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

3

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24

25

26 **Abstract**

27

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

45

46 **Keywords:** equine sarcoid – bovine papillomavirus – latency – contamination

47 **1. Introduction**

48

49 Equine sarcoids are commonly observed fibroblastic skin tumours in horses. They do not  
50 metastasise but treatment is often challenging due to frequent recurrences. The bovine  
51 papillomavirus type 1 and 2 (BPV-1, -2) is recognised as the most important etiological factor  
52 in its development. BPV DNA has been found in up to 100 % of the examined sarcoids in  
53 several studies (Otten et al., 1993; Carr et al., 2001a, 2001b; Martens et al., 2001a, 2001b).  
54 BPV mRNA and BPV proteins have also been identified in equine sarcoid tissue, providing  
55 evidence for a direct involvement of BPV in the pathogenesis (Nasir and Reid, 1999; Carr et  
56 al., 2001b; Bogaert et al., 2007).

57 The transmission of BPV to the horse and the pathogenesis of tumour development are not yet  
58 fully understood. In contrast to BPV infection in cattle, the natural host where complete virus  
59 particles are formed, a non-productive infection occurs in horses. Hypotheses for BPV  
60 infection in horses include direct or indirect contact with infected cattle or horses, as well as  
61 transmission by insects (Reid et al., 1994; Knottenbelt et al., 1995). Contact with the virus  
62 alone is probably not sufficient for tumour development: skin trauma, the immunological  
63 status and the genetic constitution of the individual horse also play an important role  
64 (Brostrom et al., 1988; Gerber et al., 1988). Furthermore, there is discussion about the  
65 occurrence of virus latency. In several species, including man and cattle, latency of  
66 papillomaviruses in asymptomatic individuals has clearly been demonstrated (Campo et al.,  
67 1994; Maran et al., 1995; Burkhart, 2004; Antonsson and McMillan, 2006). In horses, BPV  
68 DNA has been demonstrated in swabs and biopsies obtained from the normal skin of horses  
69 affected with equine sarcoid, suggesting the occurrence of virus latency (Trenfield et al.,  
70 1985; Carr et al., 2001a; Martens et al., 2001a; Bogaert et al., 2005). BPV DNA has also been  
71 found occasionally on the normal skin of healthy horses and in non-sarcoid inflammatory skin

72 conditions (Angelos et al., 1991; Bogaert et al., 2005; Yuan et al., 2007). However, the  
73 distinction between BPV DNA contamination of the skin surface and a true latent infection  
74 has never been made.

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

80

## 81 **2. Methods**

82

### 83 *2.1. Study population and sample collection*

84

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

92 From each horse, 3 samples were collected from normal skin on 4 locations: chest, axilla,  
93 ventral abdomen and medial side of the thigh. First a pre-wetted (0.9 % NaCl solution)  
94 cotton-tipped swab was rubbed on an intact skin surface of 5 x 5 cm. Next, this skin area was  
95 decontaminated using the following protocol. Hairs were clipped using a trimmer with  
96 disposable blades followed by scrubbing with povidonum iodinum 7.5 % soap and

97 disinfection with 76 % ethanol. Afterwards, the skin was tape-stripped with 3M™  
98 Transpore™ surgical tape as described by Forslund et al. (2004). Finally, another swab was  
99 rubbed over the skin surface and a full-thickness punch biopsy (8 mm diameter) was obtained.  
100 Horses from groups CS, CC and C were sedated with romifidine (0.08 mg kg<sup>-1</sup>) and/or  
101 morphine (0.10 mg kg<sup>-1</sup>) when needed and a local ring block (100 mg mepivacaine) was  
102 performed for the skin biopsies. Horses from group S were sampled during general  
103 anaesthesia required for the surgical treatment of the sarcoid. In this group a tissue sample of  
104 the sarcoid was also taken. Normal skin samples from horses in group S were removed at a  
105 distance of at least 10 cm away from the tumour(s). Skin biopsies and sarcoid samples were  
106 divided into 3 pieces: one was stored dry at -18 °C for DNA analysis, one was stored in  
107 RNAlater (Ambion) overnight at 4 °C and thereafter at -18 °C for mRNA analysis and one  
108 was formalin fixed during 24h and routinely processed for histopathological examination  
109 (HE-staining). From each horse, 10 ml of heparinised peripheral blood was collected for BPV  
110 DNA analysis. At the end of the study the owners of the horses were telephonically  
111 questioned about possible sarcoid development.

112

## 113 2.2. BPV DNA and mRNA analysis

114

115 All swabs, skin biopsies and sarcoid samples were routinely DNA extracted using the  
116 Puregene DNA extraction kit as described elsewhere (Bogaert et al., 2005). Two hundred  
117 microliter of each blood sample was DNA extracted by adding 500 µl Tris-HCl-EDTA,  
118 followed by 30 seconds of centrifuging and removal of the supernatant fluid. The pellet was  
119 subsequently washed 3 times with 500 µl Tris-HCl-EDTA. Resuspension of the pellet was  
120 obtained by adding 100 µl lysis buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.5 % Tween

121 20) and proteinase K ( $100 \mu\text{g ml}^{-1}$ ). Next, the samples were incubated for 45 minutes at  $56^\circ\text{C}$   
122 and for 10 minutes at  $95^\circ\text{C}$ . Finally, they were centrifuged for 1 minute, and stored at  $-18^\circ\text{C}$ .  
123 Multiplex real time PCR was performed using Taqman probes specific for BPV-1 and BPV-2  
124 as described in Bogaert et al. (2007), but performing 50 cycles in stead of 40 to enhance the  
125 detection limit. For each sample, 2 to 4 repeated measurements were performed. When BPV  
126 DNA was detected in at least one of the samples (swabs and/or biopsy) of a given location,  
127 RNA extraction was performed followed by real time RT-PCR of the E2 and E5 mRNA of  
128 BPV. When mRNA of one or both genes was retrieved, the same analysis was also performed  
129 for BPV E6, E7 and 3 reference genes (beta actin, ubiquitin B, beta-2-microglobulin) for  
130 quantitative analysis. All equine sarcoids were analysed for the 4 above mentioned BPV  
131 genes and the 3 reference genes. Details on the methods used have been described previously  
132 (Bogaert et al., 2006; Bogaert et al., 2007). In short, Ct values were transformed to “raw  
133 data”, taking into account PCR efficiency and all values measured during the same run. Next,  
134 obtained values were divided by a normalisation factor calculated as the geometric mean of  
135 the values of the 3 reference genes, allowing reliable comparison of different samples (both  
136 normal skin and sarcoid samples). In order to compare the two groups of sarcoid samples and  
137 normal skin samples, a ratio of the average values of both groups for each examined gene was  
138 calculated.

139

### 140 *2.3. Histopathological evaluation*

141

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



146

147 *2.4. Statistical analysis*

148

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

152

### 153 **3. Results**

154

155 *3.1. Study population*

156

157 In group S, 6 horses (S1, S2, S6, S9, S10, S11) had only one sarcoid and 5 horses had  
158 multiple sarcoids (S3: 3, S4: 7, S5: 5, S7: 3, S8: 13). The median age was 9 years (range 5-  
159 15y) and there were 7 Warmbloods, 1 Thoroughbred, 1 Frisian, 1 pony and 1 Arabian Horses.  
160 Seven of them were geldings, the other 4 were mares. In group CS, the median age was 8.5  
161 years (range 3-16y) and there were 6 Warmbloods, 1 pony, 1 Tinker, 1 Standardbred and 1  
162 Andalusian Horse. Five were geldings and 5 were mares. In group CC, the median age was 6  
163 years (range 3-27y), 10 were mares and 1 was a gelding. Six were Warmbloods, 2 were  
164 Belgian Draft Horses, 1 crossbred Standardbred x Warmblood, 1 Anglo Arabian and 1 pony.  
165 In group C the median age was 6.5 years (range 2-15y); this group consisted of 6 mares, 3  
166 geldings and 1 stallion. Seven horses were Warmbloods, next to 1 Thoroughbred, 1 Quarter  
167 Horse and 1 crossbred Appaloosa x Quarter Horse.

168

169 *3.2. BPV DNA analysis*

170

171 Eight out of 9 examined sarcoid samples were positive for BPV DNA. Two sarcoid samples  
172 (S1 and S9) as well as the biopsy of the thigh of horse CS4 were not available for analysis.  
173 BPV DNA was detected in the normal skin of 24 out of 42 horses (57 %). There were clear  
174 differences between the groups: in 73 % of the horses in group S and CC BPV DNA was  
175 detected, compared to 50 % in group CS and 30 % in group C (Table 1). The positivity for  
176 BPV DNA was independent of the type of sample obtained: globally, 17 % of the swabs  
177 before decontamination were positive, 17 % of those after decontamination and 15 % of the  
178 biopsies. BPV DNA was not detected in any of the peripheral blood samples. Tables 2-5  
179 represent the normal skin locations positive for BPV-1 and BPV-2 DNA in the 4 groups.  
180 Thirty-six percent of all 42 horses were carrying BPV-1, 12 % BPV-2 and 9 % both BPV  
181 types in their normal skin samples. All of the horses where both BPV-1 and BPV-2 were  
182 detected were living in contact with cattle having suffered recently from papillomas. The BPV  
183 type detected in the normal skin samples in group S always corresponded to the BPV type of  
184 the respective sarcoid. Significantly more normal skin swabs (both before and after  
185 decontamination,  $p=0,02$  and  $p<0,01$  respectively) were positive for BPV DNA in group S (12  
186 and 41% respectively) compared to the control group (0 and 1 %). Group CC differed  
187 significantly from the control group for the swabs before decontamination and the biopsies  
188 (10 and 13 % vs 0 and 4 %;  $p=0,01$  and  $p=0,04$  respectively). Group CS was not significantly  
189 different from the control group for any of the obtained samples. In horses with at least one  
190 positive sample, the mean number of BPV DNA positive locations was also remarkably  
191 different: horses in group S and CC had a higher number of positive locations (respectively  
192 2.6 and 2.5 out of 4) compared to group CS and C (respectively 1.6 and 1.3 out of 4) (Table  
193 1). No significant effect of location of the sampling on BPV DNA presence was observed.  
194 From all normal skin locations examined ( $n=168$ ), 11 showed BPV DNA positivity only in  
195 the swab taken before decontamination. Eight locations were positive in both swabs but not in

196 the biopsy. In 11 locations BPV DNA was demonstrated only in the biopsy and not in the  
197 swabs. In 2 locations, both the swab before decontamination and the biopsy were positive for  
198 BPV DNA. In 4 locations, the swab before decontamination was negative, but the swab after  
199 decontamination as well as the biopsy were positive. Eight locations were positive in the 3  
200 samples. Eight locations were only positive in the swab taken after decontamination but not in  
201 the two other samples.

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

206

### 207 *3.3. BPV mRNA analysis*

208

209 Seventy-nine percent of the horses where BPV DNA was detected in at least one of the  
210 samples (swabs or biopsy) were positive for the presence of BPV mRNA. This corresponds to  
211 45 % of all horses. This was mainly attributed to the horses in groups S and CC, where BPV  
212 mRNA was detected in 88 % of the BPV DNA positive horses, compared to only 60 % in  
213 group CS and 67 % of group C (Table 1). Globally, sixty percent of the samples positive for  
214 BPV DNA also harboured BPV mRNA (68 % in group CC, 57 % in group S, 50 % in groups  
215 CS and C). The mean number of BPV mRNA positive locations in BPV DNA positive horses  
216 was 1.6 in group CC, 1.5 in group S, 0.8 in group CS and 0.6 in group C. These results are  
217 shown separately per group of horses in Tables 2-5.

218 The quantity of BPV mRNA detected in the normal skin samples was much lower compared  
219 to the equine sarcoid samples. Quantitative mRNA analysis demonstrated that BPV mRNA  
220 expression of the 4 examined BPV genes was on average 3439 times lower in the normal skin

221 samples compared to the equine sarcoids. Particularly the equine sarcoid to normal skin ratio  
222 of E2 expression was low (10387:1) in comparison with E5 (659:1), E6 (1915:1) and E7  
223 (796:1). Not all examined BPV genes were expressed in all samples: E5 mRNA was most  
224 often present (93 % of samples with any BPV mRNA), followed by E7 (56 %) and E2 (52 %).  
225 E6 mRNA was only expressed in 33 % of the samples with BPV activity. The mean and the  
226 95 % confidence interval of the BPV mRNA expression levels of the 4 examined BPV genes  
227 in the 2 groups of samples are shown in Fig. 1.

228

#### 229 *3.4. Histopathological examination*

230

231 All examined tumour samples were confirmed to be equine sarcoids. Two samples (S1 and  
232 S9) were missing. Forty-nine BPV DNA positive normal skin samples originating from 23  
233 horses and 10 BPV DNA negative samples from 5 horses were examined histopathologically  
234 by 2 independent pathologists. The majority of the BPV DNA positive normal skin samples  
235 showed discrete abnormalities: minimal to mild focal epidermal hyperplasia of the stratum  
236 spinosum (acanthosis) together with slight basophilic cytoplasmic swelling were frequently  
237 observed (Fig. 2). Diffuse or focal hyperpigmentation was also commonly noticed. Several  
238 samples displayed a thickened basal membrane, with or without acanthosis. Mild perivascular  
239 dermatitis was sometimes noticed. Yet, some of these changes were also seen in 6 out of 10  
240 normal skin samples negative for BPV DNA. No correlation between histopathological  
241 abnormalities and the presence of BPV mRNA was observed.

242

#### 243 **4. Discussion**

244

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

256 Sarcoids can appear on any part of the body, but they are mostly localised on the ventral  
257 abdomen, the paragenital region, head and limbs (Torrontegui and Reid, 1994). In the present  
258 study, samples were obtained from these predilection sites to enhance the chance of finding  
259 latent carriers. On the other hand, sampling only 4 locations might be insufficient to detect all  
260 horses carrying BPV in their normal skin. Therefore, sampling on more locations might  
261 increase the percentage of horses with latent BPV infection.

262 One of the aims of the present study was to distinguish between the presence of BPV DNA on  
263 the skin surface (contamination) and in the epidermal and/or dermal layers (latency). In order  
264 to differentiate the two conditions, the skin surface was decontaminated before taking a  
265 biopsy. However, the interpretation of the results is not straightforward. BPV DNA positivity  
266 only in the swab taken before decontamination can be considered as superficial skin  
267 contamination with BPV DNA from shed sarcoid cells or with BPV produced in cattle  
268 papillomas. Locations positive in both swabs but not in the biopsy might be the result of  
269 superficial skin contamination combined with unsuccessful decontamination. BPV DNA

270 demonstrated only in the biopsy and not in the swabs can be regarded as true latent infection  
271 of the dermis or of the basal layers of the epidermis. If both the swab before decontamination  
272 and the biopsy were positive for BPV DNA, this can be seen as a combination of superficial  
273 skin contamination and latent dermal infection. If the swab before decontamination was  
274 negative, but the swab after decontamination as well as the biopsy were positive, this may be  
275 the result of latent infection of the epidermis and possibly also the dermis. Locations with all  
276 3 samples positive for BPV DNA may be the result of a combined superficial contamination  
277 and latent epidermal infection or of a combined superficial contamination, unsuccessful  
278 decontamination and dermal latent infection. Locations only positive in the swab taken after  
279 decontamination but not in the two other samples might have got re-contaminated because the  
280 horse moved with consequent contact with other parts of the body or whirling of hairs or shed  
281 epidermal cells on the decontaminated area. It is also possible that the decontamination  
282 protocol was not efficient enough. A study on HPV prevalence on the top of skin tumours  
283 compared to stripped biopsies demonstrated that the same decontamination protocol resulted  
284 in a rather good but not 100% effective removal of HPV DNA from superficial skin layers  
285 (Forslund et al., 2004). These findings can be explained by the rather resistant character of  
286 papillomaviruses against conventional disinfection protocols. The presence of BPV DNA in  
287 the swab obtained after decontamination as well as in the biopsy combined with a negative  
288 swab before decontamination can be the result of epidermal BPV infection. Equine sarcoids  
289 are fibroblastic tumours but they often have an important epithelial component. Thus far,  
290 BPV DNA has only been demonstrated in the fibroblasts and not in the epidermal cells (Lory  
291 et al., 1993). However, the presence of BPV DNA in swabs of decontaminated skin together  
292 with the observed epidermal changes could generate the hypothesis that BPV infection of  
293 equine skin might start in the epidermis, followed by shifting of the viral material to the  
294 subepidermal fibroblasts where the BPV genome can fully exert its transformational activity.

295 Another remarkable finding is that BPV mRNA was demonstrated in sample locations,  
296 positive in the swab (either before or after decontamination) but negative in the biopsy.  
297 Theoretically it is possible that the BPV mRNA in BPV DNA negative biopsies originates  
298 from the source of superficial contamination although mRNA production is not expected  
299 when intact BPV particles contaminate the skin. Another possibility is that the quantity of  
300 BPV DNA in the normal skin samples is close to the detection limit of the applied technique,  
301 which may result in a positive outcome in some samples and a negative in others. Since BPV  
302 load and mRNA expression are correlated (Bogaert *et al.*, 2007), it can be supposed that very  
303 low mRNA expression levels reflect very low levels of viral DNA in these samples.

304 Among the healthy horses, mainly those having contact with cattle with papillomas were  
305 positive for BPV DNA. These were often affected by 2 different BPV types. Even if the BPV  
306 infection in cattle had already disappeared for up to 6 months, contact horses still got a higher  
307 chance of harbouring BPV in their skin. Horses having contact with sarcoid horses did not  
308 have a significantly higher chance of harbouring BPV compared to the control group. This  
309 could be explained by the non-productive BPV infection in equine sarcoids: no capsid  
310 proteins are synthesised and thus no infectious virus particles are formed. Nevertheless,  
311 outbreaks of equine sarcoids in closed herds of equids have been reported (Ragland *et al.*,  
312 1966; Reid *et al.*, 1994; Nel *et al.*, 2006; personal observations) and BPV DNA was detected  
313 in half of the horses living in contact with sarcoid affected horses in our study. This could  
314 indicate that BPV DNA alone can be sufficient for transmission of infection between horses,  
315 although to a lower degree compared to transmission by cattle where infectious virus particles  
316 are formed. This stresses the need for hygienic measures when horses with sarcoids are  
317 present in the close surroundings of other horses.

318 In the control group, 30% of the horses harboured BPV DNA in the skin which indicates that  
319 a substantial part of the horse population is infected even without recent contact with diseased

320 animals. In many sarcoid cases, owners do not report any contact with cattle or sarcoid-  
321 affected horses. It is however possible that horses got infected by neighbouring cattle a long  
322 time ago, maybe with previous owners, and that BPV infection was latent during months or  
323 years, ultimately resulting in the development of sarcoids. In man, acquisition of HPV  
324 infection of the skin probably occurs in early infancy through close skin contact with infected  
325 individuals (Antonsson et al., 2003). Multiple studies in man have shown that a substantial  
326 proportion of the healthy population is infected with HPV on the normal skin (Astori et al.,  
327 1998; Forslund et al., 2004). It is hypothesized that in immunocompetent individuals an intact  
328 immune system inhibits the development of clinical disease (Astori et al., 1998). In horses,  
329 sarcoid susceptibility is associated with certain equine leukocyte antigen (ELA) haplotypes of  
330 the major histocompatibility complex. In the Swiss Warmblood Horse, the Irish Sporthorse  
331 and the Selle Français, horses with the ELA W13 haplotype are more susceptible to equine  
332 sarcoid development (Brostrom et al., 1988), which might indicate a role of the antigen  
333 presenting system in the host defence against BPV infection. In Arabian Horses a significant  
334 correlation exists between sarcoid development and heterozygosity for the defective DNA-  
335 PKcs allele responsible for severe combined immunodeficiency (Ding et al., 2002). The  
336 present study shows that the proportion of latent BPV carriers in the horse population (52 %  
337 of the healthy horses) is much higher compared to the prevalence of equine sarcoids, which  
338 mounts up no higher than 12 % (Gerber, 1989; Studer et al., 2007). A high clearance rate and  
339 a low progression rate towards tumour development can therefore be assumed.

340 In this study, 38 % of the horses positive for BPV DNA harboured BPV-2. This is remarkably  
341 higher than the proportion of BPV-2 induced sarcoids in Europe, which amounts not more  
342 than 20 % (Angelos et al., 1991; Otten et al., 1993; Martens et al., 2001a; Bogaert et al.,  
343 2007). However, this high percentage can be attributed to the horses living in contact with  
344 cattle having suffered a recent papillomavirus infection. When this group is excluded from the



345 calculation, the expected 80/20 proportion of BPV-1 to BPV-2 is seen. These results might  
346 indicate that BPV-2 infection in horses is more easily cleared and less likely to induce  
347 sarcoids.

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

365 It remains unclear how equine sarcoids eventually develop after BPV infection. Skin trauma  
366 can certainly play an important role because it allows direct access of BPV to the  
367 subepidermal fibroblasts which may lead, together with formation of different growth factors  
368 necessary for skin regeneration, to proliferation of the BPV transformed cells. In man it is  
369 observed that not only patients with psoriasis, which is strongly correlated with HPV-5, but

370 also patients with epidermal repair following extensive second degree burns or autoimmune  
371 bullous diseases, generate antibodies to HPV-5 (Favre et al., 2000). Healthy cattle housed in  
372 isolation can develop papillomas harbouring BPV-1 and/or -2 following immunosuppression  
373 or skin trauma (Campo et al., 1994). Also in horses, sarcoid development in wounds is  
374 frequently observed (Torrontegui and Reid, 1994; Knottenbelt et al., 1995). Nevertheless,  
375 many sarcoids appear without any clinical evidence of skin trauma, although microtrauma  
376 (e.g. insect bites) cannot be excluded.

377

## 378 **5. Conclusion**

379

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

387

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389

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- 487

488 **Tables**

489

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

492

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

493

494 Group S: horses with equine sarcoids

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

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

497 Group C: healthy control horses

498 \* in horses with at least one positive sample

499

500

501

502

503



504 Table 2: Normal skin locations positive for BPV DNA and mRNA in horses with equine sarcoids (group S).  
 505  
 506

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

507  
 508 <sup>1</sup> before decontamination

509 <sup>2</sup> after decontamination

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

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

512 Table 3: Normal skin locations positive for BPV DNA and mRNA in healthy horses living in contact with sarcoid-affected horses (group CS).  
 513

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

514

<sup>1</sup> before decontamination

515

<sup>2</sup> after decontamination

516

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

517

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

518

† biopsy of the thigh was missing

519

520

521

522 Table 4: Normal skin locations positive for BPV DNA and mRNA in healthy horses living in contact with cattle having suffered a recent  
 523 papillomavirus infection (group CC).  
 524

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

525  
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 527  
 528  
 529  
 530

<sup>1</sup> before decontamination

<sup>2</sup> after decontamination

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

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

531 Table 5: Normal skin locations positive for BPV DNA and mRNA in healthy control horses (group C).  
 532

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

533  
 534 <sup>1</sup> before decontamination

535 <sup>2</sup> after decontamination

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

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

538

539

540 **Figure captions**

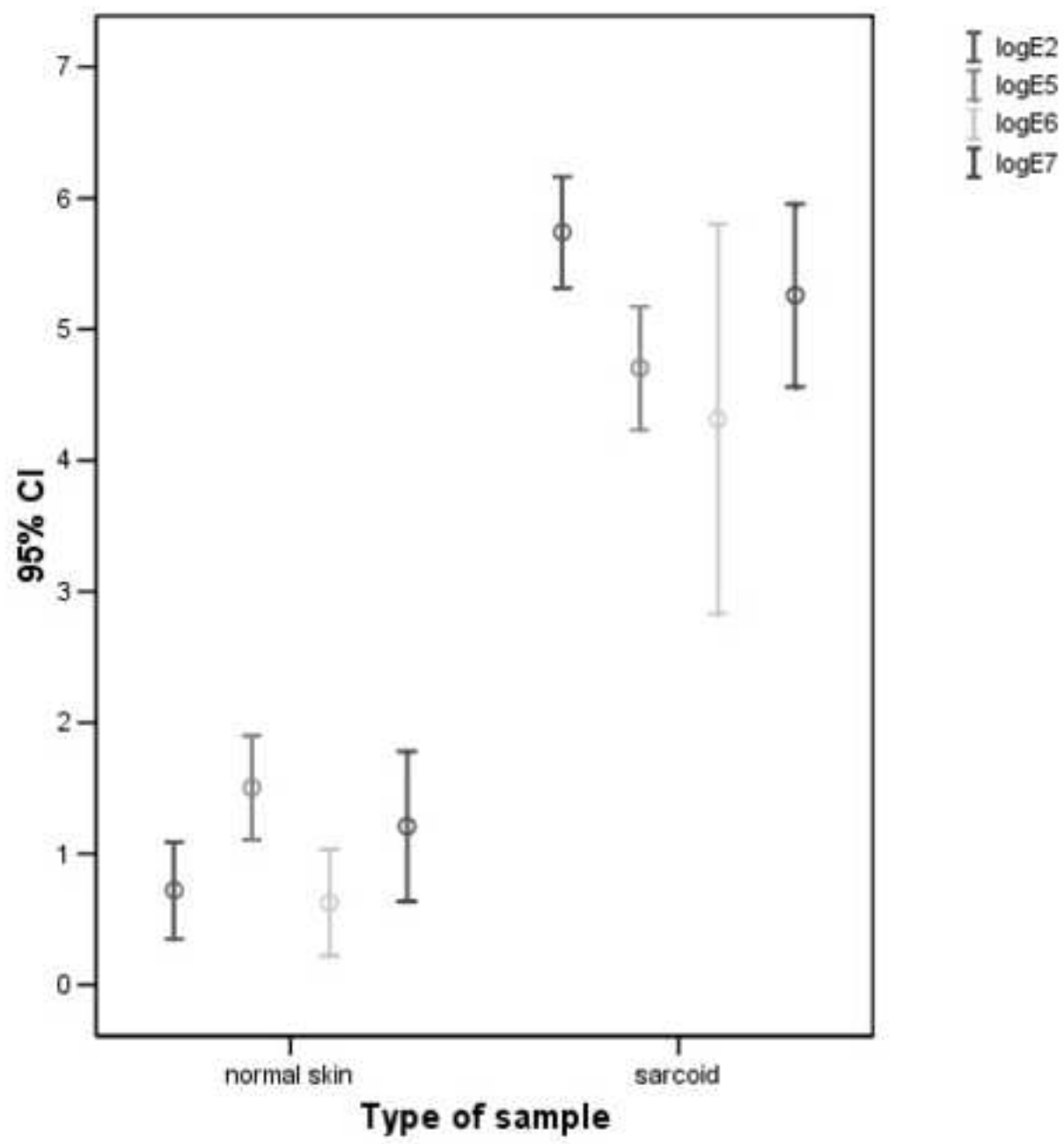
541

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

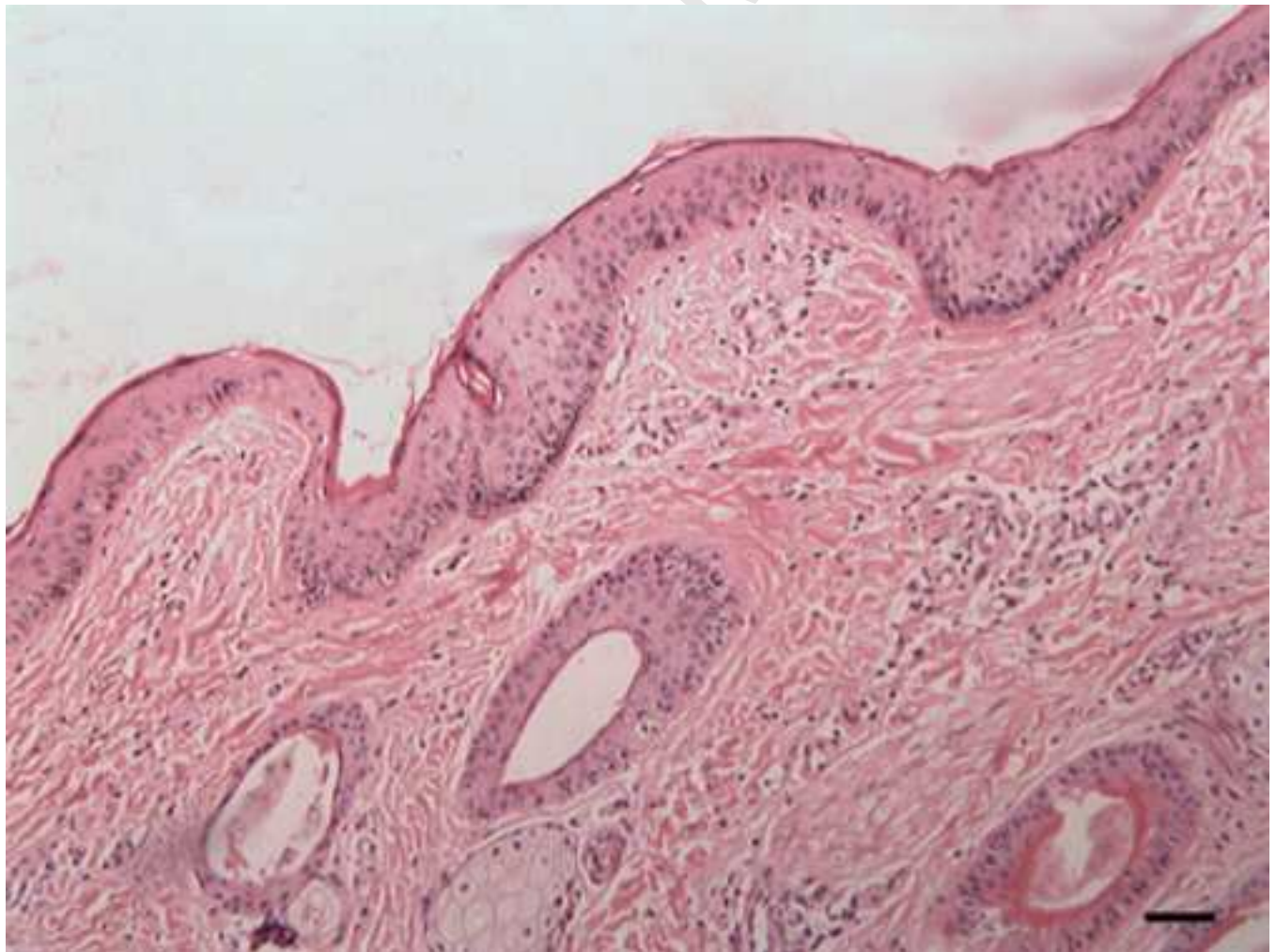
545

546 Fig. 2: Mild focal acanthosis, slight basophilic cytoplasmic swelling and parakeratosis of a  
547 normal equine skin sample positive for BPV DNA. (a) Overview of epidermal and dermal  
548 layers (bar = 50  $\mu\text{m}$ ). (b) Detail of the epidermal layers (bar = 25  $\mu\text{m}$ ).

Script



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