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CONTENTS

COLLECTION OF BLASTOMERES IN ORDER TO ESTABLISH SEX AND ISOLATE GENETIC MATERIAL-REVIEW

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Abstract

Globally, limited access to food needs in relation to meat or milk production has required the establishment of the sex of offspring from the embryonic stage. While the meat industry uses males, the dairy industry relies on females. During the period of exploitation, the number of products obtained from a female bovine is 5-6 individuals, their sex being able to be influenced by means of sexed semen. Embryo sexing programs can result in a large number of conception products, in a shorter period of time taking into account the desired sex. The use of the desired sex embryo facilitates the improvement of the genetic value. Embryonic sexing procedures involve the collection by biopsy of a minimum amount of genetic material that can ensure the determination of sex. Both invasive and non-invasive biopsy and sexing procedures can influence the subsequent viability of embryos prepared for embryo transfer. This paper highlights the methods of embryonic sexing along with the advantages and disadvantages of each technique involved in determining sex.

Key words: Embryo, biopsy, sex determination.

INTRODUCTION

Embryonic sexing has significant economic implications in the dairy and beef industries because it can meet the requirements of the producer by determining the sex of the future conception product. Thus, the use of the embryonic sexing technique can bring a greater economic potential for dairy and beef cattle farmers (Yasuhiro O. *et al*, 2015). Obtaining products by embryonic sexing, which will cover the requirements of cattle breeders is very topical (is of great relevance), by determining the sex during the formation of the (conception) product suggesting the economic importance of this technique in involving the growth of animal production. Embryonic sex is also involved in the diagnosis of genetic disorders in the prenatal stage. Embryo sexing increases the efficiency of embryo transfer, facilitates their transfer at choice, based on the desired sex (Bredbacka P., 2001;Cenariu M. *et al*, 2008). The use of sex-sorted semen is an important technique for obtaining the desired sex by artificial insemination or *in vitro* fertilization (IVF), but it is very expensive (Seidel G.E., 2007) and less effective compared to conventional, unsorted semen (Trigal B. *et al*, 2012). The use of the technique to examine the chromosome in bovine embryo cells has led to the sexing of embryos, a new approach to sex selection. The sexing of the embryos performed before the embryonic transfer, will lead to an improvement of the genetic material by determining the sex of the transferred product, thus it will generate a rapid increase of the genetic value from the respective farm (Sachan V. *et al*, 2020).

Blastomere harvesting technique

Penetration of the zona pellucida (ZP) can be done either:

- mechanical,
- chemical,
- using a laser device.

Mechanical penetration of ZP (also called partial area dissection) was the first method used to form a gap in the ZP membrane and is still applied clinically, although to a lesser extent. The method involves creating a slot in the ZP using a sharp micropipette (Kokkali G. *et al*, 2020).

Chemical perforation of the zona pellucida involves the use of an acid solution (Tyrode's acid) for its local dissolution. This method has been widely used since the first embryonic biopsies; it is used during embryonic segmentation. However, the subsequent implementation of laser technology and the disadvantages caused by the toxicity of acid substances (Tyrode's acid) on the viability of embryos led most laboratories to abandon the chemical method of perforating ZP (Kokkali G. *et al*, 2020).

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Laser is currently the most popular ZP penetration method for polar body (PB), cleavage stage and blastocyst biopsy. The method involves the use of a non-contact guided laser beam, which can be adjusted to create a ZP aperture of the desired size in a precise and fast way. Laser beam power and exposure (pulse length / width) should be carefully addressed according to the manufacturer's specifications to avoid damage to PBs or embryonic cells (Kokkali G. *et al*, 2020).

Methods for harvesting blastomeres

The method by cutting the blastocyst with the help of the microblade is applied at the stage of 7 days from artificial insemination or cultivation. After placing the blastocyst in a biopsy medium with the addition of mineral oil, the Petri dish is inserted under a microscope equipped with a micromanipulator and microblade. About a third of the blastocyst (trophoblastic cells) is removed by cutting.Embryos that have been biopsied are transferred to another environment to be washed. After washing, the embryos were cultured in a special culture medium for 2-3 hours at 38.5 ° C, in a humid atmosphere of 5% CO_2 in the air (Vieira de Sousa R., *et al* 2017**).**

In the method of pipetting or aspirating blastomeres, embryos in the morula stage are exposed for one minute in a solution (pronase) to dissolve the zona pellucida. Embryos without zona pellucida will be transferred to an environment to stop the action of the enzyme. Aspiration of blastomeres is done by light aspiration using a pipette approximately 110 μm in diameter. When the amount of blastomeres required for evaluation has been reached, the embryos will be placed in a culture medium for 48 hours at 38.5 ° C in a humid atmosphere of 5% CO2 in the air.During embryonic biopsies, the amount of DNA extracted must ensure that the sexing procedure is performed.Regarding the biopsy procedure, the main target is the care with which the embryo must be handled in order to not damage the structure and also its viability (Vieira de Sousa R., *et al* 2017**).**

Several procedures for sexing embryos in farm animals by invasive or non-invasive methods have been evaluated. These methods may or may not require embryonic biopsy (Garcia J.F., 2001). Non-invasive methods are considered less harmful to the embryo because the integrity of the embryo is not damaged and the embryos remain intact and viable (Utsumi K.,1993).

A. Non-invasive Methods

Sexation based on cleavage and development

During embryonic sexing by the PCR method and by the karyotyping method, Yindee Kitiyanant *et al*. (2000) observed that male embryos showed a faster cleavage rate than female embryos. The faster growth in male embryos may well be a consequence of the faster gene expression caused by Y-chromosomal genes. A possible effect of Y-linked genes promoting the rapid growth of male embryos has been suggested to be caused by H-Y antigen or Y-chromosome growth factors. Total glucose metabolism was found significantly greater in male than in female bovine blastocysts which might be related to more rapid development of male embryos (Yindee K. *et al*, 2000). This method of embryo sexing has still many limitations,In-vivo produced embryos cleavage time cannot be known, besides the difference in developmental rate is very small and needs high skills in separation of fast and slow embryos (Sharma M. *et al*, 2017).

Detection of X – linked enzymes

Embryos can be differentiated as male or female by measuring the dose of the X-linked enzymes gene. Glucose-6-phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyl transferase (HPRT) are involved in determining embryonic sex based on their concentrations (Kouamo J. *et al*, 2014). In normal mammals, homogametic sex (female) carries two X chromosomes (XX) whereas heterogametic sex (male) possesses only one X chromosome (XY). In female, one of the X chromosomes is in activated in each cell in embryonic life to maintain an equivalent number of genes between sexes **(**Lakshmy K. V. *et al*, 2018). Gutierrez –Adan et al. demonstrated that G6PD and HPRT are almost twice as high in female embryos as in male embryos in the early blastocyst stage (Gutierrez-Adam A. *et al*, 2000). Peippo *et al*. (2002) observed that the level of G6PD was significantly higher in female bovine embryos produced in vitro than in male ones in both the morula and blastocyst stages. The HPRT level was much higher in female embryos than in males, evaluated in the morula and blastocyst (Peippo J. *et al*, 2002). To maintain an equivalent number of genes between the sexes, one of the X-chromosomes in the female is inactivated in each cell early in the embryonic life. Although the exact timing of X-inactivation is not known, studies have suggested that there is a brief period between activation of the embryonic genome and X-inactivation in which genes from both X chromosomes in the female are transcribed **(**Sharma M. *et al*, 2017).

Detection of H-Y antigens

On the cell surface in males embryos is the surface antigen called Histocompatibility Y or HY antigen, this antigen is not present in female

embryos. The HY antigen can be used in embryonic sexing (Sachan V. *et al*, 2020). There are two tests that can detect HY antigen on the surface of embryos, namely: an immunoflorescence test and a cytotoxicity test (Sharma M. *et al*, 2017). In the fluorescent control (test) , embryos to primary H-Y antibody are incubated in the absence of complement for 30 minutes and then for another 30 minutes with secondary antibodies that are labeled with fluorescein isothiocyanate (FITC) (Sharma M. *et al*, 2017). By microscopic evaluation, male embryos show fluorescence (HY positive) and female embryos do not show fluorescence (HY negative) (Lakshmy K. V. *et al* 2018). Through the cytotoxicity test, the embryos are incubated in the presence of complement with polyclonal antiserum that will have an immunological action of cell lysis on embryos that present the H-Y antigen. Through this cytotoxicity test the male embryos will be neutralized and the female embryos will survive (Lakshmy K. V. *et al* 2018).

B.Invasive Methods

The Barr body

Based on the Lyon (1961) hypothesis, all but one X chromosome is randomly inactivated early in embryogenesis, after implantation. The end result is that, when evaluated, female cells have one Barr body, while the male cell has none. In 1948 Barr et al. proved, first in cats and then in humans, that female and not male cells consist of deeply stained body in the nucleus. The origin of the Barr body was established by Lyon (1961), who claimed that the Barr body is a heteropyknotic material (pyknosis-irreversible condensation of chromatin in the nucleus) originating from the X chromosome that is randomly inactivated and can be from either maternal or paternal origin (*O*rnfooy A. *et al*, 2020). The first blastocysts to be evaluated for the presence or absence of Barr's body were rabbits (Bondioli, K. R., 1992). Due to the granular nature of the cytoplasm, observation of the Barr body can be difficult, so some female embryos can be confirmed as male embryos (King W. A., 1984;Shetty, N. K. *et al*, 2018). The presence and detection of the Barr body depends not only on the stage of the cell, but also on the fixation procedure, so the improper stage of the cell or the improper fixation and staining procedure can give a false diagnosis for embryonic sexing. Another limitation of this technique is that due to the need for a large number of cells, embryo damage may be visible (Wakchaure R. *et al*, 2015).

Cytological methods or karyotyping

Cytogenetic sexing or karyotyping of bovine embryos from day 6 or 7 is done by analyzing chromosomes blocked in metaphase (King W. A., 1984). The technique consists in the biopsy collection of embryonic trophoblastic cells and their cultivation with mitosis-arresting agent such as (e.g. colchicines) that stop the division of cells in the metaphase stage of mitosis (Seidel G.E. *et al* 1991). These substances cause the depolymerization of microtubes and prevent the formation of the spindle through their antimitotic effect (Sharma M. et al, 2017). The cells are subjected to a hypotonic solution to osmotically lysis them so that the chromosomes can be dispersed. Giemsa solution can be used for DNA staining so that the metaphase chromosomes can be analyzed microscopically (Sharma M. *et al* 2017). As an interpretation of the results, the presence of two X chromosomes indicates the female embryo and the presence of the Y chromosome the male embryo (Lakshmy K. V. *et al*, 2018). The main advantage of the method is the accuracy of this technique. Another advantage would be the detection of chromosomal abnormalities. This method is affordable requiring a microscope and reagents that are cheap and easy to access (Yindee K. *et al*, 2000). As disadvantages, the embryo must undergo the biopsy stage and the preparation sometimes requires even 12 hours or more, so most laboratories have turned to other embryonic sex techniques (Seidel G.E. *et al* 1991).

Polymerase chain reaction (PCR) method

The polymerase chain reaction (PCR) was developed by Kary Mullis in the 1980s (Schluger N. *et al*, 1995). PCR also took shape in the embryonic sexing technique, giving the possibility to identify the embryonic sex (Mara L. *et al*, 2004). The method of sexing the bovine embryo by amplifying specific DNA sequences of the Y chromosome using PCR proves to be an effective tool for sex identification. Embryonic sexing using PCR includes embryo biopsy (1-4 blastomeres), amplification of DNA fragments and interpretation after analysis of products amplified by electrophoresis technique (Sachan V. *et al*, 2020). If only one band of the bovine-specific product is visible, on the gel, the blastomere is considered to derive from a female embryo, whereas the presence of two bands referred to a male embryo (Sharma M. *et al*, 2017). Regardless of whether the use of PCR requires technical skills for embryonic sexing, this method is almost 100% effective (Kouamo J. *et al* 2014), sensitive and fast (Gokulakrishnan P. *et al***,** 2013; Wakchaure R. *et al*, 2015) and can be achieved in several hours (Bredbacka P., 2001). With this technique, a good percentage of embryos can be sexed obtained without disturbing their capacity for development (Peura T. *et al*,1991). PCR can give false results

due to the collection of a limited amount of DNA from embryo biopsies, cross-species DNA contamination, DNA contamination during handling of DNA products in PCR procedures and electrophoresis (Geshi M., 2012). It has been reported that the accuracy of the PCR method for sexing bovine embryos gives better results (96.4%) compared to FISH (86.66%) (Cenariu M. *et al*, 2011).

Embryo sexing by fluorescence *in situ* hybridization (FISH)

The in situ fluorescence hybridization (FISH) technique can detect specific DNA sequences of individual chromosomes in a cell (Kobayashi J. *et al*, 2004). The efficiency of the technique is high and the sex of an embryo can be unequivocally determined when two X-chromosome signals in the absence of a Y-chromosome signal or one X-chromosome and one Y-chromosome signal are detected (Staessen C., 1999). By using Y chromosome-specific DNA fragments in in situ fluorescent hybridization (FISH), male and female embryos can be differentiated (Cotinot C., 1991). The evaluation of the embryonic sex by the FISH method can be performed in the morula or blastocyst stage, the collection of a small number of embryonic cells will reduce the damage to the subsequent viability of the embryo. The pregnancy rate following the transfer of biopsied embryos was 51.3% and the accuracy of the FISH sexing technique is 92% (Cenariu M. *et al*,2008). FISH is, however, expensive and has a more complex degree of operation (Xie Y., 2020).

Loop-mediated isothermal amplification (LAMP)

Sexing of embryos based on specific sequences on the Y chromosome was achieved by PCR amplification from a small number of blastomeres (Alves B.C., 2006; Garcia J.F., 2001). Hirayama *et al.* (2004) reported loop-mediated isothermal amplification (LAMP), a simpler method of sexing bovine embryos compared to PCR. LAMP is a new method of amplifying DNA that can amplify a specific DNA sequence in a temperature range of 60 to 65 ° C (Lakshmy K. V. *et al*, 2018). DNA amplification is performed under isothermal conditions using a DNA polymerase and four sets of specific DNA primers (inner and outer) that recognize a total of six distinct sequences on the target DNA (Hirayama H. *et al*, 2013). Moreover, an additional set of primer (called a loop primer) is used to accelerate the LAMP reaction. The inner primer initiates the primary DNA synthesis, and the next DNA synthesis by an outer primer releasing a single-stranded DNA derived from the inner primer (Hirayama H. *et al*, 2013). Amplification of the target DNA is estimated by measuring the turbidity due to a white precipitate of magnesium pyrophosphate, a by-product of DNA synthesis (Geshi M., 2012). This method of DNA amplification is highly specific, efficient, and rapid. Besides, gene amplification and detection can be completed in one step, and amplification could be up to 10^9-10^{10} times in 15–60 min. Since the detection of all target gene sequences can only be determined by either the presence or absence of amplification products, LAMP is considered to be highly specific (Sharma M. *et al* 2017).

CONCLUSIONS

Embryonic biopsy and embryonic sexing have improved embryos production based on the wishes of cattle breeders. The embryonic biopsy method will be chosen depending on the laboratory equipment and equipment costs, but satisfactory results can be obtained using the cutting method.Sexual methods classified as invasive and non-invasive are used in embryonic sexing. The use of invasive methods can affect the subsequent viability of embryos, which is essential in embryonic transfer, gestation and procreation (parturition). Non-invasive methods have a reduced effect on embryonic viability, they are less used. Of the invasive methods of embryonic sexing, the most used would be the PCR method with a sexing rate close to 100%, being simple and fast. The use of embryonic biopsy and embryonic sexing ensures the growth of meat production in the case of male embryos and milk production in the case of female embryos. In the future, some techniques could be improved to increase the efficiency and subsequent viability of embryos.

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PHYTOCOMPLEX WITH *ZINGIBER OFFICINALE* EXTRACT, *PIPER NIGRUM* AND *PIPER CUBEBA* OIL - *IN VITRO* ANTIMICROBIAL EFFECT

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Abstract

Vegetal compounds are known for their therapeutic actions in correlation with their antioxidant activity so that in recent times the interest in their properties has greatly increased.
The phytocomplex obtained by combining the *Zingiber officinale* extract, *Piper nigrum* and *Piper cubeba* oil is distributed and recommended in European space as a multi-benefit nutritional supplement for swine, poultry, cattle, horses and others. As the individual properties of the three compounds are known, we aimed to test the antimicrobial activity of the phytocomplex on various Gram negative pathogens.
In the time-kill assay, *in vitro* inhibitory effects were visible after 15 minutes of contact and total inhibition of the species *Samonella enteritidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* was obtained after 24 hours.
The results obtained *in vitro* showed a very good antimicrobial activity which clearly contributes to the overall beneficial effects of the *Zingiber officinale* extract, *Piper nigrum* and *Piper cubeba* oil phytocomplex.

Key words: *bacteria, phytocomplex, antimicrobial activity*

Introduction. Herbal extracts and their derivatives have received considerable attention as therapeutic agents for the prevention and treatment of health problems. Plants have always been used for human and animal health due to bioactive plant compounds (Viegi L. et al., 2003). Plants produce secondary metabolites with a role in their own metabolism but also for protection against a multitude of external aggressors. These metabolites are classified into four broad groups: terpenoids, phenolics, nitrogen-containing compounds, and sulfur-containing compounds (Susan G et al., 2007; Mazid M. et al., 2011).

In veterinary medicine, extracts and essential oils obtained from plants are known for their ability to fight certain diseases, regardless of the geographical area of the world (McCorkle, CM, 1992, McCorkle CM et al., 1998; Lev E.,2003). In the tradition medicine, herbal remedies have been strongly anchored in maintaining health being the only option of cheap and easy to administer therapy (Dilshad S.M.R. et al., 2010; McCorkle C.M., 1992). The phenomenon is so widespread that this type of medicine has been called ethnoveterinary medicine (Aziz M.A.et al., 2018) and is an alternative to allopathic medicine. (McCorkle C.M, 1986. et al., 2019). Also, the development of antimicrobial resistance to currently available semi-synthetic antibiotics is another reason why new antimicrobial formulas are sought in nature, given that approximately 25% of current drugs are also extracted from plants (Haghiroalsadat F. et al., 2011).

Medicinal plants and how they are used vary from one geographical region to another, so the reports are sometimes made from different perspectives. Studies conducted in countries in Asia, India, Pakistan, South America predominate and such scientific studies are insufficiently reported in European or North American countries (Aziz MA. et al., 2018).

The benefits of administering plant extracts or oils to animals administered as food supplements or for therapeutic purposes have been described in various studies conducted on various animal species (Dewick P.M, 2001). In pigs, there was an increase in the weight of the carcass muscle mass and improved the compositional quality of

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the meat (Szewczyk A. et al.,2006; Hanczakowska E. et al., 2007; Oyebode O. et al.,2004; Hanczakowska E. et al., 2012). Also, in cattle, phytotherapy is successfully applied in intestinal motility disorders, to inhibit pathogenic microorganisms in the rumen that can influence the rate of nutrient assimilation and thus increase production or to prevent and treat mastitis (Wynn S.G. et al., 2007; Neculai - Valeanu A.S. et al., 2021).

In birds, plant extracts have been used prophylactically as an alternative to growth promoters (antibiotics) and immunostimulants. (Hughes P. et al., 2002; Hashemi S.R et al., 2013). A statistic conducted by Tamminen LM et al., In 2018, regarding the interest of researchers in phytotherapy in veterinary medicine at European level, shows that most studies (89%) focused on the preventive effect of phytotherapeutic products administered to poultry, pigs and sheep and only 11% investigated the curative effect of phytocompounds in the treatment of specific diseases of these species, most of them infectious diseases.

Therefore, the antimicrobial activity of plant extracts and essential oils has long been known. Although most promising studies are based on *in vitro* testing, their biological potential is demonstrated, and obviously their antimicrobial efficacy has been studied and highlighted, comparable to that of antibiotics. Therefore, they could be used as an alternative to antibiotic treatment, although the scientific community remains reserved when it comes to recommending them in therapy (EMA, EFSA, 2017).

However, in the EU, medicinal plants are increasingly used in veterinary medicine as feed additives and the effects on overall health and productive performance are much more visible to farmers, which will increasingly encourage their use. (Tamminen L.M. et al., 2018).

The phytocompound based on *Zingiber officinale* extract, *Piper nigrum* and *Piper cubeba* oil is used in Europe as a flavoring or food additive administered in farm animal feed.Ginger (*Zingiber officinale*) is one of the most consumed spices in the world and also known for its medicinal properties, having anti-inflammatory, antitumor, antipyretic, antiplatelet, anti-hyperglycemic, antioxidant, antidiabetic, anticoagulant, cardiotonic, cytotoxic, etc. (Wang W.H. et al., 2005; Shahrajabian M. et al., 2019). Scientific studies on ginger extract and its various components demonstrate the medicinal, chemical and pharmacological potential of this plant (Benzie IFF et al., 2011). Ginger obviously has a multitude of other metabolites that have not yet been studied and for which specific molecular targets and mechanisms of action are not known (Wachtel-Galor S. et al., 2011). *Piper nigrum* (black pepper) and *Piper cubeba* (tailed pepper) are two of the more than 700 species of pepper in the *Piperaceae* family, plants native to South Asia but cultivated in many other regions of the world, where there is a warm climate and special lightning (Prasad Ashok K. et al., 2005). In the history of peoples, these plants have been used as traditional medicines and food flavorings. Their complex composition of proteins, fats, carbohydrates, fiber, alkaloids, essential oils, resins, vitamins, etc., has brought many benefits to human health and especially for the treatment of digestive disorders including intestinal parasitosis (Kumar S. et al., 2015,Mansurah A, 2016). Studies have shown their antimicrobial and antioxidant potential, Piper nigrum being recognized for its effectiveness in skin diseases, respiratory diseases, anemia, diabetes, etc. (Sharma MC et al., 2004; Mihăilă B. et al., 2019; Kumar S. et al., 2015) and the species Piper cubeba is recommended in venereal diseases, digestive diseases, asthma (Elfahmi KR et al., 2013). *Piper cubeba* oil is frequently used in phytotherapy, being rich in valuable bioactive compounds such as antioxidants, anti-parasites and insecticides (Yusuf A. et al., 2019).

The aim of these tests was to evaluate the antimicrobial potential of this EU-approved phytocomplex as a flavoring, given the biological properties of the three plant compounds and the therapeutic benefits observed on farms after administration of the plant suspension.

MATERIALS AND METHODS

The tested phytocomplex is marketed in Romania under the name of Respowell, and is a suspension obtained by combining *Zingiber officinale e*xtract, *Piper nigrum* essential oil and *Piper cubeba* oil. The determination of antimicrobial potential was performed by three *in vitro* techniques against four standardized Gram-negative bacterial strains: *Escherichia c*oli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076, *Klebsiella pneumoniae* ATCC 13883.

Qualitative determination of antimicrobial efficacy was performed by diffusimetric method (EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing - Version 5.0, January, 2019). For this purpose, Muller Hinton solid medium Petri dishes were seeded with bacterial suspensions whose cell density corresponded to the 0.5 McFarland Stardard scale. After dispersing the suspension, filter paper washes (5 mm) were spread on the surface of the

medium, over which 10 ul of Respowell phytocomplex was spotted. A gentamicin microcomprimate (10 ug) was used as an active control. The interpretation of the antimicrobial effect consisted in measuring the diameter of the microbial inhibition and comparing it with the inhibition zone created by the antibiotic.

The determination of the minimum inhibitory concentration (MIC) was performed by the technique of serial microdilutions in 96-well plates (CLSI, 2012). From the stock solution of the phytocomplex *Zingiber officinale* extract, *Piper nigrum* essential oil and *Piper cubeba* oil (1000 mg s.a / lt stock solution) was distributed 100 ul (100 mg s. A.) In the first column (A1-A4). In the other 11 wells (A2-A11; B2-B11, C2-C11, D2-D11) 50 ul of MH broth were distributed. Serial dilutions were performed by transferring from the first well (100 mg.s.a.) 50 ul to the second well, repeating the procedure to well 11. For each dilution, the approximate concentration (mg/ul) was: dil.1/2 (0.0005 mg/µl), dil.1/4 (0.00025 mg/µl), dil.1/8 (0.000125mg/µl), dil.1/16 (6.25E-05 mg/µl), dil. 1/32 (3.13E-05 mg/µl), dil. 1/64 (1.56E-05mg / µl), dil.1/128 (7.81E-06 mg/µl), dil.1/256 (3.91E-06 mg/µl), dil.1/512 (1.95E-06 mg/µl), dil.1/1024 (9.77E-07 mg/µl). The growth control (positive control) was distributed in the last column of wells. From the 0.5 McFarland microbial suspensions (1.8 x 10^8 cfu / ml), 100 ul of inoculum was taken and transferred to each well. The final volume was 200 ul. After distribution, the plates were stirred (State Fax 2200 Awareness). After incubation at 37°C/24h the plates were read by spectrophotometry at (450nm) using Microplate Reader Stat Fax. The MIC of the plant substance was defined as the lowest concentration of the plant substance that inhibited the growth of 80% of the bacterial culture compared to the bacterial growth of the positive control.

The time-kill method is used to test the bactericidal activity of one or more antimicrobial agents against a particular bacterial strain. This is done by counting the viability of bacterial strains at different time intervals. To obtain the time-kill curve the bacterial strains growth rate must be counted at different time intervals starting from 0 hours to 24 hours (CLSI, 1998; ASTM E2315-16, 2016). The bactericidal potential is highlighted when there is a decrease of 3log10 of cfu/ml which is equivalent to the destruction of 99.9% of the bacteria in the inoculum. The antimicrobial activity of the phytocomplex was performed at 15 min, 30 min, 60 min and 24 hours. Then, the percentage of dead cells was calculated by determining the number of living cells (cfu/ml) in each tube and reporting to the positive control (2 x 10 cfu/ml), using the counting method in solid culture plates. For this, the tested bacterial cultures were incubated at 37° C for 24 hours. This testing follows the guidelines set by the Clinical & Laboratory Standards Institute (CLSI, 1999).

RESULTS AND DISCUSSIONS

*Results obtained by diffusimetric method***:**Following the qualitative testing of the antimicrobial potential, modest inhibitory activities were observed compared to the antimicrobial activity of the antibiotic (gentamicin,10µg) (*figure 1*).

Figure 1. Testing the phytocomplex by diffusimetric method against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076, *Klebsiella pneumoniae* ATCC ATCC 13883. Muller Hinton agar medium, Gentamicin (Gn / 10 µg)

The best area of microbial inhibition (13 mm) was identified in *Escherchia coli* ATCC 25922, diameter smaller than that obtained in the positive control (20 mm). The areas of inhibition created by the phytocomplex against *Klebsiella pneumoniae* ATCC 13883 (11mm) and *Salmonella enterica* ATCC 13076 (10 mm) were much more modest compared to the inhibitory effects of *Escherichia coli* ATCC 25922 and the positive control. By diffusion method, *Pseudomonas aeruginosa* ATCC 27853, did not show sensitivity to phytocomplex.

The determination of the minimum inhibitory concentration is a quantitative method

and was performed in order to identify the best concentration of plant suspension that can inhibit the selected bacterial strains. The MIC of the plant substance was defined as the lowest concentration of the plant substance that inhibited bacterial growth (*figure. 2*). The results obtained by testing the minimum inhibitory concentration (MIC) are presented in *figure* 3

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Figure 2 Microdilution broth plate 96 well for phytocomplex *Zingiber officinale extract/ Piper nigrum /Piper cubeba* ,

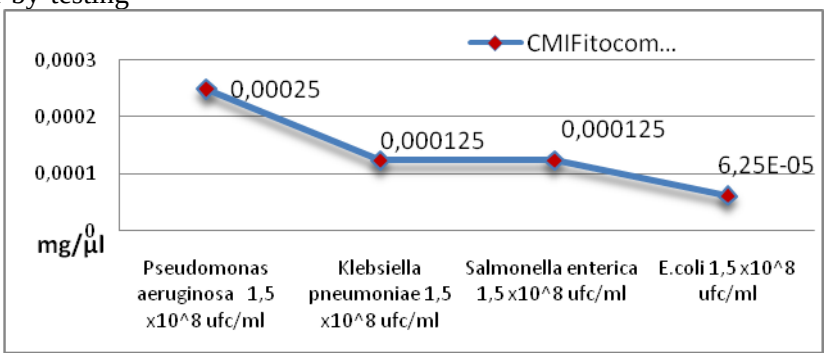

Figure 3 Determination of the minimum inhibitory concentration (MIC) for phytocomplex *Zingiber officinale extract/ Piper nigrum /Piper cubeba* (mg/ul) against the bacterial strains tested.

The results of the determinations performed showed that the phytocomplex tested had antimicrobial activity against all strains tested. As the concentration of active substances in the stock solution was very low, the calculation of the minimum inhibitory concentrations was performed keeping the ratio, so that the phytocomplex MIC for *Escherichia coli* ATCC 25922 was 6.25E-05 mg/µl, the phytocomplete MIC for *Klebsiella pneumoniae* ATCC 13883 was of 0.000125 mg/µl, the phytocomplete MIC for *Salmonella enterica* ATCC 13076 was 0.000125mg/µl, the phytocomplete MIC for *Pseudomonas aeruginosa* ATCC 27853 was 0.00025 mg/µl.

Time-killing test results. The results obtained at the time-killing test were in close correlation with the tested bacterial strain and the contact time (*figure 4, figure 5, figure 6, figure 7, figure 8, table 1*).

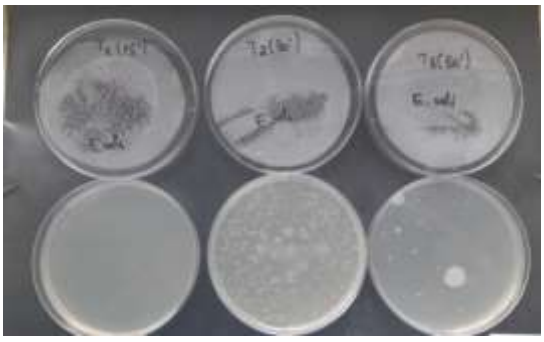

Figure 4. Time-kill assay to *Escherichia coli:* 15 min.(T1), 30min (T2), 60 min (T3)

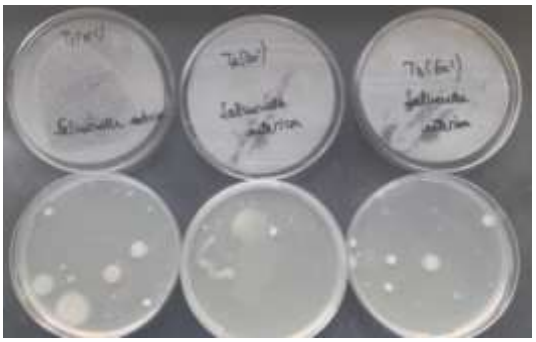

Figure 5. Time-kill assay to *Salmonella enterica*: 15 min.(T1), 30min (T2), 60 min (T3)

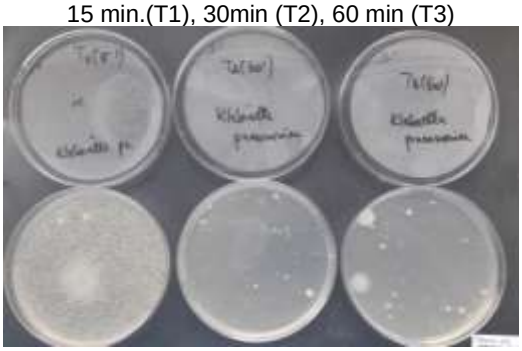

Figure 6. Time-kill assay to *Klebsiella pneumoniae*: 15 min.(T1), 30min (T2), 60 min (T3)

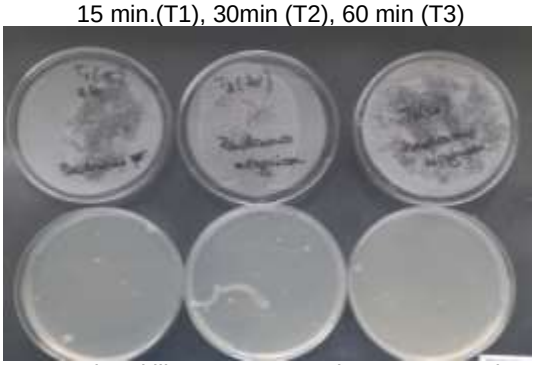

Figure 7. Time-kill assay to *Pseudomonas aeruginosa*: 15 min.(T1), 30min (T2), 60 min (T3)

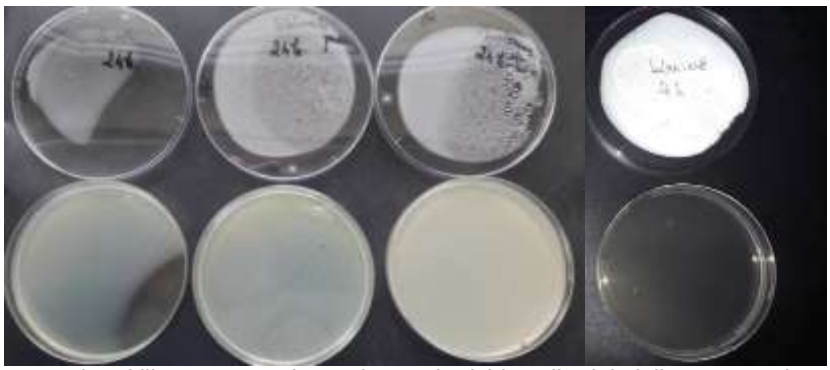

Figure 8. Time-kill assay to 24 hours for *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica*.

In the time-kill assay, the results presented in terms of the changes in the log10 cfu/mL of viable colonies indicated that the extract exhibited significant bactericidal activity.

Table 1

Time-kill test result

Bacteria species	Martor ufc/ml	Times contact with phytocomplex *Zingiber officinale extract/ Piper nigrum /Piper cubeba*			
		15 min ufc/ml	30 min ufc/ml	60 min ufc/ml	24 ore ufc/ml
Escherichia coli	1,8 x 10^8	3 x10^7	28 x10^4	3,48 x10^2	0
Log_{10} (ufc/ml)	8,26	7,48	4,45	2,58	0
LR (Log10 reduction)	8	0,7782	2,808	5.844	0
%Reduction		**83,333**	**99,844**	**100**	**0**
Klebsiella pneumoniae	1,8 x 10^8	42 x10^4	34x10^2	12 x10^1	0
Log_{10} (ufc/ml)	8,26	4,45	3,53	2,08	0
LR (Log10 reduction)	8	2,632	4,724	6,176	0
%Reduction		**99,767**	**99,998**	**100**	**0**
Salmonella enterica	1,8 x 10^8	8,2 x10^1	4,9 x10^1	4,7 x10^1	0
Log_{10} (ufc/ml)	8,26	1,91	1,69	1,67	0
LR (Log10 reduction)	8	6,974	6,564	6,583	0
%Reduction		**100**	**100**	**100**	**0**
Pseudomonas aeruginosa	1,8 x 10^8	9,2 x10^1	5 x10^1	2,9x10^1	0
Log_{10} (ufc/ml)	8,26	1,96	1,70	1,46	0
LR (Log10 reduction)	8	6,91	6,55	7,091	0
%Reduction		**100**	**100**	**100**	**0**

Logarithmic reduction (LR) is the method by which microbial reduction can be quantified and the microbicidal potential of a suspension can be determined.

The antimicrobial efficacy test by the time-kill method of the phytocomplex was performed on microbial suspensions whose microbial density was equivalent to 8.26Log10 (cfu/ml).

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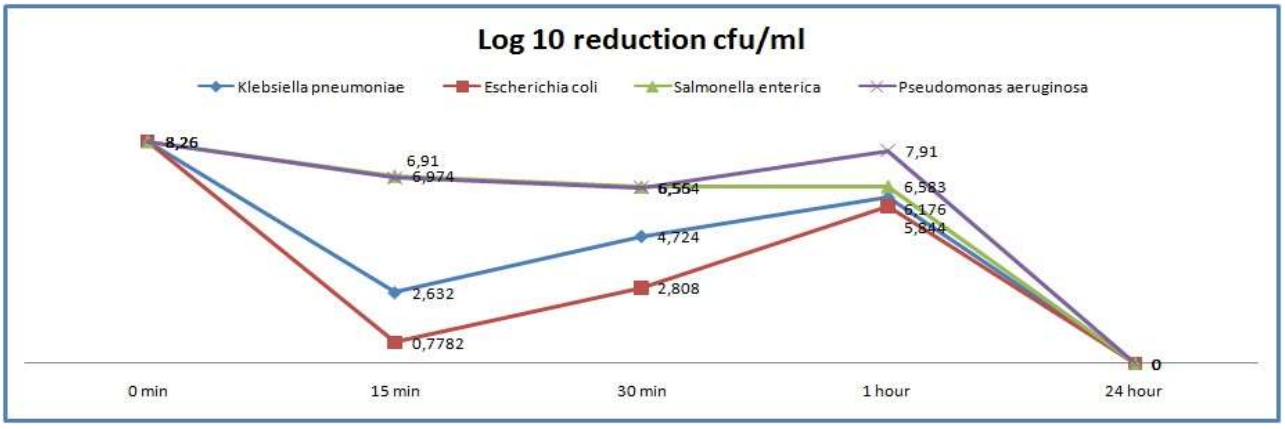

Figure 9. Microbial reduction as log reduction values (LR) when testing microbial suspensions with phytocomplex *Zingiber officinale extract/ Piper nigrum /Piper cubeba*

After 15 minutes of incubation, the best logarithmic reductions were 6.91Log10 cfu/ml in *Pseudomonas aeruginosa* and 6.97Log10 cfu/ml in *Salmonella enterica* for which a 100% logarithmic reduction was calculated. Compared to *Klebsiella pneumoniae*, a reduction of 2.632 Log10 cfu/ ml (99.767% reduction) was obtained and for *Escherichia coli* it was 0.778Log10 cfu/ml (83.33%) (*figure 9*.).

After 30 minutes of incubation, logarithmic reductions of 6.55Log10 cfu/ml (100%) were detected for *Pseudomonas aeruginosa*, 6.564Log10 cfu/ml (100%) for *Salmonella enterica*, 4.724Log10ufc/ml (99.99%) for *Klebsiella pneumoniae* and 2.808Log10 cfu/ml (99.84%) for *Escherichia coli*.

After 60 minutes of incubation, a logarithmic reduction of 5.844 Log10 cfu/ml (100%) for *Escherichia coli* and 6.176 Log10 cfu / ml for *Klebsiella pneumoniae* was identified. The other two bacterial species, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, logarithmically reduced the number of viable cells continued to increase, reaching 7.091 Log10 cfu/ml (100%) for *Pseudomonas aeruginosa* and 6.583 Log10 cfu/ml (100%) for *Klebsiella pneumoniae*.

After 24 hours of incubation, all bacterial species tested were inhibited by the phytocomplex *Zingiber officinale* extract/*Piper nigrum* essential oil/*Piper cubeba* oil. This may be an advantage, given the way this product is administered.

Various antimicrobial susceptibility tests are used in research on the antimicrobial activity of plant complexes to determine their effectiveness against various potentially pathogenic microorganisms. Plant extracts, essential oils or certain constituents extracted from plant complexes are evaluated and verified by different techniques so as to determine their therapeutic concentrations and their usefulness (Ncube NS et al., 2008)

These *in vitro* tests can also determine the sensitivity of pathogenic microorganisms that have developed resistance to semisynthetic antimicrobial agents (Wiegand I et al., 2008). Of all the techniques, dilution methods for determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (CMB) are the most widely used and can be easily determined with or without a spectrophotometer. To further facilitate the interpretation of results, colorimetric indicators such as resazurin, which is oxidized blue and turns pink when reduced by viable bacterial cells, can be added to dilution methods (Mann, C. et al., 1998).

Although the results of *in vitro* tests clearly show the antimicrobial effect of a plant product, additional studies are needed to validate their effectiveness in in vivo administration.

The use of medicinal plants in human and animal therapy has very old attestations in the history of medicine and their use is still current. Studies conducted until 2014 show that about 80% of the world's population uses plants and their extracts therapeutically. Subsequently, the incidence of studies conducted in six countries was reconsidered, also showing that their use is declining, including in the regions among the top users of natural therapies (Oyebode O et al., 2016). However, plants are an integral part of traditional medicine used as adjunctive therapy in modern medicine in countries such as India and China and are still the main source of therapy in certain diseases in humans and animals, especially when modern treatments are either unavailable or inefficient (Oyebode O et al., 2016).

Preliminary tests performed on the phytocomplex obtained by combining *Zingiber officinale* extract/*Piper nigrum* essential oil/*Piper cubeba* oil demonstrate the antimicrobial efficacy of this plant suspension, so we consider it appropriate to mention this inhibitory capacity against opportunistic Gram-negative bacteria that have the potential to be pathogens for animals. A limitation of our study is that the test was performed only on Gram-negative bacterial strains and no additional testing was possible to demonstrate the mechanism of action of the main compounds in the plant suspension.

CONCLUSIONS

The antimicrobial potential of the phytocomplex *Zingiber officinale* extract/Piper *nigrum* essential oil/*Piper cubeba* oil was demonstrated by all tests performed, compared to all Gram negative bacterial species tested.

Preliminary results of the tested Phytocomplex demonstrate its antimicrobial potential and a possible therapeutic alternative in veterinary pathology.

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BOVINE PAPILLOMAVIRUS TYPE 2 IS HARBOURDED IN CATTLE CUTANEOUS WARTS

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Abstract

Papillomaviruses (PV) are epitheliotropic double-stranded DNA viruses, known to infect animals. Up to date, more than 280 different papillomavirus types have been described in human and animals. In cattle, up to 29 genotypes of bovine papillomaviruses have been described and classified based on the nucleotide sequence identity of L1 open reading frame. The aim of this study was to detect de bovine papillomavirus type 2 in cutaneous warts. Twenty six (n=26) cutaneous lesions were collected from a cattle slaughterhouse, located in Iasi County, North-Eastern Romania. The viral DNA was extracted using PureLink™ Genomic DNA Mini Kit, following the manufacture's instruction. Detection of papillomavirus DNA was confirmed by PCR, using degenerated primers - FAP59/64 and type specific primers for BPV-2 L2 gene. PCR products were electrophoresed on 1.5% agarose gel stained with SybrSafe and each band was visualized with UV transillumination. When FAP 59/64 primers were used, PV DNA was detected in 11 (42.3%) out of 26 samples, while in 15 (57.69%) samples no PV DNA was identified. The fragment length was consisting in 478 base pairs from L1 gene. A fragment of 164 base pairs corresponding to BPV-2 L2 gene was amplified in 24 (92.2%) samples, while 2 samples were negative (7.69%). Two samples were proved to be negative when tested with both primers pairs. These results are in accordance with previous reported results. The use of type specific primers represent a useful tool in bovine papillomavirus detection.

Key words: BPV, warts, PCR

Papillomaviruses (PV) are epitheliotropic double-stranded DNA viruses able to infect human and animals. These viruses are associated with benign and malignant epithelial lesions. Up to date, more than 280 different PV types have been described in human and animals, while in cattle, up to 29 bovine papillomavirus (BPV) types have been identified and fully characterized (Yamashita-Kawanishi N., Haga T., 2020).

The bovine papillomavirus genome is divided into three different regions: early, late, and noncoding long control region (LCR). The early control region is comprising 50% of the viral genome and encodes seven early proteins: E1, E2, E3, E4, E5, E6, and E7. The late control region occupies 40% of the viral genome and encodes two late proteins or the L1 and L2 capsid proteins and LCR, which comprises 10% of the genome, with 850 bp (Zheng Z.M. and Baker C.C., 2006; Araldi R.P. *et al*, 2013). The viral oncoproteins encoded by BPV are known to be involved in several pathways of the cell transformation (Boracchiello G., Roperto, F., 2008; Lunardi *et al*, 2013).

According to criteria proposed by the International Committee on the Taxonomy of Viruses (ICTV), PVs are classified based on the nucleotide sequence identity of the major capsid protein, L1 ORF: one PV isolate is admitted as a new type if the complete genome has been cloned and the DNA sequence of L1 differs by more than 10% from the closest known PV type. Differences between 2% and 10% define a subtype and less than 2% are proposed for a variant (de Villiers E.-M. *et al*, 2004; VanDoorslaer *et al*, 2018).

According to the papillomavirus episteme (PaVE) (Bianchi R.M. *et al*, 2020), there are currently 29 fully characterized BPVs types that are classified into five genera, namely: the *Deltapapillomavirus* genus with BPV types -1, -2, -13, and -14; *Epsilonpapillomavirus* genus with BPV types -5 and -8; *Dyoxipapillomavirus* genus with BPV type -7; *Dyokappapilomavirus* genus with BPV types -16, -18 and -22 and *Xipapillomavirus* genus with BPV types -3, -4, -6, -9, -10, -11, -12, -15, -17, -20, -23, -24, -28, -29; BPV -19, BPV -21 and BPV -27 remain unclassified (Yamashita-Kawanishi N. *et al*, 2020a; Yamashita-Kawanishi N. *et al*, 2020b; Daudt *et al*, 2018). Improvement in genomic science technologies and molecular biology, such

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as viral detection and sequencing led to identify new types of PVs (Yamashita-Kawanishi N. *et al*, 2020a).

The use of PCR assays with degenerated primers, followed by sequencing has allowed the identification of several PV types in human and other animal hosts (Forslund O. *et al*, 1999; Antonsson A., Hansson B. G., 2002). The PCR primer FAP set was designed from two relatively conserved regions found in the L1 gene and has been shown to amplify PVs DNA from both papillomas and healthy tissue of many animal species, including BPVs in bovines (Carvalho R.F. *et al*, 2013). The aim of this study was to detect de bovine papillomavirus type 2 in cutaneous warts by employing degenerated primers FAP59/64 and type specific BPV-2 primers.

MATERIAL AND METHOD

The bovines with cutaneous warts were slaughtered in one slaughterhouse located in Iași County, North-Eastern Romania, from August 2020 to January 2021. Fragments of skin presenting warts were collected from 26 bovines and each sample was fixed in 10% neutral buffered formalin, routinely processed for histopathology and stained with hematoxylin and eosin (H&E).

DNAs from 26 paraffin embedded bovine cutaneous tumour samples were recovered using the PureLink™ Genomic DNA Mini Kit, following the manufacture's instruction.

Partial amplification of the PV L1 gene was performed with the forward primer FAP59 and the reverse primer FAP64 (Table 1).

amplification, it was used the protocol recommended by Araldi *et al*, 2013, as follows: 5 minutes at 95°C, followed by 35 cycles of 1 minute and 30 seconds at 98°C, 2 minutes at 52°C, and 1 minute and 30 seconds at 72°C and a final extension step of 5 minutes at 72°C. A positive control consisting in a BPV-2 positive sample was used (Bocaneti F. *el al*, 2015). PCR products were analyzed by electrophoresis in 1.5 % agarose gels stained with SybrSafe (Invitrogen) and visualized under UV light.

RESULTS AND DISCUSSIONS

Diagnosis of cutaneous warts or papillomatosis was performed by evaluating each sample. Microscopically, all samples showed specific features of BPV infections: epithelial and dermal hyperplasia, hyperkeratosis and koilocytosis.

Conventional PCR using the degenerated FAP59/FAP64 primer pair generated the expected 478 bp L1 gene fragment (*figure 1*) in 11 (42.3%) of the 26 warts samples, while in 15 (57.69%) no papillomavirus DNA was amplified (*figure 2*).

A fragment of 164 base pairs corresponding to BPV-2 L2 gene (*figure 3*) was amplified in 24 (92.2%) samples, while 2 (7.69%) samples were negative (*figure 4*). Two samples were proved to be negative when tested with both primers pairs.

Primers used for PCR amplification Table 1

Primers	Region	Sequence	Reference
FAP 59/64	L1	5" TAACWGTIGGICAYCCWTATT 3" and 5 "CCWATATCWVHCATITCICCATC 3"	Forslund O. *et al,* 1999
BPV-2	L2	5" GTTATACCACCCAAAGAAGACCCT 3" 5" CTGGTTGCAACAGCTCTCTTTCTC 3"	Araldi R.P. *et al,* 2013

For BPV-2 detection, L2 gene was targeted, resulting in a 164 amplicon. The PCR were performed with a previously described protocol (Ogawa O. *et al*, 2004, Araldi R.P. *et. al*, 2013). Briefly, 5 µL of extracted DNA was mixed with [1×] PCR mix buffer Platinum II Hot-Start Green, 20 µmol of each primer, (Invitrogen, Platinum II Hot-Start Green PCR Master Mix) in a total volume of 20 µL adjusted with water, nuclease free. For PV amplification, after an initial incubation at 95 °C for 5 min, the reaction conditions consisted of 40 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 75 °C for 1 min. For BPV-2

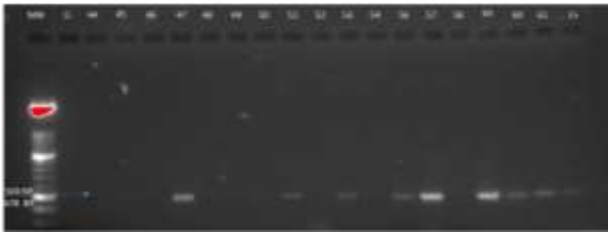

Figure 1 PCR results showing a specific band of 478 bp amplified with FAP59/64 primers. MW – 100 bp; C- negative control; C+ positive control; 44-61 bovine cutaneous warts

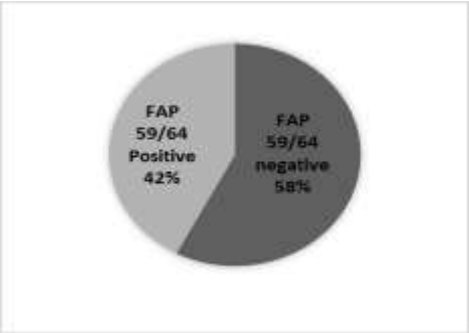

Figure 2 Result of PCR amplification using FAP59/64 degenerated primers

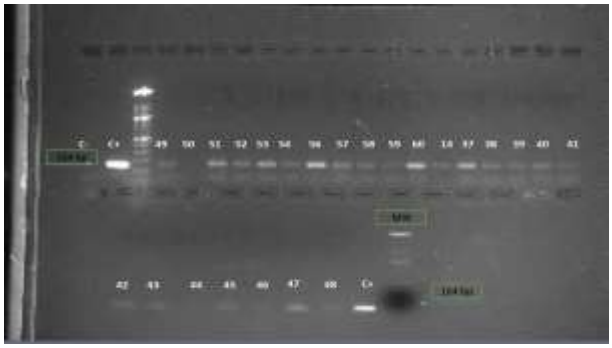

Figure 3 PCR showing a specific band of 164 bp amplified with BPV-2 L2 specific primers. MW – 100 bp; C-negative control; C+ positive control; 37-61 bovine cutaneous warts

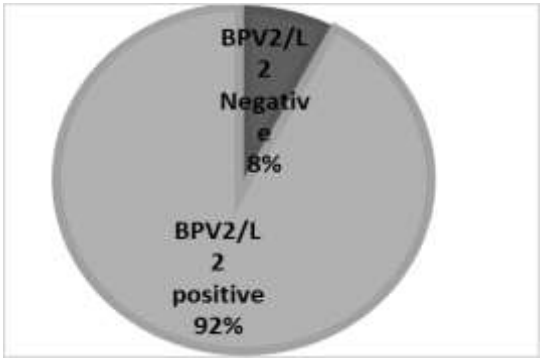

Figure 4 Result of PCR amplification using BPV-2 L2 specific primers

With respect to papillomavirus identification, these results are in accordance with previous reported results (Forslund O. *et al*, 1999; Silva M.A. *et al*, 2013). PCR assays using the FAP59/FAP64 degenerated primers to amplify partial fragments of the PV L1 gene, followed by use of specific BPV primers is an important tool that is widely used to determine the presence of different BPV types in cattle herds from different countries. Indeed, two sets of primers (FAP59/FAP64 and MY09/MY11), which were originally designed from two conserved regions of the HPV L1 gene, are widely used for identifying PVs in both humans and a wide range of animals (Manos M. *et al*, 1989; Forslund O. *et al*, 1999; Antonsson A., Hansson B.G., 2002; Ogawa T. *et al*, 2004). However, studies are showing that FAP59/64 consensus primers have a lower level of sensitivity than BPV type-specific primers (Araldi R.P. *et al*, 2013).

It is well know that both FAP and BPV type-specific primer sets are able to amplify a wide range of BPV types in cutaneous lesions, as well in blood and semen samples. However, BPV type-specific primers were more sensitive than consensus primers (Araldi R.P. *et al*, 2013). On the other hand, the consensus primers are a very suitable way of detecting new BPV types and subtypes. Therefore, the choice of the PCR primer system plays an important role in epidemiological analysis of bovine papillomavirus distribution in cattle (Araldi R.P. *et al*, 2013).

In our study, the positivity rate was for BPV-2 was 92%.This is in accordance with the results obtained by other authors who demonstrated that BPV-2 is the main BPV circulating in Moldova area, North-Eastern Romania (Balcos L. *et al*, 2008; Silva M. *et al*, 2013, Bocaneti F. *et al*, 2016).

CONCLUSIONS

The BPV type-specific primers are a sensitive method to detect papillomavirus DNA in cutaneous samples than the consensus primers.

In this study, only two sample were negative when tested with both pair of primers. However, since the rate of positivity when using degenerated primers is low, we believe that two sample is possible to harbor other type of BPV.

ACKNOWLEGMENTS

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BOVINE PAPILLOMAVIRUS TYPE 2 DETECTION IN BLOOD OF ASYMPTOMATIC LIMOUSINE BREED CATTLE

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Abstract

Bovine Papillomaviruses (BPV) are double-stranded DNA viruses, known to infect ruminants and equines. In bovines, up to 29 genotypes of BPV have been described and classified based on the nucleotide sequence identity of L1 open reading frame. The aim of this study was to detect bovine papillomavirus type 1, 2 and 4 in the blood collected from clinically healthy Limousine cattle, with history of papillomatosis. Fourteen blood samples were collected from a cattle farm, located in Suceava County, Romania. The viral DNA was extracted using PureLink™ Genomic DNA Mini Kit, following the manufacture's instruction. Papillomavirus DNA was confirmed by PCR, using type specific primers for BPV-1 L1 gene, BPV-2 L2 gene and BPV-4 E7 gene detection. PCR products were electrophoresed on 2% agarose gel and visualized with UV transillumination. None of the blood samples tested positive for BPV-1 nor BPV-4. A fragment of 164 base pairs corresponding to BPV-2 L2 gene was amplified in 10 (92,2%) samples, while 4 samples were negative (7,8%). This is the first time when in Romania, to authors best knowledge, BPV-2 is detected in blood samples collected from cattle. Moreover, BPV-2 persists and is maintained in the bloodstream of the asymptomatically cattle, representing a potential reservoir of viral infection.

Key words: BPV-2, blood, PCR

Bovine Papillomaviruses (BPV) are double-stranded DNA viruses, known to infect ruminants and equines. In cattle, up to 29 bovine papillomavirus (BPV) types have been detected and their genome fully characterized (Yamashita-Kawanishi N., Haga T., 2020).

The bovine papillomavirus genome characterized by three different regions: early, late, and noncoding long control region (LCR). The early control region encodes seven early proteins: E1, E2, E3, E4, E5, E6, and E7. The late control region encodes two late proteins or the L1 and L2 capsid proteins and LCR comprises 10% of the genome, with 850 bp (Zheng Z.M. and Baker C.C., 2006; Araldi R.P. *et al,* 2013). The viral oncoproteins encoded by BPV, especially E5, are known to be involved in different pathways of the cell transformation (Boracchiello G., Roperto, F., 2008; Lunardi *et al,* 2013; Bocaneti F. *et al,* 2016).

According to criteria proposed by the International Committee on the Taxonomy of Viruses (ICTV), BPVs are classified based on nucleotide sequence identity of the major capsid protein L1 ORF and there are described five genera, namely: the *Deltapapillomavirus* genus with BPV types -1, -2, -13, and -14; *Epsilonpapillomavirus* genus with BPV types -5 and -8; *Dyoxipapillomavirus* genus with BPV type -7; *Dyokappapilomavirus* genus with BPV types -16, -18 and -22 and *Xipapillomavirus* genus with BPV types -3, -4, -6, -9, -10, -11, -12, -15, -17, -20, -23, -24, -28, -29; BPV -19, BPV -21 and BPV -27 remain unclassified (Yamashita-Kawanishi N. *et al,* 2020a; Yamashita-Kawanishi N. *et al,* 2020b; Daudt C. *et al*, 2018).

Papillomavirus DNA is known to be detected most commonly as episomal molecules in lesions, in lymphocytes and in precursor lesions (Campo M.S. *et al*, 1994).

BPV has been detected in non-epithelial sites such as gametes and fluids in recent years (Freitas A.C. *et al,* 2003; Lindsey C.J. *et al,* 2009; Roperto S. *et al,* 2011; Silva M.A. *et al,* 2011). Since BPV DNA has been identified in other body tissues, blood has been hypothesized as a vehicle of BPV to various body parts (Freitas A.C. *et al,* 2007; Roperto S. *et al,* 2011). BPV DNA has also been found in peripheral blood mononuclear cells of sheep, the skin of wild ruminants, and the

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placenta and blood of mares (Roperto S. *et al*, 2018; Savini F. *et al*, 2019).

Recently, BPV-2 and BPV-4 DNA were identified in embryonic tissues as well as in female reproductive tissues taken from infected cows, demonstrating viral presence in tissues other than cutaneous epithelium (Yaguiu A. *et al*, 2006, 2008).

The aim of this study was to detect de bovine papillomavirus type -1, -2 and -4 in the blood collected from clinically healthy Limousine cattle, but with history of papillomatosis in the past.

MATERIAL AND METHOD

The bovines with history of cutaneous warts in the past were reared into a farm located in Marginea, Suceava County, with a flock of 62 animals. During 2015-2016, 14 bovines: 10 cows and 4 bulls, Limousine pure breed were imported from France and adapted to the climate of Bucovina. The bovines are kept in free range system during the summer, while in the winter are kept in stable and feed with dry hay (*figure 1* and *2*).

Figure 1 Limousine bull

Figure 2 Limousine cattle kept in stable

For this study, 14 blood samples were collected from animals that were known by the owner to suffer of cutaneous papillomatosis. At the moment of blood collection none of the animals showed signs of papillomatosis. From each sample, a quantity of three mL of blood was collected by coccygeal venipuncture using EDTA-containing tubes. The viral DNAs for each blood sample were recovered using the PureLink™ Genomic DNA Mini Kit, following the manufacture's instruction.

For BPV-1 detection, a fragment of 301 bp of L1 gene was amplified. For BPV-2 detection, L2 gene was targeted, resulting in a 164 amplicon, while for BPV-4 detection a fragment of 170 bp of E7 was targeted. The amplification was performed with a previously described protocol (Ogawa O. *et al*, 2004; Araldi R.P. *et al*, 2013). Briefly, 5 µL of extracted DNA was mixed with [1×] PCR mix buffer Platinum II Hot-Start Green, 20 µmol of each primer, (Invitrogen, Platinum II Hot-Start Green PCR Master Mix) in a total volume of 20 µL adjusted with water, nuclease free. For PV amplification, after an initial incubation at 95 °C for 5 min, the reaction

Primers used for PCR amplification Table 1

Primers	Region	Sequence	Reference
FAP 59/64	L1	5" TAACWGTIGGICAYCCWTATT 3" and 5 "CCWATATCWVHCATITCICCATC 3"	Forslund O. *et al,* 1999
BPV-2	L2	5" GTTATACCACCCAAAGAAGACCCT 3" 5" CTGGTTGCAACAGCTCTCTTTCTC 3"	Araldi R.P. *et al,* 2013
BPV-1	L1	5" GGAGCGCCTGCTAACTATAGGA 3" and 5" ATCTGTTGTTTGGGTGGGTGGTGAC 3"	Araldi R.P. *et al,* 2013
BPV-4	E7	5" GCTGACCTTCCAGTCTTAAT 3" 5" CAGTTTCAATCTCCTCTTCA 3"	Araldi R.P. *et al,* 2013

Partial amplification of the PV L1 gene was intended in a first step of papillomavirus surveillance with the forward primer FAP59 and the reverse primer FAP64 (*table* 1).

conditions consisted of 40 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 75 °C for 1 min. For BPV-1, BPV-2 and BPV-4 amplification, it was used the protocol recommended by Araldi *et al*, 2013, as follows: 5 minutes at 95°C, followed by 35 cycles of 1 minute and 30 seconds at 98°C, 2 minutes at 52°C, and 1 minute and 30 seconds at 72°C and a final extension step of 5 minutes at 72°C. PCR products were analyzed by electrophoresis in 1.5 % agarose gels stained with SybrSafe (Invitrogen) and visualized under UV light.

RESULTS AND DISCUSSIONS

PCR using the degenerated FAP59/FAP64 primer pair did not generated the expected 478 bp L1 gene fragment in none of the samples.

A fragment of 164 base pairs corresponding to BPV-2 L2 gene was amplified in 10 (92.2%) samples, while 4 (7.8%) samples were negative (*figure 3*). The results for each tested blood sample by PCR is shown in table 2.

Figure 3 PCR results representing a specific band of 164 bp amplified with BPV-2 L2 primers. MW – 100 bp; C-negative control; C+ positive control; 1-14 bovine blood

Table 2

PCR results for each tested blood sample

Sample	ID	Sex	DOB	FAP 59/64	B-PV-1	BPV-2	BPV-4
1	RO502009339386	F	07/04/2019	-	-	-	-
2	RO502008553961	F	26/05/2018	-	-	-	-
3	RO506008553976	F	02/04/2018	-	-	-	-
4	RO507008587840	F	05/01/2019	-	-	-	-
5	RO509009342976	F	26/04/2019	-	-	+	-
6	RO509009339387	F	06/04/2019	-	-	+	-
7	RO500000009396	F	06/03/2019	-	-	+	-
1.	FR2278711432	F	03/04/2016	-	-	+	-
2.	RO503000009717	F	29/01/2011	-	-	+	-
3.	RO500000009396	F	06/03/2019	-	-	+	-
4.	RO506007638922	M	27/04/2017	-	-	+	-
5.	RO508006920149	F	30/06/2016	-	-	+	-
6.	RO507008587840	F	05/01/2019	-	-	+	-
7.	RO50000009721	F	29/01/2011	-	-	+	-

With respect to BPV-1 and BPV-4 detection, none of the sample showed a positive response. PCR assays using the FAP59/FAP64 degenerated primers to amplify partial fragments of the PV L1 gene, followed by use of specific BPV primers is an important tool that is widely used to confirm the presence of different BPV types in cattle herds from different countries.

However, in our study, we failed to detect in the first step PV DNA when using degenerated primers. Indeed, studies are showing that FAP59/64 consensus primers have a lower level of sensitivity than BPV type-specific primers (Araldi R.P. *et al*, 2013).

With respect to BPV-2 detection, this is the first time when in Romania, to authors best knowledge, when BPV-2 is detected in blood samples collected from cattle. Accordingly, there are studies confirming the presence of BPV-2 among cattle from Moldova, where this BPV type was detected in cutaneous warts or in urinary bladder tumours (Balcos L. *et al*, 2008; Silva M. *et al*, 2013, Bocaneti F. *et al*, 2016; Bocaneti et al., 2021, in press).

CONCLUSIONS

BPV-2 persists and is maintained in the bloodstream of the asymptomatically cattle, representing a potential reservoir of viral infection.

Knowledge of BPV diversity and epidemiology is of great importance for establishing distribution, prevention strategies and understanding the evolution of this group of oncoviruses.

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BENEFITS AND USES OF LAVENDER ESSENTIAL OIL AS A COMPLEMENTARY AND ALTERNATIVE THERAPY– A SHORT REVIEW

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Abstract

The use of natural extracts as complementary and alternative therapies in human medicine to treat various ailments is presently growing, as many specialists all over the world have showcased an heightened interest in the therapeutic potential of plants. A variety of therapeutic methods are known to use essential oils extracted from various plants and herbs. Essential oils are complex mixtures of volatile compounds that are abundant in herbs and result from secondary plant metabolism. Due to their chemical composition, essential oils have many biological activities of great interest in the fields of health, food, and cosmetics.Lavender essential oil is one of the most used natural products for its anti-inflammatory, antimicrobial, relaxing, calming, anxiolytic, antioxidant, anticonvulsant, etc. therapeutic effects.In veterinary medicine, the therapeutic potential of lavender essential oil is not fully known and, therefore, is only used experimentally or sometimes as a supplement in animal feed for animals of economic interest. This has led us to publicize its beneficial effects in wound healing by presenting information from the scientific literature in order to expand the current repertoire of cost-effective wound healing options available to physicians and animal owners, especially in the current context of imposing a rational use of antibiotics in dogs and cats.

Key words: lavender, essential oils, anti-inflammatory effect, treatment of lesions

INTRODUCTION

The use of plant extracts in the treatment of human and animal diseases is an ancient tradition that began centuries ago, practiced by various civilizations around the world. All over the world there is a great variety of therapeutic plants of different shapes, colors, sizes, perfumes and active ingredients with beneficial effects on the body.

The largest proportion of active principles is found in the flowers, leaves, seeds, roots, stem, or bark of a plant. These volatile compounds, called essential oils, volatile oils, or aromatic substances, are found in high concentration and have a strong effect, sometimes even toxic, on the major functions of the body. The role of these essential oils is to give the plant its own flavor, to protect it from adverse environmental conditions, to protect it against harmful insects, but they also play an important role in pollinating the plant.

Essential oils are fatty aromatic compounds extracted from different plants and, in order to be used in various therapies, are distilled in a wide range of concentrations, from 100% pure essential oil to concentrations between 1 - 20%, which are diluted with a non-aromatic carrier oil *(Aziz Z.A.A., et al., 2018).*

Lavender has been used since ancient times for its unmistakable aroma, and later, with the discovery of its therapeutic qualities, it was used for its benefits on the body. Since ancient times, there are pieces depicting the use of this plant by the Egyptians and Romans for baths as a relaxant, for cooking due to the active principles, but also as a perfume by virtue of its strong aroma.

Lavender, scientifically named *Lavandulaangustifolia*, is part of the Lamiaceae family, related to mint, being very easy to identify due to the purple flowers and its sweet, floral scent. This plant is thought to have originated in the Mediterranean area, North Africa, the Middle East and India, and has a history dating back over 2,500 years. In ancient times, lavender was used as a sacred plant, its name coming from the Latin word "*lavare*" which means "to wash", which is why the plant was used in certain parts of the world to perfume baths, beds, clothes, and even hair. Later, its therapeutic qualities were discovered.

The active ingredient in lavender flowers is volatile oil, the content of which differs depending on the species, variety, time of harvest, or form of conditioning. Fresh flowers contain up to 0.8% volatile oil and dried flowers up to 1.5%. From a quantitative point of view, the main components of the Lavandulaeaetheroleum

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product are linalool (20-35%) and linalyl acetate (30-55%), but the aroma is also determined by a share of cineole, camphor or geraniol. Lavender acetate, cis- and trans-ocimen are the characteristic compounds of lavender oil, and peril-alcohol is important due to its pharmacological action. *(Cavanagh H.M., et al., 2002).*Lavender flowers also have slight sedative, spasmolytic, and carminative properties.

Other compounds identified in the plant product are: flavonoids (mainly accumulated in the leaves), coumarins, phytosterols and tannins (5-10%), among which the pharmacological importance of rosemarinic acid is distinguished, which is a depsid formed by the esterification of α- hydroxy-dihydro-caffeic with caffeic acid. The product also contains oleanolic acid and ursolic acid.

All these elements give it effective therapeutic properties for various ailments. Most frequently, lavender is recommended for oral administration, but it is also regularly used in aromatherapy by inhalation, massage or bath. Unlike other essential oils used in aromatherapy, *Jager et al., 1994*, suggested that lavender essential oil can be applied undiluted, being quickly absorbed through the skin.

Chemical composition of lavender essential oil

Recently, and especially in the last two decades, human medicine has renewed interest in curing diseases with natural products or essential oils from plants, using them as alternative or complementary therapies that have been accepted(*Bergh A., et al., 2021)*, becoming popular around the world.

The use of essential oils is an ancient tradition that dates back centuries, in different civilizations around the world, being thousands of years older than conventional medicines used today, proving its therapeutic effects on both the internal balance of body, as well as externally.

Lavender volatile essential oil can be extracted by various methods, the most common of which is cold pressing, but it can also be extracted by steam distillation, solvent extraction, grease extraction and filtration. It is considered that the purest essential oils are only those obtained by steam distillation or cold pressing, because by these methods the therapeutic properties of the oils remain intact and unaltered.

The chemical composition of *Lavandulaeangustifoliae* volatile oil consists of: 47.52% monoterpenes alcohols; monoterpene hydrocarbons 5.09%; hydrocarbons 5.09%; linalool 43.00%; trans-β-ocimen 1.92%; borneol 1.80%; cis-β-ocimen 1.47%; α-terpineol 1.02; esters 34.81%; sesquiterpene hydrocarbons 4.58%; linalyl acetate 32.09%; 2.80% β-caryophyllene; lavender acetate 1.29%; β-farnesen 1.46%; 1-octen-3-yl acetate 0.59%; hexyl acetate 0.40%; ketone 2.05%; 3-octanone 1.22%; camphor 0.83%(*WoronukG.,et al., 2010).*Thus, phytochemical studies *(Umezu T., et al., 2006)*have shown that the major constituents of lavender essential oil are esters (40-50%), monoterpenols (30-40%) and monoterpenic hydrocarbons (7-13%).However, the composition of lavender essential oil may vary depending on the geographical origin of the plant material and environmental factors such as geographical conditions, climate, seasonal variations and the growth stage of the plant and the methods of extraction and detection influencealso the composition*(Demasi S., et al., 2018).*

Properties and benefits of lavender essential oil

Lavandulaangustifolia extracts and essential oil have various pharmacological effects described in literature, such as anticonvulsant*(Gilani A.H. et al., 2000;Yamada K., et al., 1994),* anxiolytic*(Antonelli M., et al., 2019),*antioxidant*(Ben Djemaa F.G., et al., 2016;KozicsK.,et al., 2017),*, anti-inflammatory (*Cardia G.F.E., et al., 2018)*, antimicrobial and antifungal activities (*Ebani V.V., Mancianti F., 2020*).

Although the list of benefits of lavender essential oil is impressive, it has several valuable pharmacological properties that make it increasingly useful in alternative and complementary medicine:

Considering its antibacterial and anti-inflammatory properties, lavender essential oil is effective for opening pores and reducing acne-induced inflammation *(Sarkic A., Stappen I., 2018;Winkelman WJ., 2018)*. Due to its high content of antioxidants, it is a good skin moisturizer *(Seo YM, et al., 2015)*. Lavender oil also improves blood circulation*(Eguchi E., et al., 2016)*, which will facilitate nutrient transfer to the face, stimulating the healing process, thus reducing the inflammation caused by episodes of acne, insect bites, allergic reactions and skin diseases (*SindleA. et al., 2021*).Due to its antifungal and anti-inflammatory properties, lavender can prevent eczema, reduce depigmentation, and reduce the appearance of blemishes and redness on the face (*Blamey C., 1999; Ebani V.V., Mancianti F., 2020*).

Rai V.K. et al., 2020, confirms the relief of psoriasis, a condition that is difficult to fight and

necessitates long-term treatment with the help of lavender oil.

Known for its antibacterial properties, lavender oil has been proven effective in treating wounds for centuries. There are numerous studies (*Han X., et al., 2017;Winkelman WJ., 2018; Mori H.M.,et al., 2016*) that have proven its effectiveness in accelerating the healing process. Thanks to its antibacterial properties, lavender essential oil can speed up the healing process of cuts, burns, scratches, and wounds.A study conducted in 2016 by *Mori H.M., et al.,* showed that lavender oil accelerates the healing process of experimentally induced wounds in mice, compared to other products applied for skin healing. The conclusion of the study was that "Applying lavender oil stimulates collagen synthesis", accelerating the healing process.

The main active compounds in lavender essential oil such as linalool and linalyl acetate are known for their analgesic and anesthetic properties. At the same time, the oil also contains a small amount of beta-caryophyllene, which also induces anti-inflammatory effects. All these properties turn lavender oil into a treatment used to relieve pain and reduce inflammation from burns or injuries, also having an antiseptic role, accelerating healing and reducing scarring of the skin (*Mori H.M., et al., 2016; Samuelson R., et al., 2020*).Due to its ability to stimulate wound healing, lavender essential oil, through its role of improving blood circulation, causes the formation of new skin cells.

In various studies (*Hay I.C., et al., 1998; Lee B.H., et al., 2016*.) it has been shown that lavender oil is effective in ameliorating hair loss and stimulating its growth, which is why we find it in shampoos or hair conditioners. Due to its anti-inflammatory and anti-fungal properties, lavender oil also helps reduce dandruff and supports scalp health, while also giving the hair a pleasant smell.*Hay I.C., et al., 1998*in their study have shown that people with alopecia can be treated using a combination of lavender oil and other essential oils, all mixed with a nourishing carrier oil. It has been reported that over 40% of those tested experienced an improvement after applying this mixture. Thus, many dermatologists recommend adding lavender essential oil to the specific shampoo for scalp conditions.According to a 2016 study of mice by*Lee B.H., et al.,* it was found that lavender oil stimulated the growth of hair, which became thicker and grew faster than normal. According to these studies, lavender essential oil can be very helpful for people with alopecia (hair loss), but human studies are also needed to demonstrate this, although people can apply lavender oil to their hair without care.

Other research (*NasiriA., et al., 2016*) has shown that this oil has pain relieving effects due to its analgesic and anti-inflammatory properties, especially joint pain, muscle aches and headaches. A 2004 study by *Yip Y.B. and Tse S.H.* in Hong Kong,concluded that lavender oil acupressure reduces pain in the lower back by at least 39% and "improves travel time" and "spinal flexion capacity".Another study (*Kim J.T., et al., 2006*.), performed on 50 patients who underwent surgery, specified that 50% of them were given to inhale oxygen and the other half were given to inhale lavender essential oil. The conclusion was that "patients who received lavender essential oil had better pain control than patients who received oxygen."Another study (*Bakhtshirin F., et al., 2015*) performed on women, young students, showed that massage with lavender essential oil helped to relieve pain caused by dysmenorrhea. The result was that "Massage with lavender essential oil reduces primary dysmenorrhea and can be used as a natural remedy."

Aromatherapy with lavender essential oil calms and relaxes muscles and nerves, improving blood circulation and relieving various pains. It has been shown (*Sasannejad P., et al., 2012*) that it can significantly help relieve migraine pain if inhaled for 15 minutes. Another study (*RafieS.,et al., 2016*) showed that out of 129 people with headaches, 92 reported a total or partial relief of pain due to lavender oil. This oil acts both for its analgesic properties and for its relaxing effects, calming agitation and releasing nervous tension.

Lavender has been used since ancient Rome to relax and soothe. Due to its aroma and other properties, inhalation of lavender essential oil has been shown to be effective in maintaining the proper functioning of the nervous system, helping to reduce stress and anxiety, relieving headaches, reducing depression and fatigue, ensuring well-being.Different studies (*Generoso M.B. et al, 2017; Greenberg M.J., Slyer J.T., 2018*) showed that a placebo with lavender oil, known as Silexan, was more effective than a prescription drug, Paroxetine, when it came to anxiety. The conclusion was that "Silexan has been shown to have a strong antidepressant effect, improving mental health, overall health, and quality of life." In a study conducted in 2016 (*Kianpour M., et al., 2016*), several women with postnatal depression were treated with aromatherapy, lavender oil, for 4 weeks. The conclusion was that there was a significant reduction in postnatal depression and the aromatherapy protocol was proposed as an effective method of treatment and as a

"complementary therapy useful for both anxiety and depression". In another study (*Generoso M.B. et al, 2017*), it was shown that daily use of lavender essential oil could reduce the symptoms of depression by 32.7% in 47 patients with post-traumatic stress disorder. At the same time, lavender oil therapy has improved their mood and sleep quality.

Lavender is famous for its ability to induce relaxation. In fact, one of the most important benefits of lavender is that it can provide peace of mind without sedation. Lavender can relieve anxiety by having a positive effect on the body's response to "fight or flight". According to studies (*Donelli D., et al., 2019; Fayazi S., et al., 2011*), lavender essential oil can reduce: nervousness, anxiety, symptoms of depression, agitation, and sleep problems.A study (*Kazeminia M., et al., 2020*) has shown that lavender aromatherapy can reduce anxiety in women in labor. Lavender can have several beneficial effects that help relieve anxiety, such as: lowering heart rate, improving mood, improving sleep quality, regulating breathing, lowering adrenaline levels. Lavender essential oil, through its antioxidant content, also contributes to the detoxification of the body, being very effective in insomnia.

Research in recent years (*Sayorwan W., et al., 2012*) has shown that lavender oil can stimulate optimal brain function. Laboratory tests on mice have shown that lavender oil aromatherapy can improve cognitive function and reduce oxidative stress, which is a promising starting point in finding alternative treatments for Alzheimer's or dementia (*Kashani M.S., et al., 2011)*. A study designed by *Jimbo D. sicolab., 2009,*found that linol, an active ingredient in lavender oil, "may have the potential to be used in a drug designed to prevent cognitive impairment from Alzheimer's disease."

It has also been shown that this oil calms and induces sleep. A study (*KaradagE.,et al., 2017*) of elderly patients concluded that lavender oil has a "sedative effect" that improves sleep. The results of the study showed that their condition improved after the use of lavender oil.In another study (*KeshavarzAfshar M et al., 2015*), several women in the postpartum period were divided into 2 groups, and one of the groups was recommended to inhale lavender oil 4 times a week for 2 months, the result being that they experienced an increase in sleep quality. Unlike various medications that can have side effects, lavender essential oil is a much safer alternative for better sleep, including in terms of duration.

Lavender, through its strong aroma, has the ability to remove insects such as mosquitoes or moths, but can also reduce irritation, itching and inflammation associated with insect bites. Owing to its antibacterial, anti-inflammatory and analgesic properties, lavender essential oil is excellent for preventing infections, reducing redness and pain caused by insect bites.

Being a natural antimicrobial, it has a great cleaning power, but it also serves to refresh the air, without using harmful chemicals. The cosmetics industry uses lavender in various soaps, cleaning solutions, perfumes, because it gives a pleasant aroma to these products and they have an antiseptic effect.

With a sweet and mild aroma, lavender oil will harmonize well with various essential oils in the diffuser to refresh and purify the air. Combined with eucalyptus oil, it is especially effective during winter viruses (*Horváth G., Ács K., 2015*). This oil is useful for treating various respiratory ailments, including colds and coughs, nasal congestion, sore throats and more when inhaled.

Research (*Hamzeh S., et al., 2020; Kim S., et al., 2011; Perry R., et al., 2012*) has shown that lavender essential oil can harmonize the hormonal balance, stimulate blood circulation, treat respiratory ailments or even provide support in the fight against cancer. The oil is widely used in aromatherapy to relieve the side effects of cancer treatments.Studies have shown that lavender oil can reduce stress in cancer patients. A 2012 study (*Perry R., et al., 2012*) found that inhaling essential oils, especially lavender, can help cancer patients get over the effects of specific treatments, including nausea, pain, and exhaustion and depression. Lavender oil aromatherapy has also helped patients with malignant brain tumors feel more "relaxed" and "less tense", as well as those who perform hemodialysis (*Dehkordi A.R., et al., 2016).* In another study (*Karadag E., et al, 2017*), it was shown that 20-30 minutes of aromatherapy massage sessions with lavender essential oil help relieve pain and "significantly reduce inflammation" in breast cancer patients.

Another study (*Lee Y.-L. et al., 2011*) showed that lavender oil aromatherapy improved circulation and caused heart relaxation in 1/3 of participating young people and adults. In addition, by inhaling or massaging, the oil lowers blood pressure and is, therefore, effective for people with high blood pressure.

Lavender oil is also effective for diabetes. A 2013 study (*Sebai H., et al., 2013*) tried to show that it could be possible. In a 15-day laboratory test on mice, researchers found that lavender oil "provided increased protection against high blood

glucose levels" and helped reduce oxidative stress due to its "powerful antioxidant" properties".

Although further research is needed to understand how lavender oil can be used to prevent or alleviate the symptoms of diabetes, the study promises to prove that even natural treatments, such as those with essential oils can be helpful in combating serious ailments.

The safety and efficacy of lavender essential oil for use in veterinary medicine

In human medicine, the use of essential oils as a remedy for various pathologies is growing in popularity. In veterinary clinical practice, data on the use of lavender oil for various ailments or in vitro susceptibility testing are not as complete as in human medicine. The use of essential oils to improve the health of animals remains a controversial topic, as their benefits are not known or accepted.Some holistic veterinarians and alternative medicine practitioners use various essential oils to treat some ailments, but most veterinarians recommend pet owners to avoid them. There have always been controversies regarding the safety of the use of essential oils, but to date there are no studies showing that they are harmful to animals.

In general, essential oils are safe, with no known side effects, which is confirmed by the fact that some of them have been approved as flavoring agents in food, being considered safe by the US Food and Drug Administration (FDA). The most common side effects reported by various users are irritation to the eyes and mucous membranes in the case of permanent use and use of oils containing aldehydes and phenols.

Skin sensitization by contact with an essential oil may occur due to oxidation of monoterpenes, in conditions of improper storage. At the same time, it is possible to interfere with other essential oils or certain foods. Allergies can sometimes be caused by inhaling the aromas of essential oils.

However, data on adverse reactions depending on the degree of exposure are limited and most of them refer to perfumes rather than essential oils(*Bingham L.J.,, et al., 2019*. Essential oils are not exempt from oxidation reactions over time and changes in their chemical composition have been reported.

No side effects have been reported in humans or animals regarding lavender essential oil. Many pet owners have stated that they have been using lavender oil as an inhalation aromatherapy for a long time and have not noticed any respiratory or other conditions in animals. Many of them even underwent beneficial changes in the behavior of the quadrupeds, making them more relaxed.

There is growing evidence (*Komiya M., et al., 2009; Lans C., 2019*) which suggests that lavender oil may be effective in treating several neurological disorders in pets, which has indicated the possibility of using this oil in the form of aromatherapy, with the purpose of calming animals with nervous disorders.

Many animal experiments suggest the anxiolytic effect of lavender oil (*Lis-Balchin M., Hart S., 1999; Shaw D., et al., 2007*). This has been demonstrated in mice that have undergone an experiment by continuously inhaling lavender essential oil for 7 days.Thus, it was shown that anxiety and other depression-like behaviors were significantly inhibited, which occurred after a maze test and a forced swimming test. In another study (*De Sousa D.P., et al., 2015*), lavender oil produced significant anxiolytic effects in the Geller conflict test and the Vogel test in mice.The effects of lavender oil have been compared with anxiolytic drugs such as chlordiazepoxide (as a reference anxiolytic), and the oil has been shown to have anxiolytic properties similar to those of chlordiazepoxide (*Shaw D. et al., 2011*).The anxiolytic effect of lavender oil was also compared to diazepam in a test on the Mongolian gerbil, which underwent a stressful maze experiment. Exposure to aromatherapy by inhaling lavender oil showed a diazepam-like anxiolytic profile in gerbil females (*Bradley B.F., et al., 2007*).Investigation of the effects of lavender essential oil on anxiety, aggression and social interaction in a group of mice showed that by inhaling this oil in the form of aromatherapy proved anxiolytic properties in the light / dark test, by increasing social interaction and decreasing aggressive behavior(*Malcolm B Tallian K., 2018*).

Lans C. et al., 2007, showed the effectiveness of essential oils in treating skin infections in ruminants (cattle and goats), in the context of the legislatively enforced waiver of antibiotic therapy for the protection of the consumer and the environment.Lavender oil has been shown to have an anti-inflammatory and healing effect on skin wounds in the species in which it was used. In other studies (*Choi W.S. et al., 2002; Traboulsi A.F. et al., 2002*), lavender oil was used in herbivores in the form of skin drops administered to remove insects. Due to its repellent activity against them, lavender essential oil has proven to be highly effective as an insecticide(*Ellse L., Wal R.L., 2014)*.

The local anesthetic effect of lavender and its constituents (linalool and linalyl acetate) has been reported both in vivo and in vitro in animal experiments (*Ghelardini C. et al., 1999*). In rabbit conjunctivitis, treatment was instituted by instilling diluted lavender essential oil and a dose-dependent increase in the number of stimuli needed to provoke the reflex was observed.

An alternative to reducing antibiotic use on pig farms is essential oils. These, including lavender, have proven their effectiveness, feasibility, and potential mechanisms for their application in pig production (*Omonijo FA et al., 2018*), thus reducing the amount of antibiotic required for various infections (usually respiratory and digestive).Due to their lipophilicity, volatile oils are absorbed more quickly in the gut, and their effects on the microbiome have led to better performance in the production of animals fed essential oils in a number of studies.Although there are numerous studies (*Shahdadi H., et al., 2017*) that show that essential oils have several properties, such as antimicrobial, antioxidant and anti-inflammatory effects, improving the palatability of feed and improving growth and intestinal health, further investigations are still required to elucidate the mechanisms underlying their functions.

Lavender essential oil has been shown to have a beneficial effect on wound healing, with several reports of this effect being reported. Several experimental animal studies have been performed to investigate the role of lavender essential oil in the healing of skin lesions.*Altaei, in 2012*, showed that lavender oil helped heal the wounds of the oral mucosa in rabbits. In a randomized, double-blind study, oral lesions treated with 2% lavender essential oil twice daily for 5 days had a higher rate of mucosal repair ($p = 0.001$) compared to a group treated with another product (glycerol).Clinical and histological healing was determined by measuring the area of the ulcer and the levels of inflammation in each test group. Clinical efficacy was assessed by the level of inflammation, erythema, edema, duration of ulcer, ulcer size, mean area under the ulcer curve, healing time, and intensity and reduction of associated pain.Animals treated with lavender oil showed a significant reduction in ulcer size, an increased rate of mucosal repair, and healing within 3 days of treatment compared to baseline and placebo groups.

Another randomized clinical trial designed by *Baccaglini L., 2013*, showed similar results in topical treatment with lavender oil on canker sores that showed a significant reduction in ulcer size, suggesting the beneficial effect of lavender oil on wound healing.Furthermore, there is a report evaluating the mechanism of lavender oil's effect on skin wound healing in an animal experiments (*Kerr J., 2002*).This report showed that wound closure progressed faster with the topical application of lavender oil compared to the control group, accompanied by increased expression of epidermal growth factor (EGF), which plays an important role in the healing process wounds as well as tissue remodeling and reepithelialization.

KocaKutlu et al., 2013, induced incision wounds in rats and compared the effects of different ways of healing wounds. In four groups of rats ($n = 4$ in each group) the skin lesions were treated by transcutaneous electrical nerve stimulation, with saline, povidone/iodine and lavender essential oil.In addition, another group of rats suffered untreated lesions and another group was left without incision wounds.After the 5-day treatment period, researchers excised the incision sites and the surrounding skin to analyze the expression of growth factor, including epidermal growth factor (EGF), which is observed predominantly during active wound contraction, growth factor A. platelet-derived (PDGF-A), observed in all stages of wound healing and fibroblast growth factor-2 (FGF-2), observed during granulation tissue formation.Wound closure progressed much faster in the group where transcutaneous electrical nerve stimulation was used and in the group where the lesions were treated with lavender oil than in the control group and other study groups.

Another study by *Ben Djemaa et al., 2016*, investigated the effectiveness of healing rats experimentally induced in rats. The rats were divided into five groups, 6 per group.The test groups were treated locally with vehicle, lavender oil (4%) and a reference medicine, while the control group was left untreated. The effectiveness of wound healing was determined by monitoring morphological and biochemical parameters and histological analysis of the skin.Wound contraction and protein synthesis were also determined. At 14 days, the rate of wound contraction in the group treated with 4% lavender essential oil showed a complete closure, similar to the lesions treated with reference drugs.Untreated and vehicle-treated wounds were incompletely closed at 14 days. It has also been reported that lavender oil has anti-inflammatory and antimicrobial action, as there was no evidence of edema, suppuration or local infection.Lavender oil treatment has been found to significantly improve wound contraction rate (98%) and protein synthesis. Overall, the results provided

strong support for the effective wound healing activity of lavender oil, making it a promising candidate for future application as a therapeutic agent in tissue repair processes associated with skin lesions(*Woollard A.C., et al., 2007*).

Mori et al., 2016, used in an identical study on rats lavender essential oil in a concentration of 1%, permanently applied for 14 days on lesions. The area of lesion in rats treated with lavender oil was found to be significantly smaller compared to rats in the control group at 4, 6, 8 and 10 days after the lesion (on days 4, 6, 8: $p < 0.01$ versus on day 10: $p < 0.05$ versus control).Thus, in the group treated with lavender, compared to the untreated one or with the control group, the lesion regenerated faster and the histopathological examination on scar tissue showed the production of thinner and better-structured epidermal layers.

Kacaniova et al., 2017, conducted a study of veterinary interest, testing the antimicrobial activity of several essential oils against ten different strains of Pseudomonas grown from freshly caught freshwater fish.All isolates belonging to the species *P. agglomerans, P. antarctica, P. brassicacearum, P. frederiksbergensis, P. koreensis, P. lundensis, P. mandelii, P. proteolytica, P. synxantha* and *P. veronii*with 21 species were tested. Essential oils: *L. angustifolia, C. zeylanicum, Pinusmontana, M. piperita, Foeniculumvulgare, Pinussylvestris, Saturejahortensis, O. vulgare, Pimpinellaanisum, R. officinalis, S. officinalis, Abies alba, Citrus aurantium var. dulce, Citrus sinensis, Cymbopogonnardus, Menthaspicata var. crispa, T. vulgaris, Carumcarvi, Thymus serpyllum, O. basilicum* and *Coriandrumsativum*.All essential oils tested showed good antimicrobial activity, although *C. zeylanicum* oil was most effective against Pseudomonas spp.This investigation provided data that was useful in finding an alternative treatment against bacterial species frequently isolated from skin, gills, and intestines of fish, and in determining the spoilage processes of freshly caught and processed fish.

Bovine mastitis caused by S. aureus is a major concern in veterinary medicine for its high economic impact. *Abboud et al., 2015*, observed in an in vivo study a strong antibacterial activity of the essential oils of *T. vulgaris* and *L. angustifolia* against Staphylococcus sp. and Streptococcus sp. Intramammary application of these oils and a mixture of them has led to a drastic decrease in the number of bacteria in various milk samples after 4 consecutive days of treatment.In the same study, the strongest antibacterial activity was achieved by externally applying these oils in vaseline with a recovery rate of 100% with essential oils.

Atopic dogs affected by recurrent dermatitis were successfully treated with a mixture of Citrus aurantium and Lavandulaofficinalis 1%, with O. vulgare, Origanummajorana, M. piperita and H. italicum 0.5%, which was administered by two or daily for one month without any recurrence after termination of therapy (*Nardoni S., et al., 2014*).

A recent study, of major importance for farmers, is presented by *Adaszynska-Skwirzynska M. et al., 2021*, which determined the immunostimulatory properties and influence of lavender essential oil (added to drinking water at concentrations of 0.4 ml/L) on the production parameters for broilers.The results of the experiment showcased that lavender essential oil exerts antimicrobial and antioxidant activity and a positive effect on the production results of broiler chickens. Thus, the biological activity of lavender essential oil is a property that can be applied in the diet of birds.The use of bioactive compounds is encouraged in many areas of industry and agriculture, as these substances have similar properties to growth promoters that have been withdrawn from the market. In addition, antibiotic-resistant bacteria are one of the most important current threats to animal health.

Studies on the effectiveness of lavender essential oil in therapy are much more extensive, because in recent years there is a growing trend of using natural products in the treatment of many diseases, but in this paper we have limited ourselves to that information to highlight the effect of lavender oil in the treatment of skin wounds, canker sores, reduction of nervous disorders, stimulation of growth in poultry and pig farms, etc.

Although essential oils and plant extracts have been used for centuries, they are just as relevant today. Thanks to advanced technology, improved quality, power and safety, essential oils are now more affordable and easier to use in everyday life.Although essential oils have often been used as an integral part of past cultural practices and traditions, we now have growing scientific evidence and studies showing the effectiveness and safe nature of essential oils today (*Reddy K., et. al., 2016; Silva GL, et al., 2015*).

The pharmaceutical industry is striving to find environmentally friendly, natural and affordable alternatives that can be used for diseases associated with pathogens or metabolism.If essential oils are used, it is possible to improve the effects and bioavailability of the medicines. These essential oils, used correctly,

can have synergistic effects when used together with other drugs in case of pathologies in humans and animals.

The period in which the plant has the largest amount of a certain essential oil, which is rich in certain chemical compounds, is still being discussed. Essential oils are options to consider or can be combined with the steps already taken for certain health problems, provided that safety and quality standards are met.

The scientific community's focus on complementary and alternative medicine offers hope that the side effects of modern medicine can be reduced with the help of essential oils, and if they are properly explored to their full potential, aromatherapy can be considered a boon to anyone.

The literature suggests that lavender essential oil is a very promising substance, which has several pharmacological activities such as anti-inflammatory (*Cardia et al., 2018*), hepatoprotective (*Cardia et al., 2021*), antidepressant and anxiolytic (*Woelk and Schläfke, 2010*), cardioprotective (*Ziaee et al., 2015*), analgesic (*Silva et al., 2015*), efficient in wound healing (*Mori et al., 2016*), antimicrobial and antioxidant (*Cardia et al., 2021; Niksic et al., 2017*). Thus, with all these properties found in lavender essential oil, new research is important to expand the knowledge and use in clinical conditions to determine the therapeutic efficacy of lavender oils.

CONCLUSIONS

From the data presented it can be stated that aromatherapy is a natural and non-invasive gift for humans and animals. This form of therapy is not only used with a preventive role, but can also be used in the acute or chronic stages of a disease.

Most of the studies identified in this review support the use of lavender essential oil in the treatment of many pathologies, the most important of which is the healing of skin wounds, and suggest several unique mechanisms by which lavender oil can have beneficial effects on wound healing.

All these clinical and experimental animal studies obviously suggest the potential for the use of lavender oil and thus the benefits may increase the possibility of new approaches as complementary treatment in addition to conventional therapy.

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LUMBOSACRAL TRANSITIONAL VERTEBRA (LTV) IN CAT: A CASE REPORT

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Abstract

The observations of the study were focused on the lumbosacral region of the spine in cats in order to identify some peculiarities or abnormalities in the anatomical conformation regarding the number of the vertebra or other morphological aspects. For this purpose were analyzed the radiographs of the lumbosacral region of 63 feline patients of a veterinary clinic, both males and females, 1-9 years aged, which showed various symptoms, especially locomotor. The x-ray exam revealed sundries pathologies of the spine, but among them in a cat of European breed it was remarked an abnormal anatomical conformation of the lumbosacral spine consisting in the sacralization of the last lumbar vertebra. It was noticed that the last lumbar vertebra is fused with the first sacral one in a large degree, both vertebral bodies and the transverse processes being merged, the sacrum bone containing four sacral vertebra instead of three, as normally does. It was counted also three ventral sacral foramina, instead of two. The particular characteristics noticed conducted to the observation that the last lumbar vertebra represents in fact a lumbosacral transitional vertebra (LTV) which in cats occurs at a low rate.

Key words: cat, lumbosacral spine, sacralization, radiographs.

Along of the spine, the number of vertebrae differs from a region to another, but is relatively constant in a species. It is well known that the cervical region is the most constant, almost all the mammals having 7 cervical vertebrae (Brocal J. *et al*, 2018), few exceptions being known. The vertebral formula in domestic carnivores includes: 7 cervical vertebrae, 13 thoracic vertebrae, 7 lumbar vertebrae, 3 sacral vertebrae (fused, forming the sacrum bone) and a variable number (20-23) of caudal vertebrae (Coțofan V. *et al*, 1999; Konig H.E., Liebich H-G, 2010; Ferat-Postolache A. *et al*, 2021). Especially for the dogs, there are numerous studies which revealed that the last lumbar vertebra can be more or less fused with the first sacral vertebra, the process being named sacralization. It is reported also the lumbarization process when the first sacral vertebra is fused with the last lumbar vertebra, but in a lower rate. Lumbarization and sacralization produced similar abnormalities, so they were all considered together (Abutaher C.P., Avinash P.R., 2019; Deepa T.K., John M.K., 2014; Konin G.P., Walz D.M., 2010; Newitt L.M.A. *et al*, 2009). These processes can appear especially when exist a supernumerary vertebra in the lumbar region – 8LV, but also can occur when exist 7 lumbar vertebrae. This aspect is known as the lumbosacral transitional vertebra (LTV). It is considered a congenital anomaly and has morphological characteristics of both lumbar and sacral vertebrae (Damur-Djuric N. *et al*, 2006; Flückiger M.A. *et al*, 2009). Usually, these vertebrae have characteristics of both spinal segments, and they may be symmetrical or asymmetrical in morphology. Transitional vertebrae are considered to be an intermediate type of vertebra, formed due to the pelvis and vertebrae forming contact slightly cranial or caudal to their normal point of contact, resulting in the formative stimulus affecting the development of the transverse processes. It is postulated that if this contact is oblique, then asymmetry of the transitional vertebra will result (Newitt L.M.A. *et al*, 2009). These abnormalities can be clinically insignificant or can conduct to some pathologies of the spine, but not only (Damur-Djuric N. *et al*, 2006; Flückiger M.A. *et al*, 2006; Gong H. *et al*, 2020). If in dogs was reported a large incidence of LTV, especially in some breeds, in cats the presence of LTV is rare and there are no relevant studies in this way and for that we analyzed the morphology of lumbosacral region in cats to identify some of these abnormalities.

MATERIAL AND METHOD

The study included the cat patients of a veterinary clinic which presented various pathologies, especially locomotor symptoms. The animals were subjected to clinical examination and then to x-ray exam of the spine to identify the pathological process and for diagnosis. Were chosen those animals of whose lumbosacral spine was examined. The x-ray exam was realized in latero-lateral position and also, ventro-dorsal

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one. Were analyzed the radiographs of 63 cats, both males and females, 1-9 aged. A single animal, a cat of the European breed, 5 years, was identified with an abnormal anatomical conformation of the lumbosacral region and was selected for our observation.

RESULTS AND DISCUSSIONS

After the examination of the x-ray images of lumbosacral region, in a cat of common European breed, having 7 lumbar vertebrae, was observed that the last lumbar vertebra (7LV) is completely fused with the sacrum bone, being totally sacralized (*figure 1, 2, 3*).

From lateral view (*figure 1*) can be observed that the 7th lumbar vertebra is shorter, being almost entirely placed between the two iliac wings *(Ala ossis ilii)*, and is no evident intervertebral disk between its body *(Corpus vertebrae)* and the body of the first sacral vertebra (*Promontorium*) (N.A.V.). Ventro-dorsally (*figure 2, figure 3*) the same characteristics are evident, but also is visible that the transvers processes *(Processus transversus)* are shorter cranially and both are fused with those of the first sacral vertebra, being transformed in "wings". Because of this high degree of merging resulted another two large ventral sacral foramina *(Foramina sacralia ventralia)*, on each lateral side of the sacrum existing three such as foramina instead of two as in the normal sacrum bone (Cotofan V., 1999; Ferat-Postolache *et al*, 2021). Can be remarked the large contact between the iliac bones and the wings of the sacrum *(Ala sacralis)*, especially on the right side of the pelvic cavity. These characteristics make that the 7th lumbar vertebra to look more like a sacral vertebra. Due to these aspects we can talk about a case of transitional lumbosacral vertebra (LTV) in cat.

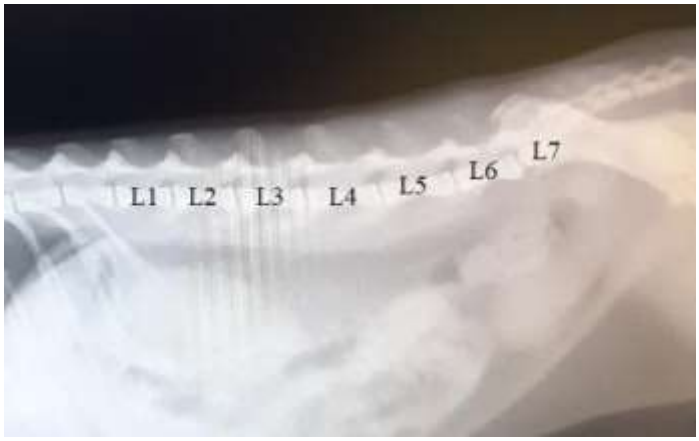

Figure 1. **European cat, 5 years – 7 lumbar vertebrae; the shortening and the sacralization of the last lumbar vertebra (L7) – no visible intervertebral disk; latero-lateral view**

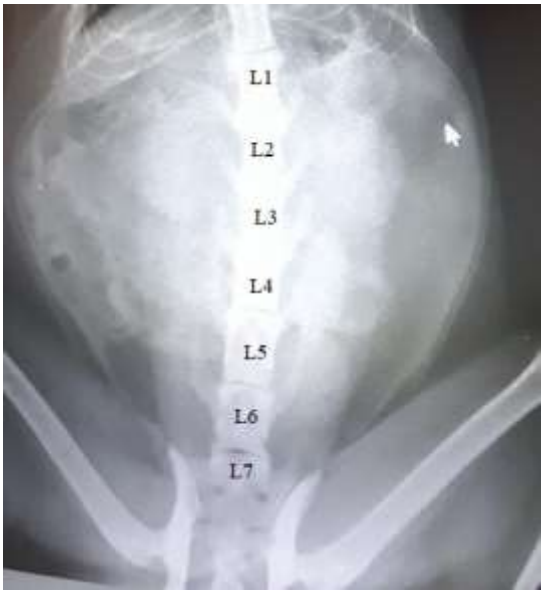

Figure 2. **European cat, 5 years – 7 lumbar vertebrae; the shortening and the sacralization of the last lumbar vertebra (L7); ventro-dorsal view**

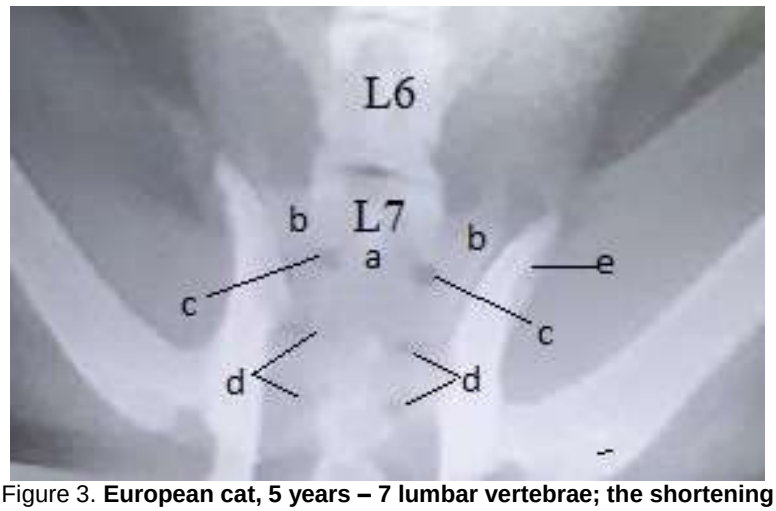

Figure 3. **European cat, 5 years – 7 lumbar vertebrae; the shortening and the sacralization of the last lumbar vertebra (L7); ventro-dorsal view – details: a-the body of the 7th lumbar vertebra; b-the transverse processes of the 7thLV-"wings"; c-"additional" ventral sacral foraminae; d-anatomical ventral sacral foraminae; e-iliac wing**

In dogs, the presence of LTV is more frequent than in cats, in particular German Shepherd being over-represented, leading to a suggestion that there may be a genetic predisposition to these abnormalities (Breit S. *et al*, 2003; Larsen J.S., 1977). In LTV, the vertebral bodies demonstrate varying morphology, ranging from broadened transverse process to complete fusion (Konin G.P., Walz D.M., 2010). These variations are affected by gender, developmental factors and race. It has been proved that this anomaly is seen to affect males more than females (Deepa T.K., John M.K., 2014). The LTV is considered a congenital anomaly, which occurs in various species of animals and in humans (Flückiger M.A. *et al*, 2006) and its presence can be clinically significant. In addition, a hereditary predisposition to LTV has been suggested (Damur-Djuric N. *et al*, 2006; Morgan J.P., 1999; Morgan J.P. *et al*, 1993; Morgan J.P. *et* al, 1999; Morgan J.P. *et al*, 2000; Wigger A. *et al*, 2009). The condition of the presence of LTV is known to predispose dogs to develop DLSS (degenerative lumbosacral stenosis) in dogs (Ondreka N. *et al*, 2013) and can lead to premature degeneration of the lumbosacral junction and cauda equina syndrome (CES). This aspect was particularly observed in German Shepherds (Flückiger M.A. *et al*, 2006; Flückiger M.A. *et al*, 2009; Morgan J.P. *et al*, 1993). There was reported that the dogs with an LTV were eight times more likely to develop CES than dogs without an LTV and German Shepherd dogs were eight times more likely to develop CES compared with other breeds. The same studies highlighted that the male dogs were twice as likely to develop CES than females. Dogs with an LTV develop CES 1-2 years earlier than dogs without an LTV (Flückiger M.A. *et al*, 2009). Asymmetric LTV types may lead to pelvic misalignment beyond the vertical axis, causing a unilateral load increase on the hip joint, which results in detrimental development of the hip joint (Damur-Djuric N. *et al*, 2006; Larsen J.S., 1977; Flückiger M.A. *et al*, 2009). Cauda equina syndrome is reported in cat (Hurov L, 1985; Hardie E.M. *et al*, 2002) but not in association with lumbosacral transitional vertebrae (Newitt A. *et* al, 2008). In cats, the studies identified no specific breed dispositions, but numbers identified in all breeds were low, so this cannot be entirely excluded. However, morphology between different breeds does not vary in the cat, unlike in dogs, and hence despite the fact that a number of breeds were used, these data are likely to be valid for the feline population as a whole (Newitt *et al*, 2009). Some studies (Newitt *et al*, 2009) described congenital abnormalities at all levels in the feline spine, predominantly transitional vertebrae, and found no association between transitional vertebrae and clinical signs. Newitt *et al.* (2008) examined the congenital (46 cases) and non-congenital abnormalities (154 cases) of the lumbosacral spine. The transitional vertebrae were the most common abnormality, being identified four cases of sacralization of L7 and 1 case of lumbarization of S1, but LTV was observed also in other regions of the spine as in the case of the thoracolumbar or sacrocaudal junction and the studies concluded that there is no evidence of association with clinical signs. Newitt *et al.* (2009), in a study that included 114 cats, identified 14 cases of LTV with a different morphology and found no significant differences in prevalence of spondylosis or hip abnormalities between all cats with lumbosacral transitional vertebrae and the control group and no association between the presence of a lumbosacral transitional vertebra and spondylosis and there

was no association between hip disease (osteoarthritis or hip dysplasia) and the presence of a transitional vertebra. Still, the study of Harris *et al*. (2018) on 13 cats with lumbosacral stenosis, revealed that despite lumbosacral stenosis is a rare spinal condition in cats, lumbosacral transitional vertebrae can be considered a risk factor for its development. In this way, seven cats (53.8%) were diagnosed with lumbosacral transitional vertebrae. In the control population of 405 cats, 24 (5.9%) were diagnosed with lumbosacral transitional vertebrae. Results indicated that lumbosacral transitional vertebrae were significantly more prevalent in cats with lumbosacral stenosis compared with the control feline population. Development of clinical signs of lumbosacral stenosis in cats with lumbosacral transitional vertebrae (mean 10.8 years) was not significantly different from that of cats without lumbosacral transitional vertebrae (mean 12.7 years). Likewise, there was no significant influence of breed or sex on the occurrence of lumbosacral transitional vertebrae.

CONCLUSIONS

As in dogs, LTV can appear in cats, but in a lower rate. The conformation of LTV in cats is also variable, in our case being present a high degree of fusion between the last lumbar vertebra and first sacral one. There are few studies that reveal the pathological implication of LTV in cats.

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CYTOLOGICAL AND HISTOPATHOLOGICAL DIAGNOSIS IN MAST CELL TUMORS IN COMPANION CARNIVORES

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Abstract

Mast cells tumours are considered a member of the round cell tumour group and are one of the most frequent cutaneous and subcutaneous neoplasms in companion carnivores (up to 21%), and are more common in dogs than in cats.They can be diagnosed both through cytopathological and histopathological diagnostic.

In 2020-2021 we diagnosed 11 mast cell tumours in dogs and 2 in cats, out of which 8 through cytological and 5 through histopathological examination. The formations were located in various parts of the body, both cutaneous and subcutaneous. The histopathological examination revealed round, oval, polygonal or even spindle-like cells that had basophilic, intracytoplasmic granules and a central, large, round nucleus. The presence of eosinophils was not observed in all the tumours. The mitotic index and anaplasia degree varied greatly, depending on the benign or malignant character of the tumour.

The cytological examination revealed round cells of a mesenchymal origin that presented basophilic granulations in various degrees. The number of granules varied from case to case, but generally speaking, these were much more easily observed in cytological slides than in the histopathological ones.

Key words: *mast cell, tumour, cytopathology, histopathology, diagnostic*

Mastocytomas are neoplasms that originate from the mast cells located in the dermis or subcutaneous fat that undergo malignant transformation (9). They are more common in dogs (10-20% of cutaneous tumours) than in cats and rarely found in other species (3, 12, 14). They can develop cutaneously or subcutaneously, the later ones sometimes displaying a higher polymorphism both in aspect and behaviour (Meuten), and are more frequent on the trunk and the limbs (6). They can also have internal localizations such as intestinal, oral, visceral or cranio-mediastinal, and may also metastasize in lymph nodes, spleen, liver and kidneys (4, 11).

Some authors even mention a generalized mast cell tumour, resembling a type of leukaemia, where solid neoplasms are associated with generalized mastocytosis (2).

MATERIAL AND METHOD

In 2020-2021 we diagnosed 11 mast cell tumours in dogs and 2 in cats, out of which 8 through cytological and 5 through histopathological examination.

The cytological examination was done following a fine needle aspiration with a 22G needle and a 5ml syringe, and staining of the obtained material using the May-Grunwald Giemsa method.

The histopathological examination was done on slides obtained through fixation of the tissue fragments in 10% formaldehyde solution, paraffin embedding, 5 µm sectioning and Masson trichrome staining.

All the slides were examined using a Leica DM750 optical microscope with an embedded camera.

The nodular formations may be single or multiple, ulcerated or not and may manifest various degrees of inflammation if traumatised (1).

The diagnostic of these tumours can be done either following a fine needle aspiration and a cytological examination of the obtained material or through histopathological examination following surgical resection. For a more in-depth diagnostic and confirmation immunohistochemistry is also a variant (9, 12).

This study aims to illustrate the major features that should be investigated when examining a mastocytoma through cytology or histopathology to set a correct diagnosis and to formulate a realistic prognosis.

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RESULTS AND DISCUSSIONS

The tumours were located in various parts of the body (head, ear pinnae, trunk), and were both cutaneous and subcutaneous. The macroscopical aspect was that of nodular formations of various sizes, relatively well delimited from the surrounding tissues. No metastases were found.

The histopathological examination revealed a homogenous population of round, oval, polygonal or even spindle-like cells that had basophilic, intracytoplasmic granules and a central, large, round nucleus.

The presence of eosinophils was observed in some tumours (Fig. 1, 3). It is considered that a normal tissular reaction would imply the presence of a relatively large number of eosinophils among proliferated mast cells due to the release of the substances contained within the intracytoplasmic granules. In some neoplasms, these additional cells were not present or were in very low numbers (Fig. 2). However, the number of eosinophils does not seem to correlate with the aggressiveness of the tumour (3).

The granulation pattern also varied between cases. We observed classical mast cells that contained a large number of cytoplasmic granules, sometimes masking the nucleus, but also situations where the granules were either poorly stained, in low numbers or lacking (Fig. 4). This one of the most important differential criteria between round cell tumours and the lack of specific granules can make for a more difficult diagnosis (10). In our practice, we found that both histology and cytology can be useful to highlight this feature.

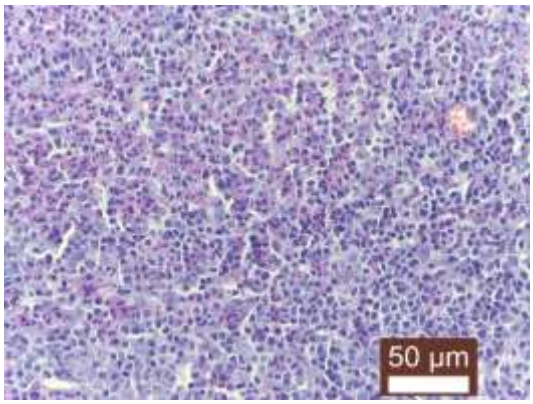

Fig. 1 – Cutaneous mastocytoma. Cords of mast cells and numerous eosinophils. Masson trichrome stain

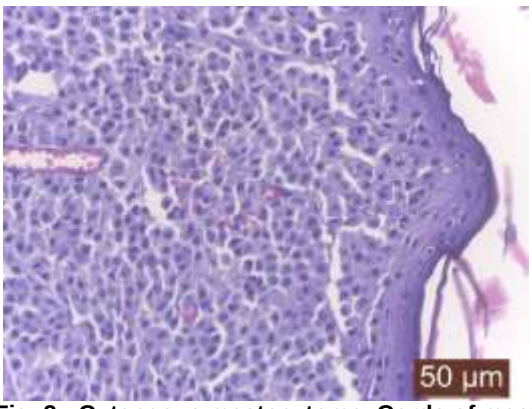

Fig. 2 - Cutaneous mastocytoma. Cords of mast cells without eosinophils. Masson trichrome stain

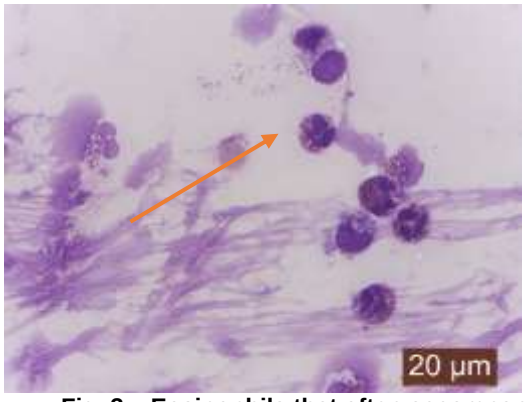

Fig. 3 – Eosinophils that often accompany mast cells in tumoral proliferation. MGG stain

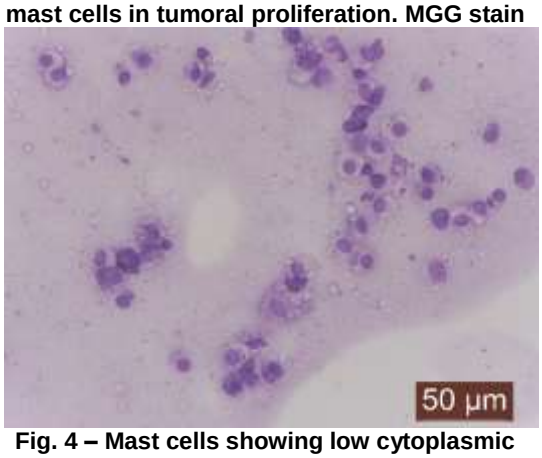

Fig. 4 – Mast cells showing low cytoplasmic granulation. MGG stain

Another feature that aided in evaluating the grade of the tumours is the consistency of cytological characteristics. We observed in some cases that the proliferated mast cell displayed marked anisokaryosis, different cellular shapes (epithelioid, spindle), variable number of nucleoli, nuclear indentations, and inconsistent chromatin pattern (Fig. 5). These features were seen both in histological and cytological preparations.

Also, in some slides, multinucleated cells were present (Fig. 6, 7, 8, 9) with two or even five nuclei. The binucleation is not usually used as a criterion for the grading of tumours, but it is a feature that may indicate an abnormal behaviour of the cells and may be observed both through

histology and cytology (3). The variation of cytological characteristics is considered an important indicator of malignancy (3).

The proliferative behaviour of the tumours varied greatly, with some neoplasms showing almost no mitotic figures whilst others displayed up to 6 mitotic figures on just one microscopical field with 1000x magnification power (Fig. 10, 11, 12).

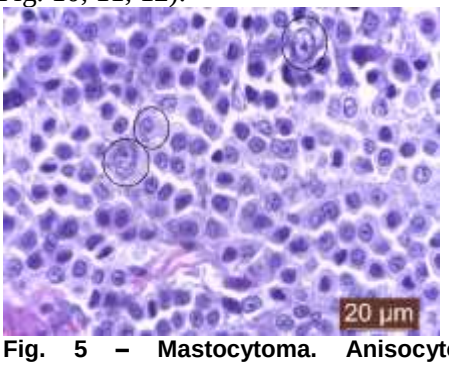

Fig. 5 – Mastocytoma. Anisocytosis, anisokaryosis, anisonucleoliosis. Masson trichrome stain

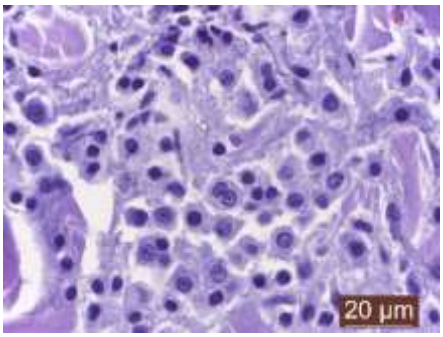

Fig. 6 - Binucleated cell. Mastocytoma. Masson trichrome stain

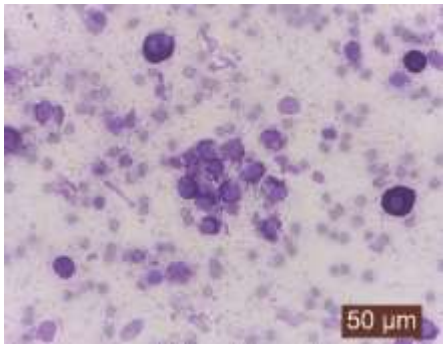

Fig. 7 – Binucleated cell. Mastocytoma. MGG stain

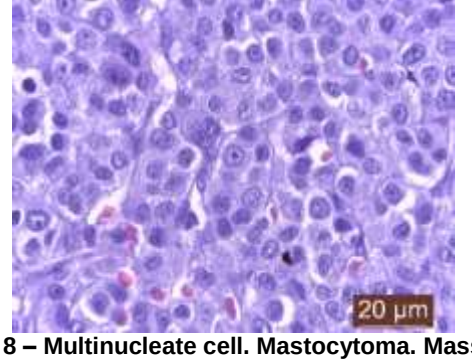

Fig. 8 – Multinucleate cell. Mastocytoma. Masson trichrome stain

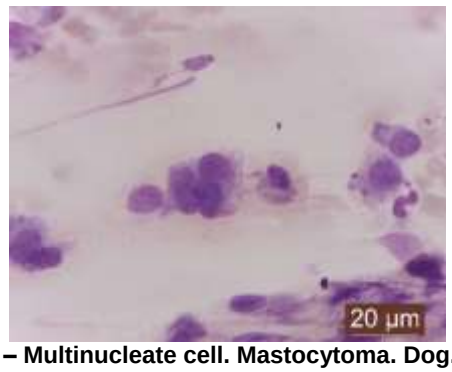

Fig. 9 – Multinucleate cell. Mastocytoma. Dog. MGG stain

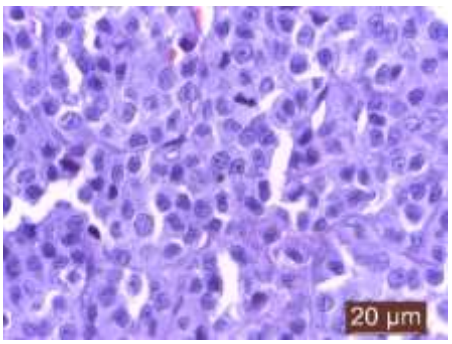

Fig. 10 – Mitosis. Mastocytoma. Masson trichrome stain

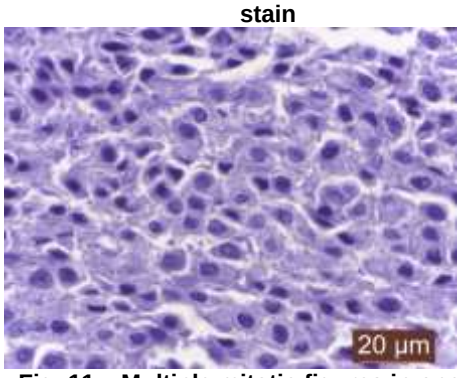

Fig. 11 – Multiple mitotic figures in one field with 1000x magnification power. Mastocytoma. Dog. Masson trichrome stain

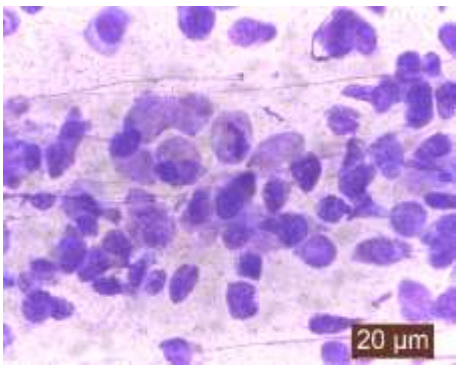

Fig. 12 – Mitotic figure observed through cytology. Mastocytoma. Dog. MGG stain

The cytological examination revealed round cells of a mesenchymal origin that presented basophilic granulations in various degrees. The number of granules varied from case to case, but generally speaking, these were much

more easily observed in cytological slides than in the histopathological ones.

For mast cell tumours a grading system is used to evaluate de malignancy of the neoplasm which categorizes as follows: grade I – well-differentiated, uniform cellular population, cytoplasmic granules present, low mitotic index; grade II – moderately differentiated cells, moderate mitotic index, infiltrative behaviour; grade III – high number of cellular atypia, high mitotic index, lack of cytoplasmic granules, deeply infiltrative behaviour (5). The grading system is meant to be applied on histological slides, but many features can be observed also through cytology, some authors considering that cellular atypia and multinucleation may even be more easily noticeable by using this technique (13).

Other authors have proposed a different grading system with is based only on the mitotic index, the presence of multinucleated cells and abnormal nuclei (7). Mitotic figures can be observed through cytology, as also the other characteristics.

Generally, the interpretation of this grading scheme is heavily influenced by the subjectivity of the examiner and can be inconsistent (8). However, grade II mast cell tumours are generally the most diagnosed type and most of them resolve through surgical excision with only 8% rate of recurrence (15).

CONCLUSIONS

The diagnosis and grading of cutaneous and subcutaneous mast cell tumours can be done both through histopathology and cytology, these techniques allowing for the identification of the necessary criteria.

We consider that when cytological samples are of good quality, the diagnostic and grading of a mast cell tumour can be done to a satisfactory level and with fewer costs or trauma to the patient

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MACROSCOPICAL LESIONS OF DIAGNOSTIC VALUE IN BOVINE PATHOLOGY

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Abstract

Beef is one of the most consumed meats in the world, preceded by pork and poultry. Thus, bovine health directly influences public health and there are risks to food biosecurity when diseases of a zoonotic, infectious or parasitic nature are detected in slaughterhouses.

During the 2 years of study carried out in the period 2018-2020, several cattle from the entire territory of Romania were examined necropsically or at the slaughterhouse, especially from the region of Moldova. Most cases were diagnosed with fasciolosis found in 11 cases of cattle, dichrocelliosis (was diagnosed in 7 cases of cattle), hydatidosis (5 cases), bovine tuberculosis (2 animals).

We found numerous acino-nodular foci of the caseous type affecting the lungs and the retropharyngeal, submandibular and mediastinal lymph nodes in tuberculosis; hypertrophic cirrhosis and severe angiocolitis. enlarged gallbladder, dilated bile ducts highlighted in the form of whitish cords on the visceral surface of the liver in fasciolosis; increased consistency of the liver, whitish trajectories consisting of ectasia of superficial bile ducts, lesions of chronic perihepatitis, cholangitis and pericanalicular cirrhosis in dichroceliosis and hydatids on the surface of the lung and liver in hydatidosis. All diagnostics were confirmed through histopathological and microbiological examinations.

The control and examination of the carcass and organs in the slaughterhouse is a very important action and contributes to public health, especially if zoonoses are discovered that can endanger human health.

Key words: bovine, slaughterhouse, zoonosis, tuberculosis, fasciolosis

Bovine tuberculosis is an infectious and contagious disease, with a zoonotic character, which has a nonspecific symptomatology and granulomatous lesions in tissues and organs.

The causative agent of cattle disease is *Mycobacterium bovis*. It is a Gram-positive bacillus, thick and short, straight or curved, not sporulated and not encapsulated.

The representative tinctorial affinity is acid-resistance, highlighted by the Ziehl-Neelsen staining. The cultivation is done in aerobiosis, and the most used media are: Lowenstein, glycerinated potato, Petroff, Petragnani, Dubos, Sauton.

Fasciolosis is a hepato-biliary trematodosis, clinically manifested by anemia, weight loss, jaundice, subcutaneous edema, diarrhea, ascites, and anatomopathologically angiocolitis, cirrhosis and traumatic hepatitis.

The etiological agent of parasitosis is *Fasciola hepatica*, with a foliaceous appearance, measuring 20-30 x 8-15 mm.

Bovine dictyocaulosis is a geohelminthosis, enzootic, evolving acutely and chronically, which parasitizes young in the trachea and bronchi, clinically manifested by severe respiratory syndrome and morphopathology by tracheobronchitis and bronchopneumonia.

The etiological agent is *Dictyocaulus viviparus*, which has an elongated, white body.

The pathogenetic mechanism depends on both the degree of infection and the age of the animals. The larvae on their way to the lymphatic system enter the intestinal barrier and traumatize it, causing inflammation and transient digestive disorders.

In the lung, the passage of larvae through the alveolar septa causes microhemorrhages and destruction of the alveolar walls. Inflammation is complicated by association germs.

Adult parasites by exerting mechanical action cause inflammation of the tracheobronchial mucosa and obstruction of the bronchi, causing respiratory disorders. Therefore, dyspnoea, atelectasis, suffocation attacks and emphysema are found.

Bovine hydatidosis is a severe parasitic zoonosis represented by hydatid cysts, of variable dimensions, which parasitize in the internal organs of both herbivores and omnivores and humans, clinically manifested by weakening syndrome, decreased production and disorders of parasitic

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organs, and morphopathologically by the presence on the surface of the organs of vesicular cystic formations.

The causative agent is the larvae of *Echinococcus granulosus*, which parasitize mainly in the liver and lungs of cattle, but also in other organs.

Hexacanthus embryos reach the intestinal mucosa and then traumatize the passenger to reach the target organs.

The parasitic vesicle develops in the organs of choice through mechanical and compressive action, causing atrophy of adjacent tissues, necrosis and fibrosis.

The development of specific vesicular formations in the mass of the liver and lungs leads superficial hydatids in the lung rupture, hydrothorax occurs as a result of pulmonary hypofunction.

Myocardial hydatids cause severe dysfunction due to fibrosis of the heart muscle.

The rupture of hydatid cysts in various organs due to trauma or exertion of animals leads to death.

The studies performed had as primary purpose the highlighting of macroscopic lesions with diagnostic value in the main pathologies encountered in cattle. In order to achieve the objectives of this work within a period of 2 years, a number of 25 cattle were examined after slaughter or autopsy.

MATERIAL AND METHOD

The studies aimed primarily to highlight macroscopic lesions with diagnostic value in the main pathologies encountered in cattle.

The research was carried out over a period of two years, the cattle were slaughtered and examined in the slaughterhouse of SC EMANUEL COM SRL, Botoșani County, Răchiți, Răchiți County, respectively SC SAMCOM MEAT SRL, Botoșani County, Cătămărăști-Deal, Mihai Eminescu County.

The studies were also performed in the Department of Pathological Anatomy, Forensic Medicine and Necropsic Diagnosis of the Faculty of Veterinary Medicine, Iasi, where the carcasses of cattle were necropsied and examined, but especially the samples collected from the above slaughterhouses. The necropsy room is located in Pavilion VI and is composed of: necropsy laboratory for the conduct of Forensic Medicine classes, corpse reception room, cold room.

RESULTS AND DISCUSSIONS

Most cases were diagnosed with fasciolosis, disease found in 11 cases of cattle (Fig. 5, 6), dichrocelliosis (was diagnosed in 7 cases of cattle – Fig. 7, 8), hydatidosis (5 cases – Fig. 9, 10), bovine tuberculosis (2 animals – Fig. 1-4).

We found numerous acino-nodular foci of the caseous type affecting the lungs and the retropharyngeal, submandibular and mediastinal lymph nodes in tuberculosis; hypertrophic cirrhosis and severe angiocolitis, enlarged gallbladder, dilated bile ducts highlighted in the form of whitish cords on the visceral surface of the liver in fasciolosis; increased consistency of the liver, whitish trajectories consisting of ectasia of superficial bile ducts, lesions of chronic perihepatitis, cholangitis and pericanalicular cirrhosis in dichroceliosis and hydatids on the surface of the lung and liver in hydatidosis. All diagnostics were confirmed through histopathological and microbiological examinations.

Fig. 1 Cattle, Simmental breed, age 9, slaughtered on 09.10.2018: Generalized bovine tuberculosis

Fig. 2 Cattle, Black spotted romanian, age 8 years, slaughtered on 10.07.2019: Nodal tuberculosis - tuberculous granulomatous lymphadenitis

The control and examination of the carcass and organs in the slaughterhouse is a very

important action and contributes to public health, especially if zoonoses are discovered that can endanger human health.

Fig. 3 Cattle, Black spotted romanian, age 8 years, slaughtered on 10.07.2019: Nodal tuberculosis - tuberculous granulomatous lymphadenitis

Fig. 4 Cattle, Black spotted romanian, age 8 years, slaughtered on 10.07.2019: Nodal tuberculosis - tuberculous granulomatous lymphadenitis

Fig. 5 Bovine, Black spotted romanian, 16 years old, slaughtered on 13.08.2019: Bovine fasciolosis

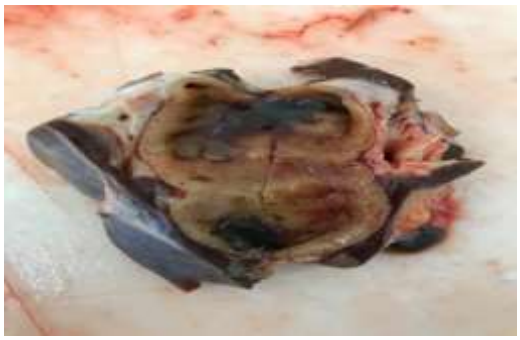

Fig. 6 Bovine, Black spotted romanian breed, 16 years old, slaughtered on 13.08.2019: Bovine fasciolosis

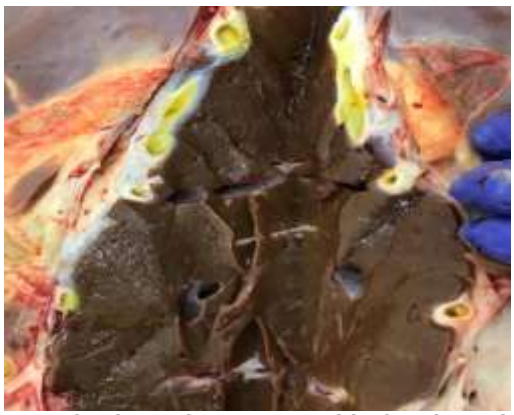

Fig. 7 Holstein cattle, 13 years old, slaughtered on 16.09.2019: Bovine dichrocelliosis

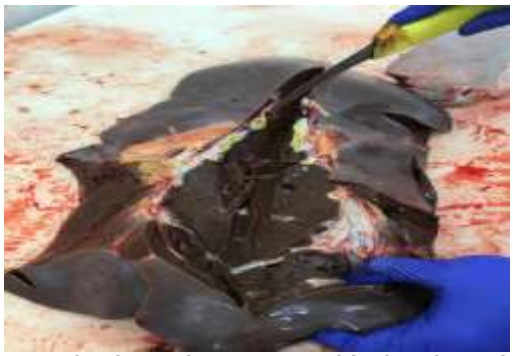

Fig. 8 Holstein cattle, 13 years old, slaughtered on 16.09.2019: Bovine dichrocelliosis

Fig. 9 Cattle, Mixed breed, age 15, slaughtered on 12.02.2020: Bovine hydatidosis

Fig. 10 Cattle, Mixed breed, age 15, slaughtered on 12.02.2020: Bovine hydatidosis

CONCLUSIONS

In the case of diseases with parasitic etiology, the macroscopic lesions were sufficient to elucidate the diagnosis, however, for a detailed description of the changes encountered, samples were taken for histopathological examinations.

The parasitosis identified in most cases does not cause mortality, but causes a decrease in the productive yield of cattle, and at the time of slaughter the affected organs are confiscated, confirming their economic importance.

The control and examination of the carcass and organs in the slaughterhouse is a very important action and contributes to public health, especially if zoonoses are discovered that may endanger human health.

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INVESTIGATIONS INTO THE PRESENCE OF VIRAL INFECTIONS IN ANIMALS OF HUNTING INTEREST IN NORTHEASTERN ROMANIA

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Abstract

Forest ecosystems are characterized by a great diversity but at the same time by a great capacity for self-regulation, self-reproduction and stability. They still have dominance of the bioecology and pathology of the animals still incompletely elucidated and, respectively, controlled. The following species of wild animals of hunting interest were studied: deer (*Dama dama*), wild boar (*Sus scrofa ferus*) and fox (*Canis vulpes*) and the diseases studied are: African swine fever in wild boars, specific diseases of deers and Rabies in foxes. The study provides support for preventive management actions aimed at protecting the public health and the economy.

Key words: public health; economy; African swine fever; hunting

INTRODUCTION

Romania's integration in the economic and social structures of the European Community is not possible without the alignment of the Romanian scientific research to the community priorities and, especially, to the methodology of the true research. In the fields of biology, human health and veterinary health, the identification of problems can be done by small research teams or even by isolated researchers, but solving them requires, however, broad collaborations between different research and development entities, economic units and public administration.

Forest ecosystems are characterized by a great diversity but at the same time by a great capacity for self-regulation, self-reproduction and stability. They still have dominance of the bioecology and pathology of the animals still incompletely elucidated and, respectively, controlled.

PURPOSE OF THE WORK

The mai purpose of the research is to analyze the epidemiological and zoonotic impact associated with some species of hunting interest in the N-E area of Romania. The following species of wild animals of hunting interest were studied: deer (*Dama dama*), wild boar (*Sus scrofa ferus*) and fox (*Canis vulpes*) and the diseases studied are: African swine fever in wild boars, specific diseases of deer and Rabies in foxes.

We consider the wildlife in Romania an important source of public income that is superficially exploited, partly due to the fact that the biological and health needs of different species of animals of hunting interest are not fully resolved. In addition, many of the wild species are the natural reservoir of many domestic animal diseases, to which is added the presence in wildlife populations of some very serious zoonoses, which make the wild animal a factor of biological pollution of the environment and of permanent risk to public health.

MATERIAL AND METHOD

The research in this study was carried out in Neamt county. The DSVSA database was accessed to obtain informations.

Compared to 2020, if we talk about materials and methods, this year were analyzed for rabies surveillance 157 samples by direct immunofluorescence (IFD) and 5 samples by the intracerebral inoculation test of mice (bioprob). Also for rabies surveillance, 152 mandibular samples from shot foxes were analyzed for biomarker determination control. 152 thoracoabdominal fluid samples for serological control ELISA postvaccine antibodies were also analyzed.

For African swine fever, 744 sets of organs from wild boar were tested by ELISA and 734 blood samples on EDTA for RT-PCR examinations.

For the surveillance of classical swine fever, 254 sets of organs from wild boar were analyzed by ELISA and 254 blood samples on EDTA for examinations in the direction of RT-PCR.

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For the spongiform encephalopathies, 30 blood samples on EDTA were analyzed for examinations in the direction of RT-PCR.

Compare to 2021 (01.01.2021-30.06.2021), this year were analyzed for the surveillance of rabies 100 samples by IFD. In 2021, the oral vaccination campaign for foxes in Romania was not started.

For African swine fever, 230 sets of organs from wild boar were tested by ELISA and 230 blood samples on EDTA for RT-PCR examinations.

For the surveillance of classical swine fever, 251 sets of organs from wild boar were analyzed by ELISA and 230 blood samples on EDTA for examinations in the direction of RT-PCR.

For the spongiform encephalopathies, 7 blood samples on EDTA were analyzed for examinations in the direction of RT-PCR.

RESULTS AND DISCUSSION

Following the examinations of the serum and organs samples collected from the animals hunted on the territory of the Neamt county during 01.01.2020-30.06.2021, the following were found:

1. The number of serum and organ samples examined was significant and the examinations performed were the most reliable and recommended for supervised diagnoses (Table no.1 and table no.2);
2. Both the serological examination by ELISA and the examination of organ samples by RT-PCR were negative (Table no.1 and table no.2);
3. Rapid serological examination by ELISA for surveillance of spongiform encephalopathies in cervids was also negative (Table no.1 and table no.2).

CONCLUSIONS

1. Wildlife tank zoonoses are a major public health problem affecting all continents. Hundreds of pathogens and many different modes of transmission are involved and many factors influence the epidemiology of different zoonoses. The importance and recognition of wildlife as a reservoir of zoonoses are growing. Cost-effective prevention and control of these zoonoses requires an interdisciplinary approach and international cooperation. Surveillance, research, training, education and communication are key elements.
2. Throughout history, wildlife has been an important source of infectious diseases transmitted to humans. The importance of such zoonoses is increasingly recognized and more attention is needed in this area.

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Ordinul nr. 35/2016 al Președintelui ANSVSA pentru aprobarea Normelor metodologice de aplicare a Programului acţiunilor de supraveghere, prevenire, control şi eradicare a bolilor la animale, a celor transmisibile de la animale la om, protecţia animalelor şi protecţia mediului, de identificare şi înregistrare a bovinelor, suinelor, ovinelor, caprinelor şi ecvideelor, precum şi a Normelor metodologice de aplicare a Programului de supraveghere şi control în domeniul siguranţei alimentelor;
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Regulamentul (CE) nr.338/97 al Consiliului Europei din 9 decembrie 1996 privind protecția speciilor de faună și floră sălbatică;

Table no.1

01.01.2020-31.12.2020

Nr. crt.	Animals examined	Diseases investigated	Number of samples		Results		
			Blood samples	Organs	ELISA	RT-PCR	IFD
1	Wild boar	CSF	254	254	Negative	Negative	-
		ASF	734	744	Negative	Negative	-
2	Deer	Spongiform encephalopathies	30	-	Negative	Negative	-
3	Foxes	Rabies	-	155	-	-	Negative

Table no. 2

01.01.2021-30.06.2021

Nr. crt.	Animals examinated	Diseases inveestigated	Number of samples		Results		
			Blood samples	Organs	Test ELISA	RT-PCR	IFD
1	Wild boar	CSF	230	251	Negative	Negative	-
		ASF	230	230	Negative	Negative	-
2	Deer	Spongiform encephalopathies	7	-	Negative	Negative	-
3	Foxes	Rabies	-	100	-	-	Negative

CORRELATIONS REGARDING THE DIAGNOSIS AND THE OPTIMAL THERAPEUTIC PROTOCOL IN CANINE BABESIOSIS

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Abstract

Canine babesiosis is one of the most important vector-borne diseases worldwide that affects dogs regardless of age, breed or gender. The aim of this study was to corroborate the clinical signs of canine patients confirmed with babesiosis, the results of paraclinical investigations, as well as the choice of the therapeutic protocol. The present study was performed on 42 dogs referred to the Clinic of Parasitic Diseases from the Faculty of Veterinary Medicine of Iasi with similar symptoms to canine babesiosis. After recording data regarding age, breed and gender of all dogs and the clinical examination of the patients, two peripheral blood samples were collected from each patient for the following investigations: Diff-quick stained blood smears and blood tests (hematological, biochemical, serologic). After analysis of blood smear, all the dogs (42/42) were positive for *Babesia* spp. and the most common clinical signs identified were: fever – 37/42 (88,1%), pale mucous membrane – 31/42 (73,8%) and hemoglobinuria – 31/42 (73,8%). The results of hematologic tests revealed thrombocythopenia – 40/42 and moderate to severe anemia. Further serological tests detected *Babesia gibsoni* antibodies in 2/42 blood samples. Depending on the results of the blood tests, the therapeutic dose of imizole was administered in a single dose or divided into two doses, administered within a maximum of 12 hours. In conclusion, the present study emphasizes the importance of paraclinical investigations in order to identify possible co-infections and adjust treatment in infected dogs.

Keywords: canine babesiosis;diagnosis; treatment;

INTRODUCTION

Canine vector-borne diseases include several types of pathogens (bacteria, parasites, viruses) that cause a variety of health problems for dogs (Irwin P.J., 2014). In Romania, one of the most important vector-borne diseases is represented by babesiosis. Canine babesiosis is caused by a protozoan from the genus Babesia, transmitted through many species of ticks (Grey J.S. et al., 2019). There are several, genetically different species of babesia: large where merozoites measure between 3-5µm (Babesia canis canis, Babesia canis vogeli, Babesia canis rossi) and small, where merozoites measure between 1,5-2,5 µm (Babesia conradae, Babesia gibsoni, Babesia microti-like) (Imre M. et al., 2013; Tudor P. et al., 2008). Dermacentor reticulatus and Ixodes ricinus are the species of ticks incriminated for the development of the disease in the Eastern part of Romania. The increase in spreading of arthropod vectors globally, as well as the association of canine vector-borne diseases can be explained by the change in climatic and ecological factors but also by the increase of the mobility of humans and animals, thus determining the global spread of canine vector-borne diseases (Andersson M., et al., 2017).

Vector diseases are considered real challenges for veterinarians, as clinical signs can sometimes be diffuse or overlapping with other vector-borne pathogens (Andersson M., et al., 2017; Grey J.S. et al., 2019). The diagnosis of babesiosis in dogs is based on corroborating the anamnesis, clinical signs (pale mucous membranes, hemoglobinuria, hyperthermia, icterus) and identification of intraerythrocytic piroplasmas in the cytological blood smears (Ionita M., et al., 2012).

MATERIALS AND METHODS

This study included 42 dogs suspected of babesiosis based on the anamnesis and clinical signs. After general clinical examination of the patients and transcription of medical history, two peripheral blood samples (EDTA and Clot activator) were collected from each patient for the following investigations: microscopic examination of the blood smear, hematological test, blood biochemical examination and two serological tests: SNAP 4DX Plus (IDEXX), respectively Babesia gibsoni antibody test (WellTest).

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Blood collection can be performed in two ways, in order to highlight hemoparasites: in vacutainer tubes, from the peripheral veins (brachycephalic, saphenous or jugular) or the capillary circulation (from the nose) (only for blood smears).
The smears are stained immediately after drying and fixation, to reduce the risk of erythrocyte modification. We used Diferential Quick stain kit (Modified Giemsa) according to the manufacturer's instructions for identification of babesia merozoites.

After confirmation of the diagnosis, the two blood samples collected were analyzed to determine the degree and the type of anemia, as well as to check the renal and hepatic function, frequently affected in canine babesiosis. These indicators are very important for adjusting the optimal therapeutic protocol in order to recover patients. The serum has also been used to check for possible co-infections, often found in canine tick-borne diseases. Therefore, we chose to use the SNAP 4DX Plus (IDEXX) test, for identification of 4 possible pathogens: Ehrlichia canis, Anaplasma platys/Anaplasma phagocythophillum, Borrelia burgdorferi and Dirofilaria immitis while for the detection Babesia gibsoni, we used Babesia gibsoni antibody test (WellTest). Both rapid tests were used according to the manufacturers' instructions.

RESULTS AND DISCUSSION

The results of the study showed that the most affected dogs were common or mixed breed - 40.48% (17/42), followed by the Pekingese - 11.9% (5/42), and with 7,14% (3/42): Husky, German Shepherd and Caucasian Shepherd.

Dogs can become infected with *Babesia spp.* regardless of age. It can be seen in Table 1 that the differences between the number of cases in different age categories are small.

Regarding the sex of dogs, the number of infestations in males was higher (66.66%) compared to females (33.34%).

According to the data obtained from the anamnesis reported in Table 2, we found that 76.19% (32/42) of the dogs participating in the study were not externally dewormed according to the recommendations of veterinarians. The lack of awareness of the owners by postponing external deworming, leads to an increase in the cases of vector-borne diseases, which can be fatal in some situations.

Among the patients included in this study, 88.1% of the dogs were brought in consultation for apathy, 83.3% for lack of appetite and 73.8% for the presence of dark urine.

Clinical examination is very important for an accurate diagnosis. The clinical signs in canine babesiosis are not specific, but the data from the anamnesis corroborated with the clinical examination and the cytological examination of the blood, are sufficient for diagnosis.

In babesiosis, the main clinical signs are: lethargy, depression, fever, pale mucous membranes, jaundice, hemoglobinuria, splenomegaly, hepatomegaly. The most common clinical signs found in the 42 dogs studied were: fever - 37/42 (88.1%), pale mucous membranes - 31/42 (73.8%) and hemoglobinuria - 31/42 (73, 8%), followed by dehydration and jaundice (Figure 1). Morphological examination of the blood smears is the fastest method of diagnosis. It is also often used due to its low cost.

To confirm parasitic infestation with small species of *Babesia spp. (Babesia gibsoni, Babesia microti)* the definite diagnosis is given only by using molecular biology techniques. For all blood samples collected, between 3-5 smears were performed, and at least 50 microscopic fields were examined x100 objective. All blood samples were positive for *Babesia canis* (Figure 2).

Due to the pathogenesis, the most important change in the blood count is found in the red blood cells, as canine babesiosis often develops with moderate to severe anemia. Thus, red blood cells, hemoglobin and hematocrit will have low values and following the hemorrhagic diathesis, a decrease in platelets can also be observed. These aspects can also be noticed in the case of the 42 dogs studied, presented in Table 3, where 30/42 of the patients had low red blood cell counts, and 34/42 had hematocrit below normal limits. In patients with hematological values within normal limits, the degree of parasitaemia was low and the clinical signs were unstable. Thrombocytopenia was found in 40/42 canine patients.

Analyzing the 42 biochemical blood results, we found increased values of renal parameters (BUN - 24/42), liver enzymes (GGT - 25/42), alkaline phosphatase (29/42) and total bilirubin (22/42). Also, Table 3 shows normal values of creatinine (CRE - 32/42) or alanine aminotransferase (ALT - 25/42), which led to a faster recovery of patients after imizole administration.

The 42 serum samples were analyzed using the SNAP 4DX (IDEXX) and *Babesia gibsoni* Atc rapid tests (WellTest) to identify possible co-infections with other pathogens transmitted through ticks.

Table 1 - Prevalence of positive cases of canine babesiosis in relation to breed, age and sex

	Number of positive dogs/total dogs	%
Breed		
Mixed/common breed	17/42	40,48%
Husky	3/42	7,14%
Pekingese	5/42	11,9%
Labrador	1/42	2,38%
Shar-pei	1/42	2,38%
English Setter	1/42	2,38%
Caucasian Shepherd	3/42	7,14%
Samoyed	2/42	4,76%
Bichon	2/42	4,76%
Akita Inu	2/42	4,76%
Yorkshire terrier	1/42	2,38%
German Shepherd	3/42	7,14%
Bernese Shepherd	1/42	2,38%
Age		
0-1 year	7/42	16,7%
1-5 years	12/42	28,6%
5-7 years	11/42	26,2%
7-15 years	12/42	28,6%
Sex		
Females	14/42	33,34%
Males	28/42	66,66%

% - percentage

Table 2 - The informations obtained from the owners (the anamnesis)

	Number of positive dogs/total dogs	%
Without external deworming	32/42	76,19%
Apathy	37/42	88,1%
Inappetence	35/42	83,3%
Dark urine	31/42	73,8%
Ticks found on the body / observed by the owner and removed	28/42	66,7%
Vomiting	18/42	19,05%
Locomotor problems	5/42	11,9%
No medical history	10/42	23,81%

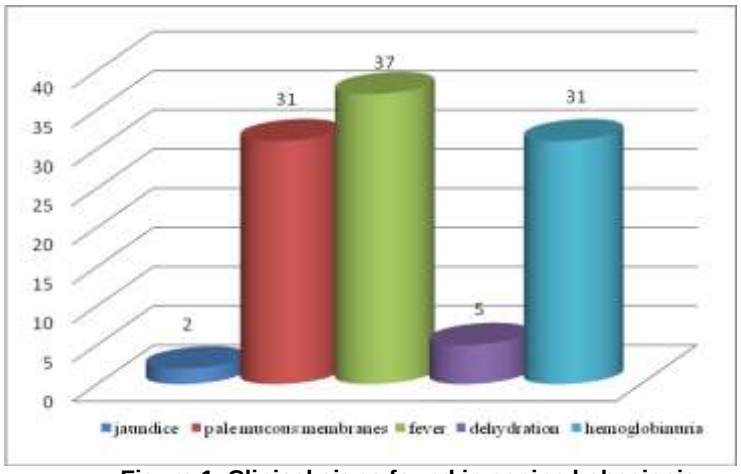

Figure 1 Clinical signs found in canine babesiosis

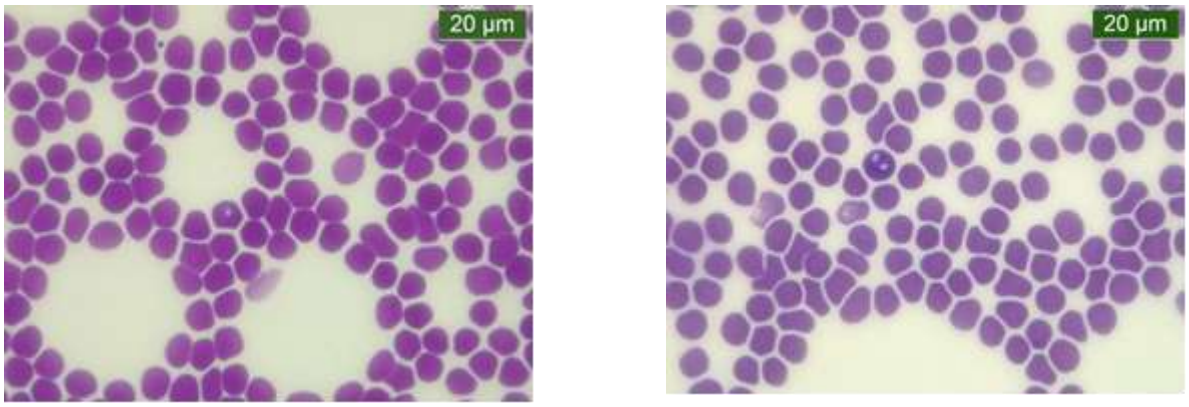

Figure 2 Babesia canis – blood smear, x100

Table 3 - The results of blood tests

Parameter	Low values	Normal values	High values	Total number of analyzes
		Hematological examination		
WBC	22	16	4	42
LYM	19	22	1	42
MON	11	31	0	42
NEU	12	27	3	42
EOS	0	32	10	42
BAS	0	42	0	42
RBC	30	11	1	42
HGB	25	15	2	42
HCT	34	8	0	42
MCV	7	35	0	42
MCH	2	28	12	42
PLT	40	2	0	42
		Biochemical examination		
ALP	0	13	29	42
ALT	0	25	17	42
TBIL	0	20	22	42
BUN	2	16	24	42
CRE	0	32	10	42
TP	17	25	0	42
GLOB	20	21	1	42
GLU	0	19	23	42
GGT	5	12	25	42

Corroborating the results of serological tests, we found the presence of *Babesia gibsoni* antibodies in 2/42 blood samples (Figure 3) and 0/42 positive samples for infections with *Ehrlichia* spp., *Anaplasma* spp. or *Borrelia* spp.

Figure 3 *Babesia gibsoni* Atc. test - positive

Therapeutic protocol

All patients diagnosed with canine babesiosis entered the following protocol, after receiving the written consent of the owners:

- Rehydration with NaCl, depending on the degree of dehydration;
- Administration of atropine sulfate (Atropine sulfuric 1%), in a dose of 0.1-0.2 ml and waiting 10 minutes or hydrocortisone hemisuccinate;
- Administration of Imidocarb dipropionate (Imizol) at a dose of 6.6 mg / kg (Table 4);
- 30 minute monitoring in case of side effects;
- Nutritional supplements, vitamins.

Table 4 - Therapeutic protocol in canine babesiosis

Activ substance	Dose (mg/kg)	Route of adm.	Interval (hours)	Duration of treatment (days)	Pathogen	
					B. canis	*B. gibsoni*
Imidocarb dipropionate	5-6,6 7,5	IM/SC IM/SC	1 adm. 1 adm.	Repeat in 14 Not necessary	+++	+
Diminazene aceturate	3,5-5	IM	1 adm.	Not necessary	+++	++
Azithromycin and Atovaquone	10 13,3	PO PO	24 8	10 10	+++	+++
Clindamycin and Doxycycline and Metronidazole	25 5 15	PO PO PO	12 12 12	90 90 90	+	+
Phenamidine isethionate (Lomadin, Fenamidin)	15-20	SC	24	2	+++	++

References: *Sykes, J. E., 2013*; *Greene, C. E., 2012*
Adm. - Administration

Some dogs may develop a number of side effects, such as parasympathetic symptoms after administration of imidocarb dipropionate (Imizol): sialoree, vomiting, general weakness. These symptoms can be prevented by administering a dose of atropine sulfate.

Depending on the results of blood biochemical tests, the therapeutic dose of imizole was administered in a single dose or in two divided doses, administered within a maximum of 12 hours.

The fractional therapeutic dose was administered only in the following cases: geriatric patient, liver or kidney failure, gestation or patients with chronic pathologies.

Improving of the general condition of positive patients with babesiosis usually occurs in 24-72 hours, but some dogs can heal in 4-7 days. Continuous monitoring of hematological parameters is performed until they return to normal limits.

In dogs infested with Babesia gibsoni, the PCR test should be performed 60-90 days after treatment with atovaquone and azithromycin (Irwin P.J., 2014).

CONCLUSION

The study was performed on a group of 42 canine patients suspected of babesiosis, which were presented to the Medical Clinics and Parasitic Diseases Clinics within the Faculty of Veterinary Medicine Iasi. The most affected dogs were those of common or mixed breed - 40.48%, followed by the Pekingese - 11.9%, regardless of age. The most common clinical signs were: fever - 37/42 (88.1%), pale mucous membranes - 31/42 (73.8%) and hemoglobinuria - 31/42 (73.8%).

We observed changes in hematological tests - moderate to severe anemia (decreased red blood cells, hematocrit and hemoglobin) and thrombocytopenia - 40/42 canine patients; changes in renal and hepatic parameters, resulted from the biochemical blood examination. We detected Babesia gibsoni antibodies in 2/42 blood samples positive at canine babesiosis, which showed a moderate form of infestation with Babesia canis and responded to treatment with Imidocarb dipropionate.

Depending on the results of biochemical blood tests, the therapeutic dose of imizole was administered in a single dose or in two divided doses, administered within a maximum of 12 hours.

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THE ANTI-INFLAMMATORY EFFECTS OF TURMERIC IN LOCOMOTOR DISORDERS: A SHORT REVIEW

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Abstract

Pain and inflammation management in the medical field are necessary in every pathology regardless of the origin. When it comes to the veterinary field, pain and inflammation reduction is the key to full recovery, considering the animal patient's behavior in such cases. Also, because the medication may in some cases be inaccessible or hard to be administered, veterinarians and owners should take alternative medicine into account, as many literature researches had indicated high efficacy. This research aims to analyze the latest discoveries regarding the inflammation and pain management through curcumin treatment.

Keywords: musculoskeletal; pathology; alternative; medicine; curcumin; inflammation; pain.

INTRODUCTION

Ethno-veterinary practices research is a burgeoning area of inter-disciplinary research with enormous potential for learning many aspects of folk knowledge on domesticated animals. Medicinal plants and their therapeutic benefits in livestock care are attracting the attention of an increasing number of natural and social scientists, veterinary practitioners, livestock owners, and field workers in developing nations. The community-based local or indigenous knowledge and techniques of caring for, healing, and managing animals is a simple definition of ethno-veterinary medicine. This encompasses social practices as well as the integration of animals into systems. Ethno-veterinary medicine is the knowledge of local people about folk beliefs, skills, procedures, and practices related to animal health and production. Close observation of animals and/or oral transmission of experience from one generation to the next provide the foundation for this knowledge. Although it is or well nourished among pastoral nomads all over the world, most rural and tribal communities have this rich reservoir of local knowledge on practically all aspects of animals [Misra et al., 2004; Dejonckheere et al., 2016].

Curcumin is the yellow pigment found in turmeric (*Curcuma longa L.*), India's most popular spice and a key component of curry powders.

Turmeric has a rich history of medical usage, particularly for the treatment of inflammation, and many of its traditional uses have been mechanistically verified in cellular systems and animal disease models. Curcumin is one of the most researched botanical compounds in the biomedical literature, with about 3,000 preclinical studies. Curcumin acts as a master switch of inflammation, regulating pro-inflammatory enzymes (cyclooxygenases [COX] and lipoxygenases) as well as inflammatory transcription factors (nuclear factor-kappaB [NF-kB] and signal transducer and activator of transcription 3 [STAT3]) and their genomic expression, according to these studies. The majority of curcumin's therapeutic effects are suggested by epidemiological studies, corroborated by animal model studies, and extrapolated from in vitro studies, but not clinically validated [Misra et al., 2004; Clutterback et al., 2013].

Curcumin's poor stability, which is particularly unstable at gastrointestinal pH (half-life at pH 7 <10 min), and limited oral absorption contribute to this perplexing predicament. After oral administration of doses as high as 12 g/day, plasma concentrations of phase II metabolites (glucuronides and sulfates) hardly approach 50 ng/mL. Curcumin, once in the plasma, has a surprising stability and even permeability to hard-to-reach regions like the brain [Misra et al., 2004; Wynn et al., 2007].

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Like most dietary phenolics, curcumin is water and fat soluble in small amounts. It has polar groups (two phenolic hydroxyls and one enolic hydroxyl) that can engage with a complimentary group via hydrogen bonds and polar interactions, similar to phospholipid polar heads. Phenolics have a high affinity for biological membranes and, once complexed with phospholipids, are incorporated in a lipid matrix that protects them from hydrolytic degradation while allowing them to take advantage of the rapid exchange of phospholipids between biological membranes and extracellular fluids, which can lead to increased cellular uptake. These principles underpin the phytosome method for increasing phenolic bioavailability, and they have now been effectively applied to curcumin,5,6 a patented compound with phosphatidylcholine known as the Curcumin-phosphatidylcholine Complex [Misra et al., 2004].

This research assessed the long-term efficacy and safety of this curcumin in the management of different musculoskeletal pathologies, conditions in need of novel therapeutic options, based on very promising results in terms of improved hydrolytical stability, human pharmacokinetics, and previous clinical studies [Misra et al., 2004].

RESULTS

Turmeric has anti-inflammatory and antioxidant qualities, which help to eliminate free radicals, which are responsible for cell damage in the body. People who suffer from arthritis can benefit greatly from utilizing turmeric because of this feature and the same may be mentioned for the veterinary field [Verma et al., 2018].

[Caterino et al., 2021] has done a research upon the effect of turmeric in combination with boswellic acid in the treatment of osteoarthritis (OA) in dogs. There were twenty dogs included in the study (11 male, out of which only 1 neutered and 9 females, out of which 5 were neutered).

The dogs were separated into two groups at random: treatment (A) and control (B). Five dogs in the A group had elbow joint OA and five had stifle OA; four dogs in the B group had elbow joint OA and six had stifle OA. The mean body weight (BW) for groups A and B was 35.04 ±4.89 kg and 35.36 ±4.66 kg, respectively. In group A, the mean standard deviation OA grade was 2.3 0.48, while in group B, it was 2.2 0.63. There was no statistical difference in GFRs (glomerular filtration rates) or BW between the two groups at T0, indicating that the groups were homogeneous at that time [Caterino et al., 2021].

Because curcumin and boswellic acid are chemically distinct, their targets are likely to be distinct as well, therefore a combination of the two could explain their synergistic activity, which has been documented in both human and veterinary literature. The clinical significance of our findings implies that curcuma and boswellic acid have a key function in inflammation and pain alleviation, which is also supported by the literature [Caterino et al., 2021].

OA is a chronic degenerative disease for which there is no cure. The most typical treatment combines NAISDs (Non-steroidal anti-inflammatory drugs) and nutraceuticals in a multimodal manner. Although, in veterinary medicine, the use of NSAIDs is limited due to long-term adverse effects. They treated dogs with a mild degree of OA exclusively with nutraceuticals administration for 90 days in this clinical research, with objectively satisfactory results observable after 60 days from the end of the treatment [Caterino et al., 2021].

Because forelimb PVF is generally higher than hind limbs, comparing gait analysis data from the thoracic and pelvic limbs in lame dogs may make data interpretation more complex. Data analysis at T0 revealed that the two groups were homogeneous in terms of OA localisation, score, GFRs, and BW, avoiding the need for rescaling, harmonization, or normalization, which is required in comparison research. Peak Vertical Force (PVF) and vertical impulse (VI) are two useful indices for assessing limb function. PVF is defined as the greatest force exerted perpendicular to the surface during the stance phase (ST), while VI is the computed area under the vertical force curve over time [Caterino et al., 2021].

As a result, in a lameness dog, a lower PVF indicates a lower bear weight and, as a result, a lower ST and VI. Despite the lack of statistical significance, we saw an improvement in PVF in 16/20 (80%) of patients in our clinical experiment, indicating that the nutraceutical can help with pain perception and hence lameness. Furthermore, the dogs given curcumin and boswellic acid had larger and more consistent overall PVF percent BW mean values until the study's end point than the control group [Caterino et al., 2021].

Furthermore, a statistically significant improvement in VI percent BW and ST in treated dogs over time indicates an improvement in limb function, demonstrating the therapeutic potential of curcuvet® and boswellic acid. The rising slope (RS) and falling slope (FS) of the force curve are defined as the time between the baseline value at ground initial contact and the maximal force, and the time between the maximal force and when

contact with the ground ceases, respectively. Because of the careful initial bear load and a faster release of weight from the limb in lame dogs, the RS is lowered and the FS is enhanced. In this clinical research, it was discovered that the RS was steeper in treated dogs, indicating faster bear loading, whereas the FS was less steep, indicating slower weight offloading [Caterino et al., 2021].

[Dejonckheere, 2016] mentions in a review that curcumin's anti-inflammatory benefits have been tested in people and rats in the majority of large-scale clinical trials. There are a couple of large horse and dog trials. A randomized, double-blind, placebo-controlled parallel group research using turmeric extract for the treatment of osteoarthritis in dogs failed to produce statistically meaningful results. Curcumin reduced macrophage multiplication, substantially downregulated TNF, and activated fibrinolysis, according to a small study involving 12 osteoarthritic dogs. These promising results will need to be confirmed in larger studies.

The pharmacodynamics of liposomal curcumin intravenous injection in beagles has been examined, although there are no practical uses for the average pet owner. A study demonstrated that feeding a dietary supplement including curcumin and boswellia extract to thoroughbred horses reduced pro-inflammatory cytokine expression and thereby improved exercise adaption. Large-scale investigations are required to confirm these conclusions once again. A study conducted at the University of Udine in Italy found that giving a phytosome complex of curcumin to seven mares with verified osteoarthritis and five foals with osteochondrosis alterations for fifteen days had some positive benefits. Gene expression was measured before, during, and after the treatment for four, eight, and fifteen days [Dejonckheere, 2016].

Even while only the downregulation of IL-1 and IL1RN was significant, curcumin suppressed the expression of COX-2, TNF-, IL-1, IL1RN, and IL6 in mares. Curcumin decreased COX-2, TNF-, and IL1RN expression in foals while significantly increasing IL6 expression [Dejonckheere, 2016].

These findings suggest that curcumin has potential, but this is a limited study. Curcumin at doses less than 25μM has a substantial antiinflammatory impact on cartilage explants in vitro, according to [Clutterbuck et al., 2013] [Dejonckheere, 2016].

The researchers cautioned against extrapolating the findings and suggested more research to determine curcumin's bioavailability and physiologically appropriate serum and synovial concentrations in people and animals [Dejonckheere, 2016].

[Kobatake et al., 2021] also conducted a study on 40 dogs with degenerative myelopathy (DM). There were 26 male dogs, 21 of whom had been castrated, and 14 female dogs, nine of whom had been spayed. The average age of onset was 10 years and 8 months (range: 7 years and 7 months to 14 years old). The median age at death was 13 years and nine months (range: 10 years and three months to 16 years and one month), with a median surviving time of 36 months (from symptom onset to death) (range: 18 to 52 months). The c.118G>A mutation in the SOD1 gene was found in all of the dogs [Kobatake et al., 2021].

All of the cases began with spastic upper motor neuron (UMN) paresis and later proceeded to flaccid tetraplegia due to increasing widespread proprioceptive ataxia of the pelvic limbs. Thirty-one dogs had urinary incontinence, with 17 of them also having fecal incontinence. Urinary incontinence was found concurrently or after the beginning of fecal incontinence. Eleven dogs had dysphonia, particularly hoarseness, however the start of symptoms varied greatly between them. Three of the four dogs with dysphagia died within two months of the onset of dysphagia, while the fourth dog lived for seven months following the onset of dysphagia. Five dogs developed tongue spasms. The sense of the face in one dog deteriorated, indicating trigeminal nerve paralysis. Another dog suffered facial nerve paralysis, which meant he couldn't move its face. Patients that lived longer than the median survival period developed tongue spasms as well as facial and trigeminal nerve paralysis. The study also shows the occurrence and development of symptoms other than gait disturbance [Kobatake et al., 2021].

A large number of the dogs examined had dyspnea (34 of 40 dogs; 85.0%). The median time from the onset of dyspnea to death was 1.5 months (range: 0–22 months), with 11 of the 34 dogs dying within one month of beginning. Meanwhile, after the onset of dyspnea, five of the 34 individuals lived for more than a year. The correlation coefficient between the beginning of dyspnea and survival time was 0.76 ($p < 0.0001$), indicating a link between respiratory impairment and death. Symptomatic treatment, such as oxygen inhalation, was given in these patients when their usual veterinarian felt it necessary [Kobatake et al., 2021].

All of the incidents began with increasing pelvic limb paresis, which proceeded to flaccid tetraplegia. These findings imply a link between the start of dyspnea and survival time ($R = 0.72$, $p < 0.0001$) [Kobatake et al., 2021].

It was discovered that dogs with DM who were given curcumin had a considerably higher survival period than those who were not. Curcumin is a physiologically active polyphenolic molecule with a variety of effects on the central nervous system, including neuroprotective and anti-inflammatory properties. Furthermore, a prior study found that curcumin molecules bind tightly to the aggregation-prone areas of mutant SOD1 proteins and block the exposed aggregation site, preventing the development of unstructured SOD1 aggregates. Curcumin also has three documented favorable effects on muscles: (1) it promotes protein synthesis, (2) it reduces muscle protein breakdown, and (3) it reduces exercise-induced muscle damage. In the curcumin and control groups, there were no statistically significant changes in the time from onset to thoracic limb paresis. Nonetheless, in the curcumin group, the time from onset to nonweight bearing of the hind/thoracic limbs was greater. This shows that curcumin could help dogs with diabetes prevent neurodegeneration and muscular atrophy. Despite the uncertain benefit of curcumin in DM, dog owners who choose to administer curcumin to their pets may be more engaged in conservative care, such as physical therapy. As a result, extended survival may be due to the dog owner's approach to care rather than curcumin's therapeutic effects. In addition to nerves and muscles, curcumin has anti-inflammatory and antioxidant properties in other organs. As a result, improvements in motor function may be attributable to curcumin's action on joints rather than the DM disease itself [Kobatake et al., 2021].

CONCLUSION

Curcumin's use in the treatment of joint inflammation is supported by a large amount of clinical observational and anecdotal evidence, as well as some encouraging in vitro and in vivo research. However, large-scale clinical trials in dogs, as well as clinical veterinary research, are urgently needed to find strategies to improve the bioavailability and clinical usefulness of supplemental curcumin in the treatment of musculoskeletal pathology. Curcumin's relative safety should allow it to be used as a supplement to treat osteoarthritis and other muscular and skeletal pathologies in the vast majority of patients. For dogs, the recommended dose ranges from 50 to 250 mg curcumin three times per day [Wynn et al., 2007]. The active ingredients of veterinary nutraceutical products may vary significantly due to a lack of uniformity in extraction techniques and quality monitoring. More veterinary clinical trials with more bioavailable forms of curcumin, on the other hand, will provide more precise dosing guidelines and standardised products.

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