Peripheral blood mononuclear cells represent a reservoir of bovine papillomavirus DNA in sarcoid-affected equines

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Bovine papillomaviruses (BPV-1 and -2) chiefly contribute to equine sarcoid pathogenesis. However, the mode of virus transmission and the presence of latent infections are largely unknown. This study established a PCR protocol allowing detection of \( \leq 10 \) copies of the BPV-1/-2 genes E5 and L1. Subsequent screening of peripheral blood mononuclear cell (PBMC) DNA derived from horses with and without BPV-1/2-induced skin lesions demonstrated the exclusive presence of E5, but not L1, in PBMCs of BPV-1/2-infected equines. To validate this result, a blind PCR was performed from enciphered PBMC DNA derived from 66 horses, revealing E5 in the PBMCs of three individuals with confirmed sarcoids, whereas the remaining 63 sarcoid-free animals were negative for this gene. L1 could not be detected in any PBMC DNA, suggesting either deletion or interruption of this gene in PBMCs of BPV-1/-2-infected equines. These results support the hypothesis that PBMCs may serve as host cells for BPV-1/-2 DNA and contribute to virus latency.

Bovine papillomaviruses types 1 and 2 (BPV-1 and -2) are chiefly involved in the pathogenesis of locally aggressive cutaneous lesions termed sarcoids that frequently affect horses, donkeys and mules (Olson & Cook, 1951). In contrast to BPV-induced bovine papillomas, which usually resolve spontaneously, equine sarcoids mostly persist and tend to recrudescence following treatment. In general, sarcoids do not metastasize, yet their localization and progression to more aggressive types can compromise the use and welfare of affected animals (Scott & Miller, 2003).

An aetiological association of BPV with sarcoids was first suspected when inoculation with bovine wart extract produced transient sarcoid-like lesions in horses (Olson & Cook, 1951; Voss, 1969). BPV DNA was first demonstrated in sarcoids by Southern blotting (Lancaster et al., 1979; Amtmann et al., 1980; Trenfield et al., 1985). Using the more sensitive PCR, BPV DNA has been detected in up to 100% of investigated sarcoids (Otten et al., 1993; Bloch et al., 1994; Carr et al., 2001a, b; Martens et al., 2001a, b; Bogaert et al., 2005, 2008) and also in apparently intact skin of sarcoid-bearing individuals, leading to the speculation that BPV might reside latently in fibroblasts until factors such as trauma initiate its transcriptional and hence transforming activity (Carr et al., 2001a). There is evidence that BPV DNA cannot be found in sarcoid-free horses or non-sarcoid equine tumours (Otten et al., 1993; Carr et al., 2001a, b). However, the presence of viral DNA in skin swabs of unaffected horses has been reported recently (Bogaert et al., 2005, 2008). In addition, BPV-1 DNA (Angelos et al., 1991; Chambers et al., 2003a) and early gene transcripts have been demonstrated in some cases of dermatitis (Yuan et al., 2007).

Although viral genes are intraleesionally transcribed (Nasir & Reid, 1999; Carr et al., 2001b; Nixon et al., 2005; Bogaert et al., 2007), intact virions have not been detected in sarcoids so far. Hence, the disease is understood as the result of an abortive infection where BPV exists episomally (Amtmann et al., 1980; Lancaster, 1981).

The mode of BPV transmission within and between animals is still unclear. Virus may be propagated by direct contact or via contaminated habitual surroundings such as tack, barns or stable walls (Chambers et al., 2003b; Bogaert et al., 2005). The predilection for sarcoid development at wound sites also suggests that insects infesting sites of trauma may act as vectors. Indeed, viral DNA has been
detected in face flies feeding on sarcoids (Kemp-Symonds, 2000). Blood might be another reservoir of viral DNA contributing to the propagation of the disease. BPV DNA has been demonstrated in whole blood of infected cattle (Campos et al., 1994; Campo, 1998; De Freitas et al., 2003; Wosiacki et al., 2005), suggesting vertical virus transmission via the blood stream. Following blood transfusion from papilloma-bearing to virus-free cattle, BPV DNA was detected in peripheral blood mononuclear cells (PBMCs) of recipient cows and their progeny, supporting the concept that BPV can be transmitted in utero (Stocco dos Santos et al., 1998). Human papillomavirus (HPV) DNA has been detected in PBMCs (Pao et al., 1991; Bodaghi et al., 2005), serum (Liu et al., 2001) and plasma (Dong et al., 2002) of human patients affected by HPV-associated cancers. This finding was initially interpreted as originating from disseminated tumour metastases. However, HPV DNA has also been detected in PBMCs of clinically unremarkable children that had acquired a human immunodeficiency virus (HIV) infection via blood transfusion or vertical transmission, thus suggesting that HPV had been co-transmitted by PBMCs (Bodaghi et al., 2005).

BPV DNA has not been demonstrated in the blood of BPV-infected equids so far (Angelos et al., 1991; Nasir et al., 1997; Bogaert et al., 2008). Based on the hypothesis that PBMC-derived BPV DNA may have escaped detection due to extremely reduced virus loads, we reassessed this issue by establishing a highly sensitive PCR protocol for detection of the BPV-1/-2 genes E5 and L1. Cloned full-length BPV-1 DNA (plasmid BPV1-pML) with an initial concentration of 108 ng μl-1, equivalent to 1010 molecules μl-1, was serially diluted 10-fold (109–101 genome copies) in virus-free genomic DNA (170 ng μl-1) obtained from an ~3 mm3 skin biopsy of a healthy horse using a DNeasy Tissue Extraction kit (Qiagen). Subsequently, 1 μl sample aliquots were subjected to E5 and L1 PCR using BPV-1/-2 consensus primers selected from published BPV-1 (GenBank accession no. X02346) and BPV-2 (GenBank accession no. M20219) sequences. E5-specific primers (5′B1/2-E5: 5′-CTAACACCTCTGGAATGAAACATTTC-3′; 3′B1/2-E5: 5′-TCACCTTGWGTATCACTCTGTTG-3′) were designed for amplification of a 499 bp (BPV-1) or 497 bp (BPV-2) fragment spanning the E5 open reading frame (ORF). L1-specific primers (5′B1/2-L1: 5′-GCTAAAGCAACACAGATCTGCAGTCC-3′; 3′B1/2-L1: 5′-TCAGCCATTTTGAATGATCCTGG-3′) were designed for amplification of a 266 bp region of the major capsid gene L1. PCR was carried out in 0.5 ml MultiII Ultra PCR tubes (Sorenson Bioscience), each containing 9.5% DMSO, 10 mM Tris/HC1 (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 1.5 mM each dNTP, 100 pmol sense and antisense primer, and 1 μl DNA template in a volume of 49 μl, using two drops of mineral oil as a top layer. Reaction tubes were placed unlocked in an Eppendorf Mastercycler. Following a manual hot start at 95 °C for 5 min and the addition of 1 U Taq polymerase (Roche Life Science), tubes were closed and an amplification program consisting of seven touch-down cycles [92 °C for 30 s/65–56 °C for 45 s (~1.5 °C per cycle)/72 °C for 45 s], followed by 40 standard cycles (92 °C for 30 s/56 °C for 45 s/72 °C for 45 s) was performed. PCR products were visualized on 2% Tris/acetate agarose gels by ethidium bromide staining. As shown in Fig. 1(a), both reactions were positive for all plasmid dilutions (109–101 copies), thus revealing a PCR detection limit of ≤10 copies for E5 and L1. A plasmid-free negative control reaction (dilution 0) was included in each experiment.

In the next step, whole blood (2 × 8 ml per individual) was collected in Vacutainer CPT Cell Preparation Tubes with sodium citrate (Becton Dickinson) from four sarcoid-bearing horses (horses A, B, D and E), one mare suffering from an histologically confirmed vaccination abscess.
(horse C) and one healthy pony (horse H), the latter serving as a PBMC-negative control. Tissue was collected from a sarcoid of horse B, distant intact skin of sarcoid-bearing horse E and from lesions of two sarcoid-free horses F and G used as controls, comprising an equine papillomavirus type 1-induced juvenile wart (Fw) and a melanoma (Gm). Following PBMC isolation according to the instructions of the manufacturer (Becton Dickinson), blood cells and tissue specimens were subjected to DNA extraction as described above. β-Actin PCR performed under the conditions described above (primers 5′EBA 476: 5′-TCACCACACTGTGGCATCTACG-3′; 3′EBA 1090: 5′-CGTCRTACTCCTGCTTGCTGATCC-3′; GenBank accession no. AF035774) confirmed successful DNA purification for all isolates (results not shown). Subsequent E5 PCR from 2 μl DNA aliquots were positive for tumour (Bsa) and distant skin DNA (Eds) of sarcoid-affected horses B and E, respectively, as well as for PBMCs of sarcoid-bearing individuals A, B, D, E and the abscess-bearing mare C. PBMC or tissue DNA of control animals F, G and H was negative (Fig. 1b; Table 1). Sarcoid DNA of an individual with a determined BPV-1-infection (Jsa) was used successfully as positive control. To confirm specificity, one PBMC-derived amplicon (Fig. 1b, lane 2) was cloned and sequenced, revealing four silent point mutations compared with BPV-1 E5 laboratory clones with 100% identity to the predicted BPV-1 E5 protein sequence Swiss III (GenBank accession no. AY232259; Chambers et al., 2003a).

Subsequently, DNA was purified from sarcoid tissue (Ksa), perilesional skin (Kps) and distant intact skin (Kds) of a sarcoid-bearing horse K, from a penile squamous cell carcinoma (SCC) from horse L (Lscc), from a periorcular SCC (horse M; Mscc) and from skin from the margin of the vaccination-induced abscess of horse C (Cva). Following successful β-actin PCR (not shown), DNA isolates were screened for the presence of E5 and a 266 bp region of L1. PBMC and tissue DNA from sarcoid-bearing individuals B and E were included in the reaction to determine the reproducibility of the E5 PCR results and presence of L1. PCR detected E5 in PBMC, skin and tumour DNA of sarcoid-affected individuals B, K and E, in tissue DNA derived from the vaccination abscess (Cva) and also in the periorcular SCC (Mscc). Sequencing of the SCC-derived E5 amplicon revealed three point mutations compared with laboratory clones with 97.7% identity to the predicted BPV-1 E5 protein sequence Swiss III. The penile SCC (Lscc) was E5-negative, as were the negative controls (Fw, Gm and Hb) and the no-template control (sterile water). L1 PCR revealed 266 bp amplimers for affected and unaffected skin of sarcoid-bearing horses (B, E and K), whereas E5-positive DNA isolates from PBMC (Bb), abscess (Cva) and SCC (Mscc), and the E5-negative penile SCC (Lscc), were negative for L1 (Fig. 1c; Table 1).

Finally, blind PCRs for E5 and L1 were carried out from enciphered PBMC DNA isolates (1–66) obtained from 66

<table>
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<tr>
<th>Horses</th>
<th>Disease</th>
<th>Sample type</th>
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<th>E5 PCR</th>
<th>L1 PCR</th>
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<td>Cb</td>
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<td>Mscc</td>
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co-stabled horses (donated by Monika Seltenhammer, our laboratory). The success of DNA purification was confirmed by β-actin PCR (not shown) and photometry, the latter revealing a mean DNA concentration of 320 ng μl⁻¹. E5 PCR from 2 μl DNA aliquots was positive for 3/66 DNA specimens (PBMC DNA isolates 20, 24 and 33), as well as for sarcoid- (Jsa) and PBMC-derived (Bb, Db and Eb) positive controls. No amplification products were obtained for the negative (Ff and Hb) and no-template controls (Fig. 2; Table 1). The L1 PCR was negative for all tested DNA specimens except for the sarcoid DNA-positive control (not shown). Following decoding, clinical examination and analysis of the medical records provided revealed the exclusive presence of sarcoids in E5-positive individuals 20, 24 and 33, whereas no BPV-related malignancies could be determined for the remaining 63 individuals. Sample specifications and the PCR results are summarized in Table 1.

To our knowledge, this report is the first to demonstrate BPV DNA in PBMCs obtained from sarcoid-affected horses. Controls derived from virus-free individuals were consistently negative for BPV-1/-2 E5 and L1, thus demonstrating the absence of cross-contamination. Sequencing performed for two of the E5 amplimers revealed their specificity and deviation from laboratory strains. Our findings were reproducible and strengthened by a blind E5 PCR from PBMCs, which detected the three sarcoid-affected horses in a cohort of 66 individuals. As sarcoinds are mostly non-metastasizing tumours, it appears unlikely that BPV-specific amplification products originated from sarcoid cells circulating in the blood.

Quantitative E5 PCR recently carried out by us has revealed <100 E5 molecules per 1.2 × 10⁶ PBMCs. The occurrence of such small amounts of E5 in white blood cells (mean of ~1 copy per 1000 cells) in combination with less sensitive detection or PBMC extraction methods may explain previous failure to demonstrate them.

The intralesional presence of BPV-1/-2 E5 DNA and transcripts has been well documented (Nasir & Reid, 1999; Carr et al., 2001b; Chambers et al., 2003a; Bogaert et al., 2007), supporting an active role of E5 in sarcoid formation. We have detected E5 DNA in the PBMCs of BPV-1/-2-affected horses, suggesting an as yet unknown concurrence with morbidity. E5 was also identified in a histologically diagnosed periocular SCC. A novel equine papillomavirus termed EcPV-2 was recently identified in genital (Scase, 2007) but not in ocular SCC, suggesting an association of as yet unidentified papillomaviruses with ocular SCC. BPV-1/-2 E5 DNA was detected equally in PBMCs and perilesional tissue of a sarcoid-free mare affected by a vaccination abscess. It is not yet clear whether infection in this horse was accidental or contributed to abscess formation.

The L1 capsid gene-specific 266 bp sequence could not be detected in E5-containing PBMCs, the absence or the periocular SCC, suggesting that L1 may be partially or totally deleted from the viral genome. Absence of the cottontail rabbit papillomavirus L1 ORF has been shown to compromise papilloma formation (Nasseri et al., 1989). However, transfection of murine cells with an L1-deleted BPV-1 genome resulted in tumorigenic transformation (Lowy et al., 1980). Moreover, in vivo transforming activity has been observed for naturally occurring L1 deletion mutants of BPV-1 (Angelos et al., 1991), HPV-6a, HPV-5 and HPV-8 (Ostrow et al., 1982, 1987; Deau et al., 1991; Suzuki et al., 1995), suggesting that L1 may not be required for episomal maintenance and transforming functions in abortive papillomavirus infection.

Alternatively, L1 might be interrupted or lost due to integration of viral DNA into the host cell genome. Integration of cancer-associated HPV types is assumed to correlate with tumour malignancy, possibly because integration occurs at oncogene sites or disrupts tumour suppressor genes (Popescu et al., 1990; Greenspan et al., 1997; Ferber et al., 2003a, b). However, viral integration has never been observed in BPV-infected ungulates (Lancaster, 1981). In an experimental model, BPV-1 transgenic mice developed fibropapillomas harbouring viral episomes, whereas normal tissue contained integrated BPV-1 DNA (Lacey et al., 1986), suggesting that the BPV-1 genome can undergo excision events correlating with tumour formation. By analogy, it is conceivable that BPV integrants may be maintained in PBMCs until excision occurs following interaction with as yet unknown factors.
The presence of BPV DNA in PBMCs suggests a possible contribution to virus spread. Given that whole infectious virus is assumed to produce de novo infection (Ragland & Spencer, 1969) and that horses are considered to be non-permissive hosts for BPV (Chambers et al., 2003b), it seems unlikely that infected PBMCs are involved in horizontal BPV transmission. However, virus may spread in utero from infected mares to their foals, as has been shown in cattle. Moreover, infected PBMCs may propagate disease within one individual, as they migrate to sites of inflammation where they may take up the virus and function as a carrier. Alternatively, PBMC infection may trigger disease by negatively affecting their immunological functions. Further studies are warranted to elucidate the biological and pathologic significance of our findings.

References


