Papillomavirus DNA detection in non epithelial tissues: a discussion about bovine papillomavirus

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Papillomaviruses are associated with different carcinogenesis process in human and other animals. Human papillomavirus (HPV) has been tightly dentified as the major etiologic agent of neoplasia of cutaneous and mucosal epithelia. Among the 100 or more types of HPV that have been discovered, approximately 35 of them are associated with cancer. In cattle, bovine papillomavirus (BPV) is the etiologic agent of neoplasia of upper gastrointestinal tract and urinary bladder. Eight types of BPV have been identified. They are related to cutaneous fibropapillomas and, squamous papillomas of the skin and esophagus and carcinomas (BPVs 1, 2 and 4). Infection by papillomavirus requires epidermal or mucosal epithelial cells. Initial infection occurs in basal cells and the access to these cells occurs through micro-abrasions caused by various forms of physical trauma. The correlation of BPV and cancer has been evaluated not only because of the economic repercussions of virus infection, but also because the system is an attractive experimental model to study HPV infection and carcinogenesis. In studies performed in bovines, our group showed that despite the description of papillomavirus as epitheliotropic, its genetic material was found in several different types of tissues (reproductive tract, embryonic tissues, gametes and blood) in a same animal and also in the peripheral blood of its offspring. We suggest that blood could act as a pathway for papillomavirus dissemination to other tissues and body fluids, in special for reproductive tract and gametes, leading to important considerations about dissemination of BPV related diseases and artificial insemination and embryo transfer.

Keywords: Bovine papillomavirus; Bloodstream; body fluids; Peripheral blood mononuclear cells

1. Introduction

Papillomavirus are found in a wide variety of proliferative lesions of epithelial origin and are associated with different carcinogenesis process in human and other animals such as bovines [1]. They are nonenveloped double-strand DNA viruses about 55 nm in diameter with an approximately 8Kb genome in a nucleohistone core. Viral capsids are composed of two proteins, L1 and L2. L1 is the major capsid protein, which is arranged in 72 pentamers and has the capacity to self-assemble in virus-like particles (VLPs). L2 is a DNA-binding protein and it is necessary to viral genome encapsidation. The genome encodes another seven or eight nonstructural proteins, E1-E8 (Early genes), which are vital for the replication and transcription of viral DNA [1, 2].

Human papillomavirus (HPV) has been firmly identified as the major etiologic agent of neoplasia of cutaneous and mucosal epithelia [3]. Among approximately 100 types of HPV that have been discribed, nearly 35 are associated with cancer. Cervical cancer is the second most common cause of cancer-related

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deaths in women worldwide. More than 450,000 cases are diagnosed each year, resulting in nearly 250,000 deaths. HPV-16 is the most prevalent, accounting for more than half of cervical cancer cases [4].

In cattle, bovine papillomaviruses (BPV) are the etiologic agent of upper gastrointestinal tract, urinary bladder and cutaneous papillomatosis tumors. Eight types of BPV have been identified and they are related to cutaneous fibropapillomas, squamous papillomas of the skin and esophagus and carcinomas [5, 6, 7].

Initially papillomavirus infection leads to hyperplasic lesions defined as warts, papillomas or condilomas [8]. These lesions are usually benign, but environmental cofactors (such as the fern *Pteridium aquilinum v. aracnoideum* consumption) have been described as inducing cell modifications resulting in neoplasic transformation [5, 9, 10]. The oncogenic viral action involves the expression of viral oncogenes (Early genes) inside the host cell. These oncoproteins interfere in the cell cycle control throughout interactions with specific proteins (p53 and pRB) resulting in genetic abnormalities [1]. The natural regression of papillomas has been described in humans and other animals, and the histological examination of these lesions revealed an intense lymphocytary infiltrate in both dermis and epithelium [11, 12].

The pathogenesis of papillomatosis in cattle is related to decay of immunological response associated to the presence of BPV [3, 11]. Among the several compounds of the fern, the flavonoid quercetin bears immunosupressive and mutagenic properties and was described as presenting probable carcinogenic synergic action associated to BPV-4, leading to malignation of the lesions [13]. Cytogenetical studies showed clastogenic action of natural chemical agents of the fern in peripheral blood lymphocytes [9]. Although reports of cytogenetic studies in lesions due to BPV are scarce, we have described chromosomal abnormalities in a cell line obtained from bovine palate fibroblasts submitted to a specific protocol of transfection with active Ha-*ras,* BPV-4 protein E7 and exposed to a quercetin pulse [14]. Our group founded a high rate of chromosomal aberrations after treatment with quercetin, with peculiar rearrangements originating from possible centric fusions [14].

It has been widely accepted that HPV are not disseminated by blood, i.e., there is no viremic phase in the course of papillomavirus infection. However, the detection of papillomavirus DNA sequences in peripheral blood mononuclear cells (PBMC) of women with urogenital infection by HPV was also reported [15]. In cattle, the presence of BPV DNA has been detected in the peripheral blood of animals affected by diseases related with papillomavirus infection [16, 17, 18, 19]. Increased levels of chromosomal aberrations in short term lymphocyte cultures of bovines affected by papillomatosis were also described as evidence of the virus presence in these cells and the presence of BPV-2 genome in lymphocytes isolated from peripheral blood was confirmed [16; 17]. Thus, Stocco dos Santos *et al* (1998) have suggested the possibility that the lymphocyte may be a site for viral latency.

In recent studies developed in bovines, our group showed that despite the description of papillomavirus as epitheliotropic, its genetic material was found in several different types of body fluids and tissues [18, 19, 20]. In the present report we selected samples from animals affected by cutaneous papillomatosis to evaluate the presence of BPV DNA in non epithelial sites, including whole blood, plasma, milk, colostrum, placenta and amniotic liquid, and discuss their correlation with BPV detection in cutaneous lesions.

2. MATERIAL AND METHODS

2.1. Animal Selection

We selected 19 cows affected by cutaneous papillomatosis and one newly-born calf from one of these cows. The samples were obtained after cleaning the area with water and soap and decontamination with 70% ethanol.

2.2. Sample Collection

All procedures for sample collection were performed in order to reach the suitable conditions for handling of biological material for use in molecular protocols.

WART: segments of warts were removed by parallel incision in the surface of the skin using a disposable sterile scalpel and kept in sterile tube containing 10% formaline.

BLOOD: 10mL of blood were collected using disposable syringe and needle with anticoagulant (EDTA, for molecular analysis or heparin for cytogenetical procedures).

PLASMA: 5mL of the collected blood were centrifuged at 3500 rpm/15min. About 1 mL sample from the supernatant was collected and kept at – 20º C.

PLACENTA AND AMNIOTIC LIQUID: samples of placenta and amniotic liquid were collected by veterinary professionals in the Veterinary Hospital of Universidade de São Paulo (FMVZ-USP);

MILK: 20mL of milk were collected in a 50mL Falcon sterile tube;

COLLOSTRUM: 10mL of collostrum were collected in a 50mL Falcon sterile tube.

2.3. DNA Extraction

All collected samples were submitted to DNA extraction by "Qiagen DNA easy Blood and Tissue kit" (Qiagen), in accordance with the manufacturer protocol. About 300ng of the extracted DNA were used for analysis by PCR. Various precautions were taken to minimize sample-to-sample cross-contamination, including limited sample processing and DNA extraction to a maximum of 5 samples per day.

2.4. BPV-1 Detection

PCR procedures were achieved with specific primers for L1 gene of BPV-1 (Fw- nt: 5721-5742 – Gene Bank access X02346): 5'-GGAGCGCCTGCTAACTATAGGA-3'; (Rev -nt: 6021-6001 - Gene Bank access X02346): 5'-ATCTGTTGTTTGGGTGGTGAC-3'), that produce an amplification product of 301bp. The conditions for PCR were the following: 94°C/3min (denaturation) followed by 35 cycles at 94°C/50seg (denaturation), 67°C/1min (annealing) and 72°C/1min (extension), with a final cycle at 72°C/5min (final extension). The analysis of the amplified products was carried out in agarose gel containing ethidium bromide and the photodocumentation was done in the Biometra device using the "Bioanalysis software".

2.5. β-globin Detection

Each plasma DNA sample was screened for the presence of bovine β-globin DNA by PCR amplification using the primers: Fw: 5'-AACCTCTTTGTTCACAACCAG-3' and Rev: 5'-CAGATGCTTAACCCACTGAGC-3'. This primer set amplifies a 450bp product and provides an indication of a appropriated DNA quality. The conditions of PCR, analysis and photodocumentation were the same described above.

2.6. *DdeI* Digestion

Samples of the PCR product of BPV-1 L1 gene (301bp) were digested with *DdeI* enzyme following the protocol of the manufacturer. This enzyme cuts the amplified fragment just in one specific site (nt: 63) resulting in two fragments of 237bp and 64bp.

2.7. Southern blot for BPV-1

The BPV-1 viral genome cloned in pAT153 (kindly provided by Dr. Maria Saveria Campo, University of Glasgow, Scotland) was released by the digestion with *HindIII* and marked with peroxidase by using the ALKAPHOS kit (Amersham), according to the protocol of the manufacturer. The BPV-1 PCR products

were separated in 2% agarose gel and transferred to a nylon membrane. The membrane was hybridized overnight with the probe and submitted to high stringency washes, developed with CDP-star (chemoluminescent reagent) in accordance with the protocol of the manufacturer (Amersham), and exposed to the X-Ray film.

3. RESULTS

3.1. BPV-1 DNA was detected in warts and in the peripheral blood of the same animals

All wart samples collected from the 19 animals showed a 301bp amplified product corresponding to the expected L1 gene fragment of BPV-1 segment, compared to the positive control. In 17 of 19 studied animals were detected the amplified product from L1 gene of BPV-1 (300bp) in peripheral blood samples. The results obtained were confirmed by enzymatic digestion of the PCR products using *Ddel* enzyme (data not shown). A summary of the results of BPV-1 detection in warts and whole blood is shown in the Table 1.

3.2. BPV-1 DNA sequences can be carried out by bloodstream cells

In order to evaluate if the detected virus DNA sequences could be hosted by any cell in bloodstream or eventually free in the plasma, the analysis for the presence of BPV-1 DNA sequences was carried out in samples of isolated plasma from 13 of 19 samples of peripheral blood. DNA was extracted from the plasma and evaluated if it was preserved enough to allow PCR amplification of bovine β-globin gene sequence. This strategy was based upon reports of the presence of circulating cellular DNA product of apoptotic and/or necrotic processes [21]. All plasma samples, except number 5, showed a 450bp amplified product corresponding to expected β-globin gene sequence amplification, confirming the viability of the DNA sample for PCR (data not shown). The results confirming the detection of BPV-1 DNA sequences in the plasma by PCR are also shown in Table 1. These results obtained by PCR procedures were confirmed by Southern blot (data not shown).

3.3. BPV-1 DNA detection in different biological samples in the same animal and its offspring

The presence of BPV1 DNA sequences was verified by PCR in samples of warts, blood, placenta, amniotic liquid, milk and collostrum from a cow affected by papillomatosis and in the blood of its newly-born calf (Fig.1a and b) and confirmed by Southern blot (Fig. 1c). This result highlights the possibility that blood can act as a pathway for viral dissemination and also provides evidence of vertical transmission for BPV-1 DNA sequences.

Table 1 – BPV-1 detection in wart, blood and plasma samples of bovines affected by papillomatosis and the control for DNA quality for PCR procedures (β-globin gene amplification) in plasma sample. The animal samples 8, 10, and 11 indicate the presence of intracellular BPV genetic material.

	BPV-1 detection			PLASMA
ANIMAL	WART	WHOLE BLOOD	PLASMA	β-Globin
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	-	-	-
6	+	+	ND	ND
7	+	+	+	+

8	+	+	-	+
9	+	-	-	+
10	+	+	-	+
11	+	+	-	+
12	+	+	+	+
13	+	+	+	+
14	+	+	ND	ND
15	+	+	+	+
16	+	+	ND	ND
17	+	+	ND	ND
18	+	+	ND	ND
19	+	+	ND	ND
20 (Calf)	ND	+	ND	ND
	19/19 (100%)	**18/20 (90%)**	**7/13 (51%)**	**12/13 (99%)**

ND: not determined + : Presence - : absence

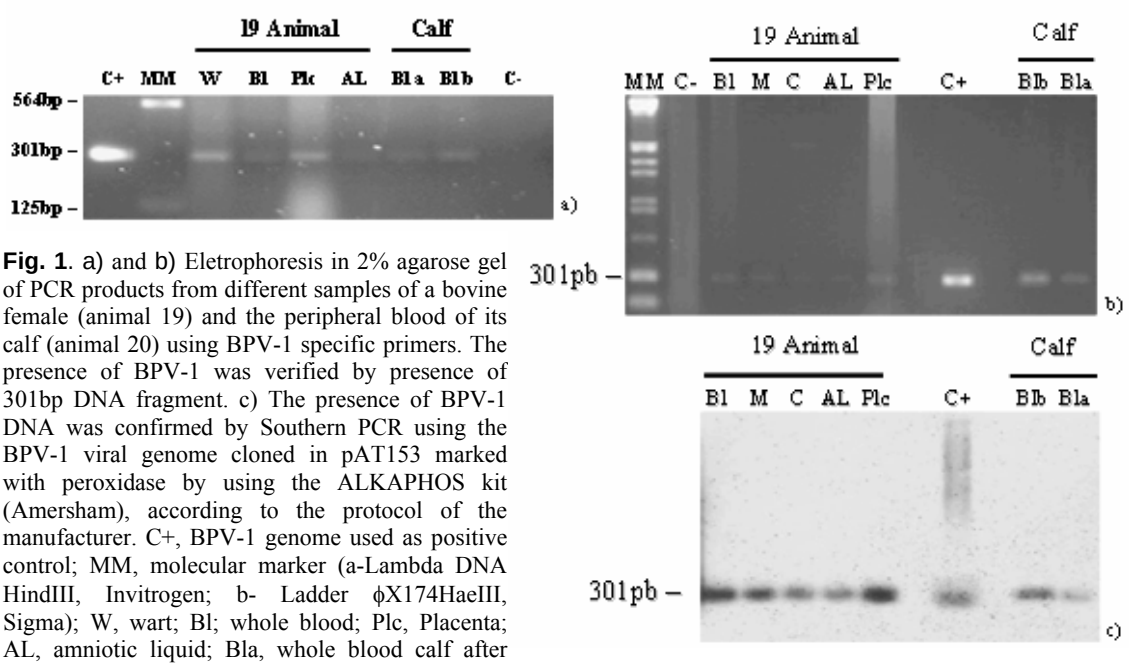

Fig. 1. a) and b) Eletrophoresis in 2% agarose gel of PCR products from different samples of a bovine female (animal 19) and the peripheral blood of its calf (animal 20) using BPV-1 specific primers. The presence of BPV-1 was verified by presence of 301bp DNA fragment. c) The presence of BPV-1 DNA was confirmed by Southern PCR using the BPV-1 viral genome cloned in pAT153 marked with peroxidase by using the ALKAPHOS kit (Amersham), according to the protocol of the manufacturer. C+, BPV-1 genome used as positive control; MM, molecular marker (a-Lambda DNA HindIII, Invitrogen; b- Ladder ϕX174HaeIII, Sigma); W, wart; Bl; whole blood; Plc, Placenta; AL, amniotic liquid; Bla, whole blood calf after colostrums ingestion; Blb, whole blood calf before colostrums ingestion.

4. Discusion

Recent studies suggest that HPV infection can induce oral, head and neck, esophageal, lung, and colorectal cancers [21]. In addition, other reports document the presence of HPV DNA in prostatic tissue, sperm cells, and breast cancer tissue [22]. In the case of BPV, its importance in urinary bladder and gastrointestinal tract cancers of bovine was verified [1].

This study reports evidences that the same type of BPV genetic material present in the epithelium lesion (wart) can also be found inside the cells in the bloodstream of animals and other non epithelial sites. The detection of BPV-1 genetic material in warts, blood, milk, and collostrum of a cow affected by cutaneous papillomatosis, as well as in the placenta, amniotic liquid, and blood of its newly-born calf, suggests that the peripheral blood can be a dissemination pathway to other tissues and provides evidence

for vertical transmission of BPV-1. We have studied 19 animals affected by cutaneous papillomatosis and a newly-born calf from one of these animals. All the animals showed BPV-1 DNA sequences in the warts and 17 of the animals have shown also BPV-1 DNA sequences in peripheral blood samples. The identification of samples positive for BPV-1 DNA sequences in whole blood but negative in the plasma are indicative of intracellular presence of viral DNA sequences in the bloodstream. However, it is important to evaluate the positive samples for BVP-1 DNA sequences in both whole blood and plasma, which can suggest the presence of free DNA sequences in bloodstream originating from death of the virus carrier cell.

The presence of BPV DNA in peripheral blood, reproductive tract, and gametes of the bovines were documented [17, 18, 19, 20]. The latter observations raise questions as how papillomavirus could be localized in these tissues considering the abscence of direct infection and the historical presumption that papillomavirus viremia and hematogenous dissemination do not occur. Perhaps there is no better interpretation to the finding of papillomavirus DNA in PBMCs than to adress how the virus can spread and infect epithelial cells in other organs.

The process of regression of papilomavirus lesions depends on a cell immune response, mainly infiltrate of T lymphocyte [11]. These results raise the hypothesis that the virus may become hosted in the blood cells during the immunological response to lesion. The natural regression of the papillomavirus lesions is followed by a remarkable infiltration of lymphocytes [11, 22]. Several types of T lymphocytes (CD4+, CD8+ and γδ-WC1+) are present in the lesions in regression process. In many cases, due to the destruction of the basal membrane, these cells penetrate in the suprabasal layer of the lesion. However, the stimulus that allows incoming of lymphocytes to the sub-basal space and their infiltration into the lesion remains unknown [11]. The cellular receptor for papillomavirus is reported as being widely expressed and evolutionarily conserved [23, 24, 25]. The receptor FcγRIII is proposed as a helping molecule in the cell infection by papillomavirus in the epithelium. This was proposed due to the fact that there is a high expression of this receptor in the glandular epithelium of the endocervix, transformation zone, prepuce and rectal epithelia, also target sites for mucosotropic papillomavirus. However, FcγRIII is also present in several types of cells of the immunological systems in humans and rats and seems to be able to achieve the function of viral "binding" and internalization [24].

The presence of genetic material of BPV-1 in samples of wart, blood, milk and collostrum from a cow affected by papillomatosis and peripheral blood, placenta, and amniotic liquid of its offspring was verified in this study. We are suggesting that the viral DNA reached these tissues and fluids by bloodstream. The role of the peripheral blood as a pathway for dissemination was already verified [15, 26]. In the case of the BPV, its infectious capacity of peripheral blood was experimentally evaluated [17]. However, it is not yet clear the importance of the simple presence of BPV DNA sequences to cell dysfunction.

Our group focused this question using the cytogenetical approach. The cytogenetic analysis performed in short term peripheral blood lymphocyte cultures showed chromosomal aberrations similar to those found in transfected cells with viral oncogene E7 of BPV-4 [14]. Similar chromosomal alterations were also previously seen in animals infected with BPV-2 [17]. In HPV, the chromosome fragility in lymphocytes was reported in women with cervical lesions [27] and the increase of micronucleous (MN) frequency in epithelium cells and lymphocytes with the progression of cervical cancer was studied, and the presence of the HPV can be seen as the possible cause [28]. Connelly *et al.* (2001) experimentally showed that fragments of HPV genes E6 and E7 can lead to the fragmentation of DNA of spermatozoa. These data and the findings reported here can support the hypothesis that the genetic material of the papillomavirus is not passively found in the cell, but it interacts directly (DNA-DNA) or indirectly (DNA-Protein) with the chromatin of the host cell leading to chromosomal fragility in the case of cattle, resulting in centric fusions. Efforts are being driven to better understand this mechanism.

The presence of genetic material of BPV-1 in samples of the amniotic liquid and placenta indicates that the virus can eventually cross the placental barriers, as previously described for the HPV, which raised the hypothesis that the transplacental infection would be due to viremia [30, 31). In the case of

HPV, several authors [31, 32, 34] have suggested such possibility, in spite of being not well documented [34]. An outstanding factor in the maternal-fetal transmission is the viral load, which can also be important, but not the determining factor for this transmission [33]. Generally, the contact of the embryo or fetus with the genetic material of papillomavirus does not show an immediate effect [34]. An explanation to this fact is that the virus can infect embryonic or fetal cells and remain under a latency period due to the repressing action of the genes of the host cell and, afterwards, a change in the environment could attack the cellular organization leading to the loss of its suppressive capacity (Armbruster-Moraes, *et al.*, 1994). However, there are data in the literature suggesting a linkage between the infection by HPV and abortion [32, 34], but there is no report in this subject related to BPV infection. Thus, the BPV infection can be responsible by low pregnancy rates and spontaneous abortions.

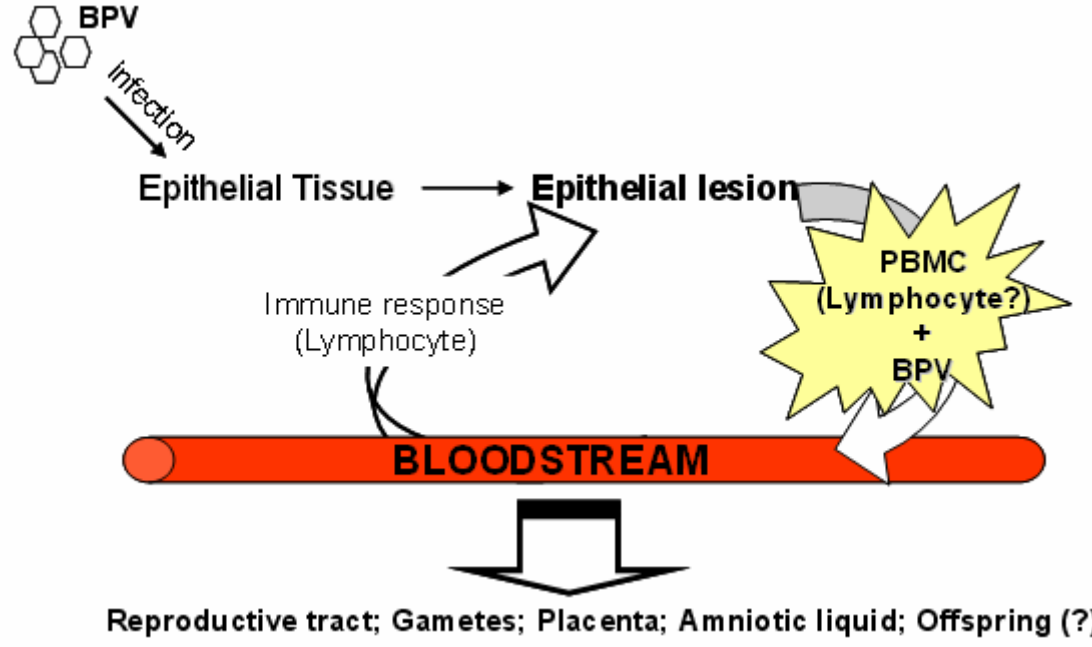

Fig. 2 Proposed BPV virus transit between tissues/body fluids throughout bloodstream after infection of epithelial tissue.

We verified the presence of BPV-1 in the bloodstream of a bovine female and its calf suggesting vertical transmission of the virus. Studies developed with HIV, which infect T CD4 lymphocytes, could demonstrate its placental transmission and suggests that the leukocytes present in the decidua, as well as the presence of the mother's blood in the spaces among the vilosities, as possible sources of infecting virus [26]. The same places could be the sites for BPV-1 transplacental transmission, since its presence in the peripheral blood, free or inside the cell was verified in this work (Fig. 2).

Thus, the present investigation collects a strong evidence for BPV-1 presence in non epithelial sites in bovines and the correlation among the viral type detected in wart and blood/plasma sample from the same animal. Since during the immune-response against papillomavirus the immune cells present in bloodstream interacts with papillomavirus, we speculate that peripheral blood mononuclear cells, possibly lymphocytes, can be acting as a pathway to spreading/transmission of BPV-1, that might serve as a source of BPV-1 in infection of epithelial cells and contribute to their dissemination.

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