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Research Article

CHROMOSOME ABERRATIONS AS A BIOMARKER FOR GENOMIC INSTABILITY IN CELL CULTURES ORIGINATED FROM BOVINES, CANINES AND EQUINES INFECTED WITH PAPILLOMAVIRUS

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Abstract

Chromosomal abnormalities or aberrations were investigated in ten bovines, three canines and three equines presenting cutaneous papillomatosis. The aim of the present study was to evaluate possible chromosomal abnormalities as a biomarker of genomic instability induced by the interaction of the viral genome with the chromatin of the host cell in peripheral blood lymphocytes and skin warts lesion cultures. Genomic DNA was extracted from blood samples and epithelial biopsies. Amplification products from PCR using degenerated primer pair FAP59/FAP64 were sequenced. Peripheral blood lymphocytes and tumor cell cultures were performed. Structural chromosomal aberrations were verified as associations, chromosomal fragments, centric fusions, open chromatids, gaps, rearrangements and rings. Several levels of premature chromosome condensation were observed in both cultures. Numeric abnormalities as aneuploidy were also detected and hipoployd cells were found in a larger number than the hiperployd cells. This cytogenetic study in detecting premature chromosome condensation in the three species investigated describes by the first time the structural and numerical abnormalities in canines and equines presenting papillomatosis lesions. These abnormalities are similar of the three groups of animals, but virus specific.

Key words: papillomavirus; peripheral blood lymphocytes; tumor cell cultures; chromosome aberrations; genomic instability

Introduction

The papillomaviruses belong to the *Papillomaviridae* family and new genera have been described on the basis of the interspecies phylogenetic similarity. The papillomavirus is a DNA virus that induces the formation of benign and malignant tumors in a vast range of hosts.

Previous studies have reported the occurrence of chromosomal aberrations in peripheral blood lymphocytes cultured from cattle infected by *Bovine papillomavirus* (BPV) after consumption or not of the carcinogenic substances of the fern *Pteridium aquilinum*, regardless of the development of bovine enzootic hematuria (Stocco dos Santos *et al*., 1998). Presence of DNA sequences of the *Bovine papillomavirus* 2 (BPV-2) was detected in the cattle with bladder cancer. The correlation data suggested that the BPV-2, together with *Pteridium aquilinum*, acts synergistically to produce the chromosomal instability (Lioi *et al*., 2004).

Experimentally, BPV DNA sequences have been detected in peripheral blood of non-clinically affected cattle by papillomatosis, presenting high levels of chromosomal aberrations or abnormalities (Campos *et al*., 1994; Stocco dos Santos *et al*., 1998). Cytogenetic abnormalities and numerical and structural chromosome aberrations, such as endoreduplications, gaps, break chromatids, centric rings and dicentric chromosomes were observed in human keratinocytes and mouse cells transfected with E7 oncogene HPV-16. Chromosomal fragility has been detected also in lymphocytes of women with lesions in the cervix after submitted to a treatment against HPV (Paz-y-mino *et al*., 1992).

Campo *et al*., (1994) and Campo (2002) suggested that the viral latency site occurred in lymphocytes when studying BPV DNA sequences detected in lymphocytes of bovines with or without papillomatosis, as well as HPV DNA in blood cells of women with HPV urogenital and cervical cancer infection.

These HPV infected cells express viral oncogenes (E6 and E7) that inactivate the function of the tumor suppressor genes and induce the formation of chromosomal aberrations, interfering in checkpoint control mechanism G1 and G2, and braking cell division (Chang *et al*., 1997; Coursen *et al.*, 1997). In the case of the BPV, the mechanism is different. Experimental data showed that the BPV is unable to associate with mitotic chromosomes by E2 protein, being this mechanism less studied. The mitotic activity of epithelial cells in mucosa and skin tissue of persistent infections is able to maintain the viral genome with a low number of copies (Mc Phillips *et al*., 2006).

Papillomavirus oncoproteins interfered in DNA mitotic checkpoints which are normally activated when an event occurs, such as breaks in DNA, triggering cells to enter the S phase or mitosis, to repair cell damage (Thompson *et al*., 1997).The first observation of chromosomes condensed prematurely or premature chromosome condensation (PCC) was recorded in virus-infected cells, mediating cell fusion in the interphase of mitotic cells (Kato and Sandberg, 1967; Gotoh and Durarte, 2006).

The mechanisms of chromosome condensation have been intensively investigated. The appearance of the sprayed chromatin has been associated with chromosomal fragments induced by viruses (Kato and Sandberg, 1967 in Gotoh and Durante, 2006). From the mid-70s, the PCC has been recognized as a tool for the investigation of chromosomal dynamics in radioactive cytogenetic and chromosome replication studies (Gotoh and Durante, 2006). Condensinas, histone H1, kinase and topoisomerase II (TOPO II) are some molecules occurring in cell induction mechanism of PCC (Lee *et al*., 2007).

Cyclin-dependent kinases (cdks) govern cell cycle progression in eukaryotes. In mammalian cells, the transition from the G1 / S and G2 / M phase are regulated by complex distinct cdk / cyclin. In conjunction with cdc2, cyclin A and B regulate the entry into cell mitosis (Pines, 1996 cited Thompson *et al*., 1997). The activation of cdc2 is mediated by a complex starting with the increase of cyclin B1 in the S and G2 phase in the presence of Epstain-Barr virus (EBV) (Lee *et al*., 2007). The cyclin-dependent kinase activity in the S phase increases during viral replication, while the cell DNA replication is inhibited. Genetic and biochemical evidence suggests that the condensed complex of protein subunits and DNA topoisomerase II, essential for mitotic condensation, both developed important role in the assembly and organization of mitotic chromosomes (Lee *et al*., 2007).

The increase of cdc2 kinase activity associated with histone H1 (Thompson *et al*., 1997) has been detected in cell populations expressing E6 of HPV-16. The ATR enzyme belongs to the kinase family and is able to prevent cell induction by checkpoint regulation of G1. However, cancer cells and the loss of p53 function causes the inhibition of ATR and thus interfere in the checkpoint G1, sensitizing the cells to the formation of PCC (Nghiem *et al*., 2001).

The aim of the present study is to evaluate possible chromosomal abnormalities induced by the interaction of the viral genome with the chromatin of the host cell in peripheral blood lymphocytes and skin wart lesion cultures of bovines, canines and equines infected with papillomavirus

Material and Methods

Clinical and histopathology examination

Skin warts lesions of cutaneous papillomatosis were detected in biopsy fragments of ten bovines, three canines and three equines samples, and kept in RPMI medium to tumors cell cultures for cytogenetic studies. Tumor samples were fixed in 10% phosphate-buffered formalin solution, embedded in paraffin and stained with haematoxylin and eosin (H&E) described by Marins *et al*. (2010).

Cytogenetic analysis

Blood collection was carried out using syringes with heparin or heparinized vacutainer tubes for each species. Peripheral blood lymphocyte cultures were realized at 37^0C for 72 hours and 5% CO^2. Cytogenetic preparation of the tumor cell cultures obtained from skin biopsy was reported by Marins *et al*., (2012). The karyotypes were assembled using the *Photoshop* CS_2 *version* 9 software. After selecting the metaphases, the karyotypes constructed were classified about the number of chromosomes in complete, incomplete and overlaps (not considered appropriate for analysis).

Molecular screening

DNA extraction was carried out from blood samples and epithelial tissues of cattle, canines, and equines using QIAamp DNA blood and tissue mini kit following the manufacturer`s protocol (Qiagen). Polymerase chain reaction (PCR) was performed with β-globin gene PCR and generic primer pairs PCR FAP59 (forward: 5´- TTA CWG TIG GIC AYC CWT ATT – 3`) and FAP64 (reverse: 5´- CCW ATA TCW VHC ATI TCI CCA TC – 3`), performed according to Marins *et al*., (2012). In sequence, 5µl of the purified PCR product using the GFX PCR DNA purification kit (GE Healthcare), were used. Subsequently, 2µl of the purified PCR product was quantified. The sequences were analyzed using the Sequence Analyser software of Cimarron Base Caller 3.12. The quality of the sequences obtained was evaluated by Chromas program version 2.33 and / or the software Biological Sequence Editor Aligment (BioEdit). The identities of the products were searched using the Basic Local Alignment Search Tool program (Blast).

Statistical Analysis

Chromosomal analysis was performed by the program Statistical Package for the Social Sciences (SPSS), version

13.0. Wilcoxon test was applied to compare two variables of a same animal and Friedman test was computed to analysis the parameters of a same group of animals.

Results

The microscopical patterns were characteristic of carcinoma and papilloma infection presenting hyperproliferation in epidermal layer showing inflammatory cells specially lymphocytes. Hyperkeratosis, hypergranulosis and basophilic intranuclear inclusion bodies were detected by histopathology.

L1 DNA sequences were detected using degenerate primers FAP59/FAP64. Samples sequenced of the *Bovine papillomavirus* (BPV) and *canine oral papillomavirus* (COPV) showed similarity with the sequences of the HPV by molecular analysis.

Chromosome aberrations were detected in the peripheral blood lymphocyte and tumor cell cultures of the bovines (Fig. 1 - 2), canines (Fig. 3) and equines (Fig. 4) infected with papillomavirus. Viral DNA amplification was detected in the samples of skin biopsy and blood of animals with cutaneous papillomatosis. In this study, canines carrying adenocarcinomatosa lesions and bovines with papilloma infection showed genetic similarity with the HPV DNA sequence.

Structural chromosome aberrations were verified as associations, chromosomal fragments, centric fusions, open chromatids, gaps, rearrangements and rings. In both types of cell cultures several levels of premature chromosome condensation was observed.

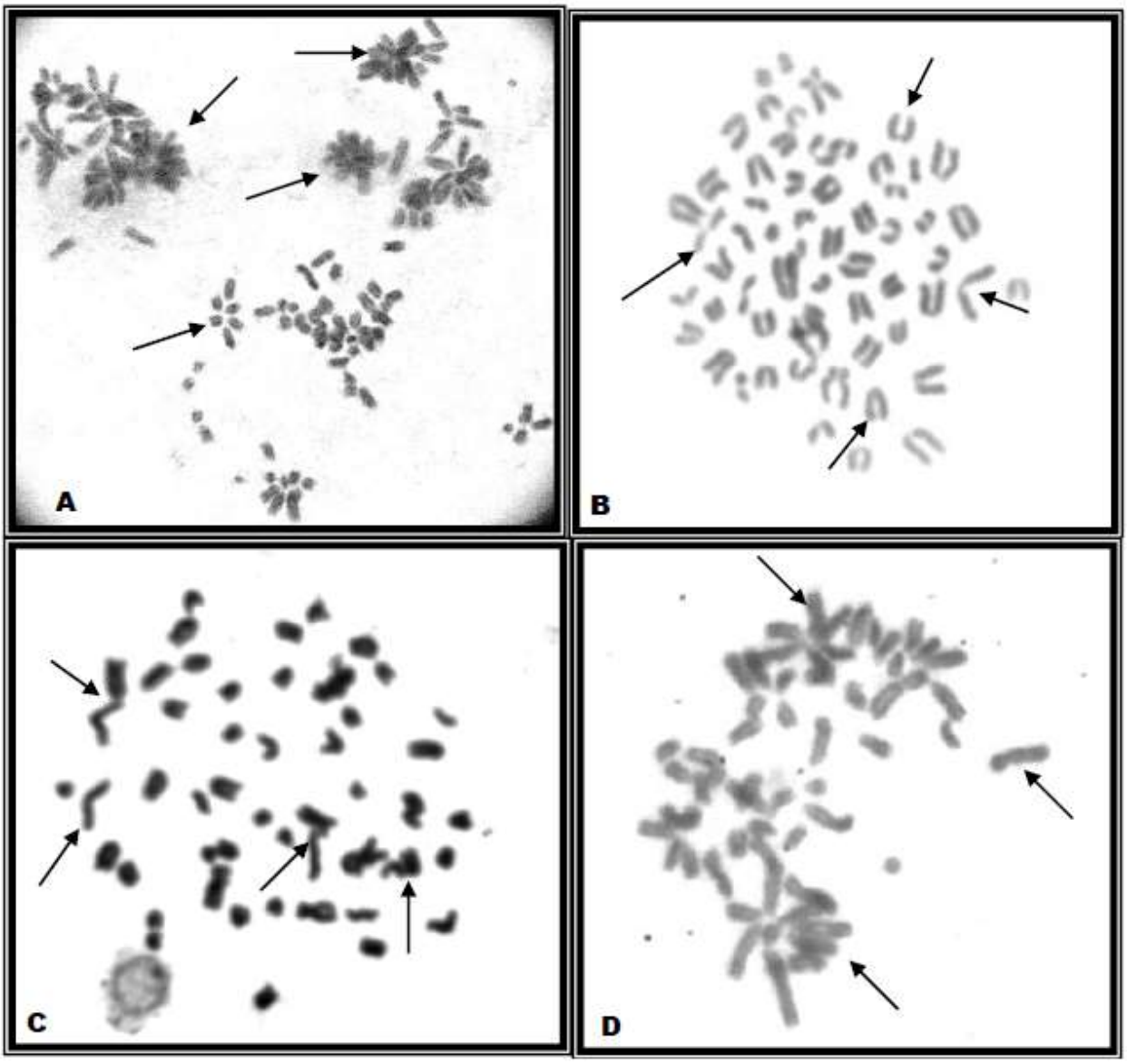

Fig. 1: Chromosome rearrangements observed in bovine peripheral blood lymphocytes culture. The suggestive lesions are (arrows): A) chromosome association; B) chromosomes with rearrangements and open chromatids; C) Fusion of telomeres with centromeres and telomeres fusion; D) metaphase with chromosome associations and possible centric fusion.

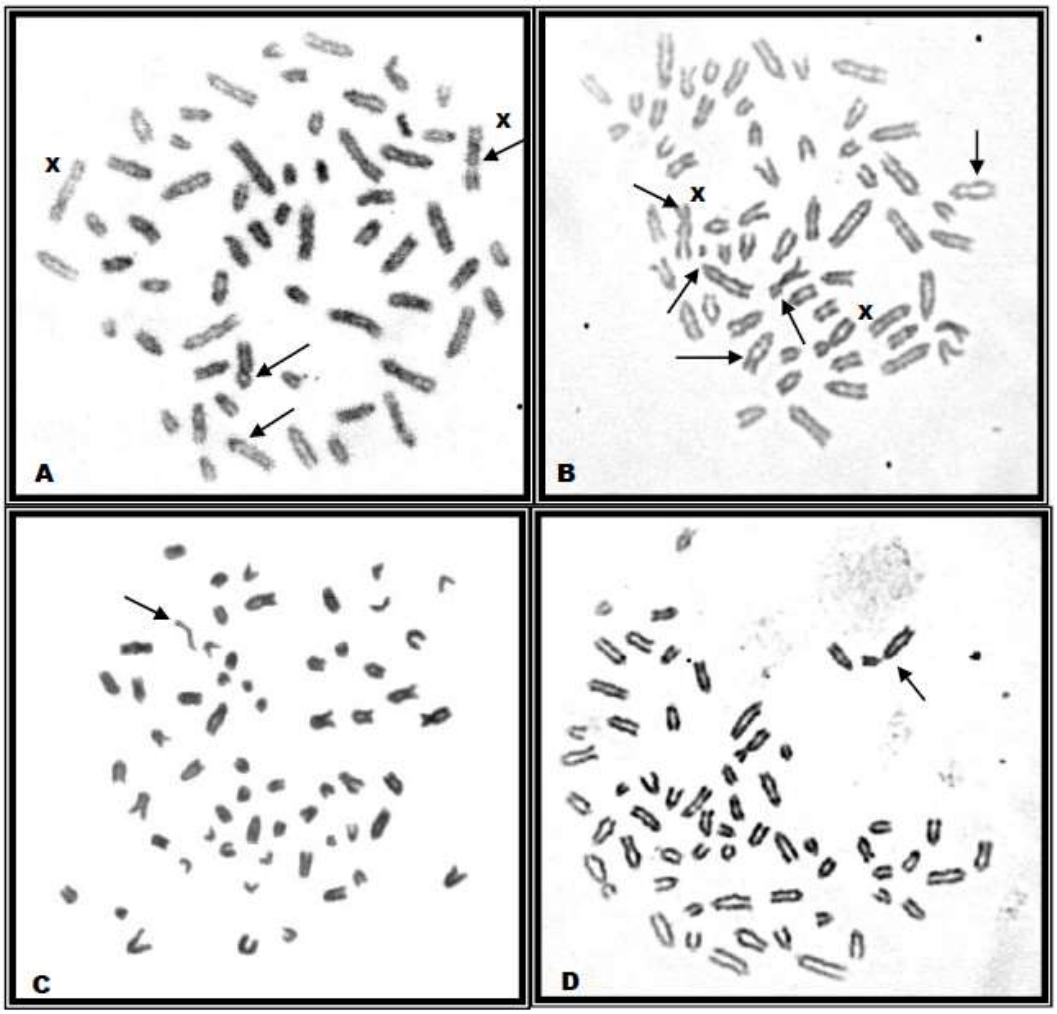

Fig. 2: Chromosome structural aberrations of bovine cultured peripheral blood lymphocytes. The arrows indicate possible chromosomal damage: A) metaphase with female sex chromosome (X) presenting chromosomal fragility and chromosomal rearrangement (x); B) Female sex chromosome (X) presenting possibly gaps, rearrangements, centric fusion and fragments (x); C) open chromatid; D) lesion indicative of centric association.

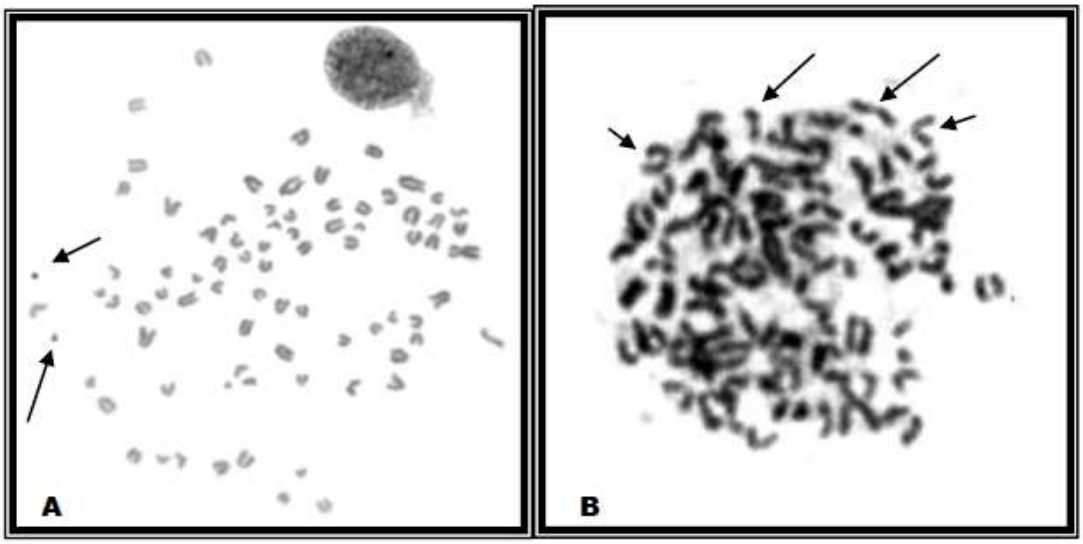

Fig. 3: Chromosome abnormalities in peripheral blood lymphocytes from canines. The arrows indicate lesions suggestive of: A) chromosome fragments (Long & short arrows); B) Cell in the G1 phase with only one chromatid (long arrows) and G2 phase with PCC chromosomes showing double chromatids (short arrows).

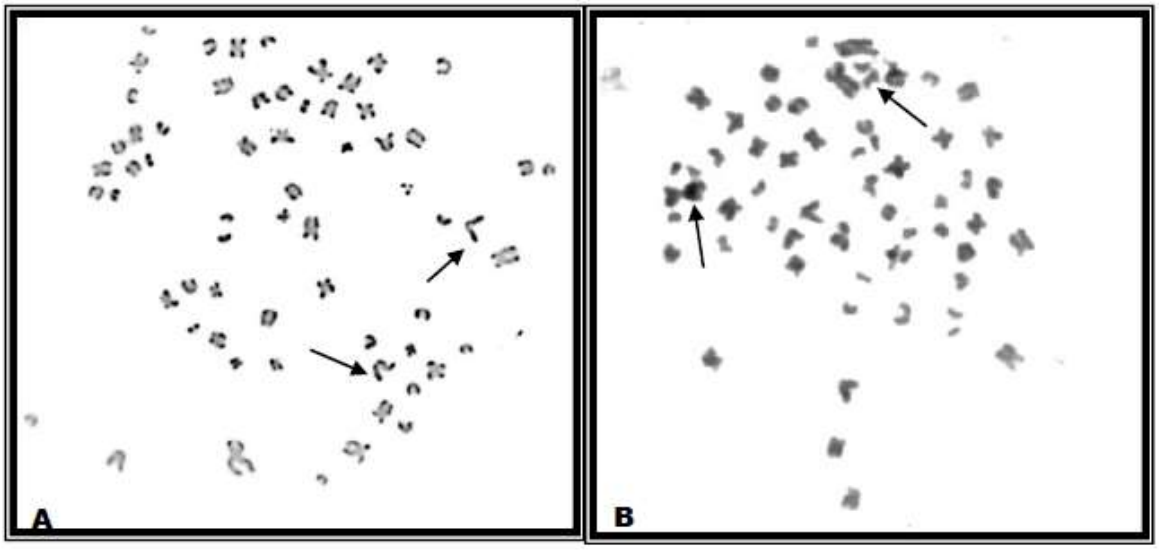

Fig. 4: Chromosome damage in peripheral blood lymphocytes from equines. The arrows indicate chromosomal lesions suggestive of: A) open chromatids during metaphase with chromosomal fusion; B) centric fusion.

Table1. Percentage of cells with numerical-type aberrations in the animal groups investigated

Species	Completed metaphases (%)	Hypoploid cells (%)	Hyperploid cells (%)	Numerical changes (%)
Bovines	**28,68** ± 16,12	**67,29** ± 16,06	**4,01** ± 4,04	**71,31** ± 16,12
Canines	**15,28** ± 13,25	**48,46** ± 42,05	**36,22** ± 55,24	**84,71** ± 13,25
Equines	**45,24** ± 26,33	**50,89** ± 22,95	**3,86** ± 3,38	**54,76** ± 26,32

Among the metaphase in bovines, the average proportion of whole cells with diploid number of chromosomes was 28.68% ± 16.12% (CV = 0.56). Averaged 71.31% of the cells showed numerical changes with a standard deviation of 16.12% (CV = 0.23). It was concluded that the differences between the whole cell percentage and the numeral changes are not significantly different by Wilcoxon test (p-value = 0.007). There are significant differences between the percentages of whole cells, hypoploid (67.29% ± 16.06) and hyperploid cells (4.02% ± 4.04) in cattle by the Friedman test (p-value = 0.000). The differences between the mean proportion of metaphases and PCC are not significant, with a p-value of 0.173 (Wilcoxon test).

In the canines, it was found that an average of 15.28% ± 13.31% (CV = 0.87) corresponded to complete metaphases and 84.72% ± 13.31% (CV = 0.16) to numerical changes. Among the cells with numerical changes, hypoploid cells presented an average of 48.46% and hyperploid cells of 36.26%. Comparing the percentage of metaphases of total cells analyzed with the PCC percentage, an average of 54.06% ± 50.28% (CV = 0.93) corresponded to metaphases and 45.94% ± 50,28% (CV = 1.09) to PCC cells.

In the equines, an average of 45.24% ± 26.33% (CV = 0.58) was found in complete metaphases and 54.76% ± 26.33% (CV = 0.48) of numerical changes. Among the numerical changes, hypoploid cells showed an average of 50.89% ± 22.96 (CV = 0.45), and hyperploid cells of 3.87 ± 3.38 (CV = 0.87). An average percentage of metaphases of 62.22% ± 7.70% (CV = 0.12) was found when total cells were evaluated, and 37.78% ± 7.70% (CV = 0.20) of PCC cells (Table 1)

Discussion and Conclusion

Chromosomal instability

In general, viruses have the ability to enter cells by different mechanisms, leading the cell chromosomal instability, and some of them contribute to the development of cancer cells (Duelli and Lazebnik, 2007). The interaction with the host cell chromatin, which occurs when the circular DNA is associated with histones of the cell and being able to express the ORF genes is the most accepted mechanism to justify the chromosomal instability of HPV. The viral genome is integrated into mitotic chromosomes through the action of the E2 gene, allowing segregation of the episomal genome inside the host daughter cells. Thus, the replication and maintenance of the viral genome in the host cell and during cellular proliferation is maintained (Mc Phillips *et al.*, 2006). In this context, the present study reports the occurrence of chromosome aberrations as a biomarker for

genomic instability in peripheral blood lymphocytes and skin wart lesion cultures of bovines, canines and equines. Studies are required for a better understanding of the cell cycle mechanism in the papillomavirus infection.

Karyotypes of bovines, equines and canines with skin lesions suggests the presence of chromosomal fragility during metaphases in peripheral blood lymphocyte cultures presenting similar chromosomal changes as in HPV infections, like chromosome associations, fragments, centric fusions, open chromatids, gaps, rearrangements, rings and aneuploidies. It is known that the E6 and E7 genes, involved as crucial factors in cancer development, interferes in the normal epithelial differentiation and induce failure in cellular control of viral transcription (Mansur and Androphy, 1993). These genes are consistently expressed in cells infected with PV, according to Thompson *et al*. (1997).

Chromosomal fragility was detected in the cattle not exposed to *Pteridium aquilinum* diet, considered by Leal *et al.,* (2003) as a carcinogenic agent which interferes in the initial and late levels of the papillomavirus infection.

The results of chromosomal analyzes found in the present study are consistent with those of Mansur and Androphy (1993), Thompson *et al*., (1997) and Recouso *et al*., (2003), referring to the increase and decrease in the number of normal chromosomes, as well as the presence of possible structural abnormalities that are common findings observed in populations of neoplastic cells, including human infected by HPV and positive for cervical cancer. Recouso *et al*., (2003), described only 7% of total cells analyzed presented some type of structural abnormality, considering the presence of only a chromosomal aberration until nine structural abnormalities in the cells analyzed. Duelli and Lazebnik (2007) cited that the change in the number of chromosomes (34-184) always was indicative of chromosomal instability. In the present study, the number of abnormalities occurred in excess when compared to the structural aberrations.

Fragmented cells probably aroused due to the high amount of viral load, capable to induce cell lysis. Chromosomal fragments observed also correspond to the description of the appearance of pulverized chromosomes during the interphase in cultures of lymphocytes from peripheral blood of cattle, equines and canines in the present study. The same observation was made in a study in which cells were treated with methanesulfonate (MMS) chemically and camptothecin (CPT) (Garcia *et al*., 2001).

Such pulverizaded configurations were also detected in the lesions of cell cultures of the animals studied, characterizing the presence of PCC. The statistical analysis in cattle, equines and canines used non-parametric tests. Friedman test which compares more than two groups and the Wilcoxon test for evaluation between two groups or two variables used in the present study also were described by Fillipin *et al*., (2006).

Chromosome numerical and structural aberrations

The present data show many variations in the diploid number, smaller or larger than normal number of chromosomes (hypoploid and hyperploid), in peripheral blood lymphocytes of bovines, canines and equines investigated. Numerical abnormalities in immortalized epithelial cells of the HPV 16 have been described as triploydias (3n) and tetraploydias (4n) by Yasumoto and Hashida (1991). High incidence of hypoploid cells and hyperploid was also described in studies in bovines by Leal *et al*. (2003).

According to Reis *et al*. (1984) cited Mansur and Androphy (1993) the aneuploid (hyperploid and hipoploid) cells percentage ranged from 33% to 82% in cases of cervical intraepithelial neoplasia in women infected with HPV. Similar findings were found in cattle with an average percentage of 67.29% in cells with hypoploid and 4.01% in the cells with hyperploid. According to Leal *et al*., (2003), the average percentage was also higher with hypoploid in cells experimentally infected with BPV-4 E7 oncogene. The number of hypoploid cells of equines and canines were also higher than in hyperploid cells in this present study.

Chromosomal aberrations such as: associations, fusions, fragments and gaps described in bovines lymphocytes, according to previous reports are consistent with our results. In the karyotypes of bovines, equines and canines, in this study, it was found that not all karyotypes showed a number of chromosomes exceeding the normal diploid cells (2n). According to Nicholas (1997), abnormal karyotypes arise from errors in their chromosomal replication.

Structural aberrations detected with chromosome markers have been found also in bovine and human populations (Recouso *et al*., 2003). These markers identify abnormal and morphologically different chromosomes. They have a centromere and are generally derived from a chromosome breakdown with loss of an acentric fragment (Richardson, 1991). In the present study, changes were found suggestive of chromosome fragments in metaphases of lymphocytes from cattle with cutaneous papillomatosis. Breaks and gaps which occur at various stages of cell division have been found also. The chromatid breaks or gaps occur only on one chromatid, for the other hand breakage or chromosomal gap refers to structural abnormality that occurs in both chromatids at the same point in the chromosome (Richardson, 1991).

Numerous chromosomal overlaps and a large variation in the number of chromosomes in metaphase, higher than structural abnormalities were observed when analyzing lymphocyte cultures and neoplastic lesions of the studied groups of animals. Due to the large number of cattle and

canine chromosomes, possibly, some may be lost during the chromosomal preparation.

However, this finding may also be related to the presence of PV, as described in cattle by other authors (Stocco dos Santos *et al*., 1998; Leal *et al*., 2003). Typically, these chromosomal abnormalities are observed in high levels in bovine lymphocyte cultures. However, based on cytogenetic results, chromosomal abnormalities were observed in cattle, canines and equines lymphocytes, described as chromosome associations, chromosome fragments and numerical aberrations (hypoploid and hyperploid) with predominance of hypoploid cells for cattle.

Premature chromosome condensation (PCC)

There was a variation in the number of cells with PCC in our study, particularly in lesion cultures, causing a possible chromosomal instability as a result of fusion between cells in the mitotic or interphase with pre-mitotic cells. The PCC findings, mostly performed in the S phase, and according to studies of Duelli and Lazebnik (2007), refer to multiple DNA breaks resulting in multiple chromosomal fragments attesting the pulverized appearance of the cell. According to reports of Lee *et al*., (2007), the chromatin of nasopharyngeal carcinoma cells became highly condensed when positive *Epstein–Barr Virus* (EBV). Similarly, the chromatin of adenocarcinoma cells in canine and bovine papilloma infected cells showed high levels of PCC. Similar findings were reported also by Marins *et al*., 2010 in peripheral blood lymphocytes and skin warts lesions cultures of the bovines and by Ito *et al*., (2002), after analysis by confocal microscopy of condensed chromatin in interphase in EBV infected cells.

The high PCC index observed in the present study was similar to the observation of Lee *et al*., (2004), treating the lymphocytes with calyculin A, experimentally inducing the formation of PCC, and in accordance also with Lee *et al*., (2004) to verify that the number of PCC cells ranged from 160 to 1.040 the each sample of patients with lung cancer treated chemically with calyculin A.

Cultures of lesions and lymphocytes from cattle, equines and canines showed cellular phenotype similar to prophase, in agreement with the study of the Marins *et al*., (2010), Marins *et al*., (2012) and Trimborn *et al*., (2004), referring to the PCC aberrations found as genetic mutations.

Many cells showed also highly fragmented chromosomes, indicating breaking of lymphocytes of the investigated animals. In experiments with lymphocytes treated with methyl methanesulfonate for chemical induction of PCC, chromosomal breakage with the appearance of highly fragmented chromosomes was found, and detected only in high chemical doses and related to the dose-dependent chromosomal breaks (Garcia *et al*., 2001). These findings from cultures of skin lesions of cattle suggest different stages of chromatin condensation, corresponding to the specific nomenclature of premature chromosome condensation (PCC). Rao *et al*., (1981) described the chromosomal interphase morphology consistent with studies of human leukemia (Hittelman *et al*., 1981). In the present study, the PCC found in the S phase with chromosomal pulverizations indicates failure in cell cycle subject to genomic instability and are according to Thompson *et al*., (1997) as the uncontrolled cellular mechanisms.

The resulting PCC of the fusion of interphase cells and mitotic cells presented here in was firstly described in the 70s by Rao and Johnson. To date, the PCC had not been registered with the papillomavirus infection but with *Sendai Virus* (SV) infections (Rao *et al*., 1981) and more recently with EBV (Ito *et al*., 2002; Lee *et al*., 2007). Thus, for the first time the presence of PCC in cells infected with PV in bovines, canines and equines were demonstrated.

In the tissue of skin lesions in bovines and canines, we detected the presence of viral DNA nucleotide sequence by PCR. Although the phenomenon of PCC can be induced by chemical processes (Thompson *et al*., 1997; Garcia *et al*., 2001; Trimborn *et al*., 2004; Gotoh and Durante, 2006; Lee *et al*., 2004), other studies have shown the occurrence of PCC resulting of SV (Rao *et al*., 1981) and EBV infection (Ito *et al*., 2002; Lee *et al*., 2007).

PCC was found in bovine lymphocyte cultures in equines and canines infected with PV without intervention or chemical therapeutic process, which can likely be attributed to the presence of the virus. The PCC found in this study probably resulted from an exacerbated PV action inducing cell proliferation and promoting the fusion of pre-mitotic cells, as formerly described by Rao *et al*., (1981), Ito *et al*., (2002) and Lee *et al*., (2007).

This finding for cattle was similar to those described by Leal *et al*., (2003) in cell lines of the palate male fetal bovine transfected with E7 gene BPV4, specific oncogene activation and after treatment with quercetin (5,7,3`,4`-tetrahydroxyflavone), cofactor that acts in synergy with viral action.

However, these tests do not showed a correlation between the amount of PCC observed in the culture of lymphocytes and bovine lesions, demonstrating that this were probably independent factors. By statistical analysis it was demonstrated only that the occurrence of cells with hypoploid was higher in all species examined, as found by Leal *et al*., (2003) in the case of bovine cells experimentally infected E7 oncogene BPV-4.

This cytogenetic study is pioneer in the detection of premature chromosome condensation in the three species investigated. In addition, it described at the first time the structural and numerical chromosome abnormalities in canines and equines with papillomatosis lesions. Therefore, chromosome condensation, numerical and structural

aberrations are similar in bovines, canines and equines infected with papillomavirus, but are virus species dependent.

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