

Bovine papillomavirus transmission and chromosomal aberrations: an experimental model

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Enzootic haematuria and urinary bladder cancer in cattle are associated with feeding on bracken fern and bovine papillomavirus (BPV) infection. An increased rate of chromosomal aberrations in peripheral blood lymphocytes from chronically affected haematuric cows raised in bracken fern pastures has been reported, suggesting the presence of BPV in the peripheral blood of afflicted animals. The purpose of the present investigation was to examine the role of peripheral blood as a potential BPV-transmitting agent and search for clastogenic effects in experimentally infected animals kept on a bracken fern-free diet. Healthy cows were inoculated with blood samples of haematuric animals every two weeks for 18 months. Recipient cows, their offspring, donor animals and a control group were kept on a bracken fern-free diet throughout the experiment. Clinical and molecular analyses for

detection of BPV infection were carried out periodically in all groups. Short-term lymphocyte cultures were performed to assess chromosomal aberration levels. The donor cows, the recipient cows and their offspring presented increased levels of chromosomal aberrations. BPV-2 DNA was identified by Southern blotting, PCR and cycle-sequencing of PCR products in peripheral blood of donor and recipient animals and in the progeny of recipient animals. Data support both the concept that BPV can be transmitted through blood and the hypothesis that infection with the virus causes the clastogenic alterations observed in the present experimental model. The presence of BPV-2 DNA and chromosomal alterations in peripheral blood of offspring at the moment of birth is evidence for vertical transmission of BPV.

Introduction

Papillomaviruses are associated with different types of carcinogenesis. The development of malignancy from benign tumours is often associated with additional factors (Jackson *et al.*, 1993). In bovines, the correlation of bovine papillomavirus (BPV) and cancer has been evaluated extensively (Campo *et al.*, 1980), not only because of the economic repercussions of virus infection, but also because the system is an attractive experimental model to study environmental synergism in cancer aetiology. The present model was initially developed by injecting a suspension of haemangiomas, obtained from a bovine urinary bladder, into healthy cows; this

resulted in development of multiple bladder haemangiomas (Kalkus, 1913; Olson *et al.*, 1959). Subsequently, it was shown that injection of BPV suspension, with or without the addition of carcinogenic compounds, into bovine bladders produced tumours in 13 of 15 animals (Olson *et al.*, 1965), suggesting that BPV may be the infective agent in the development of urinary bladder tumours.

Consumption of bracken fern (*Pteridium aquilinum*) has also been associated with haematuria (Rosenberger *et al.*, 1960). Bracken fern is ubiquitous in most tropical and subtropical and some temperate climate countries. Cattle may resort to eating bracken fern during dry seasons. The fern has been reported to be carcinogenic when ingested by cattle or rats (Campo *et al.*, 1992; Campos Neto *et al.*, 1975; Döbereiner *et al.*, 1967; Santos *et al.*, 1987, 1990, 1992). However, data are not unanimous and reports of negative findings may be found. The

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Table 1. Chromosomal aberrations and presence of BPV-2, BPV-4 and BLV in donor animals before the experiment began and after 12 and 18 months

Animal	Breed*	Age (years)	No. of cells with chromosomal aberrations†			Presence of virus in peripheral blood‡					
			Before	12 months	18 months	BPV-2			BPV-4/BLV		
						Before	12 months	18 months	Before	12 months	18 months
D1	N	3	5	16	15	+	+	+	-	-	-
D2	H	4	10	12	21	+	+	+	-	-	-
D3	H	5	10	11	9	+	+	+	-	-	-
Average no. of cells with chromosomal aberrations (confidence interval):§			8 (0-18) ^a	13 (5-2) ^{a,b}	15 (4-24) ^{a,b}	3 out of 3			0 out of 3		
Proportion of animals with virus in peripheral blood:						3 out of 3			0 out of 3		

* H, Holstein; N, Nelore.

† Fifty cells were analysed per animal; out of these 50 cells, the number with chromosomal aberrations is given.

‡ Determined by PCR (BPV-2 and BPV-4) and immunoprecipitation (BLV).

§ *a*, Differs significantly from control group at any time ($P < 0.001$); *b*, differs significantly from before blood inoculation ($P < 0.02$).

relationship between BPV and environmental factors has been further documented: BPV-4 is one of the aetiologic agents in alimentary tract carcinoma (Pennie & Campo, 1992) and BPV-2 DNA is found in 46% of natural bladder cancers and in 69% of experimental bladder lesions (Campo *et al.*, 1992) of animals raised on bracken fern. Enzootic haematuria is endemic in various areas common to bracken fern (Döbereiner *et al.*, 1967). The virus is believed to interact with bracken fern compounds such as quercetin (5,7,3',4'-tetrahydroxyflavone) in the malignant transformation of primary bovine cells *in vitro* (Pennie & Campo, 1992). Quercetin alone induces mutations in bacteria (Walter-Moura *et al.*, 1997), has clastogenic effects in cultured mammalian cells (Caria *et al.*, 1995; Carver *et al.*, 1983), and, in eukaryotic cells, intercalates into DNA, causing single-strand breaks as a consequence of free radical generation (Popp & Schimmer, 1991). However, quercetin failed to induce cancer in controlled *in vivo* experiments (Zhu *et al.*, 1994). Investigations show that 99% of ingested quercetin is degraded in the digestive tract (Gugler *et al.*, 1975) and the absorbed compound is rapidly methylated (Zhu *et al.*, 1994) or eliminated in urine and breath (Gugler *et al.*, 1975). High frequencies of structural chromosomal aberrations in cultured peripheral blood lymphocytes of chronic haematuric bovines raised on bracken fern pastures have been described (Walter-Moura *et al.*, 1988).

Papillomavirus oncoproteins, which are involved in several cell transformation steps, have been associated with clastogenicity. Ploidy variations have been found in HPV-16-immortalized human epithelial cells (Hashida & Yasumoto, 1991). Also, cytogenetic abnormalities and numerical and structural aberrations (e.g. endoreduplications, chromatid gaps and breakages, dicentric chromosomes and centric rings) were observed in keratinocytes transfected with the HPV-16 E7 gene and chromosome fragility was detected in lymphocytes from women with cervical uterine lesions with HPV infection

after treatment with aphidicolin (Paz y mino *et al.*, 1992). These findings suggest that papillomavirus infections, *per se*, may cause chromosome fragility. The objective of the present study was to verify the possibility of virus transmission through blood and its clastogenic activity in cows not exposed to a bracken fern diet. The clastogenic effect of bracken fern compounds has been assessed *in vitro* and *in vivo* (Caria *et al.*, 1995; Carver *et al.*, 1983); however, the potential contribution of BPV to clastogenicity has not been clarified. In this report, papillomavirus infection was achieved by intramuscular injection of blood samples taken from haematuric cows, since peripheral blood lymphocytes have been reported to harbour BPV (Campo *et al.*, 1994). Clinical and molecular analyses for detection of BPV infection, as well as cytogenetic evaluation, were carried out in haematuric donor animals, healthy controls, and recipient cows and their offspring.

Methods

■ **Selection of the animals.** Three groups of bovines (donors, recipients and controls) of comparable ages were selected. The fourth group (progeny) resulted from mating of some of the recipients. With the exception of the donors, all animals were verified as healthy. All animals, including the donors, were verified as free of bovine leukaemia virus (BLV) (Burny *et al.*, 1985; Miller *et al.*, 1975; Burny & Mammerickx, 1987) which could cause chromosome aberrations (Castro *et al.*, 1988). Analyses for BLV were carried out routinely every 6 months in all the animals of the farm (Burny *et al.*, 1985). Experimental animals (donors, controls, recipients and progeny) were shown to be free of BLV up to 14 months after the end of the experiment.

■ **Handling.** The groups were kept in separate open stables throughout the experiment. Animals were maintained on an alfalfa and grass diet supplemented with a balanced mineral chow and fed *ad libitum* in suspended troughs. No grazing was allowed.

Table 2. Chromosome aberrations and presence of BPV-2, BPV-4 or BLV in control animals before the experiment began and after 18 months

Animal	Breed*	Age (years)	No. of cells with chromosomal aberrations†		Presence of virus in peripheral blood‡			
					BPV-2		BPV-4/BLV	
			Before	18 months	Before	18 months	Before	18 months
C1	H	4	2	1	—	—	—	—
C3	H	4	2	2	—	—	—	—
C4	H	6	1	3	—	—	—	—
C5	H	7	2	0	—	—	—	—
C6	H	5	2	0	—	—	—	—
C7	H	4	1	1	—	—	—	—
C8	H	4	1	1	—	—	—	—
C9	H	5	1	1	—	—	—	—
C10	H	5	1	0	—	—	—	—
Average no. of cells with chromosomal aberrations (confidence interval):			1 (0–4)§	1 (0–3)§				
Proportion of animals with virus in peripheral blood:					0 out of 9§	0 out of 9§	0 out of 9	0 out of 9

* H, Holstein.

† Fifty cells were analysed per animal; out of these 50 cells, the number with chromosomal aberrations is given.

‡ Determined by PCR (BPV-2 and BPV-4) and immunoprecipitation (BLV).

§ Differs significantly from donor animals ($P < 0.001$).

■ **Group 1 (donors).** The donor cows (two Holsteins and one Nelore; Table 1), chronically affected by haematuric disease, were brought from a herd exposed to bracken in pastures. The animals were kept on a bracken-free diet 36 months before the beginning and throughout the experiment. This period was deemed sufficient for elimination of any bracken fern compounds by the animal. The period of clinical observation was also considered sufficient to discard the possibility of undetected infections. No epidermal warts or signs of BPV-1, BPV-4 or BPV-6 infection were observed in donor cows before or during the experiment. They were submitted to clinical, cytogenetic and endoscopic analysis to verify the extension of the bladder lesions and the stage of the disease. The three animals excreted erythrocytes in the urine. They were kept under controlled conditions, without contact with bracken fern or other animals, during the entire experiment.

■ **Group 2 (controls).** The controls were nine Holstein cows (Table 2) that had never been fed bracken fern and were raised on the farm where the experiment was conducted. These animals were clinically and cytogenetically normal and were kept on a bracken fern-free diet during the whole experiment.

■ **Group 3 (recipients).** The recipients were eleven cows (seven Holsteins and four Nellores; Table 3) from the same herd as the control animals, selected according to the criteria used for the control group and handled likewise. These animals were inoculated with donor blood samples.

■ **Group 4 (recipient progeny).** This group consisted of two calves (Table 4) born to recipient females mated to a normal bull. The group was kept in the same controlled conditions as the other groups.

■ **Clinical evaluation.** Clinical evaluation consisted of physical examination and endoscopic, haemogram and urine analyses. All subjects were examined at regular intervals. Clinical examination was performed at the beginning of the experiment and then every week. Haemogram and urine analyses were performed at the beginning of the experiment and then at 3-monthly intervals.

■ **Blood samples.** Blood was collected by placing a 9-gauge disposable needle in the jugular vein. After removing the syringe and letting blood flow for a few minutes, a new disposable syringe was connected to the needle and 10 ml blood was collected. Samples were cooled (4–8 °C); 1 ml was used for lymphocyte culture and 9 ml was for DNA extraction.

■ **Inoculation.** A sample of peripheral blood was collected every fortnight from the donors, under aseptic conditions as described above, pooled and immediately injected intramuscularly (15 ml per animal) into the recipient animals.

■ **Cytogenetic studies.** Cytogenetic analyses were carried out at the beginning of the experiment before inoculation in all groups and followed at 6, 12 and 18 months in the recipient group, at 12 and 18 months in donors, and at 18 months in the control group. The progeny of two of the recipient cows were analysed at birth, before and after colostrum ingestion. The studies were performed on short-term peripheral lymphocyte cultures [72 h; TC199 medium, 10% foetal calf serum, 2% phytohaemagglutinin (Gibco BRL)]. Fifty metaphases per animal were analysed at each step of the experiment with conventional Giemsa staining. The frequency of cells with structural chromosome aberrations was scored according to the occurrence of breaks, acentric

Table 3. Chromosome aberrations and presence of BPV in recipient animals before inoculation and at 6, 12 and 18 months post-inoculation with peripheral blood of haematuric cows

Animal	Breed*	Age (years)	No. of cells with chromosomal aberrations†				Presence of virus in peripheral blood‡					
			Before	6 months	12 months	18 months	BPV-2				BPV-4/BLV	
							Before	6 months	12 months	18 months	Before	6/18 months
R1	N	3	2	4	5	8	—	+	+	+	—	—
R2	H	4	3	5	5	7	—	+	+	+	—	—
R3	H	3.5	2	4	7	21	—	+	+	+	—	—
R4	H	5	1	4	6	13	—	+	+	+	—	—
R6	H	4	1	5	ND	ND	—	+	ND	ND	—	—/ND
R7	N	3	3	17	3	8	—	+	+	+	—	—
R8	H	2.5	1	4	4	11	—	—	+	+	—	—
R9	H	2	1	8	10	13	—	+	+	+	—	—
R10	H	5	2	11	2	13	—	+	+	+	—	—
R11	N	3	2	7	6	10	—	+	+	+	—	—
R12	N	4.5	2	5	7	5	—	—	ND	ND	—	—
Average no. of cells with chromosomal aberrations (confidence interval):§			2 (0–5)	7 (2–12) ^{b,c}	6 (4–8) ^{b,c}	11 (4–18) ^{b,c}						
Proportion of animals with virus in peripheral blood:§							0 out of 11	9 out of 11 ^a	9 out of 9 ^a	9 out of 9 ^a	0 out of 11	0 out of 10

* H, Holstein; N, Nelore.

† Fifty cells were analysed per animal; out of these 50 cells, the number with chromosomal aberrations is given.

‡ Determined by PCR (BPV-2 and BPV-4) and immunoprecipitation (BLV).

§ *a*, Differs significantly from before blood inoculation and control group at any time ($P < 0.02$); *b*, differs significantly from before blood inoculation and control group at any time ($P < 0.001$); *c*, differs significantly from control animals ($P < 0.001$).

|| Accidental death. ND, Not determined.

Table 4. Chromosomal aberrations and presence of BPV-2 or BPV-4 in progeny of two recipient (P) and three control (CP) animals

Animal	Recipient	Breed*	Sex	No. of cells with chromosomal aberrations†		Presence of virus in peripheral blood‡			
				At birth	After birth	BPV-2		BPV-4/BLV	
						At birth	After birth	At birth	After birth
P1	R1	N/H	M	4	4	+	+	—	—
P2	R11	N/H	M	9	5	+	+	—	—
Average no. of cells with chromosomal aberrations (confidence interval):				7 (0–19)§	5 (0–15)				
Proportion of animals with virus in peripheral blood:						2 out of 2§	2 out of 2	0 out of 2§	0 out of 2
CP1	C2	H	M	2	ND	—	ND	—	ND
CP2	C3	H	M	3	ND	—	ND	—	ND
CP3	C7	H	M	2	ND	—	ND	—	ND
Proportion of animals with virus in peripheral blood:						0 out of 3		0 out of 3	

* H, Holstein; N, Nelore.

† Fifty cells were analysed per animal; out of these 50 cells, the number with chromosomal aberrations is given.

‡ Determined by PCR (BPV-2 and BPV-4) and immunodiffusion (BLV). ND, Not determined.

§ Differs significantly from control progeny at any time ($P < 0.05$).

fragments and rearrangements (Swierenga *et al.*, 1991). Chromosome or chromatid gaps alone were not considered. The results were analysed statistically using a Chi-squared test appropriate to low frequency (Pereira, 1991) and a Mann's trend test (Lehmann, 1975).

■ **BPV-2 and BPV-4 detection.** DNA samples were extracted from 9 ml peripheral blood. As BPV genomes are maintained in episomal form (Campo *et al.*, 1981), two sequential extractions were carried out (Vande Pol & Howley, 1992). Undigested viral DNA was detected by Southern

blotting and hybridization with ^{32}P -labelled BPV-2 and BPV-4 probes (Amersham Redprime Labelling kit). The probes were constructs of the 7.9 kb genome of BPV-2 DNA or the 7.2 kb genome of BPV-4 DNA cloned in plasmid vector pAT153 (Campo & Coggins, 1992).

PCR analysis (Innis *et al.*, 1990) was performed using specific primers for BPV-2 (L1 region: nt 1318–1341, 5' GTTATACCACCCAAAGA-AGACCCT 3', and nt 1490–1466, 5' CTGGTTGCAACAGCTCTCT-TTCTC 3') and BPV-4 (E7 region: nt 642–661, 5' GCTGACCTCC-AGTCTTAAT 3', and nt 812–792, 5' CAGTTTCAATCTCTCTTCA 3'). PCR conditions were as follows: denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing for 40 s at 55 °C, and extension at 72 °C for 1 min, run on a Gene Atycycler (Pharmacia Biotech). DNA (10 ng) was added to each reaction in standard PCR buffer (Gibco BRL) with 1.5 mM MgCl_2 , 2.5 ml *Taq* polymerase and 2 mM of each primer. The final volume was 50 ml. A 1 ml aliquot of the reaction mix was resolved by 12.5% PAGE and visualized by silver staining (Phast system; Pharmacia Biotech). The sensitivity of the PCR reaction was estimated to be between 10 and 10^2 DNA copies per reaction.

Standard precautions were taken to avoid sample contamination. Blood collection and DNA extraction from blood were carried out simultaneously in control and recipient animal samples. The PCR reaction was prepared in a different room from DNA extraction and by a different investigator. The reaction was run and amplified products were analysed by electrophoresis in different areas from amplification and reaction preparation. Control and recipient DNA samples were analysed simultaneously and negative DNA controls were added. The PCR was performed in a laboratory in which BPV PCR had not previously been performed.

■ Cycle-sequencing of PCR products. Amplification products from PCR reactions were sequenced. Purified amplification products (50–150 ng) were used for the cycling reaction (Amersham RPN 2438) with either of the above primers, labelled at the 5' end with fluorescein, under the following conditions: denaturation at 95 °C for 5 min followed by 25 cycles at 95 and 55 °C (30 s each). Reactions were run on an ALF automatic DNA sequencer (Pharmacia Biotech). Two full double-stranded sequences were obtained for each sequenced amplification product.

Results

Haematuric donors had bladder lesions that were detected by endoscopic examination. Clinical evaluation of the animals throughout the experiment allowed appropriate management in order to secure survival and maintain their status condition as donors. BPV-2 DNA was detected in peripheral blood samples used for inoculation by Southern blot and PCR analyses (Table 1). The donor animals did not show clinical symptoms of alimentary tract lesions, epidermal warts, or signs of BPV-1, BPV-4 or BPV-6 infection before or during the experiment. The frequency of chromosomal aberrations detected in the peripheral lymphocytes of the donor group, verified at the beginning and 12 and 18 months later, was similar to that described for haematuric animals (Walter-Moura *et al.*, 1988) and significantly greater than in the control group (Tables 1 and 2). Control animals were free of clinical signs related to papillomatosis as assessed by endoscopic and urine analyses. Endoscopic examination of bladders performed throughout the experiment did not reveal any alterations. The frequency of chromosome aberrations in cultured lymphocytes

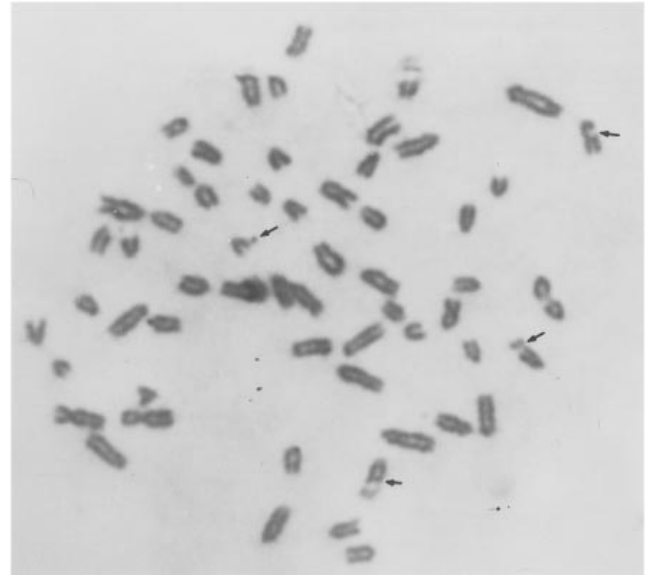


Fig. 1. Structural chromosome aberrations in metaphase plate from short-term cultured peripheral blood lymphocytes of a recipient animal (R11). The arrows indicate the chromosome lesions.

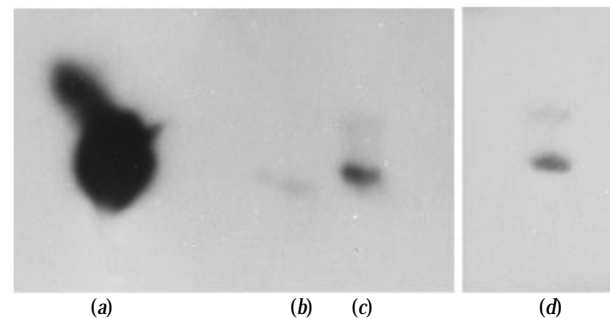


Fig. 2. Southern blot and hybridization detecting the presence of undigested BPV-2 DNA in the peripheral blood of animals studied. (a) Control BPV-2 11.5 kb DNA construct (3.6 + 7.9 kb); (b) sample from a donor animal (D3); (c) sample from recipient animal (R11); and (d) sample from the calf (P2) born to recipient animal R11.

of the control group at the beginning of the experiment and 18 months later was significantly lower than that of the donor group. The control animals were free of BPV-2 and BPV-4 DNA throughout the experiment (Table 2).

The recipient group, before blood inoculation, was similar to the control group in terms of clinical condition, chromosome aberration rate, and absence of BPV-2 and BPV-4 DNA. The animals did not show clinical symptoms of alimentary tract lesions, epidermal warts, or signs of BPV-1, BPV-4 or BPV-6 infection before or during the experiment. Following the initiation of blood inoculation, examination of recipient animals did not reveal any signs of bladder lesions, with the exception of one animal (R4) which after 18 months showed haematuria and signs of bladder lesions. Haematuria increased in this animal 6 months after the first observation of erythrocytes in

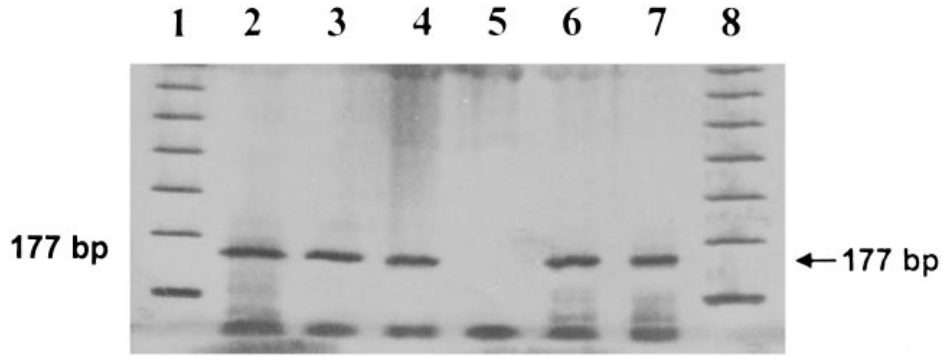


Fig. 3. PCR analysis with BPV-2 primers. Lanes: 1 and 8, 100 bp ladder molecular mass marker; 2 and 3, samples from recipient animals R9 and R10, respectively; 4, sample from offspring (P2); 5, sample from control animal C3; 6, sample from recipient animal R7; and 7, BPV-2 DNA control. Amplification products were resolved by 1.2.5% PAGE and stained with silver.

6890	TGTTATACCA	CCCAAAGAAG	ACCCTTATGC	AGGGCTTAaG	TTTTGGAGCA	TAGACTTAAA	BPV2 genome sequence
1	TGTTATACCA	CCCAAAGAAG	ACCCTTATGC	AGGcCTTAGG	TTTTGGAGCc	TAGACTTAAA	Donor cow (D1)
	TATACCA	CCCAAAGAAG	ACCCTTATGC	AGGcCTTAGG	TTTTGGAGCA	TAGACTTAAA	Recipient cow (R7)
	CCAAAGAAG	ACCCTTATGC	AGGGCTTAaG	TTTTGGAGCA	TAGACTTAAA		Progeny (P2)
6950	AGAAAAGCTG	TCTTTGGACT	TAGACCAATT	TCCCTTGCGA	AGAAGATTCT	TAGCTCAGCA	BPV2 genome sequence
61	AGAAAAGCTG	TCTTTGGACT	TAGACCAATT	TCCCTTGCGA	AGAAGATTCT	TAGCTCAGCA	Donor cow (D1)
	AGAAAAGCTG	TCTTTGGACT	TAGACCAATT	TCCCTTGCGA	AGAAGATTCT	TAGCTCAGCA	Recipient cow (R7)
	AGAAAAGCTG	TCTTTGGACT	TAGACCAATT	TCCCTTGCGA	AGAAGATTCT	TAGCTCAGCA	Progeny (P2)
7010	AGGGGCAGGA	TGTTCAACTG	TGAGAAAGAG	AGCTGTTGCA	ACCAG		BPV2 genome sequence
121	A_GGGCAGGA	TGTTCAACTG	TGAGAAAGAG	AG_TGTTGCA	ACCAG		Donor cow (D1)
	AGGGGCAGGA	TGTTCAACTG	TGAGAAAGAG	AGCTGTTGCA	ACCAG		Recipient cow (R7)
	AGGGGCAGGA	TGTTCAACTG	TGAGAAAGAG	AGCTGTTGCA	ACCAG		Progeny (P2)

Fig. 4. Sequences generated by automatic cycle-sequencing of amplification products of DNA samples. Top line: BPV-2 DNA from GenBank (D. E. Groff, R. Mitra & W. D. Lancaster, unpublished results). Line 2: sequence obtained for donor cow (D1); 5' → 3', from nt 1–163, 98% homology to BPV-2 DNA. Line 3: sequence obtained for recipient cow (R7); 5' → 3', from nt 1–162, 97% homology to BPV-2 DNA. Line 4: sequence obtained for progeny (P2); 5' → 3', from nt 1–154, 100% homology to BPV-2 DNA.

urine. Upper alimentary tract lesions were not detected in the recipient animals and none of them harboured BPV-4 DNA in peripheral blood as assessed by Southern blot and PCR analyses. Before inoculation, the recipient animals showed levels of chromosomal aberrations similar to those seen in control animals, whereas 6, 12 and 18 months after inoculation there was a significant increase in the frequency of aberrations (Table 3, Fig. 1). A significant increase in the rate of chromosome aberrations is evident when the results obtained before and after the inoculation of the recipient and from the control group are compared. BPV-2 DNA was assessed by Southern blotting in five recipient animals 18 months after the

beginning of the experiment (Fig. 2). PCR results were positive for BPV-2 in nine out of ten animals at 6 months and in ten animals at 12 and 18 months (Table 3, Fig. 3). Table 4 presents the results of the study conducted in two calves generated from the mating of recipients R1 and R11 with a healthy bull. At birth, the progeny presented increased levels of chromosome aberrations compared to three calves born to control cows in the same period. There was no significant difference between the frequency of chromosome aberrations before and after the ingestion of colostrum. Both calves had BPV-2 DNA, revealed by PCR and Southern blotting, in the peripheral blood (Figs 2 and 3), but were negative for BPV-4 (Table 4). All

animals were verified to be free of BLV throughout the experiment (Tables 1, 2, 3 and 4) and up to 14 months after the end of the experiment (not shown).

The identities of PCR fragments obtained from samples of all experimental groups were confirmed by cycle-sequencing of amplification products. Automatic sequencing generated 150–160 base DNA sequences of donor and recipient cows and of progeny with 97–99% homology with the published sequence of the conserved L1 region of the BPV-2 genomic sequence (Fig. 4).

Discussion

This study reports the occurrence of both horizontal and vertical transmission of BPV in bovines under controlled experimental conditions. Horizontal transmission was achieved by inoculating healthy cows with peripheral blood from animals affected by fully fledged chronic enzootic haematuric disease. Offspring of two of the experimentally infected animals, mated to a healthy bull, had BPV-2 DNA in blood samples collected immediately after birth. These findings are relevant to the understanding of the dynamics of BPV-2 transmission in farmlands. The presence of BPV-2 in the peripheral blood of afflicted animals supports the concept that infection may result from grazing in pastures contaminated by haematuric animals. The verification of vertical BPV-2 transmission as an infective mechanism will contribute to cattle breeding and handling strategies in endemic areas.

Dramatic increases in chromosome aberrations associated with transmission of BPV were observed in the experimentally infected animals. The chromosome aberration rate in the recipient animals was similar to that observed in the chronic haematuric donor animals and occurred in the absence of synergistic factors related to bracken fern. Structural chromosomal aberrations were described in bovines chronically affected by haematuric disease feeding on bracken fern pastures (Walter-Moura *et al.*, 1988). The latter findings were originally correlated with the possible clastogenic action of the bracken fern compounds. Quercetin, the most probable candidate, has been described as mutagenic (Walter-Moura *et al.*, 1988) and clastogenic (Caria *et al.*, 1995; Carver *et al.*, 1983; Popp & Schimmer, 1991). The present data show that chromosomal damage occurs in the absence of bracken fern carcinogens and precedes or occurs independently of the development of haematuric disease and is probably due to BPV and its effect on chromosomal stability. Cytogenetic instability and chromosomal damage have been documented in both human keratinocytes and mouse cells containing the E7 oncogene of HPV-16 (Hashida & Yasumoto, 1991; DiPaolo, 1992). Similar findings have been described for simian virus 40 infection of human keratinocytes (DiPaolo, 1992) resulting in chromosomal aberrations and aneuploidy events. Aneuploidy was not detected, probably because short-term cultures of lymphocytes

(72 h) were used. Severe abnormalities would be eliminated prior to mitosis in the latter system.

The following points are to be considered. Donor animals had been exposed to bracken in pastures and were affected by chronic haematuria, but were kept on a bracken fern-free diet for 36 months before the beginning of the experiment, thus ensuring the elimination of any residual bracken fern compounds. Their lymphocytes presented chromosomal abnormalities as previously described (Walter-Moura *et al.*, 1988), including breaks, acentric fragments and rearrangements. Earlier reports of the presence of BPV DNA in lymphocytes of calves (Campo *et al.*, 1994), and of HPV DNA in lymphocytes of women with cervical cancer (Pao *et al.*, 1991) support the possibility that papillomaviruses may be the causal agents of chromosome fragility. These animals did not present any upper alimentary tract lesions attributable to BPV-4, epidermal warts or signs of BPV-1 or BPV-6 infection (Campo *et al.*, 1981). The recipient animals had never been fed bracken and had no haematuria; at the beginning of the experiment, they had no chromosomal abnormalities and no BPV DNA was detected in their lymphocytes. Nevertheless, 6 months after inoculation with blood from diseased animals they presented chromosomal aberrations similar to those observed in the donors. At this stage, the blood cells of nine recipient animals were shown to contain BPV-2 DNA. These observations suggest that BPV can be transmitted horizontally through blood. On the other hand, the absence of any sign of BPV-2 infection in control animals kept under controlled conditions in proximity to infected animals lends support to the hypothesis that infection may occur as a result of exposure to grazing in contaminated areas. This hypothesis gains support from recent findings of large amounts of BPV-2 DNA in urinary sediments of haematuric cows (unpublished results).

Haematuria developed in one recipient cow approximately 18 months after the first inoculation. This case implies that bracken fern is not essential for development of the disease. The absence of urinary tract lesions in the recipient group, with the exception of one animal, up to 18 months after blood transfusion, could suggest that the development of chronic haematuric disease may be facilitated by synergistic factors, such as dietary bracken fern (Campo *et al.*, 1992). However, considering that the latency for development of the disease is believed to be 18–24 months (Döbereiner *et al.*, 1967), the contribution of synergistic factors remains speculative.

At birth, both the progeny of two recipient cows presented chromosomal abnormalities and BPV DNA in blood samples. The identity of BPV-2 DNA was confirmed by automatic cycle-sequencing of amplification products in the infected progeny, donor and recipient cows. Amplification products were shown to have 97–99% homology to the highly conserved L1 region of the BPV-2 genome sequence. In contrast, calves born to healthy control animals had no detectable BPV DNA in their blood or signs of chromosomal damage. This finding is convincing evidence of vertical

transmission of BPV and substantiates the hypothesis which suggests a virus-mediated aetiology for the reported chromosomal aberrations. Vertical transmission has also been suggested for HPV in a number of cases (Sedlacek *et al.*, 1989).

This is the first report of the transmission of BPV through blood and of the clastogenic action of BPV *in vivo* in the absence of bracken fern. These results point to novel aspects of BPV biology and have important implications for human and veterinary medicine.

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