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

L. Bogaert,1 M. Van Poucke,2 C. De Baere,1 J. Dewulf,3 L. Peelman,2 R. Ducatelle,4 F. Gasthuys1 and A. Martens1

Correspondence
A. Martens
ann.martens@UGent.be

1Ghent University, Faculty of Veterinary Medicine, Department of Surgery and Anaesthesiology of Domestic Animals, Salisburylaan 133, B-9820 Merelbeke, Belgium
2Ghent University, Faculty of Veterinary Medicine, Department of Nutrition, Genetics and Ethology, Heidestraat 19, B-9820 Merelbeke, Belgium
3Ghent University, Faculty of Veterinary Medicine, Department of Obstetrics, Reproduction and Herd Health, Salisburylaan 133, B-9820 Merelbeke, Belgium
4Ghent University, Faculty of Veterinary Medicine, Department of Pathology, Bacteriology and Avian Diseases, Salisburylaan 133, B-9820 Merelbeke, Belgium

Equine sarcoids, the most common skin tumours in horses, are induced by bovine papillomavirus (BPV). Their clinical appearance varies from small stable patches to aggressively growing masses. Differences in BPV load and mRNA expression and Ki67 and p53 immunostaining among four clinical types (fibroblastic, occult, nodular and verrucous sarcoids) were evaluated to test the hypothesis that the clinical behaviour of equine sarcoids correlates with BPV activity. Viral load and expression of the BPV E2, E5, E6 and E7 genes were determined using quantitative real-time PCR. The proliferative fraction (PF) of the tumours was determined by Ki67 immunostaining and expression of p53 was analysed by immunohistochemistry. Nodular sarcoids showed a significantly higher viral load than the other types. A significant overall difference among the four types was observed for E2, E5, E6 and E7 mRNA expression. Nodular sarcoids showed the highest expression level for each BPV gene examined, followed by verrucous, fibroblastic and occult tumours. Viral DNA and mRNA outcomes correlated with each other, indicating a similar transcription pattern in each type of sarcoid. The PF was significantly higher in the superficial layers of verrucous and fibroblastic sarcoids compared with occult and nodular types. No significant difference was observed for the PF in the deep layers and for p53 expression.

These results clearly demonstrate the omnipresence and active transcription of BPV in equine sarcoids. However, the hypothesis that the clinical behaviour of an equine sarcoid can be explained on the basis of differences in BPV activity could not be demonstrated.

INTRODUCTION

Equine sarcoid is the most common skin tumour in horses. The clinical aspect can vary remarkably, ranging from small, stable patches to aggressively growing tumours up to more than 10 cm in diameter. A sarcoid can appear as a single tumour, but horses often show multiple lesions. Although equine sarcoids do not metastasize, they recur frequently if not treated properly. Different clinical types have been described (Pascoe & Knottenbelt, 1999). The fibroblastic type, with a typically ulcerated epidermis, usually presents an aggressive clinical behaviour (Fig. 1a). Occult types are flat, alopecic patches, sometimes with hyperkeratosis or small nodules on their surface (Fig. 1b). These tumours will often not evolve, even over several years. Nodular sarcoids are round masses with an intact epithelium (Fig. 1c). The overlying skin can be loose from the stromal tissue or (partially) attached to the fibroblastic portion of the tumour. Verrucous sarcoids have a typically warty appearance (Fig. 1d). Nodular and verrucous sarcoids display a clinical behaviour in between the fibroblastic and the occult sarcoid, mostly with moderate growth. Mixed sarcoids, composed of two or more of the described types, are also commonly observed. Occult sarcoids, and even nodular and verrucous sarcoids, can remain stable for many years and then suddenly, without any apparent reason, change dramatically into a rapidly growing tumour, often fibroblastic, that can be difficult to treat.
The most important aetiological factor in the development of equine sarcoids is the presence of bovine papillomavirus types 1 and 2 (BPV1/2). BPV DNA has been found in up to 100% of examined sarcoids in several studies (Teifke & Weiss, 1991; Otten et al., 1993; Teifke et al., 1994; Carr et al., 2001a, b; Martens et al., 2001a, b, c). In addition, BPV mRNA and BPV proteins have been detected in equine sarcoid tissue (Nasir & Reid, 1999; Carr et al., 2001b), providing evidence for a direct involvement of BPV in equine sarcoids.

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

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

**METHODS**

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

**Quantitative BPV DNA and mRNA analysis.** DNA extraction of the first sample of each sarcoïd and subsequent PCR analysis for BPV DNA detection, with a threshold of 20 copies per sample, was performed as described by Bogaert et al. (2005). Positive controls were sarcoïd samples formerly confirmed by sequencing. dH2O served as a no-template control. To identify the BPV type, a restriction fragment length polymorphism assay was applied as described by Martens et al. (2000). The viral load of BPV1 and BPV2 was determined by quantitative real-time PCR using Taqman probes.

Total RNA isolation and subsequent first-strand cDNA synthesis were performed on the second sample of each sarcoïd as described previously (Bogaert et al., 2006). Briefly, RNA was isolated using Total RNA Isolation Reagent (Abgene), followed by DNase treatment and a control minus reverse transcriptase with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to check for successful removal of all contaminating DNA. Next, first-strand cDNA synthesis was carried out with Superscript II Reverse Transcriptase (Invitrogen) and a combination of random primers (Invitrogen) and oligo(dT)20 primers (Invitrogen) in a total volume of 20 μl. After this step, a PCR was performed with primers for GAPDH to check for the presence of cDNA.

Primers and probes were designed using PRIMER 3 software (Rozen & Skaltsky, 2000) for E1 (BPV load analysis) and for E2, E5, E6 and E7 (BPV mRNA analysis) of both BPV1 and BPV2. Specificity was tested using BLAST analysis against the genomic NCBI database. The amplicon and surrounding sequences were characterized using MFOLD (Zuker, 2003) to take into account possible secondary structure at the primer and probe binding sites that might influence the PCR efficiency. The PCR products were cloned into pCR2.1 (Invitrogen) and sequenced for verification (Thermo Sequenase the PCR efficiency. The PCR products were cloned into pCR2.1 (Invitrogen) and sequenced for verification (Thermo Sequenase the PCR efficiency. The PCR products were cloned into pCR2.1 (Invitrogen) and sequenced for verification (Thermo Sequenase SuperMix UDG (Invitrogen)) and oligo(dT)20 primers (Invitrogen) in a total volume of 20 μl. After this step, a PCR was performed with primers for GAPDH to check for the presence of cDNA.

JUV.8 reaction volume on an iCycler iQ Real-Time PCR Detection System (Bio-Rad) using the iQ Supermix (Bio-Rad) for DNA analysis and the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) supplemented with 0.02 μM fluorescein (Bio-Rad) for mRNA analysis. Samples were diluted 1:1000 (DNA) or 1:100 (mRNA). First, UDG treatment was done at 50 °C to prevent cross-contamination. The initial denaturation was performed at 95 °C for 3 min to activate the iTag DNA polymerase (DNA) or for 2 min to activate the Platinum Taq DNA polymerase (mRNA), followed by 40 cycles of denaturation at 95 °C for 20 s and a combined primer annealing/elongation at the specific annealing temperature for 40 s during which fluorescence was measured. During analysis of mRNA samples, a melting curve was generated to confirm a single gene-specific peak and to detect primer-dimer formation by heating the samples from 70 to 95 °C in 0.5 °C increments with a dwell time at each temperature of 10 s, whilst continuously monitoring the fluorescence. Each reaction was run in duplicate, and two no-template controls and a dilution series for BPV1 and BPV2 were included in each run. Additionally, two normal skin samples from sarcoïd-free horses, two samples of equine melanomas and two samples of exuberant granulation tissue were included as controls. During optimization of the protocol, the PCR products were loaded on a 3 % agarose gel after each run to confirm specific gene amplification and the absence of primer-dimer formation. PCR efficiencies were calculated using a relative standard curve (a 10-fold dilution series with five measuring points) derived from pooled DNA obtained from a mixture of 13 equine sarcoïds for BPV DNA analysis and pooled cDNA from a mixture of 10 sarcoïds for BPV mRNA analysis, for both BPV1 and BPV2. Ct (threshold cycle) values were obtained at the time the fluorescence exceeded the threshold value. Ct values were subsequently transformed to 'raw data', taking into account PCR efficiency, and all values were measured during the same run.

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

**Immunohistochemical staining procedures.** Cryosections of the third sample of each sarcoïd were cut at 6 μm and mounted on APES-coated slides (StarFrostPlus). Sections were allowed to dry at 37 °C for 30 min and then fixed in acetone for 5 min at room temperature. After drying, slides were stored at -20 °C until staining. Slides were mounted in disposable immunostaining chambers (Shandon) and endogenous peroxidase activity was blocked using a solution of 3 % H2O2 in methanol. After rinsing, sections were pre-incubated for 30 min with 30 % goat serum (Dako) at 25 °C. Next, the appropriate primary monoclonal antibody (mAb) was applied. The mAb 297M (BioGenex) was employed to detect Ki67 at a 1:20 dilution (2 h at 37 °C) and the mouse DO-7 mAb (Novocastra) against p53 at a 1:50 dilution (2 h at 25 °C). After rinsing, biotin-conjugated goat anti-mouse Ig (Dako) served as the secondary antibody (30 min at 21 °C). Signal amplification was obtained by incubating the slides for 30 min at 21 °C with a horseradish peroxidase-conjugated streptavidin–biotin complex (Dako). Diaminobenzidine tetrahydrochloride (Sigma) was used as the chromogen and H2O2 as the substrate, and the slides were counterstained with haematoxylin. Positive control tissues were equine papilloma for Ki67 and equine squamous cell carcinoma for p53. Normal equine skin served as a negative control. On the Ki67-stained slides, eight high-power fields (×640) of the superficial and eight of the deep layers of each tumour were randomly chosen to count all nuclei and the number of Ki67-positive nuclei. The PF was calculated separately for the superficial and the deep tumour portion, as well as globally. The p53-stained slides were evaluated for the presence or absence of p53 expression.

**Statistical analysis.** All numerical data were log-transformed to achieve a normal distribution. Correlations between outcomes were investigated using Pearson’s correlation coefficient (r). For all different outcome variables (viral load and mRNA of E2, E5, E6 and E7), differences between the sarcoïd types, BPV type, localization, size, time of onset, breed, sex and recurrence after former surgery were analysed using a linear mixed effect model, with ‘horse’ as a random effect. Differnces in the PF between the superficial and deep layers of the sarcoïds were analysed using a paired-sample t-test. Differences in p53 expression in relation to the sarcoïd type were analysed by means of logistic regression. For all tests, differences were considered to be statistically significant if P values were below 0.05. All statistical analyses were performed in S-PLUS 7.0 for Windows (Insightful Corp.).
RESULTS

Sample population

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

Quantitative BPV DNA and mRNA analysis

All tumours showed the presence of BPV DNA. The majority of the sarcoids (88%) harboured BPV1, whilst the remaining 12% had BPV2.

Fig. 2(a) illustrates that nodular sarcomas had a more than fourfold higher viral load than fibroblastic, verrucous and occult sarcomas. The BPV load of the latter three sarcoma types was not significantly different. No-template controls, samples of normal skin, melanomas and exuberant granulation tissue were all negative for BPV DNA.

Fig. 2(b) represents the means and the 95% confidence intervals of the log-transformed values of all samples quantitatively analysed for the four BPV early genes (E2, E5, E6 and E7) in each group of equine sarcomas. For each BPV gene examined, nodular sarcomas showed the highest expression, followed by verrucous, fibroblastic and occult sarcomas. Differences between nodular and fibroblastic and between nodular and occult sarcomas were always statistically significant. On the other hand, the distinction between nodular and verrucous sarcomas was too small to be significant. Verrucous and occult tumours varied significantly for each BPV gene examined, except for E6. Verrucous and fibroblastic sarcomas only showed a statistically significant difference for E7 expression and not for the other BPV genes. Finally, fibroblastic and occult sarcomas showed a significant difference for E2 and E5, but not for E6 and E7. None of the negative controls contained BPV mRNA. No significant correlation was found between outcome of any examined BPV gene and BPV type, localization, size, time of onset, breed, sex or recurrence after former surgery (data not shown). Tumours originating from the same horse showed related outcomes, despite a sometimes different clinical appearance.

Immunohistochemical staining

The mean PF for all equine sarcomas was 2.41%. The log-transformed PF for the superficial portion of each sarcoaid was significantly higher (P<0.001) than the log-transformed PF for the deep portion. Verrucous and fibroblastic sarcomas showed a significantly higher PF in the superficial
layers compared with occult and nodular tumours. No significant difference for PF in the deep layers could be observed. These data are represented graphically in Fig. 3. Perinuclear p53 expression was present in 36.1 % of the tumours. A relatively high proportion of the verrucous and fibroblastic sarcomas showed p53 expression (47 and 41 %, respectively), in occult sarcomas the expression was present in 35 % of the tumours and only 19 % of the nodular sarcomas showed p53 expression. However, these differences were not statistically significant.

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

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

As expected, fibroblastic tumours, which show an aggressive character, have a high PF. This is in contrast to the observed low BPV mRNA expression in these tumours. In contrast, human papillomavirus (HPV) mRNA expression increases in human cervical cancer when lesions progress from low-grade dysplasia to invasive carcinoma (Wang-Johanning et al., 2002; Kraus et al., 2004; Scheurer et al., 2005). Also, viral load was not in the expected concordance with the clinical behaviour, with nodular sarcoms showing a much higher viral load than the three other types. In human medicine, it has been shown that women with low-grade cervical intraepithelial lesions accompanied by a high HPV load are at higher risk for progression to high-grade lesions (Ho et al., 2006) and that a higher HPV load is seen in high-grade lesions compared with low-grade lesions (Caropino et al., 2006; Kovacic et al., 2006; Lai et al., 2006). On the other hand, Dahlgren et al. (2006) could not determine any influence of HPV load (except for HPV16) on survival in patients with early-stage cervical carcinoma. It is possible that the role of BPV becomes less important in tumour progression once certain cellular abnormalities have been established, which might indicate a 'hit-and-run' role for BPV.

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

Carr et al. (2001b) found expression of the BPV E5 protein in all sarcoms examined, with the highest expression observed in biologically aggressive fibroblastic variants. However, this expression was not quantified. Another explanation of the discrepancy between their and our
results is that there was no linear correlation between mRNA expression and protein expression due to possible post-transcriptional regulation under varying cellular conditions. The PF of the equine sarcoids examined in this study was low. This is in accordance with other studies, where the observed PF varied between 0 and 11% (Martens et al., 2000; Nixon et al., 2005). This might reflect the sometimes very stable character of sarcoids. However, even fast-growing and clinically agressive tumours still showed a rather low PF. It is possible that only highly malignant tumours, such as squamous cell carcinomas, show a high PF.

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

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

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