S)		Complete skin thickness
2	Type (N, S)		Skin subset thickness
3	Type (N, S)	Complete skin thickness	AC in complete skin
4	Type (N, S)	Skin subset thickness	AC in skin subset
5	Skin subset (DD, SD, E)	Skin subset thickness	AC in skin subset
6	Type (N, S)	Complete skin thickness	AC in RF by time
7	Type (N, S)	Complete skin thickness	J_{ss}
8	Type (N, S)	Complete skin thickness	Q_{48h}
9	Type (N, S)	Complete skin thickness	$K_{p,v}$
10	Type (N, S)	Complete skin thickness	Lagtime

Results

Equine sarcoid diagnosis was confirmed histologically for all sarcoid samples. One sarcoid was located on the neck, one at the level of the axilla, two on the abdominal wall, three at the inside of a hind leg and five around the praeputium or teats. One sarcoid sample was omitted from all calculations except for skin thickness because of reduction of the RF level during the experiment, indicating an artefact.

Skin thickness

Table 2 shows the mean thickness of complete skin and skin subsets for both normal and sarcoid skin, along with the associated F- and P-values. Sarcoid skin was significantly thicker compared to normal skin, due to the dermis of sarcoid skin being significantly thicker than normal dermis (Table 2). Because of these differences, skin or subset thickness was included as a covariate in further models comparing normal and sarcoid skin.

Table 2 - Thicknesses in mm of complete skin and skin subsets of normal and sarcoid skin along with associated F- and P-values. SE = standard error; DD = deep dermis; SD = superficial dermis; E = epidermis. * $P \leq 0.05$.

	Normal skin (n=12) Mean value (± SE)	**Sarcoid (n=12)** Mean value (± SE)	**F- and P-values**
Complete	1.95 (± 0.12)	2.53 (± 0.16)	$F_{(1;17)} = 9.07$; $P < 0.01$*
DD	0.58 (± 0.06)	0.85 (± 0.08)	$F_{(1;22)} = 7.37$; $P = 0.01$*
SD	0.60 (± 0.06)	0.84 (± 0.08)	$F_{(1;21)} = 6.12$; $P = 0.02$*
E	0.75 (± 0.11)	0.85 (± 0.12)	$F_{(1;21)} = 0.80$; $P = 0.38$

Acyclovir in skin

In Table 3, the concentrations of acyclovir and associated P-values are listed for complete skin and skin subsets of normal and sarcoid skin. While there was no significant difference between normal and sarcoid skin in the concentration measured in total skin, significantly lower concentrations of acyclovir were detected in DD of sarcoid skin compared to normal skin.

In sarcoid skin, there was a significant difference between different skin layers for the concentration of acyclovir ($F_{(2;29)} = 15.06$; $P < 0.01$), which was not present in normal skin ($F_{(2;30)} = 1.82$; $P = 0.18$). Post-hoc tests revealed that in sarcoid skin, significantly higher concentrations of acyclovir could be measured in E compared to SD ($P = 0.03$) and DD ($P < 0.01$), and acyclovir concentration was significantly higher in SD compared to DD ($P = 0.03$).

Table 3 - Concentration of acyclovir in ng/mm³ for complete skin and skin subsets of normal and sarcoid skin along with associated F- and P-values. SE = standard error; DD = deep dermis; SD = superficial dermis; E = epidermis. * $P \leq 0.05$.

	Normal skin (n=12) Mean value (± SE)	**Sarcoid (n=12)** Mean value (± SE)	**F- and P-values**
Complete	755.68 (± 122.71)	576.49 (± 92.40)	$F_{(1;19)} = 1.28$; $P = 0.27$
DD	459.41 (± 73.48)	197.62 (± 25.11)	$F_{(1;20)} = 10.01$; $P < 0.01$*
SD	664.03 (± 113.83)	450.02 (± 90.84)	$F_{(1;19)} = 3.59$; $P = 0.07$
E	1031.97 (± 242.95)	983.59 (± 156.53)	$F_{(1;19)} = 0.05$; $P = 0.83$

Acyclovir in receptor fluid

Figure 1 shows the mean cumulative concentration (ng/ml) of acyclovir found in RF at each sample point for normal skin and sarcoid skin. At each time point, significantly more acyclovir had permeated through normal skin compared to sarcoid skin ($P \leq 0.05$).

Acyclovir permeation parameters through normal and sarcoid skin are listed in Table 4, along with the associated F- and P-values. All permeation parameters except lag time were significantly higher in normal skin compared to sarcoid skin.

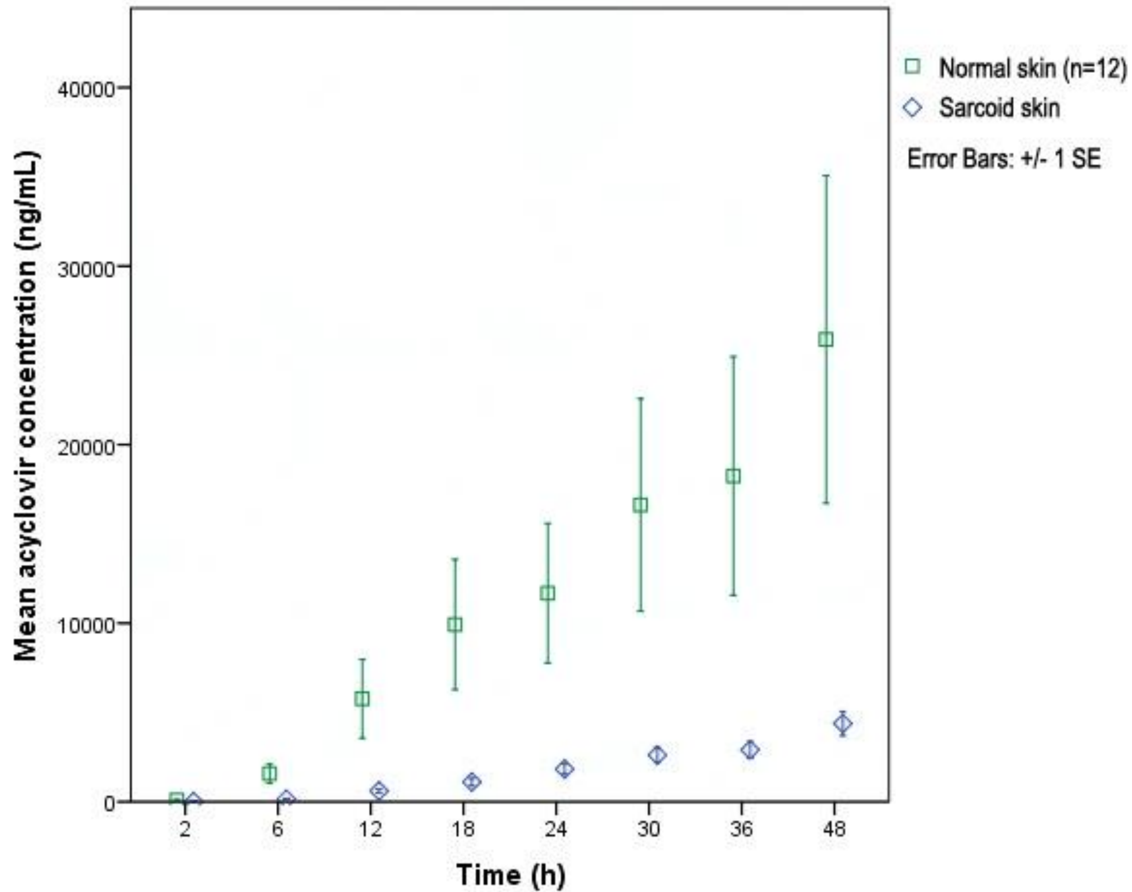

Figure 1 - Mean ± standard error (SE) acyclovir concentrations determined in the receptor fluid at each sampled time point for both normal and sarcoid skin.

Table 4 - Permeation parameters for normal and sarcoid skin and the associated F- and P-values. SE = standard error. * $P \leq 0.05$.

	Normal skin (n=12) Mean value (± SE)	**Sarcoid (n=12)** Mean value (± SE)	**F- and P-values**
J_{ss} (ng/cm²*h)	4958.56 (± 1735.39)	924.31 (± 147.54)	$F_{(1;19)} = 4.83$; $P = 0.04$*
Q_{48h} (%)	0.97 (± 0.34)	0.16 (± 0.03)	$F_{(1;19)} = 5.36$; $P = 0.03$*
$k_{p,v}$ E^{-5} (cm/h)	9.90 (± 3.47)	1.8 (± 0.3)	$F_{(1;19)} = 4.83$; $P = 0.04$*
Lag time (h)	3.54 (± 0.66)	6.8 (± 1.08)	$F_{(1;19)} = 2.64$; $P = 0.12$

Discussion

Sarcoid skin was thicker than normal skin and this difference was due to thickening of the dermis (Table 2). These findings can be related to the distinct proliferation of fibroblast-like cells in the dermis which occurs in all equine sarcoids (Martens et al., 2000). While epidermal hyperplasia is not uncommon in equine sarcoids, the epidermis of equine sarcoid skin was not significantly thicker compared to normal skin in this experiment(Table 2). The mean normal skin thickness (1.95 mm) was in the lower spectrum of the reported range of equine skin thickness (1.0 – 7.7 mm) (Wong et al., 2005; Scott and Miller, 2011). As normal skin samples were matched to the body location of sarcoid skin samples, this confirms earlier statements of sarcoids occurring more frequently at body locations where the skin is thinner (Pilsworth and Knottenbelt, 2007).

Less acyclovir penetrated to DD of sarcoid skin compared to normal skin. Within sarcoid skin, higher concentrations of acyclovir were measured in E than in SD and DD, and more acyclovir was present in SD than in DD (Table 3). In normal skin, no such differences could be found. Moreover, all permeation parameters, except for lag time, also indicated that the permeability of sarcoid skin was lower compared to normal skin. This filter effect of sarcoid skin is probably due to the thicker and more dense dermal layers and the presence of hyperkeratosis at the level of the epidermis (Martens et al., 2000). Nevertheless, a certain amount of acyclovir still reached the dermis, where viral transcription occurs and causes the fibroblastic proliferation typically present in equine sarcoids.

The minimal acyclovir dose that leads to 50% inhibition of viral cytopathic effect of HSV is 0.35 – 0.79 ng/mm^3 (Crumpacker et al., 1979; Declercq et al., 1980). In human epidermis (which is the target skin layer in humans), concentrations of 7 ng/mm^3 were reported after topical acyclovir application (Parry et al., 1992). The mean concentration found in the sarcoid DD in this experiment was 197.62 ng/mm^3 (Table 3), which would be sufficient to treat a HSV infection. Of course, the viral target in this case is BPV and to the author's knowledge, no information is available on the susceptibility of the BPV DNA polymerase for acyclovir.

In humans it has been shown that more acyclovir was present in E after topical administration compared to oral administration, while in the deeper basal epidermis, the acyclovir concentration was 2 – 3 times higher after oral administration (Parry et al., 1992). Since the target region for treating equine sarcoids with acyclovir is located even deeper in the skin, oral acyclovir administration could increase the drug concentration at this target location. However, oral acyclovir administration resulted in low plasma concentrations and a low absolute oral bioavailability (< 5%) in adult horses, pointing to intravenous acyclovir administration or oral valacyclovir administration as better alternatives (Garré et al., 2007). Other techniques improving permeation of acyclovir through human skin (e.g. Solid Lipid Nanoparticles (Gide et al., 2013) or iontophoresis (Volpato and Nicoli, 1998; Shukla et al., 2009)) could also be applied to horses.

In conclusion, the results show that after topical acyclovir administration on sarcoid skin, concentrations in the deeper dermal layers were significantly lower compared to normal skin. Nevertheless, concentrations in the DD of sarcoid skin were still considerable. If BPV is susceptible for those acyclovir concentrations, a therapeutic effect at the level of the target skin region could be obtained after topical administration. To find out whether the obtained concentrations are sufficient to interfere with BPV DNA replication, a DNA polymerase assay can be carried out to test the susceptibility of the BPV DNA polymerase for the effects of acyclovir. Further, the question remains how much acyclovir is being triple phosphorylated in the equine dermis.

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Topical use of 5% acyclovir cream for the treatment of occult and verrucous equine sarcoids : a double-blinded placebo-controlled study

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Results of this study were presented at the 25th ECVS annual scientific meeting, Lisbon, July 7-9 2016.

Summary

Previous studies mention the use of topical acyclovir for the treatment of equine sarcoids. Success rates vary and since the bovine papillomavirus (BPV) lacks the presence of a kinase necessary to activate acyclovir, there is no proof of its activity against equine sarcoids.

Twenty-four equine sarcoids were topically treated with acyclovir cream and 25 with a placebo. Both creams were applied twice daily during six months. Before the start of the treatment and further on a monthly basis, photographs and swabs were obtained. On the photographs, sarcoid diameter and surface area were measured and verrucosity of the tumours was quantified using a visual analog scale (VAS). The swabs were analysed by PCR for the presence of BPV DNA and positivity rates were calculated as the number of positive swabs divided by the total number of swabs for each treatment group at each time point. Success rates were not significantly different between both treatment groups. There was also no significant effect of treatment on sarcoid diameter, surface area or VAS score. For the swabs, a significantly higher BPV positivity rate was found for acyclovir treated tumours compared to placebo treated sarcoids only after one month of treatment and not at other time points.

None of the results indicate that treatment with acyclovir yields any better results compared to placebo treatment.

Introduction

Acyclovir (acycloguanosine) is an antiviral drug developed for the treatment of herpes simplex virus (HSV) infections in humans (Elion et al., 1977). The drug relies on competitive inhibition of the viral DNA polymerase, but needs to be phosphorylated by a HSV specific thymidine kinase and then further by cellular enzymes to a triphosphate form to exert its action (Miller and Miller, 1980). Nevertheless, inhibition of viral replication also occurs for other viral species than HSV (Datta et al., 1980), suggesting phosphorylation of the drug may also occur in cells where the HSV thymidine kinase is not present, albeit to a lesser extent.

Equine sarcoids are tumours originating in the dermal layers of equine skin. The pathogenesis is not entirely clear yet, but there is agreement in literature that the bovine papillomavirus (BPV) (mainly type 1 and type 2) most likely plays an important role in the development of these tumours (Nasir and Campo, 2008). Many treatments have been reported, but no universal treatment has been found to cure all sarcoids on all body locations.

Topical treatment with acyclovir has been described to result in complete regression in 68% of occult, verrucous, nodular or mixed equine sarcoids (Stadler et al., 2011). In addition, a recent *ex vivo* study has shown that acyclovir concentrations reached in the dermal layers after topical administration on sarcoid-affected equine skin are high enough to possibly achieve an antiviral effect (Haspeslagh et al., 2016a). However, the thymidine kinase necessary for initial phosphorylation of the drug is missing in BPV DNA (Baker and Howley, 1987) and the susceptibility of the BPV DNA polymerase for acyclovir is unknown. A retrospective study further described complete regression after topical acyclovir treatment in only 53% of the cases (Haspeslagh et al., 2016b).

The goal of the present study was to establish if topical treatment of occult equine sarcoids with a 5% acyclovir cream is more effective compared to the application of a placebo cream following the same administration protocol.

Materials and methods

Subjects

A power analysis revealed that at least 15 unrelated sarcoids were needed in each treatment group to be able to show a difference between acyclovir and placebo treatments (power = 0.8; type-1 error = 0.05). Because multiple sarcoids on the same horse would be treated the same way, a minimum of 15 horses was necessary in each treatment group.

All horses that were presented to the Department of Surgery and Anaesthesiology of the Faculty of Veterinary Medicine of Ghent University for the treatment of previously untreated occult or partly verrucous equine sarcoids were considered for inclusion in the study. Horses that had fibroblastic or nodular sarcoids in addition to the occult and/or partly verrucous tumours and horses that received concurrent medical treatment for other indications were excluded. The diagnosis of equine sarcoid was made by clinical examination by an experienced veterinarian. As a compensation for taking part in the study, the treatments and consultations were offered free of charge and sarcoids that would have been treated with placebo during the study would be treated afterwards without additional costs. When owners were willing to participate, an informed consent was signed in which the owners also committed to apply the topical treatment as instructed.

Treatment and sampling

Sarcoids were topically treated with either a generic 5% acyclovir cetomacrogol cream or a placebo consisting of the same cetomacrogol cream without active component. The choice between both treatments was made at random and owners were blinded to the treatment. Packaging of the creams was identical and the labels were coded. When multiple occult or partly verrucous sarcoids were present on the same horse, all tumours were treated with the same product.

The sarcoids of both treatment groups were completely covered with cream twice daily by the owner. If cream remnants were still present, the lesion was cleaned with water and dried with a paper towel before applying more. Owners were instructed and demonstrated to beforehand how to apply the cream. Treatment continued for six

months or until the sarcoid had disappeared completely. The experiment was stopped if sudden aggressive growth of the sarcoid would occur.

Before the start of the treatment (T0) and further at monthly intervals (T1 until T6), a close-up photograph of the sarcoid was taken with a ruler next to, but not covering the tumour. To gain more insight in the antiviral effect of acyclovir on BPV in equine sarcoids, a swab sample for BPV DNA analysis was taken at the same time as the photographs by rubbing a sterile cottontip swab soaked in sterile distilled water over the surface of the sarcoid (Martens et al., 2001). Swabs were stored in separate containers at -20 °C until further processing. When the horses were stabled too far away from the clinic, private practitioners were responsible for obtaining the swab samples and photographs and provide them to the clinic. When this was the case, the private practitioners received clear instructions on how to do this to ensure a high sample quality.

All pictures and samples were processed together at the end of the experiment. Pictures were given a random coded file name and put in random order to ensure blinded processing. On all pictures, sarcoid maximal diameter and surface area were measured twice using image measuring software (ImageJ). The mean of two measurements was used for further analysis. Severity of the sarcoid was determined by three diplomates of the European College of Veterinary Surgeons. A visual analog scale (VAS) was used ranging from no visible abnormalities of the skin (score 0) to distinct skin verrucosity (score 1000) (Figure 1). The mean of three scores was used for further analysis.

Swabs were examined for the presence of BPV DNA. DNA was extracted using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen). Swabs were first incubated for 12 hours in 180 µl buffer ATL and 20 µl proteinase K at 56 °C. The samples were then vortexed and 200 µl buffer AL was added. After vortexing again, swabs were removed from the vials and discarded. Further DNA extraction was continued as described in the manual of the kit, following the tissue protocol. After DNA extraction, real-time PCR analysis was performed using general BPV primers and BPV-1 and -2 specific TaqMan probes as described by Bogaert et al. (Bogaert et al., 2007). All samples were processed in duplicate and when quantification cycle values between repeats differed by more than one, the PCR was repeated. Positive controls consisting of a mixture of

known BPV-1 and BPV-2 samples and negative controls consisting of distilled water were included in each run. All samples were also tested for the presence of equine interferon beta (IFNb) DNA to confirm successful DNA extraction (Haralambus et al., 2010). Samples were considered positive when either BPV-1 or BPV-2 DNA was detected. Samples were only considered negative when IFNb DNA could be detected, but no BPV DNA. When no IFNb DNA was detected, samples were marked as missing data for further analysis.

Statistical analysis

All data analysis was performed using statistical software (SPSS 20, IBM). Statistical significance was set at $P \leq 0.05$. To estimate the effect of treatment on the number of fully regressed sarcoids, a Fisher exact test was used. For all continuous data (sarcoid diameter, sarcoid surface area and VAS score), the effect of time and treatment on the dependent variables was determined by repeated measures ANOVAs with horse as a blocking factor. When sphericity could not be assumed, a Greenhouse-Geisser correction was applied. Additionally, a “change parameter” was calculated for continuous data from all sarcoids as the difference between the measurement at T0 and the last measurement. The effect of treatment on this “change parameter” was estimated by a generalized linear model, corrected for follow-up time and with horse as a blocking factor. For each time point, the effect of treatment on the number of samples positive for BPV DNA was tested using a binary logistic regression with horse as a blocking factor. To test the effect of time on the number of samples positive for BPV DNA, a binary logistic regression with horse as a blocking factor was used for both treatments with T0 as the reference category.

Results

Twenty-eight horses and three ponies were included in the study. In total, 24 sarcoids on 15 individuals were treated topically with 5% acyclovir cream and 25 sarcoids on 16 individuals were treated topically with placebo cream. Multiple sarcoids were present in four horses in the acyclovir group, and in five horses in the placebo group. For both groups, median treatment time was six months (min: one month, max: six months). The study was stopped early because of sudden aggressive tumoural growth in one horse, which was part of the acyclovir treatment group. For three placebo treated horses and one acyclovir treated horse, the study was stopped early because of

complete sarcoid regression. No side effects were observed in any of the treated horses.

Complete regression during treatment occurred in two of the acyclovir treated sarcoids (8.3%; 95% CI: 1.0% - 27.0%), while this was the case in four (16%; 95% CI: 4.5% - 36.1%) of the placebo treated sarcoid. This difference was not significant ($P = 0.67$).

Figure 2 shows the mean measurements along with the 95% confidence interval of sarcoid diameter (A), sarcoid surface (B) and VAS score (C) at each time point for both treatment groups. The intraclass correlation coefficient of the raters for average measures of VAS was 77.0%. There was no significant effect of treatment or time on any of these variables. The mean calculated "change parameters" are listed in Table 1 along with the P-values for the effect of treatment on them. A positive "change parameter" indicates a decrease in measurement between T0 and the last sample point whereas a negative change indicates an increase. The mean largest sarcoid diameter of acyclovir treated tumours increased during treatment, while it decreased for the placebo treated sarcoids (Table 1). Mean surface area increased during the treatment for both groups (Table 1). Mean VAS score decreased, indicating that sarcoids were found to be less verrucous towards the end of the treatment (Table 1). Differences in "change parameters" between both treatment groups were never significant.

No genomic DNA was present on the swab in 15.9% of the samples in the acyclovir treated group and in 22.8% of the samples in the placebo group. Figure 3 shows the percentage of positive PCR samples in each treatment group at each time point. Only at T1, a significantly higher percentage of samples was positive for the presence of BPV DNA in the acyclovir group compared to the placebo group ($P = 0.005$). At all other time points, there were no significant differences between groups. In the acyclovir group, the percentage of positive samples was significantly higher at T1 compared to T0 ($P = 0.004$). At all other time points, the percentage of positive samples was not significantly different from T0. In the placebo group, the percentage of positive samples was significantly lower at all time points except for T1, compared to the percentage of positive samples at T0 (T2: $P = 0.06$; T3: $P = 0.03$; T4: $P = 0.04$; T5: $P = 0.04$; T6: $P = 0.04$).

Table 1 - Mean "change parameters" and associated p-values for the difference between both treatment groups (SE = Standard Error; VAS = visual analog scale).

Change	Acyclovir (± SE)	Placebo (± SE)	p-value
Diameter (mm)	-6.10 (± 4.63)	2.02 (± 5.67)	0.52
Surface area (mm²)	-314.26 (± 203.44)	-60.25 (± 247.67)	0.87
VAS score	146.43 (± 53.75)	166.58 (± 39.41)	0.65

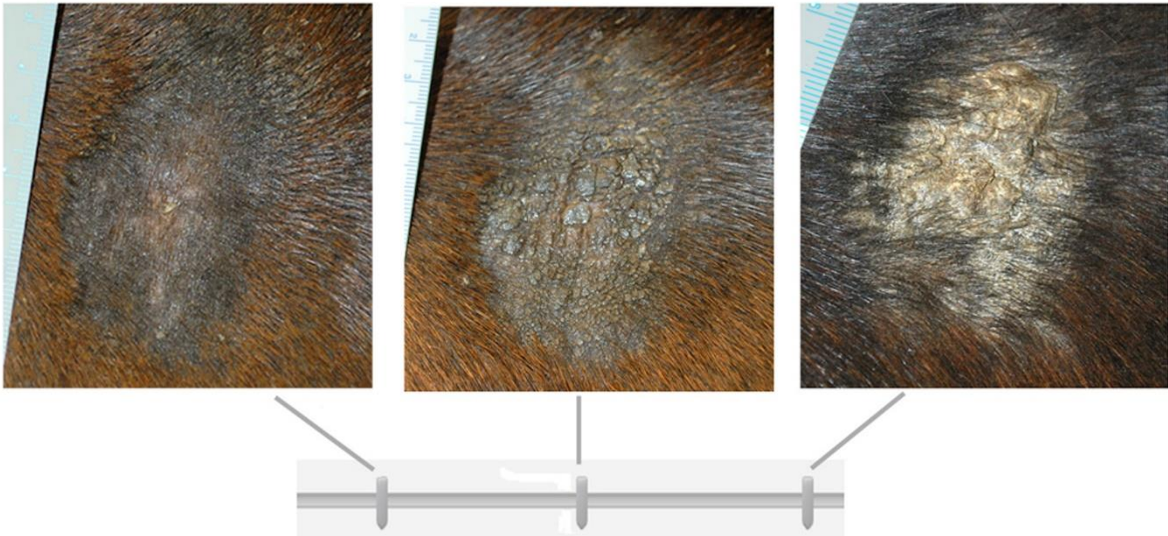

Figure 1 - Example of how a slider bar was used to determine severity of a sarcoid on a visual analog scale (VAS), based on verrucosity.

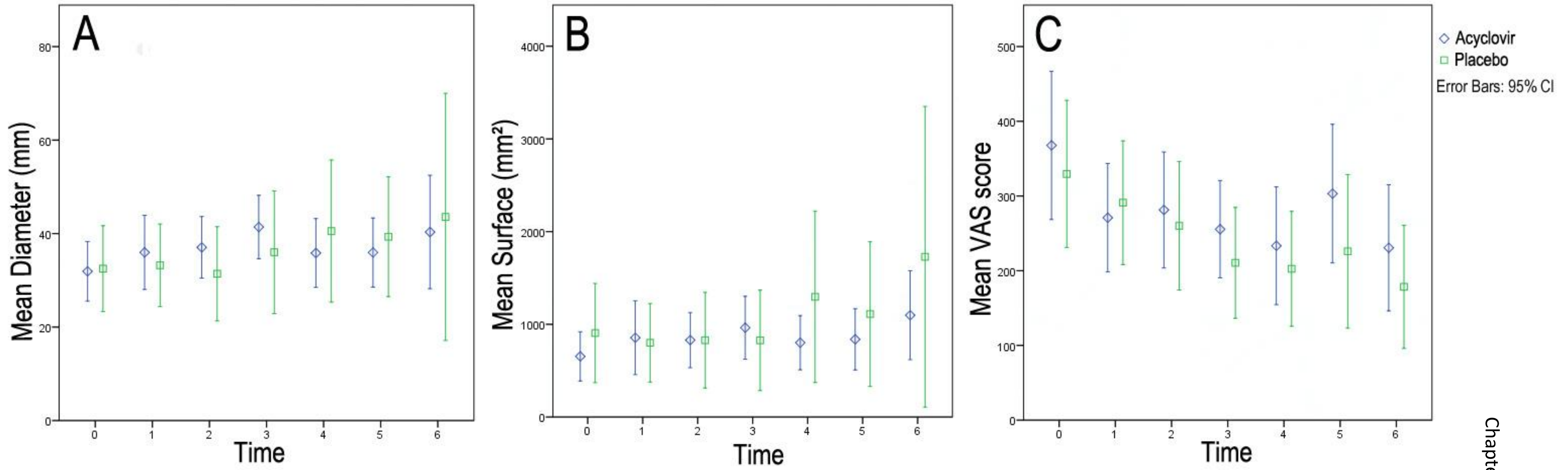

Figure 2 - Mean measurements along with the 95% confidence interval of sarcoid diameter (A), sarcoid surface (B) and visual analog scale (VAS) score (C) at each time point for both treatment groups.

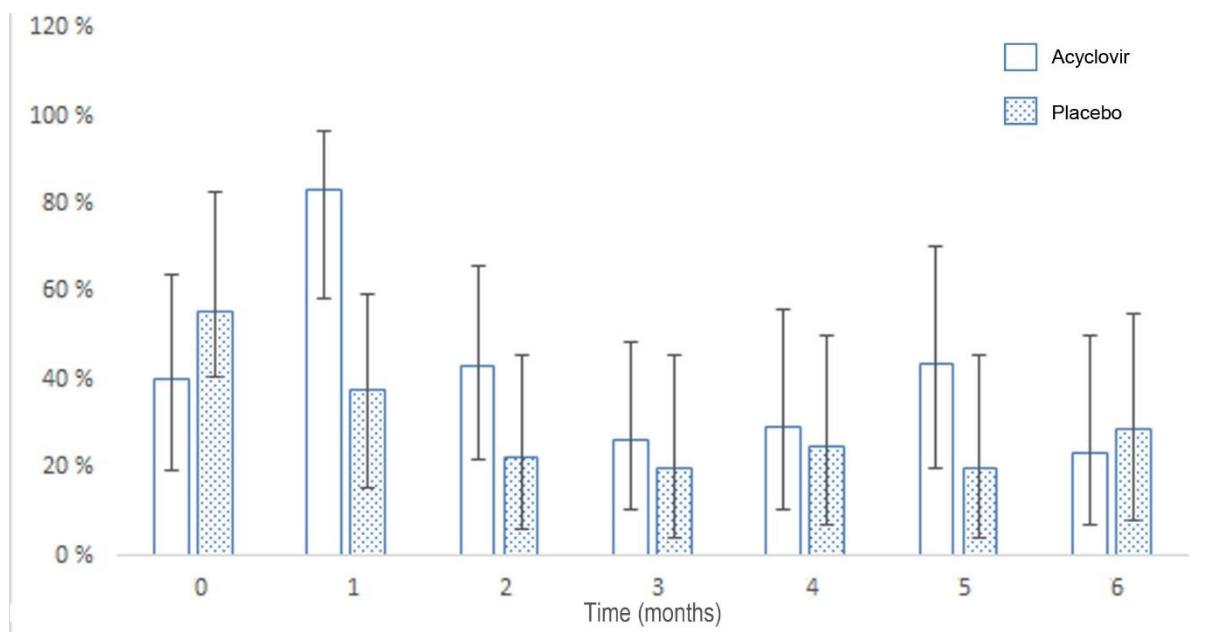

Figure 3 - The percentage of samples positive for the presence of BPV DNA at each time point (months after start of the treatment) and corresponding 95% confidence intervals. No fill: acyclovir; dotted fill: placebo.

Discussion

This is the first study on equine sarcoid treatment which is placebo controlled and double blinded. Results of this study are therefore valuable for treatment selection in practice. Because of the long study duration, the horses were cared for by their owners at home and this implicated that it was hard to check for treatment compliance. However, all owners signed a commitment to the experiment beforehand and were contacted regularly by phone to monitor the course of the study. Because the horses were stabled at home and this was in many cases too far away to allow for visits to the clinic, monthly visits were often performed by trusted private practitioners. While this implicates a possible variation in sample quality, practitioners received clear instructions on how to take pictures and swabs to maximize uniformity. A benefit of this was that the persons evaluating the pictures and analyzing the samples never saw the patients in real life, which enabled an unprejudiced evaluation.

The success rate for acyclovir treatment was lower compared to previously reported success rates (Stadler et al., 2011; Haspeslagh et al., 2016b). The sample population

of the present study was smaller, which could explain the lower success rates. Moreover, acyclovir treatment was stopped after 6 months of treatment in the present study, while under normal clinical circumstances it is often continued longer when the tumour has not fully regressed yet, but a beneficial effect is observed. In both previous studies, a certain number of sarcoids have indeed been treated for over six months, which could have increased the success rate. In the study of Stadler et Al. (2011), some lesions were ablated before acyclovir treatment, which could have improved the results. The sarcoids that responded well to acyclovir treatment in that study were all smaller than 5 mm in diameter (Stadler et Al., 2011), which is a lot smaller compared to the lesions that were used for the present study (Figure 2). While the concentration of acyclovir in the cream used in the present study was the same as in all earlier studies (Stadler et al., 2011; Haspeslagh et al., 2016b), the constitution of the cream vehicle used in this and earlier studies by Haspeslagh et al. (Haspeslagh et al., 2016a, 2016b) differed from the one used by Stadler et al. (Stadler et al., 2011). The cream base was altered because sedimentation occurred when using the cream formulation of Stadler et al., which lead to a heterogeneous acyclovir concentration.

Time and treatment type did not have a significant influence on mean sarcoid dimensions or mean VAS score. Mean “change parameters” were also not significantly different between treatment groups. Nevertheless, the mean VAS score decreased for both treatment groups during the study, indicating that equine sarcoids were found less verrucous towards the end of the treatment. Perhaps the previously observed benign effect is therefore not due to the application of acyclovir, but merely to the effect of a cream that keeps the skin hydrated, preventing the formation of a thick verrucous layer. Anti-keratotic creams have analogously been used to lessen the verrucosity of equine sarcoids prior to other treatments (Quinn, 2003). This hypothesis can be tested by including a third “no treatment” group, which was not done here due to ethical considerations towards the owners. The presence of a keratinous layer in equine sarcoids with a high degree of verrucosity could interfere with acyclovir penetration and the presence of highly verrucous tumours could have influenced the results. Nevertheless, the mean VAS score, based on verrucosity, was not very high at T0 and decreased during treatment. VAS scores at T0 did also not differ significantly between the acyclovir and placebo group, indicating that the comparison between both groups is valid. To evaluate the effect of a thick verrucous layer on acyclovir treatment, a

similar experiment could be performed comparing treatment of strictly occult versus strictly verrucous tumours.

In order to obtain a good indication of the BPV load in non-ulcerated equine sarcoids, a quantitative real-time PCR on tissue from a biopsy probably yields the most reliable results. However, this would have required the sarcoids to be biopsied prior to the study and further at each time point of evaluation, which could have influenced the outcome, as equine sarcoids often become more aggressive after being damaged (Knottenbelt et al., 1995; Bergvall, 2013). For this reason, swabs were obtained instead of biopsies. This also implies that histological examination to confirm the diagnosis of equine sarcoid could not be performed and that there is a chance that some lesions which regressed spontaneously were not actual equine sarcoids. Nevertheless, the clinical appearance of equine sarcoids is so typical that clinical examination by an experienced veterinarian should be sufficient to make a correct diagnosis (Knottenbelt et al., 1995; Bogaert et al., 2008) (chapter 4). While the presence of BPV DNA can be shown in up to 100% of swabs from equine sarcoids, this is only the case in ulcerated tumours where the dermis is exposed (Martens et al., 2001). As none of the tumours were ulcerated in the present study, the percentage of positive samples was lower and in range with the earlier reported positivity rate originating from occult sarcoids (Martens et al., 2001). Nevertheless, in the placebo group, a clear and significant decrease in the positivity rate could be seen over time, which was not the case for acyclovir treated tumours. No plausible explanation could be given for this observation.

In conclusion, none of the results presented in this study indicate that topical treatment of occult or partly verrucous equine sarcoids with acyclovir yields any better results compared to treatment with placebo cream.

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General discussion

Equine sarcoids are among the most common tumours in equids. Nevertheless they remain relatively unknown to the wider public and their repercussions on animal value and well-being are often underestimated. Because of the complexity of the pathogenesis of equine sarcoids, research in this field is difficult and advances at a slow rate only. In addition to the research which has been presented in the previous chapters of this work, the author had the opportunity to devote a significant amount of time to clinical work, treating over 250 equine sarcoid cases. Thanks to this unique immersion in all facets of the disease, a broader vision could be developed on what is necessary to advance equine sarcoid treatment. Many aspects of this disease remain uncertain and these shortcomings in our understanding form important bottlenecks for treatment or prevention of these tumours. Clearing all or even some of these bottlenecks would increase our knowledge of the disease pathways, and would give us new opportunities to interfere with those pathways or even disrupt them. This could lead to the discovery of new treatments, aimed directly at one or more aspects of the pathogenesis. Therefore, the final chapter of this work will be dedicated to addressing these bottlenecks and identifying opportunities for future research.

Etiopathogenesis and disease transmission

It is currently widely accepted that the bovine papillomavirus (BPV) is the main causative agent in equine sarcoid development (Nasir and Campo, 2008). As a consequence, equine sarcoids can be thought of as an infectious disease and some key elements are necessary for such a disease to develop. These key elements are transmission of the infectious agent and infection of the host, immune evasion, and cellular transformation.

Transmission and infection

As discussed in chapter 1, it is assumed that BPV infection in equids is abortive and the disease cannot spread from equid to equid. While this is still the main belief, evidence in favour of infectious virus production in equine sarcoids is accumulating and culminates in one electron microscopic picture of something what might be an infectious BPV virion in an equine sarcoid (Wilson et al., 2013). In the same publication, the authors however state that they do not believe that a substantial amount of infectious virus is being produced in equine sarcoids, but that further research, especially aimed at occult sarcoids, is needed to support this statement (Wilson et al.,

2013). Electron microscopy would currently be the most efficient tool for detection of infectious virus in equine sarcoids, but since BPV DNA copy numbers in sarcoids can be as low as 0.001 copy per cell (Haralambus et al., 2010), there is an urgent need for a method to concentrate intact virus in equine sarcoid cell extracts. One way to achieve this would be through the use of antibody capture techniques, for example as described by Brandt et al. (2008b). During the past years, experiments with a technique involving microscopically small magnetic beads (Dynabeads, ThermoFisher) coated with antibodies against the capsid L1 protein were carried out at our department (unpublished data). The idea is that intact virus is being captured by the antibody coated beads, which in turn are concentrated by a magnet. Once suspended in a smaller volume, the antibodies can then be dissolved and the beads removed, leaving a concentrated virus suspension. While the first results were disappointing because no virus could be visualised, this could have been due to the contrast technique for electron microscopy. The magnetic bead technique could potentially be explored more thoroughly and prove to be an elegant way of achieving higher virus concentrations in samples. Combined with the quantitative PCR that was developed for chapters 3 and 8, the technique could even be proof of presence of capsid associated viral DNA. Quantitative PCR could be carried out on paired samples, before and after concentration with the magnetic bead protocol. If after the protocol a sample contains a higher DNA concentration compared to before, this proves that the sample contains DNA in conjunction with viral capsid proteins. Other ways of concentrating virus particles in fluids include centrifugal ultrafiltration or polyethylene glycol precipitation, but both techniques require a very clean fluid sample free of debris, which is typically not the case with tumour samples.

Regardless of whether equine sarcoids are productive sources of BPV, sudden onsets of sarcoid cases have been described in isolated, formerly sarcoid free, populations (Nel et al., 2006; van Dyk et al., 2009). This indicates that the virus is being transmitted in another way than by direct contact. In chapter 3, it was established that the stable fly (*Stomoxys calcitrans*) can become positive for BPV DNA in controlled conditions and the possible role of the stable fly in sarcoid transmission was discussed. There are however other insects (for example *Haematobia irritans* or different tabanid species) that are capable of disease transmission and have not been investigated yet. The methods established in chapter 3 can easily be adapted to accommodate other vector

research. When one or more possible vectors are identified, it will be important to define exactly how the disease is being transmitted. It is not because a certain insect is capable of becoming positive for BPV DNA that infectious virus will be present and that the insect is capable of actually inducing equine sarcoids. The possible advances in electron microscopy that were mentioned earlier can assist in identifying infectious virus in vectors and transmission experiments will be necessary to establish whether a biting vector is needed, or if skin lacerations need to be present in the case of a non-biting vector.

Immune evasion and cellular transformation

The current knowledge on the pathogenesis of equine sarcoids is discussed in chapter 1. Evasion of the immune system seems to be one of the key factors for the BPV to be able to infect and transform cells. A better understanding of the mechanisms involved in immune evasion could identify key pathways for the virus to escape the immune system and finally result in new ways to prevent equine sarcoid disease by interfering with these pathways. The predisposition of sarcoids to develop in horses carrying certain alleles of the equine leukocyte antigen (ELA) gene suggests that breeding associations could potentially be able to select against sarcoid susceptibility and even if heredity of sarcoid susceptibility would be proven to be polygenic this would still remain true, as a heritability of 21% on the liability scale has been demonstrated. (Christen et al., 2014).

Transformation of fibroblasts is most likely mainly driven by viral E5 expression as discussed in chapter 1. While the effects of E5 expression are well understood in bovine epithelial cells, this is not the case in equine fibroblasts and *in vitro* experiments on equine fibroblast cell lines are needed to better understand the effects of E5 on cytokine expression and cell growth. Blocking the expression of viral oncogenes by using small interfering RNA (SiRNA) could be an innovative way to treat or even prevent sarcoid disease, although certain hurdles will need to be taken first (see *treatment* further in this chapter).

Equine sarcoids are mostly believed to be the result of a localized BPV infection and recurrence after treatment is being chalked up to incomplete treatment of the affected tissues. If BPV infection occurs through insects as suggested in chapter 3, the occurrence of multiple sarcoids can be the result of individual *de novo* infections at

different body sites by the same vector. Another explanation for the fact that horses tend to have multiple sarcoids on different body sites is found in the discovery of BPV DNA in PBMCs of sarcoid affected horses (Brandt et al., 2008a). This has led some to believe that equids go through a viraemic phase before sarcoids develop (Hartl et al., 2011) or that BPV persists in a latent form in PBMCs. Curiously, only E5 DNA and no L1 DNA could be detected in equine PBMCs, suggesting an altered viral DNA at least in equine PBMCs (Brandt et al., 2008a). In cattle, BPV2 has been shown to reside in PBMCs as well, and E5 oncoprotein and L1 capsid protein were also detected in bovine PBMCs (Roperto et al., 2011). E5 expression was not tested in equine PBMCs and doing so could enhance our understanding of BPV infection in horses, as E5 expression could induce transformation of PBMCs, which in turn could enhance immune-evasion by the virus. Using the same protocol as published earlier (Brandt et al., 2008a), it was at our facilities not possible to detect BPV DNA in PBMCs of any of the 7 sarcoid affected horse that were tested (unpublished data). This could indicate that the viraemic phase is short or viral latency in PBMCs does not always occur, which in turn could be related to equine immunity and genetics.

Diagnosis

In sarcoid research, a correct and reliable diagnosis is paramount: it needs to be certain that the lesions under investigation for an experiment actually are equine sarcoids.

Histopathology remains the gold standard for equine sarcoid diagnosis, but even histologically, a correct diagnosis is not always easy to make and depends heavily on the experience of the pathologist. As discussed in chapter 1, typical histological properties of the equine sarcoid include picket-fence formation at the dermo-epidermal junction, long rete ridges and a cell-rich dermis of densely stacked fibroblasts in whorls or bundles. While these properties are indeed typical for equine sarcoids, they are not always present in all cases (Martens et al., 2000) and to make matters even worse, extensive ulceration or secondary inflammation in samples can make it even more difficult to come to a diagnosis (Wobeser, 2017). Combined with the fact that other (neuro)fibromas can closely resemble equine sarcoids both clinically and histologically, misdiagnosis is not uncommon, even by experienced pathologists. Therefore, there is a need for immunohistological markers that are discriminatory for equine sarcoids. The

combination of presence of BPV DNA and absence of S-100 protein in equine sarcoid tissue samples has been proposed to discriminate them from schwannomas (Bogaert et al., 2011), but further investigation revealed that the combination of these characteristics was not enough to positively identify equine sarcoids from other skin tumours in the horse (Epperson and Castleman, 2017). Because BPV interferes with ELA functionality in the host cells, it can be assumed that antigen presentation at the cell surface is limited and the search for specific markers should be aimed at intracellular antigens such as the early oncoproteins E5, E6 and E7. P38 is overexpressed in equine sarcoid cell lines (Yuan et al., 2011b), as is p53 (Finlay et al., 2012), but overexpression of these proteins is not very specific for equine sarcoids. Recently, the complete transcriptome of equine sarcoid derived fibroblasts has been compared to that fibroblasts originating from healthy equine skin and over 900 transcriptional differences were found (Semik et al., 2017). Perhaps the key to a sarcoid specific marker can be found there.

Taking biopsies of suspected equine sarcoids is associated with risk of sudden growth (Scott and Miller, 2011) and it is therefore not always advisable to do so in cases where owners are not immediately willing to treat. In sarcoid research, when experimenting on clinical cases, a confirmed diagnosis is often needed. However, for reasons mentioned above, the act of taking a biopsy can in itself affect the outcome of such experiments and obtaining a histological diagnosis is therefore out of the question. For those cases, the detection of BPV DNA in superficial swabs (Martens et al., 2001) can be an elegant and reliable solution, although BPV DNA has also been detected in healthy equine skin (Bogaert et al., 2005) and sarcoid unrelated lesions (Brandt et al., 2011). Besides, in non-ulcerated occult and nodular sarcoids, detection of BPV in superficial swabs is not always possible (Martens et al., 2001). In order to obtain a diagnosis for those clinical cases, fine needle aspirates of the lesions can be taken. Because equine sarcoids cannot be diagnosed histologically on fine needle aspirates, the samples can be analyzed for the presence of BPV DNA by PCR. Fine needle aspirates are far less invasive compared to biopsies and therefore, the risk of tumoural exacerbation is limited. By this method, 18 histologically confirmed sarcoids were positively identified and 9 histologically confirmed non-sarcoids were negatively identified (unpublished data). Of course, a higher sample number and more elaborate research protocol are needed to validate this diagnostic technique, but these

preliminary results are promising. Fine needle aspirates could be useful for diagnosing non-ulcerated lesions by PCR and at the same time prevent false positive results from non-sarcoid lesions contaminated on the surface by BPV DNA.

Private practitioners and non-university clinics are not always aware of PCR techniques for sarcoid diagnosis, or do not opt for them for logistical reasons and are therefore limited to histological examination of the lesion. In cases where taking a biopsy is unwanted, there are no further diagnostic tools available and one has to rely on the clinical diagnosis. In chapter 4, the ability of veterinary professionals of different expertise levels and undergraduate students to correctly diagnose equine sarcoids was tested. Clinical diagnosis of equine sarcoids proved to be highly reliable, with an overall sensitivity of 83.3% and an overall specificity of 79.6%. Clinical diagnosis by equine sarcoid experts yielded an even higher sensitivity and specificity. While sensitivity and specificity were also high for other levels of expertise, there was room for improvement. Therefore, a tool to aid inexperienced veterinarians with equine sarcoid diagnosis was developed and tested. The results described in chapter 5 prove that the use of this tool significantly increases the performance of clinical diagnosis, especially for the least experienced veterinarians. The tool needs to be developed further and tested on more cases, but it has the potential to become a benchmark to make clinical diagnosis more objective. This will ultimately lead to clinical diagnosis being accepted as a validated diagnostic method in equine sarcoid research, especially when the diagnosis was made by veterinarians fulfilling the requirements to meet the equine sarcoid expert level, or when less experienced veterinarians use the diagnostic protocol, as explained in chapters 4 and 5.

The current categorisation of equine sarcoids into 5 subtypes (occult, verrucous, nodular, fibroblastic and mixed (Pascoe and Knottenbelt, 1999)) is theoretically very straightforward, but sometimes difficult to apply on clinical cases. Especially for mixed sarcoids, it is not clear in the current nomenclature how the different aspects of the sarcoid are proportioned. In addition, most of the sarcoid cases have characteristics of more than one sarcoid type. This can lead to confusion when describing cases, both in research and in clinical practice. Figure 1 shows an example of such a case where categorizing the lesion can be confusing: by strict definition this would be a mixed nodular fibroblastic lesion, although the fibroblastic portion is very small and the lesion is actually of the nodular type. To streamline clinical descriptions and to avoid

confusion in communication on equine sarcoids, it could be helpful to avoid using the term “mixed equine sarcoid” altogether and instead to clearly describe the lesion using the remaining 4 clinical types. The example in Figure 1 would then be a nodular equine sarcoid with local ulceration in an area of 5 by 5 mm. The term “mixed sarcoid” can then be reserved for those cases where two or more sarcoid types are present in approximately equal proportions in the same lesion.

Figure 1 - Describing this lesion according to the classic nomenclature can be confusing.

In order to be able to develop preventive treatments (see *treatment* later in this chapter), researchers and clinicians could benefit from a method for early detection of BPV infection, before tumours develop. First steps in this direction have already been taken by the discovery of BPV DNA on the normal skin of unaffected horses living in contact with cattle or sarcoid affected horses (Bogaert et al., 2005) and in PBMCs of sarcoid bearing horses (Brandt et al., 2008a). Nevertheless, the importance of these findings and more specifically their role in tumour development are not understood. Prospective longitudinal studies focussing on the importance of such findings in predicting tumour development will be very difficult, because they imply that a large pool of BPV positive and BPV negative horses without lesions will need to be followed and screened for lesion development, possibly during multiple years.

Treatment

Treatment options for equine sarcoids are discussed in chapter 1. In chapter 6, a methodical approach for treatment selection is proposed and tested. Currently, most - if not all- available treatments are curative: tumours are treated when they become aesthetically or functionally hindering. Ideally, treatments should evolve to become more of a preventive nature. As mentioned before, this necessitates a sound method to be developed for detection of horses that are at high risk of sarcoid development. This could be done on a genetic level, as an association seems to exist between sarcoid susceptibility and certain alleles of the ELA and possibly other genes (see chapter 1), but also on a molecular level, if the presence of BPV DNA in normal skin or PBMCs would prove to have a good predictive value for later sarcoid development. Alternatively, a prophylactic vaccine could be developed and administered to all horses without knowing if they are or will be susceptible for equine sarcoid disease. The current knowledge of the exact pathogenesis and mechanisms for immune-evasion is however insufficient to perform research aimed at specific key processes of sarcoid development and attempts of developing a prophylactic vaccine by trial and error with our current understanding is like playing darts in a dark room.

Efforts have been made to produce a vaccine based on BPV-1 virus-like particles (VLPs) to prevent sarcoid formation (Hainisch et al., 2016). The results look promising and are certainly valuable in the greater light of the development of a preventative treatment. Nevertheless, the experiment suffers from a circle argument. VLP vaccinated horses were challenged with intradermal BPV inoculations, which are known to produce nodular lesions *similar to* equine sarcoids, but they are not *actual* equine sarcoids and disappear over time (Hainisch et al., 2009), likely due to spontaneous development of an immune response (Hartl et al., 2011). If we think of these artificially induced lesions as being localized BPV infections, not sarcoids, all that is achieved by VLP vaccination is that the immune response is being developed before intradermal BPV inoculation instead of after. As a logical result, the localized BPV infection is being prevented from developing in vaccinated horses, which does not necessarily mean that the vaccine is of any use for preventing actual sarcoid development. The big culprit here is the absence of a good disease model. There is no reliable and repeatable method to induce equine sarcoids in unaffected equids and as a result, there is no good way to challenge any preventive treatment, other than to

treat a large population and observe it over time for the development of sarcoids. Since epidemic outbreaks of sarcoids in isolated populations have been described (Nel et al., 2006; Abel-Reichwald et al., 2016), preventive treatment of a number of unaffected animals in such a population could be a way to test said treatment. Nevertheless, research would greatly benefit from the development of a reliable sarcoid model, but again the development of this model will be very difficult without fully understanding the complete pathogenesis first.

Until a preventive treatment can be developed, veterinarians are stuck treating the symptoms of the disease. The currently best available treatments require surgery, which is expensive, unpractical and often implies a risk of anaesthesia and recovery (see chapter 6). Therefore, and because the horse can be treated at home, topical treatments are popular among horse owners. Topical treatment with acyclovir had the potential of becoming popular (Stadler et al., 2011), because it is relatively cheap, easy to obtain and use, and free of undesired side effects. Nevertheless, in chapter 7 and 8, it was proven that treating occult equine sarcoids topically with acyclovir did not lead to better results compared to a placebo treatment. Other topical treatments that have been described for the treatment of sarcoids (see chapter 1) have painful side effects, which are not always well tolerated in horses. Because of the demand for topical treatments without adverse effects, the value of other topical agents specifically targeting viral processes can be researched, but to date, none exist that target papillomaviruses. Potentially, the results of topical treatment with imiquimod could be improved by pre-treatment of the skin with a keratolytic cream containing tazarotene or salicylic acid. Treatment of equine sarcoids with 5-fluorouracil could be explored.

Advances in biotechnology have led to the discovery of SiRNA (Hamilton and Baulcombe, 1999; Agrawal et al., 2003). SiRNAs are small pieces of dual strand RNA which, once they enter a cell, divide into single strand RNA and form a complex with intracellular proteins, keeping them stable. These RNA-protein complexes (called RNA-induced silencing complexes) then bind to their complementary messenger RNA and inhibit its translation by degrading it. The final result is that expression of a gene is inhibited by disruption of the pathway to protein assembly. Artificial SiRNA is now widely used in genetic research as a gene silencer, but since this process is very specific, it seems to be a good candidate to treat viral diseases with minimal side effects to the host cell. In a mouse model for human papillomavirus induced cervical

cancer, SiRNAs targeting E6 and E7 successfully reduced tumour volumes (Jonson et al., 2008). *In vitro* experiments have demonstrated that BPV infected equine fibroblasts showed a lower viral load, slower and less invasive growth and more apoptosis after SiRNA treatment targeting E2, compared to non-treated cell cultures (Gobeil et al., 2009; Yuan et al., 2010). SiRNAs targeting E6 were even more efficient at inhibiting cell proliferation (Yuan et al., 2011). SiRNAs targeting E5 were less efficient (Yuan et al., 2011), which is curious, because E5 is believed to be the main oncoprotein driving cellular transformation. These results are very promising, both because SiRNA targeting of specific BPV proteins can help us to understand the specific functions of these proteins and because blocking of key proteins could prevent or even revert transformation of the host cell. There are however some difficulties that need to be overcome before SiRNA can be applied as a routine *in vivo* treatment for equine sarcoids. Most importantly, the artificial SiRNAs need to be deposited intracellularly. This is *in vitro* most often achieved by a process called transfection, for which the SiRNA is bound to a carrier that easily crosses the cell membrane. The *in vivo* efficiency of transfection, however, is low (SABiosciences, 2009). Alternatively, SiRNA can be delivered into the cell by electroporation. This has already been applied to enhance intracellular delivery of cytotoxic drugs in equine sarcoids (Tamzali et al., 2012; Souza et al., 2016), but it is difficult to separate the clinical effects of the electroporation itself from the effects of the drug being tested. A third way of getting SiRNA into a cell is by incorporating it in a viral vector (SABiosciences, 2009). Ultimately, this could lead to integration of the precursor of the SiRNA into the host genome. By doing so, the host cell would permanently produce the SiRNA and make itself resistant against the targeted viral influences.

Conclusion

The results of the research presented in this work have added important information to our knowledge and understanding of equine sarcoids. In chapter 3, it was demonstrated that airborne insects can play a role in BPV transmission. The protocol that was established to work with flies under controlled conditions can now easily be adapted to other insects and can be used to ultimately perform transmission experiments. In chapters 4 and 5, it was established that clinical diagnosis of equine sarcoids is reliable, and the foundation for a protocol for clinical sarcoid diagnosis was built. This means that clinical diagnosis should now be accepted as a valid tool in

experimental research where other diagnostic methods might interfere with the outcome. Ultimately, a guideline for equine sarcoid diagnosis will be developed, aiding equine practitioners in the decision whether or not to pursue further (invasive) diagnostics. The treatment selection protocol proposed in chapter 6 has proven to obtain a high overall success rate and can be applied in practice to improve treatment outcome and provide a realistic prognosis. In chapters 7 and 8, it was proven that topical acyclovir is not more effective to treat sarcoids compared to placebo treatment. Therefore, it is not recommended to continue with topical acyclovir treatment of equine sarcoids and for occult lesions, imiquimod can be used as a topical alternative.

In the future, efforts in equine sarcoid research should be aimed at unravelling the mysteries surrounding BPV transmission, pathogenesis and immune-evasion as this knowledge is the key to all further treatment development. A broader understanding of these topics will lead to the prevention of BPV infection and the development of treatments specifically aimed at key processes in the transformation of fibroblast cells into sarcoid cells. A technique to reliably induce equine sarcoids in healthy animals is needed to challenge studies on genetic predisposition and prophylactic vaccine development. Finally, the possibilities of novel techniques like SiRNA integration in the host genome need to be explored.

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Equine sarcoids are common tumours of the equine dermis which do not metastasize and are no immediate threat to the lives of affected animals. Nevertheless, they are often underestimated and can grow into large masses with severe implications for an animal's welfare and value. Because equine sarcoids can have different clinical presentations and because there are no specific histological markers, diagnosis is often difficult for inexperienced veterinarians. It is now widely accepted that equine sarcoids are caused by the bovine papillomavirus (BPV), but the exact mechanisms that lead to disease spread, fibroblast infection, cellular transformation and tumour development are ill understood. Therefore, it should not come as a surprise that these tumours are notoriously difficult to treat. Many treatments have been described, but as of today, no universally successful treatment exists and selection of the optimal treatment for a given sarcoid is an erratic process. In this work, a research project is described, which aims to shed light on different aspects of the disease with the ultimate goal of improving clinical management of these tumours.

In **chapter 1**, the current knowledge on equine sarcoids is reviewed. The chapter deals with the etiopathogenesis, epidemiology and transmission of the disease, as well as the clinical presentation, diagnosis and treatment. While certain of these aspects are well studied, clinical management of equine sarcoids would greatly benefit from further investigation of unexplored facets of the disease. This chapter serves to justify why this research project was undertaken and is the foundation for understanding the further chapters of this work.

Chapter 2 describes the main ambitions of this work. The general aim was to improve clinical management of equine sarcoids. This was done directly by validating the clinical diagnosis of equine sarcoids against histopathology, by developing and testing a treatment selection protocol for equine sarcoids, and by formal testing of topical sarcoid treatment with acyclovir. The knowledge gained by looking into a theory for BPV spread indirectly adds to the general aim by opening the door for disease prevention.

The often insinuated hypothesis that flies act as a vector for BPV transmission to equids was tested in **chapter 3**. The stable fly (*Stomoxys calcitrans*) was selected because of its abundance around both cattle and horses and because of its biting

mouthparts which are ideal for BPV deposition at the level of the dermis. Bovine papillomavirus negative stable flies were live caught and exposed to equine sarcoid or bovine wart tissue. The BPV viral loads of exposed flies were measured every 24 hours by quantitative real-time PCR until 7 days after exposure. Viral loads raised significantly immediately after exposure to bovine wart tissue and equine sarcoid tissue, but were higher and remained high for a longer time in the latter group. This indicates that BPV transmission by *Stomoxys calcitrans* seems possible and more likely occurs from bovines to equids as opposed to between equids. Clinical transmission experiments are necessary to establish if transmission of BPV by stable flies can indeed cause equine sarcoids in formerly BPV negative horses.

Histopathological diagnosis of equine sarcoids is not always feasible or wanted, because taking a biopsy comes with a risk of lesion exacerbation or because this interferes with the results of clinical trials. Clinical diagnosis would be a good alternative, but because its reliability has never been tested, it is not recognised as valid by the scientific community. This is remediated in **chapter 4**. Forty cases of different equine skin diseases were presented in an online examination to respondents of different expertise levels. The cases were carefully selected to realistically represent the distribution of equine sarcoids and other skin diseases in the population and respondents were asked to clinically diagnose them either as equine sarcoids or as not equine sarcoids. Clinical diagnosis of equine sarcoids proved to have a high sensitivity and specificity overall (respectively 83.3% and 79.6%) and the mean success rate for all respondents was 82.0%. Sensitivity and specificity were 89% and 86% respectively for equine sarcoid experts, while lesser experienced respondents were significantly less able to correctly diagnose equine sarcoids. Clinical diagnosis should now be accepted as valid. For inexperienced respondents, there is room for improvement.

To improve the ability of inexperienced respondents to correctly discriminate equine sarcoids from other skin lesions, in **chapter 5**, a diagnostic protocol was developed to assist them in making a correct clinical diagnosis. The diagnostic protocol was used by inexperienced respondents to diagnose the same 40 cases that were used in chapter 4 and their results were compared to the respondents who did not have access to the diagnostic protocol. Overall, respondents using the diagnostic protocol were more likely to make a correct diagnosis compared to respondents not using the

protocol. Inexperienced respondents using the protocol scored on par with equine sarcoid experts and were more confident of their diagnosis compared to their peers who did not use the protocol, proving that the tool is a useful guide to help inexperienced veterinarians to correctly diagnose equine sarcoids.

Equine sarcoids are very heterogeneous in clinical presentation and body location. This, combined with the fact that no universally successful treatment exists, makes adequate treatment selection for a given sarcoid on a given location difficult. In **chapter 6**, a treatment selection protocol is described based on tumour type and location. Included treatments were topical administration of acyclovir or imiquimod, non-touch electrosurgical excision (with or without intralesional cisplatin), cryosurgery, Bacillus Calmette-Guerin vaccine injection, and intralesional injection of platinum-containing drugs. The treatment selection protocol was applied to 230 clinical cases (614 sarcoids) and a follow-up at least 6 months after the last treatment was done. The overall success rate using this treatment selection protocol was 74.9%. Electrosurgical non-touch excision was performed most frequently and had the highest success rate (86.8%). Significantly lower success rates were obtained with topical administration of acyclovir, cryosurgery and intralesional application of platinum-containing drugs. When a sarcoid was located on a horse with multiple equine sarcoids, chances of success were significantly lower compared to when this was not the case. For sarcoids present on a horse that concurrently received immunostimulating treatment for another sarcoid, chances of successful treatment were significantly higher compared to when this was not the case. When a topical treatment was possible, owners could choose between acyclovir or imiquimod application. While significance was not reached, better results were obtained with imiquimod compared to acyclovir.

Topical treatment of equine sarcoids with acyclovir has been proposed to achieve good results, without the side effects other topical treatments have, in a limited non-controlled clinical trial. In **chapter 7**, it was tested *in vitro* how deep acyclovir penetrates into intact normal and sarcoid affected skin. Equine sarcoid skin samples were surgically removed and matched with normal equine skin samples, harvested from horses that were euthanized for unrelated reasons. After removal of the hypodermis, the samples were used in a Franz cell diffusion experiment, where they were exposed to a 5% acyclovir cetomacrogol cream on the epidermal side during 48 hours. At set time points, the acyclovir concentration in the receptor medium was measured by ultra-

performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Afterwards, the skin was frozen and cut in a cryotome. Epidermis, superficial dermis and deep dermis were separated and the acyclovir concentration in the tissues was measured by UPLC-MS/MS. While acyclovir permeated through both skin types, normal skin was significantly more permeable for acyclovir compared to sarcoid affected skin. In sarcoid skin, significantly more acyclovir was present in the epidermis compared to the superficial dermis and deep dermis, which was not the case in normal equine skin. Nevertheless, acyclovir concentrations found in the dermis of sarcoid affected skin were still high enough to treat a *Herpes simplex* virus infection in humans. Because the acyclovir concentration which is needed to treat BPV infection in equine skin is not known, we can only assume that these concentrations are sufficiently high. If the BPV is susceptible for these concentrations, a beneficial effect of topical acyclovir application on occult equine sarcoids could be possible.

In chapter 7 it was demonstrated that acyclovir is able to reach the dermis of equine sarcoid affected skin in considerable concentrations after topical application. This does however not proof that a beneficial effect can be expected when applied on equine sarcoids and in chapter 6, better results were obtained with topical imiquimod treatment. In **chapter 8**, a double blinded placebo controlled experiment was set up to test whether treating equine sarcoids topically with acyclovir yielded better results compared to a topical placebo treatment. Twenty-four sarcoids on 15 equids were treated twice daily topically with a 5% acyclovir cetomacrogol cream and 25 sarcoids on 16 equids with a placebo cream. Treatment selection was done randomly and treatment continued during 6 months, or until the sarcoid disappeared earlier. Monthly, a swab sample and a digital image were taken of the lesion. After completion of the study, the swabs were analyzed for presence of BPV DNA by PCR. Three diplomats of the European College of Veterinary Surgeons scored the images for sarcoid severity on a visual analog scale (VAS). The exact surface and widest diameter of the sarcoids were also measured on the images. There was no significant difference in lesion surface area, diameter and VAS score between acyclovir and placebo treated lesions at any time point, although the severity of the lesion decreased slightly over time for both treatment groups. There is no evidence that topical treatment of occult or partly verrucous equine sarcoids with acyclovir yields any better results compared to a placebo treatment.

In **chapter 9**, the ideas for the future of equine sarcoid research are presented, based on the clinical and scientific experience that was acquired during this project. Key topics that need to be better understood in order to develop new strategies to treat or even prevent equine sarcoid disease are BPV transmission, immune-evasion and sarcoid pathogenesis. The development of a reliable technique to induce equine sarcoids in healthy equids is needed to challenge newly developed treatments and improve our understanding of host-specific genetic influences on susceptibility for sarcoid development.

Equine sarcoïden zijn veel voorkomende tumoren van de dermis van paardachtigen. Ze metastaseren niet en vormen geen onmiddellijke bedreiging voor het leven van aangetaste dieren. Ze kunnen echter wel uitgroeien tot grote massa's die het dierenwelzijn en de waarde van het dier aantasten en worden daarom dikwijls onderschat in een vroeg stadium. Er bestaan verschillende vormen van equine sarcoïd met een verschillend klinisch uiterlijk. Daarom en omwille van het feit dat er geen histologische markers bestaan voor de aandoening is de diagnose voor onervaren dierenartsen vaak moeilijk te stellen. Het wordt momenteel aangenomen dat het boviene papillomavirus (BPV) een belangrijke rol speelt in het ontstaan van equine sarcoïden, maar de exacte mechanismen die leiden tot het verspreiden van het virus, infectie en transformatie van de fibroblasten van de dermis en verdere tumorontwikkeling zijn niet gekend. Het mag daarom geen verrassing zijn dat deze tumoren vaak erg moeilijk te behandelen zijn. Heel wat behandelingen zijn beschreven, maar tot op vandaag bestaat er geen behandeling die universeel met succes kan toegepast worden, en de keuze van de beste behandeling voor een bepaald sarcoïd is een proces dat dikwijls met tegenslag te kampen heeft. In dit werk wordt een onderzoeksproject beschreven waarin verschillende aspecten van deze aandoening verder uitgediept worden, met als uiteindelijk doel het klinisch management van de tumoren te verbeteren.

In **hoofdstuk 1** wordt een overzicht gegeven van de huidige kennis van het equine sarcoïd. Er wordt dieper ingegaan op de etiopathogenese, epidemiologie en transmissie van de aandoening, evenals de klinische vormen, diagnose en behandeling. Sommige van deze aspecten zijn goed gekend, maar er rest nog heel wat onontgonnen gebied waar meer inzicht zou kunnen leiden tot het ontstaan van een betere klinische benadering. Dit eerste hoofdstuk verantwoordt waarom dit onderzoeksproject werd uitgevoerd en vormt de basis om de volgende hoofdstukken te begrijpen.

Hoofdstuk 2 beschrijft de voornaamste ambities van dit werk. In de eerste plaats was het doel om het klinisch management van equine sarcoïden te verbeteren. Dit werd mogelijk door dieper in te gaan op de validatie van de klinische diagnose, door het ontwikkelen en testen van een beslissingsprotocol voor de behandeling van equine

sarcoïden en door het formeel onderzoeken van de topicale behandeling van de tumor met acyclovir. Het nader onderzoeken van een theorie voor het verspreiden van BPV droeg indirect bij aan het hoofddoel, door de deur voor preventie van de aandoening op een kier te zetten.

De hypothese dat vliegen verantwoordelijk zouden zijn voor de overdracht van BPV werd getest in **hoofdstuk 3**. De stalvlieg (*Stomoxys calcitrans*) werd geselecteerd voor dit onderzoek omdat ze frequent voorkomt in de buurt van zowel paardachtigen als runderen en omwille van haar bijtende monddelen, die ideaal zijn om het virus te deponeren ter hoogte van de dermis. Stalvliegen negatief voor de aanwezigheid van BPV werden levend gevangen en blootgesteld aan weefsel afkomstig van equine sarcoïd of runderpapilloma. De virale BPV load van deze blootgestelde vliegen werd elke 24 uur gemeten door quantitatieve real-time PCR tot 7 dagen na de blootstelling. De BPV load steeg significant onmiddellijk na blootstelling aan weefsel afkomstig van sarcoïd en runderpapilloma, maar was hoger en bleef langer hoog na blootstelling aan die laatste. Dit wijst erop dat BPV overdracht door *Stomoxys calcitrans* mogelijk zou kunnen zijn en wellicht plaatsvindt van rund naar paard en niet tussen paarden. Verdere klinische transmissie-experimenten zijn nodig om vast te stellen of BPV overdracht via de stalvlieg ook echt kan leiden tot het ontstaan van equine sarcoïden bij BPV negatieve paarden.

Omdat het nemen van een biopt gepaard gaat met een risico op plotse tumorale groei of een invloed kan hebben op de uitkomst van een experiment, is histologische diagnose van equine sarcoïden is niet altijd haalbaar of wenselijk. Klinische diagnose zou een goed alternatief kunnen zijn, maar is momenteel niet gevalideerd en wordt daarom niet aanvaard in de wetenschappelijke literatuur. In **hoofdstuk 4** wordt de validatie van de klinische diagnose beschreven. Veertig gevallen van verschillende huidaandoeningen bij het paard werden in een online test beoordeeld door respondenten met verschillende expertiseniveaus. De gevallen werden zorgvuldig geselecteerd om een realistische steekproef te vormen van de prevalentie van equine sarcoïd in de populatie. Aan de deelnemers werd gevraagd om de aandoeningen te categoriseren als equine sarcoïd of geen equine sarcoïd. De klinische diagnose van de aandoening had een hoge algemene sensitiviteit en specificiteit (respectievelijk 83.3% en 79.6%) en het percentage correcte diagnoses was 82.0%. Sensitiviteit en specificiteit waren respectievelijk 89% en 86% voor experten op het gebied van equine

sarcoïd, terwijl minder ervaren deelnemers significant minder goed waren in het correct discrimineren van equine sarcoïden. De klinische diagnose van deze aandoening kan nu als volwaardig aanvaard worden, al is er voor onervaren respondenten nog ruimte voor verbetering.

Om het klinisch herkennen van equine sarcoïden door onervaren personen te verbeteren wordt in **hoofdstuk 5** een diagnostisch protocol voorgesteld en getest. Het protocol werd gebruikt door onervaren deelnemers om dezelfde 40 gevallen te beoordelen die in hoofdstuk 4 gebruikt werden en hun resultaten werden vergeleken met deze van de deelnemers die geen gebruik konden maken van het protocol. Deelnemers die over het protocol beschikten waren significant beter in het onderscheiden van equine sarcoïden in vergelijking met deelnemers die het protocol niet gebruikten. Onervaren deelnemers die het protocol gebruikten scoorden even goed als experten op het gebied van equine sarcoïden en waren ook zekerder van hun diagnose in vergelijking met onervaren deelnemers zonder protocol. Dit bewijst dat het protocol een goede hulp kan zijn voor de correcte klinische diagnose van een equine sarcoïd.

Sarcoïden zijn erg heterogeen qua klinische presentatie en locatie op het lichaam. Omdat er daarenboven geen universele behandeling bestaat is het moeilijk een goede behandeling te kiezen voor een bepaald sarcoïd op een bepaalde lichaamslocatie. In **hoofdstuk 6** wordt een protocol voorgesteld om op basis van het klinisch uiterlijk van de tumor en de locatie op het lichaam een behandeling te selecteren. De behandelingen die in het protocol werden opgenomen zijn topicale toediening van acyclovir of imiquimod, non-touch electrochirurgische excisie (al dan niet met intralesionaal gebruik van cisplatine), cryochirurgie, Bacillus Calmette-Guerin vaccin injectie en intralesionale injectie van chemotherapeutica op basis van platinum. Het selectieprotocol werd toegepast op 230 paarden met in totaal 614 sarcoïden en een follow-up werd uitgevoerd ten vroegste 6 maand na het uitvoeren van de laatste behandeling. Het slaagpercentage bij gebruik van het protocol was 74.9%. Non-touch electrochirurgische excisie had het hoogste slaagpercentage (86.8%). Significant lagere slaagpercentages werden bekomen met topicaal gebruik van acyclovir, cryochirurgie en intralesionale injectie van chemotherapeutica op basis van platinum. Wanneer een sarcoïd gelegen was op een paard dat nog andere sarcoïden had was de slaagkans significant lager dan wanneer dit niet het geval was. Wanneer een

sarcoïd behandeld werd op een paard dat tegelijk voor een ander sarcoïd behandeld werd met een immunostimulerende behandeling waren de slaagkansen significant hoger dan wanneer dit niet het geval was. Wanneer een topicale behandeling mogelijk was kregen de eigenaars de keuze tussen acyclovir of imiquimod. Hoewel niet significant werden betere resultaten bekomen met gebruik van imiquimod.

Topicale behandeling van equine sarcoïden met acyclovir werd eerder beschreven in een beperkt, niet gecontroleerd klinisch experiment. Er werd een goed resultaat gerapporteerd zonder de ernstige bijwerkingen die andere topicale behandelingen hebben. In **hoofdstuk 7** werd *in vitro* getest hoe diep acyclovir kan penetreren in normale paardenhuid en paardenhuid aangetast door sarcoïd. Equine sarcoïden werden chirurgisch geëxciseerd en normale huid van dezelfde lichaamslocatie werd bekomen van paarden die geëuthanaseerd werden omwille van losstaande redenen. Nadat de hypodermis verwijderd werd, werden de stalen gebruikt in een experiment met Franz diffusie cellen waarin ze aan epidermale zijde gedurende 48 uur werden blootgesteld aan een 5% acyclovir cetomacrogolcrème. Op verschillende tijdstippen werd de acyclovir concentratie van het receptormedium bepaald door middel van ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Na afloop van het experiment werd de huid bevroren, gesneden in een cryotoom en verdeeld in epidermis, oppervlakkige dermis en diepe dermis. De weefselconcentratie van acyclovir werd ook in deze stalen bepaald door UPLC-MS/MS. Acyclovir werd teruggevonden in het receptormedium van zowel gezonde als aangetaste huid, maar in significant hogere concentraties bij gezonde huid. In de huid zelf werd meer acyclovir teruggevonden in de epidermis van sarcoid aangetaste huid dan in de oppervlakkige en diepe dermis. Dit was niet het geval bij normale huid. Desalniettemin waren de teruggevonden acyclovir concentraties in de diepe dermis van aangetaste huid nog voldoende hoog om een *Herpes simplex* virus infectie te bestrijden bij de mens. Aangezien de nodige acyclovir concentratie om BPV infectie te bestrijden niet gekend is kunnen we enkel veronderstellen dat de teruggevonden concentratie voldoende hoog is om equine sarcoïden te behandelen. Indien het BPV gevoelig is voor deze concentratie zou een gunstig effect van acyclovirbehandeling van equine sarcoïden mogelijk kunnen zijn.

In hoofdstuk 7 werd aangetoond dat acyclovir na topicale toediening een aanzienlijke concentratie bereikt in de dermis van occulte equine sarcoïden. Dit op zich is echter

nog geen bewijs dat een gunstig effect op equine sarcoïden kan bekomen worden en in hoofdstuk 6 werden betere resultaten bekomen met topicale imiquimod behandeling.

In **hoofdstuk 8** wordt een dubbel blind, placebo gecontroleerd klinisch experiment beschreven om na te gaan of topicale behandeling van equine sarcoïden met acyclovir betere resultaten oplevert in vergelijking met een placebo. Vierentwintig sarcoïden op 15 paardachtigen werden 2 keer per dag topicaal behandeld met een 5% acyclovir cetomacrogolcrème en 25 sarcoïden op 16 paardachtigen met een placebo. De selectie van behandeling gebeurde willekeurig en de behandeling werd voortgezet gedurende 6 maanden of tot wanneer het sarcoïd verdwenen was indien dit voor het einde van de behandelingsperiode gebeurde. Maandelijks werd een swab genomen en werden digitale foto's van het letsel gemaakt. Nadat de studie afgelopen was werden de swabs geanalyseerd voor het voorkomen van BPV DNA door middel van PCR. Drie gediplomeerde leden van het European College of Veterinary Surgeons beoordeelden de foto's op hoe uitgesproken het letsel was op een visueel analoge schaal (VAS). De oppervlakte en grootste diameter van de letsels werden ook gemeten op de foto's. Op geen enkel tijdpunt was er een significant verschil in een van de gemeten parameters (oppervlakte, diameter en VAS score) tussen de behandelingen, hoewel de letsels voor beide behandelingen wel minder uitgesproken werden naarmate de behandelingstijd vorderde. Er kon geen enkel bewijs gevonden worden dat het topicaal behandelen van equine sarcoïden met acyclovir betere resultaten zou opleveren dan een placebobehandeling.

In **hoofdstuk 9** worden ideeën besproken voor toekomstig onderzoek naar het equine sarcoïd, gebaseerd op de klinische en wetenschappelijke ervaring die opgedaan werd tijdens dit project. De belangrijkste domeinen waarbinnen meer inzicht zal leiden tot het verbeteren van behandeling en preventie zijn BPV transmissie, evasie van het immuunsysteem en pathogenese van het sarcoïd. Daarenboven is het noodzakelijk een betrouwbare techniek te ontwikkelen om equine sarcoïden te induceren bij paardachtigen zodat nieuwe behandelingen getest kunnen worden en inzicht verworven kan worden in de invloed van paard-specifieke genetische parameters op de gevoeligheid voor het ontwikkelen van equine sarcoïden.

Maarten Haspeslagh was born on June 7, 1987 in Nassau-Bay, Texas. After completing secondary school in Belgium, majoring in science and mathematics, he started studying Veterinary Medicine at Ghent University in 2005. In June 2011, he obtained the degree of Master of Veterinary Medicine with distinction.

After an internship in Qatar, Maarten started as an assistant at the department of Surgery and Anaesthesiology of Domestic Animals of the Faculty of Veterinary Medicine, Ghent University in February 2012, where he could combine his interests in scientific research and surgery under supervision of Prof. Dr. Ann Martens. During the six following years, he was responsible for the treatment of equine tumours, participated in practical teaching and emergency duties, was engaged in equine sarcoid research and supported other research of the department. Next to his clinical and scientific tasks, he is a member of the Editorial Board of the Flemish Veterinary Journal and represents his colleagues in the Faculty Committee for Internationalization, the Central Expert Group on Internationalization and the Advisory Committee for Educational Language.

Maarten (co-)authored several publications in international peer-reviewed journals, was a speaker at multiple national and international conferences and acted as a reviewer for The Veterinary Journal.

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I'll have to start with apologising to those who immediately skip to this section and expect to find their own name in an alphabetically ordered list of colleagues, family and friends. I deliberately made the decision to keep this section short and to the point, so I will only be explicitly thanking the people who directly contributed to the completion of this work. If you are not mentioned by name here, please do not feel offended. This does not mean I don't love you, don't like to work with you or don't like being around you.

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