ABSTRACT

Key words: Apoptosis; Bovine; BPV; Fibropapillomas; Hyperproliferation.

The breeding of large ruminants is being challenged by various skin and/or mucosal viral or bacterial disorders that maybe the cause of significant economic losses on the part of breeders (e.g. skin quality depreciation, slow growth of the animals, loss of weight and decrease in milk production). One of these infections with an economical impact for the breeders is represented by bovine papillomavirus infections. In addition to this important aspect of veterinary interest, the importance of bovine papillomaviruses (BPVs) lies in the fact that it has represented one of the most extensively studied animal modelsof viral carcinogenesisBPV-1 has the ability to morphologically transform and replicates in mouse cells in culture. This property has meant that BPV has become one of the most extensively studied animal papillomavirus model useful in understanding the oncogenic potential of the virus (Lowy et al., 1980). As a consequence of the great number of studies, the obtained results on the BPV viral mechanisms and cell transformations have provided invaluable insights into many aspects of this infection, both in human and animals infections.

With respect to the bovine papillomaviruses, these are viruses with an oncogenic potential, able to infect both the skin and mucosa of bovines. The host spectrum infected by BPV, but also the specific tropism of each type are well established, although, in the last decade these viruses were able to break this "paradigm", since unusual types were associated with different hosts and different localizations (Lohr et al., 2005; Literak et al., 2006; Kidney and Berrocal, 2008; Nasir and Campo, 2008; Silvestre et al., 2009; Pangty et al., 2010; van Dyk et al., 2011; Bam et al., 2012; Kumar et al., 2013; Lunardi et al., 2013). Thus, the BPV epidemiology and species-specificity seems to change quickly and it is expected that in few years more and more BPVgenotypes will be discovered, especially due to the medical biotechnology development (Bocaneti et al., 2014).

The doctoral thesis entitled "**The incidence and diagnosis of papillomavirus infections in bovines**" comprises 175 pages, written in XIII chapters and is structured in accordance with

current legal scientific standards in two main parts: the first part (29 pages) and the second part (90 pages), plus table of contents, introduction, summary and references list.

The first part " Current state of knowledge" is structured into 4 chapters, synthesizing the main data in the literature regarding the papillomavirus infections in bovines.

The second part „Personal contributions " consists of 9 chapters, presenting purpose, objectives and research framework, the results of the investigations conducted on cutaneous infection with bovine papillomaviruses, the materials and methods used for the experimental part, the interpretation and discussion of the results, conclusions and recommendations drawn from the experiments undertaken.

The paper is illustrated in 62 figures and 15 tables.

For the scientific documentation, 263 reference titles from national and international literature were used.

❖ Chapter I, entitled „**Bibliographic data about history and the etiology of bovine papillomavirus infections in bovines"** presents the available data about papillomaviruses. There are reviewed the history and evolution of papillomaviruses, their complex etiology and taxonomy, and the role of the viral proteins.

❖ Chapter II, entitled „**Epizootic characters and pathogenetic mechanism in bovine papillomavirus infections**" is mainly describing the characteristics of bovine papillomavirus infections and the comprehensive pathogenic mechanism.

❖ Chapter III, entitled "**Clinical signs and anatomopathological features in bovine papillomavirus infections**" describes the main lesional aspects induced by bovine papillomaviruses.

❖ Chapter IV, entitled „**Diagnosis, prophylaxis and control in bovine papillomavirus infections**" presents the laboratory diagnosis methodology and also the surveillance and control measures that are necessary in bovine lifestock.

The second part comprises in six chapter the personal contributions.
❖ Chapter V mentiones the "Purpose and objectives".

The current research aims to achieve an epidemiological survey in order to establish the prevalence of the BPV cutaneous infection in bovine livestock from eastern Moldova, to perform a clinical evaluation to better understand their anatomo-clinical and morphopatological features. Another objective was represented by the identification and characterization by molecular biology techniques the viral types involved in the occurrence of the bovine cutaneous lesions.

Given the proliferative characteristic of these lesions induced by BPV, the proliferation index was determinate. Next, an important objective was represented by the studying of some intracellular mechanisms and proteins involved in the cellular transformation of virus-infected cells.

Chapter VI entitled „**Epidemiological, anatomo-clinical and morphopathological investigations in bovine papillomavirus infections**" shows the results of epidemiological, anatomo-clinical and morphopathological investigations conducted between 2010-2014. The examined animals from which the samples were collected came from different bovine farms and households from eastern Romania. Then, from a total of 2127 examined animals, 305 bovines ranging in age from 0 to 6 years were presenting specific lesions for BPV cutaneous infection, which represent a incidence of 14.34%. A great number of affected animals was recorded in the intensive farming system, where the disease occurrence seems to be conditioned by different factors such as: poor hygiene conditions, overcrowding, season, immunosuppression, comparing with the household system, where only sporadic cases were recorded. The most significant rate of illness was recorded at the age of 6-12 months (4.94%), followed by age category 12-18 months (3.9%). The disease may be correlated with the animal age: a highest number of cases are recorded around the age of 12 months (34.43%), then it is observed a decrease of the number cases as a result of animal aging (>18 months - 16.72%). As far as lesion localization is concerned, in animals ranging between 4 and 18 months, the most frequent cutaneous localization was at head level (periocular and periauricular localization)   (50%), while in adults over 24 months, regardless of farming system, a mammary gland localization was recorded (9.51%).

Regarding the morphopathological aspects, lesions with different sizes (between 2 and 15 cm) and shapes were recorded. The general aspect was characterized by a cauliflower appearance, hyperkeratotic lesions and grey-white colour. The histopathological examination represents a method of first choice, due to the specific lesions induced by the virus; being easily differentiate from other epithelial tumours with a different etiology. From the histological point of view, the analysed tumous were characterized by epidermic hyperplasia and acanthosis, parakeratosis and othokeratotic hyperkeratosis. The presence of koilocytes in spinous and granular layer indicates a papillomaviral infection.

❖ Chapter VII entitled „**Researches regarding the identification and characterization of bovine papillomaviruses in bovine cutaneous papillomatosis**" shows the importance of the molecular biology techniques in identifing and characterization of vital types involved in the etiology of these cutaneous tumours. We tested 24 samples (20 fibropapillomas and 4 skin samples), L1-BPV-1 was detected in 18 tumour samples (90%) and in 3 out of 4 skin, while for

BPV-2 detection two sets of primers were used. The first set specific for E5 gene, amplified the gene in all tumour samples (100%) and in 3 out of 4 skin samples, while the second set amplifing the E2-BPV-2 gene showed 10 positive tumour samples (50%) and 2 out of 4 positive skin samples. 21 out of a total 24 samples (87.5%) were BPV-1 positive, while 23 (95.8%) samples were BPV-2 positive. The co-infection was recorded in 21 cases (87.5%). The primers amplifing the E5-BPV-2 gene showed to be more sensible in detection the BPV-2 positive samples. The positivity of the skin samples collected from healthy bovines might be explained by the existance of previous infection during animal life and passed in a latent from.

Another technique useful in bovine papillomavirus infection diagnosis is represented by indirect immunofluorescence. A number of 11 fibropapilloma samples and 4 normal skin samples were tested in order to detect the presence of the E5 protein, the major oncoprotein of BPV. The E5 protein was detected in all fibropapilloma samples, but not in the normal skin samples. A high E5 expression level was recorded in basal and granular layers, with a sporadic expression in the spinous layer. Within the neoplastic cells E5 was mostly recorded intracytoplasmically, often localized in a very characteristic juxtanuclear region, and/or membraneous staining pattern was also recorded. Thus, the E5 oncoprotein may be used as a marker for a BPV productive infection in cutaneous fibropapillomas, indicating the causal role of the virus.

❖ Chapter VIII was entitled „**Evaluation of proliferation indeces in bovine cutaneous fibropapillomas"**. The purpose of this experiment was the assessment of proliferation i ndex (PI) in BPV positive cutaneuos fibropapillomas, since the papillomaviruses are inducing an uncontrolled cell proliferation. After the immunohistochemical evaluation of PCNA marker, the PI for each samples was determined. A PI maximum of 98.3% and a PI minimum of 77.66% were recorded. Some of the samples were characterized by an intense proliferation rate in the basal, spinous and granular cell layers, while the normal skin was characterized by a cell proliferation limited to the basal cell layer.

❖ Chapter IX, entitled „**Investigations regarding the expression of molecules involved in the tumorigenic mechanisms in cutaneous bovine fibropapillomas"** shows the results of molecular analysis (immunohistochemical and biochemical) of the PDGFβ receptor and its phosphorylation status and other downstream molecules presented in BPV naturally induced cutaneous fibropapillomas. In order to investigate whether the same neoplastic cells from fibropapillomas are expressing both E5 and PDGFβ-R, an immunohistochemical analysis on serial sections was performed. Most of the neoplastic cells of all the analyzed fibropapillomas showed cytoplasmic co-expression of the PDGFβ-R and E5, suggesting that these two proteins may be physically bound in this kind of tumour. Analysing the status of the total and phosphorylated receptor, the mean densitometric values and standard deviations of PDGFβ-R in

bovine cutaneous fibropapillomas vs normal skin showed no significant difference between samples, while the PDGFβ-R was phosphorylated in higher amount in tumour samples and a significant difference between samples was recorded. Mean densitometric values of pPDGFβ-R downstream molecules pAkt and pErk, showed no differences between the phosphorylation status of Erk in tumours compared to normal tissue; while three out of five fibropapillomas (F2, F3, F4) showed clear pAkt overexpression, although no statistically significant difference between samples was recorded. It may be speculated that the dysregulated activity of these two pPDGFβ-R downstream molecules might be a consequence of PDGFβ-R activation by E5 oncoprotein in cells in which these two proteins are co-expressed.

❖ Chapter X entitled „**Expression of apoptosis mediating proteins bcl-2 and p53 in cutaneous bovine fibropapillomas**" presents the results obtained during the immunohistochemical and biochemical analysis of anti-apoptotic bcl-2 protein and tumour supressor p53 in BPV1-1/-2 positive bovine cutaneous fibropapillomas. In order to verify the specificity of primary antibodies in reconizing the bcl-2 and p53 antigens, proteins extracts from tumour and skin samples were analysed biochemically. By Western blot analysis the specificity of antibodies was confirmed, since bands with expected molecular weight (bcl-2=25 kDa and p53=53 kDa) were recorded both in protein lysates and positive controls HEK (Human Embrionic Kidney) and HeLa cell lines. Further, the expresion of these two proteins was assessed by immunohistochemistry. Nine out of twelve fibropapillomas samples (75%) stained positive for bcl-2 throughout basal and parabasal layers, with most of cells showing strong cytoplasmic and membrane immunoreactivity. The fibroblasts from the dermal component showed no reactivity, while the normal skin samples showed a weak bcl-2 cytoplasmic staining, which was confined to the cells of basal layer. Futher, to check whether the expression of bcl-2 influences the rate of cell survival, it was determined the apoptotic index (AI) comparing bcl-2 positive versus bcl-2 negative samples. An AI of 43% (±5.2) was observed in bcl-2 negative fibropapillomas, whereas in bcl-2 positive samples showed an AI of 37% (±2.57). Regarding the p53 expression, in normal skin samples, the expression of p53 was weak and limited to the cytoplasm of the cells of basal layer, while in nine (75%) of the 12 bovine fibropapillomas were found to be positive for p53 expression. In most samples, a strong expression of p53 protein was detected in the basal and parabasal layers, where cells with a cytoplasmic and perinuclear staining were recorded. In the spinous and granular layers few cells with an intense perinuclear staining were observed. The presence of these two proteins in the bovine cutaneous fibropapillomas may indicate that bcl-2 expression may promote the survival of the neoplastic cells possibly depending on a p53 impaired expression.

❖ Chapter XI, entitled "**Expression of cyclooxygenase-2 enzyme in naturally occurring bovine cutaneous fibropapillomas**" shows the expression of cyclooxygenase-2 enzyme, which is known to be implicated in different physiological processes through the catalization and conversion of arachidonic acid into bioactive lipids, while its expression was associated with pathological conditions, inflammatory reactions and cancer development, among others. By immunohistochemistry, COX-2 immunoreactivity was not detected in normal skin samples. Eight of twelve tumour samples (67%) were COX-2 positive and the immunosignal was recorded in the cytoplasm of the basal cells layer, with increased intensity in perinuclear area. The ovexpression of COX-2 in cutaneous fibropapillomas may suggest a contribution of this enzyme in epithelial tumour development.

❖ Chapter XII, entitled „**General considerations on prophylaxies and control in cutaneous infections with bovine papillomaviruses**" briefly describes the necessary prophylaxies measures that are required for the maintenance of disease-free status, and additionally the efficient control measures that must be taken where the disease is present The importance of the surgery (as a correct treatment) should not be omitted, since it has a major contribution in removing the main sources of infection. The best prophylaxys in my opinion is to keep animals in a good and healty status.

❖ Chapter XIII briefly presents a number of 17 final conclusion, and based on the results obtained during the experimental work, a strategy of biology molecular diagnosis techniques was recommended.