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A subset of equine sarcoids harbours BPV-1 DNA in a complex with L1 major capsid protein

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Abstract

Bovine papillomavirus type 1 or 2 (BPV-1, BPV-2) are accepted causal factors in equine sarcoid pathogenesis. Whereas viral genomes are consistently found and expressed within lesions, intact virions have never been detected, thus permissiveness of sarcoids for BPV-1 replication remains unclear. To reassess this issue, an immunocapture PCR (IC/PCR) was established using L1-specific antibodies to capture L1-DNA complexes followed by amplification of the viral genome. Following validation of the assay, 13 sarcoid-bearing horses were evaluated by IC/PCR. Samples were derived from 21 tumours, 4 perilesional/intact skin biopsies, and 1 serum. Tissue extracts from sarcoid-free equines served as controls. IC/PCR scored positive in 14/24 (58.3%) specimens obtained from sarcoid-patients, but negative for controls. Quantitative IC/PCR demonstrated <125 immunoprecipitable viral genomes/50 µl extract for the majority of specimens. Moreover, full-length BPV-1 genomes were detected in a complex with L1 proteins. These complexes may correspond to virion precursors or intact virions.
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Keywords: Bovine papillomavirus; Equine sarcoid; Virions; Immunocapture PCR

Introduction

Bovine papillomaviruses (BPV) are of considerable veterinary and agricultural importance. In their natural bovine host, they induce hyperproliferative lesions of cutaneous or mucosal epithelia that usually regress spontaneously without treatment. In some cases however, lesions undergo malignant transformation to squamous cell carcinomas (Campo, 2002, 2006).

Like other papillomaviruses, BPV virions are non-enveloped icosahedral structures of 55–60 nm in diameter harbouring circular, double-stranded DNA of 7.3 (BPV-3, -4, -6) or 8 kb (BPV-1, -2, -5). The genome consists of a long control region containing the *cis*-regulatory elements required for replication and transcription of viral DNA, an early gene region coding for non-structural proteins including viral oncoproteins E5, E6, and E7, and a late gene region coding for the major L1 and the minor L2 capsid proteins. Infectious BPV virions have been isolated from bovine papillomas demonstrating productive infection in the habitual bovine host (Campo, 2006).

In addition to causing disease in cows, BPV-1 and BPV-2 are aetiologically involved in the development and maintenance of benign, yet locally aggressive cutaneous lesions termed sarcoids, which frequently affect equids all over the world (Sullins et al., 1986). This etiologic association represents the only example of a natural papillomavirus cross-species infection documented so far (Amtmann et al., 1980; Angelos et al., 1991; Bloch et al., 1994; Carr et al., 2001a,b; Chambers et al., 2003a; Lancaster et al., 1979; Lory et al., 1993; Nasir and Reid, 1999; Otten et al., 1993; Reid et al., 1994; Trenfield et al., 1985).

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Sarcoids occur as single or multiple skin lesions and often develop at sites of previous injury and scarring (Ragland et al., 1970; Torrontegui and Reid, 1994). In contrast to bovine papillomas, sarcoids usually persist, are refractory to therapy and tend to recrudesce following excision (Chambers et al., 2003a). According to their presentation tumours are classified as occult, verrucous, nodular, fibroblastic or mixed type lesion (Pascoe and Knottenbelt, 1999). A causal role for BPV was first suspected when experimental inoculation of equine skin with sarcoid extract or purified BPV led to the development of transient sarcoid-like lesions (Olson and Cook, 1951; Ragland and Spencer, 1969; Voss, 1969). Subsequently, Southern blot revealed BPV DNA in sarcoids from horses and donkeys (Amtmann et al., 1980; Lancaster et al., 1979). Using more sensitive PCR technology, BPV-1 or BPV-2 DNA has been detected in up to 100% of lesions (Bloch et al., 1994; Carr et al., 2001a,b; Martens et al., 2001a,b; Otten et al., 1993) and also in intact skin of sarcoid-bearing individuals (Carr et al., 2001b). Conversely, BPV DNA cannot be detected in horses without sarcoids or in non-sarcoid equine tumours (Carr et al., 2001a,b; Otten et al., 1993; personal observation). However, BPV DNA has been shown in skin swabs of some unaffected horses living in close contact with sarcoid-bearing animals (Bogaert et al., 2005). In addition, episomal BPV-1 genomes (Angelos et al., 1991; Chambers et al., 2003b) and expression of viral early genes have been demonstrated in dermatitis-affected equine skin (Yuan et al., 2007a).

In sarcoids, BPV DNA resides as multiple episomal copies within the nuclei of infected cells (Amtmann et al., 1980; Campo, 2002; Yuan et al., 2007b). Viral early and late genes have been demonstrated to be intralesionally transcribed (Carr et al., 2001a; Nasir and Reid, 1999; Nixon et al., 2005)

Table 1
Investigated sample specimens: specifications, PCR, IC/PCR and IC/qPCR results

Horse	Sample No.	Sample type	Collection site	E5 PCR	L1 PCR	IC/PCR	Number of complexes/50 µl extract±SD
Patient group							
A	A1	Verrucous sarcoid	Penis	1	0	0	
	A2	Verrucous sarcoid	Penis	1	1	0	
B	B3	Fibroblastic sarcoid	Ear	1	1	1	*n.d.*
	B4	*Fibroblastic sarcoid*	*Ear*	*1*	*1*	*n.d.*	
C	C5	*PBMCs*	*Whole blood*	*1*	*0*	*n.d.*	
	C6	*Intact skin*	*Neck*	*1*	*1*	*n.d.*	
	C7	Fibroblastic sarcoid	Prepuce	1	1	1	*n.d.*
D	D8	Mixed sarcoid	Prepuce	1	1	1	<125
	D9	Mixed sarcoid	Prepuce	1	1	1	<125
	D10	Mixed sarcoid	Prepuce	1	1	1	<125
	D11	Serum	Whole blood	*n.d.*	*n.d.*	0	
E	E12	Verrucous sarcoid	Crown	1	1	0	
F	F13	Fibroblastic sarcoid	Stifle	1	1	1	118,500±10,610
	F14	Fibroblastic sarcoid	Anterior chest	1	1	1	<125
G	G15	Fibroblastic sarcoid	Left ear	1	1	1	<125
	G16	Nodular sarcoid	Right ear	1	1	1	<125
	G17	Intact skin	Neck	1	1	0	
H	H18	Mixed sarcoid	Fetlock	1	0	0	
J	J19	Mixed sarcoid	Hind leg	1	1	0	
	J20	Mixed sarcoid	Hind leg	1	1	0	
	J21	Mixed sarcoid	Hind leg	1	1	0	
	J22	Perilesional skin	Hind leg	1	1	1	<125
K	K23	Fibroblastic sarcoid	Nostrils	1	*n.d.*	1	Not detectable
	K24	Intact skin	Neck	1	*n.d.*	1	<125
L	L25	Verrucous sarcoid	Chest	1	1	0	
M	M26	Fibroblastic sarcoid	Elbow	1	1	1	<125
N	N27	Verrucous sarcoid	Neck	1	1	1	<125
	N28	Nodular sarcoid	Prepuce	1	1	1	52,800±2970
	N29	Perilesional skin	Prepuce	1	1	1	254±30
O	O30	Dermatitis	Hind leg	1	0	0	
	O31	Intact skin	Neck	1	*n.d.*	0	
Control group (-c; sarcoid-free individuals)							
P	P32	Intact skin	Scrotum	0	0	0	Not detectable
Q	Q33	Intact skin	Umbilicus	0	0	0	Not detectable
R	R34	Intact skin	Scrotum	0	0	0	Not detectable
S	S35	Mastocytoma	Fore leg	0	0	*n.d.*	
T	T36	Melanoma	Peritoneum	0	0	*n.d.*	
	T37	Cartilage	Peritoneum	0	0	*n.d.*	

1: positive; 0: negative; n.d.: not done; SD: standard deviation of duplicates.
Italic: only DNA isolation performed, no extract available due to small sample size.

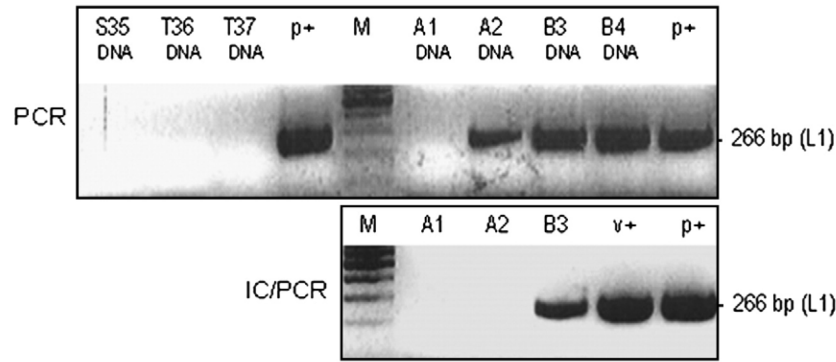

Fig. 1. IC/PCR scores positive in case of an ulcerated fibroblastic sarcoid. Top: PCR from purified DNA revealed presence of L1 in case of a verrucous (A2) and two fractions of a fibroblastic (B3, B4) sarcoid, whereas DNA of another verrucous sarcoid (A1) derived from patient A scored negative. DNA obtained from a mastocytoma (S35), a melanoma (T37) and cartilage of the melanoma-bearing mare (T36) tested negative, as anticipated. Bottom: When using IC/PCR however, extract of both verrucous sarcoids of same origin (A1, A2) scored negative, whereas extract from fibroblastic tumour B3 tested positive by this method, thus suggesting the presence of capsid protein-complexed BPV-1 DNA in this lesion. M: GeneRuler™ 100 bp DNA Ladder Plus (Fermentas); p+: plasmid positive control (BPV1-pML plasmid, PCR step only); v+: BPV-1 virion positive control.

providing additional evidence of viral involvement in sarcoid pathogenesis. Infectious virions have not been detected in equine sarcoids so far (Amtmann et al., 1980; Lancaster, 1981), leading to the assumption that horses are non-permissive hosts for BPV (Campo, 2002; Chambers et al., 2003a).

However, previous failure to demonstrate BPV virions in sarcoids may be due to very low particle concentrations. Alternatively, virion formation may be restricted to particular developmental stages of the tumour (Campo, 2002), or remain incomplete in horses. To reassess this issue, a previously established single tube immunocapture PCR (IC/PCR) protocol (Brandt et al., 1993) was adapted for detection of L1 capsid protein-associated BPV-1 DNA in samples from various equine sarcoid types and skin of affected horses. Given that IC/PCR combines the specificity of an immune-affinity capture step with the sensitivity of PCR for selective amplification of antigen-associated viral nucleic acids (Jansen et al., 1990), this method is suitable for detection of extremely low amounts of virions or premature assemblies against the background of crude tissue extract.

Results

PCR reveals E5 in 100% and L1 in 84% of DNA samples from equine patients

In the first step, samples were obtained from 13 sarcoid-bearing horses and 1 individual affected by dermatitis (patient group). As a control biopsies were obtained from 3 healthy horses and 2 with BPV-unrelated tumours (control group) (see Table 1 for details). Whole cellular DNA was isolated and subjected to PCR amplification using BPV-1/-2 consensus primers specific for E5 and L1. Hundred percent (30/30) of DNAs from the patient group tested positive for E5 by PCR, including one peripheral blood mononuclear cell (PBMC) sample (C5) (Table 1). In contrast, E5 was not detected in skin of 3 healthy individuals, and 2 horses with a mastocytoma and a melanoma, respectively. Sequencing of E5 amplimers obtained from sarcoid A2 and B3 DNA revealed 100% identity to BPV-1 variant Swiss III E5 (Genbank accession number AY232259).

An L1-specific 266 bp region was amplified from 85% (23/27) of DNA isolates from the patient group. One penile verrucous

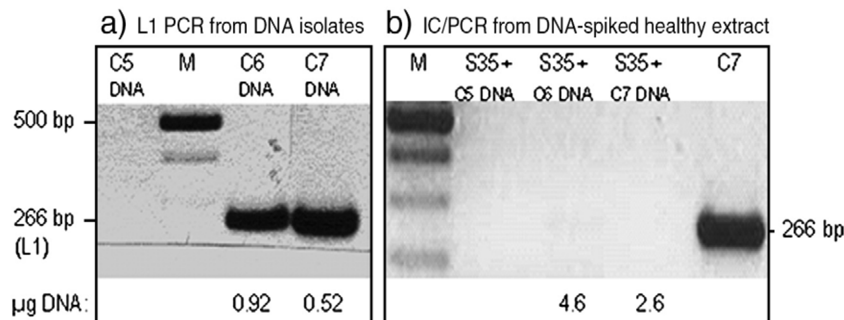

Fig. 2. IC/PCR specifically detects BPV-1 major capsid protein-associated viral DNA. DNA was isolated from PBMC (C5 DNA), intact distant skin (C6 DNA) or from a fibroblastic sarcoid (C7 DNA) of one affected individual. a) PCR revealed L1-specific amplimers in skin and tumour DNA (lanes 3 and 4), but not in PBMC DNA (lane 1). b) Skin extract was prepared from a virus-free horse (S35) and spiked with C6 DNA or C7 DNA (5× the amount used in 1a). S35 spiked with L1-negative C5 DNA served as control. IC/PCR scored negative for all DNA-spiked extracts (lanes 2–4), whereas the reaction produced high yields of L1 amplicon when using extract supernatant of the fibroblastic sarcoid (C7) as IC/PCR template. Amounts of L1-positive DNA used as template in (a) and for extract spiking in (b) are indicated in µg. M: GeneRuler™ 100 bp DNA Ladder Plus (Fermentas).

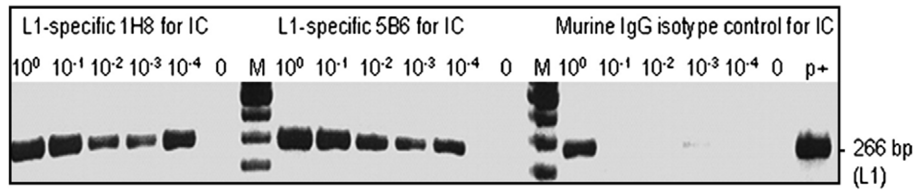

Fig. 3. Sensitive IC/PCR-mediated detection of capsid protein-complexed viral DNA requires L1-specific antibody. Sarcoid extract (C7) was left undiluted (10^0) or serially 10-fold diluted (10^{-1}–10^{-4}) in extraction buffer as indicated. Following incubation with monoclonal antibodies 1H8 or 5B6 directed against BPV-1 L1, or murine IgG as control, L1-specific PCR was carried out. The reaction scored positive for all extract dilutions when using L1-specific capture antibody (lanes 1–5 and 8–12), but only for undiluted extract, when coating unspecifically (lane 15). 0: extraction buffer (no template control); p+: positive control (BPV1-pML plasmid, PCR step only); M: GeneRuler™ 100 bp DNA Ladder Plus (Fermentas).

sarcoid (A1), 1 mixed sarcoid (H18), 1 PBMC sample (C5) and 1 dermatitis-affected skin (O30) scored negative for L1, as did all DNAs isolated from the control group. E5 and L1 PCR results are summarised in Table 1.

A pilot IC/PCR suggests the existence of L1 protein complexed with BPV-1 DNA in a fibroblastic sarcoid

Single tube IC/PCR has been proven valuable for sensitive detection of RNA and DNA viruses for which virion antibodies are available. Virus in crude extract supernatant is captured by virion-specific antibody coated to a reaction tube thus concentrating the virion target. Following washing under gentle non-denaturing conditions to avoid complex disruption, viral DNA is released by heating and PCR amplified. In the herein applied IC/PCR, BPV-1 L1 major capsid protein-specific capture antibodies and PCR primers spanning a 266 bp region of the viral L1 gene were used.

In the first attempt, IC/PCR was applied to extracts of 2 verrucous sarcoids (A1, A2) and 1 ulcerated fibroblastic sarcoid (B3). Standard PCR from corresponding DNA isolates had revealed E5 in 3/3, and L1 in 2/3 (A2 and B3) tumours (Table 1; Fig. 1). Subsequent IC/PCR scored negative for extracts of both verrucous lesions (A1, A2), despite the fact that PCR had detected L1 in A2 DNA. However, IC/PCR scored positive for the ulcerated fibroblastic sarcoid (B3) suggesting the presence of capsid protein-associated BPV-1 DNA (Fig. 1). The reaction was verified using BPV-1 virions (IC/PCR) and cloned BPV-1 DNA (PCR only) as controls (Fig. 1).

BPV-1 L1 capsid protein-complexed DNA is required for IC/PCR-mediated detection

To validate the method, we investigated whether IC/PCR can distinguish BPV-1 capsid protein-complexed DNA from viral episomes that may be released during mechanical tissue disruption, adhere non-specifically to antibody and/or tube walls and resist to washing. To this aim, BPV-free tissue extracts prepared from a mast cell tumour (S35) were spiked with L1 gene-containing DNA isolated from intact skin (C6) or a fibroblastic sarcoid (C7). Spiked extracts as well as sarcoid extract (C7) were subjected to IC/PCR. The reaction scored positive for the sarcoid, whereas no amplicons were obtained for the mastocytoma spiked with L1 gene-positive DNAs (Fig. 2) ruling out detection of viral DNA adhering non-specifically to the reaction tube.

To further study specificity of antigen trapping, serial ten-fold dilutions of sarcoid extract (C7) were analysed in reaction tubes that had been alternatively coated with two different capsid protein-specific antibodies, 1H8 and 5B6, or murine IgG as a control. Undiluted or serially 10-fold diluted (10^0 to 10^{-4}) sarcoid extracts scored positive by IC/PCR using L1-specific antibodies, whereas only undiluted extract scored positive in murine IgG-coated tubes (Fig. 3). Taken together these results indicated that detection by IC/PCR requires the presence of viral genome in a complex with BPV-1 L1 capsid protein.

IC/PCR detects L1 capsid protein-associated BPV-1 DNA in 58.3% of sarcoids, perilesional skin or intact skin of sarcoid-bearing horses

We next examined 21 sarcoids classified as verrucous ($n=5$), nodular ($n=2$), fibroblastic ($n=7$) or mixed types ($n=7$) by IC/PCR. Two perilesional and 2 intact skin biopsies from sarcoid-bearing horses were also analysed, as were dermatitis-affected skin and intact skin from an otherwise healthy individual. IC/PCR scored positive for all tested nodular (2/2) and fibroblastic (7/7) sarcoids, 3/7 mixed, and 1/5 verrucous sarcoids, and also for 1/2 perilesional skin specimen (N29) (Table 2). In contrast, capsid protein-associated BPV-1 DNA was only detected in 1/5 (N27) verrucous lesions. For 7 mixed sarcoids collected from 3 horses only 3 tested positive, all derived from one individual with ulcerated tumours of the prepuce (D8–D10). Serum (D11) of this latter patient was negative by IC/PCR. Mixed sarcoids

Table 2
Presence of L1 protein-complexed BPV DNA in sarcoid-affected horses per sample type analysed

Tissue type collected	Number of samples	IC/PCR positive
Verrucous sarcoid	5	1
Nodular sarcoid	2	2
Fibroblastic sarcoid	7	7
Mixed sarcoid	7	3
Perilesional skin	2	2
Intact skin	2	1
Serum	1	0
Σ	26	16 (61.5%)

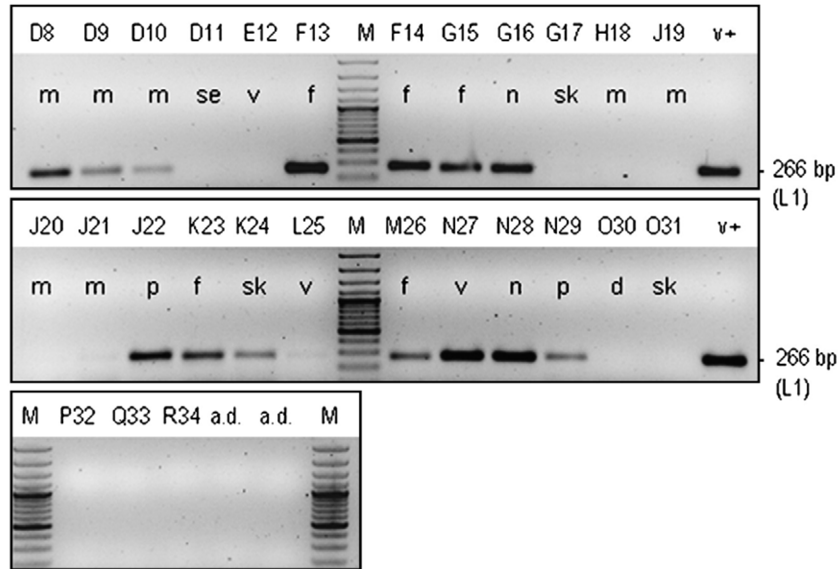

Fig. 4. IC/PCR reveals capsid protein-complexed BPV-1 DNA in 14/24 samples derived from sarcoid-bearing horses. Tissue extract supernatants (and 1 serum; D11) derived from 10 sarcoid-affected individuals (D–N), and three healthy equines (P–R) as controls were analysed by IC/PCR. L1-specific 266 bp amplimers obtained for 14 out of 24 samples derived from the patient group indicate presence of L1-containing DNA in a complex with immunopurified major capsid protein in these specimens. Healthy equine controls scored negative, as expected. m: mixed, f: fibroblastic, v: verrucous, n: nodular sarcoid; se: serum; sk: intact skin; p: perilesional skin; d: dermatitis; v+: BPV-1 virion positive control; a.d.: sterile water (no template control); M: GeneRuler™ 100 bp DNA Ladder Plus (Fermentas).

from two other horses (H18, J19–J21) scored negative, whereas perilesional skin (J22) tested positive. Interestingly, L1-complexed DNA was also detected in distant intact skin (K24) of a horse with an ulcerated fibroblastic sarcoid of the nostrils, whereas intact skin (G17) was negative from a horse with fibroblastic and nodular sarcoids of the ears. Both affected (O30) and normal skin (O31) from an individual with advanced granulomatous dermatitis of the hind legs (E5-positive by standard PCR) tested IC/PCR negative, which had been expected on the basis of negative L1-standard PCR. As expected, tissue extracts from the control group (P–T) also scored negative by the reaction. IC/PCR using BPV-1 virions or water served as the appropriate controls. Results are shown in Fig. 4 and summarised in Table 1.

Quantitative IC/PCR reveals low concentrations of capsid protein–DNA complexes in the majority of tissue specimens

We further determined the amount of capsid protein-associated BPV DNA in equine sarcoids using quantitative IC/PCR (IC/qPCR). IC/PCR-positive tissue extracts and control specimens were subjected to IC, washed, and trapped complexes were analysed by qPCR using serially diluted plasmid BPV1-pML as standard. IC/qPCR revealed protein–DNA complexes at concentrations of <125 DNA molecules per 50 µl tissue extract for the majority of tested specimens (D8–D10, F14, G15, G16, J22, K24, M26 and N27). 254 complexes (±30 standard deviation [SD]) were detected per 50 µl perilesional skin extract N29. A higher number of DNA molecules per 50 µl extract was

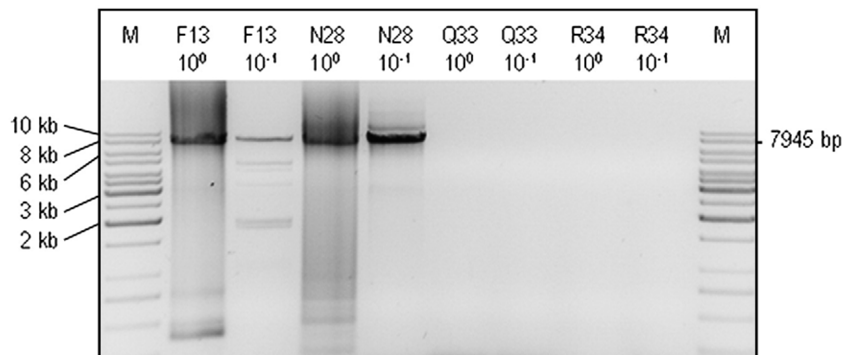

Fig. 5. IC/PCR detects capsid protein-complexed full-length BPV-1 DNA in extracts of two sarcoids. Extract supernatants prepared from a fibroblastic (F13) and a nodular sarcoid (N28), or from skin of healthy equines (Q33, R34) as controls were left undiluted (10^0) or diluted 1:10 (10^{-1}) in extraction buffer. Subsequent IC/PCR performed with BPV-1-specific back-to-back primers and highly processive polymerase resulted in amplification of the entire BPV-1 genome (7945 bp) from undiluted and 1:10 diluted sarcoid extracts (lanes 2–5), whereas healthy equine controls scored negative, as anticipated. M: GeneRuler™ 1 kb DNA ladder (Fermentas).

detected in a nodular sarcoid (N28; 52,800±2970 SD), or a fibroblastic sarcoid (F13; 118,500±10,610 SD). Large amounts of capsid protein-complexed BPV-1 DNA in these two sarcoid specimens were associated with severity of disease. Individual N suffered from multiple sarcoids — a rapidly progressing mass on its prepuce and verrucous tumours of the neck. Individual F was affected by hundreds of verrucous, nodular and fibroblastic sarcoids on the thorax, and a large ulcerated fibroblastic tumour on the stifle (F13) for which the highest amount of complexed DNA was determined. As expected, intact skin specimens (P32, Q33, R34) and no template controls (extraction buffer, TE buffer) scored negative in the assay. For fibroblastic tumour K23 complex amounts could not be determined, which may be due to repeated freezing and thawing, negatively affecting the quality of this sample. IC/qPCR results are listed in Table 1.

Sarcoids contain full-length viral genome complexed to L1

Finally we determined whether full-length BPV genomes are present in the L1-DNA complex in sarcoids. Immunocapture with antibody 5B6 was performed on undiluted and 1/10 diluted extracts of two sarcoids (F13, N28), or skin of two healthy individuals (Q33, R34) as controls. The amplification reaction was carried out with BPV-1-specific back-to-back primers and highly processive DNA polymerase allowing reliable detection of the entire BPV-1 genome. As shown in Fig. 5, amplimers of about 8 kb were detected for undiluted and 1 / 10 diluted sarcoid extracts, whereas skin extracts derived from healthy individuals scored negative. These results strongly support the presence of full-length viral genomes in a complex with L1 in these two sarcoid specimens.

Discussion

It is widely accepted that infection with BPV-1, or less frequently BPV-2, is causally involved in the pathogenesis of equine sarcoids, which represent the most common skin tumour in horses. However, biology and epidemiology of BPV infection in the foreign equine host are in large part unknown. Intradermal injection of horses with cell-free supernatant from minced bovine papillomas has led to the development of fibroblastic tumours. Unlike naturally occurring sarcoids, experimentally induced lesions regressed spontaneously, indicating that additional factors may contribute to disease progression. As an exceptional case, a single induced lesion has persisted and cell-free inoculum of this tumour has produced a similar lesion in another animal (Olson and Cook, 1951). In addition, BPV-1 L1 transcripts have been detected in 50% (10/20) of sarcoids, indicating the possibility that BPV-1 infection may at least occasionally be productive in horses (Nasir and Reid, 1999).

Arguing to the contrary, studies of sarcoids using electron microscopy or antibody-based techniques have failed to demonstrate BPV virions (Amtmann et al., 1980; Lancaster, 1981). In cattle and other species, the papillomavirus life cycle is strictly linked to the state of epithelial cell differentiation. Whereas early gene expression and DNA replication occur in undifferentiated basal and suprabasal cell layers, expression of late genes is restricted to terminally differentiated cells, where new virions are formed and released. In horses, BPV infects fibroblasts that may not provide the cellular environment required for the final steps of virion production.

Assuming that virions are required to infect horses, BPV may spread from infected cattle, e.g. by insect vectors such as face flies (Kemp-Symonds, 2000), or via contaminated habitual surroundings such as tack, barns or stable walls (Bogaert et al., 2005; Chambers et al., 2003a) as virus resists desiccation for days without loosing infectivity (Roden et al., 1997). However, if sarcoids are productive lesions horses may spread BPV infection also directly to their stable mates. This possibility is supported by the occasionally successful experimental passage of the virus in horses, and the occurrence of sarcoids in clusters (Ragland et al., 1966). Moreover, in a study of more than 4000 donkeys, sarcoid disease has mostly affected animals kept in close contact (Reid et al., 1994).

Prior to IC/PCR, DNA specimens were examined for viral genes E5 and L1 by standard PCR, demonstrating BPV infection in 100% of our patients. Sequencing of E5 amplified from two sarcoids revealed BPV-1 E5 variant Swiss III which has been proposed to be associated with equine sarcoids. In comparison to bovine isolates, this E5 variant contains nucleotide substitutions G3921T and A3938G, the latter of which may promote an increase in codon usage (up to 5-fold) and thus more efficient translation of the E5 oncogene in equine cells (Chambers et al., 2003b; Nasir and Reid, 2006). In contrast to previous reports (Angelos et al., 1991; Nasir et al., 1997) BPV E5 DNA was also detectable in PBMC from sarcoid-affected individual C. This finding has been corroborated in an additional cohort of sarcoid-bearing horses (Brandt et al., in press). However, attempts to demonstrate L1-DNA in blood cells of sarcoid-bearing equines and to detect L1 protein-associated BPV DNA via IC/PCR have failed (not shown). Thus it appears unlikely that infectious virions are produced by PBMCs of BPV-infected equines.

Following experiments conducted by standard E5 and L1 PCR, a highly sensitive IC/PCR method (Brandt et al., 1993, 1995) was applied in a pilot experiment and scored positive in case of 1 / 3 tested sarcoid extracts. To confirm authenticity of this preliminary result indicating the presence of capsid protein-complexed BPV-1 DNA in a sarcoid, we evaluated the possibility that IC/PCR detects free viral DNA adhering to coated tube walls, rather than L1-complexed DNA. However, virus-free extract spiked with up to 4.6 µg of BPV-positive DNA (containing 4×10^7 copies of E5 gene by real-time PCR) scored negative by IC/PCR. Furthermore, when sarcoid extracts were diluted over a wide range and reacted either with L1-specific antibody or unspecific control-IgG, IC/PCR scored positive for all dilutions using antibody directed against L1, whereas IC/PCR amplification product resulting from unspecific binding to isotype was only obtained for undiluted sarcoid extract.

Given that IC/PCR proved specific, the method was used to screen a series of different sarcoid types and skin biopsies. IC/PCR scored positive for 14 sample specimens derived from horses with verrucous, nodular, fibroblastic or mixed sarcoids out of 24 tested. Since low target concentrations were assumed, 5B6-mediated immunopurification was combined with 47

amplification cycles as to enhance sensitivity of the test. Subsequently, quantitative IC/PCR from positive extracts demonstrated that the majority of tissues contained only limited amounts of capsid protein-complexed viral DNA. This finding could explain previous failure to detect them by less sensitive methods. High levels of DNA-L1 complexes were detected in a fibroblastic sarcoid (F13) from a horse bearing multiple progressive, therapy-resistant tumours for many years, indicating a possible association with severity of disease.

Finally, we have demonstrated the presence of full-length BPV1 circular genome in a complex with L1 in 2/2 tested sarcoids. This finding supports the possibility that whole virions might occasionally be produced in equine sarcoids. However, capture antibody 5B6 reacts with both, intact BPV-1 capsid and L1 pentamer subunits, thus IC/PCR cannot distinguish between pre-viral structures, e.g. DNA associated with L1-pentamers (capsomers) at an early stage of virus assembly, or fully assembled virions. Additional studies are warranted to elucidate the nature of these complexes and their contribution to equine sarcoid biology.

Materials and methods

Sample collection and processing

The here described study was conducted in a cohort of 19 horses divided into a patient group and a control group. The patient group comprised 13 horses with clinically diagnosed sarcoids (individuals A–N) and one individual with granulomatous dermatitis (individual O). The control group consisted of 5 sarcoid-free individuals that were clinically unremarkable (P, Q, R) or affected by non-sarcoid tumours (S, T). Tumour material was collected from each sarcoid-bearing horse of the patient group. From some affected individuals, we equally took biopsies from distant intact skin (C6, G17, K24) and perilesional skin (J22, N29). In one case, serum (D11) was isolated. In another, PBMCs were purified from whole blood (16 ml) via Ficoll gradient centrifugation (C5). In addition, affected (O30) and intact skin (O31) were collected from a horse with granulomatous dermatitis. Supposedly BPV-free tissue material comprising intact skin specimens (P32, Q33, R34), a mastocytoma (S35), a grey horse melanoma (T36) and cartilage (T37) of the melanoma-affected horse were collected from individuals of the control group. Sample specifications are itemised in Table 1.

Tissue specimens (except B4 and C6 that were too small) were grossly bisected into ~3 mm^3 fractions as to allow simultaneous DNA isolation for PCR and extract preparation for IC/PCR. DNA purification was carried out from all tissue samples and from PBMCs using DNeasy™ Tissue extraction kit (Qiagen). β-actin PCR was routinely performed from thus obtained DNA isolates as to confirm their successful purification.

Extracts were prepared from tissue fractions (except B4, C6) by mincing them mechanically in 2 ml reaction tubes each containing 1 ml 0.5 M Tris–HCl pH 8.2, 2% (w/v) PVP K25, 1% (w/v) PEG 6000, 0.14 M NaCl and 0.05% Tween 20. Following centrifugation for 5 min at 13,000 rpm and 4 °C, thus obtained tissue supernatants were kept on ice for immediate use or stored at −20 °C.

To generate an IC/PCR virus control, cow warts (kindly provided by Richard Roden, Johns Hopkins University, Baltimore, MD) were minced with a scalpel, re-suspended in PBS and frozen in liquid nitrogen. Subsequently, tissue was disrupted mechanically for 3 min using a Mixer Mills MM 200 (Retsch, Haan, Germany) and centrifuged at 10,000 g, 4 °C, for 5 min. Thus obtained supernatant containing BPV-1 virion was stored at −70 °C before use.

BPV-1/2 E5 and L1 standard PCR

Prior to immunocapture-sustained PCR analyses, tissue derived DNA isolates and PBMC DNA in one case were screened for presence of viral genes E5 and L1 by standard PCR. DNA samples K23, K24 and O31 were subjected to E5 PCR only. BPV-1/-2 consensus primers 5′E5 (5′-CACTACCTCCTGGAATGAA-CATTTCC-3′)/3′E5 (5′-CTACCTTWGGTATCACATCT-GGTGG-3′) were used for amplification of a 499 bp region spanning the entire E5 oncogene. BPV-1/-2 consensus primers 5′ L1 (5′-GCTAAGCAACAACAGATTCTGTTGC-3′)/3′ L1 (5′-TCAGCCATTTTGAGGTAGTCTGG-3′) were designed for amplification of a 266 bp sequence of the L1 capsid gene. Both primer pairs were selected according to published BPV-1 and BPV-2 sequences X02346 and M20219, respectively. PCR was carried out in a final volume of 50 µl, containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 9.5% DMSO, 1.5 mM of each dNTP, 100 pmol of sense and antisense primer, 2 µl of DNA template and two drops of mineral oil as top layer. After a hot start at 95 °C for 5 min and addition of 1 U of *Taq* polymerase (Roche Diagnostics GmbH, Vienna, Austria), a reaction program consisting of seven touch-down cycles {denaturation at 92 °C for 30 s; primer annealing at 65 °C–56 °C (L1) or 67 °C–58 °C (E5) for 45 s (−1.5 °C/cycle); primer elongation at 72 °C for 45 s} followed by 40 standard cycles {92 °C for 30 s; 56 °C (L1) or 58 °C (E5) for 45 s; 72 °C for 45 s} and a final elongation step {72 °C for 5 min} was conducted in an Eppendorf Mastercycler (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Cloned full-length BPV-1 DNA (BPV1-pML; p+) diluted 1:500 in sterile water and/or BPV-1-positive sarcoid DNA (c+) were used to confirm success of PCR reactions. 16 µl aliquots of resulting amplification products were visualized on 1.5% Tris-acetate agarose gels by ethidium bromide staining.

Pilot IC/PCR assay

For a pilot IC/PCR assay, four 0.65 ml MµlTI Ultra PCR tubes (Sorenson™ BioScience, Inc., Salt Lake City, Utah, USA) were coated with 50 µl of L1 monomer-specific mouse monoclonal BPV-1-1H8 antibody (Abcam plc, Cambridge, UK) diluted 1:50 in 0.1 M sodium carbonate buffer pH 9.6 and incubated at 4 °C overnight. Subsequently, tubes were washed twice with PBS-Tween (phosphate buffered saline pH 7.2, 0.1% Tween 20) and rinsed thrice with PBS. Then, 50 µl aliquots of tissue extract supernatants A1 (L1-negative control), A2, B3 and purified BPV-1 virion diluted 1:100 in extraction buffer as IC/PCR-positive control (v+) were applied to pre-coated tubes

and incubated at 4 °C overnight. After washing thoroughly as described above, L1 PCR was carried out in a total volume of 50 µl/tube under defined conditions, including cloned full-length BPV-1 DNA (p+) as additional positive control for PCR only. 16 µl aliquots of resulting amplification products were visualized on 1.5% Tris-acetate agarose gels by ethidium bromide staining.

Specificity of IC/PCR

To investigate whether IC/PCR products might have resulted from unspecific attachment of free viral DNA to antibodies or tube walls, IC/PCR was simultaneously performed from sarcoid extract supernatant (C7) and from 50 µl aliquots of virus-free skin extract supernatant (S35) spiked with L1-positive (4.6 µg DNA of skin sample C6; 2.6 µg DNA of sarcoid C7) or L1-negative DNA (10 µl DNA of PBMC isolate C5) obtained from sarcoid-bearing individual C. L1 monomer-specific murine monoclonal anti-BPV-1-1H8 (Abcam) served as capture antibody in this experiment.

To assess specificity of antibody trapping, reaction tubes were alternatively coated with antibody 1H8 (Abcam), L1 capsomere-specific murine monoclonal antibody 5B6 or isotype-matched murine IgG (donation from Armin Saalmüller, Veterinary University Vienna, Austria). Subsequent IC/PCR was carried out from tumour extract supernatant C7 serially diluted from 10^0 to 10^{-4} in extraction buffer, the latter equally serving as negative control. Purified BPV-1 virion 1:100 was used to confirm successful IC/PCR-mediated virus detection.

Screening of tissue specimens by IC/PCR

Extract supernatant from 17 tumour specimens comprising 3 verrucous (E12, L25, N27), 2 nodular (G16, N28), 5 fibroblastic (F13, F14, G15, K23, M26) and 7 mixed sarcoids (D8, D9, D10, H18, J19, J20, J21), a serum sample (D11), extract supernatant from 3 intact skin biopsies (G17, K24, O31), 2 perilesional skin samples (J22, N29), 1 dermatitis specimen (O30) and 3 skin biopsies obtained from clinically unremarkable horses (P32, Q33, R34) as negative controls were subjected to IC/PCR using antibody 5B6 for antigen trapping. Purified BPV-1 virion diluted 1:100 in extraction buffer served as positive control. Sterile water was used as no template control in this experiment.

Quantification of capsid protein-complexed BPV-1 DNA

Extract supernatants from IC/PCR-positive fibroblastic (F14, F14, G15, K23, M26), nodular (G16, N28), verrucous (N27) and mixed sarcoids (D8–D10), perilesional skin (J22, N29) and intact skin (K24), as well as three IC/PCR-negative extract samples (P32, Q33, R34) from the control group were subjected to immunocapture, using L1 capsomer-specific antibody 5B6 as described. Extraction buffer was included in this step as no template control. Following washing and addition of 50 µl buffer TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA)/tube, samples were incubated at 95 °C for 10 min. Subsequently, 5 µl aliquots of thus heat-released DNA were subjected to 25 µl-qPCR performed in duplicate each containing 80 mM Tris–HCl, 20 mM $(NH_4)_2SO_4$, 0.02% w/v Tween 20, 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM primer (5′EG-L1 and 3′EG-L1), 0.4 × EvaGreen™ dsDNA-binding dye (Biotium, Hayward, CA, USA), 1 U of a *Taq* DNA polymerase chemically modified for "hot start" (HotFIREPol®, Solis Biodyne) and 5 µl sample or standard DNA. qPCR consisting of an initial hot start at 95 °C for 15 min followed by 50 amplification cycles (95 °C for 15 s, 59 °C for 30 s, 72 °C for 30 s) was conducted in the LightCycler 480® System (Roche Applied Science). A subsequent amplicon melting analysis confirmed the absence of primer dimerisation and the BPV-1 L1 amplicon-specific melting temperature, as determined by MeltCalc© (Schütz and von Ahsen, 1999). The non-linearised plasmid pML-BPV-1 was 10-fold serially diluted in TE (10^8 to 10^3 copies). To improve the accuracy of quantification in samples with low copy numbers, two-fold serially diluted standards (200, 100, 50, 25 and 12.5 copies) were included. The amount of capsid protein-complexed BPV-1 DNA per 50 µl tissue extract supernatant was calculated based on standard curve analysis and the quantitative detection limit of 12.5 copies of the L1 qPCR assay. Primers 5′ EG-L1 (5′-GCCCGTCCATGTGTTACTGAT-3′) and 3′EG-L1 (5′-TTTTGAATGTCAAGAGGTAGATCTGATT-3′) designed with the Primer Express software 1.5 (Applied Biosystems, Foster City, CA, USA) for amplification of a 164 bp BPV-1 L1 fragment were selected according to the BPV-1 reference sequence X02346. Copy numbers per 5 µl qPCR template were determined by the LightCycler 480® software release 1.3.0.075 using the Absolute Quantification/2nd Derivative Maximum modus. Finally, copy numbers were given per 50 µl tissue extract.

IC/PCR-mediated detection of capsid protein-associated full-length BPV-1 DNA

Undiluted and 1/10 diluted extract supernatants of two sarcoid specimens (F13, N28) and two healthy skin samples (Q33, R34) were incubated in 0.5 ml reaction tubes pre-coated with antibody 5B6 as described. Following washing, PCR was carried out in a total volume of 50 µl, each containing 5 µl of 10× Expand Long Template buffer 1 (Roche), 9.5% DMSO, 1.5 mM of each dNTP, 100 pmol of primers 5′BPV1-6046 and 3′BPV1-6045 and two drops of mineral oil as top layer. After a hot start at 95 °C for 5 min and addition of 4 U of Extend Long Template Enzyme mix (Roche), a reaction program consisting of seven touch-down cycles {denaturation at 92 °C for 30 s; primer annealing at 65 °C–56 °C for 45 s (−1.5 °C/cycle); primer elongation at 68 °C for 8 min} followed by 40 standard cycles {92 °C for 30 s; 56 °C for 45 s; 68 °C for 8 min} and a final elongation step {68 °C for 10 min} was conducted in an Eppendorf Mastercycler (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Back-to-back primers 5′BPV1-6046 (5′-GCTAAGCAACAACAGATTCTGTTGC-3′) and 3′BPV1-6045 (5′-ATCTAGGCCTGTTTGTTTCCTGTCATC-3′) were selected from sequence X02346 and designed for amplification of a 7945 bp sequence corresponding to the entire BPV-1 genome. 16 µl aliquots of resulting amplification products were

visualized on a 1% Tris-acetate agarose gel by ethidium bromide staining.

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