

Canine latent papillomavirus infection and chromosomal instability studies in peripheral blood lymphocytes and tumors cells cultures from lesions biopsy

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In the present study, we reported about papillomavirus (PV) clinical infection and viral latent investigation in canines. Clinical and molecular analyses for detection of DNA PV infection, as well as histopathology examination and cytogenetic evaluation were carried out in clinical lesions canines. Blood sample from the both epithelial infection and the asymptomatic dogs had been collected for molecular analysis. Amplification of DNA samples with the L1 gene from generic primers were detected in skin biopsies and blood samples from canines healthy clinically or not. To investigate the chromosomal fragility, we researched by cytogenetic technique in the peripheral blood lymphocytes cultures and in the tumors cells cultures from canines presenting oral and cutaneous warts lesions. The histological examinations of the skin biopsy specimens were diagnosed like adenoma, carcinoma and papilloma. In some cases, besides the surgical removal, autogenous vaccine derived from fresh warts of the same animal was indicated. The findings documented as mucosal and cutaneous tropism, which the PV viral infection not confined only epithelial sites, but also involved intracellular genome of host cell.

Keywords: *canine papillomavirus*, peripheral blood lymphocytes cultures, tissue cell cultures and PCR.

INTRODUCTION

Papillomaviruses (PVs) research involved the study of cancer and molecular virology in the Veterinary and Human Medicine fields (Onions et al., 1997). *Human papillomavirus* (HPV) infections have been reported numerous lesions resulting several morphological types associated with pathogenicity level in low risk (e.g.; HPV 6 and 11) or high risk (e.g.; HPV-16 and 18) that can to progress to malignancy. Recently, PV types

were classified in 18 genera, based on their nucleotide sequence identity and biological and pathological properties (Tomita et al., 2007).

In many cases, there is absence of signs clinical indication of previous infection but the tumour formation is possible. This effect suggests viral latent infection into the circulating lymphocyte (Campo et al., 1994). *Canine oral papillomavirus* (COPV) DNA has been detected in oral mucosa samples after spontaneous papillomaviruses, COPV may enter a state of latency (Nicholls et al., 2001).

Tables 1. Epidemiological dates test animals *Canis familiaris* for the viral latent infection study

Animal	Sex	Breed	Age
C1	F	SRD*	2 years
C2	F	Labrador	1 year
C3	F	SRD	8 years
C4	M	Poodle	8 months
C5	F	SRD	2 years
C6	F	SRD	6 years
C7	F	Boxer	5 years
C8	M	Pastor	10 years
C9	F	Poodle	6 years
C10	M	Bloodhound	2 years

RD: Indeterminated breed

Table 2. Epidemiological dates test animals *Canis familiaris* for the clinical infection study

Animal	Sex	Breed	Age (years)	Form	Sites
CPV 01	M	Duchound	7	Flat / Plan	Snout Head /Snout
CPV 02	F	Poodle	9	Plan / Pedunculated	Eyes /Members Back /Neck /Tail
CPV 03	M	Poodle	11	Flat / Florid Pedunculated	Head /Snout Eyes /Members Back /Neck /Tail
CPV 4	M	São Bernardo	4	Flat / Florid Pedunculated	Oral mucosal cavity

The replication cycle of the papillomavirus begins in the nucleus of basal layer and consisted in high level expression of viral proteins, synthesis of capsid proteins and viral assembly to production of infectious virus particles in differentiating squamous epithelial cells (Nicholls et al., 1999; Howley and Lowy, 2001). To investigate wart life cycle from papillomavirus infection and to study about cellular immune response, chronological series of lesions obtained from canines

and bovines, were used how a new strategy (Campo, 1997; Nicholls et al., 1999; Nicholls et al., 2001).

There is no adequate in vitro model of papilloma development, so many animals experimental studies has been employed mouse, dogs and bovines models to available the efficacy of potential therapeutic vaccine (Moore et al., 2003). Canine model has been used in the development of papillomavirus treatment, including L1 virus-like particles, early genes, synthetic peptides,



Figures 1 and 2: Clinical oral lesions warts in mucosal canine

fusion proteins, recombinant viruses and DNA vaccines (Campo, 1997; Onions, 1997; Dillner et al., 1991; Nicholls et al., 2001; Moore et al., 2003).

Nucleotide sequence analysis of L1 gene has been utilized for the identification of human and animal papillomaviruses (Tanabe et al., 2000). In the present study, we detected using degenerate primers FAP59/FAP64 sequences DNA PV in clinical biopsies and blood samples from group of canines healthy clinically and group of canines presenting cutaneous and mucosal lesions. Polymerase chain reaction (PCR) analysis was performed with generic primers provided by Ogawa et al., 2004. Chromosomal instability was also investigated once the PV infection has induced increased mitotic activity in the epithelium.

MATERIALS AND METHODS

Clinical evaluation and animals selected

For PV viral latent infection study, ten canines were assisted in the practices medicine of small animals of the Veterinary Hospital for routine exams were verified as free clinical lesions (table 1) and four animals showed epithelial lesions similar to papillomavirus in different locations (table 2).

The animals belonged to proprietors' peculiar with handlings, historical and differentiated alimentary habits. One of them presented many warts lesions into oral mucosa cavity and it has not observed the presence of cutaneous PV lesions (figure 1 and 2). Mucosal and cutaneous papillomatosis were diagnosed on the basis of clinical history and these animals were selected for the clinical disease infection study. After the identification of each animal, they were documented in registration records, each lesion examined as form, dimension and corporal location.

Epithelial biopsy preparation

All symptomatic animals were directed for the surgical removal partial and/or total of the lesions and subjected to sedation and local or general anesthesia to samples preparations. Biopsies of the epithelial tissues were taken using a punch or scalped, after asepsis with iodized alcohol, followed by suturing. Part of the tumors samples were fixed in 10% neutral buffered formalin to histological examination. Others fragments of biopsies were embedded in RPMI medium to tumors cell cultures for the cytogenetic studies.

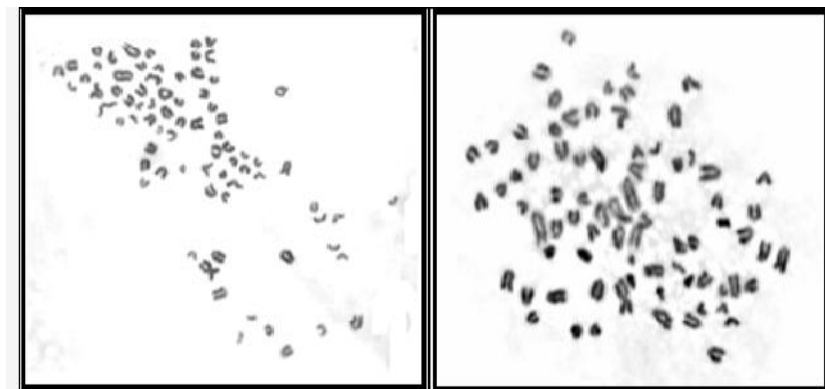
Blood samples

Approximately 5 mL of whole blood samples, in duplicated, were collected aseptically from the cephalic vein of symptomatic and asymptomatic canines, using heparin and EDTA vacutainer tubes. Blood samples contained EDTA were frozen at - 20°C until DNA extraction and molecular analysis. Blood samples contained sodium heparin were refrigerated at 4-8°C and transported to the laboratory for cytogenetic studies. Less than 24 h after sample collection, aliquots were added to cultures medium to prepare cultures.

Diagnostics Methods

Histopathology examination

The laboratorial procedure consisted of cleavage in small fragments, histotechnic, paraffin emblocked by TP1020 (Leica), thickness sections 0,5 um processing by semi-automatic microtom RM 2145 (Leica), sections haematoxylin and eosin-stained for routine, analysis and photo documented of histological alterations by MDC



Figures 3 and 4

Structural chromosome aberrations in metaphase from peripheral blood lymphocytes culture of canine with clinical lesions. The arrows indicates the chromosome anomalies (centric rings, open chromatids and associations)

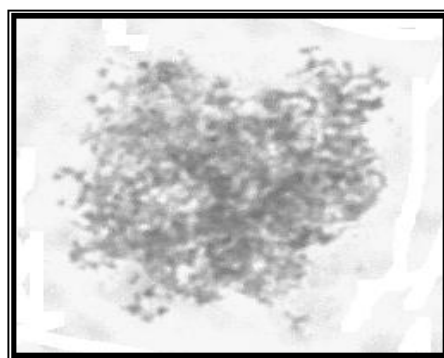


Figure 5 Premature chromosome condensation (PCC) with pulverization appearance obtained from tumors cells cultures of canine presenting cutaneous lesions

LENS COOLPIX 995, accopplated in optical microscopy Nikon. Sections were examined by light microscopy and immersion objective. The histological examination of skin biopsy specimens from dogs was diagnosed like adenoma, carcinoma and papilloma.

Cytogenetic analyses

Cytogenetic analyses were carried out on short-term peripheral blood lymphocyte cultures for 72 hours using Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal calf serum and 2% phytohaemagglutinin. In addition, tumors cells cultures were done starting from lesions of skin biopsy. More than fifty metaphases per animal with conventional Giemsa staining were analysed and the karyotypes were constructed using the *Photoshop CS₂* version 9 software. Chromosome banding techniques were also performed. In metaphases analyzed it was possible to observe the patterns of chromosomal banding G and C by the

method of banding, was identified marking the constitutive heterochromatin in the centromeric region of chromosomes autosomes and sex of dogs investigated.

Molecular analysis

DNA Extraction

The frozen specimens were homogenized and DNA was extracted from whole blood and papilloma lesions samples using the Kit QIAamp DNA blood mini kit following the manufacturer's protocol (Qiagen). Approximately 200µl of whole blood of each sample characterized from clinical cases of canine oral and cutaneous papillomatosis and dogs without lesions were aliquoted in previously identified eppendorfs tubes. The extraction method of DNA from tissue of the skin biopsy was reported by Freitas et al., (2003). Approximately 200µl elution buffer was added and

Table 3 Statistical analysis of counted cellular with or not chromosomal under position in the different classifications

Cells counted*	Percentage of cells with chromosomal under position *	Percentage of cells without chromosomal under position*
2n = 78	26,41 ± 21,62	22,95 ± 19,87
Aneuploidy	35,41 ± 22,60	77,05 ± 40,24
Total Metaphases	54,25 ± 50,33	50,59 ± 49,83
PCC	45,75 ± 50,32	49,40 ± 49,83

* The significance were considered differs statistically when p -value < 0,05

centrifugation to 8000 rpm per 1 minute was done. DNA each sample was eluted and stored at -20°C until next step. The products were analyzed by 1% agarose gel electrophoresis along a 1Kb DNA ladder (Promega).

Genomic DNA

Genomic DNA quality was available following 1,2 µl Blue/orange stained (Promega), 3 µl test sample and 2 µl 1Kb DNA ladder. DNA extraction products were analysed in red-stained 1% agarose gels in horizontal cube Amersham Pharmacia Biotech a 60v e 100ma. Bands were visualized by a Bio Doc Analyze System (Biometra Ti 5) during 2 hours. Samples were quantified by spectrophotometer Gene lector.

β-globin gene PCR

β-globlin gene was used to verify the quality of DNA for PCR procedures using primer pairs: β-globlin fw (forward; 5' - AAC CTC TTT GTT CAC AAC CAG - 3') and β-globlin rev (reverse; 5' - CAG ATG CTT AAC CCA CTG AGC - 3'). The PCR reaction was prepared in a different room from DNA extraction to avoid sample contaminations. The aliquots were insured in Programmable Thermal Controller (PTC-100) and analysed conform described protocol by Stocco dos Santos et al., (1998). For the primer β-globlin, after an initial denaturation at 94°C for 3 min, the PCR consisted of 35 cycles of 50 seg at 94°C, 1 min at 60°C and 1 min at 72°C, followed by extension at 72°C for 5 min. DNA bands amplified were visualized by size determination under UV light.

Generic primers PCR

DNA was used for Polymerase chain reaction (PCR) analysis using generic primers pairs: FAP59 (forward; 5' - TTA CWG TIG GIC AYC CWT ATT - 3') and FAP64 (reverse; 5' - CCW ATA TCW VHC ATI TCI CCA TC - 3') (Ogawa et al., 2004). PCR conditions were as follows: initial denaturation at 94°C for 10 min, followed

by 45 cycles of denaturation at 94°C for 1,5 min, and annealing for 1,5 min at 50° C, initial extension at 72°C for 1,5 min and extension at 72°C for 5 min. Approximately DNA 3 µl was added to each reaction in mix PCR buffer with 1,0 U Taq DNA Polymerase and 2µl of each primer 0,5 µM, in a final volume of 25 µL. The reaction was run and amplified products were identified by electrophoresis on a 2% agarose gel in TBE buffer, followed by red-stained.

Statistical Analysis

The collected data of the cytogenetics technique for chromosomal populations' studies were analyzed by the program Statistical Package for the Social Sciences (SPSS), version 13.0. No parametric tests were applied in the total cells counted. To compare the variables of a same group of animals the Friedman test was used and for two variables of a same animal, Wilcoxon test was computed.

RESULTS

Cytogenetic analysis

Cytogenetic analysis of the lymphocyte cultures and warts cells cultures showed cells presenting differed numbers chromosomal and some chromosomal aberrations. We detected numerical cytogenetic abnormalities (e.g. hiperploidy and hipoploidy) and structural aberrations (e.g. chromatid gaps, fragments and centric rings) in the lymphocytes cultures (figure. 3 and 4) and premature chromosome condensation (PCC) presenting pulverized configuration was identified in the tissues cultures from canine's lesions biopsies (figure. 5). Furthermore, the present investigation addresses not only question related to the clinical lesions features but especially intracellular cytogenetic findings for the integration of the viral genome in the chromatin cell host. In cultures of tumor cells were found strong evidence of premature chromatin condensation, showing different stages of the proliferative potential index (PPI).

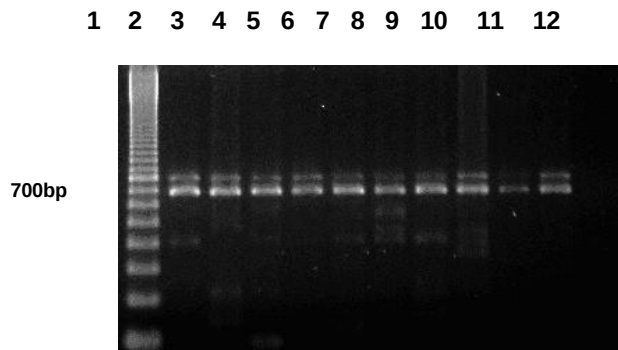


Figure 6: PCR analysis with generic primers. Lines 1: 100 bp ladder molecular mass marker; Lines 2 -11: samples from asymptomatic canines; Line 12: sample from negative control. Amplification products were resolved by 2% agarose gel

For the chromosomal study, more than 50 metaphases per animal, (*Canis familiaris* $2n=78$ chromosomes with 38 pairs of acrocentric chromosomes and one metacentric for female and one acrocentric for male sexual chromosomes), were scored. The chromosomal put upon not included in the calculation in the percentage of aberrant cells (table 3).

Histological and clinical evaluation

Histological examination of haematoxylin and eosin-stained sections reveals hyperkeratosis and acanthosis, intense proliferative activity of lymphocytes and plasmocytes cells was observed in the layers and vacuolated typical of the productive papillomavirus infections. The lesions were characterized by epithelial hyperplasia, acanthosis, hyperkeratosis and connective tissue proliferation. Examination of material removed confirmed the presence of PV infection. In the clinical evaluations, papillomas were observed on the haired skin of the face, pinna and forelimbs.

Molecular analysis

Viral DNA sequences in peripheral blood in canines infected or not by PV were detected using degenerate primers FAP59/FAP64 (figure 6). The amplification of DNA bands specific for PV were analyzed by electrophoresis on 2% agarose gel showing in all samples tested positive bands for papillomavirus infection.

DISCUSSION AND CONCLUSIONS

Studies reported chromosomal instability in peripheral blood lymphocytes of bovines and humans with or not the effect resulting of the carcinogenic agents (Stocco dos Santos et al., 1998; Recouso et al., 2003), since

PV viral gene products induce intracellular morphologic changes (Howly & Lowy, 2001).

Similar ours findings, chromosome fragility was detected in lymphocytes from women with HPV infection after treatment and high frequencies of numerical and structural chromosomal aberrations have been described in keratinocytes cells transfected with the HPV-16 E7 gene (Mansur & Androphy, 1993). Morphologically, mucosal lesions from canine oral papillomavirus (COPV) have been described similar to mucosotropic anogenital HPVs infections (Moore et al., 2003).

Studies reported severe or generalized papillomatosis in dogs immunosuppressed by drugs associated with deficiency of the immune system. Persistent and generalized PV infection have been described how refractory to treatment. Similar associations are documented in humans' immunosuppressed or immunodepressed and in epidermodysplasia verruciformis patients or by human immunodeficiency virus (HIV) co- infection (Campo et al., 1994).

The degenerated primers FAP59/FAP64 based L1 ORF, the most highly conserved of the PV ORFs with approximately 40% of the amino acid residues being identical in distantly related PVs (Dillner et al., 1991), have been used to detect all the papillomaviruses in whole blood, swab samples and biopsy specimens from humans and animals species (Ogawa et al., 2004; Tomita et al., 2007).

Earlier studies reported that the transformation of human keratinocytes with HPV-16 E6 and E7 genes and papillomavirus associated cervical cancers was sufficient to induce the increase or decrease in the normal number of chromosomes (aneuploidy) (Mansur and Androphy, 1993). This chromosomal instability may be result of viral oncoproteins induced cell growth stimulation (Mansur and Androphy, 1993).

This study demonstrated for the first time chromosomal abnormalities in peripheral blood lymphocytes cultures and tumors cells cultures from

canines PV infections cases. In spite of the cultures conditions have been similar compared with study in bovine lymphocyte cultures (Walter-Moura et al., 1988; Recouso et al., 2003), to date, chromosomal aberrations were only demonstrated in studies of the cattle with chronic enzootic haematuria (CEH), with or not access to bracken fern (Walter-Moura et al., 1988), in bovines with urinary bladder cancer by reported Stocco dos Santos et al., (1998) and uncommon rearrangements were detected in cells lines of bovines (Leal et al., 2003).

PCR analysis suggested that the canines probably have evaluated the virus in episomal form and is probably integrated into the chromosome of the host cell and producing a latent or asymptomatic animal typical of sub-clinical.

Cytogenetically, the presence of frequent findings of PCC with different morphologies metaphase chromosome culture of the lesions, believed to be the cell cycle arrest in Interphase by inhibiting the role of protein kinase due to such uncontrolled proliferation of cancer cells infected by the virus.

We concluded that cytogenetic analyses determined the interactions of viral genome with host cells. The immunological pathways, therapeutic strategies will have to design to control persistent and recurrent papillomavirus infections. Our findings are compatible with the productive infection of cells by the papillomavirus and contribute with the understanding of the canine PV infections but more studies are required to explain better the functions of cellular genes active at different stages of the cell cycle.

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