SCIENTIFIC WORKS
SERIES C. VETERINARY MEDICINE
VOLUME LXI (1), 2015

University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Veterinary Medicine

# SCIENTIFIC WORKS
## SERIES C
## VETERINARY MEDICINE

Volume LXI (1)

2015
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Address: 105 Splaiul Independentei, District 5, Zip code 050097, Bucharest, Romania
Phone: + 40 21 318 04 69,
E-mail: veterinarymedicinejournal@usamv.ro, Webpage: www.fmvb.ro

**CERES Publishing House**
Address: 1 Piața Presei Libere, District l, Zip code 013701, Bucharest, Romania
Phone: + 40 21 317 90 23, E-mail: edituraceres@yahoo.com, Webpage: www.editura-ceres.ro



*To be cited: Scientific Works. Series C. Veterinary Medicine, Vol. LXI (1), 2014*



**ISSN 2065-1295, ISSN 2343-9394 (CD-ROM), ISSN 2067-3663 (Online), ISSN-L 2065-1295**

**International Database Indexing:**
Index Copernicus; CABI; Google Scholar; Scipio; OCLC; PNB (Polish Scholarly Bibliography);
Cite Factor; Research Bible; Universal Impact Factor

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# ANATOMO-HISTOLOGICAL ASPECTS OF THE CORONARY ARTERIES IN PIGS (SUS SCROFA)

**Alexandra BLENDEA[1], Ioana CHIRILEAN[1], Irina IRIMESCU[1], Nicolae CORNILĂ[2], Mihai CORNILĂ[2], Aurel DAMIAN[1]**

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***Abstract***

*Compared to known anatomical data, we appreciate that our observations underline certain particularities of the coronary arteries and their superficial and deep branches in the pig. The study was conducted in the Department of Comparative Anatomy of the USAMV Cluj-Napoca, in collaboration with the Department of Histology and Embryology of Veterinary Medicine Bucharest, on 4 pig hearts samples. The clinically healthy animals aged between 2 to 4 months were commercially slaughtered by bleeding. The hearts were collected with their vessels intact. Using step-by-step dissection techniques we have harvested the 4 hearts, and in two of them we have underlined the origins of the left and right coronary arteries to inject them with a coloring agent (PALUX and red pigment), and the other two hearts were harvested for histological processing. After injecting the coloring agent in the coronary arteries the two hearts were submerged in a 10% formalin solution over a period of 24 hours, to fixate. Histological processing comprised the following steps: sample harvesting, fixation, wash, dehydration, paraffin inclusion, cutting, paraffin removal, hydration, coloring, clarification and mounting. Aside from the deep branches of the superficial coronary arteries, both the paraconal artery, the circumflex artery and the right coronary artery give off direct deep branches for the myocardium and for all of the papillary formations of the atria and ventricles. The histological aspects of the left and of the right coronary arteries are. The elastic fiber density increases with age, and the fibers are more numerous in the external half of the tunica media. In younger ages, the coronary arteries have a muscular type aspect and they present a tendency to become musculo-elastic arteries along with the ageing. The elastin is produces by the smooth muscle fibers of the internal layer of the tunica media, but also by the fibers of the adventitia, situated at the border with the tunica media.*

***Key words****: coronary arteries, pig, histological processing, pallux.*

## INTRODUCTION

Morphologically and functionally, the circulatory system is composed of two inextricably linked components: blood and lymph. Highlighting the blood circulatory system is easier because the administration of identifying substances can be done directly in their lumen, unlike lymphatic vessels that require prior identification using intra vitam dye solutions such as Evans blue (Stan F. 2008, 2014). Both components of the circulatory system can be identified in vivo using non-invasive methods, namely ultrasound, specifically using Doppler techniques, which are suited even for lymph nodes (Stan F. 2010).
In terms of blood supply, we could say that the heart is the domain of violated rules. It is a structure in which there are exceptions to every rule governing the local phenomena. Here, individual variability of the vascular distribution may be responsible for the limit between life and death, maybe more than in any other organ. The recent years have seen several technological advances in arterial assessment. Taking into account that the pig heart is very similar with the human heart, these experiments help bring new techniques for solving various cardiac problems. Literature data on coronary arteries were achieved very thoroughly, taking into account multiple artery origin variations (Wernaeky quoted by Agneoletti et al., 2005), and anatomical particularities of human blood vessels (Pop D. Popa, 1982).

## MATERIALS AND METHODS

This study aimed to highlight certain particularities of the coronary system in pigs and it was performed in two stages.
The first stage took place in the Comparative Anatomy Department of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, using stratigraphic dissection techniques to open the aortic bulb and identify the orifices of origin of the left and right coronary arteries. A catheter was placed in the lumen of the coronary arteries, and they were injected with the product PALLUX, colored with a red pigment. After injecting the coloring agent, the hearts were submerged in a 10% watery formaldehyde solution, in order to fixate them. The dissection was performed 24 hours later, gradually taking segmental photographs.
The second stage was performed in the Cellular Biology, Histology and Embryology of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca. The research was performed on samples of left and right coronary arteries from pigs of various ages. The harvesting was performed immediately after the euthanasia of the animal by exsanguination and the anatomical dissection. Histological processing required compulsory steps such as: sampling, fixation, washing, paraffin inclusion, cutting, staining. The staining techniques used in our histological study were: Hematoxylin-Eosin staining, Orcein staining and Mallory staining.

## RESULTS AND DISCUSSIONS

Anatomical aspects:

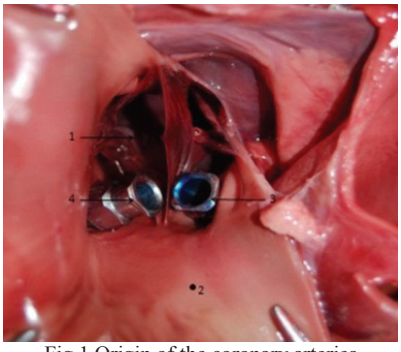

Fig.1.Origin of the coronary arteries

1. Aortic orifice of the left ventricle and sigmoid cusps; 2.Aortic bulb - opened - and sinuses of the primary aorta; 3.Left aortic sinus and orifice of origin of the left coronary artery; 4.Anterior aortic sinus and orifice of origin of the right coronary artery.

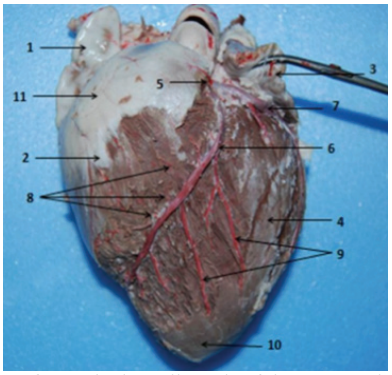

Fig.2. Left ventricular collaterals of the paraconal artery 1.Right atrium; 2.Right ventricle; 3.Left atrium; 4.Left ventricle; 5.Left coronary artery; 6.Paraconal artery (left interventricular artery); 7.Left circumflex artery; 8.Right ventricular collaterals; 9.Left ventricular collaterals (diagonal arteries); 10.Apex of the heart.

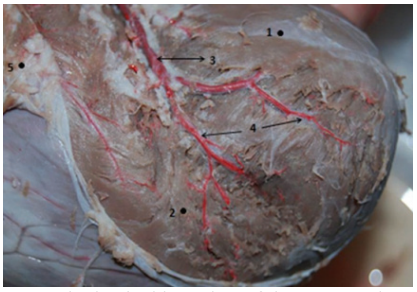

Fig.3. Terminal apical branches of the paraconal arteries 1.Left ventricle; 2. Apex of the heart; 3.Terminal segment of the path of the paraconal artery; 4.Terminal apical branches of the paraconal artery; 5.Apical region of the right ventricle.

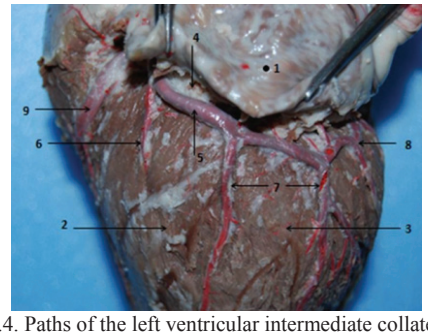

Fig.4. Paths of the left ventricular intermediate collateral arteries
1.Left atrium; 2.Left ventricle – lateral side; 3.Left ventricle - caudal border; 4.Left atrio-ventricular groove; 5. Left circumflex artery; 6.Basilar collateral branch of

the left ventricle; 7.Paths of the ventricular intermediate collateral arteries; 8.Terminals of the left circumflex artery; 9.Paraconal artery.

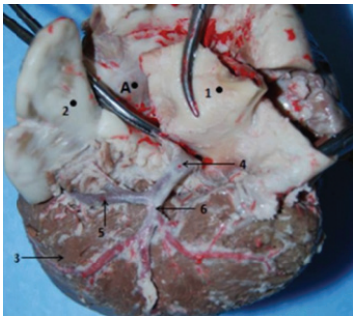


Fig.5. A - Anatomical aspect of the base of the heart
1.Aorta - region of the aortic bulb; 2.Right atrium (lifted); 3.Base of right ventricle; 4.Origin of the right coronary artery; 5.Path of the right coronary artery in the atrio-ventricular groove; 6.Birufcating branch of the right coronary artery.

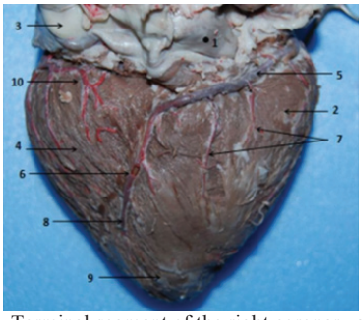


Fig.6. Terminal segment of the right coronary artery
1.Rigth atrium; 2.Right ventricle; 3.Left atrium; 4.Left ventricle; 5.Right coronary artery; 6.Descending interventricular branch of the right coronary artery; 7.Ventricular collaterals; 8.Temrinal segment of the right coronary arterial path; 9.Region of the heart's apex; 10.Terminals of the left circumflex artery.

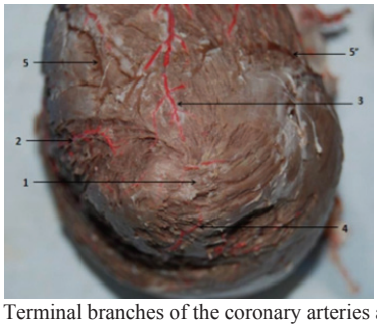


Fig.7. Terminal branches of the coronary arteries and of their collateral for the apical region of the heart.
1. Apex of the heart; 2.Terminales of the paraconal artery; 3.Branches of the intermediary (marginal) collateral arteries of the left circumflex artery; 4.Terminal branches of the right coronary artery; 5, 5'. Roots of the cardiac veins.

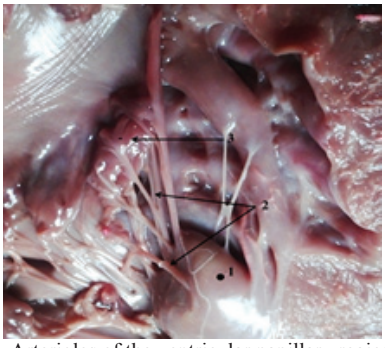


Fig.8. Arterioles of the ventricular papillary region and of the atrio-ventricular cusps
1.Ventricular papillary muscle; 2.Papillary and cuspidal arterioles; 3.Atrio-ventricular cusp.

## Histological aspects:

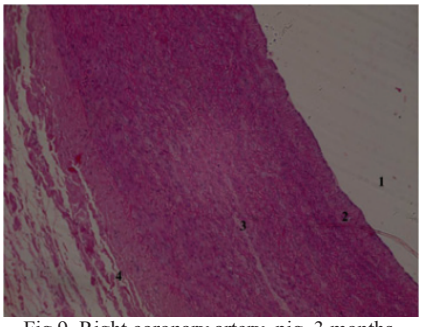


Fig.9. Right coronary artery, pig, 3 months, Hematoxylin-Eosin stain, ob. 10x.
1-lumen; 2-endothelium; 3-media; 4- adventitia
The media presents a dense juxtalumenal layer and a peripheral lesser dense layer, which is continued by the adventitia without any observable delimitation. No observable internal and external laminae.

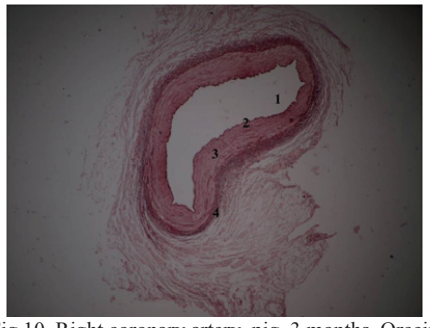


Fig.10. Right coronary artery, pig, 3 months, Orcein stain, ob. 10x.
1-lumen; 2-endothelium; 3-media; 4- adventitia

Observable agglomeration of elastic fibers at the limit between the media and the adventitia, which can be interpreted as the future external lamina.

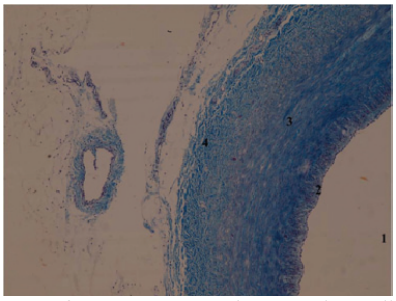

Fig.11. Left coronary artery, pig, 4 months, Mallory stain, ob. 10x.
1-lumen; 2-endothelium; 3-media; 4- adventitia
The adventitia presents a lymph vessel with an endothelial valve. Smooth muscular fibers predominate in the internal layer of the media, while conjunctive fibers are more numerous in its external layer.

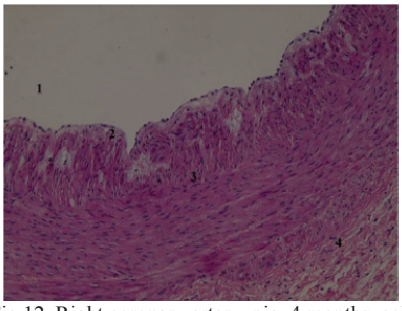

Fig.12. Right coronary artery, pig, 4 months, col. Hematoxylin-Eosin, ob. 20x.
1-lumen; 2-endoteliu; 3-media; 4- adventitia;
The endothelial nuclei appear very prominent. The subendothelial layer is poorly delimited. The media presents two layers of smooth muscular fibers. The internal (juxtalumenal) layer presents muscular fibers cut transversally or obliquely. At its periphery, the muscular fibers appear longitudinally cut.

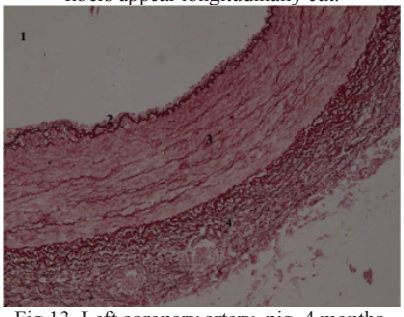

Fig.13. Left coronary artery, pig, 4 months, Orcein stain, ob. 20x
1-lumen; 2-endoteliu; 3-media; 4- adventitia;
Internal elastic lamina. Observable distribution of elastic fibers, which occupy the external layer of the media.

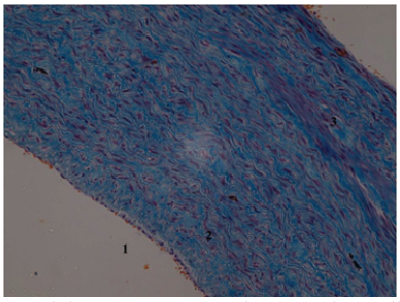

Fig.14. Right coronary artery, pig, 3 months, Mallory stain, ob. 20x.
1-lumen; 2-endoteliu; 3-media;
Observable muscular fibers and endothelium. In the external half of the media, the muscular fibers nuclei are more numerous. No observable elastic laminae.

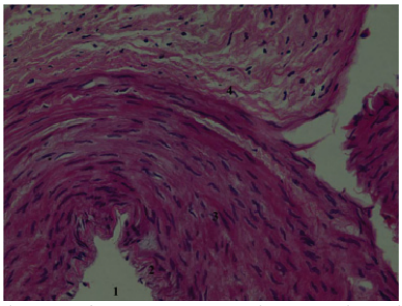

Fig.15. Left coronary artery, pig, 4 months, col. Hematoxylin-Eosin, ob. 40x.
1-lumen; 2-endothelium; 3-media; 4- adventitia.
Detail of the left coronal artery wall. Smooth muscle fibers predominate in the tunica media. The tunica adventitia presents rare conjunctive tissue nuclei (fibroblasts).

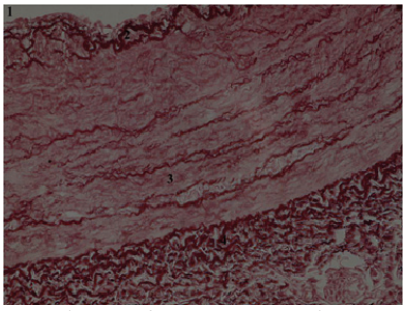

Fig.16. Left coronary artery, pig, 4 months, Orcein stain, ob. 40x.
1-lumen; 2-endothelium; 3-media; 4- adventitia
Observable distribution of the elastic fibers, which occupy the external layer of the tunica media.

## CONCLUSIONS

During the dissection of the coronary vessels, we avoided the total removal of the

subepicardial adipose tissue, which was extremely abundant and adhesive to the blood vessels. This anatomical aspect represents a natural and physiologic particularity in this animal species.

**Anatomical aspects:**

**Right coronary artery**:

1. The right coronary artery emits numerous and strong deep collaterals for the right ventricle.
2. The branches of the right coronary artery are fewer in number and have a smaller caliber. Their paths are aproximatively straight and ascending.
3. The deep collateral arteriolar branches split into a arteriolar-capillary network; the vessels of this network have a parallel arrangement.
4. The manner of distribution of the subendocardial arterioles in the ventricular papillary muscles is particular. The arterioles continue their path towards the atrio-ventricular cusps, accompanying the chordae tendineae.

**Left coronary artery:**

1. The left coronary artery emits collateral arterioles for the side adjacent to the base of the pulmonary arterial trunk and one or two arterioles for the left medial atrio-auricular wall;
2. At the level of the terminal split into the paraconal artery and the circumflex artery, there is a strong deep collateral artery for the left ventricular myocardium.
3. The deep collateral artery traverses medially the great cardiac vein.
4. The paraconal artery emits numerous collateral arterioles for the right ventricular myocardium. We consider that, in respect to the lack of this type of collaterals on the paraconal path, these particular arterioles also service the left ventricle; we have identified only one arteriole excepted from this.
5. The apex terminals of the paraconal artery emit deep arterioles for the trabeculae carnae of the area corresponding to both of the ventricular cavities.
6. Numerous deep arterioles with a perpendicular latero-medial path stem from the medial sides of the superficial diagonal collaterals of the left ventricle.
7. Numerous deep arterioles stem from the superficial collaterals of the circumflex artery.
8. The circumflex artery emits numerous deep collaterals for the atrium.

**Histological aspects:**

1. The left and right coronary arteries have similar histological aspects.
2. The frequency of the elastic fibers increases with age, being more numerous in the external half of the media.
3. At younger ages, the coronary arteries have the aspect of a muscular artery type, with the tendency to become musculo-elastic arteries as the animal ages.
4. The elastin is produced both by the smooth muscle fibers of the internal layer of the tunica media and by the fibroblasts situated in the adventitia, at the limit with the media.

## ACKNOWLEDGEMENTS

This paper was *published under the frame of* European Social Fund, *Human Resources Development Operational Programme 2007-2013, project no.* POSDRU/159/1.5/S/136893.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# EFFECTS OF THE PHOTOPERIODICITY ON THE REPRODUCTION IN SOW. II-EFFECTS ON THE REPRODUCTIVE HORMONAL SYSTEM

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***Abstract***

*The research was conducted on a breed of adult sow in different physiological states, originated from a northern EU area, during the period of adaptation in a temperate (Romanian) area. The animals were in different physiological status: gilts and sows, estrous, pregnancy or lactation. They were determined the blood plasma levels of main hormones involved in the reproductive function [17-β estradiol, progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH) and melatonin] in the days of the solstices and equinoxes. Plasma levels of 17-β estradiol for gilts and sows during the first day of the estrous showed minimal values in March, 20th, increased in June 21st, reaching a maximal values in September, 22nd, and then decreased again. The LH mean values (assayed in the first day of estrous period) were highest during the maximum photophase (June, 21st) and lowest in September, 22nd in gilts, while in sows, the higher levels of plasma LH were found during the day of spring equinox and summer solstice and the lowest levels in September, 22nd (as in gilts). Plasma progesterone of the 25-day-pregnant gilts and sows presented the highest values during the period of maximum scotophase (December, 21st), significantly higher by comparing to the other three analyzed photoperiods. Plasma progesterone of pregnant gilts was lower vs. pregnant sows for every analyzed photoperiod. The maximum amounts of melatonin blood values were found in December 21st, the lowest in June 21st and intermediate values in the two solstices. For all the assayed photoperiods, melatonin contents in midnight samplings were nearer to those taken at midday. Almost every time, the melatonin values in lactant sows were lower vs. in pregnant sows.*

***Key words***: *17β-estradiol, progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), melatonin, photoperiodicity, sows.*

## INTRODUCTION

Reproductive photoperiodicity of the sow shows a number of features related to breed physiological status, age or parity. Sensibility of the pig to different photoperiods is discussed in relation to reproductive parameters by commercial farmers. Seasonal infertility of sows for example, has a multiple causes (temperature, level of nutrition, genetic background), but this factorial complex is completed by photoperiodism. It is possible that the seasonal early disruption of pregnancy to be mediated by decreased progesterone concentrations leading to underdeveloped embryos at the time of recognition of pregnancy. This seasonal decrease in progesterone appears to be under the influence of seasonally changing melatonin secretion (Tast, 2002). Bassett *et al.* (2001, cited by Chokoe and Siebrits, 2009) found that melatonin implants were able to prevent seasonal anestrous In regions with large variation in day length during the year, the importance of photoperiod and light intensity during photo- and scotophase may be greater than the effect of high ambient temperature. (Gaustand *et al*., 2004). Transfer of animals from a region of large differences between the main photoperiods to another with low differences could involve significant modification of the reproductive hormonal system and of reproductive parameters. The purpose of this study was to identify the season effects on sow originating from a Northern EU area, during the period of adaptation in a Southern EU area.

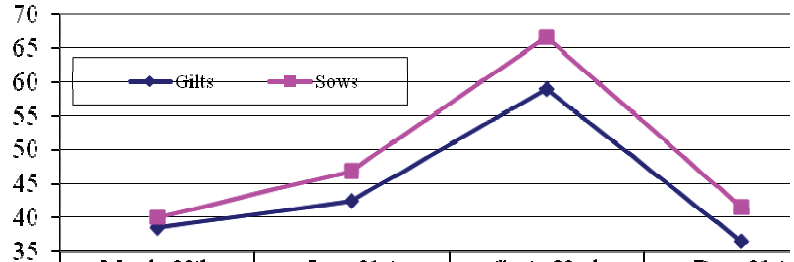
## MATERIAL AND METHODS

Researches were performed on a crossbred of Yorkshire (♀)×Landrace (♂) gilts and sows sourced from Denmark, belonging to a commercial piggery from Southern Romania. The animals were in different physiological states: pregnancy (the 25th day from artificial insemination), lactation or oestrous (first day of post-weaning oestrous). Each animal was individually labelled. The animals were fed 40% from *ad libitum* (9.5 – 10 kg/day) during the oestrous period, 50% from *ad libitum* during the first third of pregnancy period and *ad libitum* during the rest of pregnancy and lactation periods. The animals were housed in stables whose mean temperatures were 25°C (71.6 – 77.0°F) for the all monitored periods, under natural light conditions, and they had free access to water. Blood was sampled by vein puncture of *V. jugularis or Vena saphena medialis*. Each sample (approximately 8 mL) was drawn into a 10 mL syringe and immediately emptied into 15 mL plastic tubes containing 150 µL EDTA solution (250 mg/mL). Blood samples were assayed for the 17-β estradiol, FSH, LH, progesterone and melatonin contents. The blood was sampled at the equinox days (2013, Sept., 22nd, and 2014, Mars, 20th), at maximum photophase day (2013, Dec., 21st) and at maximum scotophase day (2014, June, 21st), every time at 8:30 to avoid the circadian influences, except for melatonin, to whose analyses the blood was sampled to times a day: midnight and midday. Samples were analyzed by the electro-chemiliminescence assay (ECLIA) using a COBASe 411 analyzer and using pig 17-β estradiol, progesterone, FSH, LH commercial lyophilized antisera extracted from porcine plasma provided by Abnova, and melatonin ELISA kit provided by Elabscience. The results were statistically analyzed using a commercial ANOVA software and the significance was established for $P<0.05$. The post hoc Tukey test was performed to find the statistical significance between the photoperiods inside the same category of sows. The results are presented as mean ± standard error of mean ($\bar{X} \pm s_{\bar{x}}$).

## RESULTS AND DISCUSSION

Regarding the 17-β estradiol in the first day of post-weaning oestrous gilts and sows, they were found seasonal and parity differences, with maximal values in Sept., 22nd for both gilts ($P = 0.0029$) and sows ($P= 0.0298$), but with less maximal values in gilts (58.9 pg·mL$^{-1}$) and a higher maximal value in sows (66.6 pg·mL$^{-1}$, fig. 1).

Figure 1. The plasma 17-β estradiol levels of the first day of the post-weaning estrous (in pg·mL$^{-1}$) in gilts and sows during the main photoperiods of the year




| | March, 20th | June, 21st | Sept., 22nd | Dec., 21st |
|---|---|---|---|---|
| Gilts | 38.5 | 42.5 | 58.9 | 36.5 |
| Sows | 40 | 46.9 | 66.6 | 41.5 |

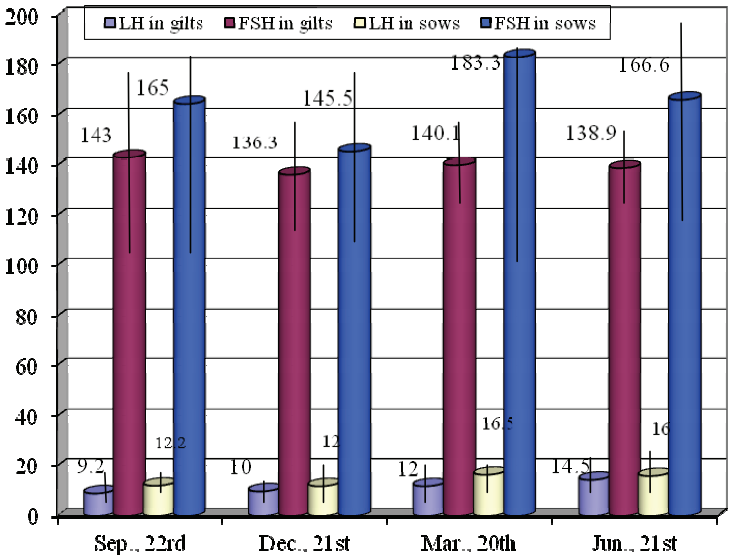
Notice: each mean is the result of 5 or 6 samples.

According to Guthrie *et al*. (1972), total serum estrogen in sows begins to increase three days before estrous (10-28 pg·mL$^{-1}$), which coincide with the time of lowest progesterone levels, and

reached maximum values of about 38 pg·mL$^{-1}$ two or one day before estrous. In our research, the plasma 17-β estradiol levels of the first day of estrous were found increased from Mars, 20$^{th}$ to June, 21$^{st}$, reaching the maximal values during the autumnal equinox day (Sept., 22$^{nd}$). This slow increase of the plasma 17-β estradiol from spring to summer could be assigned to a slow stimulatory effect of the increasing photoperiod. The same explanation for the evolution of estrogen levels from March to September. Almond and Dial (1990) reported a 17-β estradiol level of 10-19 pg·mL$^{-1}$ in anestrous sows. Estradiol-17β and progesterone blood changes were found according to breed and feeding system: Lee *et al.* (2013) investigated the changes of serum progesterone and estradiol-17b in sows of Landrace (L), Yorkshire (Y) and F1 (L × Y) fed by two ways methods, a conventional method and 3 days-flushing feed before estrous. In conventional feeding, serum progesterone and estradiol-17b levels were significantly ($p<0.01$) higher in F1 than in L and Y. In the case of flushing method, almost of hormonal levels were a little higher than that of conventional methods. The authors concluded that more studies about hormonal changes in sows according to seasonal and nutritional factors should be needed.

Plasma LH levels assayed for the first day of the estrous cycle ranged between 9.2 and 14.5 ng·mL$^{-1}$ in gilts and between 12.0 and 16.5 ng·mL$^{-1}$ in sows along the whole analyzed photoperiodicity (Fig. 2). The LH average values of gilts were highest in the moment of maximum photophase (in June) and lowest in September, while in sows, the higher levels of plasma LH belong to sows which were in oestrous during the days of spring equinox and summer solstice. Statistical analysis revealed significant seasonal differences for both gilts ($P = 0.033$) and sows ($P = 0.019$). Mean annual values of LH were 11.42 ng·mL$^{-1}$ in gilts and 14.17 ng·mL$^{-1}$ in sows.

Fig. 2. Comparative blood plasma LH and FSH levels in gilts and sows in the first day of post-weaning estrous, for the main photoperiods of the year (ng·mL$^{-1}$)




Guthrie *et al*. (1972), reported that LH concentration was significantly greater during pregnancy (3.12 ng·mL$^{-1}$) than during the early follicular phase (1.02 ng·mL$^{-1}$).

Mean annual values of FSH were 139.5 ng·mL$^{-1}$ in gilts and 165.1 ng·mL$^{-1}$ in sows, higher in sows vs. gilts for every one of the four analyzed photoperiods. In sows, highest values of FSH were found in the equinox days ($P = 0.0082$ when calculated by comparing to the lowest value, found in December). For gilts, they were no significant differences between the four analyzed photoperiods. Studies relieve a period of seasonal abortion in pig, focused on

the August, September and October (Wrathall *et al*, 1986). Smits and Almond (1991) found that frequency of LH pulses and LH pulse amplitude were higher in pregnant gilts during January and February compared to August and September. Luteinizing hormone secretion is known to be affected by seasonal variation (low in summer and high in winter) in the domestic pig (Peltoniemi *et al*., 1997) and this seasonal variation appears to be controlled by photoperiodism through melatonin secretion (Bassett *et al*., 2001; Tast *et al*., 2001, cited by Chokoe and Siebrits, 2009).

Chokoe and Siebrits (2009) hypothesized that reduced daylight will improve estrous expectancy rates of sows seven days after weaning, non-return rate, farrowing rates and litter size. They found that the supply of constant 10 h photoperiod had no effect on the proportion of sows that were served within seven days or served after seven days, neither on the number of sows that returned to service. There was no seasonal effect on the proportion of sows served or on the proportion that returned to service.

Peltoniemi *et al*. (2000) links the blood LH level variations by the phenomenon of seasonal abortion in sows. According to Peltoniemi *et al*. (2000), the endocrinological mechanism of seasonal disruption of pregnancy is yet to be determined. However, they found that LH is reduced in the summer–autumn period. These changes in LH secretion may exert a progesterone-mediated detrimental effect on the capability of embryos to produce adequate embryonic signaling. This may lead to a seasonal disruption of pregnancy and a return to estrous 25–30 days after mating.

Plasma progesterone presented the highest values in both pregnant gilts and pregnant sows during the maximum scotophase period (December), significantly higher by comparing to the other three analyzed photoperiods. Minimal values were found in June and September, and intermediates values in March (Table 1).

Table 1. The plasma level of progesterone (in $ng \cdot mL^{-1}$) in pregnant gilts and sows during the main photoperiods of the year

| Item | Sep., 22nd | Dec., 21st | Mars, 20th | June, 21st | P |
|---|---|---|---|---|---|
| Gilts (25th day of pregnancy) | 32.2± 6.8 | 36.9± 6.0 | 33.0± 7.5 | 28.0± 8.9 | 0.033 |
| Sows (25th day of pregnancy) | 36.0± 8.8 | 46.5± 6.1 | 36.3± 7.0 | 38.9± 9.0 | 0.019 |

Notice: each mean is the result of 5 or 6 samples.

According to Guthrie *et al*. (1972): the mean progesterone concentration in pig ranges from 16.1 $ng \cdot mL^{-1}$ of blood plasma to 1 $ng \cdot mL^{-1}$ or less before estrous, remains less during estrous and increases to a peak value of 35.4 $ng \cdot mL^{-1}$ during pregnancy. Peak plasma values of progesterone in December, 21st seem to be a consequence of pregnancy installation in September rather than an influence of photoperiodicity.

Wrathall *et al*. (1986) found that seasonal differences in progesterone concentrations were evident, with the concentrations rising from the lowest values in August, September and October (as in our research) but to a peak in March (not in December). Their findings are pertinent to the pathophysiology of the autumn abortion syndrome and other seasonal reproductive problems in sows.

Plasma values of melatonin for the blood sampled in the midnight showed maximal values in Dec., 21st and minimal values in June, 21st (Table 2). The two solstices presented intermediate melatonin values, between winter equinox and summer equinox, for the two physiological states of sows, lactant and pregnant, and for the two times of sampling.

Melatonin content of the blood sampled at Dec. 21st midnight vs. midday was 1.17 higher in lactant sows and 1.22 higher in pregnant sows. These differences were a bit lower in June, 21st: 1.05 and, respectively, 1.06. The plasma melatonin ranges we found in our research are in agreement with Andersson (2001) results (according to Andersson, in gilts, plasma levels

of melatonin ranges between 2.6 pg·mL$^{-1}$ in daytime and 26 pg·mL$^{-1}$ in nighttime), excepting the differences between daytime and nighttime: ten times higher in nighttime vs. daytime, but no detail is given about the season of sampling.

The same author suggests there is a genetic background involved in melatonin secretion. Seasonal variations in melatonin secretion bind the hormone levels to the phenomenon of seasonal infertility in pig. Seasonal infertility in sows has been documented to be a result of high temperature.

One of the reasons pigs are vulnerable to elevated ambient temperature is their inability to lose heat by evaporation. High ambient temperatures limit pig production (Bloemhof *et al*., 2012). There is no consensus regarding the factors that impact seasonal infertility. However, some traits involved are heat stress, genetic background and photoperiod (Love *et al*., 1993). It has been shown that pigs adjust their circadian melatonin secretion according to changes in photoperiod (Tast *et al*., 2002).

Bassett *et al.* (2001, cited by Chokoe and Siebrits, 2009) found that melatonin implants which were inserted at the spring equinox were able to prevent seasonal anestrous, thus providing further support for the contention that the duration of the daily photoperiod is the primary determinant of the reproductive seasonality.

Table 2. The plasma level of melatonin ( $\bar{X} \pm S_{\bar{x}}$ , in pg·mL$^{-1}$) in lactating and pregnant sows in the main photoperiods of the year

| Item | Time of sampling | Sep., 22nd | Dec., 21st | Mars, 20th | June, 21st | P |
|---|---|---|---|---|---|---|
| Lactant sows | Midday | 4.0±0.10 | 20.4±0.45 | 4.04±0.00 | 1.05±0.00 | 0.033 |
| | Midnight | 4.49±0.98 | 24.0±0.40 | 4.98±0.65 | 1.11±0.04 | 0.020 |
| Pregnant sows | Midday | 4.35±0.19 | 23.96±0.50 | 5.19±0.05 | 1.15±0.05 | 0.019 |
| | Midnight | 5.05±1.09 | 26.9±3.33 | 5.55±0.65 | 1.22±0.22 | 0.009 |

Notice: each $\bar{X} \pm S_{\bar{x}}$ is the results of 5 or 6 samples.

Apparently contradicting findings were observed by Kermabon *et al*. (1995) who showed that a long light duration (16 h/day) had a detrimental influence on the return to estrous after weaning, while a higher proportion of sows exposed to a short day length (8 h/day) exhibited estrous shortly after weaning. Gaustad *et al*. (2004) reported that litter size was lower after service during natural long photoperiod than during the rest of the year for both, gilts and sows from a Northern and a Southern region in Norway.

## CONCLUSION

Landrace x Yorkshire sows are subject of reproductive hormone level changes during adaptation in temperate zone, according to the seasonal photoperiodicity and different physiological states, which can be taken into account in the commercial management of this species.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# FEATURES OF THE SUBCLAVIAN ARTERIES AND THEIR BRANCHING IN THE DOMESTIC PIG

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***Abstract***

*Taking into consideration that literature asserts that pigs share common vascular and cardiac traits with humans and represent an important experimental animal model for research advances, our study addresses the need to establish if the subclavian arteries in pigs present intraspecific variations concerning their branching and topography. The study was performed using a research group of 12 carcasses of commercially slaughtered crossbreed adult pigs (7 males and 5 females), weighting between 80 and 110 kg, obtained from different breeders. We have performed a stratigraphic dissection using common techniques in order to access the heart and the origin of the main arterial vessels, followed by the injection of a colored plastic material (latex) in order to highlight the features found in the targeted arterial branching. After the injection, the dissected pieces were fixed by immersion in a 2% formaldehyde solution, for 48 hours, also allowing the colored latex to solidify. This permitted the completion of the dissection and a good examination of the arterial branching and of is topographic relations. Our results indicate that in pigs the right subclavian artery is a constant branch, which in swine emerges from the brachiocephalic trunk, while the left subclavian artery, also constant, detaches directly from the aortic arch. The caliber and lumenum of the right subclavian artery is significantly smaller by comparison to its left counterpart. None of the subjects sampled in our study presented the external thoracic artery as a branch of the subclavian arteries. Our results indicate that the subclavian arteries in pigs do not present the same intraspecific variety noted in human, which is can be accounted for by the significantly short life span of this species, due to commercial slaughter.*

***Key words****: anatomy, vascular branching, subclavian arteries, pig.*

## I NTRODUCTION

The subclavian arteries are an important topic in human cardiology, in relation to aortic arch and cardiac pathology and surgery (Xiao-Guang Tong et al., 2015; Chuankui Li et al., 2014; Rodriguez-Lopez et al., 2006; Görich et al., 2002; Bavinck etal., 1986; de Leval et al., 1981). The literature indicates the existence of many intraspecific variations of the emerging aortic branches' number and topography (Herrera et al., 2012; Muller et al., 2011; Alsai and Ramada, 2010; Bhattarai and Poudel, 2010; Jakanani and Adair, 2010; Berko et al., 2009; Jesudian et al., 2009; Fritsch, 2008; Gosling et al., 2008; Bathia et al., 2005; Carp C, 2002; Sora et al., 2002; Li et al., 2000; Papilian, 1979)

Taking into consideration that various authors (such as Zaragoza et al., 2011; Suzuki et al., 2009) assert that pigs share common vascular and cardiac traits with humans and represent an important experimental animal model for research advances, our study addresses the need to establish if the subclavian arteries in pigs present intraspecific variations concerning their branching and topography.

## MATERIALS AND METHODS

The study was performed using a research group of 12 carcasses of commercially slaughtered crossbreed adult pigs (7 males and 5 females), weighting between 80 and 110 kg, obtained from different breeders.

We have performed a stratigraphic dissection using common techniques in order to access the heart and the origin of the main arterial vessels, followed by the injection of a colored plastic material (latex) in order to highlight the features found in the targeted arterial branching.

Prior to this treatment, the pulmonary arteries were ligatured, preventing the intrusion of the latex material into the lungs.
The colored latex was introduced by direct injection into the left ventricle of the heart, until it became visible in the common carotid arteries and the antebrachial median arteries.
After the injection, the dissected pieces were fixed by immersion in a 2% formaldehyde solution, for 48 hours, also allowing the colored latex to solidify. This permitted the completion of the dissection and a good examination of the arterial branching and of is topographic relations.

## RESULTS AND DISCUSSIONS

In the examined subjects, the left subclavian artery is the second branch given off from the aortic arch. It presents a slight dorsal flexure, in between the folds of the precardiac mediastinum, along the left side of the trachea and the cranial vena cava. The origin of the left subclavian artery was identified in the dissected specimens, at 1-2 cm from the detachment of the brachiocephalic branch (Figure 25).

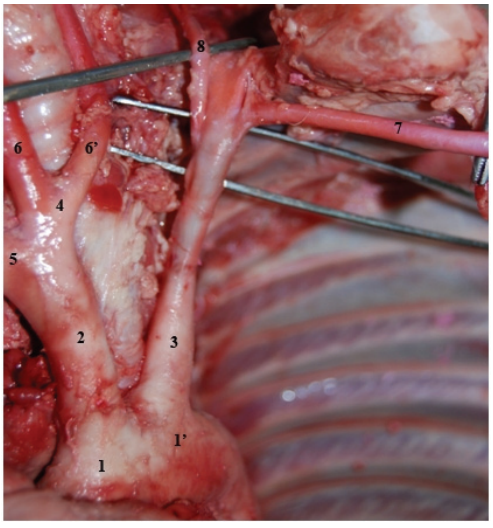


Fig.25.Aortic arch and emergence of the left subclavian artery and brachiocephalic trunk:
1.Aorta; 1'.Aortic arch; 2.Brachiocephalic trunk; 3.Left subclavian artery; 4.Bicarotic trunk; 5.Right subclavian artery; 6, 6'.Common carotid arteries; 7.Left internal thoracic artery; 8.Left omocervical artery.

The latter orients itself dorsally and slightly towards the left, and once it reaches the cranial border of the first rib it is continued, just like its symmetrical counterpart, by the axillary artery.

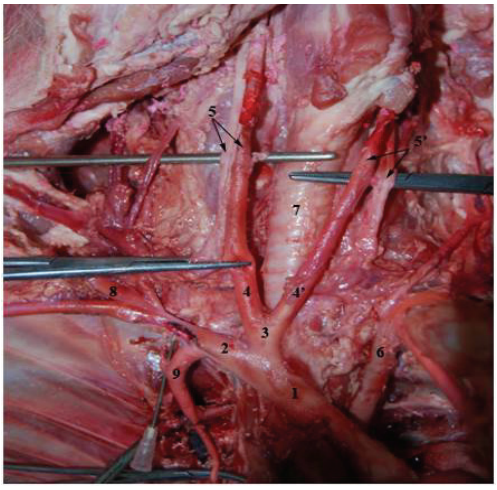


Fig.26. Left and right subclavian arteries:
1.Brachiocephalic trunk; 2.Right subclavian artery; 3.Bicarotid trunk; 4, 4'.Emergence of the common carotid arteries; 5, 5'.Common carotid arteries and vagosympathetic trunks; 6.Left subclavian artery; 7.Trachea; 8.Axillar artery; 9.Costocervical trunk.

On most subjects, the left subclavian artery gives off: the costocervical arterial trunk, the deep cervical artery, the vertebral artery, the omocervical or superficial cervical artery and the internal thoracic artery (Figure 26).

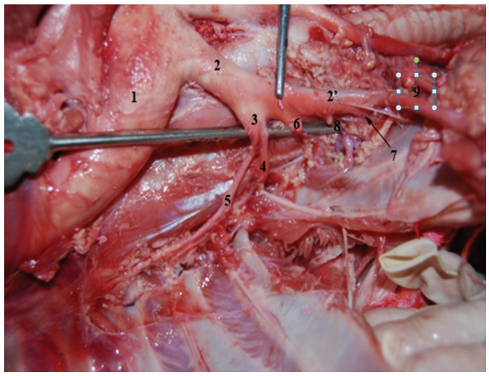


Fig.28.Dorsal branches of the left subclavian artery:
1.Aortic arch; 2, 2'.Left subclavian artery; 3.Costocervical trunk; 4.Dorsal scapular artery ; 5.Supreme intercostal artery; 6.Deep cervical artery; 7.Vetebral artery (intertransverse artery; 8.Muscular artery; 9.Internal thoracic artery.

Following the evaluation of the fixed samples, we have succeeded to identify several aspects

regarding the branches of the left subclavian artery. We have noted that three of the branches had a mainly dorsal orientation: the costocervical trunk, the deep cervical artery and the vertebral artery (Figure 28).

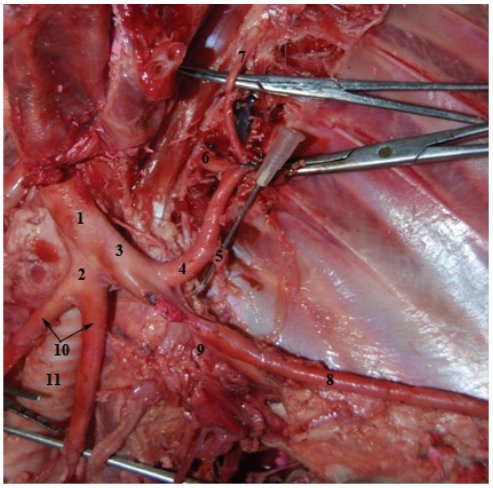


Fig.31. Left subclavian artery
1.Brachiocephalic trunk; 2.Bicarotic trunk; 3.Right subclavian artery; 4.Right costocervical trunk; 5.Right deep cervical artery; 6.Dorsal scapular artery; 7.Right intercostal artery; 8.Internal thoracic artery; 9.Dorsal muscular arterial branch; 10.Common carotid arteries; 11.Trachea.

In most subjects, the arterial costocervical trunk divided after a short path in the supreme intercostals artery and the dorsal scapular artery (Figure 26). In two cases, this was also the emerging point for the deep cervical artery (Figure 31) This terminal bifurcation is located at the level of the 2nd intercostals space.

At a short distance from the emission of the costocervical trunk, lays the origins of the deep cervical artery and of the vertebral artery (Figure 28).

It is worth mentioning that at the terminal level of the left subclavian artery and its continuation with the axillary artery, the subclavian gives off in the dorsal direction a strong arterial branching which we have named muscular artery (Figure 31).

The ventral branches of the subclavian artery are: the superficial cervical artery and the internal thoracic artery (Figure 25).

No samples of those that we have dissected the external thoracic artery, a situation in which we do not exclude the possibility of it being one of the several branches given off by the omocervical artery, which is well developed (Figure 29)

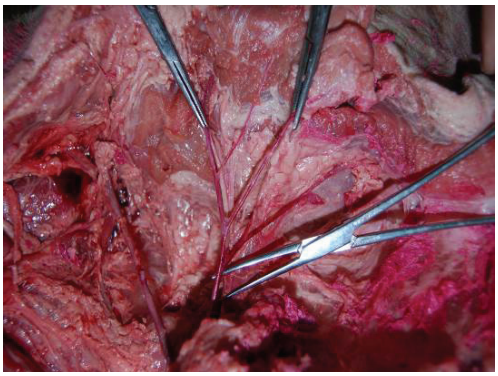

Fig.29.Branches of the omocervical artery

The right subclavian artery is the only branch given off by the brachiocephalic trunk. We have identified its origin at the level of the first rib, were this artery is emitted under a narrow angle (Figure 26 and Figure 31).

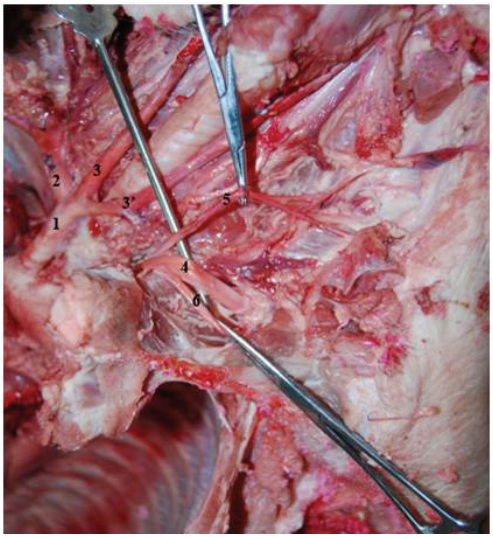


Fig.32.Branches of the left axillary artery
1.Brachiocephalic trunk; 2.Right subclavian artery; 3, 3'.Common carotid arteries; 4.Left axillary artery; 5.Muscular branch; 6.Left omocervical artery.

In our specimens, the emission of the right subclavian artery marked the beginning of a very short bicarotid trunk (3-4cm) which split into two common carotid arteries.

On its path, the right subclavian artery also

gives off, just like its left counterpart, dorsal and ventral branches (Figure 30 and Figure 31).

In addition to this, close to the origin of the subclavian right artery is located the origin of the omocervical branch and of the vertebral artery.

As seen in Figure 31, the right costocervical trunk detaches itself at the level of the great curve of the right subclavian artery.

The terminal segment of the right subclavian artery is placed at the level of the cranial border of the first rib, from which point it is continued by the axillary artery (Figure32).

Our results indicate that in pigs the right subclavian artery is a constant branch, which in swine emerges from the brachiocephalic trunk, while the left subclavian artery detaches directly from the aortic arch.

These observations confirm the general data present in literature (Chirilean et al., 2010a, 2010b; Damian, 2001; Cotofan et al., 2000; Barone, 1996; Constantinescu et al., 1982; Sisson and Grossman, 1964), according to some of which, due to this asymmetry, the brachiocephalic trunk should be called right brachiocephalic trunk.

We have also noted that the left subclavian artery gives off the internal thoracic artery when leaving the precardiac mediastinum, and, in most subject, the latter presented itself as a strong branch, which correlates to its older, functional name of internal mammary artery (Baron, 1996) (Figure 28).

Our results show that the left subclavian artery in all subjects has a relatively large calibre, which still does not surpass that of the brachiocephalic trunk, confirming previous results (Damian, 2014; Tuns, 2014).

The left subclavian artery in all of the dissected specimens presented a constantly larger calibre than that of the right homologue. This is correlated with literature data indicating the fact that the blood flow of the area of distribution of the left subclavian artery is significantly larger that of the right one (Damian, 2001; Coţofan et al., 2000; Popovici et al., 1998)

Because of the important girth presented by the right subclavian artery in all of our research samples, we ascertain that this artery can be considered the main terminal of the brachiocephalic trunk, along with the bicarotid trunk.

Consistent with previous literature data (Tuns et al., 2014; Tuns, 2014), we have also noted that the common carotid arteries have a smaller calibre than the subclavian arteries. This particularity referred mostly to the left subclavian artery and to a lesser extent to the right one. From this point of view we can also take into account the internal thoracic arteries, which had a similar calibre to that of the common carotid arteries.

When discussing the branches given off by left and right subclavian arteries, the main pattern presented by our samples confirm literature data, with the exception of some variations.

While certain authors state that the dorsal branches of the right subclavian artery (Barone, 1996) or of both subclavian arteries (Sisson and Grossman, 1964) may be joined in a common trunk, none of our subjects presented this feature.

Also, while most of our subjects presented separate left deep cervical and left costo-cervical arteries, coinciding with certain authors (Damian, 2001), two of them presented the left cervical artery as emerging from the costocervical trunk, a situation that has also been described by other authors (Barone, 1996).

As noted in previous studies (Tuns, 2013,2014) the origin of these branches do not have a strictly dorsal and ventral placement along the walls of the subclavian, thus we consider that their classification in literature (such as Damian, 2011) according to the position of emergence into dorsal and ventral branches has a purely didactic role.

Our results also suggest that, although the branches of the subclavian arteries in pigs do present some intraspecific variations, they do not attaint the level noted in humans (Gosling, et al., 2008, Fritsch, 2008; Papilian, 1979). This situation can be accounted for by the significantly short life span of this species, due to commercial slaughter, which also accounts for the lack of case reports of corresponding pathology, as in humans variability of branch emission in these arteries have direct consequences on cardiovascular pathology

## CONCLUSIONS

Our study confirms that subclavian arteries in pigs present a constant asymmetrical emergence: the left subclavian artery originates directly in the aortic arch, while the right one is given off by the brachiocephalic trunk. The right subclavian artery is the only branch detached from the arterial brachiocephalic trunk and it marks the cranial limit of this short vessel.

The calibre and lumenum of the right subclavian artery is significantly smaller by comparison to its left counterpart, but they are both larger than the common carotid arteries.

At the terminal segment of the left subclavian artery, we have noticed that this artery gives off a strong branch which we have name muscular branch.

None of the subjects sampled in our study presented the external thoracic artery as a branch of the subclavian arteries, and the left deep cervical artery presented itself either as a direct branch of the subclavian artery, or a branch of the costocervical trunk. These variations, however, are less important than in humas, a fact which possibli stems from this species short economical lifespan.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# THE EFICIENCY OF WHITHENING AND DEGREASING SUBSTANCES IN THE PROCESSING OF LEPORIDAE BONES

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***Abstract***

*Conservation of animal skeletons is necessary for various reasons - often to determine the species or as decorative items, but we are interested in terms of their use as teaching materials. Students can achieve artistic projects using bones; they can learn species identification and deducing elements of the animal's life by measuring bones. Applying the techniques of obtaining a skeleton can be an enriching experience in the educational process of any student. The aim of this study was to obtain two complete rabbit skeletons and to asses the efficiency of the whitening and the degreasing substances. The materials used were: two rabbit cadavers, dissection instruments, insect colony, hydrogen peroxide, sodium bicarbonate, acetic acid, acetone, gasoline, wooden bases, support wires and silicone gun. To obtain the skeletons we have used the technique of maceration with the help of insects, with a previous dissection of the carcasses. In both cases, the maceration process took 7 day to complete for the body and, 5 day for the cranium, respectively. The osseous pieces thus obtained were frozen at -18$^0$C to eliminates the remaining insects. The skeletons have then undergone a 24 hours whitening process using hydrogen peroxide and sodium bicarbonate, respectively, followed by 24 hours of drying at room temperature, and by 24 hours degreasing using acetone and gasoline. The processing was completed by a final drying period and the assembly on the mounts. In conclusion we can mention the fact that the process of skeleton preservation is scrupulous, time-consuming, but it yields satisfying results in terms of anatomic characteristics maintenance and didactic usability.*

***Key words:*** *bones, preservation, rabbits.*

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## INTRODUCTION

Conservation of animal skeletons is necessary for various reasons - often to determine the species or as decorative items, but we are interested in terms of their use as teaching materials. Students can achieve artistic projects using bones; they can learn species identification and deducing elements of the animal's life by measuring bones. Applying the techniques of obtaining a skeleton can be an enriching experience in the educational process of any student.

Anatomical study methods remain some of the most important ways to obtain body knowledge, because diagnosis and applying treatment starts from the knowledge of normal structures. Therefore, experimental research on animal models is based on a thorough knowledge of the morphology with direct impact on functionality. Rabbits are the most used animals for experimental models (*Stan, 2014*).

Through their peculiarities, they also present different levels of soft tissue preservation. Selecting the technique for a certain piece depends on several factors, such as the desired type of piece, the efficiency of soft tissue preservation, the degree of resemblance to the fresh piece, but also the laboratory equipment available.

Maceration is defined as a controlled form of putrefaction, a stage of decomposing in which the proteins are consumed by bacteria in anaerobic conditions (*Sommer, Anderson,1974*).

This technique necessitates the use of an incubator to ensure optimal conditions. Taking into consideration the fact that this technique involves a putrefaction process that generates a strong repulsive smell, it is indicated to use it in specially arranged rooms, with a proper ventilation system (*Sommer, et al., 1974*).

## MATERIALS AND METHODS

The experiment was performed in the Comparative Anatomy Laboratory of the Faculty of Veterinary Medicine of Cluj-Napoca.
The materials used were: two rabbit cadavers, dissection instruments, insect colony, hydrogen peroxide, sodium bicarbonate, acetic acid, acetone, gasoline, wooden bases, support wires and silicone gun.
The skeletons were obtained by applying a maceration technique using insects that required a prior dissection of the cadavers (Fig.1).

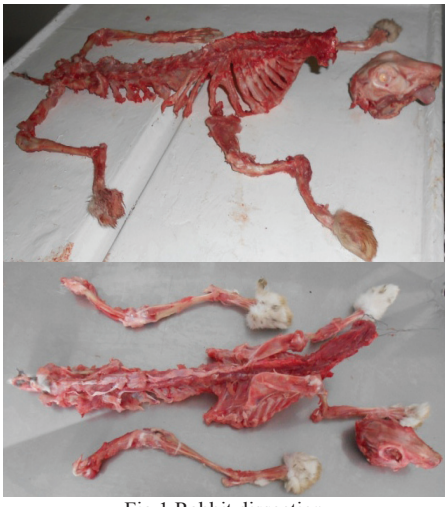

Fig.1 Rabbit dissection

In both cases, the maceration process lasted for 7 days for the body and for 5 days for the cranium (Fig.2 and 3).

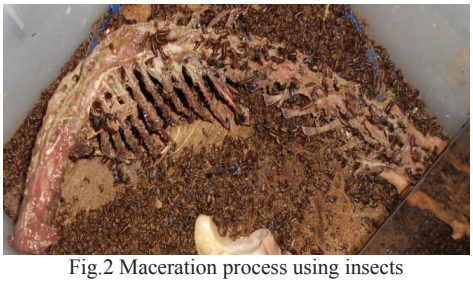

Fig.2 Maceration process using insects

Fig. 3 Maceration process

At the end of the maceration process, the pieces that we have obtained were introduced in a freezer at -18$^0$C to eliminate the insects left on them.
The skeletons thus obtained underwent a process of degreasing using acetone and gasoline, for 24 hours, followed by drying at room temperature, for 24 hours and whitened with hydrogen peroxide, and sodium bicarbonate, respectively, also for 24 hours, followed by their drying, the mounting on a base and comparing the results.

## RESULTS AND DISCUSSIONS

The experiment was carried out in view of improving the technique of osseous pieces processing.
The Acetone was efficient in the process of bone degreasing, although, from a cost value point of view, this product is very expensive. It also must be carefully handled, because it is a substance that may endanger the health of those that come in contact with it. Another risk factor constitutes its high flammability potential, a fact that is common knowledge, as demonstrated by sources such as: http://www.collectioncare.org/MSDS/Acetonemsds.pdf.
We have also obtained remarkable results in the case of using gasoline for the osseous pieces degreasing process, but we have encountered the same inconvenient: the substance is expensive and has a very high flammability degree. The latter was also reported by Green et al.,1993.
The gasoline, of course, has the advantage that it is easier to purchase.
The whitening process using hydrogen peroxide has produced a satisfying result,

corresponding to our expectations, although in the case of the small sized pieces we had to take into consideration the concentration of the substance and its action time (Fig.4). This was also reported by Hussain et al., 2007.

Fig.4 Result of acetone degreasing and hydrogen peroxide whitening

In the case of using sodium bicarbonate and acetic acid, we haven't obtained a relevant result (Fig. 5).

Fig. 5 Result of gasoline degreasing and sodium bicarbonate whitening

At the end of the experiment, the obtained pieces were assembled according to their anatomic position and mounted on wooden bases.

The results of this experiment can help us to obtain osseous pieces that are durable in time and qualitatively superior to other methods of degreasing and whitening (Fig.6 and 7).

Fig.6 Cranium degreased with acetone and whitened with hydrogen peroxide

Fig. 7 Cranium degreased with gasoline and whitened with sodium bicarbonate

## CONCLUSIONS

Following the comparison of the results yielded by this experiment, we can present several conclusions:

The applied maceration technique yielded ?remarkable results in both cases of osseous pieces obtaining process.

Both the acetone and the gasoline that we have used for the degreasing of the pieces lead to appropriately degreased items.

It must be mentioned that both substances have a high degree risk of inflammability, which entails handling them in an attentive manner.

Both products are expensive, but gasoline has the advantage that it is easier to obtain.

The hydrogen peroxide was more efficient for the process of bone whitening, by comparison to the sodium bicarbonate (NaHCO3), mixed with acetic acid.

Nevertheless, we can affirm that both skeletons are usable as didactic material, for the study of osteology in Leporidae.

## ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programe 2007-2013, project no. POSDRU/159/15/S/132765

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# ESSENTIAL TRANSCRIPTION FACTORS FOR MOUSE BLASTOCYST STAGE EMBRYOS

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***Abstract***

*Preimplantation is a process in which embryo is prepared for implantation to the wall of the uterus. This stage defines some morphological changes that occur in the first three days after fertilization during mouse development. It culminates in the generation of the blastocysts, which has a fluid-filled inner cavity and two distinct cell lineages. These two distinct cell lineages consist of trophectoderm (TE) and inner cell mass (ICM). While TE cells contribute to the placenta and the extraembryonic membranes and allow the blastocysts to implant in the mother's uterine wall, the pluripotent inner cell mass gives rise to the fetus. Some transcription factors such as Cdx2 and Oct4 (Pou5f1) have been identified in the mouse blastocyst to generate TE and ICM. Cdx2 is specifically expressed in TE and suppresses the expression of Oct4. Oct4, a key regulator of pluripotency, is strongly expressed in ICM and is essential for early lineage segregation. To present the localization of this transcription factors, we flushed 2 cell stage embryos from the oviducts and cultured to late blastocyst stage in medium. Samples were fixed and immunostained with mouse anti-Cdx2, goat anti-Pou5f1, then images were assessed using a fluorescence microscope to show the localization and presence of Cdx2 and Oct4 by immunocytochemistry. In this work, we demonstrated and reviewed the current knowledge on the Cdx2 and Oct4 in the formation of TE and ICM that is important for understanding the mechanisms of mouse embryo development.*

*Key words: CDX2, mouse blastocysts, OCT4, preimplantation, TE.*

## INTRODUCTION

Mammalian embryonic development is characterized by an initial preimplantation phase which extends from the fusion of the mammalian sperm and egg, namely fertilization and culminates in the generation of blastocysts. Fertilization and preimplantation development of eutherian embryos naturally occur in the oviduct of the mother and they can also be recapulated *in vitro* in a chemically defined culture medium. During the first three days of mouse embryogenesis, the zygote divides and changes morphologically, resulting in a blastocyst consisting of two distinct cell lineages: the inner cell mass (ICM) and the trophectoderm (TE). One or more cavities start to form and gradually expand between 16-32 cell stages to generate blastocyst, in which external cells become TE and internal cells become ICM (Marikawa Y et al., 2009; Marikawa Y et al., 2012). TE is a differentiated epithelium that is

responsible for implantation of the embryo into the mother's uterus and gives rise to placental tissues. The ICM is an undifferentiated mass of cells that will give rise to the whole embryo (Figure 1) (Bell C.E et al., 2013) .

In mouse, precursors of ICM and TE cell lineages begin to diverge very early in development and the position of the cells within the morula is important for this specification. After the fourth cleavage generates a total 16 blastomers, and this cleavage results in formation of two distinct populations of cells: the inner and outer cells (Kondratiuk I et al., 2012 ;Marikawa Y et al., 2009). According to the "Inside-Outside" Model, the cell fate in the blastocysts is established in the late morula and determined by its position; the outer cells are precursors of TE and inner cells become ICM (Kondratiuk I et al., 2012; Tarkowski et al., 1967). Another model for lineage determination is "Cell Polarity" Model, which suggests that cell fate is established at the eight cell stage, during the compaction. Another important point of this model is that cell fate is influenced by the polarity and this gives clues for the localization of the cells. A cell division parallel to the apical-basal axis (symmetric cleavage) produces two polar cells which occupy outside on the embryo and give rise to TE. A division perpendicular to the apical–basal axis (asymmetric cleavage) will produce one polar cell which is inherited to remain outside and becomes TE, and one apolar cell which stays inside and becomes ICM (Yamanaka et al., 2006). After the fifth cleavage, one or more cavities starts to form, which will gradually expand. At this point, embryo is named blastocyst (Figure 1). In addition to their polarity status, the inner and outer cells of mouse morula differ in the activity of specific genes (Kondratiuk I et al., 2012). And change in the cell fate determination at fifth cleavage is considered to be associated with an alteration in the expression of Caudal-like transcription factor (Cdx2) that is essential for normal TE development (Beck et al., 1995; Marikawa Y et al., 2009)). At around 8- to 16- cell stages, Cdx2 protein can be detectable in nuclei of all cells of the embryo. But the transition from 16- to 32- cell stage the level of Cdx-2 becomes stronger in the external blastomeres and weaker in the internal blastomeres. And Cdx2 protein is commited to external blastomers that will become TE cells at around 32 cell stage. (Marikawa Y et al., 2009). The other important gene is a POU domain trnscriptin factor, Oct4 (Yamanaka et al., 2006). Oct4 is the marker of pluripotent cells and is strongly expressed in ICM, while Cdx2 is exclusively expressed in TE (Stumpf et all. 2005; Szczepanska et al.,2011).

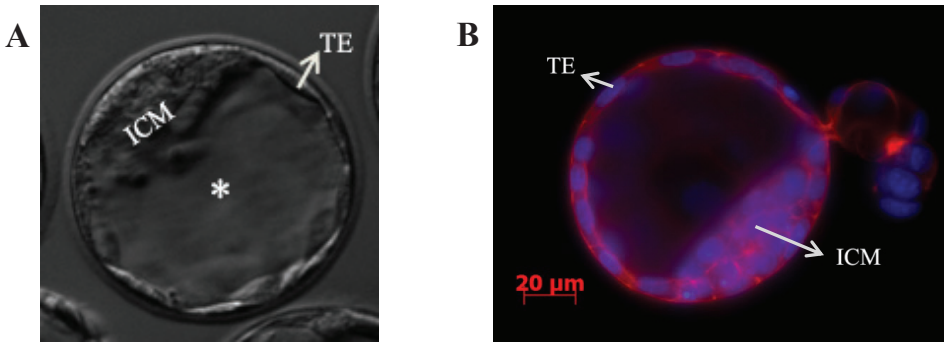


Figure 1. **A**: Image of a late stage blastocyst that is cultured in vitro. TE, trophectoderm; ICM, inner cell mass, (*), blastocyst cavity. **B:** Fluorescence microscopic images of a late blastocyst stage embryo that is stained for actin filaments (red) using fluorescently labeled phalloidin to label membranes. In the image actin filaments observed in cell-cell contact sites. Nuclei were stained with DAPI (blue). Scale bar =20 µm.

## MATERIALS AND METHOD

Embryo Collection

Firstly, B6D2F1 female mice 6-8 weeks old, were induced to superovulate by intraperitoneally injections of 5 IU of equine chorionic gonadotropin (PMSG) and human chorionic gonadotropin (hCG) at 48 hours (h) apart. Female mice were mated overnight with fertile males of the same strain. Following morning, inseminated females were selected by the presence of vaginal plug. At 44 h after hCG injection, female mice were sacrificied by cervical dislocation and embryos were flushed from the dissected oviducts with FHM HEPES-buffered medium (MR-024-D;EMD Milipore) under the stereo-microscope. After that, embryos were cultured in 20µl drops of KSOM-AA medium (MR-121-D;EMD Milipore) under mineral oil in 3,5 cm plastic dishes at 37ºC in a 5% $CO^2$ humidified air incubator.

Immunofluorescent Staining

Embryos were fixed in 4% paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS) for 30 minutes (min) at room temperature. Embryos were subsequently permeabilized in PBS containing 0.5% Triton X-100 for 15 min at room temperature. After blocking with 5% bovine serum albumin in PBS containing 0.1% Tween-20 (PBSw), samples were incubated in the primary antibody overnight at 4ºC and embryos were incubated in secondary antibody for 2-3 h at 25ºC. Primary antibodies used were, mouse anti-CDX2 (1:200; CDX2-88; BioGenex), goat anti-POU5F1 (1:200; N-19, #sc-8628; Santa Cruz Biotechnology), Secondary antibodies (1:1000; Life Technologies) used were conjugated with Alexa Fluor 546 namely rabbit anti-mouse, and conjugated with Alexa Fluor 488, namely rabbit anti-goat. F-actin filaments were visualized by adding phalloidin conjugated with Alexa 546 (Life Technologies) at a final concentration of

33 nM in the secondary antibody solution to visualize cell membranes. Stained samples were mounted in ProLong Gold antifade reagent containing 4',6'-diamidino-2-phenylindole (DAPI; Life Technologies) (Laeno AMA, 2013).

Microscopy and Image Analysis

Embryos were imaged using an Axiovert 200 fluorescence microscope (Carl Zeiss). Blastocysts were placed in the KSOM-AA drop and images were captured using Axiocam MRm digital camera, which was controlled by the Axio Vision software (Carl Zeiss).

**RESULTS AND DISCUSSIONS**

Cdx2 expression is known as a marker of TE and TE precursors (Stupf et al.,2005) and is absent from ICM in mouse blastocysts. (Stupf et al.,2005; Niwa et al., 2005). Oct-4 is a marker of pluripotent cells and is also expressed in TE cells of mouse blastocysts (Dietrich et al.,2007).

We examined the expression of Cdx2 and Oct4 in blastocyst stage embryos. Initially, embryos were incubated until blastocyst stage and were imaged using an fluorescence microscope. As expected, Cdx2 was localized exclusively to the nuclei of TE cells, while Oct4 was expressed and localized in nuclei of all cells in mouse blastocyst. We also stained blastocysts using two transcription factors (Cdx2 and Oct4) to confirm that Cdx2 can be used as a specific marker for trophectoderm cells. We did not observe any colocalization on ICM cells that showed us Cdx2 is TE specific. In blastocyst stage embryos, ICM cells exhibited strong nuclear Oct4 staining while TE cells are weakly stained.

The Cdx2 is one of the earliest transcription factors that is observed during lineage determination essential for formation and maintenance of the TE lineage in mouse blastocysts (Niwa et al., 2005; Sritanaudomchai H et al., 2009). In Cdx2, knockout embryos are capable of forming compaction, blastomere polarization and blastocyst cavity, showing that epithelization of TE is independent of Cdx2 (Marikawa Y et al., 2012, Stupf et al.,2005). At later stages, the fact that Cdx2 null embryos lose the TE and fail to sustain the blastocyst cavity shows that Cdx2 may be essential for the maintenance of epithelial integrity during this process ( Marikawa Y et al., 2009; Marikawa Y et al., 2012).

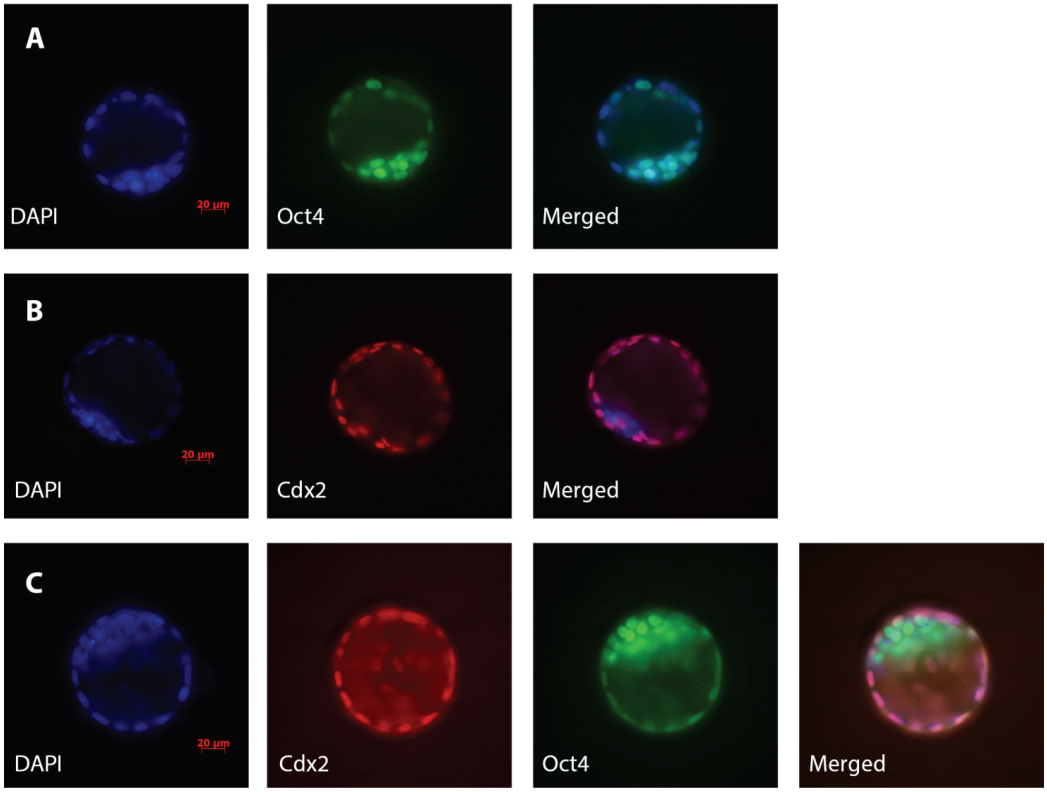


Figure 2. **A**. Fluorescence microscopic images of a late blastocyst stage embryos that is stained for nuclei with DAPI (blue), and for Oct4 protein with a specific antibody (green). **B.** Fluorescence microscopic images of a late blastocyst stage embryos that is stained for nuclei with DAPI (blue) and for Cdx-2 protein with a specific antibody (red). **C.** Fluorescence microscopic images of late blastocysts immunostained for Cdx-2 (red) and Oct-4 (green) proteins**.** Nuclei were stained with DAPI (blue). Scale bar =20 µm

Loss of Cdx-2 results in ectopic expression of ICM markers (Pou5f1 and Nanog) in the TE and an inability to maintain TE development (Stumpf et al., 2005, Stephenson RO., 2010). The early restriction of Cdx2 expression, along with its role in inhibiting the expression of ICM-specific transcription factors in the TE indicate that Cdx2 is an essential factor for the divergence of TE and ICM lineages (Stephenson RO., 2010). Although Cdx2 is required for the maintenance of TE, it is dispensable for the formation and maintenance of ICM (Marikawa Y et al., 2009). It has been suggested that Cdx2 expression is regulated by Tead4 (TEA-domain transcription factor) and Yap (Nishioka et al., 2008, 2009; Yagi et al., 2007). Tead4 is expressed in all cells of the embryo during mouse preimplantation development. Yap, which is co-activator partner of Tead4, is localized in the nucleus, and is restricted in developing TE, thus restricting CDX2 expression to outer cells of TE (Stephenson RO., 2010; Nishioka et al.,, 2009).

Oct4 is one of the cell fate determination factors during preimplantation development. It has been shown that Oct4 is essential to prevent ICM from diverting towards the TE lineage (Marikawa Y et al., 2009). Some transcriptional targets of Oct4 are Fgf4 gene and Nanaog gene. Nanog is a homeobox transcription factor that is essential for the

maintenance of pluripotency (Chambers I et al., 2003; Mitsui K et al., 2003).

## CONCLUSIONS

Blastocyst stage is critical for implantation and maintaining the pregnancy. Implantation is also critical to the survival and development of the early embryo. A complication in this stage of development will lead to defective implantation and pregnancy. Our study demonstrated and reviewed that the transcriptional factors Cdx2 and Oct4 are essential and critical regulators of cell lineage determination and these factors are important to understand mechanisims of mouse embryo development.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# COMPARATIVE EVALUATION OF SOMATIC CELLS LEVELS OF GOAT MILK FROM ALPINE AND CARPATHIAN BREEDS

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***Abstract***

*Milk is considered the most complete aliment, being abundant in high quality nutritive substances. The composition of goat milk, as well as its quality, differs between breeds and living area. Milk somatic cells are an indicator of hygienic quality as well as of the health of the mammary gland. The research aims the comparative analysis of milk quality depending on breed, as well as mammary gland health, goats being selected out of a group of 60 raised and fed in identical conditions in a private farm in Cluj County, considered clinically healthy being at the second lactation. For the determination there have been analyzed individual samples of raw milk harvested during July-September 2014 from Alpine (n=10) and Carpathian (n=10) breeds goats, using an automatic device for somatic cells count of Fossomatic type. Following the corroboration of data for 6 Alpine goats the number of somatic cells was over the maximum admitted limit (400000/ml milk), respectively 4 were under the limit. Fort the Carpathian breed 8 of them were between limits and only 2 went over this limit. The conclusion of this study highlights the acquired resistance of indigene breeds compared to imported ones.*

**Keywords:** *goat milk quality, somatic cells*

## INTRODUCTION

Caprins are usually growing in hilly and mountainous areas, often around urban places, grouped in small or large households (40-60 heads) and even in micro and specialized farms (Taftă, 2002). As Taftă appreciated in 2002, 70% of the goats of our country, belonged Carpathian race, and the remaining 30% of domestic or imported breeds. Carpathian goat herds, less improved, are still rustic, with relatively low milk production, but remarkably resistant to harsh living conditions. French Alpine is an improved breed, having a well-developed body with a very good yield at the milk and yeanlings production. Goat milk is an essential element for numerous diets used in the prevention and treatment of human diseases, is a complete balanced food containing protein, fat, carbohydrates, vitamins and minerals (Hanzen, 1994, Michel, 2001, Park et al., 2007, Ksontini et al., 2011). Milk quality is considered essential for the welfare and safety of consumers (Yadav, et al., 2014). Cell contents is also a major component of goat milk, somatic cells represents one of the most relevant parameters used to assess hygienic-sanitary breast health and hygienic quality of milk as a product for public consumption (Sabău and Rotaru, 2006).

In the case of cows, the determination of the total number of cells in milk, is one of the most widely used tests in detecting mastitis (Hicks et al.,1994, Ognean L., 2001). According to current research these tests proved to be relevant also in the case of caprins, both strategies in the prevention and combating mastitis.

In case of lactating mammary gland, the evoution of inflammatory processes causes a significant increase in the number of somatic cells in milk and significant changes in the relationship between cell populations. Such cytological developments may occur even at lactating secretions with organoleptic and physico-chemical less important changes. (Rotaru and Ognean 1998).

The counting of somatic cells in milk is used both to evaluate the quality of milk and to predict any udder infections (Poutrel and Rainard., 1981).Because of this apocrine secretion, a feature of goat milk is the presence of cellular debris, which make difficult to identify and count somatic cells, because these anucleate formations contain no DNA. Such acellular particles are normally found in goat milk, which complicates the evaluation of leukocyte response in breast inflammation. On the other hand, the number of somatic cells in the goat milk can be relatively increased, even when the

percentage of white blood cells is low, given by the epithelial cells (Roguinsky et al., 1971; Schalm et al., 1971; Kapture, 1980).

## AIMS

The research in this paper are focused on comparative cytological analysis of milk from two breeds of goats (French Alpine and Carpathian) of hilly northwest Transylvania, seeking to assess the race influence degree on the cellular content of milk . However, these investigations were aimed at evaluating the effectiveness of this major cytological test in monitoring the health of goat milk and mammary gland. Milk quality as food and keeping it in optimal consumption parameters, major were and remain objectives in its early detection of changes in case of mastitis, or other general udder diseases that cause health and milk lactation disorders.

## MATERIALS AND METHODS

Cytological investigations were conducted during July - September 2014 on individual samples of raw milk obtained from two groups of clinically healthy goats, French Alpine breed (n = 10) and Carpathian breed (n = 10). The two groups studied were selected from a herd of 60 goats of a micro-farm from a hilly area north-west of Transylvania. The animals studied were grown in household system, in compliance with bio conditions, being at the second lactation. Their feeding was based exclusively on grazing in spring, summer and autumn seasons, respectively fed fibrous feed (hay, corn cobs, roots and succulent) in winter season. Milking was performed manually by a person respecting the milking hygiene. Evolution of mastitis in micro-farm has not been registered so far. There were provided optimal conditions for sampling in sterile containers, two samples per month, the milk being tested using an automated counting system of somatic cells in milk, a Fossomatic type. This device marks by fluorescence milk cells analysed after that an optical deceleration microscope identifies them entering them automatically with an electronic counting system.

## RESULTS AND DISCUSSIONS

After corroborating data, we came to the following results. Thus, for a total of 6 of the 10 goats that belong studied French Alpine breed, calved in the farm, goats imported from three years before, animals were declared to clinical examination, as healthy. For them, somatic cell count slightly exceeded the maximum allowed by (400,000 / ml milk) and 4 of them (being numbered 2, 4, 7 and 10, were placed in the standard rules of milk, are values between 322.83 $\pm$10.4x$10^3$/ml and 388.16$\pm$7.49 x$10^3$/ml. Thus the average number of somatic cells / ml in samples obtained from goat milk with number 1 was 436.17$\pm$13.92 x $10^3$/ml. For goat number 3 was obtained a value of 441.18$\pm$11.1 x$10^3$/ml. For goat with number 5, the value obtained was 428.5$\pm$13.04 x$10^3$/ml. Goat number 6, obtained a value of 425$\pm$10.23 x$10^3$/ml mentioning the fact that this value was closest to the maximum limit considered but for goat breed is a normal value. For goat and number 8 the value obtained was 429.83$\pm$17.62 x$10^3$/ml and goat number 9 obtained 436.83$\pm$16.54 x$10^3$/ml. For the six individual determinations made for each goat studied during the three months there were no significant differences, the results are close in number as shown in Table 1.

For native Carpathian breed were studied an identical number 10 the same age and lactating goats. So for eight of them, individual milk samples analysed were within the limit of European standards, with average values between 365$\pm$16.52 x$10^3$/ml and 396.33$\pm$10.68 x$10^3$/ml. For both native breed goats studied which slightly exceeded the allowed limit of somatic cells were the number 2, which has obtained a value of 419.66 $\pm$ 7.52 / ml, respectively the number 7 to the average value was 423$\pm$7.97 x$10^3$/ml. The six individual determinations performed for ten native breed goats during the three-month period recorded no important differences, a noticing in Table 2.

Table 1. Monthly assessment of somatic cells for Alpine breed

| Somatic cell number/ml (x10$^3$ ml) | | | | | | | | Average ± DS |
|---|---|---|---|---|---|---|---|---|
| Breed | Nr. of goats | Milk samples | | | | | | |
| | | July | | August | | September | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| French Alpine | **1** | 420 | 452 | 425 | 440 | 452 | 428 | **436.17±13.92** |
| | 2 | 360 | 340 | 365 | 340 | 325 | 360 | 348.33±15.7 |
| | **3** | 450 | 425 | 436 | 446 | 438 | 456 | **441.18±11.1** |
| | 4 | 330 | 315 | 320 | 312 | 320 | 340 | 322.83±10.4 |
| | **5** | 420 | 454 | 420 | 430 | 425 | 422 | **428.5±13.04** |
| | **6** | 425 | 440 | 432 | 425 | 412 | 416 | **425±10.23** |
| | 7 | 398 | 389 | 396 | 384 | 382 | 380 | 388.16±7.49 |
| | **8** | 422 | 426 | 428 | 465 | 420 | 418 | **429.83±17.62** |
| | **9** | 460 | 440 | 426 | 452 | 423 | 420 | **436.83±16.54** |
| | 10 | 380 | 382 | 368 | 366 | 364 | 360 | 370±8.94 |

Table 2. Monthly assessment of somatic cells for Carpathian breed

| Somatic cell number/ml (x10$^3$ ml) | | | | | | | | Average ± DS |
|---|---|---|---|---|---|---|---|---|
| Breed | Nr. of goats | Milk samples | | | | | | |
| | | July | | August | | September | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| Carpathian | 1 | 400 | 398 | 365 | 368 | 365 | 389 | 380.83±16.7 |
| | **2** | 422 | 430 | 410 | 412 | 424 | 420 | **419.66±7.52** |
| | 3 | 360 | 366 | 380 | 388 | 346 | 350 | 365±16.52 |
| | 4 | 410 | 399 | 398 | 389 | 365 | 380 | 390.16±15.9 |
| | 5 | 398 | 368 | 380 | 378 | 402 | 390 | 386±12.96 |
| | 6 | 398 | 388 | 389 | 390 | 402 | 410 | 396.16±8.77 |
| | **7** | 410 | 428 | 420 | 432 | 428 | 420 | **423±7.97** |
| | 8 | 400 | 380 | 360 | 358 | 400 | 405 | 383.83±21.07 |
| | 9 | 390 | 380 | 398 | 400 | 412 | 398 | 396.33±10.68 |
| | 10 | 380 | 398 | 378 | 364 | 385 | 368 | 378.83±12.20 |

## CONCLUSIONS

The final conclusion of this study highlights and supports local Carpathian breed acquired resistance to environmental ,climatic and food conditions than race French Alpine although there were no significant differences in terms of the number of somatic cells, that is not a feature between the two breeds studied. Because lately French Alpine was imported to our country by many farmers due to increased milk production, we claim that is a breed that adapts quickly to all conditions. Thus somatic cells derived from glandular epithelium scaling without an active role in milk composition only an indicator of quality and health of mammary gland. Among the factors identified in our study that may influence the somatic cell count in milk and appearing in specialized literature can remember the young age of the animals, hand milking and the number of days in milk, knowing that the number of somatic cells increases along with the days in lactation. The observation that somatic cells in uninfected milk increased during lactation and the middle of the year, confirming the general theory that somatic cell count increases with advancing lactation (Poutrel and Lerondelle,1983). Our study supports and recommends further research in this area for assessing and maintaining health of mammary gland and to increase the quality of milk as food for yeanlings as well as food for consumption.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# ANTIOXIDANT ACTIVITY OF SUNFLOWER AND MEADOW HONEY

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***Abstract***

*Honey is a natural food produced by bees from the nectar of flowers and is a mixture of carbohydrates, amino acids, enzymes, vitamins and many bioactive compounds. Due to its special composition, honey is a functional food with antibacterial, anti-inflammatory and antioxidant properties. Studies have shown that honey's properties depend mainly on the vegetable source from which it is obtained, but also on the processing type and storage. This research aims to evaluate the antioxidant capacity of sunflower honey compared to meadow honey by assessing total phenolic content, total flavonoid content, total antioxidant activity, free radicals scavenging activity and reducing power. For the determination of total phenols and flavonoids, antioxidant capacity and reducing power, spectrophotometric methods were used. To assess free radicals scavenging activity, chemiluminometric methods were used. Meadow honey showed the highest concentration of polyphenols (143.29 ± 9.12 mg GAE / kg) and flavonoids (118.09±8.84 mg CE / kg). DPPH radical scavenging capacity was higher for sunflower honey (78.32 ± 5.11%) compared to meadow honey (45.12 ± 3.26%). The two honey types showed capacity to scavenge superoxide anion and singlet oxygen, with an inhibition rate of over 50%. In conclusion, sunflower honey and meadow honey presented important concentrations of polyphenols and flavonoids and the results suggest a relationship between honey type and total polyphenols and free radicals scavenging activity.*

***Key words:*** *antioxidant activity, chemiluminometric method, honey, phenols.*

## INTRODUCTION

Honey played an important role for human civilization since its very beginnings. Honey is used in pure form or prescribed in different preparations. Honey is used in bakery (honey cookies), in the manufacturing of beverages by mixing with alcohol or it is incorporated into cosmetics products (Saxena *et al*., 2010).

Recent years have recorded a growing interest of consumers, researchers and food industry for honey because it may help maintain human health. It is widely accepted that honey plays an important role in preventing and treating different kinds of illnesses. The importance of honey as food and nutrient is primarily based on its high content of easily absorbed constituents.

A diet that provides carbohydrates, proteins, lipids, vitamins, and minerals in sufficient quantities to satisfy the body needs, is known by the concept of "functional food" which includes the potential of food to improve and promote health, and even to reduce the illnesses risk (Nagai and Inoue, 2004).

Honey is a natural complex liquid that contains more than 200 substances, of which many are known to have antioxidant properties. These substances include phenolic acids and flavonoids, enzymes (glucose oxidase, catalase), ascorbic acid, amino acids and proteins, organic acids, carotenoid-like substances, Maillard reaction products, vitamins and minerals (Beretta *et al*., 2005). The composition of honey is variable and it depends on the floral source and processing.

In this study sunflower honey (SFH) and meadow honey (MH) were subject to analysis. Honey samples used in this research were produced in a beekeeping farm from South-Eastern Romania.

## MATERIALS AND METHODS

### *Total phenolic content analysis*

Total phenolic content (TPC) in honey samples was determined according to Beretta *et al*. (2005) and Bertoncelj *et al*. (2007), with slight modification. A mass of 1 g of honey was diluted in 5 mL of distilled water. A volume of 500 µL of honey solution was pipetted into a 10 mL test tube which contained 4.5 mL

distilled water. Then, 0.2 mL Folin Ciocalteu reagent were added and the reaction mix was vortexed and left to stand for 2 min. In the end, 0.5 mL of 20 % (w/v) $Na_2CO_3$ solution were added. After 20 min, the absorbance was measured at 725 nm using V670 UV-VIS Jasco spectrophotometer. Blind control samples were prepared (aqueous solution of sugars: 40 % fructose, 30 % glucose, 8 % maltose and 2 % sucrose). The preparation and measuring procedure was the same as the one used for honey samples. The concentration of total phenolics was expressed as mg gallic acid equivalents per kg (mg GAE/kg) of honey.

***Total flavonoid content analysis***

Flavonoids are low molecular weight phenolic compounds that are vital components for the aroma and antioxidant properties of honey. Total flavonoid content (TFC) in honey samples was determined according to Blasa *et al*. (2006) and Kim *et al*. (2003). A blank was used to eliminate the interference of reducing sugars. Briefly, 0.1 g of insoluble polyvinylpyrrolidone (PVPP) was added to 5 mL of 75 mM phosphate buffer, pH 7.0, and moisturized at 4°C for 24 h. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. A volume of 5 mL of a honey solution (5 g of honey in 25 mL of phosphate buffer, pH 7.0) was added to the residual sediment, stirred for 30 min at 30°C and then filtered. This solution was used as blank. The determination of total flavonoids in honey samples started by mixing 1 mL of sample solution with 0.3 mL of 5 % $NaNO_2$ in a 10 mL test tube. After 5 min, 0.3 mL of 10 % $AlCl_3$ were added to the solution by mixing in a vortex. After 6 min of reaction, the solution was neutralized with 2 mL of 1 M NaOH. This solution was once more mixed in a vortex and transferred to a glass cuvette. The absorbance was measured using V670 UV-VIS Jasco spectrophotometer at 510 nm. The total flavonoid content was expressed as mg of (+)-catechin equivalents per kg (mg CE/kg) honey.

***DPPH free radical-scavenging activity***

DPPH scavenging activity was based on the method proposed by Ferreira *et al*. (2009). Briefly, 0.3 mL of honey extract was mixed with ethanolic solution containing DPPH radicals (0.004 g/100 mL, 2.7 mL). The mixture was vigorously shaken and left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorbance of the mixture at 517 nm. Ascorbic acid was used as reference. DPPH radical-scavenging activity (% Inhibition) was calculated as the percentage of DPPH discoloration using the following equation:

$$\% \, Inhibition = [(A_{Blank} - A_{Sample})/A_{Blank}] \times 100$$

***Ferric reducing antioxidant power assay (FRAP Assay)***

The FRAP assay was performed according to a modified method described by Benzie and Strain (1996). Briefly, 200 µL of diluted honey (1 g/5 mL) was mixed with FRAP reagent (1.5 mL). Then, the reaction mixture was incubated at 37°C for 4 min and its absorbance was read at 593 nm against a blank that was prepared with distilled water. Fresh FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6) with 1 volume of 10 mmol TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl containing 1 volume of 20 mM ferric chloride ($FeCl_3 \bullet 6H_2O$). The resulting mixture was then incubated at 37°C. A calibration curve was prepared using an aqueous solution of ferrous sulfate ($FeSO_4 \bullet 7H_2O$) at 100, 200, 400, 600 and 1,000 µM. FRAP values were expressed as micromoles of ferrous equivalent (µM Fe [II]).

***Assay procedure for chemiluminescence emission kinetics and for quenching effects of honey samples on reactive oxygen species***

For the evaluation of chemiluminescence (CL) quenching effects, all honey samples were diluted with dimethyl sulfoxide (DMSO) in ratio 1:100 (v/v). The CL measurements were performed at room temperature for 170 s (2min 50s), in test tubes (Φ12 x 75 mm), using a Berthold luminometer. The intensity of CL is given as the relative light units per second (RLU/s). Five measurements were made and an average value was calculated. The percentage of quenching effect against the reactive oxygen species was calculated using the following equation:

$$Quenching\ effect\ \%\ (S\ \%) = [(I_0 - I)/I_0] \times 100$$

where $I_0$ is the CL intensity generated by the reference system (control) (RS) and I represents CL intensity generated by the sample.

*Hydrogen peroxide scavenging activity*

$H_2O_2$ scavenging activity was assayed according to the method described by Papuc *et al*., (2012). Briefly, the reaction mixture consisted in 100 µL of luminol ($2.5x10^{-5}$M), 440 µL 30mM Tris pH 8.5 and 10 µL sample or 10 µL Tris (as RS). 50 µL of 30 mM $H_2O_2$ were added to start the CL reaction.

*Hydroxyl radical scavenging activity*

For hydroxyl radical scavenging effect assay, HO• was generated by a Fenton-type reaction system (Parejo *et al*. 2002). 50 µL $FeSO_4$ (0.4 mmol/L) and 50 µL $H_2O_2$ (1.5%) were incubated at 30°C for 2 min. Then, 50 µL sample or PBS (in control) and 600 µL luminol ($LH_2$) (0.1 mmol/L) were added into the mixture and the chemiluminescence intensity was measured.

*Singlet oxygen scavenging activity*

Singlet oxygen scavenging activity was performed as described by Voicescu *et al*. (2010). To evaluate the quenching effect of singlet oxygen, 6 µL of sample, 300 µL of 0.4% $H_2O_2$ in 100 mM acetate buffer (pH 4.5), 300 µL of 80 mM NaBr in acetate buffer and 0.8 mM luminol in acetate buffer were added. The mixture was incubated at 37°C for 10 min. The CL intensity was measured after adding 300 µL of a 10 µg/mL solution of horseradish peroxidase in acetate buffer.

*Hypochlorite anion scavenging activity*

Hypochlorite anion ($ClO^-$) scavenging activity was evaluated as described by Wada *et al*. (2007). Briefly, 900 µL of 0.53 mM luminol in 50 mM borate buffer (pH 9.5) were added to 6 µL of sample. After incubation at 37°C for 10 min, 300 µL of 40 µM NaClO in borate buffer were added to the mixture and then the CL intensity was measured.

*Superoxide anion scavenging activity*

$O_2$•- was generated in a pyrogallol autooxidation system (Zhao *et al*., 2003). The reaction mixture contained 50 µL of pyrogallol ($3.125x10^{-4}$ mol/L), 200 µL of carbonic acid/buffer saline solution (CBSS) pH 10.2 containing 0.1 mol/L EDTA, 10 µL of polyphenolic extract (DMSO was used in control) and 400 µL of luminol ($1x10^{-3}$ mol/L). The CL intensity was measured immediately for 170s.

## RESULTS AND DISCUSSIONS

Because polyphenols are present in all plants, they are also found in honey. Honey contains complex mixtures of polyphenols depending on the climate, region, soil, pollution levels, storage and many other factors. These differences are possible because certain polyphenols are specific to particular plants and hence are found only in honey produced by bees from those plants. The TPC and TFC are presented in Table 1. It can be observed that the highest concentration of polyphenols was found in meadow honey (143.29 ± 9.12 mg GAE / kg), and over 80% of these compounds are represented by flavonoids. By comparison, sunflower honey flavonoids represent only 75% of the polyphenols concentration (84.51±6.11 mg GAE / kg).

Table 1. Total phenolic contents and total flavonoid content of sunflower (SFH) and meadow honey (MH)

| Sample | TPC (mg GAE/100 g) | TFC (mg CE/100 g) |
|---|---|---|
| **SFH** | 84.51±6.11 | 63.22±5.19 |
| **MH** | 143.29 ± 9.12 | 118.09±8.84 |

These results are similar to previous studies in which honey samples with high polyphenol concentrations also contained high flavonoid levels (Beretta *et al*., 2005; Vela *et al*., 2007; Khalil *et al*. 2011). Total phenolic content of SFH and MH are also within the reported range of Slovenian honey and Romanian honeys (Bertoncelj *et al*., 2007; Al *et al*., 2009). Al-Mamary *et al*. (2002) indicated that the determination of total phenolic content of honey is a good parameter for the assessment of its quality and possible therapeutic potential.

In evaluating the radical-scavenging potential of honey, the DPPH assay is frequently used because the antioxidant potential of honey has been shown to be directly associated with its phenolic and flavonoid contents (Beretta *et al.*, 2005).

Free radical scavenging activity by DPPH method was used to determine the antioxidant activity of honey. This method is specific because higher values mean higher antioxidant activity.

The highest percentage of DPPH inhibition was exhibited by SFH (78.32 ± 5.11 %); MH

showed a lower inhibition of DPPH free radical (45.12 ± 3.26 %) (Table 2).

FRAP is a widely used method for antioxidant determination and it has been used for the assessment of the antioxidant and reducing power of honey.

The FRAP assay gives an estimation of the antioxidants present in a sample based on its ability to reduce the Fe [III] to Fe [II]. The highest FRAP was recorded for MH (653.45 ± 49.46 µmols Fe (II)/kg), while SFH showed a lower FRAP (560.23 ± 44.71 µmols Fe II/kg) (Table 2).

Table 2. FRAP assay and DPPH scavenging activity of sunflower (SFH) and meadow honey (MH)

| Sample | DPPH (% Inhibition) | FRAP (µmols Fe [II]/kg) |
|---|---|---|
| **SFH** | 78.32 ± 5.11 | 560.23 ± 44.71 |
| **MH** | 45.12 ± 3.26 | 653.45 ± 49.46 |

Significant correlations were determined between TPC and TFC and antioxidant parameters. The strongest positive significant correlation was found between total phenolics and total flavonoids ($R^2$ =0.9287).

A strong positive correlation was also found between phenolics and DPPH ($R^2$ =0.7284), indicating that phenolics also contribute to the antioxidant capacity of honey. This statistically significant correlations are in agreement with previous findings of Saxena *et al*. (2010), Kishore *et al*. (2011), Khalil *et al*. (2011), Islam *et al*. (2012) and Maurya *et al*. (2014).

Luminol ($LH_2$) reacts with reactive oxygen species (ROS) to yield a compound in an excited electronic state which returns to ground state with production of light (chemiluminescence).

The decrease of CL intensity in time, under reference system (RS) signal, corresponds to the scavenging of ROS by an antioxidant, and the increase of CL intensity in time, upper RS signal corresponds to the formation of free radicals by a prooxidant (Papuc *et al*. 2012) .

Calculation of quenching effects 5 s after the start of luminol-superoxide anion reaction showed a remarkable antioxidant activity for SFH (85.68%) and MH (84.26%), for the dilution 1:100 (v/v) compared to ascorbic acid (AA) (23.86%) (Table 3).

Table 3. Percentage of quenching effect (Q %) against ROS, 5 s after the beginning of the reaction, of sunflower (SFH) and meadow honey (MH)

| ROS | SFH | MH | AA |
|---|---|---|---|
| **$H_2O_2$** | 42.55 | 70.93 | 39.75 |
| **·OH** | 78.79 | 17.34 | 65.81 |
| **$^1O_2$** | 54.93 | 65.20 | 67.67 |
| **ClO-** | 32.89 | 41.60 | 43.92 |
| **$O_2$·-** | 85.68 | 84.26 | 23.86 |

The results obtained after calculation of percentage of quenching effect after 5 s of reaction demonstrated that the two honey samples strongly scavenged hydrogen peroxide, the highest activity being recorded for MH extract (70.93 %).

Scavenging activity of SFH and MH and AA against reactive oxygen species, 5 s after the beginning of the reaction are shown in Figure 1 (hydrogen peroxide), Figure 2 (hydroxyl radical), Figure 3 (singlet oxygen), Figure 4 (hypochlorite anion) and Figure 5 (superoxide anion). Graphical representation of CL intensity depending on time demonstrates that, comparatively to reference system, all tested extracts have the capacity to scavenge reactive oxygen species.

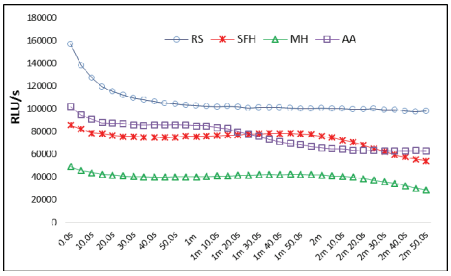


Figure 1. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by $LH_2$ - $H_2O_2$ system in Tris-HCl buffer pH 8.5

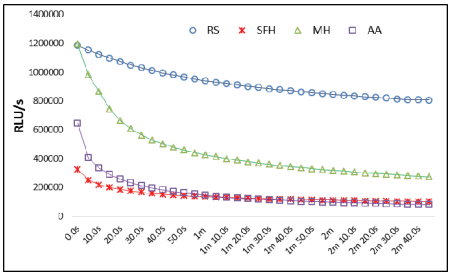


Figure 2. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by $LH_2$ - ·OH system in PBS buffer pH 7.4

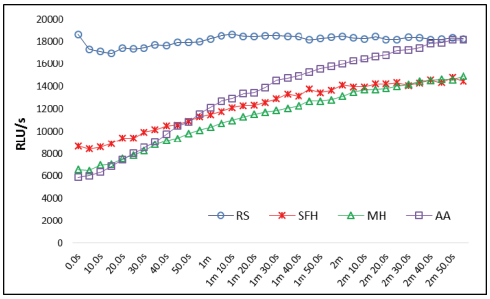


Figure 3. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by $LH_2$ - $^1O_2$ system in acetate buffer pH 4.5

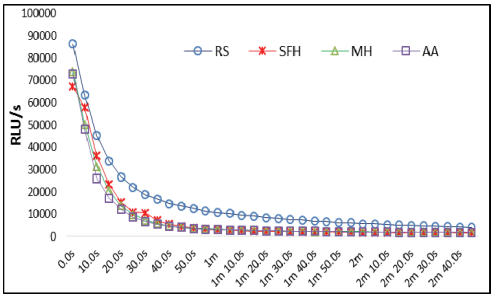


Figure 4. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by $LH_2$ – ClO- system in borate buffer pH 9.5

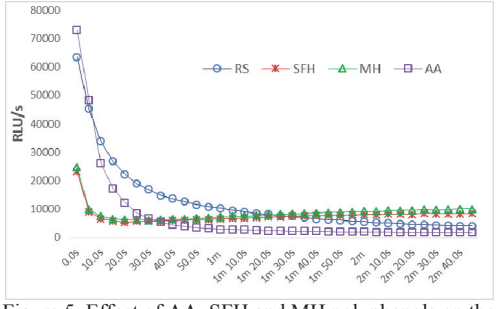


Figure 5. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by $LH_2$–$O_2^{\bullet-}$ system in carbonic acid buffer/salt solution pH 10.2

The effect of meadow honey and sunflower honey on CL emission kinetics produced by luminol – singlet oxygen reaction was similar to the one of AA. Hydrogen peroxide ($H_2O_2$) scavenging activity was higher for MH than SFH and ascorbic acid used as reference. Superoxide anion and hypochlorite anion scavenging activities were very similar for the two honey samples.

## CONCLUSIONS

In this study, the content in polyphenols and total flavonoids of two types of honey (sunflower and meadow honey) was investigated. Both honey assortments showed important concentration in polyphenols and flavonoids. Moreover, DPPH scavenging activity seemed to correlate with the concentration of honey phenolics and flavonoids.

The results suggested a relationship between honey total polyphenols and free radicals scavenging activity determined by chemiluminescent assay.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# FRACTAL CHARACTERISTICS OF ADIPOCYTE DYNAMICS IN MICE

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***Abstract***
*The functional status of adypocites is reflected in their morphology and is directly related to the metabolic state of the individual. Here, we investigate if fractal analysis is useful in highlighting the impact on adipocytes of progressive exposure to a hypercaloric diet. A total of 18 NMRI mice were assigned to 3 groups: control group (C), obesity induced by hypercaloric diet at one (M1) and two months (M2). Samples from mesenteric, omental, perirenal and inguinal subcutaneous adipose tissue samples were collected from each subject and analyzed through fractal dimension (FD) method. Within the performance range of current medical tests, FD showed differences between M1 and C (area below ROC curve > 0.9), as well as between M2 and C. Data collected from the inguinal subcutaneous site provided a statistical distinction between M2 and M1 (area below ROC curve 0.714). Thus, we concluded that FD represents a reliable method for identifying the smallest changes in the adipose tissue morphology.*

***Key words:*** *adipocytes, obesity, fractal dimension, fractal analysis, mouse.*

## INTRODUCTION

Understanding obesity may be crucial for future developments in clinical therapy and diagnosis. The mechanisms by which excess energy is managed, underlies the entire metabolic process. Both diet and genetic background of a species can influence the expansion and contraction dynamics of the adipose tissue. In our current view there are two main growth mechanisms. One of these mechanisms is represented by hyperplasia (a cell number increase) and the other by hypertrophy (cell size expansion) (Drolet et al., 2008; Spalding et al., 2008; Faust et al., 1978). Unlike other essential organs (brain, lung, heart, kidney), the adipose tissue has the widest range of expansion due to his storage function. Adipocyte hypertrophy can block the proper function of body fat by inducing mechanical stress and local inflammation, thus, initiating hyperplasia. When obesity is experimentally induced, various parameters of adipocytes can be quantified, such as diameter, perimeter, area, and other non-classical parameters. One of these non-classical parameters is represented by cell membrane contours of neighborhood adipocytes. We considered fractal analysis as the main method for quantifying such a relationship (Mandelbrot,1967; Mandelbrot, 1983). The computed fractal dimension of the microscopic picture may reveal existing relationships between hyperplasia and hypertrophy of adipocytes in different fat regions (Arner & Spalding, 2010; Britton & Fox, 2011; Smith et al., 2008; Spalding et al., 2008). Here, adipocytes from four distinct anatomical regions have been considered, namely the omental, mesenteric, perirenal, and subcutaneous inguinal region. The parallel analysis of these four regions has been the basis in elucidating different relationships between mice white adipocytes. The main objective consisted in capturing the dynamics/evolution of the adipose tissue expansion in all four regions. The secondary objective was to evaluate the

method itself, to see if it is suitable for such a task.

## MATERIALS AND METHODS

A total of 18 NMRI mice were assigned to 3 groups, each consisting of 6 individuals: control group (C), obesity induced by hypercaloric diet at one (M1) and two months (M2). Samples from mesenteric, omental, perirenal, and inguinal subcutaneous adipose tissue were collected from each subject (Figure 1).

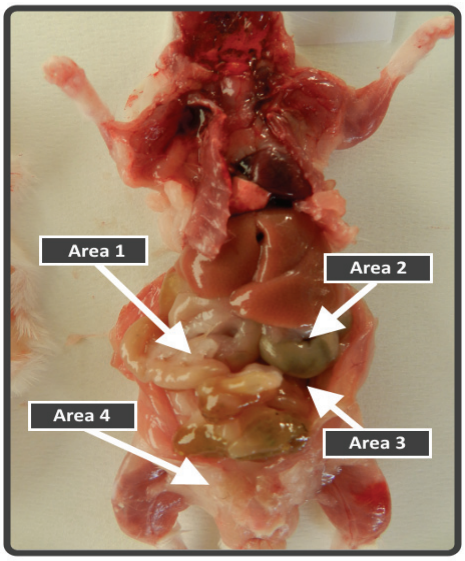


**Figure 1. Adipose tissue samples.** The samples were collected from mesenteric area (Area 1), omental area (Area 2), perirenal area (Area 3), and inguinal subcutaneous area (Area 4).

The adipose tissue samples were fixed immediately after harvesting in buffered 10% formalin solution for 24 h. Processing of the samples and paraffin embedding were made automatically by the tissue processor 120-3 Thermo Scientific STP. Onward, blocks were sectioned at 3 μm using Leica microtome RM 125RTS. All slides were stained with hematoxylin eosin using Thermo Scientific Microm HMS 70. Thus, 360 photomicrographs 400x have been made using an Olympus BX 41 microscope equipped with Olympus SP350 video camera (Figure 2A). The photomicrographs were digitally processed and their fractal dimension (FD) was calculated (Figure 2).

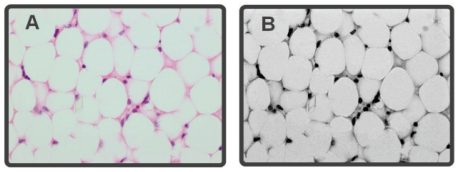


**Figure 2. Examples of photomicrographs used for fractal analysis.** (**A**) Original image of the microscopic field, (**B**) black and white image used for fractal analysis.

Prior to the image analysis, the digital processing of photomicrographs consisted of transforming each pixel color from the original image to the corresponding gray palette (Gagniuc et al., 2013). The fractal dimension for each processed picture was then calculated using FracLab 2.05 software (INRIA Saclay Research Center designed - Ile-de-France). FracLab 2.05 uses the box method (which provides a very good approximation of the Hausdorff dimension). A given set of points (pixels on the image – Figure 2B) is broken down into $\varepsilon$-sized boxes and the number $N$ of boxes which include elements of the set is counted. For various $\varepsilon$ a respective $N$ value is determined. The dependence $N(\varepsilon)$ is plotted on double logarithmic coordinates, where the slope of the dependence corresponds to the value of the fractal dimension. The fractal dimension ($D_H$) formula is defined below:

$$D_H = \lim_{\varepsilon \to 0} \frac{\log N(\varepsilon)}{\log \frac{1}{\varepsilon}}$$

where $\varepsilon$ is the size of the box, and $N$ represents the number of positive boxes. Hence, the dependence is shown by $N(\varepsilon) \sim 1/\varepsilon^D$. Thus, when the differences between means (medians) of $D_H$ for each group/anatomic site were found significant ($P<0.02$), the performance of a differentiation-test based on FD was evaluated by ROC (Receptor Operating

Curve) plot analysis (Gaiță et al., 2013). The statistical analysis used StatsDirect v. 3.0.

## RESULTS AND DISCUSSIONS

The fractal dimension remains a useful measure of the complexity of a bidimensional contour. Therefore, fractal analysis has already found widespread applications in biology, medicine, and their related fields (Losa, 2012). Here, digital image analysis has been adapted to measure the fractal dimension of adipocyte profiles (Gagniuc et al., 2013; Losa, 2009; Condrut et al., 2015). Specifically, the aim was to determine whether the complexity of the branching pattern between adipocyte cell membranes reflects their function in regard to the state of induced obesity in mice. Within the performance range of current medical tests, FD showed differences between M1 group and C group (ROC curve >0.9), as well as between M2 and C groups (Figure 3A-F). Data collected from the samples taken from the inguinal subcutaneous site provided a statistical distinction between M2 group and M1 group (area below the ROC curve 0.714).

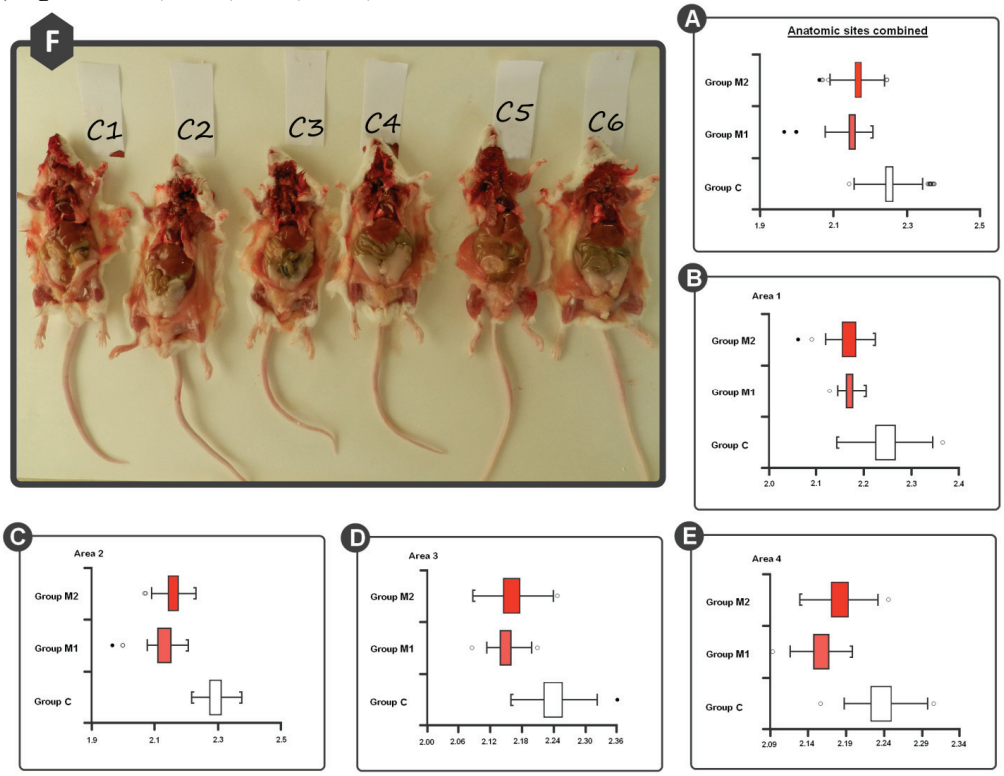


**Figure 3. Box-and-whisker plottings for fractal dimension.** (**A**) Fractal dimension of adipocytes for Group M1, Group M2, Group C (control) in all the regions (mean values for the four regions). Fractal dimension of adipocytes for Group M1, Group M2, Group C (control) in regions (**B**) mesenteric area (Area1), (**C**) omental area (Area 2), (**D**) perirenal area (Area 3), (**E**) inguinal subcutaneous area (Area 4), (**F**) individuals from group C.

Thus, these observations suggest that FD represents a reliable method for identifying the smallest changes in the adipose tissue morphology (Figure 4A-D). Although previous reports had hinted at the omental region, the inguinal subcutaneous site was

found to provide the most sensitive samples to both emergence and dynamics of changes in the adipose tissue status induced by exposure to hypercaloric diet (Figure 4C).

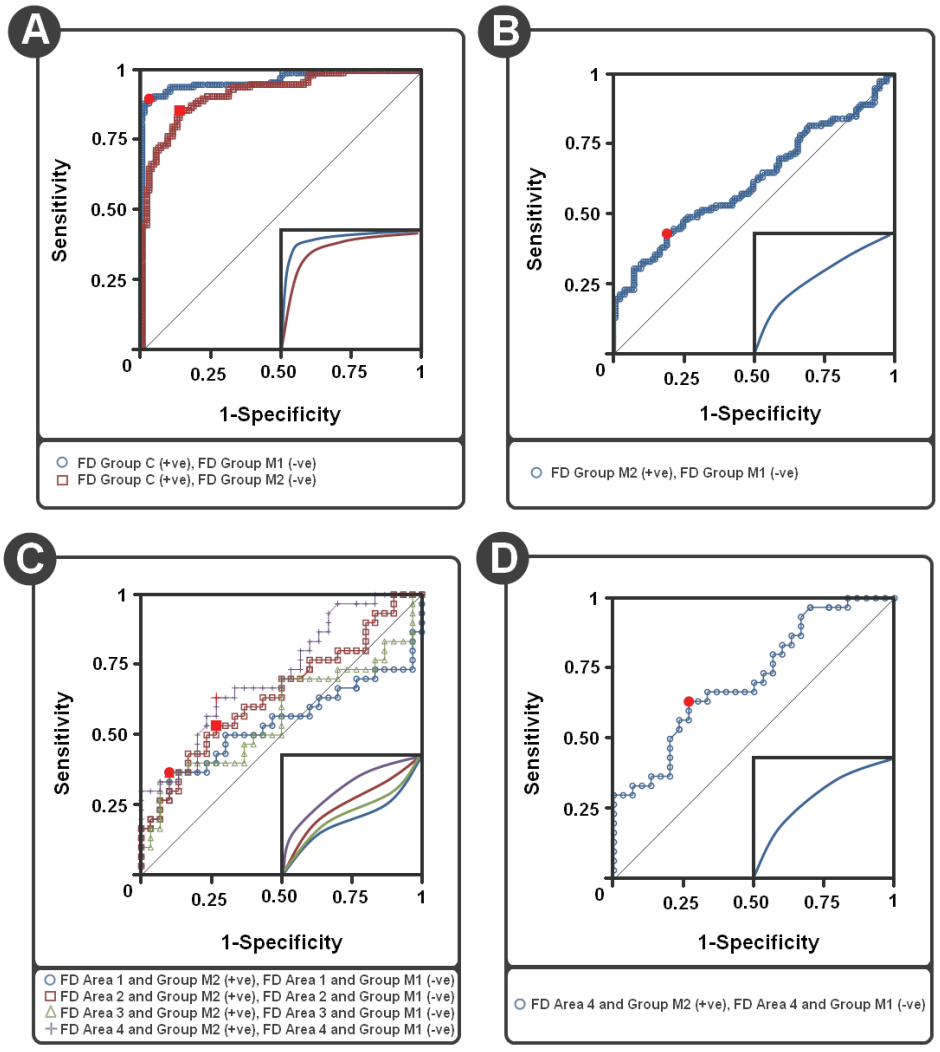


**Figure 4. ROC distributions.** (**A**) Group C/Group M1 (in all areas) and Group C/Group M1 (in all areas), (**B**) Group M1/Group M2 in all areas, (**C**) the entire group analyzed in all areas, (**D**) Group M2/Group M1. Cutoff value was the threshold for which the analysis was performed (red dots).

The wide range of the upper and lower quartile observed in M1 and M2 groups, shows that adipocytes of different anatomical areas respond differently to induced obesity in mice (Figure 3A-F). Some histopathological aspects of the adipose tissue collected from group M1 (HE stain, x400), are shown in Figure 5. An important observation was that hyperplasia and hypertrophy of adipocytes show radically different outlines (in all four anatomical regions) which are therefore interpreted differently through FD.

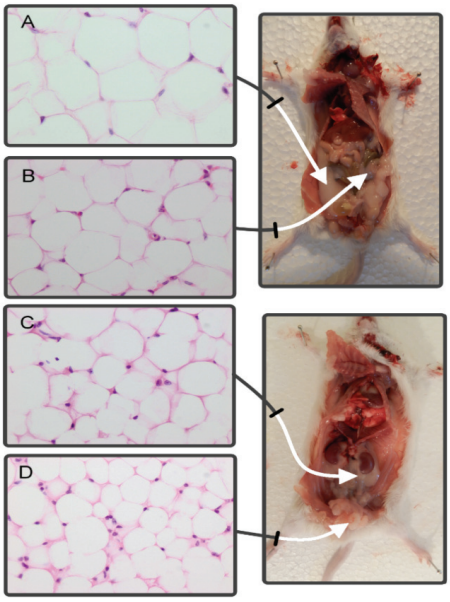


**Figure 5. Histopathological aspects of the adipose tissue in mice.** (**A**) omental area, (**B**) mesenteric area, (**C**) perirenal area, (**D**) subcutaneous inguinal area.

Even adipocytes from different anatomical sites clearly differ from each other in the context of membrane contours (Figure 5A-D).

Our initial question was if FD may or may not be used as a deterministic method on obesity-related pathology. In obese mice we observed significant structural changes in the adipose tissue compared to non-obese mice (Figure 6A-C).

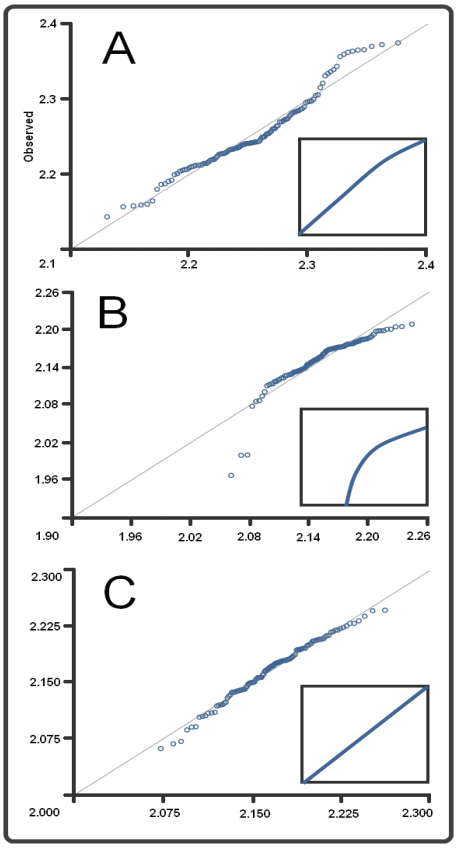


**Figure 6. Distribution of FD values**. (**A**) group M1, (**B**) group M2, (**C**) group C.

Thus, FD may be an effective histo-pathological parameter for the early detection of changes in adipocytes. However, it is difficult to predict whether this parameter can enter the diagnosis industry as a stand-alone methodology.

## CONCLUSIONS

In this study, fractal analysis has proven to be effective for an early detection of changes in fat cells. Our results suggest that adipocytes from subcutaneous inguinal area show the most sensitive changes when mice are exposed to a high calorie diet. To make specific use of the fractal dimension data as a deterministic tool, a more complex approach is required in the future. By correlating different clinical and/or biochemical data with observations made through the fractal dimension method, new

diagnostic and/or prognostic models may be on the horizon.

## ACKNOWLEDGEMENT

This work was supported by "Doctoral scholarships supporting research in the field of agronomy and veterinary medicine", identification number POSDRU/107/1.5/S/76888. This work was also supported by "CERO – Career profile: Romanian Researcher", grant number POSDRU/159/1.5/S/ 135760, cofinanced by the European Social Fund for Sectoral Operational Programme Human Resources Development 2007-2013.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# HEART TOPOGRAPHY AND PERICARDIC LIGAMENTS OF GUINEA PIGS

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***Abstract:***

*Small rodents are the most used experimental models in research related to cardiovascular and respiratory system. The guinea pigs occupy a leading position. However, detailed anatomical descriptions of the thoracic cavity of this specie are relatively few in the literature. Compared to mice, rats or hamsters, widely used in research, electrocardiogram waves are similar to humans, making the guinea pigs to be the choice model for studies related to cardiac arrhythmias, and in particular, pharmacological studies. Using gross dissection of the thoracic cavity of ten guinea pigs, this study aims to achieve a detailed description of the heart topography and pericardial ligaments in guinea pigs. Occupying the majority of the narrow thoracic cavity, in the middle mediastinum, in guinea pigs, like all mammals, heart is double layer coated by the pericardium. It lies in the median plane, slightly oriented to the left at the level of 2nd-4th intercostals space and approximately at 1 cm cranial to the xiphoid appendix. External thin walls of the atria are separated from the ventricles by the grooves of coronary arteries and veins, showing multiple branches in all specimens studied. Ventrally and dorsally the ventricles are separated by two shallow interventricular sulci. Pericardial ligaments are well represented and are generated by reflection of the fibrous pericardium on the neighbouring structures, making heart attachment, mechanic protection of the heart and its great vessels.*

*The following ligaments were visualized in all subjects: sterno-pericardial ligaments (cranial and caudal), in four subjects being joined by a thin blade of adipose tissue; phreno-pericardial ligaments (central-strong, left-shorter, missing in two subjects and right-long); dorsally the verterbro-pericardial ligaments which connect the pericard to the spinal cord, more developed on the left side, forming sheaths for the aorta and for the large vessels. In conclusion, pericardial ligaments achieved a dynamic balance, constantly modified in relation to the phases of the cardiac cycle, their knowledge being necessary both practitioners and researchers which uses guinea pigs as experimental models in cardiovascular studies.*

***Key words***: *heart, pericardic ligaments, guinea pigs*

## INTRODUCTION

Although in recent years there is a conservative attitude of anatomists related to the resumption of anatomical studies or acceptance of new explicit theories or morphologically documented studies, the tendency to complete anatomical descriptions, especially of the animals used as experimental model, is unquestionable and must be accepted. In animals,cardiovascular system adaptation, are referred to morphological particularities due to taxonomic affiliation,environment and physical activity (Barone 1997, Cotofan et al., 2007).It is clearly stated that the heart of mammals share many similarities, beginning from embryonic developmental evolution, as long as the heart is the first organ to fully form and function during the vertebrate development (Kent and Carr, 2001; Kirby 2002). Many of the researches findings claim the presence of the same underlying mechanisms in mammal's heart development, which are considered molecularly and developmentally similar (Harvey and Roshental 1998). However, in adult mammals the sizes, shape and positions of heart vary between species. Currently, in medical research, animal use as experimental model is fundamental in developing new therapies of cardiovascular disease, but the extrapolation of animal data requires that the animal model chosen for testing is similar in anatomy and physiology to that in humans (Paul and Paul 2001). Guinea pigs choice for cardiovascular studies must be primarily based on scientific hypothesis, the degree of species similarities to the human anatomy and the appropriate animal housing and care. This

involves a proper selection of the animal model and a detailed knowledge of its anatomy. The present study aims to provide a detailed description of topography, external conformation and pericardic ligaments of the heart in guinea pigs.

## MATERIALS AND METHODS

Ten adult guinea pigs from ages between 1 and 3 years old, both sexes (4 male and 6 female) and varying weights (370-610g) were used. The subjects were part of an ongoing study related on digestive system and were provided by the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj Napoca, Romania, bio base. Gross dissection was performed after euthanasia which was made by inhalation of an overdose of isoflurane (Baxter Health Care Corporation, USA). Thoracic cavity was opened by a double incision along the right and left side of the sternum to preserve the ligaments insertion. The ribs were carefully removed and the topography of thoracic organs and their ligaments were photographed using a Nikon D60 digital camera. Terms were used in agreement with NAV 2012. The Institutional Bioethics Committee of University of Agricultural Science and Veterinary Medicine approved the study.

## RESULTS

### *Heart topography*

In guinea pigs the heart occupies a relatively large space in the thoracic cavity, extending from the sternum to the vertebral column and leaves only a narrow space for the lungs on each side. Its base, dorsocranially oriented, lies in the midline, at the level of 2nd-4th intercostals space, the apex caudoventrally directed was situated at 1cm distance from xiphoid appendix (Fig. 1). The overall orientation was slightly to the left in caudal direction and a more ventrally tilted long axis of the heart (Fig. 2).

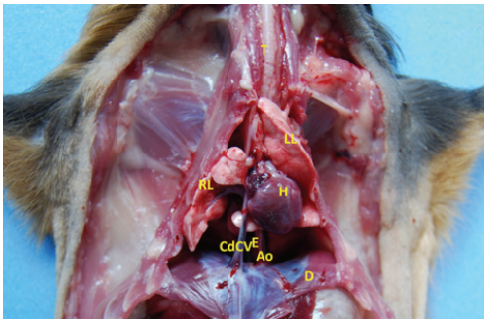


Fig. 1 Heart topography in guinea pigs. H-heart; T-trachea; LL-left lobe of lungs ; RL-righe lobe od lungs;-CdCV-caudal cava vein; E-esophagus; Ao-aorta; D-diaphragm

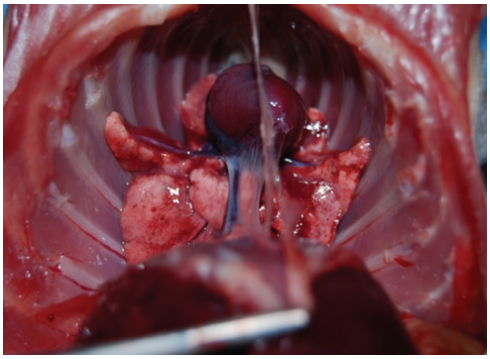

Fig. 2 Note the slightly left orientation in caudal direction and a tilted long axis of the heart

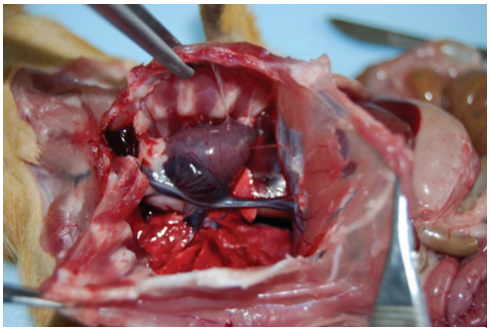

Fig. 3 Right lateral view of the thoracic cavity in guinea pigs. Extended course of the caudal vena cava after passing the diaphragm

The tilted of the heart was limited due to the extensive attachments of the pericardium to the sternum and diaphragm. The auricles were visible on both, right and left sides with the pulmonary trunjk located between them and to the left oriented. The caudal vena cava has an extended course on the right side of median plane (Fig. 3).

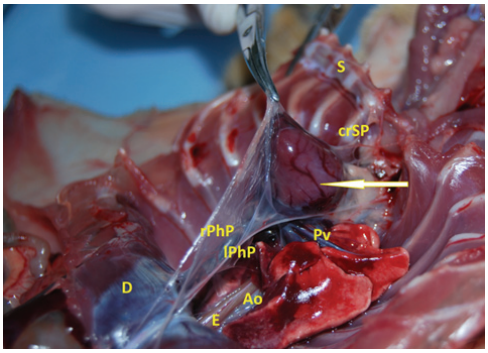


Fig. 4 Well represented arterial vascular supply and venous drainage of the heart-arrow. (S-sternum; crSP-cranial sternal pericardiclig.; rPhP-right phrenico-pericardial lig.; lPhP-left phrenico-pericardial lig.; Ao-aorta; E-esophagus; D-diaphragm; Pv-pulmonary veins)

The aortic arch projects caudally, first on the right then to the left being accompanied by the pulmonary trunk.

In all subjects, the coronary arteries were well observed on the external aspect of the heart running into the shallow coronary groove, with an extensive collateralization of the arteries. Also, an extensive network of intercommunicating veins provides the venous drainage of the heart (Fig 4).

***Pericardic ligaments***

The heart was enclosed by the fibrous pericardium (*Pericardium fibrosum*), which in cranial direction was fixed at the base of the heart, extends upward on the great arteries and veins, enclosing these vessels; ventrally the fibrous pericardium was attached to the sternum and to the diaphragm by several and obvious ligaments.

Using NAV we systematized the pericardic ligaments in ventral, cranial, dorsal and caudal ligaments.

In all subjects, ventrally, the pericardium was attached to the sternum through the cranial and caudal sterno-pericardic ligaments (*Lig. sternopericardiacum*), which was continuous in seven subjects, showing a small amount of adipose tissue within. The *cranial sterno-pericardic ligament* emerges from the ventral cranial region of heart to be inserted on the dorsal side of the manubrium and on the two small cylindrical vestigial clavicles on the each side. This ligament was a direct continuation of the vertebro-pericardic ligament (right and left), delimiting a space which houses a small amount of adipose tissue, a reminiscent of the thymus in adult animals. The *caudal sterno-pericardic ligament* was detached from the ventral margin of the heart, above the interventricular sulcus to be inserted of the xifoid appendix of the sternum (Fig. 5). This ligament too, was fulfilled with adipose tissue.

Cranially, the parietal pericardium (*Lamina parietalis*) surrounds the aorta and the pulmonary trunk, the inferior and superior vena cava together with the pulmonary veins, being the reflection on visceral layer (*Lamina visceralis-epicardium*) of serous pericardium (*Pericardium serosus*) creating the *pericardial cavity (Cavum pericardii)*. The fusion of the parietal serous pericardium with the fibrous pericardium creates one layer with two surfaces. The fibrous pericardium has little elasticity and by its fusion with the base of the great vessels creates a closed space in which the heart is disposed.

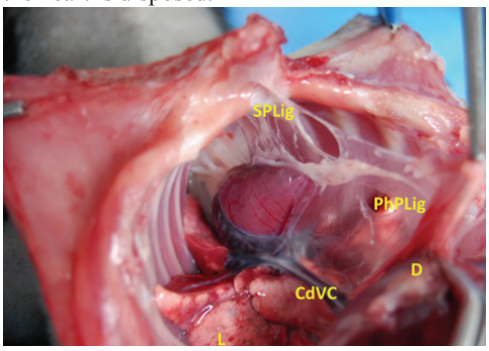


Fig. 5 Overall patern of fibrous pericardium. SPLig-sternal pericardial lig.; PhPLig. Phreno-pericardial lig.; CdVC-caudal cava vein; D-diapragm; L-lungs

In all subjects, dorsal cranial pericardial ligament was short being represented by the *aorto-pericardic ligament* which anchors the pericardium from the aortic arch in cranial direction. This ligament was almost imperceptible due to the relative large place occupied by the heart in thoracic cavity and the close proximity of the heart to the thoracic

inlet. Also, in the middle mediastinum the fibrous pericardium cover the descending aorta making the connection of the pericardium to the vertebral column.

Between the dorsal pericardium and the lungs hilum, covering the vascular and the bronchial elements, the tiny but well visualized *pericardo-pedicular ligaments* were noted. Besides the mentioned ligaments which stabilize relations between the mediastinal organs, in all subjects were identified the connection ligaments between the esophagus and trachea, esophagus and bronchi, and between the esophagus and fibrous pericardium.

The caudal pericardic ligaments were represented by the obvious *phreno-pericardic ligaments, (Lig. phrenicopericardiacum* which connect the caudal margin of the heart with the diaphragm (Fig. 6).

Three ligaments were observed in eight subjects, while in two subjects the left phreno pericardial ligament was missing. The *right phreno-pericardial ligament* was detached from the cranial right ventricle to be inserted on the tendineous center (*centrum tendineum*) of the diaphragm. The *left phreno pericardial ligament* emerges from the apex having an oblique direction for its insertion half divided: one part on the tendinous diaphragm and one part on the costal muscular diaphragm close to the 8$^{th}$ intercostals space. The *central phrenico-*

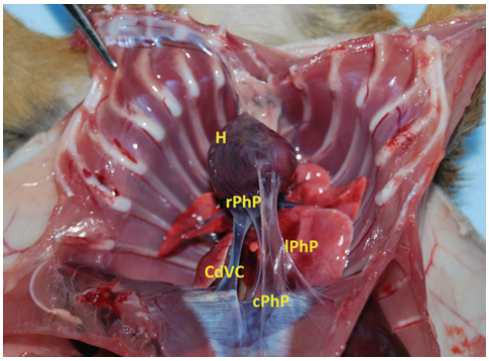


Fig. 6 Phreno-pericardial ligaments and their insertion.rPhp-rightphreno-pericardial lig. ; lPHp-leftphreno-pericardial lig.; CdVC-caudal cava vein; cPhP-centralor ventral phreno-pericardial ligament.

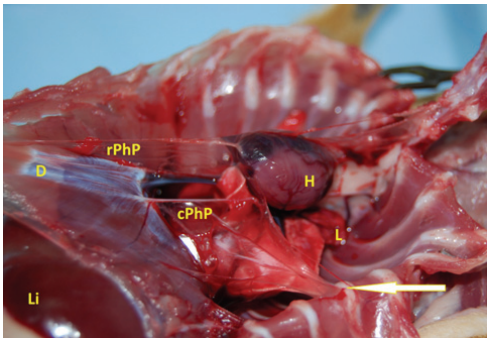


Fig. 7 The left insertion on the 7$^{th}$ rib of pericardium-white arrow

*pericardial ligament* connected the central part of the tendinous diaphragm with the apex.

This ligament was attached to the right pericardial ligament in five subjects. In two subjects in which the left phrenopericardial ligament was missing,it was well defined a *left lateral pericardial ligament* which emerges from the apex to be inserted on the 7$^{th}$rib and intercostals space (Fig 7).

***Division of the mediastinum*** A clear division of mediastinum was possible due to the accurate topography of the composing structures in all examined subjects. The cranial mediastinum (*Mediastinum craniale*) was the space between the dorsal side of the sternal manubrium, cranial mediastinal pleura on each side, upper pericardium and the first thoracic vertebrae. It contains several important structures like aortic arch, the final course of superior vena cava, the tracheea, the esophagus, thoracic duct, vagus and phrenic nerves. Also, in the cranial mediastinum a small amount of adipose tissue, a reminiscent of involuted thymus, lying close to the thoracic inlet was found in all subjects. The middle mediastinum

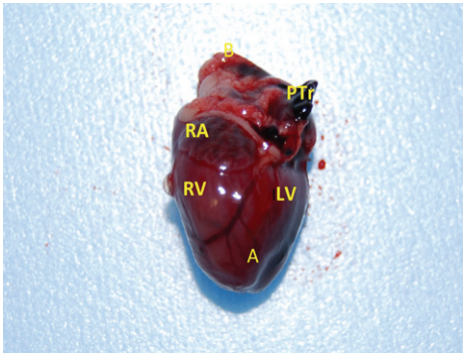


Fig.8 Size, shape and external conformation of guinea pig heart.B-base of the heart; RA-right atrium under the right auricule; RV-right ventricle; A-apex; LV-left ventricle; PTr-pulmonary trunk

(*Mediastinum medium*) was further divided into three spaces: ventral, middle and dorsal. Ventrally, the ventral mediastinum (*Mediastinum ventrale)* was bounded by the sternum and the pericardium dorsally. In this space the sterno-pericardial ligaments, internal thoracic vessels, small lymph nodes were found. The central (middle) space of middle mediastinum was occupied by the great vessels (superior and inferior vena cava, aorta, pulmonary trunk and pulmonary vessels), pericardium and heart. The caudal vena enters into the right atrium after an extended course after passing the diaphragm and caudal mediastinum: the same long trajectory was present of cranial vena cava after the confluence of the right and left brachiocephalic veins. In all subjects the pulmonary veins were well individualized emerging from the well delineated pulmonary lobes. The pulmonary trunk ascends from right ventricle, dorsal and to the left oriented, passing in front of the aorta (Fig. 8). The aorta leaves the left ventricle primary on right oriented, curves dorsally to the left becoming the aortic arch. After the aorta exit the pericardium it arches over the right pulmonary trunk, passing to the left of the trachea and esophagus and entering into the dorsal mediastinum as descending aorta. Dorsal mediastinum (*Mediastinum dorsale)* was the space between the dorsal pericardium and the posterior thoracic walls containing descending

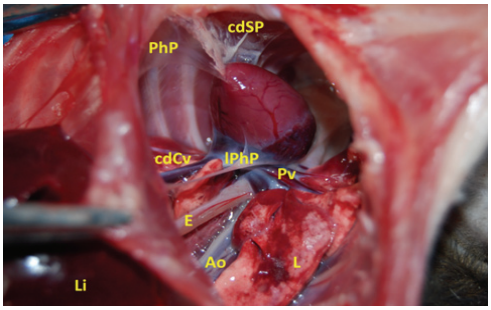


Fig. 9 Caudal view of the thoracic cavity in guinea pigs and its principal structures. cdSP-caudalsterno-pericardiclig.; PhP-phreno-pericardiclig.; cdCv-caudal cava vein,.; Ao-aorta; L-left caudal lung lobe; Pv-pulmonary veins; lPhP-left phreno-pericardiclig.

aorta, thoracic duct, thoracic sympathetic trunk and the thoracic splanchnic nerves. Esophagus passes along the right side of the descending aorta in dorsal mediastinum.

The caudal mediastinum (*Mediastinum caudale*) was a relative large space between the caudal sagital plan which passes under the apex, and diaphragm. It contains the caudal thoracic segments of caudal vena cava, descending aorta, esophagus guarded by the vagus branch (Fig. 9).

## DISCUSSION

The heart is located in ventral part of middle mediastinum in large mammals, tend to have a less pronounced left side orientation and a more ventral tilted long axis (Getty 1995; Barone 1997; Crick et al., 2001) if we compare to humans (Goss 1949; Barone 1997). Also, the heart of most animals tends to be elongated having a pointed apex. This feature is absent in dogs which have an ovoid heart with a blunt apex (Evans, 1993), ruminants, which have a pointed apex and a conical shape heart, compare with the blunted apex in sheep (Ghoshal, 1975, Kent 2001) and pigs in which the blunt apex is medially oriented (Cotofan et al., 2007). The conical, elongated heart and pointed apex in rabbit (Schiffmann, 2002) is similar to the guinea pigs heart, but in guinea pigs the hearttend to be more large related on thoracic cavity size, occupying a

disproportionally large part of the thorax and leaves only a narrow space for the lungs on each side.This is in agreement with the report of other studies about the differences that exist in the ratio of heart weight to body weight, which show that adult sheep and adult pigs have a smaller ratio of heart weight to body weight compare to adult dogs, whom ratio was as much as twice the heart weight to body weight than in mentioned animals (Ghoshal et al., 1975; Evans, 1993). The earliest literature data show that the body weight is inversely related to the heart rate and directly related to blood volume and heart weight (Holt 1970; Getty 1975).

In all mammals the apex is formed only by left ventricle, but differences exist in heart orientation. In quadruped standing animals, the heart long axis is oblique, ventro-caudally oriented and slightly to the left. Due to the quadruped posture of animals, the apex of the heart is more ventrally titled toward to the sternum, than in humans, but in guinea pigs this tilting is limited because of extensive attachment of the pericardium both to the sternum and to the diaphragm. Most quadruped mammals tend to have a less pronounced left side orientation and a more tilted long axis of the heart compare to humans, in which the heart is situated with the right atrium on the right, the right ventricle anterior, the left ventricle to the left and posterior, and the left atrium entirely posterior. The apex is projected inferiorly and to the left.

All mammalian hearts lies in the middle mediastinum being enclosed into the pericardium which creates the pericardial cavity around the heart (Barone 1997; Kent and Carr 2001; Cotofan et al., 2007). The mechanical function of the pericardium including the heart fixation in the thorax, prevention of the heart dilatation by maintenance of the heart shape, preventing of excessive movement of the heart with changes in body position and a physical barrier to infection and malignancy are of great importance but not the major one. Also, the pericardium has a secretory function accomplished by visceral layer of serous pericardium, which secrete the pericardium liquid which allows the inner visceral pericardium to glide against the outer parietal pericardium. However, it was proved that the pericardium is not essential for survival, as long as the congenital absence or pericardiectomized human and animals, have no severe adversereaction to removal, or congenital absence (Hammond et al., 1992; Abel et al., 1995). Moreover, in certain condition, the presence of the pericardium, physically constrains the heart function by a depressive hemodynamic influence that limits cardiac output by affecting and reducing diastolic ventricular function (Saunders 2012, Ware 2012; Majoy 2013), feature frequent present in humans too. Equally true is that clinically pericardial disease is one of the most infrequent type of cardiac disease, but morphologically is common, both in humans (Roberts 2005) and animals (Dempsey and Ewing 2011; DeFrancesco 2013). This could be explaining by the differences related to pericardium thickness. Generally, pericardium wall thickness increases with increasing heart and cavities size between the various species. If ovine have 0.32±0.1mm wall pericardial thickness, porcine 0.20±0.1mm, dogs 0.19±0.1mm, humans have between 1-3.5mm wall pericardial thicknesses.Our description of guinea pig pericardium is in concordance with the features found in most of the mammals. Also, in animals the differences of the amount of pericardial fluid are related to the heart dimensions and animal weight. Holt (1970) reported various volume of pericardial liquid in dogs, ranging from 0.5-2.5ml or more in large breed dogs up to 15ml. In small animals, like guinea pigs, chinchilla, rat there are no reports of pericardial liquid volume, further studies are necessary.

In animals, the tiny pericardium is fixed to the great vessels at the base of the heart and is attached to the sternum and diaphragm, although the degree of attachment varies between the species. More specific, the attachment to the tendineus center of the diaphragm is firm and broad in dogs, the phreno-pericardial ligament being the only

constant pericardial ligament reported in dogs. Nevertheless, in dogs, there are reports of the presence of a tiny ligament which detached from the dorsal caudal pericardium to be inserted on the 6th costal cartilage (Kent and Carr 2001). Our results are in agreement with this reports, we describe in two subjects the presence of this pericardo costal ligaments in absence of the left phreno-pericardial ligaments. In ruminants the caudal pericardium is attached to the sternum by a strong sterno-pericardial ligament, only the apex being in contact with the sternum, compare to the extensive attachement to the sternum, in absence of the phreno-pericardial ligaments in horse (Barone 1997; Cotofan et al., 2007).

The presence of a rich coronary collateral circulation in guinea pigs revealed in this study is in accordance with that described in dogs (Ghoshal1975; Koke and Bittar 1978). In the earliest studies related to heart ischemia and in pharmacological therapies for reducing the ischemic size, the dogs were the preferred animal model, but, due to anatomical particularities of coronary irrigation these studies lead to false claim about the efficacy of medication as long as administration in humans did not produce the same results as those observed in dogs. Nowadays, it is well known that the dog have a much more extensive collateral circulation compare to sheep and pigs (Abel et al., 1995). Morphologically, the pig's heart is more similar to the human heart, due to limited collateral coronary circulation, making the swine heart, ideal for acute ischemia studies (Crik et al., 1998). In recent years, small animals are commonly used in cardiovascular disease; the rats have a sparse collateral circulation being a suitable model to heart ischemia (Chorro et al 2009). Due to the extensive collateral circulation in guinea pigs, normal perfusion of heart is maintained after a coronary artery occlusion and infarction does not develop. Another morphological feature of the guinea pigs heart is the smallest diameter of the mentioned vessels which are hard to be verified if the spontaneous or induced reperfusion appears. Nevertheless, the use of guinea pigs as experimental model remains quite important for arrhythmia studies on humans due to the similar electrocardiographic waves (Guo et al., 2009). It was demonstrated that the polarity of T waves is the same of that of QRS complex from human subjects (Watanabe et al., 1985, Roberts et al., 2003, Zaragoza et al., 2011).

Regarding the mediastinum, based on the obvious anatomical components, we realized a detailed division and description of mediastinum spaces. In human the mediastinum is divided by a transversal plane that connects the sternal angle,passes over the pericardium to the intervertebral disc of 4th and 5th thoracic vertebra, into superior and inferior mediastinum, the later being subdivided in anterior, middle and posterior (Goss 1949, Gray's Anatomy, 2008). In animals anatomical descriptions recognizes three spaces: cranial, middle and caudal, with a double division of middle mediastinum in ventral and dorsal (Barone 1997, Cotofan et al., 2007) From a morphologic point of view, and due to extensive feature of pericardic ligaments in guinea pigs, our division of middle mediastinum in ventral, middle (central) and dorsal is justified. The same simple landmarks as in humans could be made: the ventral mediastinum is dorsal to the sternum and ventral to the pericardium, the middle mediastinum contains the pericardium and its components and the dorsal mediastinum is behind the pericardium and ventral to the vertebral column.

All these considerations mentioned above are the base of using both large and small animals as experimental models for the cardiovascular studies. The advantages of large animal models are primarily based to their similarities in heart physiology to humans and ease of instrumentation, but equally true is that maintenance costs are higher which make small animal models more attractive.

## CONCLUSIONS

Apart from the differences in size the guinea pig heart is anatomically similar with the most of the mammal's heart.

Guinea pigs have a different type of heart vascularisation meaning the presence of a well developed collateralisation of heart supplying vessels offering a natural degree of protection of ischemic disease.

The tiny but obvious sterno-pericardial and phreno-pericardial ligaments give the guinea pigs heart, a strong insertion and protection into thoracic cavity.

The middle mediastinum in guinea pigs can be divided in three obvious spaces: ventral, middle (or central) and dorsal, each of them containing important and obvious anatomical structures.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# MORPHOLOGICAL DETAILS USEFUL TO IDENTIFY THE BONES OR BONE FRAGMENTS BELONGING OF CARCASS IN SHEEP OR HOMOLOGOUS REGIONS IN DOG

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***Abstract***

*Very often, veterinary professionals are faced with directly and quickly identify the bodies of animals, carcasses or carcass portions. This operation is based on morphological characters highlighting defining species, sex and even approximate age. They are very frequent cases when the soft parts are damaged bodies and carcasses of animals are partially or totally boned. In view of this, it shows the importance of examining the skeleton as a whole or its constituent parts.*

*Operation animal identification by morphological features of the skeleton is more difficult as are younger animals (presence of cartilage growth, strengthening bones insufficient, incomplete formation of characteristic details, enable scattering and fragmentation of the bones).*

*In the domestic mammals there is possibility of occurrence of confusion, especially if bones and body parts belonging to the same class animals close. Only perfect knowledge of bone morphology allows the veterinarian to determine what species undoubtedly stems from the housing or housing part, without the need for additional tests.*

*Detailed analysis presented in this paper aims to provide the most important clues so that identification of species belonging specifically to be made, even if some bones which, at first glance, seem indistinguishable. These two species can be distinguished some bones relatively easy: lumbar vertebrae, sacrum, most limb bones. However, some characters are less distinct bones, cervical vertebrae II-VII, some thoracic vertebrae, tibia, etc. However the study also insisted on the possibility of identifying all bones, because we often available only bone fragments, which prevents taking into account the most important element, namely the general appearance of the bone. The study revealed, in an original manner, details that may constitute criteria for determining the species from which the bones or fragments analyzed, largely completing a series of data described under "classical osteology"*

Key words: *sheep, dog, bones, morphological details.*

## INTRODUCTION

Examination of bone has a great importance in forensic medicine, both human and domestic animals. Most often, over time, the soft parts of the bodies are damaged and the only elements that can constitute evidence of analysis are bones. Bones are extremely useful in establishing the identity of the individual of origin. Bones help in this regard because they may be used to establish the following characteristics of the individual of origin: race, gender, age at death, dimensions and direct identification clue. Currently, an accurate identification is based on DNA analysis (Ciobotaru, 2013, Savu, Petcu, 2008).

If a body is found in burnt stage, it will be very difficult to identify physically. DNA fingerprinting comes to the rescue in such scenarios, but from where will we collect DNA on a burnt body? Depending on the level of burn, teeth or bones act as sources for DNA. Teeth have got pulp that may be protected from fire by the intrinsic properties of teeth. Similarly, bone can provide bone marrow from which DNA extraction is possible (Georgescu, 2013, Savu, 2013).

Although in some food control or forensic medicine works there are data of compared osteology, useful in identifying the species and their specific features, we believe that this detailed study, based on the methods of comparative anatomy is useful to those interested in the above areas (Ganţă et al., 2008, Gudea et.al, 2011, Predoi et al, 2011).

## MATERIALS AND METHODS

Bones were from 20 sheep and 15 dog bodies. The animals were designed for dissection and research activities in the Anatomy Laboratory of the Faculty of Veterinary Medicine,

Bucharest. Both sheep and dogs were of different races, ages and sexes. The bones were cleaned of organic debris and subjected of maceration process. The identification, description and homologation of formation were performed according to Nomina Anatomica Veterinaria - 2005.

## RESULTS AND DISCUSSIONS

The first two cervical vertebrae, atlas and axis, are relatively typical for the two species, difficult to be to confused. Problems may occur for the identification of other cervical vertebrae . We consider that the most important character, on which we can make a difference, is the presence of the muscular tubercles on the dorsal part of the caudal articular processes in dog (Fig. 1). The other elements are relative and not always helpful in identification.

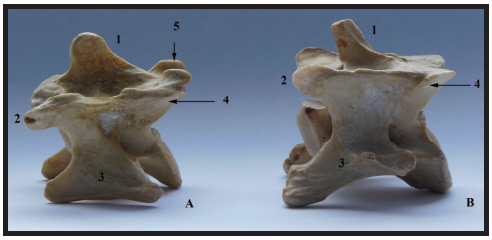


Figure 1. The 3-th cervical vertebra in dog (A) and the 5-th cervical vertebra in sheep (B) (lateral view) (original)
1-spinous process; 2-cranial articular process; 3-transverse processes; 4-caudal areticular processes; 5-muscular tubercles.

By focusing on the thoracic vertebrae, will analyze the spinous process appearance, which is narrower, thicker and finished tuberous for the first 10 vertebrae in dogs. The lateral orientation of the transverse processes articular surfaces in the dog, is different from the ventro-cranial orientation in sheep (Fig. 2). In dog, the last 4-5 vertebrae have, as a defining element, accessories processes.

Number of lumbar vertebrae is not absolutely characteristic. For this reason it will look spinous, transverse, articular and accesory processes, totally different, enough elements to easily identify the species (Fig. 3).

In all, the sacrum is easily recognizable. Examination of the cranial part allows identifying species after cranial articular processes, with concave surfaces from top to bottom in sheep and plane in dog. At the caudal extremity, transverse processes of the last sacral vertebra are characteristic, directed caudally, long and sharp in dog, exceeding the terminal face of the bone.

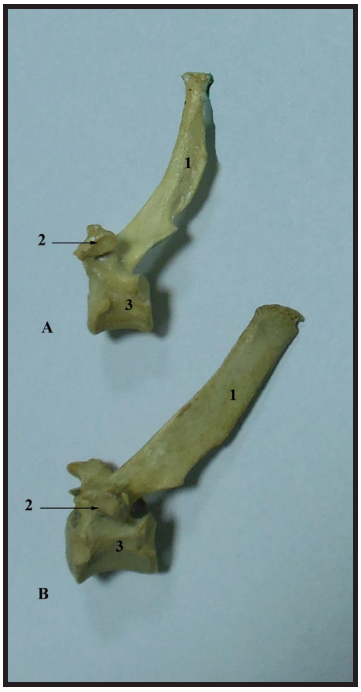


Figure 2. The 10-th thoracic vertebra in dog (A) and sheep (B) (lateral view) (original)
1-spinous process; 2 – the costotransversal articular surface; 3- vertebral body.

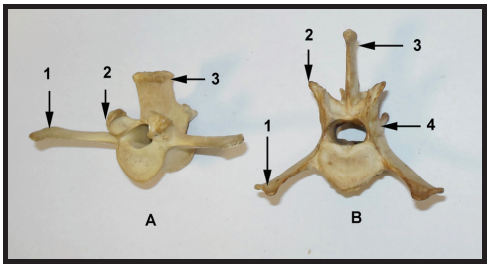


Figure 3. The 3-th lumbar vertebra in sheep (A) and dog (B) (cranial view) (original)
1-transverse process; 2-cranial articular processes; 3-spinous processes; 4- accessories processes.

In dog, ribs have a high degree of curvature, cylindroid aspect of the head and tuberous

aspect of the distal end. In sheep, the ribs are widened and the end are not tuberous.
When analyzing fragments belonging to the dorsal edge of the scapula, in dog found to be missing the suprascapular cartilage and cervical angle is rounded (Fig. 4). In the glenoid angle, tuber infraspinos is well circumscribed in dog and elongated, extended to neck of the scapula in sheep (Fig. 5).

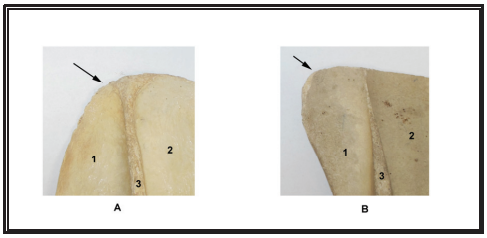


Figure 4. Aspect of the cervical angle of the scapula in (A) dog and (B) sheep (lateral view) (original)
1-supraspinous fossa; 2 infraspinous fossa; 3-scapular spine.

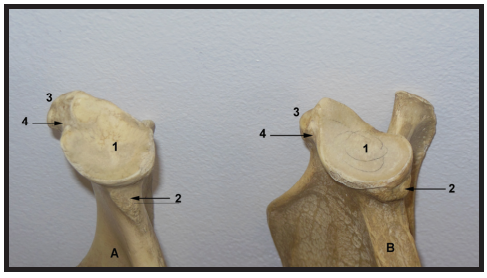


Figure 5. The appearance of the glenoidal angle in sheep (A) and dog (B) (ventral view) (original)
1-glenoid cavity; 2- infraspinous tuberosity; 3-supraglenoidal tuberosity; 4- glenoid notch.

Humerus is more difficult to identify if there is only the distal extremity. Supratrochlear hole is a characteristic element of the dog. When there is only the articular surface will be analyzed the medial lip of trochlea which is wide in sheep. In this species the condyle appears as a cylinder segment. In dogs medial lip of throchlea is sharp and it's groove is well defined. The condyle is triangular (Fig. 6).

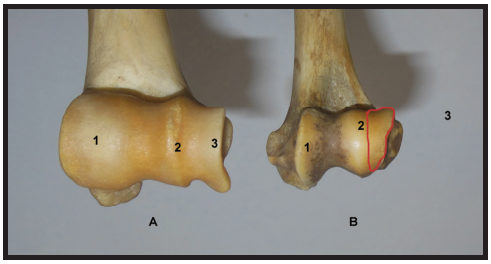


Figure 6 The appearance of distal extremity of the humerus in sheep (A) and dog (B) (cranial view) (original)
1-medial lip of the trochleea; 2-lateral lip of the trochleea; 3-humeral condyle

If the forearm region of the carcass is not complet, important data can be obtained based on the analysis olecraniene tuberosity and distal articular surfaces.
We can easily determine the species if we examine the proximal end of the femur. However, in both species, trochleea and condyles not provide sufficient differences, especially in a general examination. On the caudal side, dorsal from each condyle in dog is observed the articular surface with femoral sesamiods, which are not present in sheep (Fig. 7). Above the lateral condyle, on the shaft of the bone is observed the insertion surface of gastrocnemius muscle and superficial flexor. In sheep his is a rough and less obvious supracondylar fossa. In dog it is represented by a evident supracondylar tubercle.

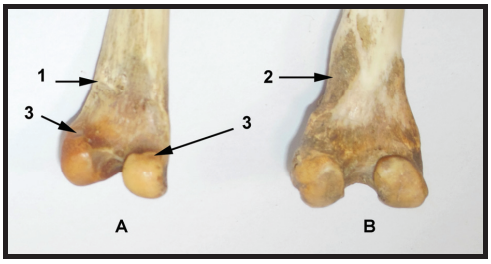


Figure 7. Distal extremity of the femur in dog (A) and sheep (B) (caudal view) (original)
1-supracondylar tubercle; 2 supracondylar fossa; 3-articular surfaces for sesamoidian bones.

If we examine the tibia, first will identify the fibular styloid apophysis in sheep. However, it is useful to identify other specific elements. The sheep tibial crest ends slowly in sheep while the dog stops suddenly, tibial groove is deeper in sheep than in dog and lateral distal end of the tibia in sheep is provided with bone malleolar articular surface (Fig. 8).

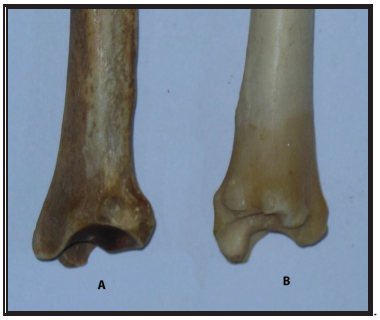


Figure 8. Distal extremity of the tibia in dog (A) and sheep (B) (lateral view) (original)

## CONCLUSIONS

On the identification of other cervical vertebrae we consider the most important character, on which we can make a difference, is the presence of the muscular tubercles on the dorsal part of the caudal articular processes in dog.

By focusing on the thoracic vertebrae, the lateral orientation of the transverse processes articular surfaces in the dog, is different from the ventro-cranial orientation in sheep and in dog, the last 4-5 vertebrae have, as a defining element, accessories processes.

In dog, ribs have a high degree of curvature, cylindroid aspect of the head and tuberous aspect of the distal end but in sheep, the ribs are widened and the end are not tuberous.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# EPIDEMIOLOGICAL STUDY OF THE INCIDENCE AND RISK ANALYSIS IN MAJOR DISEASES OF ANIMALS IN ROMANIA AND IN THE WORLD IN THE PERIOD 2007-2014

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***Abstract***

*Epidemiological dynamics in major animal diseases, suffered extensive changes since the year 2007 and was dominated by vesicular disease, foot and mouth disease and Sheep and goat pox, to the detriment of diseases such as classical swine fever or avian flu, which dominate global epidemiological picture in the beginning of the years 2000. This paper aims to highlight the dynamics of major diseases in the last decade, the factors that led to their reappearance in areas that were eradicated for a long time, and the impact of national programs accelerated eradication of diseases like equine infectious anemia, classical swine fever, and others.*

***Keywords****: major diseases, epidemiological dynamics, accelerated eradication, incidence*

## INTRODUCTION

The history of recent years shows that we assist to extensive changes in the global epidemiological situation in terms of major diseases of animals. Thus, diseases such as classical swine fever, highly pathogenic avian influenza H5N1, which in 2004-2006, dominated epidemiological picture world, were replaced by other diseases, some of which (ex. Dourine, sheep and goat pox, peste des petits ruminants, vesicular diseases) eradicated for decades. Also, the epidemiological situation of Romania has suffered dramatic changes in recent years, she confronted with a number of diseases until then considered exotic, such as avian flu, scrapie, blue-tong or West Nile meningitis. The causes of these diseases are various in Romania, one of them referring to the intensification of trade in animals and animal products after 2007, the existence of these diseases on the borders of Romania, and the ineffectiveness of the monitoring and prevention programs the ingress of these diseases from the outside. In the context of the European Union, Romania is its eastern border, which implies an increased risk for all major diseases of animals and, of course, surveillance and control programs more extensive than in other Member States within the European Union.

## MATERIALS AND METHODS

Analysis of the appearance and propagation of major diseases, epidemiological statistics and epidemiological surveillance, have always been a priority for specialized international organizations such as the World Organization for Animal Health, World Health Organization, the European Food Safety Authority or the United Nations agriculture and Food. This paper proposes a pertinent analysis of all these issues in consultation documents produced by these organisms in recent years (WTO. Uruguay Round Final Act, 1994; OIE /WTO, 1995).

We also studied the risk analysis regarding potential threats to Romania, made in recent years by the Romanian national veterinary administration that showed the following (Government Decision no. 1189/2009):
-bluetongue in 2010-2011 was considered the greatest threat, which was also true in 2014;
-classical swine fever believes it will continue to constitute a danger due to at least three factors: Romania is the border southeast of the European Union; limited control of feral pig populations and breeding of pigs in population households lacking biosecurity conditions. The most important general methods to prevent the introduction swine fever virus in commercial farms with pigs remain strictly comply with stringent biosecurity conditions and interdiction staff who work with these animals, to hold pigs in their own households, or having a hobby hunting;
-african swine fever, foot and mouth disease (FMD) and other vesicular diseases, will continue to pose a major threat to all European countries;
-Newcastle disease seems to be dominate avian pathology in the near future;
-meningitis West Nile will continue to evolve in Europe. Evolution asymptomatic at animals, except horses, the large number of species susceptible, virus transmission by mosquitoes, favors diffusivity disease.
Of all Member States of the European Union, now Italy is epidemiologically like a "reservoir" in which interacting both diseases removed / extinguished in the years 1960-1965 (ex. dourine) and "modern" disease of this started century (OIE General Session, 2014). On the other hand, all these changes related to the global epidemiological context, which is subjected to constant changes, so what exist today, tomorrow may be considered history, but that we must draw lessons.

**RESULTS AND DISCUTIONS**

Qualitative risk analysis carried out mainly on epizootic dynamics and respecting the other links of the "chain epizootic" reveals the following situation:

-out of European countries in which *classical swine fever* has evolved in the past five years include: Russia, Croatia, Lithuania, Bulgaria, Serbia, Hungary, the last three countries are located in close proximity of Romania. However, except Russia facing with numerous active outbreaks of classical swine fever in both the European and the Asian part, the incidence of this disease in Europe has declined; however, it is found an increased frequency in Asia and Africa.
In Romania, the last outbreak of classical swine fever was notified in 2007, and this led to the inclusion of Romania in the Decision 2008/855/EU. This allows trade in meat and meat products produced in Romania, opening prospects for the Romanian producers to put on the European market quality products in order to market them in the Community. The next desiderate, would be the status of classical swine fever free country, where vaccination is not practiced, and the continuation of the national epidemiological surveillance programs, approved and financed by the European Commission.
Vaccination of domestic pigs in all commercial farms in Romania was prohibited in April 2007, and vaccination of domestic pigs from non professional holdings on 31 December 2009. Vaccination of wild boars of hunting funds continued until the fall of 2011, as a "buffer belt "of 10 km along the state border with Moldova and Ukraine (EU Commission Decision 2008/855).
*African swine fever* continues to dominate the Epidemiological map of Asian and African countries, but also in Europe, in countries like Russia and Italy (Sardinia where evolves endemic) and Ukraine.
The first outbreak of African swine fever were reported in Transcaucasia (Georgia, Armenia, Azerbaijan, Russia) in 2007-2008, but the current epidemiological situation in the Caucasus region is relatively stable, with no new outbreaks. Concern is the situation in the Russian Federation where the situations regularly reported to the OIE, shows that the disease has become endemic in some regions, it constitutes a permanent threat to the

surrounding areas, including EU member states in Eastern Europe. So far, the disease has spread in Belarus (2 outbreaks in 2013), Ukraine (1 outbreak in 2012, two in 2014), Poland (2 cases in 2014), Lithuania (2 cases in 2014) (FAO Empress, 2013; Commission Decision 2005/176/EC).

Member States of the European Union have a common policy of not vaccinating against ASF, applying, in case of suspicion or confirmation legislative provisions of Council Directive 2002/60/ EC which one establishes specific provisions to combat African swine fever. African swine fever has never been diagnosed in domestic pigs and wild in Romania. However, the lack of information in the computer system of the World Organization for Animal Health (OIE) on the evolution of any outbreak of classical swine fever and African swine fever in Moldova, is a great unknown against which Romania must implement effective measures. Also in the case of Romania, are at risk domestic pigs and wild boars and pigs reared in semi-liberty in some areas (Danube Delta, Balta Brailei) which may come into contact with sick animals or carriers or non heat treated products or by-products coming from them. Respecting the rules of animal farm biosecurity, establishing minimum rules for non-professional holdings, the risk constituted of introducing disease in the country, can be reduced significantly. Even if the risk of an outbreak of African swine fever in Romania, at present, is low to medium, introduction of disease in Romania would have major consequences on the national economy by spending huge posed by the eradication costs and restrictions trade and export of live pigs and products and by-products derived from them (Council Directive 425/90/EC). Once entered in the territory, because the percentage maximum of mortality due to evolving, following the application of measures to eradicate incorrect coordinates, the disease may become endemic in a short time, blocking the entire pig sector's growth, causing significant economic losses to pork producers and economy in general(Council Directive 2002/60/EC).

*Fever disease* developed in 2007-2011, in 70 countries, most Asian and African countries. Very important is the evolution of FMD in 2011 in Europe, in Bulgaria, Russia and Turkey, which leads to the conclusion that we assist to an expansion of this pandemic; also, the evolution of swine vesicular disease in Italy shows slight tendency to return this vesicular disease in Europe. FMD virus currently circulate in parts of Europe and in the vicinity of about 100 countries in Africa, the Middle East, large parts of Eurasia and parts of South America. European Commission pays special attention to the Balkans, a region which one includes both Member States and non-EU states, area being closer to the infected countries in South and East. Consequently, FAO has helped these countries to develop and test emergency plans, and recently they have been tested in Bulgaria, Serbia and Macedonia, where government veterinary services took part in a computer-aided simulation concerning simultaneous appearance of more outbreaks of FMD in these three countries (ANSVSA Order no. 113 of 27.04/200).

Still 1998, *bluetongue* began to spread in Europe, including territories increasingly stretched. In late 2001, several Mediterranean countries and neighboring area, were confronted with the appearance of this disease. New serotypes reported that occasion were 2, 4, 9 and 16 along the south-east borders of the countries in Europe, to which were added serotypes 6, 10 and 13 earlier diagnosed. The top of disease in Europe, was the 2006-2009 period when there was a progressive increase from 2479 cases in 2006-2007 to 63,182 cases in 2007-2008, 39 737 cases in 2008-2009, followed by a decrease in the period 2009-2010, when there were 219 cases.

Bluetongue has evolved in the past 5 years in 31 countries, over 50% of which are European countries such as Belgium, Cyprus, Switzerland, Denmark, Germany, Greece, Italy, Luxembourg, Netherlands, Portugal, Spain, Sweden, United Kingdom, Turkey. In July of 2014, Bulgaria, and Greece, notified by codified system ADNS of EU and on the OIE WAHIS system, disease recurrence after about

ten years of absence, and in august the disease recurs in the Republic of Macedonia. Thus, a risk analysis carried out by the central veterinary administration of Romania, it considered that risk of developing this disease is very high in Romania, which was also true a month later. In the near future bluetongue will dominate the epidemiological situation of European countries, which requires a new approach, including the aspects of immunoprophylaxis susceptible animals, with live vaccines (Council Directive 2000/75/EC; Commission Decision 2005/176/EC).

*Bovine spongiform encephalopathy (BSE*), is a disease that develops predominantly in Europe -Austria, Czech Republic, Switzerland, Italy, Ireland, Germany, Netherlands, United Kingdom, France, Spain, Slovenia and Portugal, and outside Europe have reported cases, is really very rare in Canada, Brazil, USA, Japan and Israel; however no cases of BSE have been notified in Asian and African countries; this epidemiological situation is an argument to continue to maintain severe restrictions on the marketing of live animals of species susceptible to BSE and products derived from them. In the period 1987-2013 the United Kingdom has registered more than 184,000 cases of BSE. From the data analysis, is observed there is a maximum number of cases in 1992 to 37,280 cases of BSE, but after this peak annual number of BSE cases began to decline, while in 2012 and 2013 to be declared only by 3 cases annually. Between 1987-2014 period, in the world, except in the United Kingdom, were declared 6044 cases of BSE, which is about 3.2% of total cases in the United Kingdom.

At the present time the global epidemiological situation, is relatively stable due to preventive actions and corrective actions to reduce the risk from BSE to an acceptable level.

In Romania, the disease has not been reported until now and the level of this risk is unacceptable, the main preventive action applied our country is implementing annual programs for the eradication and monitoring of animal transmissible spongiform encephalopathies (BSE and scrapie), co-financed by the European Commission, and specific preventive measures, required by Community legislation directly applicable in national law (Regulation (EC) no. 999/2001). Regarding the evolution of *scrapie*, in the world has been a constant evolution of the disease in 25 countries of which 20 are European countries, in 2007-2011. This trend was maintained in the coming years, with a slightly higher incidence from year to year. According to the OIE Bulletin No. 3/2013, published on 16 October, Romania has declared as free from scrapie for 19 counties. Although the information sources indicate the presence of disease in the world for over 200 years, scrapie has been officially confirmed in Romania in 2002, by the Institute of Diagnosis and Animal Health, only in one case (Regulation (EC) no. 999/2001/).

*Regards equine infectious anemia (EIA),* except for Romania where evolve and where it runs a program of accelerated eradication of this disease of equidae, the disease evolves in other European countries such as Croatia, Germany, Greece and Italy. However, experts believe that this chronic disease evolves in several European countries, but the absence of screening disease programs and use of equine mainly as pets, make real epidemiological situation, may not be known (ANSVSA Order no. 46/2014).

As for the evolution of *avian influenza* in Europe in recent years, it was a sporadic and, in all cases the origin of avian influenza viruses have been in wild birds, an avian influenza H5N1 highly pathogenic continuing to generate alerts major in Asian countries. Over time, the disease has evolved somehow sporadically global, after World War II, it was believed to have disappeared, but she appeared abruptly in 2004, covering 11 countries in East Asia and Southeast, and affecting more than 150 million birds. In the period 2004-2014, bird flu has evolved in Asia, Africa, Europe and Middle East, with a peak of 56 countries in 2006. Besides, the disease first appears in Romania in 2005, and in neighboring countries like Russia, Ukraine, Turkey, Croatia, but top disease is registered in 2006, and includes other European

countries like Austria, Bulgaria, Croatia, Denmark, France, Greece, Hungary, Italy, Poland, Bosnia, Serbia, Slovakia, Slovenia, Spain, Sweden and Switzerland.
In Romania, during 2005-2014, 152 outbreaks were recorded as follows: in 2005-10 outbreaks, in 2006-109 outbreaks in 2007-one outbreak of disease, and two outbreaks in 2010. Thanks control measures applied in Romania and in Europe, after 2008 has regressed disease recorded in the three countries in 2009 and 201, and in one country, Italy, in 2013(Law no. 221 of 31/05/2006).
If avian pathology in recent years has been dominated by avian influenza, currently there is an exacerbation of *Newcastle disease* in poultry in both the developed European countries (France, Switzerland, Sweden, Turkey) and in Asian countries (Israel where the disease progresses endemic) in Australia and the United States, and because it has an extremely high diffusivity, Newcastle disease it is provided that will continue to evolve over the next years worldwide (ANSVSA Norm of 27/06/2006).
Regarding *West Nile meningitis,* in 2007 has evolved in seven countries, located in Central America and Asia; in 2009 W. N. cases doubled, in 2010 have tripled, and 50% of cases were diagnosed in European countries. In the last years, the disease was diagnosed in 11 countries: Canada, Cuba, Israel, Italy, Macedonia, Romania, Spain, Hungary, USA, Guatemala, Haiti, which means maintaining in "plateau" of the disease, especially in Europe. In november 2014, 74 human cases of West Nile fever have been reported in the EU and 136 cases have been reported in neighbouring countries since the beginning of the 2014 transmission season, of which 27 in Romania.
*Variola, dourine, peste des petits ruminants, equine encephalomyelitis*, although are diseases that are no longer threats recently, confirming their evolution in European countries (Italy, Greece, Turkey) and their character readily diffusible, should be an alarm signal in disease management animals.

**CONCLUSION**

In the context of the European Union, Romania is its eastern border, which implies an increased risk for all major diseases of animals and, of course, surveillance and control programs more extensive than in other Member States within the European Union. Origin of risk regarding the introduction of these diseases in Romania, can be represented by:
-evolution of the disease in third countries bordering the northern, northeastern, eastern and south-eastern part of Romania,
-the epidemiological statute unknown of the third country, in the immediate vicinity of Romania (according OIE)-illegal traffic with animals,
-movement of people and vehicles, especially at border of crossing points. At none of crossings point the borders of north-east, Romania does not have appointed road disinfectors and any other means of disinfection, which significantly increases the possibility of penetration of the disease in the country. Romania's central veterinary authority must decide the opportunity to arrange disinfection facilities on these boundaries. They serve both, protection against all major diseases in animals (FMD, avian influenza, etc.), in those areas where, often, the epidemiological situation is delicate,
- hunting action (Council Directive 662/89/CEE).
Inside Romanian origin of the risk may be represented by:
-lack of implementation biosecurity conditions in farm,
-lack of implementation minimum biosecurity conditions in households population,
-uncontrolled movement of animals,
-direct or indirect contact from households, with wildlife animals.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# STUDIES ON THE SUPPLY AND PRICE MANAGEMENT IN VETERINARY PHARMACEUTICAL UNITS

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***Abstract***

*Nowadays, veterinarian's activity takes place in a highly dynamic and changing environment; thus, the managers of economic units having as main activity the sale of veterinary products should have leadership and organizational qualities that allow them to develop a successful business in a competitive environment. The purpose of this study was to highlight the main aspects related to veterinary pharmaceutical units supply, and the manner in which price management is done. The study was conducted in 25 veterinary pharmaceutical units (pharmacies and pharmaceutical points) in Bucharest and consisted of a market survey in order to identify the factors that influence supply, the criteria by which suppliers are selected, rhythm and mode of supply, as well as aspects related to stocks' management and control, pricing criteria, markup and its influencing factors. The results showed that the supply of veterinary pharmaceutical units is typically performed daily or weekly and is influenced by season, prices and profitable offers from suppliers. In an overwhelming percentage, veterinary pharmaceutical units' stocks are electronically managed by using software that facilitates staff's work, and most of the surveyed veterinary pharmaceutical units keep a safety stock. The most important factors that influence price levels are mainly represented by the competition, discounts, offers and benefits from suppliers, and the most common markup for veterinary pharmaceutical units is between 20-50%. Following the studies, it was concluded that modern management and marketing principles related to the supply, stocks and prices are properly applied in the surveyed veterinary pharmaceutical units.*

***Key words:*** *markup, prices, stocks, supply, veterinary pharmaceutical units.*

## INTRODUCTION

Nowadays, veterinarian's activity takes place in a highly dynamic and changing environment; thus, the managers of economic units having as main activity the sale of veterinary products should have leadership and organizational qualities that allow them to develop a successful business in a competitive environment.

At present, in Romania, the conditions of organization and functioning of veterinary pharmaceutical units, as well as the procedures of sanitary veterinary registration/authorization of veterinary pharmaceutical units and activities are regulated by ANSVSA Order no. 83/2014.

Thus, veterinary pharmacy is a veterinary pharmaceutical unit which holds and retail sells veterinary medicinal products, animal feed, medicated feed and other veterinary products, instruments and medical equipments, medical devices and accessories for animals. Veterinary pharmaceutical point is a veterinary pharmaceutical unit having as main activity the retail sale of veterinary medicinal products which are not subject to prescription (OTC), other veterinary products, instruments and medical equipments, medical devices, accessories for animals and feed.

The supply is defined as the provision of appropriate technical and material items, at the place and time required, with a minimum cost

and a maximum profit (Băşanu and Pricop, 2001). Thus, the main objective of supply is to search for the best materials at the lowest costs and from the best sellers (Moga, 2009). The functioning of a veterinary pharmaceutical unit can only be cost-effectively, by using material resources at the best economic level; thus, irrational use of materials, dissipation and unjustified costs must be avoided (Băşanu and Fundătură, 1993).

In terms of stocks, it is advisable to adopt a rational approach related to the optimal amount to store, optimal amount to purchase and optimal resupplying interval (Băşanu and Pricop, 2001). Stocks represent the amounts of veterinary pharmaceuticals products that accumulate in veterinary pharmaceutical units' warehouses, in a certain volume, with a specific structure, for a fixed period of time and with a particular purpose (Băşanu and Pricop, 2001). At the current juncture of supply, stocks of medicinal products in danger of expiring greatly diminished, and therefore supply costs decreased significantly (Cernea, 2004).

The price can be defined as the monetary expression of the exchange value of a product (Moga, 2009). In a market economy, the general rule is that the prices and fares are freely determined through competition, based on supply and demand rule.

The markup is the parameter by which a product is bought at a purchase price, to which a certain percentage is applied, obtaining this way the selling price of the product. The dimensions of markup should ensure both operating costs and obtaining profit (Carată, 2008; Moga, 2009).

The purpose of this study was to highlight the main aspects related to veterinary pharmaceutical units (veterinary pharmacies and veterinary pharmaceutical points) supply, and the manner in which price management is done.

## MATERIALS AND METHODS

The study was conducted in 25 veterinary pharmaceutical units (pharmacies and pharmaceutical points) in Bucharest and consisted of a market survey in order to identify the factors that influence supply, the criteria by which suppliers are selected, rhythm and mode of supply, as well as aspects related to stocks' management and control, pricing criteria, markup and its influencing factors.

The used method was the quantitative assessment using questionnaires that included questions about involved issues, each question having multiple possible answers (Table 1).

The research questionnaire is an investigation method consisting of a series of written questions, logically and psychologically ordered, which determine from the surveyed persons answers to be recorded in writing; the aim is to collect data that generates information intended to meet the objectives of research.

Table 1. The questionnaire used in the study

| No. | Question | Possible answers |
|---|---|---|
| 1 | Which is the most important criterion on which you choose the suppliers that you collaborate with? | a) cuts and discounts;<br>b) extended payment deadlines;<br>c) reliability, punctuality;<br>d) low prices. |
| 2 | At what time frame do you perform the supply of your pharmaceutical unit? | a) daily;<br>b) weekly;<br>c) bimonthly;<br>d) monthly. |
| 3 | What are the factors that influence the rate of supply? | a) market demand;<br>b) season;<br>c) convenient prices from the supplier;<br>d) supplier's offers. |
| 4 | What method to control stock accounts do you use? | a) electronic management;<br>b) classical management;<br>c) other method: ….……<br>d) is not performed. |
| 5 | Do you use a safety stock? | a) yes (go to question 6);<br>b) no (go to question 7). |
| 6 | Based on what criteria you have decided to implement safety stock? | a) current offers;<br>b) problems with the suppliers;<br>c) seasonal sales;<br>d) other reason: …… |
| 7 | Based on what criteria do you establish the prices? | a) competition;<br>b) discounts, offers and benefits from suppliers;<br>c) customers' feedback;<br>d) other reason: ...…… |
| 8 | What is the average markup applied in | a) 0 – 10 %;<br>b) 10 – 20%; |

| | | |
|---|---|---|
| | your unit? | c) 20 – 50 %;<br>d) 50-100%;<br>e) over 100 %. |
| 9 | Based on what criteria do you establish markup for a product? | a) product value;<br>b) availability;<br>c) market demand;<br>d) other criterion: …..… |

## RESULTS AND DISCUSSIONS

**Question 1.** Following the research, the most important criteria on which veterinary pharmaceutical units select their suppliers are: cuts and discounts (7 units; 28%), low prices (7 units; 28%), extended payment deadlines (6 units; 25%) and reliability, punctuality (5 units; 20%). This question has caught a great deal of attention from the questioned staff because, from their point of view, the supplier is the starting point in a business. As veterinary pharmaceutical units closely interact with the suppliers, the suppliers must meet certain eligibility and competitiveness criteria.

**Question 2.** Most veterinary pharmaceutical units (18 units; 72%) perform the supply daily, while 6 units perform the supply weekly (24%). One unit (4%) reported bimonthly supply; monthly supply was not reported. The units that reported a daily supply mentioned that they collaborate with many suppliers which deliver the merchandise on different days of the week.

**Question 3.** After processing the data, it has been found that the rate of supply is influenced by season (9 units; 36%), supplier's offers (8 units; 32%), market demand (4 units; 16%) and convenient prices from the supplier (4 units; 16%). The season influence the rate of supply by increasing the sales of drugs (e.g. external and internal antiparasitic products), cosmetics (shampoos) and accessories (clothes for dogs, collars, leashes), resulting in an accelerated supply rate. In relation to supplier's offers, they are generally constant, because there will always be some products on offer, even if they are not always the same. The suppliers apply favorable prices when launching new products to market, when stocks are too high in certain products with short shelf life, or when launching a new product on the market that will replace another well-known existing product.

**Question 4.** In terms of the methods to control stock accounts, 84% of the surveyed units reported the use of electronic management of stocks (21 units), and only 16% (4 units) control stocks by classical management. The other two possible answers were not chosen, because all veterinary pharmaceutical units prefer to record inputs and outputs for better evidence, which helps them for future orders. Thus, these methods can avoid situations as overstocks, stocks-run out or slow-moving or motionless stocks, and therefore long periods of material and financial resources immobilization. Veterinary pharmaceutical units perform electronic management of the stocks using billing and inventory management software, such as Facturis$^{TM}$, Pharmec$^{TM}$ or Farma$^{TM}$, while classical management is achieved by products' subtracting based on fiscal receipt at the end of each day. Electronic management is preferred because it is much easier to use, working time is reduced, but there are also disadvantages such as high costs, especially in case of pharmaceutical units whose profits are not high.

**Question 5.** After processing the data, the following results were obtained: 80% of the surveyed units use a safety stock (20 units), and the remaining 20% (5 units) do not operate a safety stock. Safety stock is specifically designed to ensure sales continuity in the event of supply pauses caused by legal holidays or supplier problems, or when current stock is finished. Most of the surveyed veterinary pharmaceutical units opted to create a safety stock, because it is useful in critical situations, and it is renewed from time to time to prevent the possibility of veterinary products to reach their shelf life.

**Question 6.** Following the survey, it was found that veterinary pharmaceutical units implement the safety stock based on the following reasons: problems with the suppliers (11 units; 44%), seasonal sales (6 units; 24%) current offers (4 units; 16%) and other reasons (e.g. legal holidays) (4 units; 16%). Regardless of the relationship between veterinary pharmaceutical

units and suppliers, there may be situations when problems occur, and the delivery can not be made for various reasons (vacation, lack of ordered products in stock, etc.). When suppliers make advantageous offers to veterinary pharmaceutical units, the managers of these units tend to buy a larger amount, and thus create their safety stock, because it is much profitable to buy the same product for a lower price, or buy more and as a gift to receive certain products for free, compared to another period of the year when the same product costs more. Depending on the season, veterinary pharmaceutical units create a safety stock meant to be helpful when the demand is high (e.g. internal and external antiparasitic products, antiallergic drugs, accessories, etc.). As for legal holidays, sometimes veterinary pharmaceutical units provide services within these days, and customers may request different veterinary products; as a general rule, suppliers do not operate on public holidays.

**Question 7.** Pricing criteria were as follows: competition (13 units; 52%), discounts, offers and benefits from suppliers (9 units; 36%) and customers' feedback (3 units; 12%). In a market economy, the price of a product can not be much higher than the competition, because customers will hesitate to buy from that unit. This is the reason why a commercial unit takes into account the price of the same product sold by the competition. When suppliers make offers or discounts, pharmaceutical units are able to turn these discounts and offers to their customers, this resulting in a lower price for the final buyer. Customers' feedback is also important, any customer wishing for lower prices. In this regard, there is always a minimum price limit because the prices must ensure the recovery of investments, full coverage of costs and a satisfactory profit.

**Question 8.** The average markup applied in the surveyed veterinary pharmaceutical units was determined at the following levels: 20 – 50% (20 units; 80%), 10 – 20% (3 units; 12%), and 50 – 100% (2 units; 8%). The applied markup must cover both the operating costs of pharmaceutical units, and making a profit. Thus, most of veterinary pharmaceutical units achieve this balance by using a markup ranging between 20-50%, with an average of 25-30%.

**Question 9.** After processing the data, it was found that the markup for a product is established depending on the value of the product (13 units; 52%), market demand (7 units; 28%) and product's availability (5 units; 20%). The value of the product influences its markup by the value of purchase price from the suppliers. As a general rule, if a product is purchased at a lower price, the markup will be higher, and vice versa. However, some pharmaceutical units practice the same markup, regardless of the product's value. Market demand is an important factor in establishing the markup. Customers are the ones who, indirectly and unwillingly, decide the implementation of a markup different than usual. The products are more popular, and sales grow, the markup increases, resulting in increased profit. As for availability of products, when some items are rare or difficult to purchase, the markup established by veterinary pharmaceutical units' managers is higher than the markup for products without purchase difficulties.

## CONCLUSIONS

The results showed that the supply of veterinary pharmaceutical units is typically performed daily or weekly and is influenced by season, prices and profitable offers from suppliers.

In an overwhelming percentage, veterinary pharmaceutical units' stocks are electronically managed by using software that facilitates staff's work, and most of the surveyed veterinary pharmaceutical units keep a safety stock.

The most important factors that influence price levels are mainly represented by the competition, discounts, offers and benefits from suppliers, and the most common markup for veterinary pharmaceutical units is between 20-50%.

Following the studies, it was concluded that modern management and marketing principles

related to the supply, stocks and prices are properly applied in the surveyed veterinary pharmaceutical units.

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***A.N.S.V.S.A. Order nr. 83 from 2014 approving the sanitary veterinary norm regarding the organization and functioning conditions of veterinary pharmaceutical units, as well as the procedures of sanitary veterinary registration/authorization of veterinary pharmaceutical units and activities.

# CLINICAL SCIENCES

Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# BIOLOGICAL OSTEOSYNTHESIS: MINIMINAL INVAZIVE PLATE OSTHEOSYNTHESIS VS. CASTING/SPLINTING

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***Abstract***

*In Romania, in the orthopedic veterinary practice, splinting/casting was considered an acceptable treatment of some types of fractures, namely, stable, closed reductible fractures.The greatest advantage of this type of asset is the biological osteosynthesis, favored by indirect reduction, preservation of the blood supply of all fragments, including small ones, which easily can turn into bone sequesters when regional vasculature is impaired. External fixation provided by casts and splints has several significant advantages compared with internal fixation methods: no need for implants, low postoperative infection rate, minimal disruptions of the fracture hematoma and the low cost of the procedure. Complications that can occur, usually due to improper selection of cases, inaccurate application technique and / or poor postoperative management can be minimized by using the minimally invasive plate osteosynthesis, maintaining the pros of bone healing.*

***Key words****: cast/splint, MIPO, biological osteosynthesis*

## INTRODUCTION

The biological method of fracture treatment limits the effect of rigid stability and highlights the rapid healing due to conservation of soft tissues adjacent to the fracture site (Palmer, 1999).
When preoperative radiographs show the impossibility of perfect anatomical reduction, priority changes from an absolute bone reconstruction to an acceptable spatial alignment and blood resource preservation (Aron et al., 1995; Hulse, 1997; Johnson et al., 1998; Palmer and Aron, 1996).
In this paper, we intend to present a statistical report of orthopedic cases in our clinic, , to mention the main methods used in recent years, with advantages and disadvantages, to highlight the latest trends in the field of veterinary orthopedics and put in balance two methods of biological osteosynthesis: casting/splinting (more in this paper) and MIPO –Minimally Invasive plate Ostheosyntesis. Having completed all the above objectives, we want to highlight the importance of veterinary orthopedics and the need to improve treatment techniques.

## MATERIALS AND METHODS

To achieve our aim, records from 2007 to 2011 in our clinic were considered for the study and statistically analyzed using basic methods and indices, registering the types of treatment, and complications during monitoring.

## RESULTS AND DISCUSSIONS

Between 2007 and 2011, we received for consultation and/or treatment 5987 patients, of which 20% aimed the orthopedic field. 9% were diagnosed with fractures, 6% with fractures of the long bones of the appendicular skeleton (humerus, radius and ulna, femur, tibia and fibula).
Basically, the average was 1197 ± 248 clinical cases per year, of which 72 ± 14 were diagnosed with fractures of long bones (Tabel 1).

Tabel 1. Summary of records taken into study

| Year | Total | Orthopedics | Fractures | Long bones fractures |
|---|---|---|---|---|
| **2007** | 902 | 192 | 82 | 56 |
| **2008** | 1418 | 252 | 129 | 72 |
| **2009** | 968 | 240 | 102 | 67 |
| **2010** | 1423 | 277 | 121 | 94 |
| **2011** | 1276 | 240 | 115 | 70 |
| Total | **5987** | **1201** | **549** | **359** |
| Mean | 1197 | 240 | 110 | 72 |
| StDev | 248 | 31 | 18 | 14 |

Of the 359 cases representing fractures of long bones of the appendicular skeleton, 58% received a biological osteosynthesis method (mostly splinting/ casting, also external fixators, interlocking nails inserted percutaneously) and the remaining 42% - open reduction internal fixation method (Table 2).

Tabel 2. Summary of categories of treatment

| Year | **BOS**[1] | **ORIF**[2] | **TOTAL** |
|---|---|---|---|
| **2007** | 41 | 15 | 56 |
| **2008** | 36 | 36 | 72 |
| **2009** | 41 | 26 | 67 |
| **2010** | 53 | 41 | 94 |
| **2011** | 37 | 33 | 70 |
| Total | **208** | **151** | **359** |

External cooptation provided by casting has several significant advantages compared with internal fixation methods: no need for implants, postoperative infection rate is minimal, no disruptions in fracture outbreak and low cost of procedure (Oakley, 1999; Tomlinson, 1991). Complications that can occur, usually due to improper selection of cases, incorrect application technique and/or poor postoperative management, include: delayed union, malunion, nonunion, joint laxity/ankylosis, dermatitis, soft tissue swelling and pressure sores (the term sometimes downplay the severity of injuries - "pressure ulcers") (Tomlinson, 1991; Oakley, 1999; Weinstein and Ralphs, 2004; Campbell, 2006).
Indications of external fixation by casting is limited to soft tissue injuries - minor pinpoint wounds, fractures that occurred within 8 hours and stable fractures of the distal radius and ulna and the extremities of the fore- and hind limbs (Piermattei et al., 2006).
Casts and splints are contraindicated in the treatment of distal diaphyseal fractures of mini and toy breeds because of the high incidence of nonunions and also, in the treatment of giant dogs breeds fractures of as unique method of fixations (Toombs, 2005).
Application of casts/splints requires closed reduction of fracture, under fluoroscopy. Closed reduction is typically obtained and maintained by applying traction and contertraction movements, ideally, with minimal soft tissue trauma (Piermattei et al., 2006).
Open reduction method aimes especially fractures located distal to the elbow and stifle, where soft tissues are not an impediment to assess the degree of reduction by palpation. casts have the greatest applicability on these sections. Indirect reduction has a higher success rate in smaller animals and in those with long limbs compared to large breeds, to chondrodystrophic or those with very well highlighted muscular mass (Piermattei et al., 2006).
Indirect reduction should be performed as soon as the state of the animal allows a safe general anesthesia because any delay increases muscle spasm and thus the difficulty of reduction. It is not recommended to wait for decreasing of the swelling as this will happen only after the local circulation will normalize. Primary, the contracture is originated by the muscles is likely to answer to phisical traction, general anesthesia and muscle relaxants. After 2-3 days, inflammation and proliferative changes produce a permanent contracture and difficult to overcome (Piermattei et al., 2006).
Applying a cast/splint is often seen as a minor procedure. However, if the fracture is not properly aligned, if the bandage is not applied properly or if the postoperative care is not appropriate, major complications may occur with severe implications on the functionality of the limb (angular deformities) or even amputation (Altizer, 2004).
The most frequent complications of casting/splinting are the pressure sores due to technical deficiencies or to loose enforcement. Casts that ends on the proximal phalanx region

[1] BOS – Biological Osteosynthesis
[2] ORIF – Open Reduction Internal Fixation

or those demaged by the patients can act as a tourniquet for the fingers causing swelling (Figure 1), at first, followed by ischemia and wet gangrene (Denny and Butterworth, 2000).
Most of our patients (84%) presented at least one soft tissue complication after casting/splinting: 64% - minor lesions (superficial sores), 12% - moderate lesions (superficial septic wound), 6% - severe lesions (deep wound to wet gangrene/amputation of the limb) (Figure 2).
Regarding bone tissue healing, the complication we encountered are: nonunion, malunion, angular deformity, hypertrophic callus, failure of reduction and/or mantining the reduction of fracture (Figure 3).

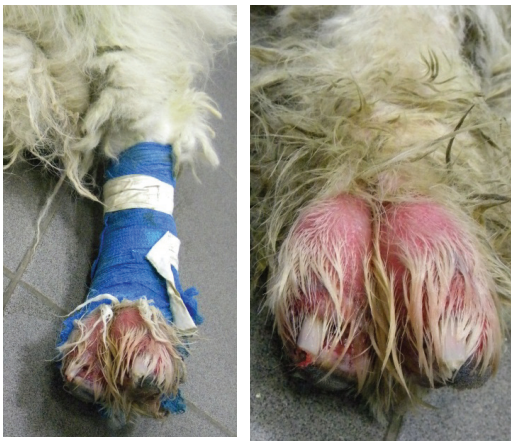

Figure 1. Swelling of the toes due to improper splinting in an 1 year old Romanian Mioritic Shepard.

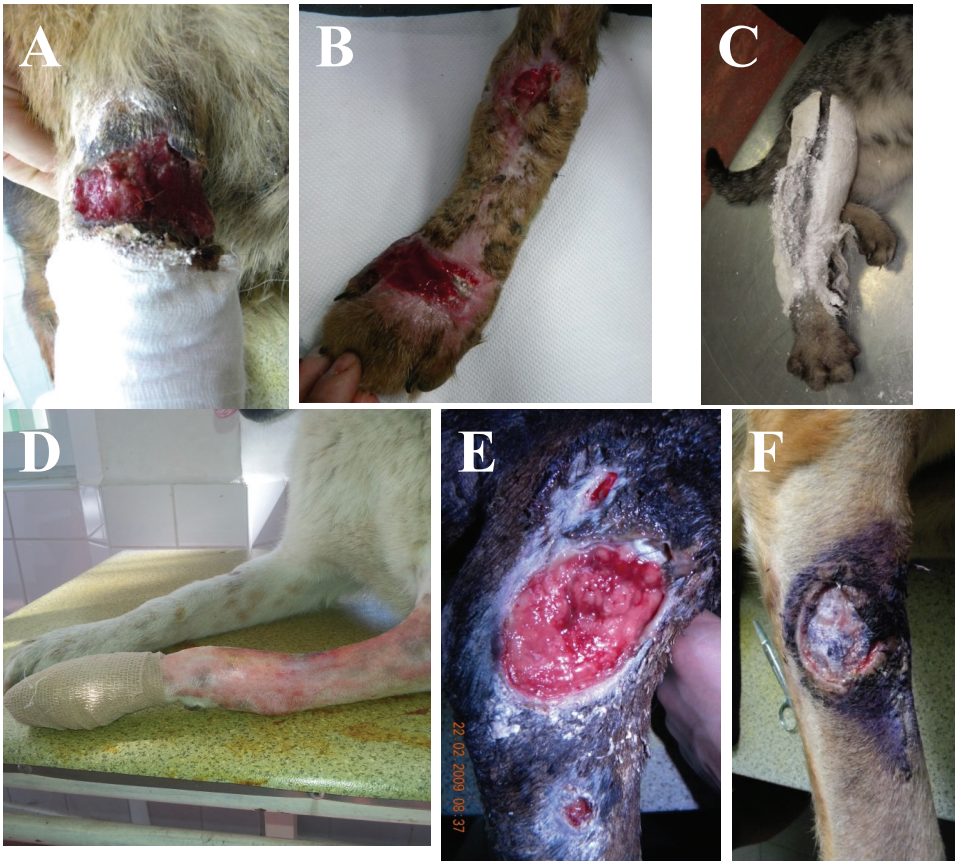


Figure 2. Soft tissue complications after splinting casting. A. Deep wound – proximal end of cast. B. Superficial wound – proximal and distal. C. Swelling of the extremity in a cat. D. Clinical aspect of angular deformity. E. and F. Presure ulcers.

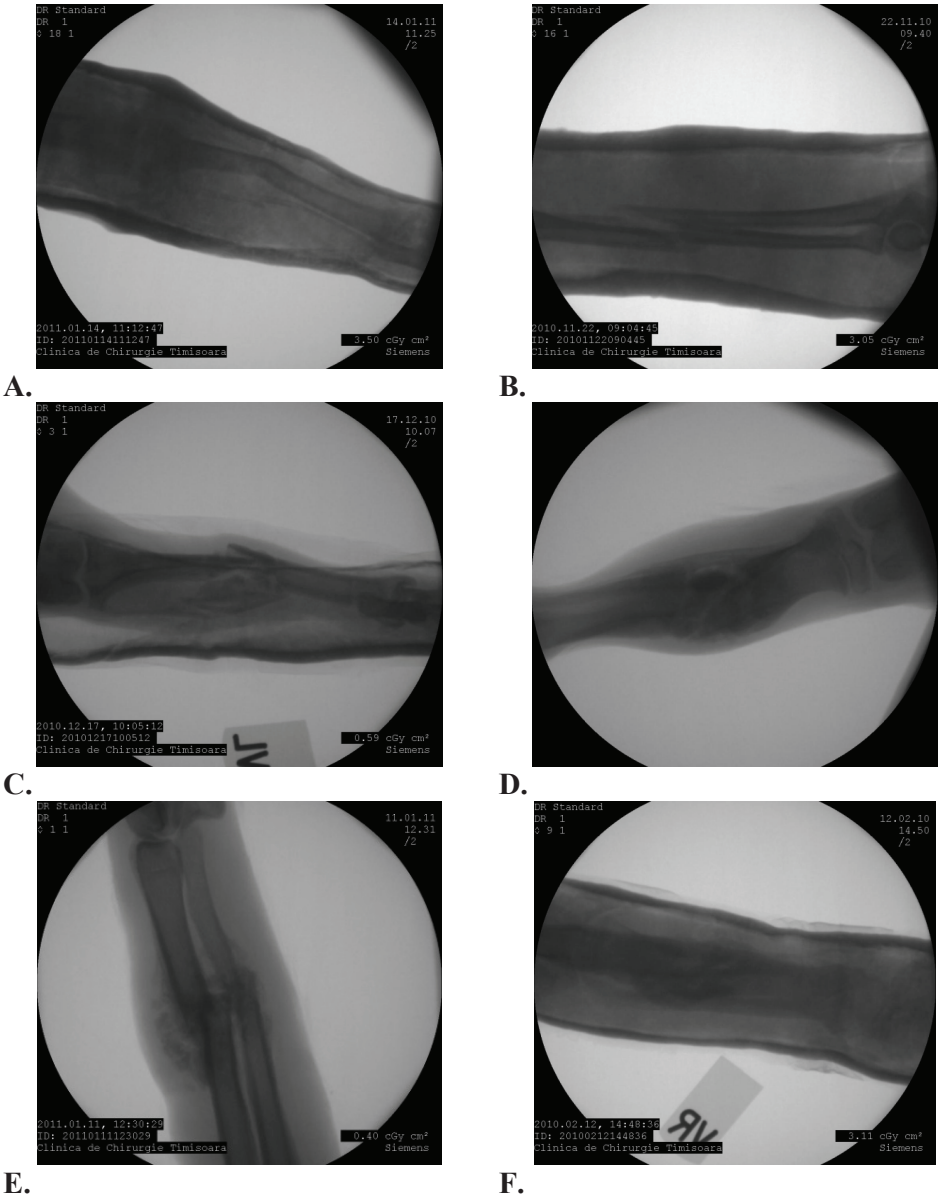


Figura 3. Complications of casting. A. Malunion with angular deformity. B. Failure of fracture reduction (predisposed to la defect nonunion). C. Opening of the fracture site under the cast due to lack of stability D. Hyperthrophic nonunion. E. Failure of fracture reduction – improper case selection; nonunine. F. Vicious callus after improper fracture reduction

A 2011 published research regarding the complication of casting/splinting showed that 60% of patients showed minor lesions – erythema, swelling, sores, without any sign of infections, 20 % presented moderate lesions as superficial septic wound requiring specific treatment and 20 % of patients had a severe alteration of general state, fever, lameness, skin necrosis, gangrene (Meeson et al., 2011). During the past three decades, internal fixation has become increasingly popular for fracture management and limb reconstruction.

As a result, during their training, orthopaedic surgeons receive less formal instruction in the art of extremity immobilization and cast application and removal (Halanski and Noonan, 2008). In this regard, we bring in discussion a method of internal fixation which retains all the advantages of casting/splinting but also of rigid internal fixation, overcoming most difficulties, namely, the minimally invasive plate osteosynthesis.

## CONCLUSIONS

20% of cases presented in our clinic required an orthopedic treatment, 9% being diagnosed with fractures.

Most (84%) patients that underwent a casting/splinting procedure suffered a soft tissue complications even minor.

Regarding bone tissue healing, the complication we encountered are: nonunion, malunion, angular deformity, hyperthrophic callus, failure of reduction and/or mantining the reduction of fracture.

MIPO retains all the advantages of casting/splinting but also of rigid internal fixation, overcoming most dezadvanteges.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# DIODE ENDOSCOPIC CYCLOPHOTOCOAGULATION IN VETERINARY OPHTHALMOLOGY

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***Abstract***

*Endoscopic cyclophotocoagulation (ECP) is a relatively new method of cyclodestruction in the treatment of glaucoma, by lowering IOP through aqueous suppression. Although the coagulation of the ciliary processes using laser is well documented, this new endoscopic approach has numerous benefits comparing to the transscleral cyclophotocoagulation. The paper aims to present a review of the indications, techniques and efficacy of diode ECP. ECP uses a diode laser equipped with an endoscope which permits direct localization and photocoagulation of the ciliary processes. The procedure can be combined with phacoemulsification in patients with coexisting cataract. Other indications of diode endolaser are iridal mass, ciliary body neoplasia, uveal cysts and retinopexy in retinal detachment cases. The available clinical evidence reviewed suggest that ECP is a safe and effective procedure in veterinary ophthalmology.*

***Key words****: endoscopic cyclophotocoagulation, diode laser, retinopexy, glaucoma.*

## INTRODUCTION

Glaucoma is one of the most common causes of irreversible blindness in dogs and cats. The treatment is focused on lowering the intraocular pressure (IOP) by either improving aqueous outflow (filtering) or suppressing inflow (cyclodestructive). Many surgical techniques have been described for the treatment of glaucoma, but, recently, the use of endoscopic cyclophotocoagulation is becoming more accepted and is no longer reserved for end-stage cases (Falkenberry et al., 2009; Berke, 2006).

Traditionally, cyclophotocoagulation was performed with a diode laser through the sclera (transscleral cyclophotocoagulation, or TSCP) targeting the ciliary body without direct visualization and was reserved to refractory or end-stage glaucoma (Falkenberry et al., 2009). The benefits of using an endoscopic tool for laser application (termed endolaser cyclophotocoagulation, or ECP) include the direct visualization of the ciliary body, eliminating destruction of surrounding tissues, with the use of a minimal amount of laser energy (Bras et al., 2005; Lutz et al., 2008).

ECP uses a diode laser with a wavelength of 810 nm for photocoagulation, a 175 W xenon light source for illumination and video imaging for visualization of intraocular structures. The internal structures of the eye from the posterior aspect of the iris, ciliary body, pars plana, peripheral retina and more posterior retina may be imaged (Operator's manual for the E2 Laser and Endoscopy system, EndoOptiks).

## MATERIALS AND METHODS

A review of published articles on ECP was conducted using PubMed database and Google search engine. The search words used included: *ECP*, *endoscopic cyclophotocoagulation*, *diode laser*, *transscleral cyclophotocoagulation*, *endolaser*, *glaucoma*, *retinopexy*. Both human and veterinary medicine articles were searched.

## RESULTS AND DISCUSSIONS

Transscleral cyclophotocoagulation (TSCP) as a surgical treatment for glaucoma has been used in veterinary medicine.

In a study, TSCP was used as a treatment of primary glaucoma in 18 dogs. Adequate control of IOP was achieved in 92% of the cases in this study, and 50% of potentially sighted eyes regained vision and was maintained over 6 and 12 months follow-up. Complications were reported, such as cataract formation (25%) and corneal disease (45%) (Hardman et al., 2001, Cook et al., 1997).

The main disadvantage of TSCP is the lack of direct visualization of the ciliary body during the procedure that may lead to an increased risk of collateral damage to nontargeted tissues. (Harrington et al., 2012).

Described first in 1992 by Martin Uram, ECP permits visualization and photocoagulation of ciliary processes (in contrast with the TSCP which estimates the location of the ciliary body).

Although both techniques achieve aqueous suppression through cycloablation, there is a clear distinction between the two treatments when evaluating the extent of tissue destruction (Seybold et al., 2015).

Histopathologic studies have confirmed that ECP causes less damage to the ciliary body compared with TSCP, while still destroying the ciliary body epithelium (Pantcheva et al., 2007). A study of rabbit eyes conducted by Lin et al. showed that both transscleral and ECP are associated with occlusive vasculopathy, but the endoscopic route is associated with late reperfusion and therefore less chronic poor perfusion (Lin et al., 2006; Falkenberry et al., 2009). Immediately and 1 day after laser, both TSCP and ECP eyes demonstrated severely reduced or nonexistent blood flow in the areas of treatment. TSCP treated processes essentially remained non-perfused at the 1 week and 1 month time points. ECP treated processes showed some reperfusion at 1 week and greater reperfusion by 1 month. Histopathology confirmed the overall greater vascular occlusion seen with TSCP. Other associated side effects of TSCP are injury to the sclera, inflammation, hypotony, and phthisis (Lin et al., 2006; Mastrobattista et al., 1996).

The use of ECP is recommended in patients with uncontrolled glaucoma (>25 mmHg) on medical therapy or with an IOP> 20 mmHg on preoperative cataract screening. The procedure can be combined with phacoemulsification in patients with coexisting cataract. Other indications are iridal mass, ciliary body neoplasia, uveal cysts, retinopexy in retinal detachment cases (Bras, 2013).

ECP uses an endoprobe with endoscopic view and a diode laser treating each individual ciliary process until whitening and shrinkage is observed.

The pupil is dilated and the ciliary sulcus is expanded using a viscoelastic substance.

The probe is inserted intraocularly through a limbal incision (in the phakic, pseudophakic and aphakic eye) or pars plana (in the pseudophakic or aphakic eye, not recommended in the phakic eye) (Bras, 2013).

The amount of energy applied is titrable and the amount of cyclophotocoagulation is therefore calibrated for each patient. Laser energy is applied to each process until shrinkage and whitening occur (Bras et al., 2005; Lutz et al., 2008).

The entire ciliary process must be ablated in order to render it nonfunctional and thereby lower IOP (Uram, 1992). Bras et al. recommend that one third of the posterior ciliary process should be spared to avoid retinal edema. The ECP treatment zone can vary from 90° to 360°, depending on the hypotensive effect that is wanted to be achieved. Tissue explosion, “popping” or bubble formation should be avoided (Bras, 2013).

Following treatment, viscoelastic is removed using irrigation followed by wound closure (Falkenberry et al., 2009).

Endoscopic cyclophotocoagulation in veterinary ophthalmology has been reported, but no peer-reviewed studies on its use have been published.

ECP efficacy in treatment of bovine and equine glaucoma has been demonstrated by Harrington et al., according to studies published in 2010 and 2012.

Lutz et al. evaluated the use of ECP in pseudophakic and aphakic dogs with secondary glaucoma following primary cataract removal. A total of 15 dogs (n = 17 eyes) with secondary glaucoma were treated with a limbal approach endoscopic cyclophotocoagulation (ECP). He reported a 94% success of decreasing IOPs and 60% maintenance of vision over a 10 month period post-op. (Lutz et al., 2009).

Bras et al. described the successful use of ECP in 112 canine cases with >91% success of decreasing IOPs for a period larger than 12 months (Bras, 2013). In a study performed on 15 canine patients, Bras et al. reported the following complication: hypotension and retinal detachment (7%), uncontrolled IOP (5.5%), corneal disease (5.5%), hypertension and retinal detachment (2.7%), optic nerve degeneration (2.7%), SARDS (1.8%), normotensive and retinal detachment (0.9%), bacterial endophthalmitis (0.9%) (Bras, 2013). Another study reports as postoperative complications superficial corneal ulceration (1/17), recurrence of glaucoma (5/17), and phthisis with blindness (1/17) (Lutz et al., 2009).

Concerning the feline patients, Bras et al. used ECP successfully in 11 cats with glaucoma with a 92% success of decreasing IOP up to 1 year post-operatively and a 100% success of preserving sight. 50% of patients were off glaucoma medications. Complications included corneal ulcers (41.67%) and sequestrum (25%) (Bras et al., 2009).

Postoperatively, all patients received topical and oral CAI's and medications were decreased over time as IOP remained normal (Bras et al., 2005).

Bras recommends a treatment area of 180-200° as a prophylactic therapy or in cataract cases with IOP between 20 and 30 mmHg. As a therapeutic procedure, an area of 270-360° should be treated.

Some authors do not recommend the use of ECP in phakic patients because of the high risk of damage to the crystalline lens during the procedure (Morales et al., 2013)

Additional uses for diode endolaser include endolaser ablation of iridal melanoma (8 cases, 100% success rate in preventing growth, glaucoma or destruction of the globe), surgery of ciliary body neoplasia, endolaser uveal cyst coagulation, retinopexy in retinal detachment cases (with a high success rate for preventing further retinal detachment: 14/16 cases are reported sighted long term) (Bras, 2013).

## CONCLUSIONS

Endolaser cyclophotocoagulation offers the advantage of direct localization and photocoagulation of the ciliary processes.

The available clinical evidence reviewed suggest that ECP is a safe and effective procedure in veterinary ophthalmology, that results in a therapeutic reduction of IOP and eliminates or reduces the use of glaucoma medication.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# MEASURING THE HEART SIZE OF DOGS WITH VHS METHOD

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***Abstract***
*In small animal practice thoracic radiography is a useful diagnostic tool providing important information about heart disease. The purpose of this study was to determine heart size of six clinically healthy dog breeds by computerized radiographic technique. The study was taken into account 90 dogs: 15 Pekingese, 15 Bichon, 15 German Shepherds, 15 Labradors, 15 German Brack and 15 Mongrel. Ages ranging from 1 to 15 years. Following the evaluation of VHS of the six dog breeds were obtained the following values: German Brack had an VHS mean value of 10.9 v (10.5-11.7 v), Labrador had an VHS mean value of 10.1 v (9.6-11 V), Bichon had an VHS mean value of 9.2 v (8.7-10.7 v), Pekingese had the VHS mean of 9.3 v (8.7-10.4 v), Mongrel had an VHS mean value of 9.5 v (8.8- 10.5 v) and German Shepherd had an VHS mean value of 9.8 v (9.1-10.5 v). VHS method represents a useful method for monitoring progression of heart dimensional changes over time for each individual.*
***Key words****: cardiac silhouette, computerized radiographic technique, dog, VHS.*

## INTRODUCTION

Cardiac diseases in small animals recorded an increasing frequency in recent years expressing itself through a wide range of signs. Important information about heart disease are often obtained by thorax radiography, which in small animals is a useful diagnostic tool.

A system for measuring the cardiac silhouette (vertebral heart scale) was designed for the first time by Bucheler Buchanan (1995) in a study of 100 clinically healthy dogs and a variety of breeds. This method brings additional information about clinical examination, but also can be used for monitoring cardiac disease that evolves with size and shape changes (Apetrei et al., 2014). There is a close link between race, the thorax appearance and the shape of cardiac silhouette of the animal examined by VHS method (Owens and Biery, 1999; Root and Bahr, 2002). Lamb et al. (2000, 2001) points out that when making VHS is necessarily to take into account the crosses between breeds. Ghadiri et al. (2010) adds that it's important from what incidence the radiography is made (right or left) in order to avoid any misinterpretation of cardiac dilation.

The purpose of this study was to determine heart size of six healthy dog breeds by computerized radiographic technique.

## MATERIALS AND METHODS

In this study were examined only clinically healthy dogs which were presented to Faculty of Veterinary Medicine from Bucharest, Department of Imaging during 2013. Were evaluated 90 dogs, belonging to a number of six breeds: Pekingese, Bichon, German Shepherds, Labradors, German Brack and Mongrels. Ages ranging between 1 and 15 years, including 41 males and 49 females. Radiographs were performed using a computerized radiographic system (Philips Otimus), and images were taken with a source-image distance of 100 cm. Animals were prepared according to the procedures described in the literature (Tănase and Cristescu, 2001). The films were developed using an automatic device called PCR-Eleva-S.

VHS method was used which involved measuring the long axis (L - representing the distance from the carina to the cardiac apex) and short axis (l - representing maximum diameter perpendicular to the long axis of the heart) and their value was compared with the length of the thoracic vertebrae (v) from cranial edge of thoracic vertebra 4 (T4) from right side incidence.

**Table 1.**
Cardiac silhouette values (mean, minimum and maximum) from right side incidence

| Measurements | Dog breeds | | | | | | Total (n=90) |
|---|---|---|---|---|---|---|---|
| | Pekingese (n=15) | Bichon (n=15) | German Shepherd (n=15) | Labrador (n=15) | German Brack (n=15) | Mongrel (n=15) | |
| **L - cardiac long axis (v)[1]** | 5<br>4.7-5.7 | 5.1<br>4.7-5.9 | 5.4<br>4.6-6 | 5.6<br>5.3-6 | 5.5<br>5.1-5.7 | 5.3<br>4.8-6 | 5.3<br>4.9-5.9 |
| **l - cardiac short axis (v)[1]** | 4.3<br>3.9-4.8 | 4.1<br>3.8-4.8 | 4.4<br>4-4.8 | 4.5<br>4.1-5.1 | 4.5<br>4.1-4.9 | 4.2<br>4-4.8 | 4.3<br>4-4.9 |
| **VHS (v)[1]** | 9.3<br>8.7-10.4 | 9.2<br>8.7-10.7 | 9.8<br>9.1-10.5 | 10.1<br>9.6-11 | 10.9<br>10.5-11.7 | 9.5<br>8.8-10.5 | 9.8<br>9.2-10.7 |

[1]Length measured in vertebrae

## RESULTS AND DISCUSSION

Measurements of cardiac silhouette and VHS's values are presented in Figures 1-6 and Table 1 for each breed separately. The VHS mean from all 90 dogs was 9.8 v ranging between 9.2-10.7 v.

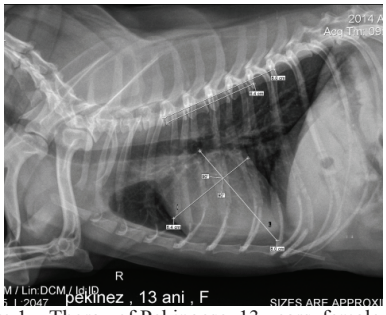

Figure 1 – Thorax of Pekingese, 13 years, female, right view

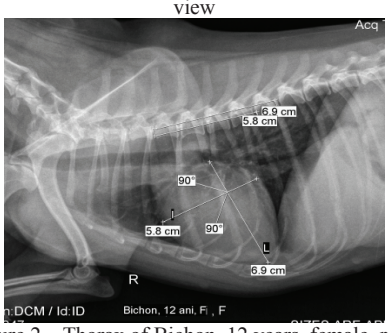

Figure 2 – Thorax of Bichon, 12 years, female, right view

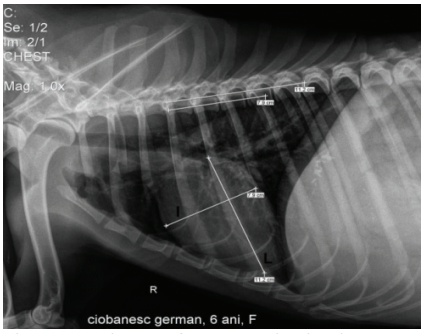

Figure 3 – Thorax of German Shepherd, 6 years, female, right view

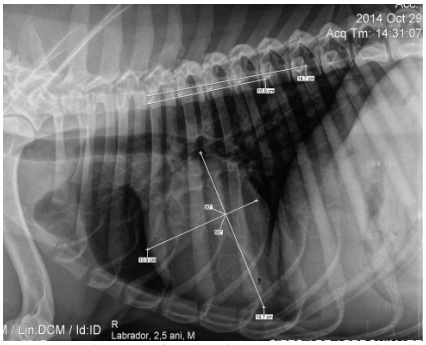

Figure 4 – Thorax of Labrador, 2.5 years, male, right view

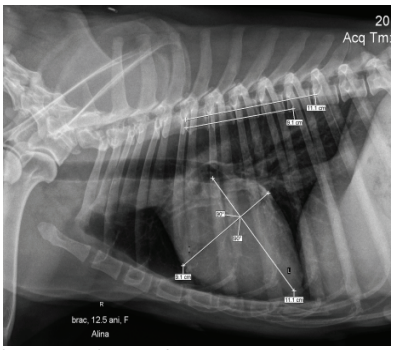

Figure 5 – Thorax of German Brack, 12.5 years, female, right view

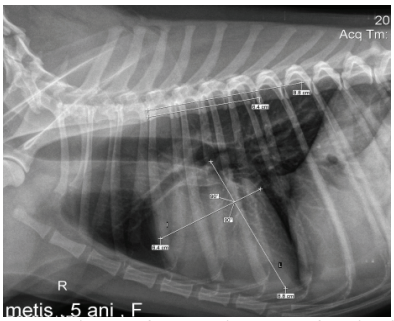

Figure 6 – Thorax of Mongrel, 5 years, female, right view

The values obtained are close to those presented in previous studies, such as Buchanan and Bucheler (1995) who conducted a study on a total of 100 dogs of various breeds, and obtained an VHS mean of 9.7 (SD ± 0.5). Ghadiri et al. (2010) obtained a VHS mean value of 9.6 ± 0.56 v from left side incidence and 9.7 ± 0.59 from right side incidence on a total of 56 dogs. Gulanber et al., (2005) have established a VHS value of 9.7 ± 0.67 v on a number of 120 dogs that ranged between 8.4 to 10.9 v. However, the differences can be attributed to many factors such as different number of animals examined, breed, crosses between animals (Lamb et al., 2000), gender, age (Lamb et al., 2001, Jepsen-Grant et al., 2013) and the incidence from which radiography was performed (Ghadiri et al., 2010).

As shown in the results, conformation of the thorax (deep, narrow, wide and intermediate) is the main factor that influenced the VHS value for this six breeds in our study. The results from this study that we conducted showed that the VHS mean value for each breed varies in proportion to the reference value obtained in previous studies (Buchanan and Bucheler, 1995). Besides, Lamb et al. (2000, 2001), for a proper assessment, he recommends limits for each breed separately.

## CONCLUSIONS

Our results indicate a relatively wide range of normal values of cardiac dimensions correlated with breed.

Evaluation of cardiac silhouette with radiological method must be correlated every time with the clinical examination of the animal.

VHS method is one of the easiest and useful tool for measuring cardiac silhouette and monitoring the heart disease at the same time must take into account the status of the individuals evaluated, age, gender, breed, and thorax conformation.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# NEUROLOGICAL AND OCULAR FORM OF TOXOPLASMOSIS IN CATS

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***Abstract***

*Toxoplasmosis is a zoonotic disease caused by the protozoan Toxoplasma gondii, an intracellular coccidian parasite. The cat occupies a central role in the life cycle of this parasite, as a definitive host. Toxoplasmosis is transmitted by consumption of infected raw or undercooked meat, consumption of oocysts from the cat feces or by transplacental transfer of tachyzoites from mother to fetus.*

*The study was conducted in the Ophthalmology Department of the Faculty of Veterinary Medicine, Bucharest, over a one year period, from September 2013 to September 2014. We examined and diagnosed with toxoplasmosis a number of 22 cats. The cases were subjected to clinical, neurological, ocular examinations and paraclinical tests. Clinical examination revealed various multifocal neurological signs such as behavioural changes, altered mentation, seizures, ataxia, blindness, anisocoria, torticollis and vestibular signs. In some cases the ophthalmological examination revealed chorioretinitis and uveitis. These results along with the history led us to the suspicion of toxoplasmosis. We performed routine haematology and serum biochemistry tests, but these tests are not specific and the results depend on the extent of systemic involvement.*

*We established the definitive diagnosis of toxoplasmosis by performing the serological specific test in order to determine the IgM and IgG levels of Toxoplasma antibodies. In the cases were the IgM titre was elevated, the acute phase of the infection was diagnosed, whereas the elevated IgG titre revealed a chronic infection.*

*The aim of this paper is to highlight the multiple and various neurological signs of toxoplasmosis, which is often misdiagnosed and incompletely investigated, and therefore improperly treated. The importance of this study stems from the fact that in recent years toxoplasmosis experienced an increase incidence in our country.*

***Key words****: cat, central nervous system, serological tests, toxoplasmosis.*

## INTRODUCTION

Toxoplasmosis represents a worldwide spread important zoonosis, in which the cats occupies a central part, are the only definitive host.

Most cats infected with *Toxoplasma gondii* will not show any symptoms, but in immunosuppressed young and adult cats the signs of disease are present, especially in cats infected with feline immunodeficiency virus (FIV), feline leukemia virus (FeLV) or other concurrent infections (Platt et al., 2013; Jaggy et al., 2010).

The clinical manifestations of feline toxoplasmosis are variable and it can cause gastrointestinal, respiratory, ophthalmological and neurological disorders. Nonspecific signs of anorexia, lethargy, depression, fever, and weight loss can be seen (Lorenz et al., 2012; Gunn-Moore et al., 2011;Platt et al., 2013).

The neurological signs may be observed alone or along with the digestive, pulmonary or ophthalmological signs (Gunn-Moore et al, 2011; Lorenz et al., 2012).

Toxoplasmosis in cats represents a common neurological problem, but only 10% from all diagnosed cases presents neurological signs, the rest of the cases representing latent infection (Gunn-Moore et al., 2011).

When toxoplasmosis affects the eyes and central nervous system, signs such as retinitis, chorioretinitis, uveitis, anisocoria, blindness, personality and behavioural changes, circling, ataxia, or seizures can be observed (Platt et al., 2013;Gunn-Moore et al., 2011; Elmore et al., 2010; Gelatt et al., 2013).

In immunocompromised patients there is a high risk in reactivation of latent infection.
Toxoplasmosis is diagnosed based on the history, clinical signs and the results of laboratory tests. Diagnostic tests for toxoplasmosis are represented by determining the antibody titers or performing the PCR technique from blood or cerebral spinal fluid. IgM antibodies reflect an acute infection, while IgG antibodies reflect chronic infection (Platt et al., 2012; Maggs et al., 2012).
The oocysts can be found in feces only for a short period of time, thus the coproparasitological test is not recommended to establish the diagnosis. The cats usually excrete the *Toxoplasma gondii* oocysts 3 days after infection, and may continue shedding up to 20 days (The Merck Veterinary Manual, 2011; Jaggy et al., 2010).

## MATERIALS AND METHODS

The study was conducted in the Ophthalmology Department of the Faculty of Veterinary Medicine, Bucharest, over a one year period, from September 2013 to September 2014. We examined and diagnosed with toxoplasmosis a number of 22 cats.
All cases were subjected to physical, neurological, ophthalmological examinations and paraclinical tests – hematology, serum biochemistry screening and serum antibody titres.
The blood samples from these cases were tested for Toxoplasma antibodies (IgM and IgG) in the Faculty of Veterinary Medicine Laboratory, performing also routine hematology and serum biochemistry screening. In some cases, the cats were also tested for FIV and FeLV, but the antibodies were not detected.

## RESULTS AND DISCUSSIONS

We diagnosed clinical toxoplasmosis in 22 cats, male and female, with ages ranged from 4 months to 17 years old, four cats were younger than one year. One of the most represented breeds was Domestic Short Haired (DSH), 19 from 22 cases; the other breeds were represented by British Short Haired, Russian Blue and Siamese.
The common clinical signs are represented by ophthalmic manifestations, unilateral or bilateral uveitis (Figure 1), presence of keratic precipitates on the corneal endothelium (Figure 2).
Among these manifestations, systemic signs such as loss of appetite, weight loss, personality and behavioral changes are also common findings of toxoplasmosis (Table 1).

Table 1. Signalment, clinical signs associated with clinical Toxoplasmosis in 22 cats.

| Nr. Crt | Age | Sex | Breed | Clinical signs |
|---|---|---|---|---|
| 1. | 9 yr[1] | F[4] | DSH[5] | Behavioural and personality changes, seizures, uveitis |
| 2. | 7 mo[2] | M[3] | DSH[5] | Unilateral uveitis |
| 3. | 10 yr[1] | F[4] | DSH[5] | Unilateral uveitis |
| 4. | 1.7 yr[1] | F[4] | British Short Haired | Behavioural and personality changes, anisocoria |
| 5. | 2 yr[1] | M[3] | DSH[5] | Keratic precipitates |
| 6. | 8 yr[1] | M[3] | DSH[5] | Bilateral uveitis |
| 7. | 1.2 yr[1] | F[4] | DSH[5] | Unilateral uveitis |
| 8. | 3 yr[1] | F[4] | DSH[5] | Unilateral uveitis |
| 9. | 2 yr[1] | M[3] | DSH[5] | Unilateral uveitis |
| 10. | 10 mo[2] | F[4] | DSH[5] | Unilateral uveitis, keratic precipitates |
| 11. | 8 yr[1] | M[3] | DSH[5] | Unilateral uveitis |
| 12. | 1 yr[1] | | DSH[5] | Unilateral uveitis |
| 13. | 7 yr[1] | M[3] | Russian Blue | Anterior uveitis, keratic precipitates |
| 14. | 2 yr[1] | F[4] | DSH[5] | Bilateral uveitis |
| 15. | 6 yr[1] | M[3] | DSH[5] | Vestibular syndrome, without ocular signs |
| 16. | 1,3 yr[1] | M[3] | DSH[5] | Unilateral uveitis |
| 17. | 9 yr[1] | F[4] | DSH[5] | Unilateral uveitis |
| 18. | 4 mo[2] | F[4] | DSH[5] | Latero-lateral nystagmus, ataxia, absent PLR, amaurosis |
| 19 | 2 yr[1] | F[4] | DSH[5] | Behavioural changes, |

| | | | | |
|---|---|---|---|---|
| . | | | | unilateral uveitis, keratic precipitates |
| 20. | 6 mo[2] | M[3] | DSH[5] | Unilateral uveitis |
| 21. | 4 yr[1] | M[3] | DSH[5] | Unilateral uveitis |
| 22. | 17 yr[1] | F[4] | Siamese | Latero-lateral nystagmus, mydriasis, bilateral chorioretinitis amaurosis |

[1]yr = years
[2]mo = months
[3]M = male
[4]F = female
[5]DSH = Domestic Short Haired

Ophthalmological signs were observed in 19 of the 22 cases examined (86 %), representing the most frequent clinical manifestation. In cases 1, 19 and 22, the ocular signs were associated with neurological signs, such as seizures, behavioural and personality changes, respectively nystagmus in case 22.

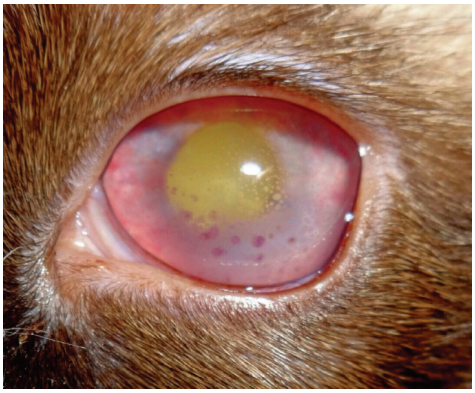

Figure 1. Ocular toxoplasmosis. Unilateral toxoplasmic uveitis, iridocyclitis in a 3 year old cat.

Anterior and posterior uveitis represents the most common ophthalmological signs. Lens luxation, absent pupillary light reflex (PLR) and chorioretinitis were also encountered.

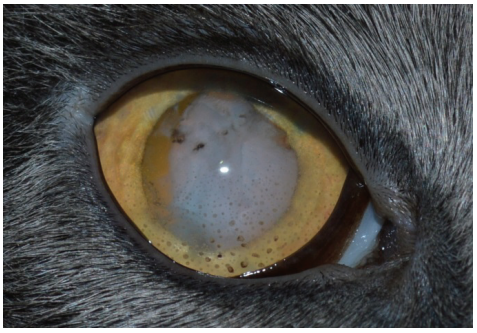

Figure 2. Ocular toxoplasmosis.Note the keratic precipitates on the corneal endothelium.

The neurological signs include a wide variety of clinical manifestations, present along or without ophthalmological signs. Six of the 22 cases presented neurological signs (27%), such as anisocoria, nystagmus, amaurosis, ataxia, vestibular signs, behavioural changes, altered mentation and seizures.

The changes of the routine hematology and serum biochemistry screening are not specific, depend on each individual case. The common changes in routine hematology include anemia, typically increased neutrophils, lymphocytes and eosinophils; while the changes in serum biochemistry screening in some cases showed liver dysfunctions, hyperglobulinemia.

Radiological examination, when performed, showed no changes.

We diagnosed toxoplasmosis by correlating the history and the clinical signs with the measured serum titre of IgM and IgG toxoplasma antibodies in all cases.

We didn't performed the coproparasitological test because doesn't represent a reliable test; the *Toxoplasma* oocysts are similar to some other parasites, and the period of excreting the oocysts is short (The Merck Veterinary Manual, 2011; Jaggy et al., 2010).

The treatment consisted in systemic administration of an antibiotic and supportive clinical treatment in all cases, for 28 days. Local treatment was performed with antibiotic and anti-inflammatory collyrium.

The antibiotic election was different, in case 4 we used Doxycycline, 10 mg/kg, once a day, during 28 days, and Azithromycin eye drops. In case 18, because of the poor general condition we used intravenous administration of Ceftriaxone, 25mg/kg, during 10 days. After this period we used Clindamycin, 25 mg/kg daily.
In rest of the cases we used oral therapy with Clindamycin, 25 mg/kg daily, for 28 days.
The therapeutic response was favorable in 20 of all 22 cases, except cases 1 and 18, which died.
The cat that presented seizures, case 1, died during the treatment, due to trauma after having a seizure.
In case 18, immunosuppression associated with secondary infection contributed to the clinical worsening, and after two months of treatment the owners chose euthanasia.

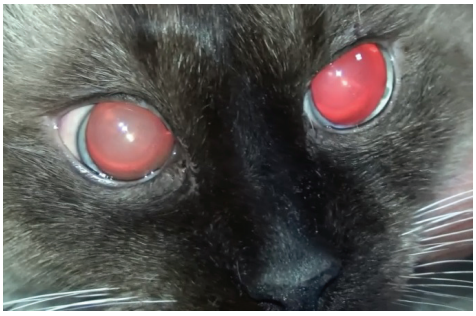

Figure 3. Bilateral chorioretinitis in a 17 years old cat – case 22, which presented latero-lateral nystagmus.

In case 22, who presented bilateral chorioretinitis, fixed mydriasis, latero-lateral nystagmus and vision loss, in addition to the antibiotic, methylprednisolone was administered. The clinical signs improved slightly during one month of treatment, so the drug therapy was lengthened with 28 days.

## CONCLUSIONS

This paper highlights the multiple and various neurological and ocular signs of toxoplasmosis, one of the most important zoonotic parasites, which is often misdiagnosed and therefore improperly treated.
Due to the predominant subclinical evolution of nervous form of toxoplasmosis, all diagnosed cases must be treated for at least 28 days, even though the clinical signs, often ophthalmologic manifestations, were resolved previously.
Diagnosis of toxoplasmosis is established by determining the serum levels of IgM and IgG antibodies, correlated with clinical signs and full history of the case.

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The Merck Veterinary Manual, 2011, 10th edition.

Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# LID SPLITTING AND POSTERIOR LAMELLAR CRYOTHERAPY FOR CONGENITAL DISTICHIASIS AND TRICHIASIS IN DOG

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***Abstract***

*Various surgical techniques have been proposed for treating distichiasis in dogs. A technique involving eyelid splitting and double freeze-thaw cryotherapy with anterior lamellar recession was evaluated. A 3 year old, female, Staffordshire bull terrier was referred for bilateral distichiasis. There were bilateral multiple distichiasis of the upper lids, more severe on the right lid with double row of cilia and two cilia on the lower lid. Under general anaesthesia, the eyelid margin was split at the gray line and a cryoprobe was used to freeze the posterior lamella. A double freeze-thaw technique was applied in both eyes. Anterior lamellar recession was performed to prevent postoperative entropion with trichiasis. The anterior and posterior lamellas were sutured with a 6/0 Vicryl suture. Bilateral upper eyelid edema was noted postoperatively. A month follow-up revealed increased bilateral granulation and depigmentation and the recurrence of one follicle on the right upper lid. Five months postoperatively there was no recurrence in the left eye but three cilia were detected in the right upper lid. The follicles have regrown due to incomplete destruction of the roots. Lid margin split with cryotherapy is an effective method for treating distichiasis but might require several attempts and increase in the cryotherapy time.*

***Key words:*** *cryotherapy, distichiasis, eyelid splitting*

## INTRODUCTION

Distichiasis represents the presence of one or more extra cilia or eyelashes arising from the free eyelid margin. They usually arise singly or with two or more hairs from the meibomian gland duct openings [1–4]. Distichia appear to develop from the ectopic hair follicles present in the tarsus as a result of an anomaly of hair follicles morphogenesis in the mesenchymal tissue of the tarsal plate. [5] Histological examination of the excised samples showed that the cilia result from metaplasia of tissues in or around the meibomian glands.[6]

Clinical signs associated include blepharospasm, epiphora, conjunctival hyperhaemia and occasionally corneal ulceration [1].

The condition is usually present bilaterally [1] and it may be inherited in many breeds with an unknown mode of transmission. Predisposed breeds include the Cocker Spaniel, English bulldog, Poodle, Boxer, St Bernard, Golden Retriever, Daschschund, Alsatian, Jack Russel Terrier, Bedlington terrier, Shetland sheepdog, Yorkshire terrier and the Pekingese [1,2].

Treatment consists of either temporary removal by manual epilation or permanent destruction by diathermy electroepilation, electrocautery, high frequency radiohyperthermia, electrolysis, cryotherapy and various surgical procedures [1]. Surgical techniques to remove or redirect the distichia follicles include partial resection of the distal tarsal plate, eyelid split, transpalpebral conjunctival dissection, Celsus-Hotz repositioning with various limitations [1,6,7].

In humans, surgical procedures have been developed to excise the distichia and follicles. These methods usually divide or split the entire eyelid skin and orbicularis oculi muscle from the tarsus and palpebral conjunctiva from the distichia follicle excision site to the conjunctival fornix. The wound is usually left open to heal by secondary intention.[1,6,7]

For the treatment of canine distichiasis was found cryotherapy non-invasive and with limited complications. A nitrous (N2O) cryounit is required and a double freeze-thaw cycle is sufficient to destroy the follicles with minimal damage to the eyelid margin.

## MATERIALS AND METHODS

A 3 year old Staffordshire bull terrier neutered female presented to the Ophthalmology Service at the Animal Medical Centre for evaluation of chronic bilateral ocular discharge and blepharospasm.
Initial ophthalmic examination revealed mild corneal oedema and bilateral distichiasis was diagnosed by adequate illumination and magnification.
There were bilateral multiple distichiasis of the upper lids, more severe on the right lid with double row of cilia and two cilia on the lower lid (Figure 1). Mild corneal oedema and vascularization was due to irritation caused by distichia.

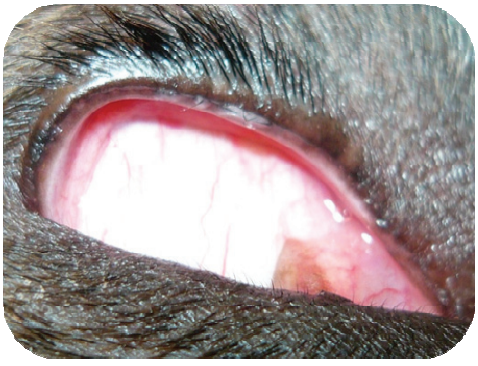

Figure 1 Upper and lower distichiasis

Under general anaesthesia, the eyelids were prepared with a 1% povidone-iodine aqueous solution. A preoperative injection of carprofen was given to reduce postoperative swelling.
A Desmarres chalazion clamp with screw lock was placed on the eyelid (Figure 2). The eyelid was grasped and held during cryotherapy by the chalazion clamp and the margin was split at the grey line using a Beaver No. 6500 microsurgical blade.

The cryoprobe was applied to the conjunctiva overlying the meibomian glands that contained the cilia. The iceball was observed under the microscope as it advanced over the line of gland openings and froze the posterior lamella. Double freeze-thaw cycles were applied bilaterally. Anterior lamellar recession was performed to prevent postoperative entropion. The anterior and posterior lamellas were sutured with a 6/0 Vicryl suture.
Topical treatment with antibiotics and corticosteroids postoperatively was initiated to minimize eyelid swelling and reduce scarring.

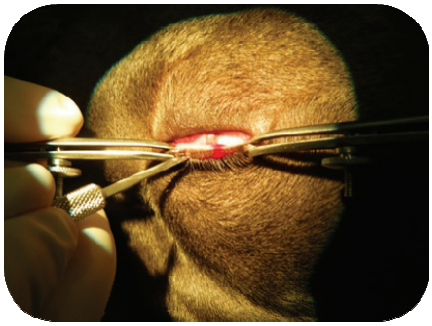

Figure 2. The eyelid was stabilized and everted using a Desmarres eyelid clamp and an incision was made at the grey line

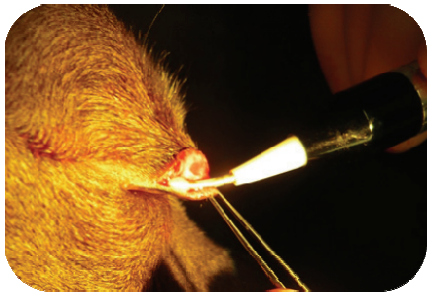

Figure 3. Cryodestruction of multiple distichiae in the conjunctiva-tarsal plate

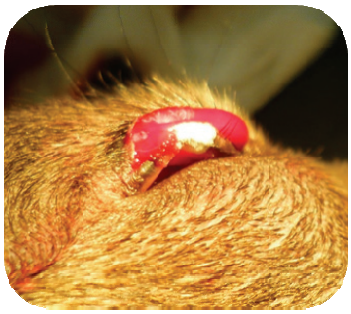

Figure 4. Cryotherapy complete prior to suture of the anterior and posterior lamellas

## RESULTS AND DISCUSSIONS

Bilateral upper eyelid and conjunctival edema was noted immediately postoperatively and lasted for two days. Systemic antiinflammatories were continued for the next week to reduce the swelling. Depigmentaton of the eyelid margin was noted after three days.

A month follow-up revealed increased bilateral eyelid scarring and focal depigmentation and the recurrence of one follicle on the right upper lid. Five months postoperatively there was no recurrence in the left eye but three cilia were detected in the right upper lid. The follicles have regrown due to incomplete destruction of the roots.

Studies showed a recurrence of 10-30% following all the distichia techniques due to complications associated with inadequate excision, eyelid fibrosis, depigmentation of the eyelid margin after cryotherapy [1].

In a study of distichiasis treatment in man including epilation, lid margin cryotherapy and eyelid splitting followed by cryotherapy to the posterior lamella the latter had shown improved ocular signs with no recurrence in 87% of the cases [8,9,10]

Eyelid splitting was associated with follicular extractions or via monopolar cautery for trichiasis and distichiasis in man with no postoperative complications [7] or the alternative technique of folliculectomy with anterior lamellar recession with 69.2% rate of success [4]

The lid margin is split at the grey line and a cryoprobe is used to freeze the posterior lamella. [8] A double freeze-thaw technique is applied, initially the time taken to register -20 C then followed by a slow thawing before re-freezing. The anterior and posterior lamellas are then sutured with a 6/0 knot vicryl suture. Anterior lamellar recession prevents the postoperative entropion with trichiasis.

Complications include eyelid edema, incomplete destruction of the roots probably secondary to inadequate temperature or duration of the freeze.[2,8–11]

## CONCLUSIONS

Various surgical techniques were proposed for treating this condition.

A technique that involves eyelid splitting and double freeze-thaw cryotherapy with anterior lamellar recession was described in man with no recurrence in a one year follow-up.[12]

The technique was used in this case where the eyelid is divided along the gray line and followed by cryotherapy on the posterior lid lamella.[6]

Lid margin split with cryotherapy is an effective method for treating canine distichiasis but might require several attempts and increase in the cryotherapy time.

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ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# BRACHYCEPHALIC AIRWAY SYNDROME IN DOGS

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***Abstract***

*Brachycephalic syndrome in dogs, also called congenital obstructive disease of the upper airway is described as a continuous process of anatomical and functional disorders of the respiratory and digestive systems.*
*The syndrome is characterized by stenotic nares, elongated soft palate, everted laryngeal saccules and in advanced stages by laryngeal collapse.*
*Clinically, dogs show signs of respiratory distress, such as: severe dyspnea, wheezing, coughing, snoring, exercise intolerance, increased respiratory effort, hyperthermia and collapse, and digestive signs, as: vomiting, regurgitation, ptyalism, pyloric stenosis and inflammation of the gastrointestinal segments.*
*Medical management includes weight control and reduced physical effort. Patients with acute respiratory syndrome should be treated as an emergency.*
*Surgical treatment requires wedge resection of stenotic nares or rhinoplasty, shortening of the soft palate or palatoplasty, and removal of laryngeal saccules.*
*Early recognition and correction of brachycephalic airway syndrome has favorable long-term outcomes for the patient.*

***Key words:*** *brachycephalic syndrome, stenotic nares, elongated soft palate, rhinoplasty, palatoplasty.*

## INTRODUCTION

Brachycephalic syndrome, also known as brachycephalic airway obstructive syndrome (BAOS), is a well described disorder of the upper respiratory tract of brachycephalic dog breeds (Wetzel J.M, Moses P., 2010). These breeds tend to have respiratory difficulties which are the direct consequence of the anatomical deformities of their head (Roedler F. et al, 2013; Lecoindre R., Richard S., 2004)

Early research on the pathology and treatment of the brachycephalic dogs with respiratory failure were published in the thirties of the last century (Oechtering G. U. et al, 2008).

Primary characteristics of the BAOS include congenital anatomic abnormalities such as stenotic nares, elongated soft palate, hypoplastic trachea and nasopharyngeal turbinates, although, hypoplastic trachea is considered independent of BAOS. Increased resistance during inspiration can cause the development of second changes that include palate and laryngeal edema, swelling, saccule and tonsil eversion, and laryngeal collapse (Meola S., 2013; Trappler M., Moore K, 2011).

All these abnormalities narrow the lumen of the upper respiratory tract and restrict breathing. Often, this ends with asphyxiation and collapse, especially during heat exposure or excitement.

Dogs with more than two of these characteristics present both respiratory and digestive signs (Meola S., 2013; Trappler M., Moore K, 2011).

Diagnosis is usually obvious and uncomplicated, based on clinical signs and physical examination.

Early correction of anatomical abnormalities such as stenotic nares and elongated soft palate may result in a significant improvement of airway dynamics, and may prevent subsequent deterioration during the animal's lifetime (Bray J.,2013).

The purpose of this article is to summarize the current knowledge about BAOS as clear as possible, starting with the genetics, physiopathology, clinical signs and ending with the diagnosis and the medical and surgical treatment.

## GENETICS, SEX, AGE AND BREED PREDISPOSITION

No specific genes have been identified. The ancestors of brachycephalic breeds seem to have Asiatic origins.
The brachycephalic dogs have a local chondrodysplasia which is the result of domestication, deliberately kept by the breeders (Koch D. et al, 2003).
The most common brachycephalic breeds are: Chihuahua, Bulldog, King Charles spaniel, Pug, Boston terrier, Maltese, Pekingese, miniature Pinscher, Shih tzu, Yorkshire terrier, and Boxer (Koch D. et al, 2003).
Regarding any possible conection between skull measurements and glottis dimensions in certain breeds, one study made in 2014 demonstrates that in Pug, French Bulldog and English Bulldog no correlation was found. The lack of correlation between skull and glottic indices does not support skull morphology as predictor of glottic morphology. On the contrary, the glottic index was significantly smaller (high and narrow glottis) in Pugs than in English Bulldogs. As a consequence of the lowest glottic index of Pugs when compared to EB and FB, it may be speculated that Pugs original narrow glottis width may predispose to further respiratory deterioration possibly as a consequence of progression to laryngeal collapse (Caccamo R et al.,2014).
Some studies made in 2002 show that there are now sex predisposition , brachycephalic syndrome affecting males and females equally, while some other studies made between 2005 and 2008 show an increased incidence (2:1) in male dogs (Meola S, 2013).
The average age for clinical manifestation of the disease is 2-3 years, although puppies less than 6 months of age have been diagnosticated with severe laryngeal collapse (Meola S, 2013).

## ANATOMY

Brachycephalic means “short, wide-headed.” (Evans H, Lahunta A, 2013).
The wings of the nostrils contains fibers of the maxillary labii and nasolabial levator muscles, thing that makes them very maneuverable. Stenotic nares are the result of congenital malformations of the nasal cartilages which cause medial collapse of the alae. This creates a smaller opening at the nostril and a decrease airflow (Koch et al., 2003).
Nasal cavities include 4 meatuses: ventral, dorsal, common and middle, created by the dorsal and ventral nasal choanae and the hard palate. In brachycephalic breeds this are shorten and may contain nasopharyngeal turbinates. This turbinates extend into the nasopharynx, are abnormal and found most in Pugs. Paranasal sinuses commonly miss in brachycephalic dogs (Ginn J. et al., 2008).
The transition from hard palate to soft palate is more caudal to the last molar than in dolichocephalic and mesocephalic dogs. The soft palates is usually elongated and can extend past the epiglottis. This would increase the air resistance at the larynx. The muscular-cartilaginous larynx controls the airflow within the trachea and takes part in vocalization. The narrowest passage of the airflow is the rima glottidis, which is formed dorsally by the paired arytenoid cartilages and ventrally by the paired vocal folds. Laryngeal saccules are located between the vocal and ventricular folds, and normally are not everted (Koch et al., 2003).

## PATHOPHYSIOLOGY

All anatomic abnormalities have modified the physiology of the respiratory system of the brachycephalic dogs. Thus, in brachycephalic dogs exhalation is forced rather than passive, because they must overcome the increase in airway resistance. According to Poiseuill's law, a 50% reduction in the radius results in a 16-fold increase in flow resistance (Meola S, 2013).
Stenotic nares (Fig.1) and nasopharyngeal turbinates are considered to be possible stenoses, which lead to an increase in negative pressure during inspiration. They are the most common primary manifestation of BAOS and are found in 17%-77%, respectively 21% of brachycephalic dogs. The negative pressure causes the drawing of the soft tissue into the lumen (stretching of the soft palate), becoming this way hyperlastic, inflamed and the swelling of the airway. Secondary manifestation, such as edema and inflammation of tonsilar tissue, eversion of the laryngeal saccules, partial

collapse of the weakened laryngeal cartilages, narrowed rima glottis, constrict the lumen even more. This way, a vicious cycle develops: collapse of the laryngeal cartilage further decreases of the radius of the airway, which increases the velocity of airflow and the negative pressure in the airway, leading to further collapse (Trappler M, Moore K, 2011;Koch D et al, 2003).

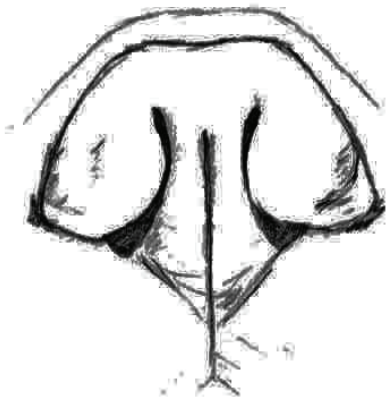

Fig. 1 – Stenotic nares

It is unknown if the elongated soft palate is a primary or secondary event, but it is clearly that the trachea is hypoplastic, due to an abnormal embryogenesis.

Although during inspiration, the soft palate may be caught dorsal to the epiglottis, inciting suffocation, a sympathetically controlled mechanism will cause vasoconstriction, reducing resistance of an acute attack. The wings of the nostrils will be actively dilated, the dog will stretch his neck upward to dilate the nasopharynx and larynx and the air will be inhaled rapidly through the nose, moistened in the conchae, and exhaled though the mouth. Some dogs will have the tendency to open mouth breathe to obtain better airflow, thing that will determine the lateral nasal salivary gland to increase production, to provide enough moisture (Stanley B, 2008).

The respiratory tract is not the only organic structure exposed to increased negative pressure during inspiration. Because of their close vicinity to the airways, the esophagus, auditory canals, central nervous system, and lower respiratory tract should also be examined. Brachycephalic breeds will almost always present an enlarged tongue, difficulty in swallowing, hiatal hernia, gastric bloating, otitis media, neurologic signs, and bronchiectasia (Koch et al., 2003).

In brachycephalic breeds, a possible explanation is the high positive abdominal pressure generated by recurrent vomiting, as well as the negative intrathoracic pressures generated by increased inspiratory work (Dupre G.,2013 ). Gastroesophageal reflux associated with regurgitation and vomiting can contribute to upper esophageal, pharyngeal, and laryngeal inflammation (White *et al*. 2002). In turn, respiratory distress could stimulate the autonomic sympathetic nervous system, which would slow gastric motility and increase gastric emptying time. Furthermore, the dilated antrum would stimulate gastrin-producing cells responsible for muscular hyperplasia (Peeters 1991; Guilford & Strombeck 1996). The correlation between respiratory and digestive disorders suggests an influence of upper respiratory tract diseases on gastroesophageal diseases, and vice versa. Gastroesophageal disorders (ptyalism, regurgitation, vomiting, and reflux) can aggravate the respiratory signs by encumbering the pharyngeal region and stimulating persistent inflammation. Conversely, chronic respiratory depression promotes gastroesophageal reflux. The close relation between respiratory and digestive problems is sustained by the fact that most of these animals “vomit” large amounts of saliva, when excited or stressed, or during respiratory distress (Dupre G, 2013)

One study made in 2011 concluded that the most important microscopic aspects were hyperplasia and intracellular edema of mucosal lining, diffuse edema, and amplified myxoid matrix in the lamina propria. The anatomy of the palatine musculature of brachycephalic dogs enrolled in the study suffered from extensive degenerative lesions involving the majority of muscular fibers. (Pichetto M et al., 2011)

## CLINIC SIGNS

Clinical signs observed in brachycephalic dogs include snoring, inspiratory dyspnea, gagging, productive coughing, difficulty swallowing, sleeping, suffocation, cyanosis and

hyperthermia. Often, they become worse after excitement, exercise, stress or increased environmental temperature. Most of dogs present a reflux esophagitis, regurgitation and vomiting.

## DIAGNOSIS

A correct diagnostic can be made only after taking a history, listening of the dog and watching his clinical presentation. Diagnostic imaging as neck and thoracic radiography, CT of the head and endoscopic examination of the upper airways and upper gastrointestinal tract is essential.

*Physical examination*

Involvement of the soft palate and laryngeal saccules often leads to inspiratory and expiratory dyspnea. Dogs with <50% reduction in airway diameter show an obstructive breathing pattern with slow inspiratory phase and rapid expiratory phase. (Trappler M, 2011)

To perform the examination of the upper airway, it is recommended to anesthetize the animal. This can be done using propofol (3-6 mg/kg) (Trappler M, 2011). The purpose of this is to evaluate the soft palat, tonsils, laryngeal saccules and laryngeal function. In brachycephalic dogs, the soft palate extend past the tip of the epiglottis, blocking the rima glottidis. Everted laryngeal saccules appear as shiny, white, convex structure, cranial to the vocal cords, along the ventrolateral surface of the laryngeal lumen. In pugs, the dorsal border of the cuneiform process of the arytenoids cartilages can invert into the laryngeal lumen. (Trappler M, 2011)

Different studies made along time revealed that the most common manifestation of the BAOS is elongated soft palate.

*Radiologic examination*

Lateral radiography of the neck can help to assess the soft palate thickness, presented as a soft tissue density between the nasopharynx and oropharynx. Thoracic radiography is made to document secondary heart or lung disease, or, sometimes, a sliding hiatal hernia. (Monnet E, 2013)

*Endoscopic examination*

Endoscopic examination allows evaluation of the conchaea, soft palate thickness and nasopharyngeal tissue hyperplasia. It is also made to appreciate the stenosis of the vestibulum. In pugs, the dorsal border of the cuneiform process of the arytenoids cartilages can invert into the laryngeal lumen. (Monnet E, 2013)

In 2004, one study made on 30 brachycehalic dogs showed, after performing the endoscopy of the upper digestive airway, that 24 patients presented an hyperplasia of the esophageal mucosa. 13 dogs showed a reflux esophagitis at stage I, 9 at stage II, 2 at stage III and 1 at stage IV compounded by a stenosis of the esophagus, while only 5 of them didn't showed any esophagitis lesions. Sixteen cases of hiatal hernia by sliding or a defective cardiotuberositary position were observed. In 21 cases, the fundic mucosa showed a typical aspect of follicular gastritis. In 13 cases, gastroscopic examination showed an abnormal hyperplasia of the antral mucosa. Five dogs presented no functional or organic abnormalities of the upper digestive tract. (Lecoindre P., Richard S, 2004) (Table 1)

Table 1 – A review based on 4 studies since 2006 to 2010, compared with a review from 2013

| Modified parts | Poncet CM, Dupre G, 2006 | RecksTW, 2007 | Fasanella F. et al, 2010 | Wetzell, Moses 2010 | Meola S, 2013 Review | Total |
|---|---|---|---|---|---|---|
| Year | | | | | | 2015 |
| Nr. of cases | 51 | 62 | 90 | 155 | | 358 |
| Elongates soft palate | | 54 | 85 | 152 | 62-100 % | 81,28% |
| Stenotic nares | 45 | 36 | 69 | 47 | 17-77 % | 55,02% |
| Everted saccules | | 36 | 59 | 63 | 53-66 % | 44,13% |
| Laryngeal colaps | 14 | 5 | | 17 | 53 % | 10,05% |
| Tonsile | | | 50 | 77 | 9-56 % | 35,47% |
| Hypoplastic trachea | | 13 | | 6 | 13% | 5,30 % |
| Complications | 13,3 | 3,5 | 10,8 | 13 | | 11,34% |

## ANESTHESIA

Before surgery, the patient should be premedicated and stress avoided. Dupré G.,

2013, recommend to performe premedication with Acepromazine (0.01–0.05 mg/kg IM, SC), Dexamethasone (0.1–0.2 mg/kg IM, SC), Opioid analgesic (eg, morphine or methadone, 0.2–0.5 mg/kg IM), Glycopyrrolate (2–10 µg/kg IM), Antiemetics & antacids (SC or IM, depending on drugs) (Dupré G, Findji L., 2013). Some authors recommend the use of dexamethasone in following doses: 0.5-1 mg/kg IV, to avoid the laryngeal edema. Before anesthesia induction, oxygen can be supplied by mask or flow-by for 5-10 minutes. Induction must be swift to allow prompt control of the airway via tracheal intubation. The pharynx should be examined and the larynx assessed for signs of laryngeal collapse.

## TREATMENT

The main condition that a brachycephalic dog owner must understand, accept and admit is that this dog breed can not be made normal.

*Medical therapy*

Medical treatment of a brachycephalic dog include both medical therapy of the upper airways and upper gastrointestinal tract.

Medical therapy of the upper airway is required almost anytime when the patient comes in crises. Hyperthermia should be treated accordingly with cold towels and tranquilizers. It is very important to administrate oxygen therapy, anti-inflammatory drugs (dexamethasone SP 0,22 mg/kg) and to intubate, as the dogs may present glottis edema and cyanosis.

Upper gastrointestinal tract should also be investigated and treated with omeprazole (0,7 mg/kg per os per 24 hours) and prokinetic medication with cisapride (0,2 mg/kg/ 8 hours), immediately after surgery. For severe gastritis and/or duodenitis with parietal fibrosis, the same treatment was advised for 3 months and corticosteroids were added (prednisolone, starting at 0.5 mg/kg per os every 12 hours). (Monnet E., 2013)

Dogs with BAS should avoid activity in warm or humid weather, walks should be kept short and taken at the cool time of day, on a harness to take pressure off the upper respiratory system. (Trappler M, 2011)

No medical therapy will treat the BAOS.

*Surgical therapy*

Surgery should proceed as soon as possible. An early relief of the proximally located obstruction should be attempted because it is postulated that early correction could prevent, or even reverse more deeply located tissue collapse (Monnet E, 2013).

*Stenotic nares*

The first procedure for correction of stenotic nares, **amputation of the alae**, was described by Trader in 1949 (Trappler M, Moore K., 2011). It has since been abandoned, because it may be easier than alaplasty technique, but mai result in a lesser opening of the nostril. (Monnet E, 2013)

**Alaplasty** is the most commonly used technique and it consist of the excision of a wedge of the ala nasi. Incisions are made with a no. 11 scalpel blade and sutured with absorbable monofilament material in 2-4 sutures placed in a simple interrupted pattern.

In the vertical wedge technique (Fig. 2), incisions are started at the apex of the wedge. The medial border of the wedge is parallel to the medial wall of the ala nasi, while the lateral border is made at an angle (40-70$^{o}$) from the medial border. The opening of the naris will be proportional to the angle chosen. The incision must be deep enough and it must include a portion of the alar fold.

Horizontal wedge resection involves the creation of a wedge in the medial to lateral direction, ending just dorsal to the mucosal edge of the nares. (Trappler M, 2011)

The lateral wedge resection technique (Fig.2) consists of the excision of a vertical wedge of tissue from the caudolateral aspect of the external nose, at the junction between the nose and the skin. The wedge can include a portion of skin or not The wedge is made deep enough to include a portion of the alar fold. When the wound edges are sutured together, the ala nasi is displaced caudolaterally and fixed in an abducted position, thereby opening the nostril. (Monnet E.,2013)

Punch resection alaplasty has been described by Trostel in 2010, in which a dermatologic punch biopsy is used to portion the ala nasi , down to the level of the alar fold. (Monnet E, 2013)

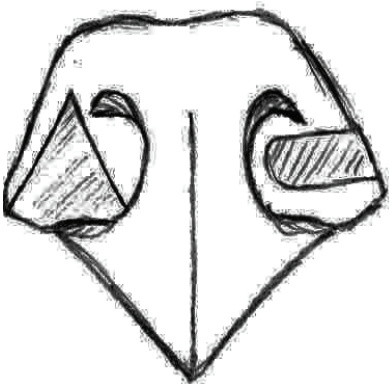

Fig. 2 - Schematic view of the vertical and lateral wedge alaplasty

**Alapexy** involves removal of the caudal edge of the nares and the adjacent skin (Fig. 3). Two elliptical incisions (5-10 mm long x 3 mm wide x 3 mm deep) are made with a no. 15 scalpel blade, one in the ventral lateral alar and one in the skin 3-5 mm lateral to the ala. The edges of the incision lying closest to each other are apposed with three to four sutures of 4/0 absorbable suture material in a simple interrupted or a simple continuous pattern skin (Fig. 4). The outer aspects of the incision are opposed with three to four sutures of 3/0 or 4/0 nonabsorbable suture material (Fig. 5). (Trappler M., 2011). Alapexy is a good alternative in dogs with excessive flaccidity of the alar cartilage.

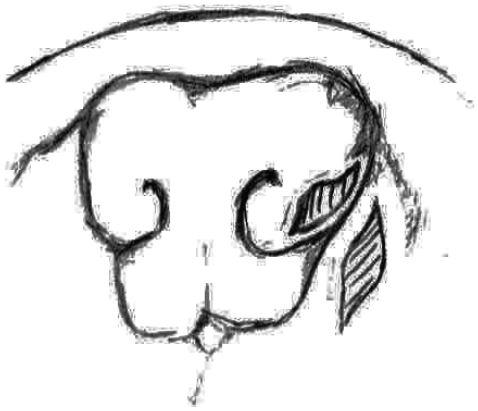

Fig. 3 – The two elliptical incisions made for alapexy

Fig. 4 – The edges of the incisions lying closest to each other in a simple interrupted suture

Fig. 5 – The outer aspects of the incisions

*Intranasal obstruction*

**Turbinectomy** is a laser-assisted technique. The aim of this procedure is removal of malformed obstructive parts of the ventral and medial nasal conchae. (Oechtering G. et al., 2008)

*Elongated or hyperplastic soft palate*

**Staphylectomy** is the main surgical technique used to treat elongated soft palate and it is described as a simple resection of its caudal portion. The soft palate should be evaluated with the head and tongue in a neutral position and without an endotracheal intubation, because this factors influence the location of the soft palate. If the palate extend past the tip of the epiglottis or the mid to caudal aspect of the tonsillar crypt, then we are talking about an elongated soft palate. When performing the staphylectomy, the caudal border of the soft palate is grasped and held with two stay sutures, placed on either side of the intended line if incision (Fig. 6). It is recommended to perform the section and the sutures in stages (Fig. 7), to avoid complete retraction of the nasopharyngeal mucosa and reduce

hemorrhage. The technique can also done using a bipolar sealing device, a diode laser or a carbon dioxide laser trocautery. Whereas use of a diode laser was found to induce more postoperative edema than a carbon dioxide laser or electrocautery (Dunié-Mérigot *et al*. 2010), no significant differences were found in clinical outcomes when staphylectomy was performed with scissors, carbon dioxide laser, or bipolar sealing device (Davidson *et al*. 2001; Brdecka *et al*. 2007). A very important aspect is that excessive shortening might result in regurgitation during swallowing. It has therefore been recommended to leave the soft palate a bit too long rather than too short (Fig. 8) (Bright & Wheaton 1983; Tobias 2010a,b).

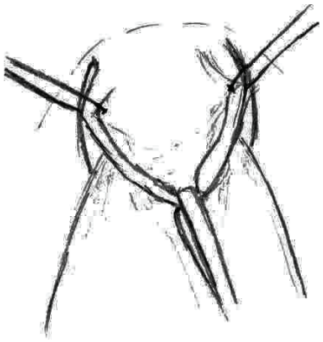

Fig. 6 - The tip of the soft palate is grasped with a forceps and the caudal border is heald with two stay sutures

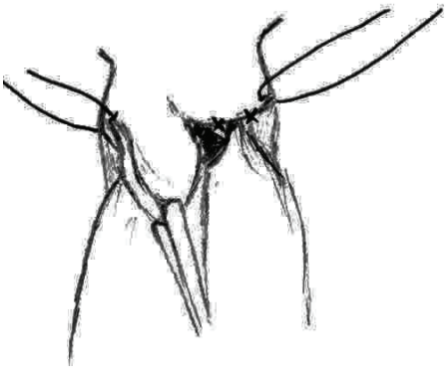

Fig. 7 - The full thickness of the soft palate is incised with a surgical blade approximately half the width of the soft palate.

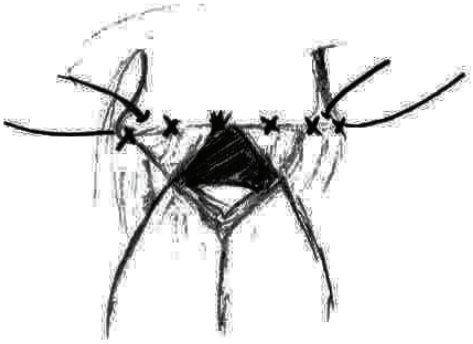

Fig. 8 – The final aspect of the soft palate after staphylectomy

**Folded flap palatoplasty** technique has been developed to correct both the excessive length and excessive thickness of the soft palate (Findji & Dupré 2008, 2009). In this technique, the soft palate is made thinner by excision of a portion of its oropharyngeal mucosa and soft tissues (Fig. 9) and made shorter by being folded on itself (Fig. 10)(Monnet E., 2013). These techniques leave only a few centimeters of soft palate by using the cranial commissure of the tonsillar crypt as a landmark for simple palatal resection (Brdecka *et al*. 2008; Dunié-Mérigot *et al*. 2010). Postoperative adverse effects or pharyngonasal regurgitation have not been observed with this technique (Monnet E., 2013).

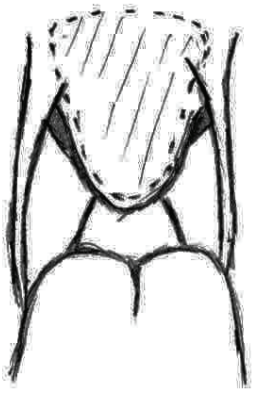

Fig. 9 - Incision lines for the thinning process of soft palate hyperplasia. End of dissection of the soft palate

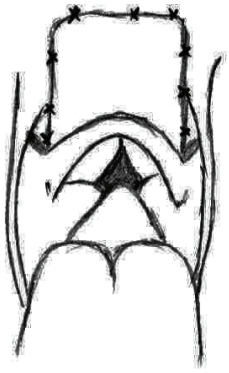

Fig. 10 - Folded flap palatoplasty: after the flap has been sutured

*Laryngeal disease*

**Everted laryngeal saccules** are considered the first stage of laryngeal collapse and, generally, are not the most frequent manifestation on BAOS. They are usually excised with a scalpel blade or a scissor. Hemorrhage is usually minimal and can be controlled with gentle pressure, and resection sites are left to heal by second intention. (Riecks TW. Et al, 2007).

Laryngeal collapse is classified into 3 stages; in advanced stages of BAOS (Stages II and III), laryngeal cartilage loses its integrity and collapses inward as a result of the excessive negative pressure created when affected dogs inspire:

• **Stage I** refers to eversion of the laryngeal saccules.

• **Stage II** indicates that the cuneiform processes have come into apposition.

• **Stage III** designation means the corniculate processes are apposed and changes associated with Stages I and II are present. (Lodato D., Mauterer J.. 2014)

It is important to make a difference between laryngeal collapse and laryngeal paralysis.

**Tonsillectomy** is recommended when they seem to contribute to the pharyngeal obstruction. Because the major palatine artery is present inside the caudal aspect of the tonsillar crypt, 1 ligature of either 4-0 Monocryl or Vicryl can be placed at the caudal edge of the crypt. (Lodato D., Mauterer J.. 2014)

*Laryngeal collapse*

Stage II laryngeal collapse is defined as loss of rigidity and medial displacement of the cuneiform processes of the arytenoids cartilage. Stage III involves collapse of the corniculate processes of the arytenoid cartilage and loss of the dorsal arc of the rima glottidis. Arytenoids lateralization and permanent tracheostomy are the recommended procedures for severe laryngeal collapse (Trappler M, 2011).

## POSTOPERATIVE CARE

The patient will stay intubated as long as he will tolerate the endotracheal tube during recovery. He should be monitored 12-24 hours postoperatively. After 12 hours he can get water in small amount (Lodato D., Mauterer J.. 2014). The diet should consist only soft food for 10-14 days to minimize irritation of the upper airway. (Trappler M., 2011).

*Pain management* include analgesia, with butorphanol (0,22-0,33 mg/kg iv) and maropitant, for nausea and regurgitation. (Lodato D., Mauterer J.. 2014)

## COMPLICATIONS

The most commonly complications are:
Inflammation that can obstruct the larynx and trachea, leading to respiratory distress;
Hemorrhage from the lateral aspect of the soft palate or caudal aspect of the tonsillar crypt;
Coughing and/or gagging;
Nasal discharge;
Voice change;
Regurgitation and/or vomiting;
Noncardiogenic pulmonary edema;
Aspiration pneumonia;
Infection;
Dehiscence (Lodato D., Mauterer J.. 2014)

It is clear that most dogs suffering from brachycephalic syndrome benefit from surgery, according to most recent studies. The proportion of dogs significantly improved by surgery is in excess of 90% (Poncet *et al*. 2006; Riecks *et al*. 2007; Findji & Dupré 2008; Dunié-Mérigot *et al*. 2010; Monnet E., 2014).

## CONCLUSIONS

To some degree BAOS is a common finding in most brachycephalic dogs. Early intervention, even as young as 3-4 months of age, should be considered to decrease progression of the

disease and life-threatening laryngeal collapse. (Meola S., 2013). Prognosis depends on the severity of the condition at the time of surgery. Partial resection of the soft palate, laryngeal saccules and nares is expected to relieve moderate to severe signs of respiratory distress in patients who do not have laryngeal collapse (Bojrab M., 2014). Treatment of concurrent GI disease may improve outcomes following corrective BAOS surgery (Trappler M., Moore K., 2011). Emergency management should focus on oxygenation, ventilation, and temperature management for initial stabilization before surgical intervention (Meola S., 2013)

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# A TRAUMA CASE REPORT AND INCIDENTAL FINDING OF HYPODERMA PARASITISM IN A FREE-RANGING ROE DEER (CAPREOLUS CAPREOLUS)

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***Abstract***

*Deer motor vehicle-crush (dMVC) trauma is a significant cause of death in free-ranging populations including tissue disruptions, organ dysfunctions and cellular damages. The present case describes the gross lesions of a Capreolus capreolus with death in suspicious circumstances. Supplementary information was provided by the police concerning the place (hunting area) and the circumstances of the accident. Radiological examination revealed multiple ante mortem (head, neck and costal) and postmortem (anterior members) fractures. The most relevant lesions observed in gross investigations were the multiple fractures and characteristic motor vehicle accident with skin abrasions and subcutaneous suffusions. Hemorrhagic effusions were remarked in the abdominal and thoracic cavities due to liver rupture (entirety transdiaphragmatic herniation) and lung perforation by rib fracture. Incidental, were found dorsal in thoracic and lumbar regions larvae of Hypoderma diana (n=93) causing myiasis in roe deer. Concerning the results from examinations, the cause of death was the hemorhagic shock due to politraumatism caused by car accident.*

***Key words****: deer, dMVC, Hypoderma larvae, trauma, case report.*

## INTRODUCTION

The roe deer *(Capreolus capreolus)* is reddish brown in colour during summer but in the winter they become grey, pale brown or even black. Males are larger than females and have short antlers, usually with three points. The roe deer is widespread and common, and is expanding in many areas (Lorenzini et. al., 2002). It occupies a wide variety of habitats, including deciduous, mixed or coniferous forests, arable land, and suburban areas with large gardens (Stubbe, 1999).

In the first half of the last century the roe deer from Southern Europe almost endangered because of habitat loss and overexploitation, but its numbers started increasing again 20-40 years ago because of countryside abandonment, improved hunting regimes and reintroductions (Lorenzini et al).

Car accidents involving wildlife animals are a serious and growing problem in Europe (Montgomery, 2012). They pose a risk for human life and may result in mortal victims, damage to vehicles and the loss of wildlife (Lagos, 2012).

The rate of these traffic accidents may be related to a number of factors such as traffic volume, technical aspects of roads, vehicle speed, time of day or the animal's motivation for crossing the road (Kusta 2014) This paper is the first to report data on deer Motor Vehicle Crush (dMVC) in Romania.

## MATERIALS AND METHODS

A free-ranging roe deer (2½-years-old male of *Capreolus capreolus*) weighting around 28 kg, body lenght of 114 cm was submitted for postmortem investigations: radiography, necropsy and parasitological exams. The person who brought the deer described that it was found dead in a forest area during a routine control. Parasitological investigations were focused on identification of the cutaneous parasites (larval stages of bot flies).

The specimens were counted, isolated and preserved in formaldehyde 10% solution.

## RESULTS AND DISCUSSIONS

Postmortem changes (relaxation, corneal opacification and hemoglobin imbibitions) prove a period of approximately 48±8 hours after the installation of death.

The radiological examination revealed fracture at the base of skull, diastase fracture of the occipito-parietal, temporo-parietal and sphenoid-temporal symphisis (Figure 1). Associated to the skull base fracture, the X-ray reveals emphysema in that zone.

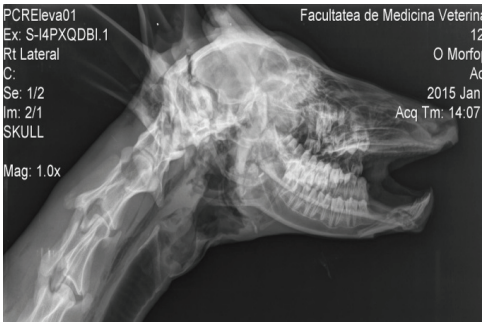


Figure 1 Radiological examination revealed diastase fracture of the occipito-parietal, temporo-parietal.

The part of the right limb shows an open spiroid diaphyseal metacarpus fracture. The left forelimb displays a spiroid diaphyseal metacarpus fracture and multiple diaphyseal fractures of the radius and ulna proximal epiphysis.(Fig. 2)

The radiographs demonstrate the presence of fluids in the abdominal and thoracic cavity and air in the connective tissue (subcutaneous emphysema).

General appearance attests a good maintenance.

The fur was gray and lusterless, presented depilated areas, with blood stains on the neck and interscapular, and a white spot near the tail (perianal-distinctive for males).

The thoracic and lumbar skin (after fur trimming) points out 89 wounds about 2-3 mm in diameter. Some of this wounds were blocked with larvae of *Diptera*: *Oestridae* (myasis-causing flies). A total number of 93 third instars larvae of *Hypoderma diana* were recovered, with size of 10-15 mm lenght and 2-3 mm width (Figures 3,4).

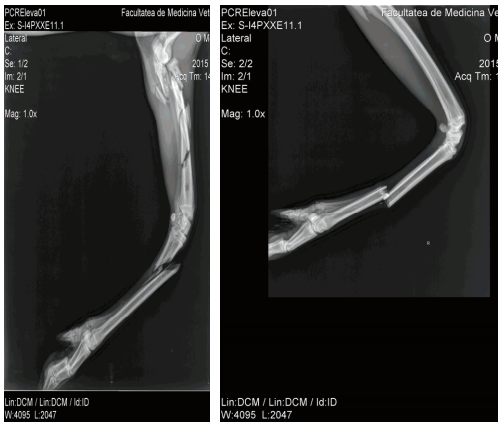


Fig. 2 –Radiological examination The left forelimb displays a spiroid diaphyseal metacarpus fracture and multiple diaphyseal fractures of the radius and ulna proximal epiphysis. The right limb shows an open spiroid diaphyseal metacarpus fracture

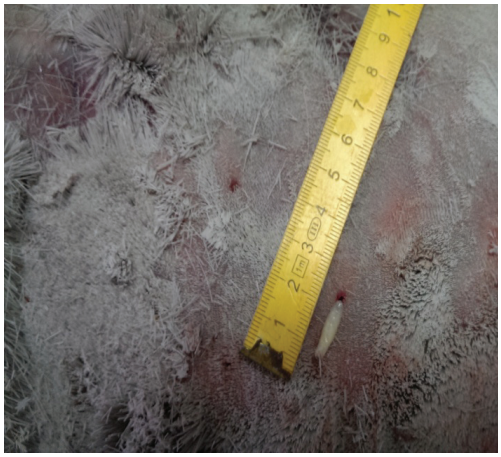

Figure 3 –*Hypoderma diana* - *larvae* in lumbar skin

The skin revealed antemortem laceration areas on the neck (dorsal and ventral), dorsal on the chest at T8-T13. Postmortem lesions were made up by abrasion areas with varied sizes from 2-3 cm to 26 cm arround the entire body surface (chest, abdomen, limbs) (Figure 4).

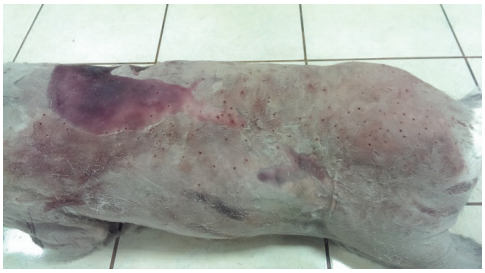

Figure 4 – Macroscopic examination lacerations and abrasions

Connective tissue presented specific morphologicaly aspects of bleeding expressed as hematoma and subcutaneous emphysema prevailing in the right thoracic and cervical area.

Muscle tissue associated to the apendicular skeleton is well developed. The thorax muscle was affected by the fractured 7$^{th}$ rib that penetrated the right hemithorax causing laceration, suffusions and subcutaneous emphysema (Figure 5).

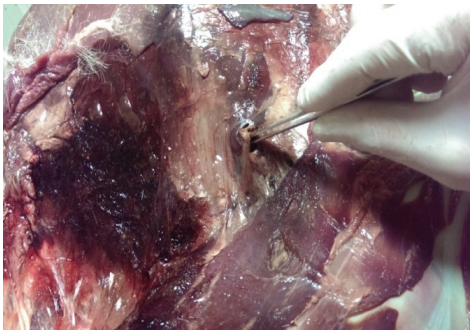

Figure 5 – The right hemithorax, rib fracture which caused laceration, suffusions and subcutaneous emphysema

Bone tissue reveals multiple fractures before death in the cervical area (atlas), costal zone (seriated ribs fracture 7-13 on the right side and 8-13 on the left side). Because the rib fractures were located on to the chondro-costal joint, it means that they were produced by crushing and it was the outcome of a compression on the breastbone or on the distal ends of the ribs (a small range from the chondro-costal joint) (Figure 6). Postmortem fractures were identified at the forelimbs (humerus, radius, ulna and metacarpus) with partially avulsion.

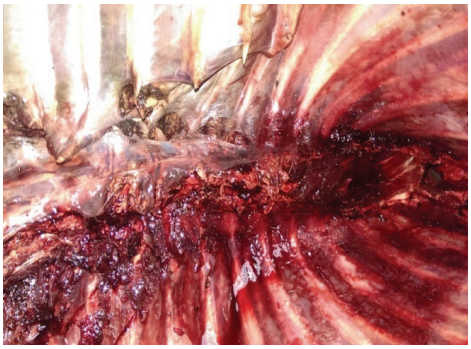

Figure 6 Rib fractures were located on to the chondro-costal joint

The diaphragm presents multiple ruptures, represented by partial circumferential rupture (insertion area on the right hypochondria), radial rupture, that creates a large communication between the thoracic and abdominal cavity, with the possibility that the organs from abdominal cavity to migrate in the thoracic space due to a powerful abdominal compression (Figure 7).

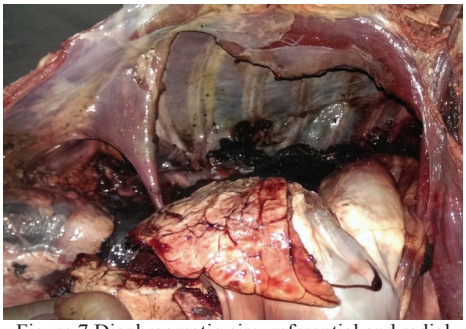

Figure 7 Diaphragmatic circumferential and radial rupture.

Thoracic cavity presented transdiaphragmatic herniation of the liver and a portion of polygastric complex. Thoracic space indicated clotted blood accumulation (pneumo-haemothorax) resulted from the rupture of the liver and lung. Larynx, trachea and the main bronchi presented huge amounts of clotted blood, resulted from the lung rupture.

The lung had multiple ruptures at the right diaphragmatic lobe caused by the broken peaks ribs with secondary pneumohemothorax.

About 75-80% of the lung expresses the lack of air from the lobes, elastic consistency, without crepitation). Mediastinal space contains a moderate quantity of clotted blood (mediastinal hematoma).
Abdominal cavity shows blood accumulation (haemoperitoneum) resulted from the liver rupture. The omentum shows blood infiltrations due to the overflow of blood in abdomen.
Liver presented multiple rupture areas, with total migration from abdominal cavity in thoracic cavity (Figure 8)

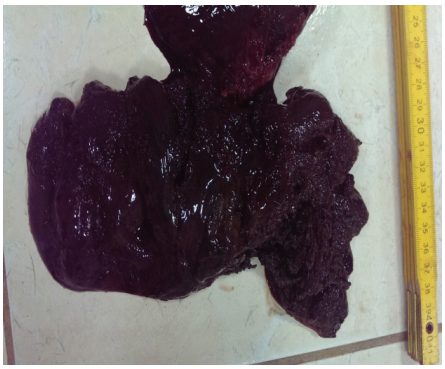

Figure 8 Liver rupture

Rumen, omasum and abomasum occupy the thoracic space. This abdominal position change is the consequence of the diaphragm rupture.
The kidneys presents discontinuous renal capsule. This lesion was produced after the death of the animal because there was not any vital reaction.
The bones that define the base of the skull show multiple fractures. Face bones shows multiple fractures with muscle laceration. The tongue points out ample laceration on the right edge, because of the teeth rupture. Pharynx and the soft palate presented ample laceration of the mucosis and underlying tissues that created a communication with the skull base. The hyoid has bilateral fracture of the epihyoid. Along with the brain injury, necropsy shows numerous issues which advocate for the traumatized individual, represented by multiple fractures. The head injury could be the primary inflict.
At the necropsy were identified several abnormalities that are part of the traumatic pathology, with multiple injuries caused by violence.
The diaphragm and liver rupture, with its total shift in the thoracic cavity and the bleeding after the rupture of organs, resulted because of the high-pressure exerted on the abdomen. It is emphasized that these lesions has the highest degree of specificity and is associated to road accidents in which the vehicle is passing over the victim's abdominal cavity.
Pulmonary rupture was the effect of the fractured ribs which resulted consecutively the thoracic compression.
In the case of kidneys the capsule rupture was made after the death of the animal, the same in the case of the fracture of the forelimbs.
Taking as reference point the trauma moment, we remarked 3 time slots: previously (head and thorax), simultaneously (thorax and abdomen) and subsequently (limbs) trauma (Ciobotaru, 2013).
The presence of parasites in the subcutaneous level hasn't played a major role in inducing the death of the animal; these are causing myasis (parasitic disease caused by the infestation with larvae of bot flies which later erupt on the skin surface). However, it is well known that hypodermosis affects livestock production and wild ruminant welfare, not only by inducing pathology in internal organs and skin but also by impairing the host's immune system.
Larvae of *Hypoderma* affect wild and domesticated ruminants including cattle, buffaloes, sheep, goats, deer and reindeer. In Europe, the most common hypodermoses in wild animals are caused by *Hypoderma diana* and *Hypoderma actaeon* in roe deer and red deer and *Hypoderma tarandi* in reindeer (Otranto et al., 2003).
In Romania, *H. diana* has been reported in roe deer, in Northwestern areas of country (Ilie et al., 2012).

## CONCLUSIONS

The necropsy revealed the presence of cranio-cerebral, thoracic and abdominal polytrauma. Given the heterogeneous nature and the varied placement of the lesions, we can affirm that the examined animal was the victim of a road accident with repeated blows.

Forelimbs and kidney lesions were produced after the death of the animal, while the remaining lesions taken separately are representing possible cause of death:

1- cranio-cerebral trauma caused damage to the central nervous system, followed by blocking the cardio-respiratory activity.

2- hemoragic shock caused by the rupture of the liver and lungs.

3- transdiaphragmatic herniation of the liver and the polygastic complex induced death by asphyxia due to compression of the lung.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# REMEDIATION METHOD OF HIP FRACTURES IN CATS USING CERCLAGE

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***Abstract***

*Hip fractures in cats occupy a significant share in traumatology and bone diverse area . Comparative advantages and disadvantages are described using cerclage fracture remedy to the method of fixation with plate and screws.*
*The results presented are obtained from the method on four cases of fracture fixation with plate and remedied by screws, 11 cases of pelvic fracture fixation remedied by cerclage and 2 cases of bilateral fracture of the iliac blade which have been corrected compared to the two methods .*
*The study shows that the method is more laborious but cerclage better protect musculature area and gives a very good resistance .*

***Key words:*** *remediation method, fractures in cats, cerclage, traumatology, pelvic fracture*

## INTRODUCTION

Traumatologia hip cat is mainly caused by road accidents or falls from heights varying empty. Fixes fractures is necessary to restore pelvic diameters to avoid unwanted consequences translated to embarrassment or the imposibility of defecation , the occurrence of dystocia due to pelvic and remedying angustiei locomotor defects .
In practice that respects the majority of indications in the literature, clinicians are using screws and plate fixation method . The authors imagined and applied cerclajelor use in remediation of hip fractures in cats with significant results.
The method is proposed as an alternative to the classical method that is intended to be introduced in practice due to its advantages .

## MATERIALS AND METHODS

In this paper were made observations on the method of fixation of pelvic bones by cerclage compared with plate fixation method and screws.

Were selected 13 cases of pelvic fracture cats presented at Pathology Clinic of the Faculty of Veterinary Medicine Bucharest. The selected animals had different ages.
Of the 13 cases , 11 were resolved through the application of minimum 2 cerclage in the outbreak of fracture and 2 cases with bilateral iliac blade fracture were operated on one side with cerclage fixation method and on the opposite side with plates and screws method .

## CLINICAL ASPECTS

Anesthesia for all cases was performed by the method of neuroleptanalgeziei anesthesia , the dose used was Acepromazine reduced to 50% of the usual dose, diluted and administered i.m. sometimes 10-20 minutes before administration of Ketamine dose 15-20 mg per kg i.m. , and the two substances , both Acepromazine 50% of the usual dose and dose Ketamine 15-20 mg per kg were administered concomitantly i.m. and mixed profoundly.
Clinical examination was performed for each case thoroughly and were found lameness, pain, crepitation bone and asymmetric

abnormal position of the pelvis, sometimes refusing to support the adoption of the hind leg position unaffected. Clinical examination was completed by radiological examination and revealed the pelvic bones fracture displacement. Animals were subjected to surgery through a surgery to interest all anatomical layers iliac blade length in the middle. It was removed the muscle insertion and fracture were performed trepanations with a drill 1-1,5mm in diameter on both sides of the fracture line at equal distance from the end of the fracture line and at a distance of at least 3 mm fracture line to have enough of bone for bone strength.

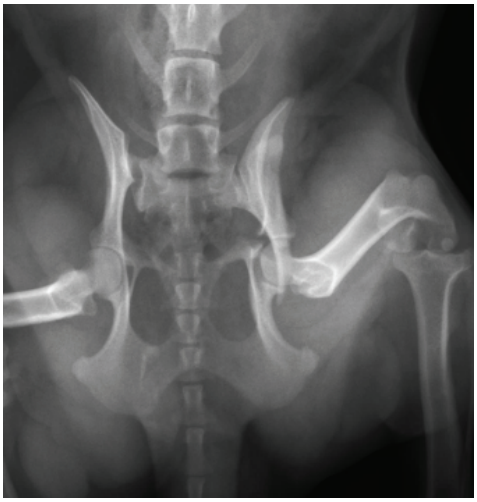

Figure 1- Pelvic fracture

Next were imagined guidance systems composed of curved tracks with inner lumen that could lead the cerclage wire through the holes made. Cerclage wire is inserted through all the holes (at least 2).
After passing through each pair of holes cerclage recourse to remedy the fracture , the fracture line recovery and collection cerclage successively. We proceeded to restore anatomical layers with absorbable suture material (PDS 3-0 or 2-0) depending on the size of the animal in separate points . The skin was sutured Evers also absorbable thread.In 2 cases were applied alternately both cerclage and screw plate .

**RESULTS AND DISCUSSION**

In all cases selected for this paper cerclages applied proved strong enough so that the fracture healed without vicious callus.
The application of cerclages was difficult especially when the fracture line was located close to the sacroiliac joint . For these cases , the burr portion hinged to the sacrum bone was performed cranial-caudal angle and medio-lateral.

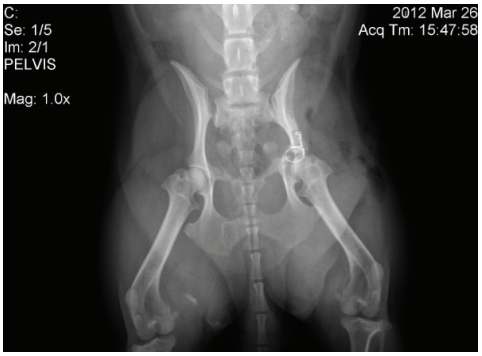


Figure 2- cerclage remediation

Crossing the cerclage wire was difficult and could not be applied without guiding system .
Cerclages must be successively tightened after placing the wire all the existing holes.
Trepanations in iliac blade protection must be done carefully so as not significant anatomical lesion formations surrounding area the fracture (obturatory muscle, femural muscle, subsacral mediate artery, obturatory artery). Protection of these formations is accomplished by placing the focus of fracture of a metal spatula and small dimensions at slightly curved front is oriented immediately below the internal iliac blade to be trepanned.
It is envisaged that the burr include both compact bone of the iliac blade. After executing the ceclage, the excess wire is removed and the remaining ends is shaped so as not to cause damage to the soft tissue .
Postoperatory, the animals were checked by X-rays in order to assess and remedy the fracture, and received antibiotics for 5 days and repose for 28 days .
Of the 13 cases, 4 were found between 5 and 9 days subcutaneous seroma due to scratching, which have been drained and

subsequently cured. In neither case were not found recurrences in the fracture line.
In 3 cases intraoperative, one of cerclage gave up due to torsion beyond resistance of wire and other cerclage had to be removed and needed to be replaced at the break.
In 2 of the cases we used double cerclage which was passed through a single burr through the proximal portion of the iliac blade and the two bone sections remote distal iliac portion of the blade, resulting in a cerclage form of the letter "V".
And this process has proven strong enough in strengthening the fracture.
Besides the method with plates and screws, the method of fixation with cerclage was more laborious but still implement both resistance and useful.

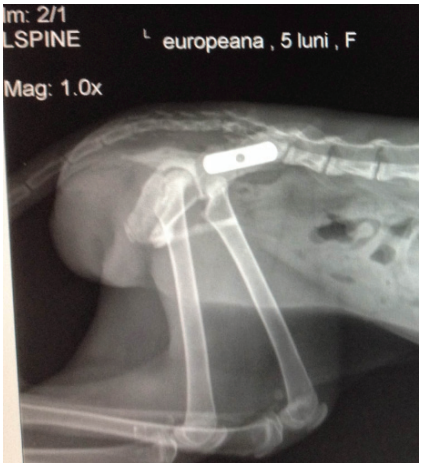

Figure 3- pelvic x-ray view, evident cerclage remediation

Besides the method with plates and screws that removes much of the soft tissues (muscles) insertion surface ,the cerclage method is less traumatic for soft tissues.
Another significant advantage of the method of fixation with cerclage for pelvic bones is that the animal does not have to be reoperated for extraction of the metal .
Fixation with cerclage has recovered the fractured pelvic bones and the animal at a rate of 85-100%.
Postoperatory, in neither case were not found symptoms of discomfort in the bowel from the pelvis.
There were no reported cases of relapse or partial separation of cerclajelor.
Restoring muscle was much faster in animals that received cerclage fixation methods to cases brought by plate fixation screws.Animals that received only osteosynthesis with cerclage were removed from the observation at 30 days after surgery by performing a radiological control.
Animals that were operated with different methods have been subject to new interventions to 60 days in order to extract metal plates. So, after the second surgery was a significant muscle atrophy .

## CONCLUSIONS

Remediation of hip fractures with cerclage fixation method is more laborious than the method plate fixation screws .
The major advantage of the method of fixation with cerclage is the fact that the animal is subjected to a second surgery .

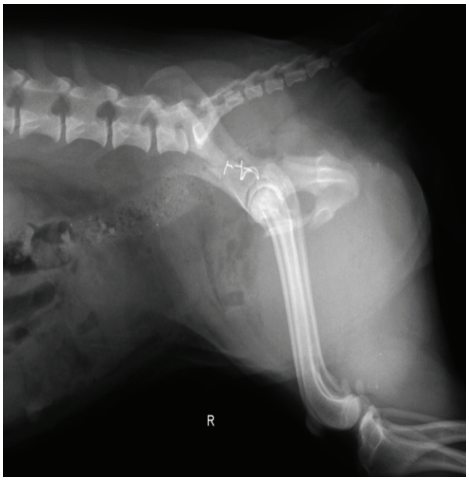

Figure 4- lateral x-ray incidence(iliac plate remediation)

Although prolongation of anesthesia in fixation with cerclage raise the cost of the intervention , in the end , no reintervention operators diminishes overall material costs .
Muscle atrophy after fixation with cerclage is much lower on glutean muscles compared to atrophy resulting from the two surgery procedures in osteosynthesis with plate and screws method.

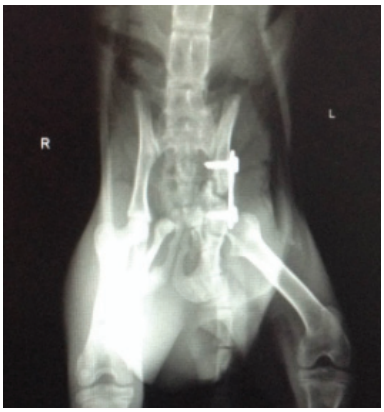


Figure 5- post operatory x-ray - plates and screws

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS DURING THE EARLY STAGES OF N-NITROSODIETHYLAMINE-INDUCED HEPATOCARCINOGENESIS IN TURKEYS

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***Abstract***

*Some haematological and biochemical parameters in turkeys, hatched from embryonated eggs inoculated with the proven hepatocarcinogen N-nitrosodiethylamine were studied. Histopathology confirmed the presence of clear-cell and basophilic foci of altered hepatocytes and hyperplasia of cholangiocytes. The application of the chemical carcinogen affected both haematological and biochemical parameters. The established conditions such as thrombocytopenia and increased levels of the major liver enzymes were associated with the process of malignancy. In addition, leukogram abnormalities (leukocytosis, lymphocytosis and neutropenia) as well as hypoproteinaemia, hypoalbuminaemia and hypoglycemia were also observed.*

***Keywords****: in ovo tests, turkeys, hepatocarcinogenesis, N-nitrosodimethilamine, haematological and biochemical parameters.*

## INTRODUCTION

Preneoplastic liver lesions have been widely used as a reliable indicator of the early stages of hepatocarcinogenesis. Sasaki and Yoshida (1935) first showed that the appearance of foci of altered hepatocytes (FAHs) preceded the onset of chemically-induced liver tumors. These preneoplastic alterations have the ability to progress to benign or malignant neoplasms and have been accepted as an early and reliable indicator for the development of the neoplastic process (Howe & Knox 2002). Data from rodent experiments suggest that the hepatocarcinogenesis is a multistage process, beginning with the appearance of clear cell and acidophilic foci, storing glycogen in excess, followed by their progression to mixed cell foci, composed of acidophilic and basophilic hepatocytes, and then to basophilic, glycogen poor foci. The later are considered as the most advanced preneopastic lesion, directly preceding the appearance of the hepatocellular carcinomas (Bannasch et al., 1989). Preneoplastic foci of altered hepatocytes have been regularly detected in the livers of experimental rodents, as well as in the livers of people with an increased risk for the development of liver tumors (Fischer et al., 1986). It should be noted that the appearance of FAHs precede the development of liver tumors, irrespective of the mechanism by which the carcinogenic process is induced. It is generally accepted that focal liver lesions are a mandatory step in hepatocarcinogenesis and, thus, can be used as reliable endpoints in the carcinogenicity bioassays (Ito et al., 1989). There exist a variety of experimental models in laboratory rodents for the assessment of the carcinogenic, mutagenic and toxic effects of different substances potentially dangerous for both humans and animals. A large part of the experiments have been focused on the mechanisms of liver carcinogenesis (Weisburger, 1999; Iatropoulos et al., 2001; Pitot et al., 2007). The duration of the *in vivo* carcinogenicity tests in rodents is usually 18-24 months (long-term tests). The neoplastic

alterations induced by the test chemical in the laboratory animals are the endpoints measured by this experimental approach (Knight et al., 2006; Williams et al., 2008). In order to shorten the duration of the carcinogenicity assays and to reduce the pain and suffering of the laboratory animals a large number of medium-term tests, with an average duration ranging from few weeks to few months, have been developed. These experiments are terminated before the appearance of solid tumors and metastases, and the induced preneoplastic lesions are used as endpoints (Hasegawa and Ito, 1992; Tsuda et al., 2010). In addition, numerous short-term *in vitro* mutagenicity and genotoxicity tests have been implemented in an attempt to reduce and/or replace the animals needed for carcinogenicity assessment (Benigni, 2013; Anadón, 2014). The ethical aspects of biomedical research and the issues related to the welfare of experimental animals has been gaining an increasing importance since the adoption in 2010 and the implementation in 2013 of the new Directive 2010/63/EC of the European Parliament and the EU Council on the protection of animals used for scientific purposes.

During the last decades, avian embryos have attracted the scientific interest as new and reliable alternative model systems (*in ovo* models) for studies on carcinogenesis. It has been shown that *in ovo* experiments can provide valuable information about the carcinogenic potential of chemical compouds and may fill the gap between the *in vivo* and *in vitro* experiments, combining some advantages of both approaches (Enzmann and Brunnemann, 1997).

The importance of avian embryos as model system for studies on different pathological processes, including biological and chemical carcinogenesis has been growing. *In ovo* carcinogenicity tests have been described in detail by Enzmann et al., (1992; 1995a; 1995b); Enzmann and Brunnemann, 1997 and Enzmann et al., 2012. It has been found that the *in ovo* exposure to chemical carcinogens resulted in the appearance of eosinophilic and basophilic foci of altered hepatocytes in the embryonal avian liver. These lesions are morphologically identical to the FAHs observed in the liver of adult rats, after treatment with hepatocarcinogens. The *in ovo* experiments are more rapid, less expensive and safer for the personnel than *in vivo* experiments in rodents. In the *in ovo* carcinogenicity studies, turkey and quail embryos were most frequently used in the experiments (Enzmann et al., 1992; 1996).

The aim of the present study is to investigate the preneoplastic liver lesions and some haematological and biochemical parameters of turkeys during the early stages of hepatocarcinogenesis, induced after *in ovo* exposure to N-nitrosodimethilamine.

## MATERIAL AND METHODS

### Eggs

Fertilized turkey (*Meleagris gallopavo*) eggs were obtained from diseases-free flocks, bred in Stara Zagora, Bulgaria.

### Carcinogen, treatment and incubation of experimental eggs

N-nitrosodimethilamine (NDMA; CAS № 62-75-9; Sigma-Aldrich) was provided by the Institute of Experimental Morphology, Pathology and Anthropology, BAS - Sofia. The carcinogen was diluted with sterile glass double distilled water and administered as single dose of 0.3 mg/per egg, with an injection volume of 0.1ml. Control eggs were injected with an equal volume of the vehicle. The eggs were inoculated during the first hours of incubation. To avoid cooling, only a few eggs were taken out of the incubator for the application of the test substance. After sterilization of the injection site with 70% ethanol, the shell was pierced at the pointed end of the egg, using a needle, making a hole with a size of 1-2mm. Test substance was inoculated into the egg white, with a syringe and then the opening was sealed with paraffin. The eggs were incubated at 37.8±0.5°C and 70±10% relative humidity in an automatic rotating incubator. At the end of the incubation, the eggs were transferred to hatcher at 37°C ± 0.2°C and 80-85% humidity.

### Experimental birds

Eight turkeys hatched from the treated and control eggs were used in the experiments. The treatment and control group consisted of four birds each. Standard fodder mixtures for

turkeys were used for feeding. Food and water were given *ad libitum*.

**Histopathology**

All experimental birds were exsanguinated 18 weeks post hatching. Tissue samples were taken from the control and treated birds and immediately fixed in 10% buffered formalin for subsequent histopathological examination. Fixed tissues were routinely dehydrated, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Histopathological lesions were observed and documented with microscope Leica DM 5000 B, equipped with a digital camera and the original software.

**Hematology**

Venous blood was taken from the wing vein of the treated and control birds at the $14^{th}$ and $17^{th}$ week post hatching. Haematological parameters (WBC, $10^9$/L; LYM, $10^9$/L; MID, $10^9$/L; GRA, $10^9$/L; HGB, g/L; RBC, $10^{12}$/L ; HCT,%; PLT, $10^9$/L) were measured in whole blood by Veterinary automatic hematology analyzer Hema Screen 18 LIHD 170, (Hospitex diagnostics – Italy).

**Biochemistry**

Biochemical parameters (total protein, g/L; albumin, g/L; ALAT, U/L; ASAT, U/L; GGT, U/L, glucose, mmol/L) were measured in the blood serum at the $3^{rd}$, $12^{th}$, $14^{th}$ and $17^{th}$ week post hatching by a semi-automatic biochemical analyzer Screen Master LIHD 113, (Hospitex diagnostics – Italy) and reagent kits for biochemical analyses (Human – Germany).

## RESULTS AND DISCUSSION

Histopathological examination revealed the presence of preneoplastic lesions in the liver of the turkeys treated *in ovo* with N-nitrosodimethilamine, during the early stages of the embryonal development. The observed lesions were classified as clear-cell and basophilic foci of altered hepatocytes (Fig. 1). In addition, a clearly expressed hyperplasia of cholangiocytes was regularly found (Fig. 2). Similar histopathological alterations were observed in turkey and quail embryos, after treatment with hepatocarcinogens (Enzmann et al., 1992; 1995a; 1996).

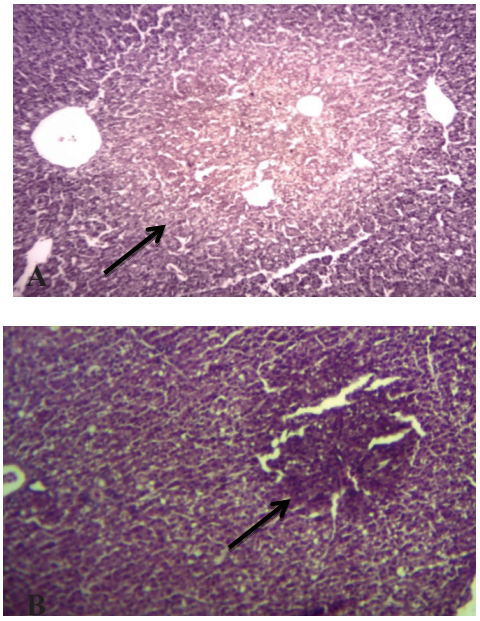



Figure 1. Foci of altered hepatocytes in the liver of turkey, after *in ovo* exposure to a single dose of 0.3 mg N-nitrosodimethilamine. **A** - Clear-cell focus of altered hepatocytes; H&E staining; Objective 20X. **B-** Basophilic focus of altered hepatocytes H&E staining; Objective 20X.

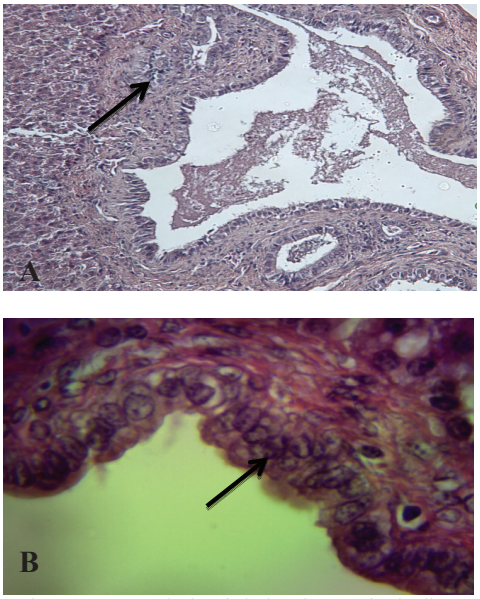
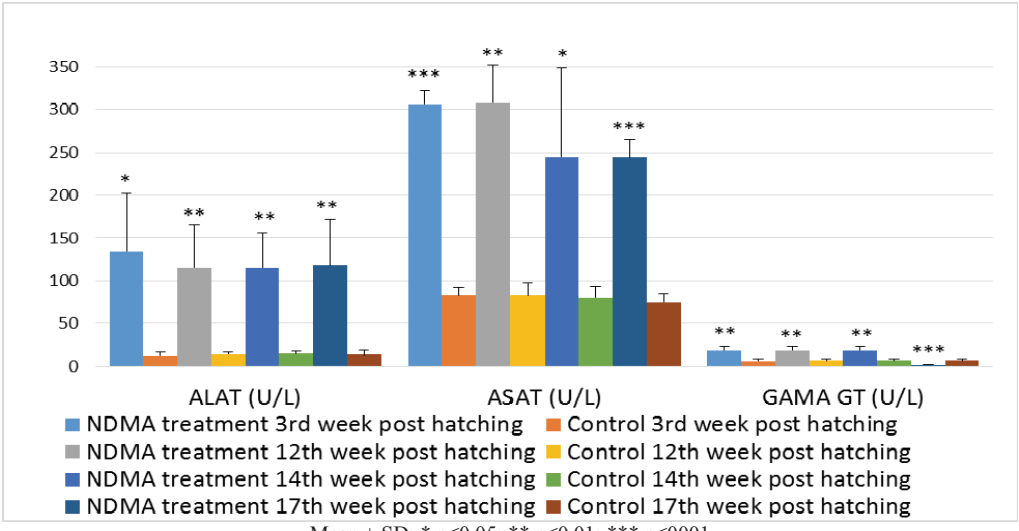


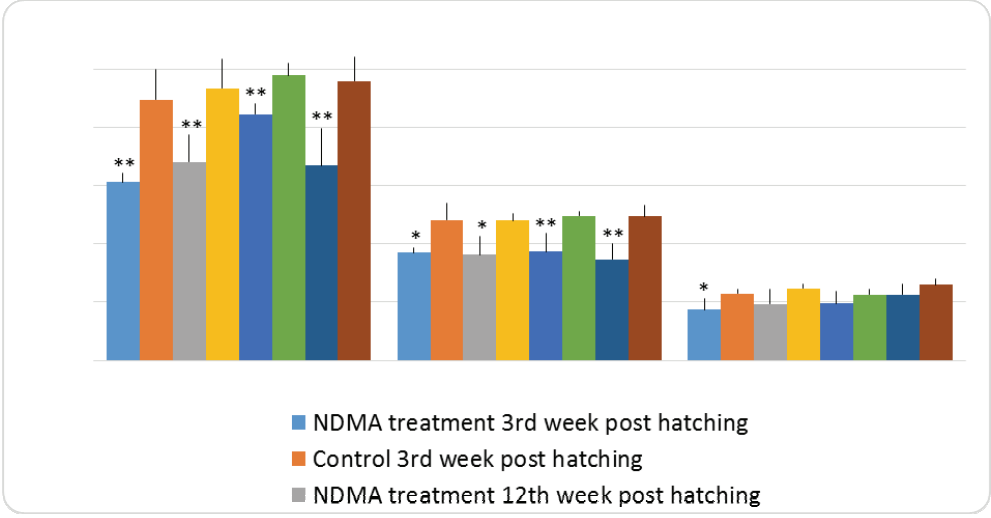

Figure 2. Hyperplasia of cholangiocytes in the liver of turkey, after *in ovo* exposure to a single dose of 0.3 mg N-nitrosodimethilamine. **A** – Hyperplasia of cholangiocytes; H&E staining; Objective 20X. **B** – Hyperplasia of cholangiocytes; H&E staining; Objective 100X.

The results of biochemical studies showed a statistically significant ($p \leq 0.01$; Fig. 3) increase in the levels of the major hepatic enzymes alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT), as well as significantly ($p \leq 0.01$) increased activity of gamma-glutamyl transferase (GGT) (Figure 3). The established hypoglycaemia in experimental birds was statistically significant ($p \leq 0.05$) as compared to the controls only at third week post hatching. Hypoproteinaemia and hypoalbuminaemia with statistical significance ($p \leq 0.01$; Fig. 4) were also registered. Data obtained from the biochemical analysis complement the observed morphological changes in the livers of experimental birds, showing a significant deterioration in the function of hepatocytes and confirms registered hyperplasia of cholangiocytes.




Mean ± SD; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0001$

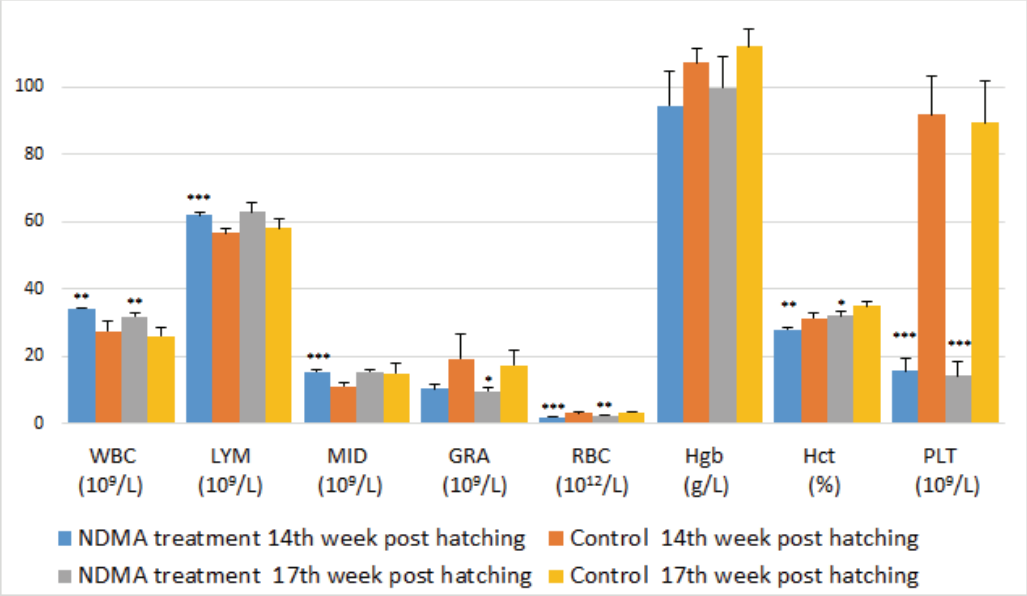
Figure 3. Some biochemical parameters in turkeys, treated *in ovo* with 0.3 mg N-nitrosodimethilamine.




Mean ± SD; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0001$

Figure 4. Some biochemical parameters in turkeys, treated *in ovo* with 0.3 mg N-nitrosodimethilamine.

Haematological investigations revealed a moderate leukocytosis with lymphocytosis, accompanied by neutropenia and prominent thrombocytopenia ($p \leq 0.001$; Fig. 5). In addition, the number of the red blood cells of the birds from the treatment group was significantly lower than those measured in the control group ($p \leq 0.001$; Fig. 5).




Mean ± SD; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0001$

Figure 5. Some haematological parameters in turkeys, treated *in ovo* with 0.3 mg N- nitrosodimethilamine.

## CONCLUSION

*In ovo* application of N-nitrosodimethilamine induced hyperplastic and preneoplastic lesions in the liver of turkeys hatched after treatment of embryos at the early stage of their development.

The results of haematological and biochemical studies are an essential complement to the observed morphological changes in the liver.

Established hypoalbuminaemia, relative anemia and hypoglycemia are not only indicators for the general changes in liver function, but they are also an essential part of paraneoplastic syndrome that accompanies the process of hepatocarcinogenesis.

## ACKNOWLEDGEMENTS

This document was supported by the grant No BG051PO001-3.3.06-0056, financed by the Human Resources Development Operational Programme (2007 – 2013) and co-financed jointly by the European Social Fund of the European Union and the Bulgarian Ministry of Education and Science.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# X-RAY EVALUATION OF PROXIMODISTAL ALIGNMENT OF CANINE PATELLA

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***Abstract***
*This study used radiographic techniques to evaluate the Insall Salvati index (ratio of the length of the patellar ligament to the length of the patella - L:P) to compare the verticality patellar position at small-medium and large breed dogs that were grouped in healthy dogs, dogs with patellar luxation and with cranial cruciate ligament ruptures. Except for the based on L:P ratio measurement at small-medium breed dogs and in large-breed there was no difference among the groups. In large large-breed dogs the LPL is associated with patella baja. No statistically significant differences of L:P ratio between dogs affected or unaffected with cranial cruciate ligament rupture are reported*
*Further studies using other imaging techniques are required in small and large-breed dogs with patella luxation (LPL and MPL).*

***Key words****: dog, Insall Salvati index, stifles*

## INTRODUCTION

Blumensaat (1938) first described in radiographic technique for measuring human patella. Over time in human medicine have been reported nine radiographic measurement techniques (Phillips et al., 2010).
In veterinary medicine, to date, there are relatively few studies on evaluation methods proximal-distal alignment of the patella in dogs. Johnson et al. (2002) presents the first data on radiographic examination of verticality patellar position on the corpses of large dogs, taking in human medicine technique Insall Salvati (1971).
Johnson et al. (2006) presents the first comparative measurements of the index Insall Salvati to healthy dogs and dogs with large medial patella luxation. Mostafa et al. (2008) shows the medium and large dogs (healthy, with medial dislocation and lateral patellar dislocation respectively) two types of assessments by radiographic techniques: the ratio of the tibio-patellar ligament length / length of the patella (index Insall Salvati) and axis length ratio of the length of the femur and patella transcondiliar (technical taken from human medicine (Carson et al., 1984) and modified by the author). Another report is of Knazovicky et al. (2012) with data obtained in normal dogs and dogs with cranial cruciate ligament rupture after evaluation index Insall Salvati. Proximal-distal alignments abnormal patellar two pathological situations include: - *patella alta* or patella high, the patella is located in the proximal portion of the femur trochlear groove and - *patella baja* (patella infera) or low patella, the patella is located in the portion distal of the femur trochlear groove.
A recent study (Mostafa et al., 2008) believes that high patella dislocation is responsible for the appearance and medial patella dislocation and patella infera for lateral nontraumatic dislocation.

## MATERIALS AND METHODS

Medical records (2012-2014) were reviewed to identify small-medium or large breed dogs that were examined by radiographs (lateral stifle radiographs). Medical and radiographic records (based on physical examination and preoperative and diagnosis registered) allowed the establishment of followings groups: group 1 - healthy small-medium dogs (< 15 kg body weight); group 2 - healthy large dogs (> 15 kg body weight); group 3 - small-medium dogs (< 15 kg body weight) with medial patellar luxation (MPL); group 4 - large dogs (> 15 kg

body weight) with lateral patellar luxation (LPL); group 5 - small-medium dogs (< 15 kg body weight) with uni- or bilateral cranial cruciate ligament ruptures; group 6 - large dogs (> 15 kg body weight) with uni- or bilateral cranial cruciate ligament ruptures.

Inclusion criteria were: skeletally mature (>1 year of age), no other orthopedic abnormalities in that stifle and presence of a good quality straight lateral radiograph of that stifle.

Single lateral stifle radiographs from each dog (1-6 groups) were examined by method described of Johnson et al. (2002) that define the vertical position of the patella as the ratio of the length of the patellar ligament to the length of the patella (L:P) – figure 1. All measurements (three for each radiograph) were performed using a digital caliper (L-4C300 Kennon-Italia).

All statistical analyses were performed using Microsoft Windows version 6.3 a computer software statistic program. The resulting data were expressed as mean ± standard deviation (±SD). The differences between groups were analyzed with a non-parametric test - Wilcoxon Signed Ranks test (IBM SPSS Statistic 2.0). For values of $p \leq 0.05$ considering significant differences and for $p < 0.001$ highly significant. The 95% confidence limits of the group were also estimated.

## RESULTS AND DISCUSSIONS

The results are presented in table 1. Sixty-six dogs were identified that fit the inclusion criteria for the group 1. Mean (± SD) body weight was 9.0 ± 3.1 kg and mean age was 3.1 ± 2.4 years. Ninety seven dogs were identified that fit the inclusion criteria for the group 2. Mean (± SD) body weight was 29.0 ± 9.0 kg and mean age was 3.3 ± 3.5 years

Eighteen dogs were identified that fit the inclusion criteria for the group 3. Mean (± SD) body weight was 8.0 ± 3.3 kg and mean age was 3.9 ± 2.5 years. Seventeen dogs were identified that fit the inclusion criteria for the group 4. Mean (± SD) body weight was 26.0 ± 3.0 kg and mean age was 4.3 ± 1.5 years.

Seventeen dogs were identified that fit the inclusion criteria for the group 5. Mean (± SD) body weight was 8.0 ± 3.2 kg and mean age was 5.9 ± 3.5 years. Twenty- eight dogs were identified that fit the inclusion criteria for the group 6. Mean (± SD) body weight was 31.0 ± 9.5 kg and mean age was 6.8 ± 3.0 years.

Means (± SD) of L:P were: group 1 = 1.71 ± 0.26, group 2 = 1.80 ± 0.2. Our results indicate that the normal vertical patellar position, based on L:P measurement, in small-medium breed dogs is between 1.64 and 1.77 (95% CI) and in large-breed dogs is between 1.76 and 1.84 (95% CI). This data, for large-breed dogs, are comparable with another reports (Johnson et al. 2006, Mostafa et al., 2008).

Difference between groups 1 and 2 found a statistically significant (p=0.039).

Means (± SD) of L: P were: group 3 = 1.79 ± 0.18, group 4 = 1.97 ± 0.24. Difference between groups 3 and 4 found a statistically no significant (p=0.109).

Means (± SD) of L: P were: group 5 = 1.62 ± 0.17, group 6 = 1.79 ± 0.23. Difference between groups 5 and 6 found a statistically significant (p=0.011).

Statistical analysis of data from healthy animals with groups of dogs with patellar luxation (medial or lateral), respectively group 1 to group 3 and group 2 to group 4, did not find statistically significant differences. Either way based on L:P measurement recorded in large large-breed dogs with LPL (1.97) are similar with values reported by Mostafa et al. (2008). (L:P=1.90).

In large large-breed dogs LPL is associated with patella baja. . Our results indicate that the MPL, based on L:P measurement, in small-medium breed dogs is between 1.37 and 2.57 (95% CI), mean 1.79. By comparing these values with data reported Mortari et al. (2009), respectively a ratio L:P between 1.84-1,9 at medium-small dogs with MPL and we can't confirm findings reports by Mostafa et al. (2008) in large breed dogs such as MPL is associated with patella alta.

Also statistical analysis of data from healthy animals with groups of dogs with cranial cruciate ligament ruptures, respectively group 1 with group 5 and group 2 to group 6 did not find statistically significant differences. Similar data of L:P ratio between dogs affected or unaffected with cranial cruciate ligament rupture was reported (Kňazovický et al., 2012).

Table 1. Mean values for the L:P ratio obtained by radiographic measurements at dogs with clinically normal stifle joints (groups 1 and 2) , dogs with MPL (group 3) and LPL (group 4) and dogs with cranial cruciate ligament ruptures (group 5 and 6)

| Group | n dogs | Weight (mean) – kg (± SD) | Age (mean)– years (± SD) | Patella length (mean) –mm (± SD) | Ligament length (mean) – mm (± SD) | L/P (mean) (± SD) |
|---|---|---|---|---|---|---|
| 1 | 66 | 9 ( ± 3.1) | 39 ( ± 2.4) | 14.07 ( ± 3.30) | 26.28 ( ± 7.54) | 1.71 ( ± 0.26) |
| 2 | 97 | 29 ( ± 9.0) | 3.3 (± 3.5) | 22.51 ( ± 3.42) | 40.62 ( ± 7.93) | 1.80 ( ± 0.20) |
| 3 | 18 | 8 ( ± 3.3) | 3.9 ( ± 2.5 ) | 11.77 ( ± 1.46) | 20.94 ( ± 2.99) | 1.79 ( ± 0.18) |
| 4 | 17 | 26 ( ± 3.0) | 4.3 (± 1.5 ) | 18.34 ( ± 4.95) | 35.43 ( ± 5.11) | 1.97 ( ± 0.24) |
| 5 | 17 | 8 ( ± 3.2) | 5.9 ( ± 3.5 ) | 13.41 ( ± 2.38) | 21.61 ( ± 4.31) | 1.62 ( ± 0.17) |
| 6 | 28 | 31 ( ± 9.5) | 6.8 ( ± 3.0 ) | 21.75 ( ± 3.34) | 38.73 ( ± 7.07) | 1.79 ( ± 0.23) |

## CONCLUSIONS

Further studies using other imaging techniques are required in small and large-breed dogs with patella luxation (LPL and MPL) due to the difficulty in obtaining a clear , well defined and precise parameter for evaluation of the proximo-distal alignment of the patella with respect to the femoral trochlea, distal aspect of the femur, and proximal aspect of the tibia.

## ACKNOWLEDGEMENTS

This research work was carried out with the support of the project *Dezvoltarea infrastructurii de cercetare, educaţie şi servicii în domeniile medicinei veterinare şi tehnologiilor inovative pentru RO 05, cod SMIS-CSNR 2669.*

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# CLINICAL FOLLOW-UP OF DOGS WITH NEUROLOGICAL DISORDERS AND POSITIVE FOR ANTIBODIES AGAINST *TOXOPLASMA GONDII*

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***Abstract***

*Toxoplasmosis is an important parasitic zoonosis caused by the protozoan Toxoplasma gondii, which is widespread in humans and animals worldwide, including dogs. Infection with T. gondii in dogs is usually asymptomatic but cases of severe clinical toxoplasmosis have been reported worldwide. Toxoplasmosis is recognized as an opportunistic disease in dogs and is characterized by neuromuscular, respiratory, and gastrointestinal signs or by generalized infection. The most common neurological signs are characterized by ataxia, circling, behavioural changes, seizures, paralysis, paraplegia, twitching and tremors. In this study we present a clinical follow-up of twenty-seven dogs admitted to the Veterinary Clinic of Faculty of Veterinary Medicine of Bucharest in 2014, presenting with neurological disorders,* mainly epilepsy-like seizures. *All of them were serological positive for antibodies against T. gondii, as follows: 63.00% (17/27) were positive for both IgG and IgM, 25.9% (7/27) were positive only for IgG, while 11.1% (3/27) were positive only for IgM. The clinical presentation and the outcome of different therapeutic regimes are discussed. The study demonstrates the importance of toxoplasmosis in dogs and represents a basis for further in-depth studies to investigate the risks for clinical canine toxoplasmosis and to confirm, including by PCR, the implication of T. gondii in the neurological pathology in dogs in Romania. In addition to its veterinary importance, toxoplasmosis is also of major zoonotic concern and dogs can serve as epidemiological indicators for local infections.*

***Key words:*** *neurological disorders, clinical investigation, Ig G, Ig M antibodies,Toxoplasma gondii, dogs*

## INTRODUCTION

Toxoplasmosis is an important parasitic zoonosis caused by the protozoan *Toxoplasma gondii*, which is widespread in humans and animals worldwide, including dogs (Doubey, 2010; Dubey and Beattie, 1988). Toxoplasmosis is recognized as an opportunistic disease in dogs, which is characterized by neuromuscular, respiratory and gastrointestinal signs, or by generalized infection (Greene, 2006).

Infection with *T. gondii* in dogs is usually asymptomatic but cases of severe clinical toxoplasmosis in dogs have been reported worldwide (Dubey et al., 2003).

The most common neurological signs are characterized by ataxia, circling, behavioral changes, seizures, paralysis, paraplegia, twitching and tremors (Greene, 1998; Da Silva et al., 2005).

Although, there are numerous serological reports of *T. gondii* infection in dogs, clinical canine toxoplasmosis is considered a rare disease (Dubey et al., 2003). Furthermore, *T. gondii* infections are epidemiologically important because the dog may be considered as a sentinel in the epidemiology of toxoplasmosis (Doubey, 2010).

In this study we present a clinical follow-up of dogs admitted to the Veterinary Clinic of Faculty of Veterinary Medicine of Bucharest in 2014, presenting with neurological disorders. The clinical presentation, serological investigations and the outcome of different therapeutic regimes have been followed.

## MATERIALS AND METHODS

Twenty-seven dogs admitted in 2014 to the Veterinary Clinic of Faculty of Veterinary Medicine of Bucharest (South-eastern Romania), presenting with nervous symptoms were included in this study. The dogs, of age between two and 12 years, of different breed.

Every dog, for which a detailed medical history was recorded, was subjected for a complete clinical and neurological examination. Additional, most of them (21/27), were subjected also for an ophthalmological exam.

For every dog, biochemical and hematological exams were performed.

Serological tests for IgM and IgG anti-*T. gondii* antibodies were carried out in the Diagnostic Laboratory. For this, serum samples were examined by an indirect fluorescent antibody test (IFAT) using a commercially available test.

## RESULTS

Twenty-seven dogs admitted to the Veterinary Clinic of Faculty of Veterinary Medicine of Bucharest presented with neurological symptoms. Their owners reported about history of epilepsy - like seizures for a period of time of a few weeks to a few years. The seizures were manifested at a variable period of time (a few per day or a few per year), not presenting a cyclical typology; in a few dogs (6/27; 22.2%.), salivation during the seizures appeared but no defecation / urination was observed.

The duration of a seizure was from 1-2 minutes to 30-40 minutes.

No changes in apetite (hydric or of food) were observed.

Unlike the epilepsy seizure, only some dogs (1/27; 3.7%) have lost their conscience, and the tonic-clonic contraction was manifested in two dogs (7.4%).

The clinical examination did not reveal significant changes, but the neurological changes were as follows:

- Status: depressed, in most patients;
- Cranial nerves:
    - Anisocoria (15/27; 55.6%);
    - No changes in face sensibility;
    - Delayed pupilar/palpebral reflex (17/27; 62.9%);
    - Delayed "menace" and "cotton ball" test results in right eye/left eye or both eyes in those presenting seizures for over 3 months;
- Posture – no changes or prefers lateral recumbence;
- Proprioception – no changes;
- Spinal reflexes – no changes;
- Panniculus – no changes;
- Perianal reflex – no changes;
- Gait: Tendency to fall on the side of the affected cerebral area;
    - tendency for dromomania after the seizure (9/27; 33.3%);
    - tendency to hold the head below the body line (13/27; 48.1%);
    - tendency to lean the head against objects.

The neurologic exam lead to the conclusion that the neuroanatomical localisation was in the area of the cerebral hemispheres.

For none of the examined dogs, ocular lesions compatible with toxoplasmosis were recorded.

Serological tests for IgM and IgG anti-*T. gondii* antibodies revealed positive results, for all dogs as follows: 63.00% (17/27) were positive for both, IgG and IgM, respectively, 25.9% (7/27) were positive only for IgG, while 11.1% (3/27) were positive only for IgM.

The chosen regime of treatment was:

- Clindamycine 10 mg x 2 times a day, for 30 days;
- Omega-3 1 g/40 kg/day, for 30 days;
- Antioxydant Wamark 1 cpr/40 kg/day, for 30 days;
- Liver support (Silimarina, Hepatiale, Essentiale) according to body weight, for 30 days;
- Probiotics- according to body weight, for 30 days;
- When seizures occurred (in two dogs; 7.4%), Fenobarbital (4 mg/kg/day) was administered;
- When the seizure was only partial/fake (especially after loud noises) we chose to administer GABA 15-20 mg/kg/day, in the evening (for one dog; 3.7%).

Fourteen days post-treatment, the dogs underwent for a check-up, including: clinical,

neurological examinations and serological tests for toxoplasmosis (IgG and IgM *T. gondii* antibodies). In a hight percentage (81.5%; 21/27), the neurological exam was significantly improved had no seizures at all, but some still went through one seizure/30 days (7.4%; 2/27) or a few partial seizures (14.8%; 4/27).

For one dog, the Magnetic Rezonance Imaging revealed asymmetry at the IV ventricule level, possibly due to a congenital reason, and multiple vascular alterations - possibly an ischemic vascular attack. In the dog presenting seizures localised on some muscle groups, it was decided to change the diet to gluten free type, which lead to a normal general state.

Overall, the post-treatment serological test results were also encouraging, all dogs being negative for Ig M. However, the serological test revealed still high titers of IgG in eleven dogs (40.7%).

For these reagents dogs, we proceeded to a new 30 day treatment with Doxicicline 10 mg/kg/day or Clindamycine 10 mg x two times/day, and we recommended to continue the previous treatment with Omega-3, Antioxydant Walmark, liver support, probiotics +/- Fenobarbital, +/- GABA).

Overall, all the dogs made a gradual recovery, with complete return of locomotor function and muscle mass within two and three months for 59.3% and 40.7%, respectively. The serological test revealed 2 positive only for IgG, low titers and they were advised for monitoring.

Toxoplasmosis is recognized as one of the most common diseases in dogs with neurological signs (Da Silva et al., 2005), and has been related to combined infections with distemper (Brito et al., 2002). Moreover, neurological signs of toxoplasmosis, distemper, and neosporosis are similar, emphasizing the importance of the differential diagnosis of these diseases (Langoni et. al, 2012). *N. caninum* infections have been described in animals affected with toxoplasmosis and it should be also considered in the differential diagnosis or with concurrent infection (Mineo et al., 2001).

In our study, the most common neurological disorders were alterations movement, mainly epilepsy-like seizures, and, for some, in consciousness. The percentage of reagents with specific IgM antibodies was higher (63.0%), indicating active infections. This was also supported by the fact that all dogs were vaccinated for distemper virus, and the age category, over 2 years, it is not known as common for neosporosis (Dubey et al., 2003).

*Toxoplasma* infection in animals has been previously studied in Romania, especially in the west, north, centre, and southeast of the country. Hotea et al. (2012) has reported high prevalence rates of *Toxoplasma* infection in cats (77.42%) and sheep (61.33%) in western Romania. For dogs, recent studies have reported sero-prevalence rates of IgG *T. gondii* antibodies from 25.46% to 50.00% in northwestern and southeastern Romania, respectively (Cozma et al., 2007; Enachescu et al., 2013). Moreover, Enachescu et al. (2013) has reported that of the dogs positives for IgG antibodies against *T. gondii*, one dog, for which a high antibody titer (S/P=291.91%) was registered but negative for *Neospora* infection, has presented neurological disorders: the dog, 14 month old, presented myoclonus and ataxia at the clinical examination, and a history of distemper - like symptoms with one month before (fever, purulent nasal and ocular discharge, dyspnea), but the rapid antigen test for canine distemper virus was negative (Enachescu et al., 2013). In a similar study, Brito et al. (2002) reported that IgM titers are more prevalent in dogs with altered consciousness.

Overall, a high seroprevalence of *T. gondii* in dogs may indicate a high pressure of environmental contamination, emphasizing the need for extended epidemiological studies (Langoni et al., 2014; Lopes et al., 2011; Schares et al., 2005; Meireles et al., 2004). Furthermore, it could emphasize potential risks to public health, pointing out potential common sources of infection for both humans and dogs (Salb et al., 2008).

## CONCLUSIONS

In the present study, the clinical follow-up of dogs with neurological disorders and positive

for antibodies against *T. gondii* emphasized a high percentage of animals seropositive for Ig M *T. gondii* antibodies, which might indicate an active infection.

Although it was not possible to be certain that every case is one of clinical toxoplasmosis, given the possibility of neurological symptomatology of other infections, these results demonstrate the importance of toxoplasmosis in dogs.

In addition to its veterinary importance, toxoplasmosis is also of major zoonotic concern and dogs can serve as epidemiological indicators for local infections.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# PREVENTIVE ECOSANITIZING OF THE BIOTOPE AND COMPARATIVE DIAGNOSIS OF MALIGNANT NHL LYMPHOPROLIFERATION IN HUMANS, DOGS AND CATS STANDARDIZATION

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***Abstract***

*Lymphomas are malignant monoclonal cell proliferation having the starting point in the organized lymphoid tissue (spleen, thymus, limfocentrii) or the diffused lymphoid tissue (gut-Payer boards and tonsils)*
*The different types of lymphomas are common regarding the symptoms, but differs depending on changes that occur at the cellular level and proliferating cell type, proliferation type and how they echo through out the body.*
*Age and sex of the animals have an importan role because this form of cancer is identified by the age of 2 (years) with maximum frequency between 5 and 7 years old, but also with cases at age 10 in both sexes. Breeds that are most often affected are: Boxer, Scottish Terrier, Airedale Terrier, German Shepherd, Poodle, Rottweiler and Golden Retriver. Purebred dogs are more sensitive than half breeds. The color black appears to be a factor due to absorption of ultraviolet radiation, dogs with dark fur being exposed to both skin tumors and NHL.*
*Lymphoma diagnosis begins with clinical examination revealing enlarged lymph nodes and sometimes the presence of general signs of illness: fever, cluster, weight loss, dyspnea, anorexia, anemia. Lymphosarcoma classification is based on location, histological criteria and immunophenotypic features.*

***Key Words:*** *ecosanitizing, markers, diagnosis, NHL.*

## INTRODUCTION

The emergence of new modern techniques and immunohistochemical phenotyping diagnostic in human medicine has helped proliferating cells of malignant lymphoma be well characterized, this contributes to the exact classification of the type of lymphoma and thereby an assessment of prognosis, closely related to the therapeutic means , taking also into account other factors such as the patient's age, associated diseases and paraneoplastic syndromes.
The frequency of these types of tumors in domestic animals is extremely high, being the most common hematopoetic neoplasm in dogs. Lymphomas are divided into two main groups: B-cell lymphomas and T cell lymphomas, according to the proliferating lymphoid cells - B or T predominant B-cell lymphomas, representing 75-80% of all lymphomas.
NHL is common in humans and pets that share the same biotope (air, water, food) and the same environmental oncofactors (natural and artificial ionizing radiation, carcinogenic chemicals - E's, aromatic hydrocarbons, chlorine, gluten, nitrates and nitrite), but mostly the well known oncogenic viruses HTLV 1 and 2 in humans and FeLV, FeSV, FIV in cats.
NHL incidence in humans is constantly growing, being more common in adults and the elderly, and representing the sixth place of cancers in men (after prostate cancer, lung, colon and rectum, bladder and melanoma) and the fifth placein women (after breast, lung, colon, rectum and uterus).
The term malignant lymphoma (NHL) in dogs is used to refer to the equivalent of NHL in humans. Due to the similarities of clinical course and therapuetic response, lymphoma in dogs is a useful model for studies with results that can be adapted to human NHL therapt. It ranks first due to the increased frequency of hematopoietic tumors in this species and the third place of all canine tumors.
Lymphoma in cats is rapidly evolving, faster than NHL in dogs, with predominantly extra

lymph node localizations, anterior mediastinum is the most common starting point when considering respiratory infections, intestinal localization is due to ingestion of the Feline Sarcoma Virus, but peripheral lymph node hyperplasia (micropoliadenopatia) rarely occurs.

## MATERIALS AND METHOD

Clinical staging and the diagnosis requires a number of clinical investigations, regardless of species, especially if the human cohabitates in thesame biotope with a domestic carnivore (dog or cat) suspected of haveing malignant lymphoma.

We have tracked, diagnosed and then treated specifically: 5 human patients (3 men and 2 women), 5 dogs (3 males and 2 females), 5 cats (3 females and 2 males) of different ages and races suspected to have malignant lymphoma, in various stages of clinical development.

Both human and veterinarian oncologists have examined all the patience, the results being written down in oncological medical charts, where we underlined if there where cases in which both the person and the animal suspected of cancer have been living together and have developed simultaneously the disease.

Complementary examinations helped staging and assessing the extent of the cancer in the body in both humans and animals using: chest radiography, computed tomography (chest and abdomen), abdominal ultrasound, exploratory laparotomy (the most reliable method for the diagnosis of intradiafragmatic acute disease), marrow biopsy, CBC, ESR, blood chemistry tests (to evaluate the function of liver and kidney, blood tumor markers, alkaline phosphatase, lactate dehydrogenase). Cytopathology exam (limfadenograma by puncture aspiration) pursus setting the type of lymphoma through the imagine and topography of the lymphoid malignant cell in the lymph nodes, and later confirmed by immunohistochemistry, the serological examination being mandatory for leukemia with suspected viral etiology.

## RESULTS AND DISCUSSIONS

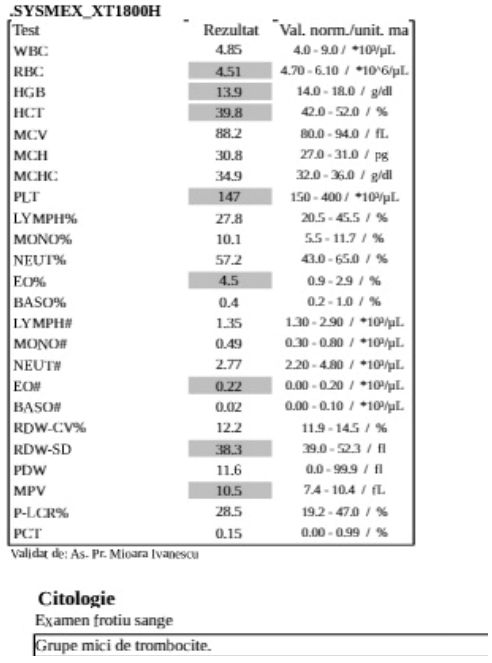
NHL clinical symptoms in both humans and domestic carnivores is polymorphic and uncharacteristic, as there are no pathognomonic signs apart from macropoliadenopathy, animal patients show anorexia, cahexie, vomiting and diarrhea. The malignant proliferation determines initially a solitary enlarged lymph node, in only one lymph node group, retropharyngeal or prescapular often with progressive enlargement accompanied by visceral lesions and bone marrow invasion expressed through late neoplastic peripheral lymphoblastic citemie. Frequent enlarging of the lymph nodes may appear: superficial or deep, single or multiple, related or not to limphistiocytic infiltrations to neighboring organs, especially in the spleen and liver.

The clinical evolution has two forms:

External adenopathy, which usually is localized mostly bilateral submandibular and/or laterocervical. But prescapulari lymph nodes may be affected. Inspection reveals animals usually develop an impressionable asymptomatic lymph nodes hypertrophy or lymphadenopathy associated with fever, night sweats, weight loss, pruritus. In humans manifestation of onset may be superior vena cava obstruction or spinal cord compression, cervical and/or supraclavicular lymphadenopathy appears in more than 70% of cases; Generalized lymphadenopathy is atypical. On palpation hypertrophied lymph nodes are irregular, painless, hard consistency.

Internal adenopathy is usually mediastinal, but can be mesenteric, myelogenous, bone or other. Mediastinal adenopathy causes mediastinal syndrome; it is common in the form of nodular sclerosis in Hodgkin's disease. It can cause coughing, blocking of the cranial vena cava, which is the most common simptome in non-Hodgkin lymphoma. Pleurisy can occur either by compression over vascular and/or lymphatic vessels or by direct invasion of the pleura. In the abdomen the spleen, the splenic and celiac lymph nodes are the first invaded. The clinical course of the spleen is misleading. Liver invasion is always associated with spleen invasion. I the final stages of the disease the bone marrow (lymphocytic

depletion and cause mixed cellularity), bone structure (jet osteoblastisa), cutis, central nervous system, meninges, Waldeyer ring and kidneys can be affected . We should not omit the sign of invasion and / or compression of various structures and the occurrence of pain.



.SYSMEX_XT1800H

| Test | Rezultat | Val. norm./unit. ma |
|---|---|---|
| WBC | 4.85 | 4.0 - 9.0 / *10³/µL |
| RBC | 4.51 | 4.70 - 6.10 / *10^6/µL |
| HGB | 13.9 | 14.0 - 18.0 / g/dl |
| HCT | 39.8 | 42.0 - 52.0 / % |
| MCV | 88.2 | 80.0 - 94.0 / fL |
| MCH | 30.8 | 27.0 - 31.0 / pg |
| MCHC | 34.9 | 32.0 - 36.0 / g/dl |
| PLT | 147 | 150 - 400 / *10³/µL |
| LYMPH% | 27.8 | 20.5 - 45.5 / % |
| MONO% | 10.1 | 5.5 - 11.7 / % |
| NEUT% | 57.2 | 43.0 - 65.0 / % |
| EO% | 4.5 | 0.9 - 2.9 / % |
| BASO% | 0.4 | 0.2 - 1.0 / % |
| LYMPH# | 1.35 | 1.30 - 2.90 / *10³/µL |
| MONO# | 0.49 | 0.30 - 0.80 / *10³/µL |
| NEUT# | 2.77 | 2.20 - 4.80 / *10³/µL |
| EO# | 0.22 | 0.00 - 0.20 / *10³/µL |
| BASO# | 0.02 | 0.00 - 0.10 / *10³/µL |
| RDW-CV% | 12.2 | 11.9 - 14.5 / % |
| RDW-SD | 38.3 | 39.0 - 52.3 / fl |
| PDW | 11.6 | 0.0 - 99.9 / fl |
| MPV | 10.5 | 7.4 - 10.4 / fL |
| P-LCR% | 28.5 | 19.2 - 47.0 / % |
| PCT | 0.15 | 0.00 - 0.99 / % |

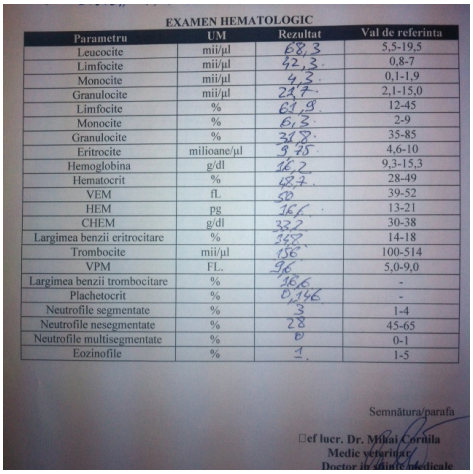
Validat de: As. Pr. Mioara Ivanescu

**Citologie**

Examen frotiu sange

Grupe mici de trombocite.

Figure 1 Blood analisys for a human patient with a splein lymphoma suspicion (Hematology lab Coltea Hospital)



EXAMEN HEMATOLOGIC

| Parametru | UM | Rezultat | Val de referinta |
|---|---|---|---|
| Leucocite | mii/µl | 68,3 | 5,5-19,5 |
| Limfocite | mii/µl | 42,3 | 0,8-7 |
| Monocite | mii/µl | 4,3 | 0,1-1,9 |
| Granulocite | mii/µl | 22,7 | 2,1-15,0 |
| Limfocite | % | 61,9 | 12-45 |
| Monocite | % | 6,3 | 2-9 |
| Granulocite | % | 31,8 | 35-85 |
| Eritrocite | milioane/µl | 3,95 | 4,6-10 |
| Hemoglobina | g/dl | 16,2 | 9,3-15,3 |
| Hematocrit | % | 42,7 | 28-49 |
| VEM | fl. | 80 | 39-52 |
| HEM | pg | 16,6 | 13-21 |
| CHEM | g/dl | 32,2 | 30-38 |
| Largimea benzii eritrocitare | % | 16,2 | 14-18 |
| Trombocite | mii/µl | 156 | 100-514 |
| VPM | FL. | 96 | 5,0-9,0 |
| Largimea benzii trombocitare | % | 16,6 | - |
| Plachetocrit | % | 0,146 | - |
| Neutrofile segmentate | % | 3 | 1-4 |
| Neutrofile nesegmentate | % | 28 | 45-65 |
| Neutrofile multisegmentate | % | 0 | 0-1 |
| Eozinofile | % | 1 | 1-5 |

Semnătura/parafa

Şef lucr. Dr. Mihai Cornila
Medic veterinar
Doctor în ştiinţe medicale

Figure 2 Blood analisys for a feline patient with a malignant lymphoma suspicion (Lad Vet Cor - Dr.Cornila)

## CONCLUSIONS

Sistemele majore de clasificare morfologica a limfomului malign uman ce au fost extrapolate limfomului malign la caine sunt: clasificarea Kiel, Rappaport, NCI Working Formulation, Lukes-Collins. Dintre acestea NCI Working Formulation s-a dovedit a fi cea mai folositoare,o punte intre diferitele clasificari.

Recomandarea rezultata din studiile noastre clince este de a acorda deosebita importanta limfonodulilor care afectati de proliferarea tumorala devin mariti in volum (uneori de 3-10 ori) duri, nedurerosi, aderenti la piele si la planurile profunde.

Simptomele cu un grad ridicat de suspiciune sunt adenopatia pronuntata unilaterala sau micropoliadenopatia externa.

Cainii cu limfom malign pot fi considerati santinele pentru potentiale situatii dezastruoase pentru om, datorita unei relativ scurte latente intre expunere si izbucnirea bolii.

S-au observat odata cu aparitia semnelor clinice de polidipsie si poliurie o scadere a ratei filtrarii glomerulare rezultand cresterea ureei sangvine si concentratiilor creatininei (in 87% din LNH-canin si respectiv 64% din cazurile de LNH-uman), anorexie (88%).

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# DIGESTIVE PARASITE FAUNA IN HARE (LEPUS EUROPAEUS) IN WESTERN ROMANIA

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***Abstract***

*During the period November 2013 - February 2014, 24 adult hunted hare (Lepus europaeus) from four hunting sites of Arad County were subjected necropsy. Of them, 13 were males and 11 females. The gastrointestinal mass from each was examined to determine the digestive parasites, macro- and microscopic examination were performed. Each segment of the digestive tube was sectioned, the mucosa and the gastrointestinal content were carefully examined and for the gastrointestinal mass successive washes method was used. The gastrointestinal content and also each segment of digestive tube (previously washed) were microscopically examined by stereomicroscope. The faeces found in the large intestine were examined by flotation method (Willis). Of the 24 samples examined, 21 were positive for gastrointestinal parasites, (prevalence of 87.50%). The most prevalent parasitism was with Eimeria spp., found in 17 samples (80.95 %), other parasites identified were: Cysticercus pisiformis found in two samples (9.52 %), Trichocephalus leporis in 13 samples (61, 90 %) and Trichostrongylus spp. in seven samples (33.33 %). The parasitism with gastrointestinal helminths and larval cestodes in hare represent risk factors for rabbits and domestic carnivores.*

***Key words****: digestive system, Lepus europeus, necropsy, parasite fauna*

## INTRODUCTION

Epidemiological aspects, the lack of references on the parasitoses in hares and also the increased health standards for hunting products, are the motivation for identifying the gastrointestinal parasites found in hares (*Lepus europaeus*) from four hunting sites from Arad County.

The hare (*Lepus Europaeus*) belong to the order Lagomorpha, Family Leporidae (http://wikipedia.org/wiki/Lepus). The living area for hares spreads through entire Europe, from fields until the alpines area. In Romania, the hare is spread from Danube Delta up the mountainous areas; higher densities are recorded at altitudes below 400 meters, in a warm and moderately dry district, avoiding marshy places with stagnant water (http://vanatoare.info/lepus-europaeus/; http://www.info-delta.ro/delta-dunarii).

Young hares live together with female hares even after weaning, this posing an increased risk of contamination with varies parasites to young hares from parasitized females.

Description: Body length varies from 48 to 52 cm, plus the tail (8-9cm), weighing between 4-5 kg. (http://vanatoare.info/lepus-europaeus/). The lifespan is 10-12 years. Hares are herbivorous and feed on grasses, herbs, twigs, leaves, buds, bark and field crops (http://www.info-delta.ro/delta-dunarii).

A study made by Afrenie et al. (2008) indicates the presence of parasitism with: *Eimeria* spp. in the faecal samples collected from European rabbits (*Oryctolagus cuniculus*) from the Zoological Garden Timisoara (Afrenie et. al. 2008).

After the examination of 24 hare gastrointestinal mass probes from a hunting place in Finland, the parasitism with *Trichostrongylus* spp., *Dicrocoelium dendriticum* and *Eimeria* spp., in 88 % were identified (Soveri T.,1987).

## MATERIALS AND METHODS

During the period November 2013 - February 2014, 24 hunted hare (*Lepus europaeus*) were necropsied. The animals originated from four hunting sites from Arad County. The hare were killed during the organized hunts made by the

hunters. Out of 24 hare, 13 were males and 11 females, all being adults. The hare were brought from Arad County, from Sofronea (8 samples), Dorobant (4 samples), Simand (6 samples) and Siria (6 samples). All cadavers have been examined at Parasitology and Parasitological diseases Clinic from Veterinary Medicine Faculty Timisoara (Figure 1).

Figure 1. Arad County map (The hunting sites studied) (http://commons.wikimedia.org/wiki/File:Harta_jud_Arad.png).

To determine the digestive parasites from each hare cadaver the gastrointestinal mass was collected and stored at -5° C until examination. Macroscopic examination was performed: the gastrointestinal mass was divided on digestive segments and each segment of the digestive tube was opened with a scissors. Longitudinal sections of the intestines were made for the content and intestinal mucosa carefully being examined. The gastrointestinal mass, divided in digestive segments, was examined then by successive washes method (Figure 2) (Dărăbuş et. al., 2013).

Figure 2. Gastrointestinal mass prepared for examination.

From each segment of the digestive tube (small and large intestine) adult parasites were collected. They were washed with 0.9 % physiological serum to remove impurities and preserved in 70 % ethanol, then by stereomicroscope or microscope were examined for identification (Darabus et. al., 2013). The stomacal and intestinal contents and also each segment of the digestive tract, following a pre-washing procedure were microscopically examined by stereomicroscope. The faeces have been examined using the flotation (Willis) method (Dărăbus et. al., 2013). Faeces were collected from the large intestine of each cadaver and examined by flotation (Willis) method, under a microscope with 10x objective. Identification of the genus *Eimeria* spp. was made in accordance with the identification keys described by Pellerdy, 1974.

## RESULTS AND DISCUSSIONS

**Macroscopic exam:**

Following the analysis of the gastrointestinal mass probes, was identified the presence of *Trichostrongylus* spp. adults, after the content of the small intestine has been emptied.

In the large intestine, was identified the presence of *Trichocephalus leporis* adults (Figure 3.)

On the serosa was identified the presence of *Cysticercus pisiformis*, larva form of *Taenia pisiformis*.

Figure 3. *Trichocephalus leporis* adults.

**Microscopic exam:**

Following coproparasitological examination using flotation (Willis) method has been identified parasitism with *Eimeria* spp. and *Trichostrongylus* spp. (Figures 4, 5).

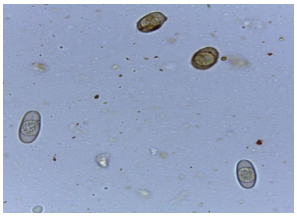

Figure 4. *Eimeria spp*. oocysts, hare faeces.

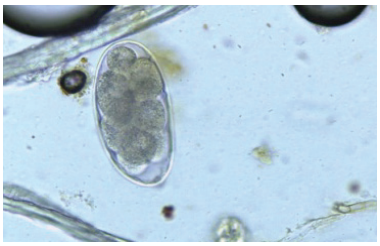

Figure 5. *Trichostrongylus* spp. egg.

Out of 24 probes, 21 were positives with an overall prevalence of 87.52 %.

In regard to the prevalence of identified parasites it has been acknowledged that parasitism with *Eimeria* spp. has been found in 17 samples (80.95 %), with *Cysticercus pisiformis* in two samples (9.52 %), *Trichocephalus leporis* in 13 samples (61.90 %), and *Trichostrongylus* spp. in seven samples (33.33 %) (Figure 6).

Regarding the sex factor was found that, out of the 24 samples examined, 13 were from males (54.61 %) and 11 females (45.83 %).

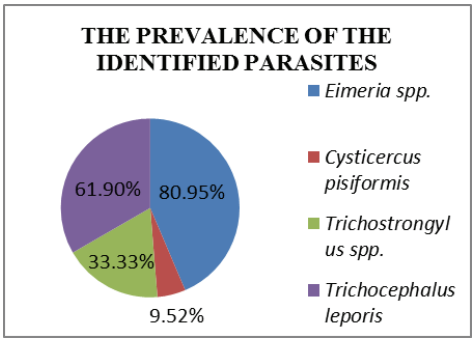


Figure 6. The prevalence of the identified parasites.

The most prevalent parasite found both in male and female was *Eimeria* spp., followed by *Trichocephalus leporis* (Table 1).

Table 1. The parasites prevalence according to the gender of the hare.

| Gender | Parasites | | | |
|---|---|---|---|---|
| | *Eimeria* spp. % | *Cysticercus pisiformis* ( %) | *Trichostrongylus* spp. (%) | *Trichocephalus leporis* (%) |
| Male | 6/13(46. 15%) | 2/13 (15.38) | 4/13 (30.76) | 7/13 (53.84) |
| Female | 11/11 (100) | 0/11 (0) | 3/11 (27.27) | 6/11 (54,45) |

Following the results obtained, the high prevalence of parasitism with *Eimeria* spp. in hare females is relevant from the epidemiological perspective: young hares live together with female hares even after weaning, which makes possible an easier and faster contamination with *Eimeria* spp. in young hares.

There is no significant difference in parasitism ratio between females and males for parasitism with *Trichostrongylus* spp., *Cysticercus pisiformis, Trichocephalus leporis.*

In this study the parasitism prevalence was: in Sofronea 75 % (6/8 analysed), in Dorobant 100 % (4/4 analysed), Simand 100 % (6/6 analysed) and in Siria 83.33 % (5/6 analysed).

The prevalence of species parasites was diferent in the hunting sites examined (Table 2).

Table 2. The prevalence of parasites according to the humting sites.

| Hunting sites | Parasites | | | |
|---|---|---|---|---|
| | *Eimeria* spp. (%) | *Cysticercus pisiformis* (%) | *Trichostrongylus* spp. (%) | *Trichocephalus leporis* (%) |
| Sofronea | 6/8 (75) | 2/8 (25) | 2/8 (25) | 3/8 (37.5) |
| Dorobant | 4/4 (100) | 0/4 (0) | 2/4 (50) | 3/4 (75) |
| Simand | 6/6 (100) | 0/6 (0) | 3/6 (50) | 2/6 (33.33) |
| Siria | 1/6 (16.66) | 0/6 (0) | 0/6 (0) | 5/6 (83.33) |

The fact that the parasitism with *Eimeria spp.* was higher in Sofronea and Dorobant to Siria, can be explained by the fact that Siria is near the hilly area of Arad County, while Dorobant and Sofronea are in a plain area with many lakes.

In the present study, *Eimeria spp.* had a prevalence of 80.95 %,in other similar study performed in Finland, *Eimeria* spp., *Trichostrongylus* spp. and *Dicrocoelium dendriticum* had a prevalence of 88 % (Soveri et.al., 1983).

The parasitism with *Eimeria* spp. in wild hares (*Lepus europaeus)* has been indentified only in those raised in freedom and not in those raised in cages. (Tacconi et.al.1995). The *Eimeria* species identified were: *E. leporis, E. semisculpta, E. robertsoni, E. townsenai, E. hungarica şi E. europeea* (Pellerdy, 1974).

Afrenie et al., 2008, indicate the presence of *Eimeria* spp. parasitism in the faecal samples collected from European rabbits (*Oryctolagus cuniculus*) from the Timisoara Zoological Garden (Afrenie, et.al. 2011).

A study conducted in Spain by Oliveros et al. (2000), showed a prevalence of 62.4 % of *Trichocephalus leporis,* very close to the prevalence found in this study at the same parasite (61.90 %) (Oliveros et.al., 2000).

McCulloch et al. (2004) have identified in one hunted hare lesions and nodules in jejunum caused by *Eimeria leporis* (McCulloch et.al., 2004).

## CONCLUSIONS

The prevalence of gastrointestinal parasitoses in hare (*Lepus europaeus*) in Arad County was 87.52 %.

The most frequent parasitism found was with *Eimeria* spp. (80.95 %).

The least common parasitism found was with *Cysticercus pisiformis* (9.52 %).

Depending on the hunting sites the most prevalent gastrointestinal parasitoses in hare (*Lepus europaeus*) was Dorobant and Simand (100 %) and the least comon in Sofronea (75 %).

The parasitism with gastrointestinal helminths and larval cestodes in hare represent risk factors for rabbits and domestic carnivores.

## ACKNOWLEDGEMENTS

This research work was carried out with the support of the project *Research, education and services development in the veterinary medicine and innovative technologies areas for RO 05*, *code SMIS-CSNR 2669.*

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# MALE GENITAL SYSTEM LESIONS IN DOGS DIAGNOSED BY CYTOLOGICAL EXAMINATION

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***Abstract***
*Understanding the pathological processes occurring in the male genital system requires advanced knowledge about its morphofunctional features. Even though the lesions of the male genital system in the dog are not as common, they constantly occur in general practice often being regarded as challenging in terms of diagnosis, treatment and prognosis. This study aims to evaluate the epidemiology, the cytological features and the efficacy of the cytological examination in achieving a definitive diagnosis in male genital system lesions in dogs.*
*This study was conducted over a 5 years period (2008-2012) in the Departament of Pathological Anatomy of the Faculty of Veterinary Medicine Bucharest and consists of 109 male dogs presenting genital lesions. The samples were obtained by fine needle aspiration, imprinting, scraping and surgical biopsy. The slides were prepared by squeezing and sliding techniques. For cytologically examined samples Romanowsky type stains were used: classic or quick May-Grünwald Giemsa and Diff-Quick.29 cases of testicular lesions were both cytologically and histologically examined.*
*During these 5 years, a total of 1872 male dogs have been specifically examined and 109 (5.8%) presented genital lesions. Of the 109 dogs considered for the study, 104 (95.4%) had testicular lesions and 5 (4.6%) had penile lesions. The 104 testicular lesions were diagnosed as follows: 20 cases (19.2%) with cryptorchidism and testicular hypoplasia, 16 cases (15.4%) with testicular degeneration, 10 cases (9.6%) with orchitis, and 58 cases (55.7%) with testicular tumours: seminoma (n=15), Sertoli cell tumours (n=13), interstitial (Leydig) cell tumours (n=15), mixed testicular tumours (n=15). The diagnosed penile lesions included acute balanoposthitis (n=1), squamous cell carcinoma (n=1) and transmissible venereal tumours (n=3). In both cytologically and histologically examined cases, cytological diagnosis was confirmed by histological diagnosis in 90% of the cases. Diagnostic errors occurred in individuals presenting testicular tumours where cytological examination did not confirm histological findings; in these cases histological examination revealed mixed tumours.*

***Key words:*** *male genital system, lesions, dogs, cytological diagnosis*

## INTRODUCTION

Male genital system lesions are important in male dogs' pathology, with neoplastic testicular tumours occurring most frequently (Moulton 1990, MacLachlan, 2002, Dinescu, 2005, Cătoi, 2008).
Testicular enlargement and testicular asymmetry, as well as the presence of sanguinolent preputial discharge warrant cytological examination as highly recommended (Dinescu 2005, Raskin, 2010).
Considering the numerous stray individuals and the potential risk of venereal disease transmission, when faced with genital lesions in male dogs, achieving an accurate diagnosis is essential in order to proceed with treatment. In this context, a definitive diagnosis can be achieved by cytological examination which is a minimally invasive and easy to perform technique, involving low costs and is commonly used in veterinary practices and diagnostic laboratories.

## MATERIALS AND METHODS

The study was conducted over a period of 5 years (2008-2012) in the Departament of Pathological Anatomy of the Faculty of Veterinary Medicine Bucharest and consists of a total of 109 male dogs presenting genital system lesions. Our study aims to evaluate the epidemiology and morphological features of such lesions, as well as the efficacy of sample collection meant for cytological examination.
The samples were obtained by fine needle aspiration, imprinting, scraping and surgical biopsy. The slides were prepared by squeezing and sliding techniques. For cytologically

examined samples Romanowsky type stains were used: classic or quick May-Grünwald Giemsa and Diff-Quick. 29 cases of testicular lesions were both cytologically and histologically examined.

## RESULTS AND DISCUSSIONS

Table 1 summarizes the data we collected during our 5 year study.

Table 1. Total of cases evaluated since 2008 until 2012

| Year | Examined male dogs | Examined male dogs presenting genital system lesions | |
|---|---|---|---|
| 2008 | 462 | 27 | 5.8% |
| 2009 | 246 | 17 | 6.9% |
| 2010 | 370 | 18 | 4.9% |
| 2011 | 425 | 22 | 5.2% |
| 2012 | 369 | 25 | 6.8% |
| Total | 1872 | 109 | 5.8% |

Of the 109 dogs evaluated in the study, 104 (95.4%) had testicular lesions and 5 (4.6%) had penile lesions.

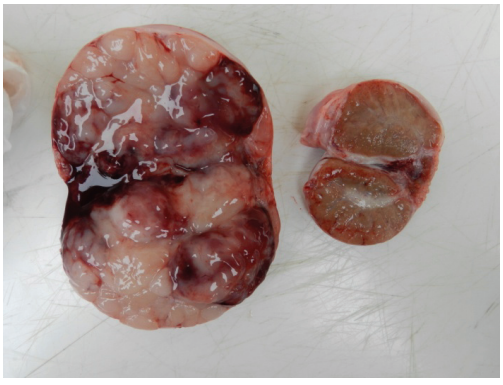

Figure 1. Testicular tumour – gross morphology. Enlarged and compact testicle, with fatty density and hemorrhagic patches on cut-section

The 104 testicular lesions were diagnosed as follows: 20 cases (19.2%) with cryptorchidy and testicular hypoplasia, 16 cases (15.5%) with testicular degeneration, 10 cases (9.6%) with orchitis, and 58 cases (55.7%) with testicular tumours.

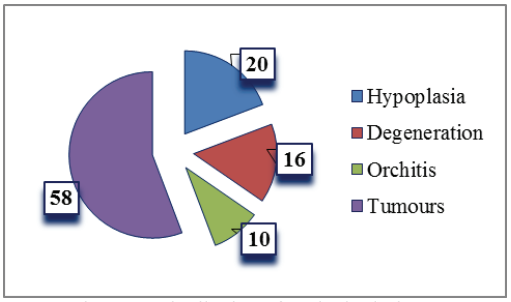


Figure 2. Distribution of testicular lesions

The samples were collected by fine-needle aspiration and imprinting techniques after orchidectomy had been performed.

Cytological examination of the hypoplastic and degenerative lesions only helped orientate the diagnosis as definitive diagnosis was achieved following histological examination.

Diagnosing inflammatory processes (orchitis, balanoposthitis) was straight-forward based on the presence of numerous inflammatory cells, particularly neutrophils.

Tumoral lesions occurred most frequently, encompassing 50% of the total number of evaluated lesions in our study. Figure 3 is a graphic representation of the various types of testicular tumours diagnosed in the 58 male dogs presenting such lesions.

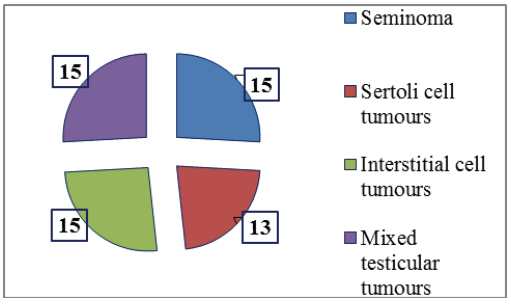


Figure 3.Diagnosed testicular tumours

As we can see, the various tumoral lesions occurred in fairly similar proportions. Even though Sertoli cell tumours occurred less, the differences were not significant.

In general cytological examination was essential for achieving a definitive diagnosis in tumoral lesions. In mixed testicular tumours, cytological examination failed to confirm the results yielded by histological examination.

Cytological diagnosis is based on cell appearance, nuclei and extracellular space assessment (Baker, 2000, Dinescu 2002).
In seminoma, cytological examination revealed a predominance of large cells, with round vesiculated central or eccentric nuclei. The nucleoli are evident and a small amount of slightly basophilic cytoplasm can be observed. The presence of bi- and multinucleated cells and a high mitotic index represent specific features of seminomas. The presence of numerous lymphocytes among the tumoral cells represents an invaluable element aiding diagnosis.

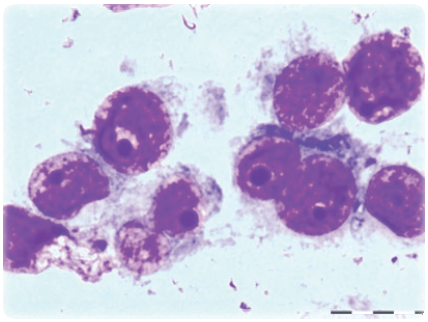

Figure 4. Seminoma. Large round cells, with large nuclei and evident nucleoli. MGG stain, x400

Cytological examination of Sertoli cell tumours revealed large round or stellate cells, presenting large nuclei with finely granulated chromatin. A specific feature is the presence of intracytoplasmic vacuoles varying in size. Free nuclei can occasionally be noticed and vacuoles are seen in the background.

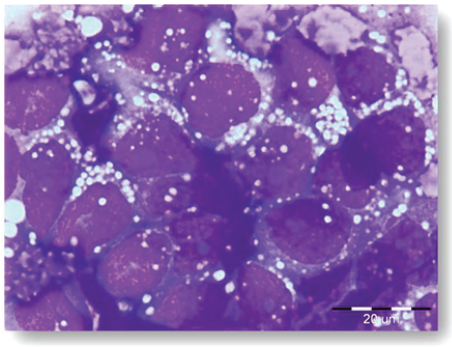

Figure 5. Sertoli cell tumour. Large cells, with vacuolated cytoplasm and reticulated nuclear chromatin. MGG stain, x1000

Interstitial cell (Leydig cell) tumours are easily diagnosed by cytological examination based on the round hyperchromatic eccentric nuclei and abundant foamy cytoplasm. Occasionally, the cytoplasm presents fine basophilic granules.

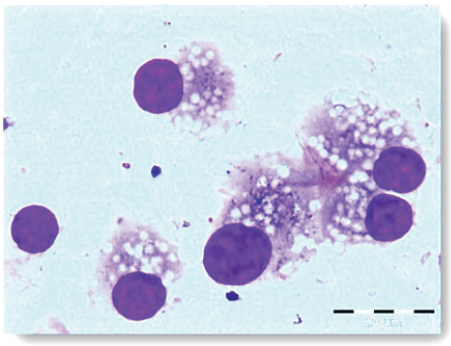

Figure 6. Interstitial cell tumour. Large cells, with reduced N:C ratio, eccentric nuclei and abundant, finely granulated, basophilic cytoplasm. MGG stain, x400

Testicular tumours generally tend to be more challenging as far as cytological diagnosis is concerned (Raskin 2010) but a judicious sample examination, identifying the specific elements of each tumour type, corroboration with epidemiology data and the clinician's level of expertise, most likely a combination of all of the above will ensure that an accurate definitive diagnosis is eventually achieved. Mixed testicular tumours did pose considerable diagnostic challenge during cytological examination and for that reason definitive diagnosis was achieved following histological examination.
The following penile lesions were diagnosed: acute balanoposthitis (n=1), squamous cell carcinoma (n=1) and transmissible venereal tumours (n=3). Sample collection was achieved by scraping and imprinting.
Penile lesions did not raise any difficulties in reaching a definitive diagnosis by cytological examination.
Penile squamous cell carcinoma presented macroscopically as a neoplastic growth located on the middle third on the dorsal aspect of the penis. Cytological examination revealed pleomorphic squamous cells, presenting moderate anisocytosis and anisokaryosis, basophilic cytoplasm and perinuclear microvacuoles.

Achieving definitive cytological diagnosis for transmissible venereal tumours was straightforward based on the presence of monomorphic round cells, with round nuclei presenting coarse nuclear chromatin and clearly defined nucleoli varying in size and number and small amounts of vacuolated cytoplasm (Fig. 7). Transmissible venereal tumours have been cytologically described as lymphocyte-like, plasma cell-like and lympho-plasmacytoid (Amaral, 2007). In plasmacell-like subtype cells are ovoid, with eccentric nuclei, abundant cytoplasm, and numerous clear cytoplasmic vacuoles. Lymphocytoid tumour subtype has a predominance of round cells, with finely granular cytoplasm and few vacuoles in their peripheral region. In our study we encountered the lymphocyte-like and the lympho-plasmacytoid subtypes.

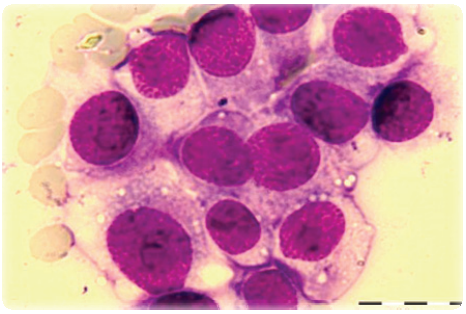

Figure 7. Monomorphic round cells, presenting round nuclei with coarse chromatin and abundant, finely vacuolated cytoplasm. MGG stain, x400

The acquired results indicated that cytological examination was essential for diagnosing male genital system lesions in dogs and in the majority of cases a definitive diagnosis was achieved only by cytological examination.
In both cytologically and histologically examined cases, cytological diagnosis was confirmed by histological diagnosis in 90% of the cases.

## CONCLUSIONS

Male genital system lesions accounted for 5.8% of the total number of male dogs that were specifically examined in the 5 year time frame.

The 104 testicular lesions were diagnosed as follows: 20 cases (19.2%) with cryptorchidism and testicular hypoplasia, 16 cases (15.4%) with testicular degeneration, 10 cases (9.6%) with orchitis, and 58 cases (55.7%) with testicular tumours: seminoma (n=15), Sertoli cell tumours (n=13), interstitial (Leydig) cell tumours (n=15), mixed testicular tumours (n=15).
The diagnosed penile lesions included acute balanoposthitis (n=1), squamous cell carcinoma (n=1) and transmissible venereal tumours (n=3). In both cytologically and histologically examined cases, cytological diagnosis was confirmed by histological diagnosis in 90% of the cases.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# DATA ON CANINE HEARTWORM (*DIROFILARIA IMMITIS*) INFECTION AND OTHER VECTOR-BORNE PATHOGENS IN DOGS IN BUCHAREST AREA, ROMANIA

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***Abstract***

*Heartworm disease is a serious cardiovascular and potentially fatal condition characterized by the presence of nematode Dirofilaria immitis in different developmental stages, found both in peripheral circulation, represented by microfilariae, and pulmonary artery and the right heart, represented by adult parasites. Diagnosis and identification of Dirofilaria species is complex involving antigen detection and microfilarial recognition. Therefore it is important for both animals and humans, improvement of rapid and efficient diagnostic protocols being a really powerful objective for epidemiological study progress.The purpose of the present study was to determine the exposure to D. immitis infection and other arthropod-borne pathogens of dogs living in Bucharest' adjacent area. For this we used modified Knott's technique and a point-of-care immunochromatographic test SNAP®4Dx® Plus. The modified Knott test is a concentration test that relies on lysing red blood cells and fixation of microfilariae for morphological examination, while, SNAP®4Dx® Plus represents an in-clinic diagnostic test that simultaneously screens dogs for 4 vector-borne diseases, including - Dirofilaria immitis antigen, and Ehrlichia canis, Ehrlichia ewingii, Anaplasma phagocytophilum, Anaplasma platys and Borrelia burgdorferi antibodies. A total of 175 dogs from Bucharest's adjacent area were included in the study. Of them, 21.14% were positive for D. immitis antigens. However, the modified Knott technique revealed a total of 16.57% samples positive for microfilariae of which 10.28% were D.immitis mf, 4.57%, D.repens mf. and 1.71% Acanthocheilonema mf. Additionally, 3.42% of dogs were positive for Anaplasma spp. antibodies, 1.14% for Ehrlichia spp, and 0.57% respectively, for B. burgdoferi.*

*In conclusion D.immitis infection in dogs from the greatest area of Bucharest is threatening high and therefore treatment and prophylaxis are needed to decrease the risks of disease since apparently healthy dogs harboring parasite serve as a reservoir of infection for other animals.*

***Key words****: Heartworm disease, vector-borne pathogens, Knott test, SNAP®4Dx® Plus test, dogs*

## INTRODUCTION

Dogs are competent reservoir hosts of several zoonotic agents and can serve as a readily available source of nutrition for many blood feeding arthropods (Otranto et.al., 2009a). The explosion of canine population and their increasingly close relationship with humans in both urban and rural areas pose new concerns for human public health (Genchi et.al., 2011).

Canine vector-borne diseases (CVBDs) represent an important group of illnesses affecting dogs around the world. These diseases are caused by diverse range of pathogens transmitted by different arthropod vectors-ticks and insects (fleas, mosquitoes, phlebotomine sandflies). In addition to their veterinary importance, some of the CVBDs are of major zoonotic concern, with dogs potentially serving as reservoirs and sentinels for human infections (Otranto et. al., 2009b).

*Dirofilaria immitis,* a filarial nematode transmitted by mosquitoes has the highest prevalence, being endemic, in southern and central European countries, especially in those with mediteranean climate as Italy, Spain, Portugal, France and Greece, but its spread has impended in central and northern European countries, as Switzerland, Germany, Netherlands, United Kingdom, Croatia, Russia, Hungary, including in Romania (Morchón et al., 2012).

Mosquitoes (*Culicidae*) represent vectors and intermediate host for *Dirofilaria* spp., fleas and lice, being vectors for *A. reconditum* and

ticks for *A. dracunculoides* (Magnis et al., 2013).

For diagnosis of heartworm infection, in dogs, can be used blood tests that detect circulating microfilariae or adult antigens, but further diagnostic procedures are usually required to determine the severity of disease and treatment options (Knight, 1995). Moreover diagnosis and identification of *Dirofilaria* species is complex, involving antigen detection and microfilarial recognition (Roth et al., 1993; Genchi, 2005).

Despite of the great concern worldwide on vector-borne diseases generally (Knols et al., 2007) and on CVBDs particularly, little is known about the occurrence and prevalence of vector-borne pathogens in dogs in different areas of Romania.

Therefore, the present study was conducted to investigate and determine the exposure to *D. immitis* infection and other arthropod-borne pathogens of dogs living in Bucharest's adjacent area.

## MATERIALS AND METHODS

A total of 175 dogs, were enrroled in the present study. The dogs were older than 1 year, without previous chemoprophylaxis or treatment by microfilaricide products and living in Bucharest's adjacent area.

All dogs were screened using a modified Knott's technique (Bowman, 2003) for microfilariae detection and by a point-of-care immunochromatographic test *SNAP®4Dx® Plus*, for qualitative detection of antigens and antibodies (IDEXX Laboratories, 2008) of different arthropod-borne pathogens (Figure 1).

*SNAP®4Dx®Plus* represents an in-clinic ELISA test, for qualitative detection of *Ehrlichia canis* and *Ehrlichia ewingii*, *Borrelia burgdorferi*, *Anaplasma platys* and *Anaplasma phagocytophilum* antibodies, as well as simultaneously detection of *Dirofilaria immitis* antigens in serum, plasma or whole blood from the dog, with a specificity of 98-99% (IDEXX Laboratories, Westbrook, ME).

Enzyme-linked immonosorbent assays are designed to detect heartworm adult antigens, which are considered highly specific, as cross reactivity with other canine parasites (i.e. *D. repens, Dipetalonema* spp.) does not occur (Venco et al., 2001).

These tests allow detection of adult heartworm antigens produced only by female worms and may provide information about worm burden (McCall et al.,1992; Knight, 1995).

The sensitivity is actually very high, but false negative results may occur in prepatent period or very light infections or when only male worms are present (Knight, 1995).

We obtained 4 ml of blood collected in EDTA tubes from all dogs, selection including a wider area and more varied places in town and its surroundings. Thus, 67 were street dogs, 22 were German Shepherd from a military unit and the remaining 86 were mixed-breed companion dogs, owned by native people, brought to the Faculty of Veterinary Medicine-Bucharest for various investigations. Blood samples and tests were kept at room temperature for 30 minutes prior to testing. We used both, whole blood and serum obtained by centrifugation. The use of serum gives an accuracy of 99.2%, a confidence of 100% and a specificity of 95% according to the manufacturers (IDEXX Laboratories, 2008).

There have been distributed 3 drops of whole blood/ serum sample to be analyzed with the pipette contained in the test kit in a 2 ml Eppendorf tube, adding above 4 drops of conjugate and mixing 2-3 times.

The SNAP was placed horizontally, then the mixture was added in the orifice for the sample content. The sample will arrive in the reading window before reaching the activation circle (30-60 seconds).The complex antigen (Ag)/ conjugate or antibody (AB) /conjugate binds to the labeled antigen or antibody. Once the activation circle has began to change color, the activator was pressed firmly to align the SNAP device body. The sample will flow back over the array. Bidirectional flow provides a second opportunity to bind to the antibody. Washing solution cleans matrix debris that could interfere with the results. Colorless substrate solution reacts with the enzyme conjugate. Each enzyme converts multiple substrate molecules from colorless to blue, amplifying

the signal. This reaction forms blue spots in the window reading device color indicating a positive result.

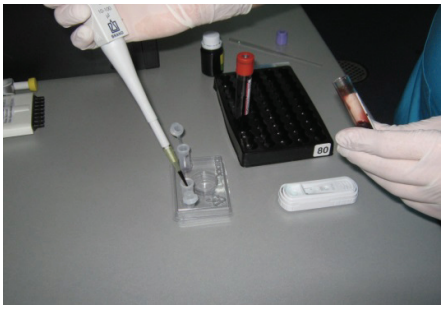

Figure 1. Comparative testing of blood samples by *SNAP®4Dx® Plus* and modified Knott test

Results were issued after 8 minutes, using SNAP shot Dx Analyzer device that records and interprets the response colorimetric enzyme activity on the surface of SNAP test. During the procedure, the analyzer records digital images of each SNAP device using an algorithm to calculate the specific test results using own bar code, thus minimizing subjective interpretation.

Along with Snap tests, we used a concentration method called modified Knott test which relies on lysing red blood cells and fixation of microfilariae for morphological examination.

For each sample obtained, we mixed 1 ml of whole blood collected in EDTA tube, with 9 ml of 2% formalin, then centrifuged 5-8 minutes at 1500 rpm. The supernatant was discarded from the centrifuge tube and the sediment was mixed with equal parts of the dye, methylene blue 1: 1000.We spread the colored sediment on a slide, put a coverslip, and examined microscopically (10X, 20X and 40X objective).

Examination of whole sediment allows finding the number of microfilariae in a milliliter of blood. We prefered this method because formalin fixed microfilariae in extension allows their measurement.

## RESULTS AND DISCUSSION

Overall, out of 175 dogs, investigated by *SNAP®4Dx® Plus*, 37 (21.14% [$CI_{99\%}$=27.6-46.4]) was positive for *D. immitis* antigens.

However, the modified Knott technique revealed a total of 29 samples of microfilariae (16.57%), of which 18 were *D.immitis* mf., 8 *D.repens* mf. and 3 *Acanthocheilonema* mf. (Table 1).

Microfilariae of *D.immitis* measured between 290 to 330 µm in length and 5 to 7 µm in diameter, with a straight tail and a spindle-shaped cephalic extremity (Figure 2).

*D. repens* microfilariae measured between 350 and 385 µm in length and 7 to 8 µm in diameter, with a curved tail and rounded cephalic extremity (Figure3). *Acanthocheilonema* species differentiation were not possible by this method (Figure 4).

However, given the variety of canine filariae, the detection of microfilariae alone does not give an accurate diagnosis, because although filaria species can be identified by an evaluation of cephalic and caudal morphologies, these features are often difficult to differentiate (Simón et al., 2012).

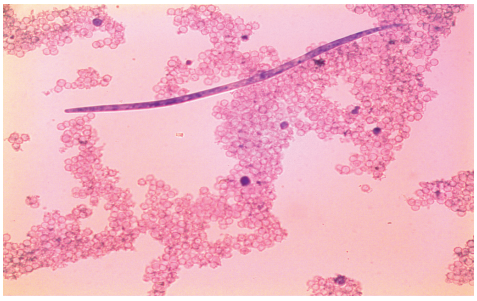

Figure 2. *Dirofilaria immitis* - microfilariae by modified Knott test, O.B x 20, (original NIKON microscope)

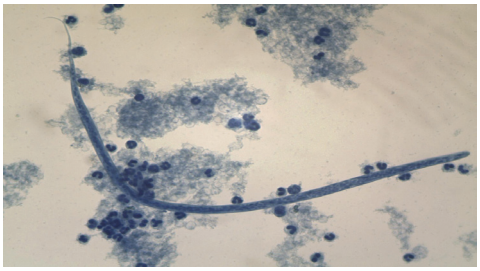

Figure 3. *Dirofilaria repens* - microfilariae by modified Knott test, O.B x 20, (original OPTIKA microscope)

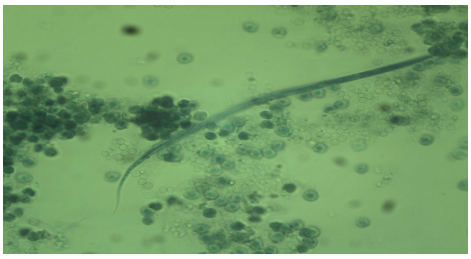

Figure 4. *Dipetalonema* spp. microfilariae by modified Knott test, O.B x 20, (original OPTIKA microscope)

Occult infections occur during prepatent period (larval stage) or unisexual infections when worms become sterile after microfilaricidal treatment, or when the body produces antibodies that cause destruction host's microfilariae. (Rawlings et al., 1982).
In this study, a case of occult infection has been reported (positive with *SNAP®4Dx® Plus*, but negative for microfilariae.)
Additionally, 6 dogs (3.42% [CI99%=1.38-10.62]) were positive for *Anaplasma* spp. antibodies, 2 dogs(1.14% [CI99%=-0.73-4.73]) were positive for *Ehrlichia* spp, and one dog (0.57% [CI99%= -0.94-2.94]) respectively, for *B. burgdoferi* (Figure 5). No mixed infection were recorded.

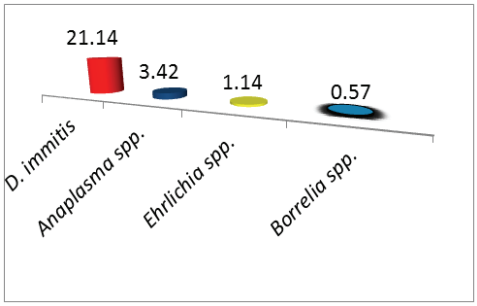


Figure 5. Seroprevalence of *D.immitis, Anaplasma* spp. *Ehrlichia* spp and *Borrelia* spp. using *SNAP®4Dx® Plus*

Table 1. Prevalence of arthropode-borne pathogens ( *Dirofilaria* spp. *Anaplasma* spp., *Ehrlichia* spp, and *Borrelia burgdoferi* sensu lato), stratified by category of dogs and detection methods

| Dog category | No.of tested | Detection methods | | | | |
|---|---|---|---|---|---|---|
| | | Microscopy (Modified Knott test) | Ag ELISA (SNAP 4DX Plus) | | | |
| | | Nos. Positive microfilariae spp. (%) | Nos. Positive *D.immitis* (%) | Nos. Positive *Anaplasma* spp (%) | Nos positive *Ehrlichia* spp (%) | Nos. Positive *Borrelia* spp (%) |
| Stray dogs | 67 | 14 (20.89%) | 19 (28.35%) | 4 (5.97%) | - | - |
| Police dogs | 22 | 4 (18.18%) | 3 (13.63%) | - | - | - |
| Pet dogs | 86 | 11 (12.79%) | 15 (17.44%) | 2 (2.32%) | 2 (2.32%) | 1 (1.16%) |
| Total | 175 | 29 (16.56%) | 37 (21.14%) | 6 (3.42%) | 2 (1.14%) | 1 (0.57%) |

These diagnostic approaches are a powerful outfit for epidemiological studies and should allow the assessment and screening of large area, considering that they have a good sensitivity and do not require invazive techniques.

Modified Knott test is more sensitive than direct smear test, because, it concentrates microfilaria, so they are less likely to be missed during microscopic examination. Nevertheless, antigen tests are very specific, but they are not always sensitive, and modified Konott test is a method that requires

time and experience, thus molecular techniques are the next step certifying the truthfulness samples.

Present finding supports similar studies of other authors, in Romania, as Mircean et al. (2012) reported seroprevalence values as follows:*D.immitis* (3.3%), *A.phagocytophilum* (5.5%), and E. *canis* (2.1%). Also, Ionita et.al. 2012, acquired the following results *D. immitis*,18.68%,*A. phagocytophilum*, 16.00%, *E. canis*, 4.00%.

Another survey conducted in 2014 by Ciucă et al. which refers to Romania's northern counties, of region Moldova, certify a seroprevalence of 2.10% of *D.immitis,* considering that most of this area has a temperate continental climate with eastern influences aridity and Baltic scandinavian influences in the north, having a cool and moist character but, reveals an expanding in the northeast areas of the country, where the climate conditions support transmission of heartworm.

Increasingly prevalence in almost all our results is due to superiority assessment tests, including new etiologic pathogens, but also by particular ecological conditions (climate, biotopes) associated with the distribution and abundance of arthropods in the studied area.

## CONCLUSIONS

These findings show that people need to be informed about the risk of zoonotic potential of dogs that harbour parasites.

In particular, *D.immitis* infection in dogs from the greatest area of Bucharest is threatening high and therefore treatment and prophylaxis are needed to decrease the risks of disease since apparently healthy dogs harboring parasite serve as a reservoir of infection for other animals.

Therefore, the findings are expected to serve as a reference for future investigations and control actions in order to protect dogs and limit the risk of transmission of vector-borne agents to humans.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# FIXED BANDAGE USE IN THE TREATAMENT OF A BAT (NYCTALUS NOCTULA) RADIAL DIAPHYSEAL FRACTURE

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***Abstract***

*In this paper we present a case of a female bat (nyctalus noctula) found with a complete closed fracture in the third middle of the right radius diaphysea. Therapeutic protocol consisted of applying a fixed bandage with a removable plastic splint covered with synthetic adhesive material after providing analgesia and anesthesia. Radiological images after immobilization show correct positioning of the fracture. Post interventional indication was to maintain the bandage for 6 weeks in order to keep the two bone segments (distal and proximal radial) in physiological anatomical relationships, to monitor his integrity and to provide analgesia since it is reported a high degree of auto mutilation. Analgesia was continued for 5 days.*

***Key words****: fracture, diaphyseal, bandage, analgesia, rat*

## INTRODUCTION:

The bat was found unable to fly, presenting a high mobility at the right wing level, at the radial level, along with a circular lesion and crepitating. Diagnostic was establish trough radiological exam: fracture in the third middle of the right radius diaphysea (Winters P., 1996).

## MATERIALS AND METHODS

The bat was received in a poor condition. After a physical exam we establish that he needed immediate intervention. The bat was premedicated with butorphanol 5 mg/kg intramuscular and induced with isofluran 5% by mask (fig.1).

Fig. 1 Mask induction with isoflurane

Anaesthesia was maintained with isofluran 2%, for 35 minutes (Grimm, K.A.,2013). Analgesia was continued every 12 hours, for 5 days (Sangster C., 2008). Therapeutic protocol consisted of applying a fixed bandage (fig.2) for a complete closed radius fracture after providing analgesia and anesthesia (James at 2001).

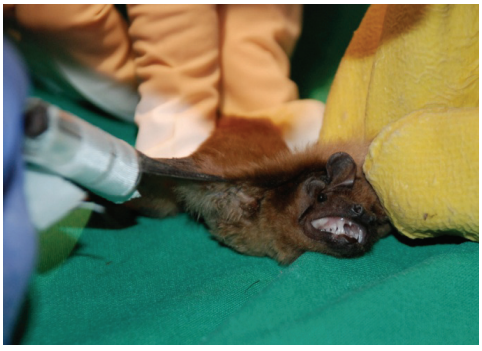

Fig. 2 Fixed bandage with a removable plastic splint

## RESULTS AND DISCUSSIONS

Anaesthesia with isoflurane by mask was well tolerate and the recovery was rapid. A fixed bandage with a removable plastic splint covered with synthetic adhesive material was design for the fracture (fig.3, fig.4). Due to the lesion existed at the fracture level we

consider to open the bandage in order to have access to this site. The lesion was cleaned daily with a high cicatrised ointment based on propolis.

Fig.3 Appling the plastic splint

Fig.4. Final aspect of the bandage

Analgesia was continued 5 days after surgery with butorphanol via intramuscular injection. The bat received Duphalyte 5ml/day pers os daily. The patient didn't present any signs of auto mutilation during the treatment.
The radiological exam performed after surgery revealed the correct repositioning of the. Complete callus formation was observed 3 weeks after the intervention.

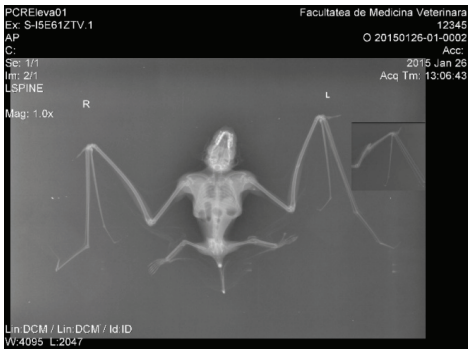


Fig.3 Radiologial exam before (small figure) and after intervention

## CONCLUSIONS

Radial fracture in bats can be treated applying a fixed bandage.It is important to choose light materials, well tolerate by the bat's tegument. Analgesia is a very important aspect, since pain can be a stimulus for auto mutilation. Propolis was used with good result for local cicatrisation.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# DETOXOFICATION ADJUVANT IN THE NUTRITIONALTHERAPY OF CANCER IN DOGS AND CATS

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***Abstract***

*The medical saying states " it is easier to prevent than to cure " thus there are many ways to prevent and the easiest way is with proper nutrition which can prevent , delay or even stop the development of cancers in humans and animals . Reducing risk factors with a balanced diet ( without pesticides and artificial preservatives ) , drinking clean water , adequate amounts of antioxidant supplementation with vitamins and minerals and regular physical activity can prevent cancer.*

*Protein malnutrition is usually associated with neoplastic disease , represented by what is called neoplastic cahexie which is the result of cumulating a deficiency in terms of the amount of protein and calories associated with metabolic effects induced by cancer. The cytostatic disease is a complex of pathological syndromes induced by chemotherapy medication that affects the whole body without discrimination. The whole complex of symptoms that appear due to chemotherapy was called „The cytostatic disease" by analogy with the disease caused by irradiation. Cahexia as paraneoplastic syndrome overlaps metabolic and immunological effects induced by chemotherapy, the detoxification and rebuilding of the cancerous body becoming a vital goal. Administration of both conventional detox agents and unconventional therapeutic products tested on dogs and cats with various forms of cancer such as PI Water ( ionized and structured ) and deuterium depleted water or DDW.*

*Proper nutrition and balanced protein - carbohydrate - lipid is able to prevent tumor growth but also to improve the quality and duration of life for both human and animal patients with cancer.*

***Key words***: *WWD, PI water, syndromes , paraneoplastic , cahexie.*

## INTRODUCTION

At a time when food is increasingly artificialized and genetically modified, cancer prevention and therapy for humans and animals through specific diets is a very important goal. Studying cancer in pets has been and will continue to be an experimental model in treating cancer in humans.

Nutritional therapy is a key component in cancer cahexia treatment, effectively helping to control malignant diseases in certain situations. Nutrients can be used as a means of adjuvant therapy in reducing the cytotoxicity associated with chemotherapy and radiotherapy and of great importance in speeding post-op healing and coping with paraneoplastic syndromes associated with advanced stages of cancer. The timing and nature of nutritional intervention should be set to start long before the patient shows signs of cancer cahexia, that being debilitating weight loss or anorexia, the aim of adjuvant therapy being improving the response to therapy and the quality of life.

## MATERIALS AND METHOD

Experiments were conducted in the Oncology Clinic of the Faculty of Veterinary Medicine Bucharest and a private veterinary medical practice.

We followed 12 cases of dogs and 12 cases of cats with various forms of cancer and in advanced stages of cancerous cahexia. Animalele benefited from both classical and unconventional therapy with PI water or DDW. Solid tumors were surgically excised after appropriate therapy.

Pi water or in Greek language life water, has an alkalined, ionized structure and was given to groups II A and II B throughout the period of the patients survival.

Deuterium depleted water (D2O DDW) is a non-radioactive, low deuterium (hydrogen isotope D2) that slows cellular metabolic

processes by substitution of the physiological water H2O, the maximum percentage beeing 30% in order not to cause harmful effects on the body both healthy and affected by thecancer disease, radiation or chemotherapy.

- Batch IA consisting of 4 dogs, 2 mammary tumors in different TNM stagesand 2 malignant lymphomas. The dogs received chemotherapy with alkilant agents - Holoxan 200 mg/m2/day and Carboplatin 1 mg/kg 14 days before and after surgical interventions. At the same time the diet therapy and deuterium depleted water DDW was administered throughout survival.
- Batch IB consisting of 4 cats, 2 mammary tumors in different TNM stages and two cats with lymphoma which received chemotherapy with alkylating agents (Ciclofofamida 50 mg/ m2/day) and anthracycline (Epidoxorubicina 1 mg/kg 14 days before and after surgical interventions diet therapy and deuterium depleted water DDW was administered. The DDW doses used were 30 ppm / kg. cats per day and 6o ppm / kg for dogs.
- Batch IIA consisting of 4 dogs, 2 females with mammary tumors in different TNM stages and 2 males dogs with lymphoma which received chemotherapy with alkilanti agents - Holoxan 200 mg/m2 / day and Carboplatin 1 mg/kg 14 days before and after surgical interventions. At the same time the diet therapy and PI water was administered throughout survival.
- Batch IIB consisting of 4 cats with lymphoma, received anthracycline chemotherapy (Epidoxorubicina 1 mg/kg before and after surgical interventions. At the same time the diet therapy and PI water was administered throughout survival.
- Batch III A consists of 4 dogs, 2 females with mammary tumors in different TNM stages and 2 males with malignant lymphomas which received the same cytostatic therapy as batches IA and IIA and ordinary water (H2O).
- Batch III B 4 cats, 2 mammary tumors and 2 lymphomas received ordinary water (H2O) along with conventional chemotherapy in groups IB and IIB.

Animals in all groups received specific treatments in parallel (chemotherapy and surgical therapy) and adjuvant diet therapy (diet food and water). Pi water and the deuterium depleted D2O- were administered throughout the survival of patients in groups IA/B and IIA/B.

Confirmation of the diagnosis on protein energy malnutrition existence in cats and dogs with neoplastic cahexie was by clinical assessment of the metabolic nutrition. We systematically evaluated the amount of protein and energy of the patient, including body condition score, routine haematological and biochemical analysis and the data obtained from the diet history.

**RESULTS AND DISCUSSIONS**

The therapy using PI Water and D2O deuterium depleted water in the diet of animals with cancer was doubled with a balanced diet and adequate clinical staging of the disease but also the cancerous body's biological needs. Favorable therapeutic effects were expressed differently depending on the type of cancer, clinical stage and type of water administered. The role of this therapy is water substitution, having detoxifying effects on animals with cancer and reducing the toxic side effects of chemotherapy.

Test results showed that deuterium depleted water had the most important therapeutic effects in the Ist batch, with both dog and cat patience with cancer, whether it was associated with low carbohydrates diets, moderate amounts of highly bioavailable protein, soluble and insoluble fibers and a moderate amount of polyunsaturated fatty acids of the omega-3 series.

Weight loss in cancer patients in the IInd batch, both dogs and cats, treated with chemotherapy and PI Water was more common, especially in individuals with advanced stage disease. Cahexia degree is an important prognostic indicator, because of the lack of asimilation and also because the actual development of cancer or complications caused by anticancer therapy. Affected patients suffer from a progressive depletion of both muscle and lipid deposits caused by abnormalities in the metabolism of carbohydrates, protein and fat. The rapid proliferation of neoplastic tissues leads to an increased demand of amino acids and energy

in the tumor cells. Patients with weight loss will live less than those without weight loss, regardless of their oncological diagnosis. The incidence of weight loss is variable, depending on the type of tumor. Neuroendocrine response (excess glucocorticoid hormones) induced by mental stress and malignant diseases caused increased protein catabolism and body energy storage.
A hereditary requirement in cats with cancer for unusually high protein may predispose animals to protein-energy malnutrition and can lead to biochemical abnormalities in the blood serum levels. An increase in serum creatinekinase concentration caused by rapid catabolism of skeletal muscles, compensating to provide the necessary amino-acids, appears in cats with mammary tumors that undergo chemotherapy. Protein deficiency may also be a factor in the pathogenesis of hypoalbuminemia that characterizes cats with high serum: liver enzymes ALT, AST and alkaline phosphatase.

Hematologie of the cancerous body shows non-regenerative anemia (anemia of chronic disease) and lymphopenia, which are often present features.
Dogs and cats with cancer from the IIIrd batch treated with chemotherapy and tap water showed biochemical abnormalities that may include decreasing concentrations of blood urea nitrogen secondary to protein decrease, decreased serum creatinine during muscle mass consumption and hypoalbuminemia due to increased protein catabolism and reduced protein synthesis.

## CONCLUSIONS

The deuterium depleted water diet resulted in significant decrease in transaminases ALT, AST and urea compared with groups treated with chemotherapy.
Deuterium depleted water is effective in improving the cancerous body's metabolism and has an adjuvant effect in detoxification and the restoration of the functions affected by the disease (paraneoplastic syndromes) and conventional therapies (cytostatic disease).
PI Water administered to the patients (dogs and cats with cancer) followed by us has proven to be a modest and inconsistent therapeutic adjuvant.
Ideal diet for a cancer patient must contain a higher amount of protein and fat than carbohydrate, ratio to be determined according to nutritionists.
The amount of proteins and their quality (rich in essential amino acids) must be maintained at a level sufficient to repair tissue, unlike carbohydrates that must be reduced to a minimum, anticancer nutrient may be used in parallel.
The cancer patients diet must consist of high quality food, with a moderate protein content (18-22%), low carbohydrate (3-13%) and an increased intake of fat (55-60%).
Diets rich in essential fats help cancer patients counteract the effects of cancer and even reduce cancer expansion.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# CLINICAL PREVALENCE OF METHICILLIN RESISTANCE STAPHYLOCOCCI IN A PIG FARM FROM ARAD COUNTY – PRELIMINARY STUDY

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***Abstract***

*Colonization with Staphylococcus aureus resistant to methicillin (MRSA) has recently been identified in pigs and that the people working in these sectors and increasing concern about the role of pigs as reservoirs of MRSA infections in humans were reported more frequently in the literature. The research had as purpose to determine the frequency of staphylococcal strains isolated from pig herds, phenotypic characterization of isolates and identification of methicillin-resistant strains and types of resistance. Samples were taken from clinically healthy pigs from a swine farm in Arad, from October to December 2013. Samples required bacteriological examinations were collected from a total of 87 clinically healthy pigs. After growth, staphylococcal isolates were identified according to their characteristics as outlined in Bergey's Manual of Determinative Bacteriology and the Manual of Clinical Microbiology. From pig farm were isolated 28 strains of staphylococci, including 20 coagulase positive strains (CoP, represented by S. hyicus and S. aureus) and 8 coagulase negative strains (CON, represented by S. haemolyticus, S. epidermidis respectively S. sciuri), isolated from clinically healthy pigs in different anatomical areas. All strains of staphylococci isolated from pigs showed sensitivity of 100% for novobiocine, rifampicine, pristinamycin, ciprofloxacin, vancomycin, ceftriaxone, cefoxitin, cefaclor and ampicillin/sulbactan, considered the drug of choice for these bacteria. As against used β-lactams (methicillin, ceftriaxone, cefoxitin, cefaclor, ampicillin with sulbactan) antibiotic sensitivity was highest, except methicillins, which were isolated 4 resistant strains, two S. hycus methicillin-resistant strains and two S. aureus strains. After testing staphylococci strains isolated from pigs, against 17 antibiotics were identified methicillin-resistant strains and several types of resistance to β-lactams, tetracyclines, macrolides and polymyxin B.*

***Key words****: staphylococci, methicillin resistance, pigs*

## INTRODUCTION

*Staphylococci are important opportunistic pathogens often found in the microflora of skin and mucosal surfaces of the upper respiratory tract of man and animals.*

*One year after the introduction of methicillin in clinical practice (1961), have been described MRSA strains (Guardabassi et al., 2006). Since then, MRSA has become a major human pathogen, responsible for considerable mortality, morbidity and healthcare expenditure in both nosocomial and community settings (Anon, 2009; Smith et al., 2013).*

*Although rarely reported in the past, the prevalence of MRSA in pigs, along with cases of possible pig-to-human transmission and vice versa, have been the subject of considerable and increasing interest over the past 2 years (Armand-Lefevreet et al., 2005; Huijsdens et al., 2006; Voss et al., 2005).*

*Methicillin-resistant Staphylococcus aureus (MRSA) has become a major nosocomial pathogen, highly prevalent in many European countries and throughout the world (Anon, 2009).*

*S. aureus* are normal inhabitants of pigs, and occur in all herds (Armand-Lefevre et al. 2005).The prevalence of MRSA in pig herds varies widely (0 to 50%) among European countries (Anon, 2009). The pig herd prevalence of MRSA in North America is uncertain, but appears lower than in many European countries (Smith et al. 2013; Weese et al. 2011). MRSA prevalence is high (>50%) in pigs in positive herds, but has minimal effect on swine health (Smith et al., 2013).

The capacity of *S. aureus*/MRSA strains of livestock origin to colonize, spread, and cause disease in humans remains uncertain (Smith et al., 2013).

## MATERIALS AND METHODS

The research was conducted in the Laboratory of Bacteriology, Department of Infectious Diseases and Preventive Medicine (Faculty of Veterinary Medicine Timisoara). Samples were taken from clinically healthy pigs from a swine farm in a village in the county of Arad during October-December 2013.

Samples required bacteriological examinations were collected from a total of 87 clinically healthy pigs (sows with piglets in the maternity, pregnant sows - breeding sector, weaners and fat pigs), are represented by samples from different anatomical areas: ear, skin, perianal and genital. Samples were collected using sterile cotton wool pads secured to the plastic rod and placed in sterile tubes (standard product). After sampling, the samples were refrigerated until bacteriological examination performance. Pathological materials were plated on agar with 5% defibrinated sheep blood poured into Petri dishes. *Staphylococcus* strains isolated and purified were tested on biochemical and pathogenic characters.

Mannitol fermentation tested on Chapman Agar (*Staphylococcus* Selective Agar, Liofilchem, Italy). Testing fermentation about other sugars, including maltose, was made using Api Staph multi test systems.

Isolates were identified as species using Api Staph system.

Pathogenic factors controlled were haemolysins and the presence of the two types of coagulase.

Highlighting coagulase was made by the two techniques used for this purpose. To highlight coagulase-related, used was Prolex STAPH Latex rapid kit produced by Pro-Lab Diagnostics. Free diffusible coagulase was highlighted from technique in tubes using four types of citrated plasma. To test the sensitivity to antibiotics, staphylococci strains isolated was used Kirby-Bauer disc diffusion method, using the following ingredients: Mueller-Hinton broth and agar, Petri dishes and bio discs impregnated with antibiotics (Oxoid). The following antibiotics were used: methicillin, ampicillin with sulbactan, amoxicillin with clavulanic acid, tetracycline, doxycycline, gentamicin, kanamycin, erythromycin, vancomycin, ciprofloxacin, polymyxin B, novobiocin, rifampicin, pristinamycin, lincomycin, ceftrioxone, cefoxitine and cefaclor. This has been tested staph sensitivity, aiming types of resistant strains and methicillin resistance frequency.

## RESULTS AND DISCUSSION

In this study were isolated 28 strains of staphylococci, including 20 coagulase positive strains (CoP, represented by *S. hyicus* and *S. aureus*) and eight coagulase negative strains (CON, represented by *S. haemolyticus*, *S. sciuri* respectively *S. epidermidis*) isolated from pigs in different anatomical areas. Bacteriological examination results are shown in Table 1.

Table 1.

The distribution of strains of staphylococci isolated from pigs

| Age category/anatomical area | Number of samples | Positive samples | Strains of staphylococci isolated | | | | |
|---|---|---|---|---|---|---|---|
| | | | ***S. aureus*** | ***S. hycus*** | ***S. epidermidis*** | ***S. sciuri*** | ***S. haemolyticus*** |
| Sows with piglets | | | | | | | |
| skin-nipples | 18 | 5 (27,77%) | 2 | 2 | - | - | 1 |
| Peri vulvar | 10 | 3 (30,0%) | 1 | 1 | - | 1 | - |
| Pregnant sows | | | | | | | |
| skin-peri vulvar | 11 | 2 (18,18%) | - | 1 | - | - | 1 |
| nipples | 9 | 3 (33,33%) | 2 | 1 | - | - | - |
| Weaned piglets | | | | | | | |
| Skin-ears | 18 | 7 (38,88%) | 3 | 2 | - | 1 | 1 |
| Fat pigs | | | | | | | |
| skin | 21 | 8 (38,09%) | 1 | 4 | 1 | 1 | 1 |
| **TOTAL** | **87** | **28 (32,18%)** | **9** | **11** | **1** | **3** | **4** |

Strains of staphylococci unexposed to the pressure of antibiotics are sensitive to these substances; however, isolates from pigs with various conditions under pressure due to antibiotic therapy may show multiple resistance phenomenon.

The results of antibiotic susceptibility testing of *Staphylococcus*strains isolated from pigs are shown in Table 2.

Table 2.

The results of the sensitivity to antibiotics of *Staphylococcus* strains isolated from pigs

| Name of antimicrobial substance (initials-MIC) | Number of susceptible isolates | | | | |
|---|---|---|---|---|---|
| | *S. hyicus* (n=11) | *S. aureus* (n=9) | *S. haemolyticus* (n=4) | *S. sciuri* (n=3) | *S. epidermidis* (n=1) |
| Methicillin - ME - 30µg | 9 | 7 | 4 | 3 | 1 |
| Gentamicin – CN – 10µg | 5 | 6 | 4 | 3 | 1 |
| Tetracycline – TE – 30µg | 4 | 5 | 2 | 3 | 1 |
| Ciprofloxacin – CIP – 30 µg | 11 | 9 | 4 | 3 | 1 |
| Kanamycin – K – 30 µg | 8 | 7 | 4 | 3 | 1 |
| Novobiocin – NV – 30 µg | 11 | 9 | 4 | 3 | 1 |
| Doxycycline – DO – 30 µg | 7 | 7 | 1 | 1 | 1 |
| Erytromycin – E – 15 µg | 6 | 6 | 1 | 1 | 1 |
| Vancomycin – VA – 30 µg | 11 | 9 | 4 | 3 | 1 |
| Ceftriaxone – CRO – 30 µg | 11 | 9 | 4 | 3 | 1 |
| Cefoxitine – FOX - 10µg | 11 | 9 | 4 | 3 | 1 |
| Polymyxin – PB – 50UI | 0 | 0 | 1 | 1 | 0 |
| Rifampicin – RA – 30 µg | 11 | 9 | 4 | 3 | 1 |
| Lincomycin – L – 30 µg | 5 | 4 | 2 | 3 | 1 |
| Cefaclor – CEC – 30 µg | 11 | 9 | 4 | 3 | 1 |
| Pristamycin – PT – 15 µg | 11 | 9 | 4 | 3 | 1 |
| Ampicillin/sulbactan – SAM – 30 µg | 11 | 9 | 4 | 3 | 1 |

Were monitored during the study the phenomenon of multiple resistances, namely resistance type frequency methicillin-resistant strains of a number of 28 strains of staphylococci isolated from pigs using 17 different classes of antibiotics (Table 3).

Table 3

The resistance type of the species of staphylococci isolated from pigs

| Species of stafilcoci | Resistant strains | The antimicrobial resistances models (no. of resistant strains) |
|---|---|---|
| *Staphylococcus hycus* | 6/11 | TE, K, DO, E, **ME**, PB (1), K, PB, TE, L (1), TE, PB, L, DO (1), PB, DO, E, L, **ME** (1), PB, TE, DO, E, L, CN (2) |
| *Staphylococcus aureus* | 4/9 | TE, CN, L, K, **ME** (1), CN, DO, E, PB (1), TE, CN, L, K, PB (1), CN, PB, E, L, **ME** (1) |
| *Staphylococcus haemolyticus* | 2/4 | CN, TE, E, L, K, PB (1), CN, PB, E, DO (1) |
| *Staphylococcus sciuri* | 1/3 | TE, CN, E, PB, L (1) |
| *Staphylococcus epidermidis* | 1/1 | PB (1) |

Analysing the results in the table it can be seen that the sensitivity to antibiotics was variable depending on the group of antibiotics.

In the case of antibiotics: novobiocin, rifampicin, pristinamycin, ciprofloxacin, vancomycin, ceftriaxone, cefoxitin, cefaclor and ampicillin/sulbactan, considered the drug of choice for staphylococci, the number of sensitive strains were 100%, all isolates were sensitive (Table 2). This suggests that isolates tested came from pigs to which these antibiotics were not used. Also, it can be said that all of these antibiotics is kit from a staphylococcal infections or typically used in humans, in the treatment of this infections in animals, respectively.

β-lactams (methicillin, ceftriaxone, cefoxitin, cefaclor and ampicillin with sulbactan), sensitivity was highest, except *Staphylococcus aureus*, which were isolated 4 methicillin resistant strains. Of these two methicillin resistant strains of *S. hycus* and two strains of *S. aureus* (Table 3). The strains tested were mostly sensitive to β-lactams other as a result of previous treatments done correctly.

The phenomenon of antibiotic resistance in the case of β-lactam is based on the type of

genetic determinants of plasmid and chromosomal governing the synthesis of β-lactamase, broad spectrum, which provides the resistance of staphylococci. Resistance to methicillin is transmitted by plasmids (R factor) having a pattern common to other β-lactams. For this reason, methicillin-resistant staphylococcal strains are considered zoonotic risk strains of staphylococci particularly with a complex circuit human-animal - human, respectively (King et al. 2006; Kluytmans et al. 2006; Vandenesch et al. 2003; Weese et al. 2011; Wulf et al. 2006).

Aminoglycosides (gentamicin, kanamycin) and macrolides (erythromycin and vancomycin), sensitivity was different, the maximum to vancomycin (Table 2). In the case of gentamicin, were isolated 9 resistant strains, 5 strains resistant to kanamycin and 9 strains resistant to erythromycin (Table 3).

Most of the strains were resistant to polymyxin B (13 strains), due to an excessive use of this antibiotic in the past (Table 3).

Sensitivity to tetracyclines (tetracycline, doxycycline) was reduced, 15 strains were resistant to this group of antibiotics, to the phenomenon of resistance is plasmid and chromosomal type (9 strains to tetracycline and 6 strains to doxycycline) (Tables 2, 3).

All strains tested were sensitive to ciprofloxacin.

The development of staphylococci resistance to different antibiotics is a consequence of wasteful use in the treatment of diseases in pigs. Antibiotics used irrationally creates a selection pressure, are selected and transmitted genetic determinants of plasmid and chromosomal type. Consequently, the phenomenon of multiple resistance that is transmitted intra and interspecific. It is important particularly because the resistance to methicillin can be associated with resistance to β-lactams and other groups of antibiotics (Gibbs et al. 2006; Guardabassi et al. 2006; Smith et al. 2013).

After testing staphylococci strains isolated from pigs against 17 antibiotics identified methicillin-resistant strains and various types of resistance against β-lactams, tetracyclines, macrolides and polymyxin B.

The data on methicillin resistance and type of resistances identified are similar to the results communicated by other authors on the phenomenon of resistance to antibiotics (Gibbs et al. 2006; Guardabassi et al. 2006; Smith et al. 2013).

*S. aureus* is an epiphyte, a normal microorganism in pigs, and occurs in all herds (Armand Lefevre et al. 2005). The prevalence of MRSA strains in pig herds varies widely (0-50%) among European countries (Anon, 2009). Actual prevalence of MRSA in pigs in North America is uncertain, but appears to be lower than in many European countries (Smith et al. 2013; Wertheim et al. 2004). The prevalence of MRSA is high (> 50%) in pigs from herds positive but has little effect on the health of pigs.

*S. aureus* is found in dust and air in the pigs farms (Gibbs et al. 2006), and healthy people working in these farms and shelters, often are carriers of *S. aureus* from pig nasal mucosa (Armand Lefevre et al. 2005; Khanna et al. 2008; Smith et al. 2013; Voss et al. 2006; Wan Mintao et al. 2013). MRSA can be detected in the case of 20-80% of clinically healthy workers operating in MRSA positive pig herds, much more than other categories of people (1.5% in the US; <0.11% in Netherlands) (Bode et al. 2011; Gorwitz et al. 2008).

The risk of exposure to MRSA from animals is largely restricted to persons who have direct contact with animals and their families, respectively (Bisdorff et al. 2012; Cuny et al. 2009; Van Cleef et al. 2010).

The ability of *S. aureus* strains/animal MRSA to colonize, to spread and cause disease in humans remains uncertain. It seems that the line ST398 persists only for a short time (hours or days) to most people, but some can colonize for months or years without developing infections (Frana et al. 2013; Graveland et al. 2011; Sun et al. 2013; Van Cleef et al. 2011; Verkade et al. 2013). In Dutch hospitals was identified ST398 line spread between people and was four times more common than MRSA strains of human origin. There have been described outbreaks of infection of MRSA ST398 line data so far. Other lines of MRSA may also occur in pigs (eg., ST9 in Asia, North America ST5), but public health implications are unknown.

## CONCLUSIONS

In one swine farm were isolated 28 *Staphylococcus* strains including 20 coagulase positive strains (CoP, represented by *S. hyicus* and *S. aureus*) and eight coagulase negative strains (CON, represented by *S. haemolyticus*, *S. epidermidis*, respectively *S. sciuri*) from clinically healthy pigs in different anatomical areas.

All *Staphylococcus* strains isolated from pigs showed sensitivity of 100% for antibiotics: novobiocin, rifampicin, pristinamycin, ciprofloxacin, vancomycin, ceftriaxone, cefoxitin, cefaclor and ampicillin/sulbactan, considered the drug of choice for these bacteria.

β-lactams (methicillin, ceftriaxone, cefoxitin, cefaclor, and ampicillin with sulbactan) sensitivity was highest, except *Staphylococcus aureus*, which were isolated four methicillin-resistant strains, two of *S. hycus* and two of *S . aureus* strains.

After testing staphylococci strains isolated from pigs, against 17 antibiotics, were identified methicillin-resistant strains and more type of resistances, against β-lactams, tetracyclines, macrolides and polymyxin B.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# PREVALENCE OF MASTITIS AND DYNAMICS OF HEALTH STATUS MAMMARY GLAND DURING LACTATION AND DRY PERIOD IN GOATS

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***Abstract***

*The purpose of this study was to investigate the prevalence of mastitis and dynamics of different mastitis forms in goats during lactation and the dry period .The prevalence of mastitis was analyzed in 250 goats. Parallel traced 52 dairy halves with different mastitis forms during lactation and 56 halves during the dry period. Express diagnosis was made on the farm with rapid mastitis test CMT-Test (Kruuse, Denmark) and test Porta SCC (Porta Check, USA). For precise determination of the health condition of all dairy halves was conducted laboratory analysis including determining the number of somatic cells by Fossomatic (Foss, Denmark) and microbiological testing for isolation of pathogenic microorganisms. Prevalence of mastitis was found within 45.8 % and results indicated that 41.67 % of the cases diagnosed with subclinical mastitis in early lactation, persisted at the end of the lactation period. At the same time the latent infection persisted in 15.38 % while secretory disorder was in 26.67 %. During the dry period the highest percentage of persisting indicated subclinical mastitis - 71.43 % and only 14.29 % were found healing, compared with secretory disorder that persisted in 42.86 % as they were healed halves. The latent infection persisted also in 42.86 %, but healing again was found only in 14.29 %. Non-clinical mastitis in the absence of the treatment are stored in 76.19%*
***Key words****: goats, mammary gland, health status, mastitis, dynamics*

## INTRODUCTION

Mastitis is inflammation of the mammary gland, which causes biochemical, physical and bacteriological changes in the milk of affected animals (Matthews,1999). Mastitis is commonly associated with poor hygienic practices as well as with trauma of the mammary tissue and papillae or other types of skin wounds, as the skin is an important barrier against infections. In goats, under the influence of stress factors, such as extreme temperature changes, unhygienic and damp rearing conditions or a sudden change in the diet, the immune system needs to respond quickly against the invasion of pathogens that cause mastitis. That is why it is of particular importance to study the dynamics of non-clinical inflammation of the mammary gland in goats during the lactation and dry period. This is commonly based on observations of the duration of infection, the shift from one form into another and cases of recovery without therapeutic intervention. Such studies are important to precisely identify the animals that should be subject to treatment and the choice of appropriate time for intracisternal infusion as well as to develop a general strategy for control of mastitis in each particular farm.

## MATERIALS AND METHODS

### Animals included in the study

The prevalence of mastitis was analyzed in 250 goats. To study the dynamics of mammary gland inflammation, the animals were grouped into experimental groups consisting of a different number of animals depending on the condition of the mammary gland. The study included farms located in four different administrative districts in Bulgaria.

### Sample collection

Milk samples were collected aseptically from all udder halves. Prior to sample collection, the udder and papillae were washed and then disinfected with 70° ethanol. The first portions of milk were discarded, then duplicate samples were collected from each udder half. Samples for microbiological analysis (10 ml) were collected in sterile tubes and those for somatic cell counts (50 ml), in milk containers. Milk samples were transported to the laboratory in an ice chest at 4°C and all analytical procedures

were performed within 16 hours from sample collection.

***Cytological analysis***

Cell counts were indirectly determined by the rapid mastitis tests CMT-Test (Kruuse, Denmark) and Porta SCC (Porta Check, USA). The results from the CMT-Test were interpreted based on the Smith and Sherman (2009) scale, and those from the Porta SCC test, based on the manufacturer's instructions.

Direct determination of somatic cell counts was performed according to BS EN ISO 13366-2/IDF 148-2:2006 at the National Reference Laboratory for Milk and Dairy Products, Regional Directorate for Food Safety–Sofia (Bulgaria), using Fossomatic (Foss, Denmark).

***Microbiological analysis***

Isolation and identification of microorganisms causing mastitis in goats was carried out according to routine methods (National Mastitis Council, 1999). Ten-microliter aliquots from each sample were inoculated on elective and selective culture media for bacteria and fungi and were incubated at 37°C and 28°C for 24–72 h in aerobic conditions. Bacterial isolates were taxonomically identified based on Gram and Pfeiffer staining, cultural characteristics and biochemical properties determined using Polymicrotest (National Center for Infectious and Parasitic Diseases–Sofia) and additional tests for oxidase, catalase activity etc. using reagents from Antisel – Scharlau Chemie S. A. (Spain). Identification of bacterial isolates was done using Bergey's Manual.

***Statistical analysis***

Statistical analysis was performed using the program SPSS 16.0.

**RESULTS AND DISCUSSIONS**

It was shown that, of the 500 studied udder halves, 54.2% were found to be healthy, and 45.8%, with a different type of mastitis (Table 1). These results are similar to those observed by Contreras et al. (1999) but higher than the 36.4% reported by Whiteand Hinckley (1999) in the USA. There are, however, data from other goat farms showing even higher percentages ranging from 45.25% to 82.51% (Mhase et al. 2007). This considerable prevalence of affected udder halves highlights how significant the problem is in goats.

Detailed data about the relative share of different mastitis forms in the studied animals are presented in Table 2. There was subclinical mastitis (SM) in 31.6% of the examined udder halves, whereas udder secretion disorder (SD), in 8%, and latent infections (LI) and clinical mastitis (CM), in 3.4% and 2.8%, respectively. The prevalence of subclinical mastitis in our study was similar to the 33% reported by Hall and Rycroft (2007) in the United Kingdom and the 29%, by Boscos et al.(1996) in the region of Thessaloniki (Greece). However, Kostelić et al.(2009) determined 20% prevalence, and **Persson and Olofsson (2011),** 18% in Sweden, suggesting that there are high variations in the prevalence of subclinical mastitis.

The prevalence of CM observed by us, 2.8%, is in agreement with the results of Bergonier et al. 2003, who showed it to be under 5%. A slightly higher percentage (6.33%) has been reported by Ameh et al. (2000), unlike Jensen et al. (1996), who observed 37% prevalence of CM. In previous studies in Bulgaria, Bozhkova et al. (2000) showed 7.26% and 39.05% prevalence of clinical and subclinical mastitis in goats, respectively, which, compared to the results from our study, indicates a reduction in these two types of mastitis over the last 15 years.

Together with the prevalence of mastitis, we studied the dynamics of different forms of mammary gland inflammation during a lactation period and its subsequent dry period. The dynamics of subclinical mastitis during the lactation period is presented in Table 3. Of all SM cases diagnosed at the beginning of the study, 41.67% persisted until the dry period. In comparison, this percent in sheep is twice as high, 86.11% (Koleva, 1998). Despite this, with time, there was a steady trend for gradual self-healing during lactation, the percentage of self-healed udder halves at the end of lactation being 20.83%. These results indicate that the self-healing ability of the mammary gland in goats could be considered higher than that in buffalo, where the self-healing rate is 12.7% (Parvanov,2000).

Table 1. Prevalence of mastitis in goats

| State dairy halves | n (500) | P (%) | Δ |
|---|---|---|---|
| **Healthy** | 271 | 54,2 | 4,76 |
| **With mastitis** | 229 | 45,8 | 3,86 |

Table. 2. Prevalence of various forms of mastitis in goats

| State dairy halves | | n (229) | P (%) | Δ |
|---|---|---|---|---|
| **With mastitis** | **clinical mastitis** | 14 | 2,8 | 1,12 |
| | **subclinical mastitis** | 158 | 31,6 ** | 3,64 |
| | **secretory disorder** | 40 | 8 ** | 1,85 |
| | **latent infection** | 17 | 3,4 ** | 1,23 |

* p<0.05, ** p<0.01, *** p<0.001

Table. 3. Dynamics of subclinical mastitis during lactation in goats

| | 1 month | | 3 month | | 5 month | | 7 month | | 9 month | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | n | % |
| **Self-healing** | | | 1 | 4,17 | 3 | 12,5 | 3 | 12,5 | 5 | 20,82 |
| **Secretory disorder** | | | 2 | 8,3 | 3 | 12,5 | 4 | 16,67 | 3 | 12,5 |
| **Latent infection** | | | | | 1 | 4,17 | 1 | 4,17 | 1 | 4,17 |
| **Subclinical mastitis** | 24 | 100 | 20 | 83,3 | 14 | 58,33 | 12 | 50 | 10 | 41,67 |
| **Clinical mastitis** | | | 1 | 4,17 | 2 | 8,33 | 1 | 4,17 | 1 | 4,17 |
| **Cured** | | | | | 1 | 4,17 | 3 | 12,5 | 4 | 16,67 |

Table. 4. Dynamics of latent infection during lactation in goats.

| | 1 month | | 3 month | | 5 month | | 7 month | | 9 month | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | n | % |
| **Self-healing** | | | 1 | 7,69 | 1 | 7,69 | 1 | 7,69 | 2 | 15,38 |
| **Secretory disorder** | | | 3 | 23,08 | 3 | 23,08 | 2 | 15,39 | 2 | 15,38 |
| **Latent infection** | 13 | 100 | 6 | 46,15 | 5 | 38,46 | 4 | 30,77 | 2 | 15,38 |
| **Subclinical mastitis** | | | 3 | 23,08 | 3 | 23,08 | 4 | 30,77 | 5 | 38,48 |
| **Clinical mastitis** | | | | | 1 | 7,69 | 1 | 7,69 | | |
| **Cured** | | | | | | | 1 | 7,69 | 2 | 15,38 |

Table. 5. Dynamics of secretory disorders during lactation in goats

| | 1 month | | 3 month | | 5 month | | 7 month | | 9 month | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | n | % |
| **Self- healing** | | | 4 | 26,67 | 5 | 33,33 | 6 | 40 | 5 | 33,32 |
| **Secretory disorder** | 15 | 100 | 8 | 53,33 | 7 | 46,67 | 4 | 26,67 | 4 | 26,67 |
| **Latent infection** | | | 1 | 6,67 | | | 1 | 6,67 | 1 | 6,67 |
| **Subclinical mastitis** | | | 2 | 13,33 | 2 | 13,33 | 3 | 20 | 4 | 26,67 |
| **Clinical mastitis** | | | | | 1 | 6,67 | | | | |
| **Cured** | | | | | | | 1 | 6,67 | 1 | 6,67 |

Table.6. Dynamics of the non-clinical forms of mastitis in goats during the dry period

| Status of dairy halves before drying | dairy halves | Health status of dairy halves in early lactation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Healthy | | Subclinical mastitis | | Latent infection | | Secretory disorder | | Non-clinical mastitis (SM + LI + SD) | |
| | n | n | % | n | % | n | % | n | % | n | % |
| **Healthy** | 14 | 11 | 78,57 | 1 | 7,14 | 1 | 7,14 | 1 | 7,14 | 3 | 21,43 |
| **Subclinical mastitis** | 14 | 2 | 14,29 | 10 | 71,43 | | | 2 | 14,29 | | |
| **Latent infection** | 14 | 2 | 14,29 | 5 | 35,71 | 6 | 42,86 | 1 | 7,14 | | |
| **Secretory disorder** | 14 | 6 | 42,86 | 1 | 7,14 | 1 | 7,14 | 6 | 42,86 | | |
| **Non-clinical mastitis (SM + LI + SD)** | 42 | 10 | 23,81 | | | | | | | 32 | 76,19 |

Another important characteristic of the SM dynamics was the transition to SD at the end of lactation in 12.5% of the cases. Throughout the lactation period, the percentage of CM remained relatively constant at 4.17%. These cases were all treated in a timely manner and were presented in the results as having been cured. Thus, at the end of lactation their relative share was 16.67%. A percentage as low as that indicates that the mammary gland could be considered resistant to clinical forms of inflammation despite the presence of subclinical infection. Regarding the transition of SM to LI, the latter became observable as late as the 5$^{th}$ month of lactation in just 4.17% of the cases and remained at this level until the end of lactation.

One of the main characteristics of LI dynamics in goats is its gradual but steady decrease with time during lactation. At the end of lactation, LI was diagnosed in 15.38% of the studied udder halves (Table 4). Most of the udder halves showing LI at the end of lactation were diagnosed with subclinical mastitis (38.48%). These dynamics are clinically important, especially at the end of lactation, and should be taken into account in the drying-off of udder halves. This is also supported by the results for the relative shares of self-healed udder halves and those with SD at the end of lactation, which were both 15.38%.

Regarding the dynamics of SD (Table 5), it is noteworthy that there is still no universal global standard about the somatic cell counts per 1 ml milk from healthy mammary glands in goats. That is why for the purpose of the present study we assumed that there was SD in the udder halves showing over $500\times10^3$/ml somatic cell counts. Compared to all other forms of non-clinical mastitis, SD showed the highest self-healing percentage at the end of lactation, 33.32%. At the same time, the percentage of udder halves diagnosed with SM (26.67%) was the same as that with SD. It was only in rare cases that SD showed complications and developed into CM (6.67%) and this was only observed in the 5$^{th}$ month of lactation. The trend for LI, which was also diagnosed in 6.67% of the udder halves, was, however, different in that it occurred throughout the whole lactation period. These results in goats are in agreement with data in sheep, where according to Koleva (1998), SD persisted in 40% of cases, was self-healed in30%and developed into CM in another 30%.

The dynamics of the non-clinical forms of mastitis in goats during the dry period (Table 6) showed that they remained persistent in 76.19% of the cases and were self-healed in 23.81%. Of the udder halves diagnosed with SM at drying-off, 71.43% showed the same condition at the beginning of the next lactation period. There was self-healing and SD in 14.29%. LI remained persistent in 42.86%, developed into SM at the beginning of lactation in 35.71%, or into SD, in 7.14%. The rate of self-healing in this form of non-clinical mastitis was 14.59%. SD showed a tendency to persist in 42.86% and to develop into SM in 7.14% and into LI in another 7.14%.Of all forms of non-clinical mastitis, the self-healing rate was highest in SD, 42.86%.

The obtained results also showed that 78.57% of the udder halves that were diagnosed as healthy at drying-off remained healthy until the beginning of the next lactation. There were a total of 21.43% new cases of non-

clinical mastitis, including equal shares (7.14%) of each of the three forms of non-clinical mastitis.

The total relative share of self-healed udder halves was 23.81%, which indicates that the dry period has a positive effect on the mammary gland health. At the same time, this percentage only insignificantly exceeds the new cases of non-clinical mastitis. This insignificant difference between the percentages of self-healed cases and new ones, from a clinical point of view, suggests that the dry period could be considered an appropriate time for treatment and prophylaxis of non-clinical mastitis in goats.

## CONCLUSIONS

The results from this study showed a high prevalence of mastitis in goats. This indicates that it is necessary to perform regular screening of the mammary gland health in goats. The analysis of the dynamics of different mammary gland conditions during the lactation and dry period showed that special attention needs to be paid to subclinical mastitis. What is more, proper prophylaxis and treatment of non-clinical mastitis during the dry period in goats would reduce the losses due to mammary gland inflammations during the following lactation period.

## ACKNOWLEDGEMENTS

This research work was carried out with the support of University of Forestry and project BG051PO001-3.3.06-0056 "Support for the development of young people in University of Forestry", Operational Programme "Human Resources Development" financed by the European Social Fund of the European Union.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# EPIDURAL DELIVERY OF LIDOCAINE AND TRAMADOL TO CONTROL PAIN DURING OVARIOHISTERECTOMY IN THE BITCH

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***Abstract***

*Epidural anesthesia is a simple, safe and effective way to administer anesthesic and analgesic drugs for caudal abdominal surgeries in canines. The aim of the study was to compare the analgesic effect of lidocaine or lidocaine-tramadol association administered epiduraly during ovariohisterectomy in the bitch. 14 bitches, ASA status 2 to 4, were premedicated with midazolam 0,2 mg/kg, butorphanol 0,2 mg/kg, induced with propofol 5 mg/kg iv and mantained with isoflurane. For 7 bitches lidocaine 2 mg/kg was administered epiduraly (L batch), while for the other 7 lidocaine was associated v:v with tramadol (LT batch). Analgesia was monitored intraoperatory (pulse frequency, non invasive blood presure, muscle relaxation) and postoperatory for the first 4 hours using the Glasgow pain scale. Blood pressure remained constant for both groups. There was a deduction of volatile agents in both groups, the bitches being extubated soon after stopping the delivery of anesthesic gas. Glasgow pain score was higher for the L batch (10) ompared to the LT batch (5), showing a higher analgesic capacity for the lidocaine-tramadol association. There were no complications, adverse effects or technique related difficulties for the epidural anesthesia in the bitches included in this study. In conclusion, the association between lidocaine and tramadol for epidural anesthesia represents an efective, cheap and simple alternative for analgesia during ovariohisterectomy in the bitch.*

***Key words:*** *lidocaine, tramadol, bitch, epidural anesthesia*

## INTRODUCTION

Research on pain concept and the importance of its alleviation brought regional anesthesia in plane sight, in order to complete general anesthesia. Regional anesthesia presents many advantages: is less aggressive than general anesthesia, especially towards the cardio-vascular system and is cheap, with an easy technique. Epidural anesthesia is recommended in bitches for C-section because it does not negatively affect the puppies, while the mother stays awake and can take care of the newborns immediately after the intervention (Tranquilli et al., 2007). It is also recommended for interventions on the genital area or for orthopedics surgeries (Jones, 2001; Pohl et al., 2012; Sarotti et al., 2014).

Epidural anesthesia requires administration of local anesthetic substances outside dura mater at the sacro-lombar space (Gregori et al., 2014). In dogs it has been reported a maximal spread of anesthetic blockade until to fourth or sixth lumbar vertebra following lumbosacral extradural lidocaine which is usually unsatisfactory for ovariohisterectomy, because the ovarian pedicle is innervated by third and fourth lumbar afferent nerves. Thus, some studies have proposed the extradural administration of an opioide in combination with lidocaine in an attempt to spread the sensory blockade, optimizing the anesthesia to lower abdominal surgical procedures in dogs (Cruz et al., 1997; Diniz et al., 2013; Gasparini et al., 2007; Saritas et al., 2014).

In dogs, reported adverse effects following epidural anaesthesia include delayed hair re-growth in 11%, urinary retention in 3–44% and pruritus affecting the lumbosacral area, with an incidence of < 2% (Campoy et al., 2012; Kalchofner Guerrero et al., 2014).

The purpose of this study was to compare the analgesic effect of simple lidocaine and of a lidocaine-tramadol mixture administered epidurally during ovariohisterectomy in bitches. Also, we assumed that epidural anesthesia will reduce the requirement of inhalatory agent, reducing the cardio-respiratory depression and leading to a faster recovery.

## MATERIALS AND METHODS

14 client-owned bitches undergoing surgery of the genital apparatus and classified as ASA (American Society of Anesthesiologists) category II to IV were included.

Dogs were assigned to one of the two treatment groups by block randomization: group L (n = 7) received an epidural injection of lidocaine 2 mg/kg and group LT (n = 7) received epidural lidocaine associated v:v with tramadol, not exceeding 6 ml per bitch..

Dogs were fasted for 10–12 hours before surgery. On the day of surgery an intravenous catheter was placed and dogs were premedicated with with midazolam 0,2 mg/kg and butorphanol 0,2 mg/kg intravenously (IV). Ten minutes later, anaesthesia was induced with propofol 5 mg/kg IV to effect. Following endotracheal intubation, anaesthesia was maintained with isoflurane. Lactated Ringer's solution (LRS) 5 ml/kg was administered to all dogs during the procedure.

The lumbosacral area was clipped and surgically prepared with povidone-iodine solution. Epidural anaesthesia was performed with the dog placed in sternal recumbency and fully extended hind limbs. A 21 gauge spinal needle was introduced in the lombo-sacral space (figure 1). Correct needle placement was confirmed by the hanging drop test.

Analgesia was monitored intraoperatory (pulse frequency, non invasive blood presure, muscle relaxation) and postoperatory for the first 4 hours using the Glasgow Composite Pain Scale (Holton et al., 2001: Murrell et al., 2008). Maximum Glasgow pain score that can be obtained is 20. Administration of an analgesic is recommended for all the Glasgow scores above 5.

Statistical analysis was performed using a commercial software (Statview® 5.1, Software SAS Inc. Cary). Significance was identified at $p < 0.05$. Data are expressed as mean ± standard deviation (SD).

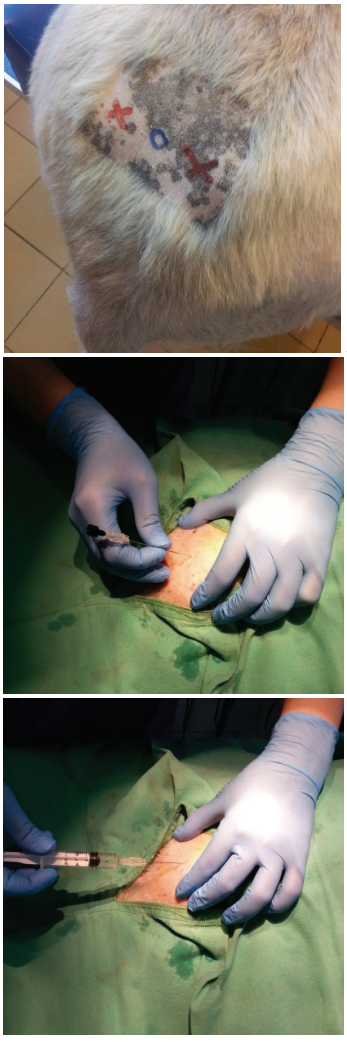


Figure 1. Identification of the lumbo-sacral space (A) and epidural administration of anesthetic agents (B and C).

## RESULTS AND DISCUSSIONS

14 bitches were neutered in the Clinic of Obstetric and Ginecology of the Faculty of Veterinary Medicine of Bucharest due to different affections (figure 2). The age of the bitches varied between 1,5 and 17 years,with a mean age of 8,7 years (table 1).

Table 1. Distribution of breeds and age of the bitches in the experimental groups.

| Nr. | Breed | Age (years) | Weight (kg) |
|---|---|---|---|
| 1. | Mioritic romanian shepherd | 9 | 47 |
| 2. | Caucasian shepherd | 1,5 | 60 |
| 3. | Poodle | 17 | 10 |
| 4. | Cane Corso | 2 | 49 |
| 5. | Siberian husky | 12 | 36 |
| 6. | Pekinese | 11 | 6,1 |
| 7. | Labrador | 7 | 27 |
| 8. | American Staffordshire Terrier | 3 | 22 |
| 9. | Mix breed | 1,5 | 36 |
| 10. | Mix breed | 12 | 6 |
| 11. | Mix breed | 15 | 13,6 |
| 12. | Mix breed | 15 | 7,5 |
| 13. | Mix breed | 7 | 4,5 |
| 14. | Mix breed | 9 | 14 |

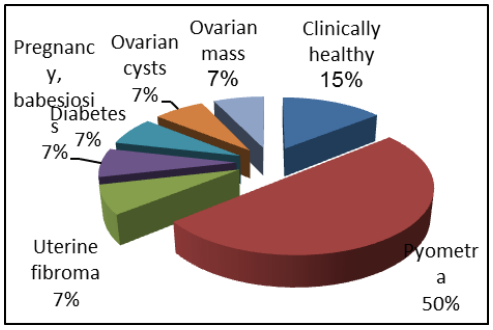


Figure 2. Distribution of affections in the experimental group.

The mean time duration from epidural anesthesia administration to sitting position was 35 minutes for the L group. For the LT group, after surgery, the bitches did not move their tale or hind limbs, the anus was relaxed, showing the persistance of epidural anesthesia. Sitting position was adopted in 50 minutes since the administration of lidocaine-tramadol. The mixture of lidocaine with tramadol prolonged the anesthetic effect of lidocaine with 15 minutes.

Blood pressure was relatively constant throughout the surgical procedure. Hypotension was noted in just one case during the postop time and it could be related to the epidural anesthesia.

Mean Glasgow score for the L group was 5,28 compared to 3,71 for the LT group. Rescue analgesia was required for 2 bitches (28,57%) in the L group. This shows a superior analgesic effect for the lidocaine-tramadol mixture compared to simple lidocaine.

Extubation time after ceasement of the inhalatory agent varied between 3 and 15 minutes for the L group and 2 – 15 minutes for the LT group. There is no significant diference between the two groups regarding extubation time.

There were no complications, adverse effects or technique related difficulties for the epidural anesthesia in the bitches included in this study.

## CONCLUSIONS

Blood pressure was not significantly influenced by the type of substance administered epidurally.

The lidocaine-tramadol mixture ensured a longer and more powerful anesthetic and analgesic effect compared to single lidocaine.

Epidural anesthesia reduced the requirement of volatile agents for maintanance of anesthesia, allowing a faster extubation.

In conclusion, the association between lidocaine and tramadol for epidural anesthesia represents an efective, cheap and simple alternative for analgesia during ovariohisterectomy in the bitch.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# THE PREVALENCE OF GASTROINTESTINAL PARASITES IN RED FOXES (*VULPES VULPES*) FROM WESTERN ROMANIA – PRELIMINARY STUDY

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***Abstract***

*In this study, the gastrointestinal parasites and prevalence of infestation in the red fox (Vulpes vulpes), were investigated at the laboratory of Parasitology of Veterinary Medicine Faculty Timisoara. During period December 2011 - January 2012, 20 red foxes from 12 hunting grounds of Arad County were necropsied. Animals were killed by shooting for establishing the effectiveness of rabies vaccination. Faecal samples and gastrointestinal masses were collected and examined for parasites. The presence of endoparasites was detected in 12 of 20 samples, and overall prevalence was 60%. Of the examined samples poliparasitism was observed in 10 (50%), and monoparasitism in 2 (10%). There were no significant differences between sex groups and intensity value. Male and female red foxes were infected with five genera from three classes of parasites: fluke - Alaria alata (40%), tapeworm (55%) - unidentified tapeworm (10%), Taenia taenieformis (10%), Taenia hydatigena (15%), Mesocestoides lineatus (40%), Taenia pisiformis (15%), roundworm (30%) - Ancylostoma spp., (25%), Pterygodermatites affinis (5%).*

***Keywords****: parasites, gastrointestinal, red fox, Romania*

## INTRODUCTION

Red foxes (*Vulpes vulpes*) is a common carnivorous, widely distributed throughout the world, considered also as opportunistic omnivorous (Dybing et al., 2013; Lahmar et al., 2014), with an important role in the transmission of diseases, some with zoonotic importance (Henderson, 2009; Wolfe et al., 2001).

Foxes alimentary habit makes them potential hosts for many species of gastrointestinal parasites that can be harmful to both humans and animals (domestic and wild) (Willingham et al., 1996).

It is well known that parasitic digestive infestations decreases performance in domestic animals and wildlife worldwide. The main effect is often subclinical with the reduction of appetite, reproduction, performance etc. However most studies focused on parasitological investigation of livestock, and recently, there was determined that parasitic infestations are as common and important also in wildlife which may serve as a potential reservoir of parasites.

The aim of this study was to determine the prevalence of infestation with gastrointestinal parasites in foxes from hunting grounds in western Romania.

## MATERIALS AND METHODS

### 1.1. Description of the sampling area

Arad is located at the western Romania, in the high plain of Arad, 30 km from Zarand Mountains, part of the Western Carpathians, with a landscape characterized by the presence of a tiered relief from east to west, a well distributed river network, most of them affluent of two major rivers Mures and White Cris, the presence of a temperate continental climate with oceanic influences and not least the presence of flora and fauna with high value items (www.wikipedia.org).

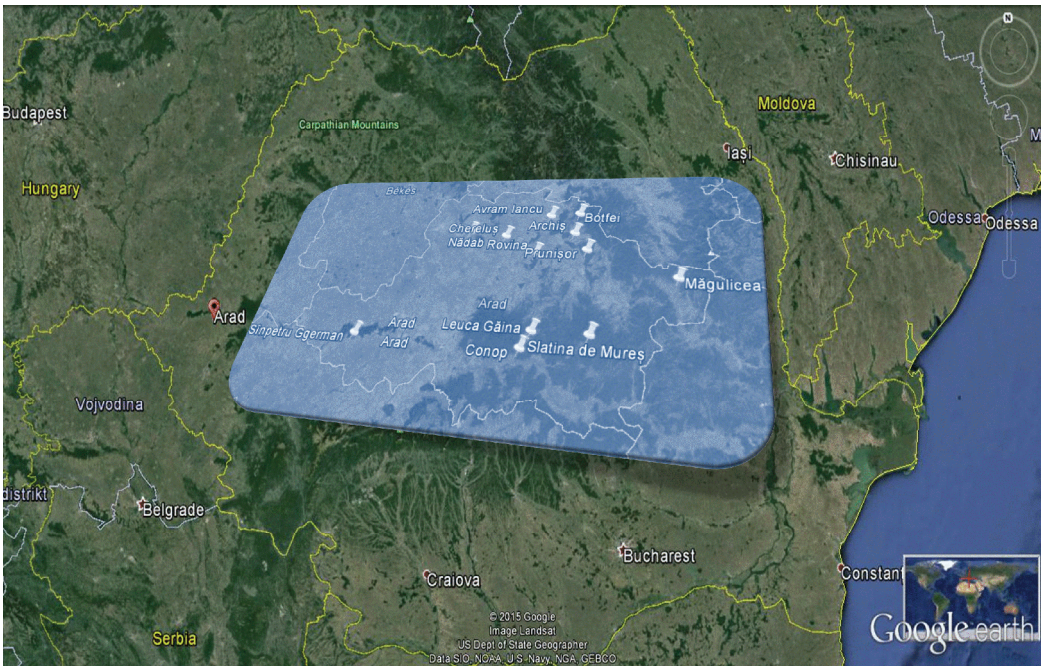


Fig. 1. The area of origin for sampled foxes

### 1.2. Animals studied, parasitological examination and epidemiological investigation

The presence of gastrointestinal parasitism was investigated in 20 foxes killed by shooting to determine the effectiveness of rabies vaccination in 12 hunting grounds between December 2011 and January 2012.

To identify the species of parasites coproscopic flotation methods and successive washings of faeces collected from gastrointestinal masses were performed.

Faecal samples were collected directly from the gastrointestinal mass of shot animals.

Identification of parasite species or genus was made in accordance with determination keys for trematodes, cestodes and nematodes, from the literature (Anderson, 2000; Dărăbuş et al., 2006, 2013; Dunn, 1978; Euzeby, 1963, 1971; Gibson et al., 2002; Khalil et al., 2004; Skryabin, 1992a, b).

For each animal, data on the origin of hunting, registration number and gender, were recorded thus compiling epidemiological investigation. Data were statistically analyzed using Fisher's test. Differences were considered statistically significant when p values were less than 0.05 ($p \leq 0.05$).

## RESULTS AND DISCUSSIONS

Coproparasitological exams revealed parasites divided into three classes and five genera, among which: trematodes (40 %) - *Alara* spp., Tapeworms (55 %) - *Taenia* spp., *Mesocestoides* spp., Nematodes (30 %) - *Ancylostoma* spp., *Pterygodermatites* spp. (table 1**).**

Among the 20 foxes examined, 12 were positive on the parasitological examination and the overall prevalence was 60 %. The results are shown in table 2 and figure 2. Of the samples examined 10 samples (50 %) were found with poliparazitism and 2 (10 %) with monoparasitism. Monoparasitism was found with *Alara alata* (1/20 – 5 %) and *Mesocestoides lineatus* (1/20 – 5 %). Poliparasitism was encountered in 10 animals, being the combination of trematodes, cestodes and nematodes.

The prevalence and intensity were calculated for gender group. The prevalence was not significantly different ($p = 0.7128$) between the

number of positive males (8/11, 67 %) and female foxes (4/9, 23 %). No statistically significant differences were found between gender of animals and intensity value.

Table 1. Synoptic data on foxes studied

| *Crt. No.* | *H.G. AR* | *Registration No.* | *Gender* | *Internal parasites species found* |
|---|---|---|---|---|
| **1** | Rovina | 164469 | F | Negative |
| **2** | Năbab | 202503 | F | Negative |
| **3** | Nadăş | 180092 | F | Negative |
| **4** | Archiş | - | M | *Taenia pisiformis* (n=11), *Taenia hydatigena* (n=1), *Alaria alata* (n=54), *Mesocestoides lineatus* (n=1) |
| **5** | Archiş | - | M | *Taenia taeniaeformis* (n=1), *Heterakis gallinae* (n=3) (Chicken legs found in the stomach) |
| **6** | Leuca Găina | 146380 | M | *Alaria alata* (n=10), *Mesocestoides lineatus* (n=58), *Ancilostoma* spp. (n=1), *Pterygodermatites affinis* (n=2) |
| **7** | Chereluş | 37986 | M | *Alaria alata* (n=1), *Mesocestoides lineatus* (n=12), *Ancilostoma* spp. (n=5), *Taenia pisiformis* (n=15), *Taenia hydatigena* (n=3) |
| **8** | Slatina de Mureş | 8 | M | *Alaria alata* (n=20), *Mesocestoides lineatus* (n=5), *Ancilostoma* spp. (n=5) |
| **9** | Conop | 202483 | M | *Mesocestoides lineatus* (n=5), Unidentified cestodes, *Heterakis gallinae* (n=3), *Ascaridia galli* (n=1) |
| **10** | Conop | 202282 | M | *Taenia pisiformis* (n=2), *Alaria alata* (n=99) |
| **11** | Avram Iancu, Măgulicea | 149259 | F | Negative |
| **12** | Avram Iancu, Măgulicea | 149302 | M | Negative |
| **13** | Avram Iancu, Măgulicea | 149209 | M | Negative |
| **14** | Slatina de Mureş | 7 | M | Negative |
| **15** | Botfei | - | F | *Alaria alata* (n=12) |
| **16** | Sînpetru German | 81937 | F | Negative |
| **17** | Sînpetru German | 81889 | F | *Alaria alata* (n=2), Unidentified cestodes |
| **18** | Prunişor | - | F | *Alaria alata* (n=5), *Mesocestoides lineatus* (n=5), *Ancylostoma* spp. (n=3), *Taenia hydatigena* (n=2), *Heterakis gallinae* (n=1) |
| **19** | Prunişor | - | F | *Mesocestoides lineatus* (n=18) |
| **20** | Prunişor | - | M | *Ancylostoma* spp. (n=3), *Taenia pisiformis* (n=2), *Alaria alata* (n=32) |

Legend: H.G. – hunting ground; F – female; M – male.

Table 2. Epidemiological data recorded in foxes in western Romania

| | | **No. of animals** | **No. of positive samples** | **P value** | **Prevalence %** |
|---|---|---|---|---|---|
| | Monoparasitism | 20 | 2 | | 10 |
| | Poliparasitism | 20 | 10 | | 50 |
| Gender | Male | 11 | 8 | 0.7128 | 73 |
| | Female | 9 | 4 | | 44 |
| **Total** | | **20** | **12** | | **60** |

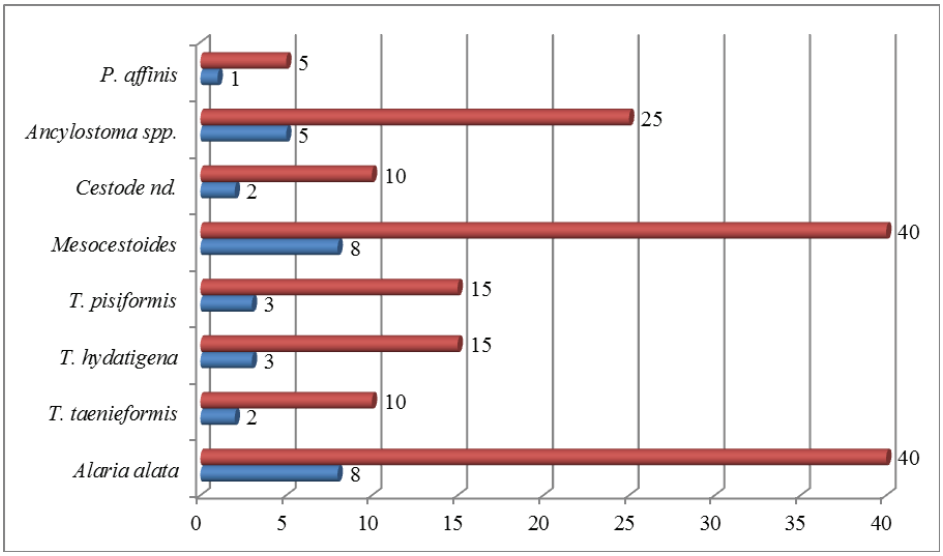


Fig. 2. Prevalence of gastrointestinal parasites in foxes in western Romania

Regarding the prevalence of parasites identified (figure 2) there have been found that *Alaria alata* was identified in eight foxes (40 %), unidentifiable cestodes in two animals (10 %), *Taenia taenieformis* two foxes (10 %), *Taenia hydatigena* in three animals (15 %), *Mesocestoides lineatus* in eight (40 %), *Taenia pisiformis* in three animals (15 %), *Ancylostoma* spp., five animals (25 %), *Pterygodermatites affinis* in one fox (5 %).

In a study conducted in Saudi Arabia, of 58 faecal samples examined there were found oocysts of coccidia (*Eimeria* and *Isospora*) *Tricocephalus* eggs spp., *Ancylostoma* spp., *Trichosomoides* spp. and oncosphera of *Taenia* spp. On the other hand after larva cultures there were revealed the presence of larvae species: *Ancylostoma caninum*, *Pterygodermatites affinis* and *Trichocephalus vulpis.* Of tapeworms there were identified *Joyeuxiella echinorynchoides, Diplopylidium nölleri, Taenia hydatigena,* and of acantocephali *Macracanthorhynchus catalinus (Alagaili et al., 2011).*

*Compared to our study there was observed in addition, Eimeria and Isospora, Tricocephalus* spp., *Joyeuxiella echinorynchoides, Diplopylidium nölleri and Macracanthorhynchus catalinus.*

Our study is the first to indicate nematode *Pterygodermatites affinis* infestation in Romania.

*Prevalence of helminths in red fox was studied in Western Australia, where there were highlighted: Dipylidium caninum (27.7 % of foxes) Uncinaria stenocephala (18.2 %), Toxocara canis (14.9 %), Spirometra erinaceieuropaei* (5.4 %), *Toxascaris leonina* (4.7 %), *Taenia serialis* (1.4 %), *Taenia hydatigena* (0.7%), *of* unidentifiable *Taenia species (4.1 %), Brachylaima cribbi* (0.7 %), *Plagiorchis maculosus* (0.7 %) *and an acantocephalus form Centrorhynchidae family (2.1 %)* (Dybing et al., 2013).

A study conducted by Barabasi et al., in 2007-2010, on 561 foxes in 15 counties in Romania revealed nematode parasitism (91.4 %) followed by cestodes (90.7 %) and trematodes (15 %). A total of 17 species of intestinal helminths were found: *Alaria alata, Dipylidium caninum, Echinococcus multilocularis, Mesocestoides lineatus, Taenia polyacantha, T. hydatigena, T. Multiceps, T. pisiformis, T. serialis, T., taeniaeformis T. crassiceps, T. ovis, Ancylostoma caninum, Uncinaria stenocephala, Toxascaris leonina, Toxocara canis* and *Trichuris vulpis* (Barabasi et al., 2010).

## CONCLUSIONS

The results of this study underline the importance and spread of gastrointestinal parasites in foxes in Romania, previously confirmed by researchers in other countries.
Foxes in Western Romania are simultaneously parasitized with trematodes, cestodes and nematodes of the genera *Alaria, Taenia, Mesocestoides, Ancylostoma, Pterygodermatites*.
It was reported for the first time in Romania, infestation with nematode *Pterygodermatites affinis* in foxes. However, for a more comprehensive picture of these parasites etiological further studies are still needed to identify species common with domestic animals, and also zoonotic species.

## ACKNOWLEDGEMENTS

The study was supported by the project Development of the research and education infrastructure, and services in the fields of veterinary medicine and innovative technologies for RO 05, code SMIS-CSNR 2669. This paper was published during the project POSDRU/159/1.5/S/132765.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# RISK OF EBOLA VIRUS DISEASE SPREAD OUTSIDE OF AFRICA: REVIEW OF NATURAL RESERVOIR AND TRANSMISSION

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***Abstract***

*The Ebola virus is one of the most virulent pathogen of humans. Until 2014 there have been reported 35 outbreaks, of which 25 in Africa, three in Asia (Philippines), three in America (USA) and four in Europe (Russia, UK, and Italy). Several outbreaks affected multiple countries. The most non-African human cases were accidentally produced in laboratories (researchers) and hospitals (medical staff). The largest outbreak of Ebola is still ongoing across West Africa (Guinea, Liberia, and Sierra Leone). Sporadic cases of illness and deaths have been reported outside of West Africa, in USA, Spain, UK, Nigeria, Senegal, and Mali. During February, 2014 - January 5, 2015were recorded 20,691cases of human illness and 8,168deaths (data are constantly evolving). The paper aims to present the epidemiologic characteristics of Ebola outbreaks that occurred from 1976 to 2014, in order to identify the source of infection and the route of transmission. The major source of Ebola virus infection identified in outbreaks with human casualties was the close unprotected physical contact with casualties. Another important source of human infection was wildlife. The natural reservoirs of Ebola virus are considered fruit bats (Hypsignathus monstrosus, Epomops franqueti,Myonycte ristorquata, Rousettus aegyptiacus) that spread the virus through urine and saliva. In two major human outbreaks with several casualties, (Zaire virus; Gabon; 1996-1997) the first sources of infection were chimpanzees. In seven outbreaks with asymptomatic human infections (Reston virus; Philippines, USA, Italy, 1989-2008) were involved apes and pigs, but the source of animal infection weren't identified. As a conclusion, the risk of Ebola virus disease spread outside of Africa is mainly associated with the international travel and the trade of live exotic animals. Ebola isn't an airborne disease, but direct exposure (percutaneous or mucous membrane) of people to infected blood or body fluids leads to the rapid transmission of the virus.*

***Key words****: epidemiologic risk factors, infection, transmission, Ebola virus disease outbreaks.*

## INTRODUCTION

The ebolaviruses is one of the most virulent pathogen of humans. It is a rare and deadly disease of humans caused by ebolaviruses (EBVs). EBVs has been included in the family *Filoviridae*, genus *Ebolavirus* (CDC, 2014; WHO, 2015).

Ebolavirus etymology derived from the headstream name of the Mongala River (Democratic Republic of the Congo), where the virus was encountered for the first time (Wildy, 1971).

There are five identified Ebola virus species, named from the region where each was originally identified: *Ebola virus* (EBOV) (formerly *Zaire ebolavirus)*, *Sudan ebolavirus* (SUDV), *Taï Forest ebolavirus* TAFV) (formerly *Côte d'Ivoire ebolavirus*), *Bundibugyo ebolavirus* (BDBV), and *Reston ebolavirus* (RESTV). The last one is not pathogenic for humans (Kuhn et al., 2010; CDC, 2014; WHO, 2015)

EBVs are pathogenic for humans and nonhuman primates (monkeys, gorillas, and chimpanzees) (CDC, 2014). The natural reservoir host remains unknown, but the most likely reservoir are different bat species (e.g. *Hypsignathus monstrosus, Epomps franquet, Myonycte ristorquata, Rousettusa egyptiacus*), most of them native to Africa (Leroy et al., 2005; Pourrut et al., 2009). One exception was reported, RESTV might have the bats from Philippinesas the reservoir host (e.g. *Rousettusamplexicaudatus*) (Taniguchi et al., 2011).

Depending of EBVs susceptibility, the animals can be classified in three epidemiological categories: (1) Accidental

hosts (e.g. humans) - develop serious disease, often fatal, and are the main sources of infection for humans and other animals. (2) Optional hosts – develop silent infections and are not sources of infection for humans or other animals. (3) Natural hosts –maintain the virus on the field (natural reservoir) byintra-species contagious infections (CDC, 2014).

The paper aimes to present the epidemiologic characteristics of Ebola outbreaks that occurred from 1976 to 2014, in order to identify the source of infection and the route of transmission.

## MATERIALS AND METHODS

In order to present the epidemiologic characteristics of Ebola outbreaks we've studied official reports of WHO, CDC, ECDC and scientific papers published after 1976.

The Ebola outbreaks included in this study are presented in table 1.

Table 1. Ebola outbreaks used in epidemiological study

| Year | Country | Ebolavirus |
|---|---|---|
| 2014 (Aug-Nov) | Democratic Republic of the Congo* | Zaire |
| Feb 2014-Present | Multiple countries | Zaire |
| Nov 2012-Jan 2013 | Uganda | Sudan |
| 2012 | Democratic Republic of the Congo* | Bundibugyo |
| 2012 | Uganda | Sudan |
| 2012 | Uganda | Sudan |
| 2011 | Uganda | Sudan |
| 2008 | Democratic Republic of the Congo* | Zaire |
| 2007 | Uganda | Bundibugyo |
| 2007 | Democratic Republic of the Congo* | Zaire |
| 2005 | Congo | Zaire |
| 2004 | Sudan | Sudan |
| 2003 (Nov-Dec) | Congo | Zaire |
| 2003 (Jan-Apr) | Congo | Zaire |
| 2001-2002 | Congo | Zaire |
| 2001-2002 | Gabon | Zaire |
| 2000 | Uganda | Sudan |
| 1996 | South Africa | Zaire |
| 1996 (Jul-Dec) | Gabon | Zaire |
| 1996 (Jan-Apr) | Gabon | Zaire |
| 1995 | Democratic Republic of the Congo* | Zaire |
| 1994 | Cote d'Ivoire | Taï Forest |
| 1994 | Gabon | Zaire |
| 1979 | Sudan | Sudan |
| 1977 | Democratic Republic of the Congo* | Zaire |
| 1976 | Sudan | Sudan |
| 1976 | Democratic Republic of the Congo* | Zaire |

* FormerRepublic of Zaire

## RESULTS AND DISCUSSIONS

Until 2014 have been reported 35 outbreaks, of which 25 in Africa, three in Asia (Philippines), three in America (USA) and four in Europe (Russia, UK, and Italy). Some outbreaks were included in different epidemiological studies as parts of the same event (non-African cases were liked with the outbreak that started in West Africa in 2014) (CDC, 2014; ECDC, 2015). The most non-African human cases were accidentally produced in laboratories (researchers) and hospitals (medical staff) (WHO, 2015).

The largest outbreak of Ebola is still ongoing across West Africa (Guinea, Liberia, and Sierra Leone). Sporadic cases of illness and deaths have been reported outside of West

Africa, in USA, Spain, UK, Nigeria, Senegal, and Mali. During February, 2014 - February25, 2015 were recorded 23,729 cases of human illness and 9,604deaths (data are constantly evolving) (CDC, 2015; WHO, 2015).

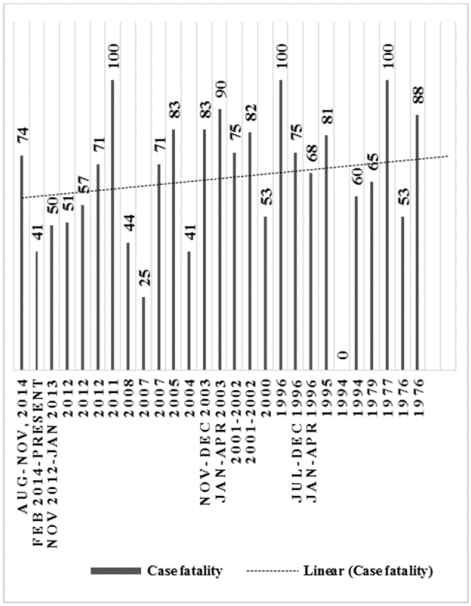


Figure 1. The proportion of Ebola case fatality in 27 outbreaks recorded from 1976 to February 2015. Linear trend line of case fatality has descending trend.

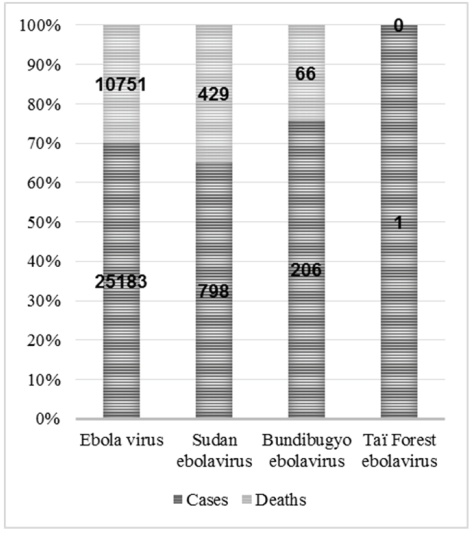


Figure 2. Comparative study of the case fatality in Ebolavirus species. It were compared only species proved to be pathogenic for humans. Datawere recorded from 1976 to February 25, 2015. *Sudan ebolavirus* has the highest proportion of fatality.

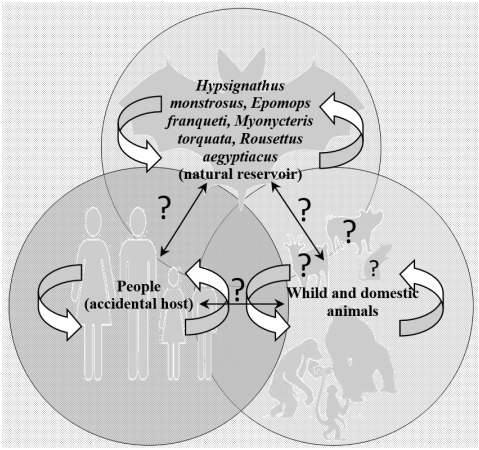


Figure 3. Sources and routes of infections in ebolaviruses infections

The major source of Ebola virus infection identified in outbreaks with human casualties has been the close unprotected physical contact with casualties (WHO, 2015).

Another important source of human infection is wildlife. The natural reservoirs of ebolaviruses are considered fruit bats (*Hypsignathus monstrosus, Epomops franqueti, Myonycteris torquata, Rousettusa egyptiacus*) that spread the virus through urine and saliva. In two major human outbreaks with several casualties, (Zaire virus; Gabon; 1996-1997) the first sources of infection were chimpanzees. In seven outbreaks with asymptomatic human infections (Reston virus; Philippines, USA, Italy, 1989-2008) were involved apes and pigs, but the source of animal infection weren't identified. (Leroy et al., 2005; Pourrut et al., 2009; Taniguchi et al., 2011; CDC, 2014, WHO, 2015, ECDC, 2015).

## CONCLUSIONS

The risk of Ebola virus disease spread outside of Africa is mainly associated with the international travel and the trade of live exotic animals.

The virus is not transmitted through dogs or cats but it's not recommended that they get into contact with casualties. Insects (e.g. mosquitos, flies) have no implications in spreading the virus.

Ebola is not an airborne disease, but direct exposure (percutaneous or mucous membrane) of people to infected blood or body fluids leads to the rapid transmission of the virus.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# PSEUDOACHONDROPLASIA IN DOGS – A CASE REPORT

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***Abstract***

*Here we present the radiographic findings of limb and spine in a 15-month-old female Bichon with pseudoachondroplasia (PSACH). PSACH is a rare form of osteochondrodysplasia, the main clinical characteristics are disproportionate short stature, abnormalities of the limbs and spine, abnormal walk, joint laxity and early osteoarthrosis. The animal was evaluated by physical and radiographic exams. Radiographic images of the skull, spine and limbs were obtained, and showed normal appearance of cranial structure and multiple changes in the limbs and spine, including generalized dysplasias of epiphyses and metaphyses of the long and short bones. Epiphyses were incomplete developed and had irregular appearance, separated by a radiolucent line, and metaphyses of the long bone were prominent flared appearance. Carpal and tarsal bones were also affected, showing shape changes.*

***Key words:*** *dog, pseudoachondroplasia,skeletal dysplasia.*

## INTRODUCTION

Locomotory apparatus includes bones, articular and muscular structures in which are present all categories of tissues used for growing, mecanic suport, vital organs protection, movement and mineral and blood cells reservoir (Predoi et al., 2012; Georgescu and Raita, 2014). Skeletal components of vertebrates have their origins in mesoderm (Docheva et al., 2014) which is the second embryo layer. In ontogenetical development the mesoderm generates the mesenchyme out of which arise all types of conjunctive tissue of adult animals (Cornilă and Manolescu, 1995). Conjunctive tissues contain conjunctive cells and extarcellular matrix (ECM) (consisting of fundamental substance and conjunctive fibres), then after metaplastic processes those components are suffering adaptive phenomena causing appearance of supportive conjunctive tissues, respectively cartilage and bone tissues (Georgescu and Raita, 2014). Under the influence of physical, chemical, nervous and humoral factors, conjunctive cells perform multiple functions such as mechanical, defense (by phagocytosis, antibody release), cytogenetics (generating other cell types) and synthesis of various protein and carbohydrates range composing the ECM (Georgescu and Raita, 2014). Collagens are one of the most important structural proteins which compose the ECM having widespread inside the organism and determinating specific features of tissues depending on the collagen type (Krakov and Rimoin, 2010). Bone formation starts in fetal period with two osteogenetic mechanisms, inside matrix ossification on a conjunctive pattern, the way it happens in forming the bones in cranial vault, maxilla, part of the clavicle and pubis, or on a hyaline cartilage pattern, the way it happens with formation of long bones (Georgescu and Raita, 2014). The bone formation mechanism is under specific genetic and direct control (Colnot, 2005). Mutations in genes encoding collagen fibres formation and cartilage ECM proteins, such as cartilage oligomeric matrix protein (COMP), proteoglycans aggrecan and perlecan usually result in various skeletal dysplasia (Ionita C., 1999; Carter et al., 2009; Krakov and Rimoin, 2010; Cao et al., 2011; Kyöstilä and al., 2013). Skeletal dysplasia found in humans and dogs alike are disorders of varying severity characterized by abnormally skeletal shape and size and long bones, spine and skull disproportion (Warman et al., 2011). Currently in humans based on radiological and clinical criteria are described more types of skeletal dysplasia (Alanay and Lachman, 2011), while in dogs include more entities which sometimes can look similar clinical and radiological but histological and biochemical they are representing a heterogeneous group of disease (Wisner and Pollard, 2007). Among them is

pseudoachondroplasia (PSACH), a rare form of osteochondrodysplasia whose main characteristics are disproportional waist shortening, limbs and spine abnormalities, abnormal walk, articular laxity and early osteoarthritis (Radlović et al., 2013). The purpose of this report is to describe radiographic findings of limb and spine PSACH in a 15-months-old female dog.

## MATERIALS AND METHODS

A 15-months-old female Bichon was presented for evaluation with walking disorders, pain and deforming of limb articulation. Faulty movement has been seen since she was 4 months old and in time became more obvious. The animal was evaluated by physical examination (inspection and palpation) and radiographic as previously described (Tudor, 2002; Papuc and Lăcătuş, 2013). The Radiological exam was conducted from orthograde incidents using a computed radiography machine set to 66 kV and 6.3 mAs with a radiation source – film distance of 100 cm. Were obtained radiographic images of the skull, spine and limbs.

## RESULTS AND DISCUSSION

Pshysical examination has shown that the limbs were shorter than the normal size with a high sensibility in articular palpation with a grown laxity and a slow movement. There were not found cutaneous, ocular or craniofacial changes. Radiographic images have indicated normal appearance of cranial structures and multiple changes in limb and spine bones. The reduction in dimension of bone rays was accompanied by important bone changes in all ephihyses both vertebral bodies and long bones. The ephiphyses were incompletely developed and had irregular appearance still separated from the shaft by a radiolucent line. The long bone metaphyses were deformed especially the distal humerus, radius and ulna which had a flared apperence (Fig. 1 and 2). Bony growths were noticed at the crest deltoid of humerus and medial humeral epicondyle level. Vertebral end plates have had irregular appearance, distictly separated from the vertebral body. Moreover, the ventral borders of the vertberal bodies were lost the pleated appearance and vertebrae have been shorter than normal. Tarsal and carpal bones were affected also showing shape changes. It was also found deformation of shoulder glenoid cavity bilaterally.

In the pelvis it has been found acetabular cavity and proximal femoral epiphyses agenesis (Fig. 3). Limb articulations were modified mostly in the elbow, knee and hip. As a result of bone ends incongruity involved in the articulation were produced degenerative processes especially in the elbow. Since onsteochondral changes incuded only the axial and appendicular skeleton without affecting craniofacial structures (such as achondroplasia does) it was diagnosed with PSACH.

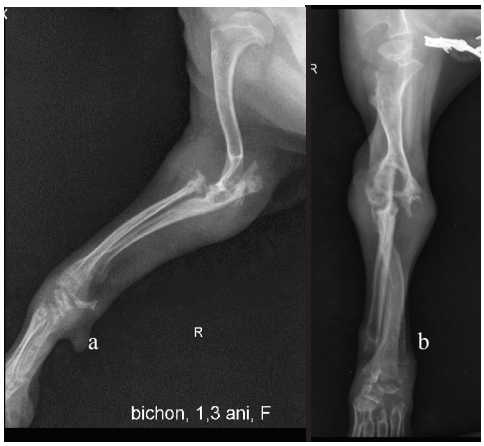


Figure 1. Lateral (a) and craniocaudal (b) views of the right limb. In the lateral view it can saw severe changes on bones: incomplet growth of the femural head; the proximal humerus metaphysis is wider; the elbow joint incongruency is faulty development of the distal epiphysis of the humerus and the proximal epiphysis of radius and ulna; the distal metaphysis enlargement and the incomplete growth of the distal epiphysis of the radius and ulna; carpal bones and proximal epiphysis of the metacarpal bones have incomplete growth. In the craniocaudal view it can be observed the normal appearence of the proximal and distal extremities of the humerus, malformation of the elbow joint, the new bone formation on the medial epicondyle of humerus, the medial angular deformity of the ulna, enlargement of the distal metaphysis of ulna, incomplete growth of the distal epiphysis of radius and ulna like carpal bones and proximal etremities of metacarpal bones.

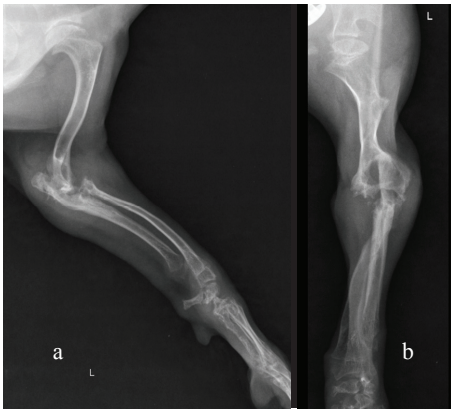


Figure 2. Lateral (a) and craniocaudal (b) views of the left limb. Can see bone changes similar to those of the right forelimb.

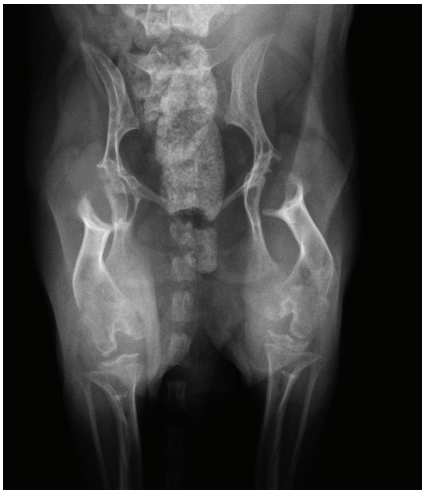

Figure 3. Ventrodorsal view of pelvis and hind limbs. It can be observed the epiphyseal alterations of the endplate in vertebral bodies. On hind limbs it can be observed bilateral bone severe changes: the acetabular cavity and the femural head are not formed, femural diaphysis have angular deformity; the proximal tibial epiphysis is incomplet formed and the proximal tibial metaphysis is wider.

Generally osteoachondrodysplasia occurs as a result of faulty endochondral ossification causing disproportionate dwarfism and morphological defects of appendicular and axial skeleton (Tanase and Craciunescu, 2003; Wisner and Pollard, 2007; Ionita L., 2008; Cao et al., 2011). Traditionally some dog breeds were classified as chondrodysplastic breeds based on their phenotypic aspect such as Basset Hound, Dachshund, Bulldog etc. (Hoelen, 2010), and gene intervention in producing these defining breed features was demonstrated (Parker et al., 2009). It was reported that osteochondrodysplasia appears also in some non-chondrodysplatic breeds such as Alaskan Malamutes (Sande et al ., 1982), Norwegian Elkhounds (Bingel and Sande, 1982), Great Pyrenees (Bingel and Sande, 1994), Scottish Deerhounds (Breur et al., 1989), German Shepherd (Mosallanejad et al., 2007), Miniature Poodles (Riser et al., 1980) and Iris Setter (Hanssen et al., 1998). This condition is considered to be hereditary having different phenotypic expressions (Wisner and Pollard, 2007). Besides skeletal defects the disease can affect other organs like the eye causing blindness as is was pointed in Labrador Retrievers (Goldstein et al., 2010) and Samoyeds (Meyers et al., 1983).

Radiographic features of skeleton in the case described here were represented by generalized dyspalsia of limb bone epiphysis and metaphysis and vertebral bodies without involving craniofacial bones are compatible with PSACH (Wisner and Pollard, 2007; Radlović et al., 2013). All limb bones were shorter than they normally are and the most affected were humerus and femur typical changes for rhizomelic dwarfism (Radlović et al., 2013). Moreover, were indicated angular deformities of the limbs showing asychronous growth of bone rays (Tanase, and Craciunescu, 2003). Thus the femur and radius have presented the most obvious changes. Radiographic images have shown articular deformation with the production of new periepifizar bone and the articular laxity seen in palpation sugests affecting tendon and ligament structures. PSACH diagnosis is based firslty on familly history of the individual and characteristic findings pshysical and radiographic. When possible genetic verification of the skeletal disorder must also be made. Skeletal changes met in this case were simillar with those described previously at dog and man (Wisner and Pollard, 2007; Radlović et al., 2013).

PSACH is a condition framed in the osteochondrodysplasia group which contains more than 150 distinct conditions characterized

by abnormal developement of bone and cartilage (Spranger, 1992). Previous studies established that in humans this condition happens consequently to a structural gene mutation positioned on 19p12-13.1 choromosome (Briggs et al., 1995; Cao et al., 2011) and encodes COMP, an ECM protein primarly expressed in cartilage, tendon and ligament, but also in many other tissues (Kracov and Rimoin, 2010). In dogs PSACH has been described in Miniature Poodle (Riser et al., 1980) and Scotish Deerhound (Breur et al., 1989) beeing considered by some authors to be a form of multiple epipyseal dysplasia (Riser et al., 1980), but skeletal abnormalities from PSACH are more generalized than in MED (Jezyk, 1985). In this study were described skeletal adnormalities of limbs and spine in Bichon dog, which is a rare case.

The causes of the appereance of this condition in the dog from the present report are still unclear because of the unknowingly of any data about the dogs parents or brothers or the conditions in which the female gestation took place. The involvement of genetic mutations in the appereance of this skeletal abnormality were previously presented (Carter et al., 2009; Goldstein et al., 2010; Frischnecht et al., 2013; Neff et al., 2012).

PSACH was differentiated from other condrodysplasic abnormalities such as chondrodysplasia of Alaskan Malamutes, chondrodysplasia of Norwegian Elkhounds or ocular chondrodysplasia of Labrador Retrievers and Samoyed dogs based on the radiographic and clinical features. Where in the case of Alaskan Malamutes skeletal changes affect limb bones without involving the skull and the spine. The psysis of all limb bones can be affected but obvious radiographic changes are pointed in the distal physis and metaphysis of ulna and radius. Asychronous growth is causing angular deformations of the limbs (Sande et al., 1982). Chondrodysplasia of Norwegian Elkhounds is characterized by disproportionate dwarfism but the forelimbs are more frequently affected than the hindlimbs. In addition radiographical are found abnormalities of the vertebrate bodies and constochondral junction whithout affecting the skull (Bingel and Sande, 1982). In Labrador Retriever breed skeletal expression of the limbs, varied in this breed (Smit et al., 2011; Frischknecht et al., 2013), may be accompanied sometimes by ocular manifestations such as cataracts, retinal dysplasia and retinal detachment (Goldstein et al., 2010).

## CONCLUSIONS

PSACH represents a form of osteochondrodysplasia characterized clinically by dwarfism and abnormal locomotion and radiographic by generalized dysplasias of epyphyses and metaphyses of the long and short tubular bones.

Avoiding the appereance of such compounds should be traced by breeding control and inbreeding avoidance.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# CORRELATION BETWEEN CLINICAL SIGNS AND DIFFERENT LABORATORY INVESTIGATIONS IN DOGS DIAGNOSED WITH LEPTOSPIROSIS

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***Abstract***

*Leptospirosis represents a scattered zoonosis determined by antigenically distinct serovars of Leptospira interrogans, a sporadic bacterial disease which causes severe clinical illness in dogs and humans. Leptospira thrive directly within hosts, dogs and humans, and reservoirs hosts, rodents, and indirectly within the environment.*
*Leptospirosis is an odd disease, with a large variety of symptomatology, or, in some cases, shows no signs or symptoms at all. That can be explained by the dog's organism defense mechanisms against infection. Although, in other cases, the disease may be life threatening.*
*Even when symptoms and signs are quite specific, in order to confirm the diagnosis it is compulsory to perform laboratory tests, such as dark-field microscopy examination (DFM) and microscopic agglutination test (MAT).*
*In this study we highlighted the request to link the clinical history with the clinical signs and paraclinical specific tests. The purpose of this paper is to show how to relate the results of different test with the clinical stage of the illness.*

***Key words****:dark-field microscopy examination, dog, leptospirosis, microscopic agglutination test.*

## INTRODUCTION

Leptospirosis is a significant clinical ilness present in canine pathology, known to cause hepatonephric syndrome characterized by acute hemorrhagic diathesis, subacute jaundice or subacute uremia (Greene et al., 1990; Adin et al., 2000).
In the first phase of the disease, leptospira organisms enter the bloodstream causing bacteremia, then the spirochetes may multiply in the kidney, liver, spleen, central nervous system, ocular tissue and genital tract. There are three main forms of the disease, represented by the hemorrhagic, renal and hepatic form. In the hemorrhagic form, the infection is localized in the bloodstream and, usually, causes bleeding. In the renal form, the spirochetes are localized mainly in the kidney, they multiply in the renal tubular epithelial cells causing acute nephritis. When the bacteria is mainly localized in the liver, it causes centrilobular necrosis and bile duct occlusion, inducing the jaundice obstructive syndrome, representing the hepatic form (Barr et al., 2002; Hartmann et al., 2005).
In Bucharest, in the last several years, the diagnosed cases of Leptospirosis in dogs has increased dramatically. This is due to the poor control of rodents and especially of the stray cats population that has reached a high number. It is a well known fact that cats develop an asymptomatic infection. Also, it is important not to neglect the zoonotic aspect of the disease, which intimately is correlated with the improper control isolation and strict measures of infected dogs.

## MATERIALS AND METHODS

The study was conducted in the Department of Internal Medicine, Faculty of Veterinary Medicine Bucharest, over a period of 2 years, from October 2012 to October 2014. Six dogs of different ages, genders and breeds

were diagnosed with Leptospirosis, presenting various clinical signs.

The dogs taken for study presented a history of illness during the past days, before they being examined in the Department of Internal Medicine, Faculty of Veterinary Medicine Bucharest. Firstly they were misdiagnosed and an ineffective treatment was started, that led to a worsening of the animals state and disease outcome.

The dogs were clinically examined, blood tests - coagulation profile test, haematology and biochemistry profiles were performed, also urinalysis and imagistic exams, in order to evaluate the internal organ damage. Signalment and results of this tests fully corroborated with the history, led to the clinical suspicion of an infection with *Leptospira* species.

The urine and blood serum were examined under dark field microscopy in all cases in order to provide a strong suspicion diagnosis. The motile organisms were detected in these samples, using dark-field microscopy. In the presented cases, Penicillin was administered at a dose of 40,000 IU/kg I.M.. At the same time, all dogs were hospitalized in an isolation room and supportive and symptomatic treatment was provided. The urine samples were obtained by cystocentesis, in order to avoid the bacterial contamination.

Dark-field microscopy may be useful for observing leptospires in fluids such as culture medium, blood or urine, particularly when they are present in large numbers. The results of dark-field microscopy of clinical material should always be confirmed by specific tests. To confirm a certain diagnostic, microscopic agglutination test (MAT) was performed in all cases. The microscopic agglutination test is a specific serodiagnostic method and represents the an important test for the diagnosis of leptospirosis (Harkin, 2003).

The MAT technique was performed with the following 4 serovars of *Leptospira* as antigen: *pomona*, *icterohaemorrhagiae*, *canicola* and *sejroe*.

## RESULTS AND DISCUSSIONS

The highest incidence of the dogs with Leptospirosis is found in adult dogs, ranging from one to five years of age, mean age of 5.05 years. The result of MAT showed 4 cases infected with serovars *L. canicola,* and 2 cases with *L. icterohemorrhagiae*; the samples are considerate positive when the titres are higher than 1:800.

Four of 6 cases were diagnosed between September and December and it seems to be a correlation between the frequency of the cases and seasonality, during the rainfall season.

In cases diagnosed with serovars *L. canicola,* all 4 dogs presented clinical signs of renal dysfunction associated with subacute and acute renal failure. From these 4 cases, 2 of them presented only signs of renal failure, another one presented also clinical signs of hepatic dysfunction and the other case presented also signs of clinical muscle dysfunction (Table 1).

Dogs infected with *L. canicola* presented initially nonspecific signs of lethargic depression (n=4), appetite loss (n=3), dehydration (n=3) and vomiting (n=3). Other signs included polyuria/polydipsia (n=2), lymphadenopathy (n=2), macroscopic hematuria (n=2), microscopic hematuria (n=2), weight loss (n=2), lumbar pain (n=1), intermittent fever (n=2) and muscle pain (n=1) (Table 2). Nephromegaly was detected following abdominal ultrasound examination (n=1).

Serovars *L. icterohemorrhagiae* were diagnosed in 2 dogs, which showed clinical signs of hepatic dysfunction, one of them also developing renal dysfunction. These two dogs initially presented signs of lethargy (n=2), appetite loss (n=2), dehydration (n=2), jaundice (n=1), fever (n=1), diarrhoea (n=1), microscopic hematuria (n=1) and vomiting (n=1). Ascites was observed following abdominal ultrasound examination (n=1).

Table1. Organ injury as indicated by serovars and serum biochemical analysis in studied dogs

| | Breed | Gender | Age (yrs.) | Serovars | Clinical syndrome |
|---|---|---|---|---|---|
| 1 | Labrador | M | 1.6 | *L. canicola* | Renal |
| 2 | "mixed breed" | M | 3.8 | *L. canicola* | Renal/Hepatic |
| 3 | "mixed breed" | F | 5.8 | *L.. canicola* | Renal |
| 4 | Doberman | M | 6.9 | *L. canicola* | Renal/muscle |
| 5 | Golden Retriever | M | 9.7 | *L. icterohemorrhagiae* | Renal/Hepatic |
| 6 | German shepherd | F | 2.5 | *L. icterohemorrhagiae* | Hepatic |

Table 2. Predominant clinical signs observed in studied cases

| Case nr. / Clinical signs | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Lethargic depression | • | • | • | • | • | • |
| Fever | | | | | • | |
| Intermittent fever | | • | | • | | |
| Appetite loss | • | | • | • | • | • |
| Dehydration | • | | • | • | • | • |
| Vomiting | • | • | | • | • | |
| Polyuria/ polydipsia | • | | • | | | |
| Lymphadenopathy | | • | | • | | |
| Diarrhoea | | | | | • | |
| Macroscopic hematuria | • | | • | | | |
| Microscopic hematuria | | • | | • | • | |
| Lumbar pain | • | | | | | |
| Muscle pain | | | | • | | |
| Jaundice | | | | | | • |

Fever was observed in one case (case 5) when the spirochetes probably were present in the bloodstream. Further complications may arise when spirochetes are localized in the kidney, where the bacteria reproduces, causing inflammation, kidney and liver failure. Other symptoms of the disease are vomiting, hematuria, jaundice.

Almost all dogs showed increased urea and creatinine, as well as leukocytosis with neutrophilia and decreased hemoglobin level. The mild decreased hemoglobin, increased packed cell volume and total leukocyte counts can be attributed to toxins released by leptospira organisms, which cause damage to red blood cells. Normal or high leukocyte counts and lower haemoglobin values could potentially indicate a diagnosis of leptospirosis. Two cases presented increased levels of alkaline phosphatase, may indicate hepatic cytotoxicity, which may be caused by the leptospiral endotoxins. Two other cases had evidence of hepatocellular and cholestatic disease, and none had evidence of

Table 3. Biochemical and hematological panel

| Parameter | Reference ranges | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 |
|---|---|---|---|---|---|---|---|
| **Blood urea nitrogen (mmol/L)** | 3.5-9.0 | **27** | **15.8** | **56.9** | **149** | **12.9** | 8.2 |
| **Creatinine (µmol/L)** | 20-150 | **440** | **398** | **347.7** | **452.6** | **275.9** | 62 |
| **Total bilirubin (µmol/L)** | 0-4 | 2 | 3 | 3 | 2 | **6** | **608** |
| **Alkaline phosphatase (U/L)** | 22-143 | 33 | **148** | 56 | **456** | **289** | **1190** |
| **Gamma glutamyl transferase (U/L)** | 0-7 | 4 | **68** | **45** | **8** | **99** | **145** |
| **Alanine aminotransferase (U/L)** | 19-107 U/L | 45 | 89 | 57 | 106 | **120** | **445** |
| **Leukocytes ($\times10^9$/L)** | 4.9-15.4 | **18.9** | **16.2** | **15.6** | **38.3** | **18.2** | **17.7** |
| **Neutrophilis ($\times10^9$/L)** | 2.9-10.6 | **36.7** | **19.8** | **12.3** | **17.5** | 9.7 | **13.1** |
| **Monocytosis ($\times10^9$/L)** | 0.0-1.1 | 0.13 | 0.56 | **1.59** | **1.89** | **4.08** | **2.45** |
| **Trombocytes ($\times10^9$/L)** | 117-418 | **56** | **78** | 159 | 175 | 123 | 229 |

hepatocellular damage only, showed by the bilirubin and transaminases levels. The coagulation profile test revealed mild increasing values in all cases probably correlated with thrombocytopenia. Patients which presented early neutrophilia and thrombocytopenia developed severe infection with leptospirosis.

The urine specific gravity value was isosthenuric, with values between 1.004 to 1.010, in all cases. Analysis of urine by dipstick method revealed the presence of occult blood in cases 1, 2, 3, 4 and 5, bilirubinuria in cases 5 and 6, and trace of protein in cases 1, 3 and 5. Urinary sediments included granular casts (cases 1, 4 and 6) and erythrocytes (cases 1 and 5). These values are secondary and reveal the renal injury.

Table 4. Table showing the correlation of dark field microscopy and Microscopic agglutination test results. (* after 7 days from the first tests)

| Test / Case nr. | Dark-field microscopy Blood | Dark-field microscopy Urine | Microscopic agglutination test |
|---|---|---|---|
| 1 | Negative | Positive | Positive |
| 2 | Positive | Positive | Positive |
| 3 | Negative | Positive | Positive |
| 4 | Positive | Positive | Positive |
| 5 | Positive | Negative | Negative |
| 5* | Negative | Positive | Positive |
| 6 | Negative | Positive | Positive |

Leptospiras may be visualized by dark-field microscopy in clinical material, blood or urine, in correlation with the stage of the disease. Thus, in the first week of infection, the spirochetes are observed in blood, but not in urine and MAT test was negative. This suggest that the dog was brought into the clinic in the first stage of infection, when the detection of group-specific antibodies was not possible. In this case the MAT test was repeated after 7 days, the result was positive for *L. icterohemorrhagiae.* Also we repeated dark-field microscopy and it was positive in urine sample and negative for the blood sample. Clinically, the dog presented now jaundice. However, there are two cases, case 2 and 4, in which the dark-field microscopy results are positive both in blood and urine, which can explains the intermittent fever.

This study reveals the importance of correlation between clinical stages of infection and different diagnostic tests, whenever there is a suspicion of leprospirosis, therefore the diagnostic is more accurate when dark-field microscopy (urine and blood samples) and MAT are combined.

The dark-field microscopy test is important to justify the quickly use of antibiotics in order to clear the leptospiremic phase and/or sterilize the urine in first stages of infection, when the MAT has a low sensibility. In this way the use of antibiotics in earlier stages increase the rate of favorable outcome.

The clinical signs and evolution seems to be diverse between different serovars of *Leptospira* species, the virulence and the

**CONCLUSIONS**

organ targeted by the bacteria, most of them presented signs of renal disease.

Because canine leptospirosis has become increasingly common in recent years and due to the poor control of rodents and asymptomatic cats, the vaccination with a canine vaccine is recommended, especially for dogs which present a high risk of infection.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# PROSTATE CANCER DIAGNOSTIC PROTOCOL - WHAT ARE THE PIECES OF THE PUZZLE

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***Abstract***

*Prostate cancer is a malignant disease with high incidence in dogs aged over 10 years, favored by sexual hyperfunction associated with a high protein diet. Chronic inflammatory lesions (prostatitis, cysts, etc.) are precancerous lesions and undiagnosed and treated early can degenerate into malignant lesions.*
*Diagnosis is based on laboratory tests: ultrasound, urine cytology, contrast radiography, puncture aspiration through ecography or trans-perineal, blood works and specific detection of tumor markers.*
*By using several diagnostic methods we are trying to establish the baseline of malignization with the beneficial consequences of establishing early, curable therapy protocols with consequent prolongation of survival and increased comfort.*
*Early screening for diagnosis and treatment of various diseases, like chronic inflammatory lesions (prostatitis, prostatic cysts, etc.) may prevent malignancy or even block or allow the detection of the process of cancerization in early stages with decreasing tumor metastasis and reduce the chances of suffering patients.*

***Key words***: *prostate, cancer, biopsy, endoscopy, ultrasound.*

## INTRODUCTION

Clinical diagnosis began with patient history: race / age on clinical signs , clinical signs observed by the owner , difficult urination , hematuria .
In general the digital rectal exam will be correlated with ultrasound data and this will help with the differential diagnosis of prostate tumors , cyst or abcess , prostatic hypertrophy , so surgery would br performed solely for exploratory or harvesting a piece of parenchyma in order to perform a histopathological examination if indicated.
Multidisciplinary diagnosis is based on laboratory tests: ultrasound, urinalysis, cytology, contrast X-ray, fine-needle aspiration, transperitoneal ultrasound, hematology and especially blood biochemistry
detect specific tumor markers, namely acid phosphates, PSA (prostate specific antigen) and fibrinogen.

## MATERIALS AND METHOD

We had a number of 33 male patients (dogs) of different breeds, medium and large built, with ages between 6 and 12 years. The dominant symptoms were: hematuria, kyphosis and constipation.
Starting with the clinical exam and recording the information in the oncology medical charts. Besides the classic history, very important is the life style of the patient (food, water, food supplements, if the animal lived in the house or outside, the city where the patient lives, if it was vaccinated, de-wormed or neutered)
22 out of 33 patients were submitted to complete blood tests, a spine and legs rx, an ultrasound of the pelvic area and urine testing.
8 patients underwent a rectal exam, complete blood test, several ultrasounds and a biopsy
2 patients underwent abdominal ultrasound, blood tests and rectal examn
1 patient had an MRI, an abdominal ultrasound, complete blood tests and urine testing.

## RESULTS AND DISCUSSIONS

Our patients that underwent complex tests had a more personalized treatment protocol. Begining with the rectal exam, complete blood tests, including specific blood tests (PSA, prostatic acid phosphatase and total prostatic phosphatase ),ultrasound, Rx (for possible bone metastasis), urine tests, fine needle-aspiration biopsy and a contrast MRI, every investigation is a piece of the puzzle and help for a better imagine for a treatment for the whole body.

Inspection and palpation of the prostate gland through a rectal exam is done so you can appreciate the size of the male genital annexe glands . The slipping of the prostate beyond the pubic threshold signifies an appreciable increase in its volume . Also modified contour ( regular , irregular ) and endured structure may signify the development of a tumor process .

P.S.A. (Prostatic Specific Antigen) / Arginin esteraza is a glycoprotein, a serine protease biochemically formed exclusively in the prostate gland, increasing its level suggesting the presence of prostate cancer.A small percentage is free, being called P.S.A. unbound (free). In prostate cancer, the report free P.S.A. / total P.S.A. decreases, lower it is, the higher the risk of prostate carcinoma.

Although ultrasound can not provide data about the function of the prostate, providing valuable information about the morphology of the prostate, which has been useful in determining the size, shape and internal architecture of the prostate gland. In addition, ultrasound has the advantage of not using ionizing radiation, contrast substances, and if the animal is not cooperating and if biopsy is not necessary, there will be no need to sedate the patient. Ultrasonography enables the early identification of changes in the parenchyma, differentiation between solid neoformations and fluid-filled cavities.

Although it is not decisive and does not directly contribute to the diagnosis of prostate cancer, radiography brings useful data for differential diagnosis and detection of metastases. It facilitates the differential diagnosis of prostatic nodules palpable by rectal examination, as well as highlights bone metastases at some stage of their development. Putting together the blader and kidney images and a chest x-ray with the knowledge of possible bone metastasis in pelvic bones, vertebrae, femur bones and ribs, so almost the entire skeletal potentially exposed to cellular invasion from the prostatic neoplasm we have a complete imagine. Cytological investigation is the method that is suitable for inflammatory lesions, tumors with epithelial origin and tumors with abundant stroma Puncture aspiration was performed on awake animals, anesthesia is required only for uncooperative patience

Histological examination was performed by scraping the surface of prostate tissue samples obtained by biopsy, displayed as colored smears with the May-Grunwald-Giemsa method. Remaining fragments were then fixed in 10% formaldehyde solution and paraffin embedded. Obtained sections were stained with Masson trichrome method (hematoxylin-eosin methylene blue).

Biopsy is an invasive surgical technique indispensable as a diagnostic tool in case the other diagnotic methods were not sufficient to assess the evolutionary stage of an animal affected by cancer. Biopsy results must be interpreted with caution and in combination with the results of other diagnostic procedures, such as blood tests, X-rays and other medical imaging techniques.

Animal: Specie Canină, Rasa Boxer, Sex M, Varsta 10 ani

| Parametru | UM | Rezultat | Val de referinta |
|---|---|---|---|
| Fosfatază acidă totală | U/l; 37°C | 148 | Sub 135 |
| Fosfatază acidă prostatică | U/l; 37°C | 72 | Sub 65 |

Figure 1 Blood analysis for a patient with prostatic carcinoma (Dr. CornilaMihai)

Animal: Specie Canină, Rasa Metis, Sex M, Varsta 11 ani

| Parametru | UM | Rezultat | Val de referinta |
|---|---|---|---|
| Fosfatază acidă totală | U/l; 37°C | 163 | Sub 135 |
| Fosfatază acidă prostatică | U/l; 37°C | 91 | Sub 65 |

Figure 2 Blood analysis for a patient with prostatic carcinoma (Dr. Cornila Mihai)

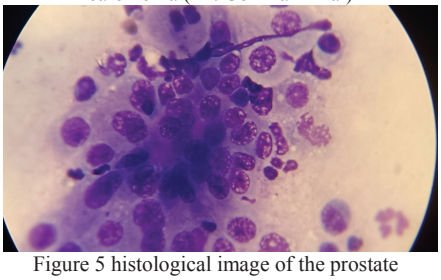

Figure 5 histological image of the prostate

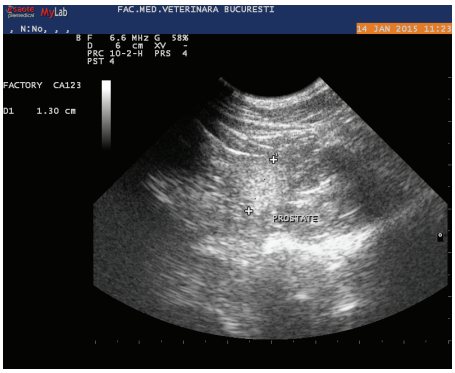

Figure 3 Paraprostatic cyst – ecography
(Dr. Constantinescu Radu)

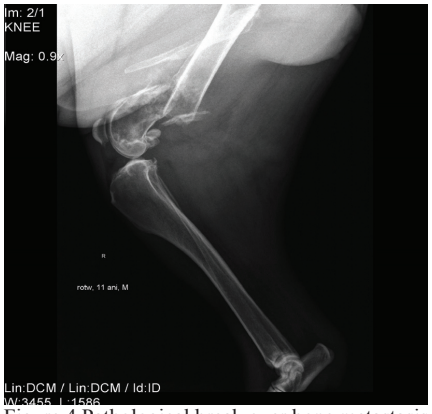

Figure 4 Pathological break over bone metastasis

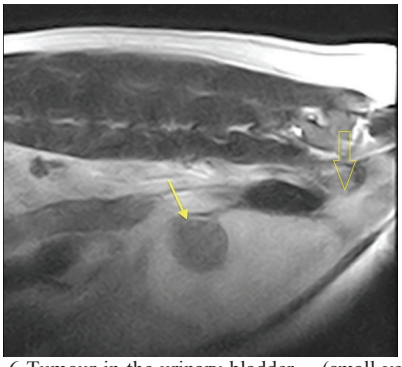
Figure 6 Tumour in the urinary bladder (small yellow arrow) and prostate benign hypertrophy (larger yellow arrow) - MRI (Dr. Grosu Florin)

## CONCLUSIONS

Chronic inflammatory lesions (prostatitis, cysts, etc.) are precancerous lesions and undiagnosed and treated early can degenerate into malignant lesions.

Screening for early diagnosis and treatment of various diseases, from chronic inflammatory lesions (prostate hypertrophy, prostate cysts, etc) can prevent or block the malignancy process or allow the detection of early stages with decreasing tumor metastasis and reduce the chances of suffering for the patients.

Investigations with specific markers allow the identification of neoplastic cells thus the early diagnosis of prostate cancer, before emphasizing the clinical symptoms.

By using several diagnostic methods we are established the baseline of the malign process with beneficial consequences related to the establishment of early, curable therapy with consequent prolongation of survival rates and increased comfort

Without proper investigations we are blind to the correct treatment protocol

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# THE INFLUENCE OF DISTOCYA ON THE TYPES OF INDICES OF BREEDING IN DAIRY COWS

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***Abstract***

***Breeding technology of dairy cows represents a set of organizational and technical measures, including** sanitary - veterinary**, what have the final result, on the one hand, a large number of products from an animal and, on the other hand, getting some of the greatest productions of milk. To develop a proper breeding activity, breeders, with the help of experts in the field, you have to know and abide by certain principles and certain factors that influence this activity, known as the upward growth of the herd of cattle from the farms is the result of breeding work carried out.** One of these factors is rather frequent, distocya completed, sometimes with loss of calf mortality, influenced by maintenance conditions, irrational diet pregnant female, inadequate sanitary - veterinary conditions. Breeding activity must have the priority objective getting each year from each cow, a normal calving because milk production occurs after birth. This is achieved through an appropriate interval between calving and designed to prevent a disturbance of reproductive function of dairy cows. This paper presents a study carried out in a professional holding, on the zooeconomics implications of gestations and parturitions complicated that degenerates into various types of distocya, some quite serious, having major repercussions on both indices of breeding and milk production of cattle on the farm.*

***Key words:** distocya, dairy cattle, farm, breeding*

## INTRODUCTION

Calving may evolve is a complex phenomenon eutocic or dystocia. Dystocia is defined as birth difficult or impossible. Dystocia can be of fetal, maternal or mixed (10) and may influence the puerperium and reproduce further clues which will lead to loss of production and economic course dairy farms. The incidence of dystocia in dairy cows can vary between 5.15% (8) and 23% (7). Other authors reported a lower frequency - between 3-10% of total births, but they also show that the frequency of dystocia are the cow can be much higher (4).
Complications dystocia, which causes economic losses may occur immediately after parturition: the death of the bull (2) or a cow (5) and away: reduced fertility (6; 9), milk production (1) and reform. Because of these considerations arising parturition how to become an important economic trait in dairy farming.

## MATERIAL AND METODS

The research was conducted during 01.01.2013-01.01.2014 a dairy farm Holstein located in southern Romania.
Were studied 560 births divided into three groups:

- ✓ Lot E1 - dystocia births with dystocia of maternal origin
- ✓ Lot E2 - dystocia births with dystocia of fetal origin
- ✓ Lot M – eutocice births

The items followed were represented by:

- the total percentage of dystocia
  - ➢ fetal dystocia
  - ➢ maternal dystocia
- frequency of various types of dystocia
- mortality at birth;
- puerperal uterine infection rate;
- interval calving-first oestrus;
- service-period's duration;
- the total percentage of gestation;
- average number of IA / pregnancy

Cows were followed for 450 days post-partum, after which they were removed from the study and those that did not get pregnant have been reformed.

## RESULTS AND DISCUSSION

Following studies conducted on 560 births was found that 67 (11.96%) were dystocia births and 493 (88.04%) eutocice. Percentage of births dystocia in farms studied was an average, compared with literature values are between 5.15% and 23% (7, 8). Of the 67 births dystocia, 41 (61.20%) were the home fetal dystocia and 26 (38.80%) of maternal origin, finding a higher frequency of dystocia compared with maternal fetal origin. Dystocia maternal origin were represented by: feto-maternal disproportion in excess of fetal volume 19 cases (46.34%), presentations dystocia 8 cases (19.51%), retention of fetal varying degrees of different body segments 12 cases ( 29.27%), hydrocephalus 1 case (2.44%) and fetal ascites 1 case (2.44%).

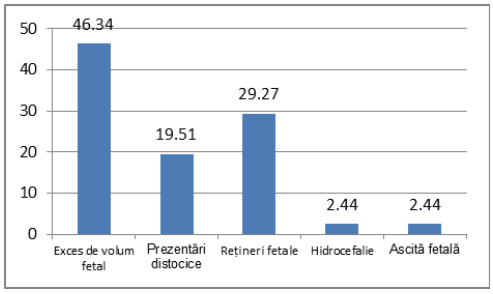


Figure 1. Frequency of fetal dystocia

The most common fetal dystocia were due by feto-maternal disproportion due to excess fetal volume (46.34%), this being due in most cases to determine seeding cows calving bulls heavy due to excessive development of the conceptus. Dystocia of maternal origin were represented: non cervical dilatation 4 cases (15.38%), uterine inertia 9 cases (34.62%), uterine torsion 5 cases (19.23%), pelvic angusta 5 cases (19, 23%) and 3 cases of hypoplastic vulva (11.54%).

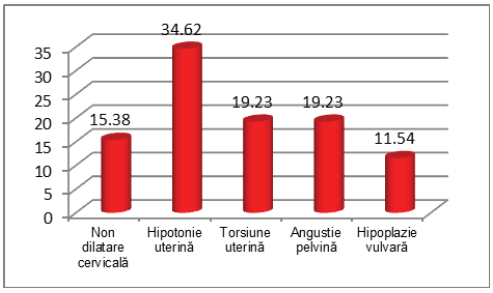


Figure 2. Frequency of maternal dystocia

From the above it appears that the highest frequency had a maternal dystocia by uterine inertia, which can be correlated with the production of milk, showing a depletion massive Ca2 + and therefore reducing the ability of contractile myometrium. In addition to depletion of Ca2 + other causes of hypotonia uterine Primary been expanding exaggerated uterine wall where births twins, weakening exaggerated some cows for decreasing tone and response uterine action oxytocin, and fattening exaggerated cows during weaning which resulted in reducing uterine contractility by fatty infiltration of the myometrium.

Of the 67 births dystocia, died five cows (7.62%) and 8 calves (11.9%). Mortality in group E1 calving with fetal dystocia was 7.31% (3 cows) and in group E2 - births by maternal dystocia was 8.69% (2 cows). Another followed in this study was the percentage of puerperal uterine infections. For the remaining 39 births in the study group were diagnosed E1 origin fetal dystocia, uterine puerperal infections were diagnosed at a rate of 87.17% (34 births) while the E2 group of 24 births in the were diagnosed dystocia of maternal origin, uterine infections were 79.16% percentage (19 births), a lower percentage compared to group E1. The control group - eutocice calving, calving in the 493, 34 (6.89%) were complicated with puerperal uterine infections.

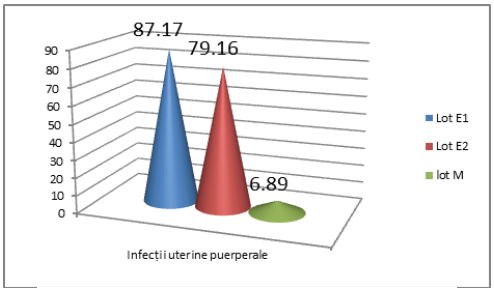


Figure 3. Frequency of puerperal uterine infections in cows

Uterine infections extensively in the groups that presented dystocia compared to the group eutocice calving may be due to uterine subinvoluției emphasized that enables and multiplying bacterial population during remediation of polluted uterus dystocia (11). Also, trauma during remediation and retention roof fetal dystocia that can be causes of puerperal uterine occurrence of these infections (3; 11).

Resuming sexual activity after calving, it was realized later in the experimental groups compared with controls. The average interval calving-first oestrus was 99.5 days in group E1, E2 104 days in group and only 65.4 days in the control group. The delay in resuming sexual activity after calving, lots experimental compared with controls was due to several factors such as the presence of uterine infection that causes the inflammation of the lining of the uterine prostaglandin F2 alpha secretion unable to luteolytic role, allowing the maintenance of the corpus luteum of pregnancy on one of ovaries. On the other hand special efforts dystocia during parturition and puerperal uterine infections then the presence of large numbers after birth reduces appetite or the existence of a selective appetite which will allow entry into a negative balance and even augmentation eenrgetică so not being able to achieve growth of ovarian folicululilor and the emergence of the first estrus p.p.

Service - Recurrent site (calving interval - fertile) is one of the most important indicators of breeding, showing the efficiency of breeding at the farm level. In our study, the average service-period was in group E1 (births with fetal dystocia) of 174 days and in group E2 (births by maternal dystocia) of 181 days, much higher compared to group M (calving eutocice) to which this index was 86 days.

If you look at this index we find that if the efficiency of breeding dystocia is influenced by the dystocia, they having decisive influence on this activity both directly and remote complications that they induce.

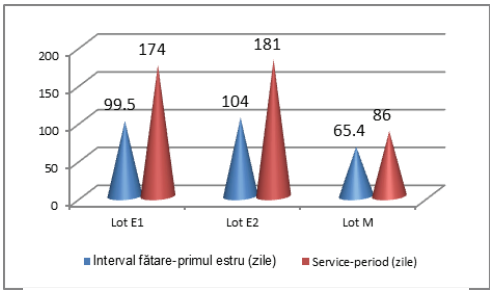


Figure 4. The average interval calving-first oestrus and service-period the cows

It also notes that there is a correlation between the proportional average calving interval during the first estrus and service-period's average.

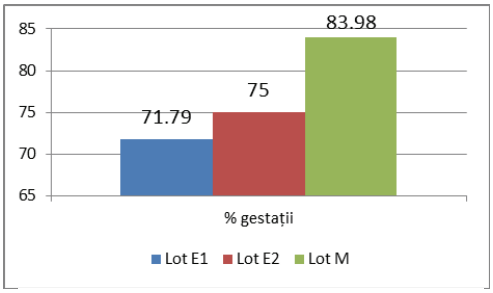


Figure 5. The average gestation in cows with dystocia births versus normal calving

The average percentage of gestation during the study period was 71.79% in group E1 (28 gestation) lower than that in group E2 was 75% (18 gestation). The average percentage of gestation control group was 83.98% (414 gestation). If we look at the average length of service compared to site-period and the average gestation is found that the E2 group to which service-period's duration was longer (181 days) compared to group E1, the average gestation was higher in group E2 (75%). The control group although service-period's duration was lower than the experimental groups, the

average gestation was much higher, which suggests a major influence on the parameters of dystocia in dairy cattle breeding.

The average number of IA / index pregnancy was another monitor. In group E1 is found to have been made 2.8 IA / gestation while in group E2 were carried 3,0 IA / gestation and 1.6 in the control group IA / gestation. And the analysis of this parameter is found dystocia influence on reproductive parameters in dairy cows.

## CONCLUSIONS

The frequency of dystocia in cattle in this study was 11.96%, falling within the average values from the literature. Dystocia of fetal origin showed a higher frequency (61.20%) compared to dystocia of maternal origin (38.80%). Dystocia of fetal origin were the fetomaternal disproportion in excess of fetal volume (46.34%), presentations dystocia (19.51%), retention of fetal varying degrees (29.27%), hydrocephalus (2.44%) and fetal ascites (2.44%), the fetal origin are represented by non cervical dilation (15.38%), uterine inertia (34.62%), uterine torsion (19.23%), pelvic angusta (19.23%) and vulvar hypoplasia (11.54%).

Cow mortality was at a rate of 7.62% and 11.9% in calves. Puerperal uterine infections were diagnosed at the rate of 87.17% in group E1, E2 group and 79.16 to 6.89% in group M (eutocice calving). Resuming sexual activity after calving was achieved on average 99.5 days in group E1, E2 later in group (104 days) and much faster in group M (65.4 days).

Service-period site followed the same trend as the interval calving-first estrus, being 174 days in group E1, E2 181 days in group and 86 days in group M. The average percentage of gestation showed the lowest values in group E1 (71.79%) followed by E2 group (75%) and control group (83.98%). There was a positive correlation between duration proportional's service-period and the average calving cows with dystocia pregnancy. The average number of IA / pregnancy was higher in groups E1 (2,8I.a. / Gestation) and E2 (3.0 IA/gestation) comaparativ control group which was 1.6 IA / gestation.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# PREVALENCE OF ZOONOTIC STAPHYLOCOCCI IN DOGS – PRELIMINARY STUDY

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***Abstract***

*This study was conducted to investigate the prevalence of zoonotic staphylococci isolated from dogs in Western Romania. Samples were obtained from adult dogs of both sexes submitted to the University Veterinary Clinics Timisoara and private veterinary practice. Animals selected for this study had no known history of previous antibiotic treatment. Samples were identified and labeled as to source, male or female, adult and the anatomical area of harvesting. A total of 51 samples were obtained from different anatomical sites such as nose, eyes, ears, extremities reproductive and skin. After growth, staphylococcal isolates were identified according to their characteristics as outlined in Bergey's Manual of Determinative Bacteriology and the Manual of Clinical Microbiology. 35 samples were positive for staphylococci, being isolated both positive and coagulase-negative species. The species most frequently isolated were S. (pseud)intermedius, S. aureus, S. hycus, S. epidermidis and S. haemolyticus. The prevalence of staphylococcal infections in veterinary medicine is increasing worldwide. Staphylococci have shown a frequent and rapid development of nosocomial infections. Unfortunately, these studies have not been documented continuously in veterinary medicine. The present investigation has examined the clinical prevalence of zoonotic staphylococci in the dogs that may constitute a reservoir for these bacteria.*

***Key words***: staphylococci, zoonotic, dogs, nosocomial infection

## INTRODUCTION

Staphylococci are one of the most important groups of commensal bacteria that are isolated from dog skin and mucous membranes. Moreover, they are responsible for opportunistic infections acquired in hospitals and communities, affecting mostly the skin and ears and other anatomic areas. Coagulase-positive staphylococci (CoPS), *Staphylococcus aureus*, *S. intermedius*, *S. pseudintermedius*, *S. delphini* and *S. schleiferi subsp. coagulans*, are the most common cause of staph infections in dogs.

Both groups of staphylococci are characterized alarming rates increasing antibiotic resistance, the problem belongs to the most important aspects of the management of staphylococcal infections worldwide. In particular, the problem of resistance is important for multidrug-resistant strains (MDR) and methicillin-resistant (MR). Staphylococci are frequently isolated from areas porting clinically healthy dogs. It is not surprising, because the nostrils, mouth and anus are considered sources of colonization and infection with staphylococci other areas. Frequency porting staphylococcal infections in dogs, especially skin infections up to 100%.

## MATERIALS AND METHODS

Methods-Animals and sample collection

Samples were obtained from 112 dogs belonging to both sexes, from September to December 2013. The dogs were from owners of Timisoara, which were submitted for routine checkup or vaccination clinics University Veterinary Faculty of Veterinary Medicine Timisoara.

Samples were collected by buffering after the animals have passed through a preliminary primary medical attention (thermometry, general clinical examination) which excludes the existence of a local or generalized

infection. All samples were collected by a veterinarian.
The study included 51 healthy dogs completely without symptoms of infection or history of antibiotic treatment prior to sampling. Three swabs were collected from each dog in this group. With one buffer pharyngeal exudate collected a sample was taken from the ear canal, the second mouth (gingival area) and third in the perineal region (buffer was introduced approximately 0.5 cm into the anus).

Phenotypic Identification of staphylococci isolated

Isolates were sub-cultured on 5% blood agar incubated at 37°C for 24 hours aerobically. Suspected staphylococcal isolates were identified based on colony characteristics, pigment production, appearance on Gram stain and hemolysis. Results were confirmed using the Vitek 2 system (bioMeriux, France) according to the manufacturer's recommendations.
To identify the prevalence and antibiotic susceptibility of pathogenic staphylococci, investigations were carried out on 51 clinically healthy dogs.
For identification of pathogenic staphylococci work using the following protocol: harvesting biological material by tapping sub gingival area or on the surface of teeth and skin areas (perianal); seeding on selective culture media, blood agar ram 5 and 10%, Chappmann agar - selective for staphylococci - examining and reading the plates was performed after 24 hours, incubated at 37°C under aerobic conditions.

Identification of strains - using GP identification card

The test bacterial suspension was adjusted to a value of 0.5 McFarland standards in 2.5 ml of 0.45% sodium chloride, with a tool VITEK 2 system (DensiChek; bioMerieux). Time between inoculum preparation and filling card was always less than 30 minutes. GP card format includes 64 wells on a plastic card, which contains 43 tests.

## RESULTS AND DISCUSSION

Research has established the prevalence of zoonotic risk strains of staphylococci, in clinically healthy dogs at the same time allowed for routine examinations to isolate and identify these bacterial species.
Bacteriological examinations carried out of the 51 samples collected from clinically healthy dogs, allowed the isolation of several bacterial species, the largest share with a staphylococcus; the results are shown in table 1.

Table 1Total number of strains of staphylococci isolated according to the anatomical areas

| Anatomy cal areas | Strains of staphylococci isolated | | | | |
|---|---|---|---|---|---|
| | *S. (pseud)intermedius* | *S. aureus* | *S. hycus* | *S. epidermidis* | *S. haemolyticus* |
| External auditory canal | 22 | 2 | 2 | 1 | 1 |
| Oral cavity | 9 | 3 | 3 | - | - |
| Anus | 12 | 1 | 2 | 2 | - |
| Total | **18** | **6** | **7** | **3** | **1** |

Bacteriological examinations carried out on a total of 51 samples from clinically healthy dogs, allowed the isolation of several strains of staphylococci belonging to the species *S. (pseudo)intermedius* and *S. aureus*, *S. hycus*, *S. epidermidis* and *S. haemolyticus*.
Strains of staphylococci unexposed to the pressure of antibiotics are sensitive to these substances; on the other hand, strains isolated from dogs with various conditions, under pressure due to antibiotic therapy, can provide multiple resistance phenomenons.
The results of antibiotic susceptibility testing of staphylococci strains isolated from clinically healthy dogs are shown in tables 2.
The 35 zoonotic risks of staphylococci strains isolated of clinically healthy dogs were tested against 20 antibiotics belonging to several classes of antibiotics against methicillin respectively.
The results of antibiotic resistance 35 staphylococcal strains isolated from clinically healthy dogs shown in Table 2.

Table 2. The results of antibiotic resistance of 18 strains of *S. (pseudo)intermedius* isolated from clinically healthy dogs

| Antimicrobian agents | Number of isolates of *S. (pseudo)intemedius* (%) | | |
|---|---|---|---|
| | Resistant | Intermediate | Sensitive |
| Benzylpenicillin | 6 (33,33) | 2 (11,11) | 10 (55,55) |
| Ampicillin | 12 (66,67) | 6 (33,33) | 0 |
| Ampicillin/sulbactan | 18 (100) | 0 | 0 |
| Oxacillin | 10 (55,56) | 2 (11,11) | 6 (33,33) |
| Imipenem | 18 (100) | 0 | 0 |
| Gentamicin | 10 (55,55) | 1 (5,56) | 7 (38,88) |
| Kanamycin | 4 (22,22) | 1 (5,56) | 13 (72,22) |
| Enrofloxacin | 18 (100) | 0 | 0 |
| Marbofloxacin | 18 (100) | 0 | 0 |
| Erythromycin | 5 (27,78) | 2 (11,11) | 11 (61,11) |
| Clindamycin | 12 (66,67) | 6 (33,33) | 0 |
| Vancomycin | 18 (100) | 0 | 0 |
| Tetracycline | 5 (27,78) | 3 (16,66) | 10 (55,55) |
| Nitrofurantion | 8 (44,45) | 10 (55,55) | 0 |
| Fusidic acid | 16 (88,89) | 2 (11,11) | 0 |
| Mupirocin | 14 (77,78) | 4 (22,22) | 0 |
| Chloramphenicol | 15 (83,34) | 3 (16,66) | 0 |
| Rifampicin | 17 (94,44) | 1 (5,56) | 0 |
| Trimethoprim/sulfamethoxazole | 5 (27,78) | 3 (16,66) | 10 (55,55) |
| Methicillin | 16 (88,89) | 0 | 2 (11,11) |

If antibiotics: ampicillin, ampicillin/sulbactan, rifampicin, enrofloxacin, marbofloxacin, clindamycin, nitrofurantion, fusidic acid and chloramphenicol mucopirinul sensitivity was highest, these antibiotics are considered of choice for staphylococci. This suggests that isolates and tested from animals to which these antibiotics were not used, except lincomycin. Also, we can say that these four antibiotics for staph kit is used, usually men, lincomycin is used only in animals.

Compared used β-lactams (methicillin, benzylpenicillin, ampicillin, ampicillin/sulbactan, oxacillin, imipenem, rifampicin) was antibiosensibilitatea maximum ampicillin, ampicillin/sulbactan and rifampicilină. The strains tested were largely resistant to β-lactams other. The distribution of resistant strains was as follows: 6 strains benzylpenicillin, 10 strains oxacillin and 2 strains were resistant to methicillin (Table3).

Table 3Results of antibiotic resistance for 2 methicillin resistant strains of *S. (pseudo)intermedius*

| Antimicrobian agents | Number of isolates of *S. (pseudo)intemedius* (%) | | |
|---|---|---|---|
| | Resistant | Intermediate | Sensitive |
| Benzylpenicillin | 1 (50) | 1 (50) | 0 |
| Ampicillin | 2 (100) | 0 | 0 |
| Ampicillin/sulbactan | 0 | 0 | 2 (100) |
| Oxacillin | 0 | 1 (50) | 1 (50) |
| Imipenem | 0 | 0 | 2 (100) |
| Gentamicin | 0 | 1 (50) | 1 (50) |
| Kanamycin | 0 | 1 (50) | 1 (50) |
| Enrofloxacin | 0 | 0 | 2 (100) |
| Marbofloxacin | 0 | 0 | 2 (100) |
| Erythromycin | 1 (50) | 1 (50) | 0 |
| Clindamycin | 0 | 0 | 2 (100) |
| Vancomycin | 0 | 0 | 2 (100) |
| Tetracycline | 1 (50) | 0 | 1 (50) |
| Nitrofurantion | 0 | 0 | 2 (100) |
| Fusidic acid | 0 | 0 | 2 (100) |
| Mupirocin | 0 | 1 (50) | 1 (50) |
| Chloramphenicol | 0 | 1 (50) | 1 (50) |
| Rifampicin | 0 | 0 | 2 (100) |
| Trimethoprim/sulfamethoxazole | 1 (50) | 1 (50) | 0 |

The phenomenon of antibiotic resistance in the case of β-lactams is based on the type of genetic determinants of plasmid and chromosomal governing the synthesis of β-lactamase, broad spectrum, which provides the resistance of staphylococci. Resistance to methicillin is transmitted by plasmids (R factor) having a pattern common to other β-lactams. For this reason, methicillin-resistant staphylococcal strains are considered zoonotic risk strains of staphylococci particularly with a complex circuit human-animal-human, respectively (Devriese et al. 2009; Dufour et al. 2002; Guardabassi et al. 2004; Lee 1994).

Compared to aminoglycosides (gentamicin, kanamycin) and macrolides (erythromycin and vancomycin), antibiotic sensitivity was different to the maximum and minimum vancomycin against kanamycin, erythromycin and gentamicin. These classes of antibiotics has been an increased resistance to antibiotics commonly used in pet therapy, as 4 strains resistant to kanamycin, 5 to erythromycin and 10 to gentamicin.

Antibiotic sensitivity to tetracyclines (tetracycline) was reduced 5 strains were resistant to this group of antibiotics, the phenomenon of resistance is plasmid and chromosomal type.

All strains tested were sensitive to enrofloxacin, marbofloxacin because these fluoroquinolones are used infrequently or not at all pets. Also to clindamycin (lincosamide) nitrofurantion, fusidic acid (other classes of antibiotics) mucopirin (class monoxycarbolic acid) and chloramphenicol (chloramphenicol) all isolates were susceptible to dogs.

The development of resistance staphylococci to different antibiotics, it is a consequence of wasteful use in the treatment of diseases in dogs and cats. Antibiotics used irrationally creates a selection pressure, are selected and transmitted genetic determinants of plasmid and chromosomal type. Consequently, the phenomenon of multiple resistance that is transmitted intra and interspecific. It is important particularly because the resistance to methicillin can be associated with resistance to β-lactams and other groups of antibiotics (Guardabassi et al. 2004).

As a result of the test strains of staphylococci isolated from clinically healthy dogs community, compared to 20 antibiotics were identified methicillin-resistant strains and several type of resistance, to β-lactams, tetracyclines, macrolides, aminoglycosides. The isolated strains were methicillin susceptible.

The data on methicillin resistance and type of resistance identified are similar to the results communicated by other authors on the phenomenon of resistance to antibiotics (Bywater et al. 2004; Bywater 2004; Fluit et al. 2006; Frank et al. 2009; Guardabassi et al. 2004; Kawakami et al. 2010).

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# THE PREVALENCE OF *SALMONELLA* SPP. IN PIGS DURING THE FATTENING PERIOD AND DURING THE SLAUGHTER

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***Abstract***

*Salmonellosis is one of the most reported zoonotic diseases, constituting a major problem worldwide. In the interests of sound management of this zoonosis, attributed to the consumption of meat and pork products, control measures must be taken simultaneously at all levels of production from farm to consumer. The purpose of this study was to identify the carrier status of Salmonella spp. starting with analyzing the fat pigs before slaughter, and analyzing each stage of the process flow within the slaughterhouse.*

*In order to identify the Salmonella carrier status 50 samples were collected from faeces in the pig farm and 50 samples from the different technological processes within the slaughterhouse, starting from pigs reception to chilling. Samples were analyzed by classical method SR EN ISO 6579: 2003 / AC: 2007.*

*The following results were obtained: the Salmonella spp. load of the faecal samples collected from pigs before slaughter was 67% (33/50, while after transportation of pigs to the slaughterhouse, at reception the load was 73%. In the slaughterhouse, it was found that the load varied at each checkpoint of the process flow.*

*Interpreting the results obtained in each checkpoint in the slaughterhouse and making comparisons between them, differences of Salmonella spp. load that ranged from 100% before slaughtering to 12.5% after carcass refrigeration were found.*

*It was concluded that carriers pigs are a carcass contamination source, because they cannot be identified at the time of their reception in the slaughterhouse, and to be able to control the level of contamination of carcasses, control measures should be applied at farm level and compliance with the HACCP principles during the technological flow in the slaughterhouse should be followed.*

*Keywords: Carcasses, identification, faecal, pigs, Salmonella spp.*

## INTRODUCTION

*Salmonella* spp. is a factor of concern for the pork industry worldwide, due to its impact on public health and high costs. Annually 80.3 million cases of food poisoning are caused by *Salmonella spp.* (Majowicz et al., 2010).

In the US, *Salmonella* spp. is the second leading cause of poisoning and the leading cause of death and hospitalization (Scallan et al., 2011). Although it was reported that the number of cases of human salmonellosis can be directly attributed to the consumption of pork, they are difficult to determine, estimates varying between 1% and 25% (Guo et al., 2011).

Although *Salmonella* spp. has been identified in all links of the pork production chain, there has been increasing focus on the pre-harvest phase (i.e., on-farm). However, in order to be able to develop effective intervention measures, it is essential that risk factors for the occurrence of *Salmonella* infections in swine herds be identified. By identifying and quantifying the effects of risk factors, interventions can be developed and applied to reduce *Salmonella* infection and carriage in the pigs at the herd level, which will reduce the contamination pressure at the abattoir (Rostagno et al., 2012).

*Salmonella* infections in swine herds are much more common than the clinical disease (i.e., salmonellosis). Asymptomatic intestinal carriage and intermittent shedding of *Salmonella* characterize most subclinically infected pigs, which represents a very common scenario in swine herds around the world. Pigs can potentially remain a risk to food safety for *Salmonella* long after they have been infected, excreting the bacteria in faeces and/or harbouring it in several tissues, particularly the intestinal tract, and associated lymph nodes (Boyen et al., 2008).

In addition, surveillance programs aim to improve the understanding of the sources and the prevalence of *Salmonella* starting from the primary production. Although the eradication of the pathogen is rarely achievable, a

reduction of the pathogen load by identifying which factors influence the animal carriage status could help in reducing the risk to human health in an integrated food chain plan (Milnes et al., 2009).
The aim of this study was to determine the carrier status at farm level and the carrier pigs' influence on the carcass contamination during slaughtering.

## MATERIALS AND METHODS

The study was conducted in a production farm, where 50 pigs were monitored during the finishing stage, where faecal samples were collected before delivering the pigs to the slaughterhouse.
In order to highlight the importance of transportation for transmission of the *Salmonella* spp., the same number of samples was collected from vehicles after unloading pigs at the slaughterhouse.
Subsequently, in order to make an analysis of the impact of each stage of the process flow within the slaughterhouse with reference to the presence of *Salmonella* spp. the same number of samples were collected in each stage of the processing flow, from the waiting area to the carcass chilling.
Samples were analyzed in the hygiene laboratory using the method SR EN ISO 6579: 2003/AC: 2007.

## RESULTS AND DISCUSSIONS

After analyzing the faecal samples collected from fat pigs collected a week before delivery a prevalence of 67% with *Salmonella* spp. was found.
After analyzing the faecal samples collected from the transportation vehicles, at receiving the pigs to the slaughterhouse, a 5% increase in *Salmonella* spp. load was observed.
Analysis of samples of faeces collected from the lairage area or after stunning showed only positive samples due to the presence of *Salmonella spp*. carrier pigs, which became a source of contamination for the pigs free of *Salmonella* spp.
Non-significant differences ($p > 0.05$) were found between the lairage area and stunning, as well as before carcass chilling (test $\chi^2$) were found, but there were significant differences ($p < 0.001$) between the lairage area, depilation, polishing, and after chilling.
Distinct significant differences ($p < 0.01$) were found between refrigeration and scalding, while the differences were highly significant ($p < 0.001$) between stunning and scalding.
After depilation and polishing an increase to 24% in the presence of *Salmonella* was found, possibly due to contact with depilatory machine which causes the elimination of faeces in the rectal ampulla of the pigs. Statistically, the differences were not significant ($p > 0.05$) between depilation and polishing stages, as well as between evisceration and after chilling.
After evisceration an up to 72% increase of *Salmonella spp.* load was observed and at the final stage, before the carcass delivery, only 12% positive samples were found, the difference being statistically significant ($p<0.05$).
*Salmonella* carrier pigs during the fattening stage constitutes the main source of contamination of the carcass and pork (Beloeil et al., 2004).
In the USA the prevalence of *Salmonella* spp. in fattening pigs was between 3.4% and 48% (Rostagno et al., 2012).
Following the completion of a study in a Portuguese slaughterhouse, Viera-Pinto et al., 2006 concluded that there are three ways in which *Salmonella* spp. could be transmitted among pigs: farm that the pig come from, during transportation and lairage time to be slaughtered.
Before referring to what happens in the slaughterhouse, a brief description of the role that fattening farms and transportation has on the pig contamination have to be described.
According to the data available at present, it is certified that 70% of carcass contamination is due to the swine origin, while other studies have shown that 100% contaminated carcasses could be reached due to cross-contamination in the slaughterhouse (Argüello et al., 2012).

In countries with a low prevalence, such as Denmark, where *Salmonella spp. cases* arriving at the slaughterhouse are limited, the significance of farms is consistent. In other areas (Spain), where about one third of the farms are infected with *Salmonella spp*., the other two stages have a special significance because during the lairage period the risk is very high that *Salmonella*-free pigs to be contaminated by coming into contact with pigs coming from farms with this disease (Argüello et al., 2012)**.**

A study conducted in 2005 monitoring the transportation of pigs from farm to slaughterhouse, an increase in the final contamination of carcasses was observed by 7 times higher than the one determined on live animals. In the same study, it was shown that 50% of carcasses were contaminated because of the process flow (cross-contamination) (Argüello et al., 2012).

As a conclusion, hygiene measures compliance is mandatory for maintaining an environment conducive to achieving *Salmonella*-free carcasses (Vangroenweghe et al., 2009).

In the slaughterhouse, after receiving the pigs, the lairage area is a source of *Salmonella spp*. contamination, because with increasing lairage time the risk of contamination of the *Salmonella*-free pigs increases (EFSA 2010).

Survival of *Salmonella spp.* during the scalding phase occurs when the water temperature falls below 62°C (Hald et al., 2003) and/or if the amount of organic material is high enough to determine *Salmonella* to protect against heat (Sörqvist et. al., 1990).

Depilation is a source of carcass contamination with faeces (Borch et al., 1996). Silva et al., 2012 found an increased number of *Salmonella*-positive carcasses after depilation stage compared with evisceration stage.

The high degree of carcass contamination before singeing was associated with failure of washing stage before cutting pigs, scalding water contamination or depilation process itself (Letellier et al., 2009; Hald colab., 2003; Pearce et al., 2004).

Polishing step determines carcasses recontamination (Rivas et al., 2000; Yu et al., 1999), while Gill and Bryant 1993 found a reduction of the contamination level of during this phase. Carcass contamination during the next stages of the slaughterhouse technological flow may occur due to non-compliance with sanitation stages of work equipment, of which the sterilization water temperature is crucial (Borch et al., 1996; De Sadeleer at al., 2008).

During the gutting process a contamination of carcasses by 55-90% occurs compared to the initial contamination (Berends et al., 1997). Compliance to animal dietary requirements before slaughter, correlated with evisceration operations compliance determines the reduction of the carcass contamination risk in this stage.

## CONCLUSIONS

Comparing the last values before transport, with those from the reception at the slaughterhouse, there was an increase in the degree of contamination (transport stress).

During slaughtering process, analyzing the samples from the surface of carcasses, significant differences between the control points from the dirty area and the clean area were found, so it was found a high degree of contamination in the clean area, in the evisceration stage, decreasing significantly in the chilling stage of the carcass, which inhibits the growth of microorganisms of the genus *Salmonella.*

## ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

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# PUBLIC HEALTH, ANIMAL PRODUCTION AND FOOD CONTROL

Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# STUDY REGARDING THE USE OF TETRACYCLINES IN THE TREATMENT OF CHICKENS AND THE IMPLICATIONS OF ACTIVE SUBSTANCE IN FOOD SAFETY

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***Abstract***
*The correlation between food and the population health, they have led to an increasing demand for the chicken and chicken products, as a possible successfully alternative to the dissatisfactions offered by other food categories. In this study, the samples were collected from chicken, which were administered various veterinary products based on antibiotics, as a treatment of different diseases. The chickens they were slaughtered after the waiting period mentioned by the manufacturer in the leaflet. The samples were represented by muscles, liver, kidney and gastrointestinal mass and they were analyzed for levels of oxytetracycline, chlortetracycline and tetracycline. After the results integration, the samples analysis has not revealed the presence of these compounds. The study confirmed the need to use the antibiotics only for therapeutic purposes and to having the obligation to complying the time waiting to remove the active substance from the chicken body.*

***Key words:*** *antibiotics, chicken, food safety, residues, tetracyclines.*

## INTRODUCTION

The use of antibiotics should not be a first reflex for people when they get sick. The same principle should be applied to the farm animals, which some breeders they "treated" with antibiotics, even if they do not suffer from any illness, the justification of these antibiotics use is considered a preventive measure (Van Eeckhout, 2001).

If antibiotics are prescribed to humans to treat serious infections, in animals, drugs such as penicillin and tetracycline they were added constantly to their food as a cheap way to make them grow faster (Fuoco, 2012).

These practices endanger the animal health, developing their immunity to antibiotics, immunity which can be transmitted to humans from eating meat and other products derived from these animals.

The use of antibiotics for growth promotion was banned in the EU since 2006, animals are administered antibiotics only to treat the illness.

Even if there are advantages of using these antibiotics, translated by increasing production and improving the quality of food production, there is also concern about the cumulative effects of antibiotics or their excess.

As a result of consumption of food containing residues of tetracyclines in small or large quantities, their absorption is partial, some remaining in the intestines. Once in the blood, tetracyclines form complexes with plasma proteins. The high absorption has tetracycline, oxytetracycline and followed lastly by chlortetracycline (Marie, 2000). The tetracycline diffusion in tissue is uneven. In gall bladder were found concentrations 10 times higher than in the blood. A part of the active substance arrived in gall bladder, is intestinal resorbed, which helps to maintain for a longer period of time in elevated plasma levels.

Since the main elimination pathways of tetracyclines in the body are the digestive and renal pathways, at these levels appear and develop specific symptoms following incorrect administration of these drugs, translated by changing the intestinal flora and marked renal insufficiency (Corneci, 2009). Malfunction of these organs, increases the concentrations of these active substances in the body (Adriana Catarig, 2012).

After treatment with antibiotics, producers should expect a waiting period before slaughter birds, because the body can metabolize and eliminate completely the active substance (Al-Ghamdi, 2000).

## MATERIALS AND METHODS

Samples were collected during 2014 from chicken, which were administered various veterinary products based on antibiotics, as a treatment of different

diseases. Were collected five samples batches, each batch including muscles, liver, kidney and gastrointestinal mass, total 60 samples. Samples were subjected to a qualitative and quantitative levels of oxytetracycline, chlortetracycline and tetracycline using the Charm II or radioimmunoassays (RIA). RIA is a highly sensitive and specific test method which is based on the principle of antigen-antibody reaction using the interaction of a radio-labeled compound and the unlabeled compounds, which are required to determine their concentration. The method is very fast and enables screening for different types of residues of antibiotics likely to be present in the sample.

Samples were collected in containers represented by plastic bags or sterile collection tubes and refrigerated thermally conditioned at 0-4$^{o}$C. The tissues were then cleaned of fat and weighed by 10 g, placed in a centrifuge tube, above which was added 30 ml MSU extraction buffer. The tubes were homogenized and heated to 80°C for 30 minutes, cooled 10 minutes over a water bath with ice flakes and then centrifuged at 15.000 rpm. for 10 minutes. The supernatant was further exposed to measurements following the procedures outlined in the kit protocols for use of each test that consisted in incubation of the binding agent, reincubare after the addition of the marker, and then testing.

## RESULTS AND DISCUSSIONS

Throughout the period of study, none of the 60 samples collected and analyzed for the residues of oxytetracycline, chlortetracycline and tetracycline, has not revealed the presence of these compounds.

## CONCLUSIONS

The presence of antibiotic residues in foods of animal origin may be reduced by educating everyone involved in the food chain from farmer to producer and processor. They should be aware of the potential hazards associated with the presence of antibiotic residues in food, health hazards for both consumers and financial loss as a result of such refusal marketing inadequate food.

Some antibiotics are eliminated from the body, while others have an affinity for certain organs (liver, kidneys, lungs, brain), where they accumulate, which imposes a stricter control of medical treatments administered to animals on compliance protocols of administration, dosage and waiting times, and testing of foodstuffs for human consumption to detect this threat to public health.

## ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# BIOSECURITY OF TRADITIONAL PRODUCTS OBTAINED FROM SHEEP AND COW GRAZING IN ALPINE CONDITIONS OF THE COM. DOFTANEI VALLEY, JUD. PRAHOVA

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***Abstract***

*Alpine Grazing is a common practice used in sheep and cattle breeding. Milk from this number is processed and converted into local traditional products. Biosecurity products is ensured mainly by the short time between milking and processing , which prevents the multiplication of germs of any kind. Laboratory determinations ; TPC(total plate count) made for control and detection of pathogens (E. coli ) show that TPC(total plate count) is lower than for analyzes of milk from farms organized and collected , harvested at the factory for processing*

***Key words:*** *biosecurity, traditional products, conditions, Alpine Grazing, milk.*

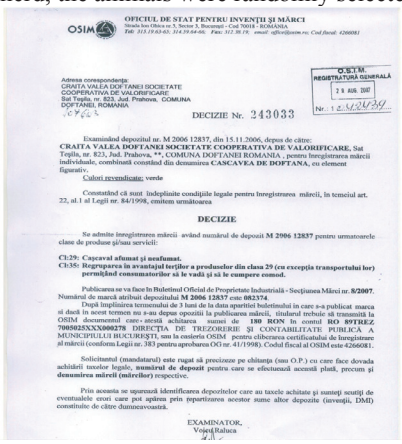
## INTRODUCTION

Alpine grazing is a practice commonly used in Romania , which exploits both feed resource exploitation and maximum biosecurity conditions resulting from livestock products . ValeaDoftanei ranks first, as a territory in Prahova County. The exploitation of animals in summer especially sheep and cattle herd is widely practiced with satisfactory economic results . Products of processed milk that comes from these animals is mainly represented by a traditional product registered as *,OSIM Nr.1042439/29 August 2007"*that is alsocalled traditional cheese and bellows cheese .

## MATERIAL AND METHODS

Operating conditions of sheep and cattle grazing system were studied and also the conditions of biosecurity alpine and alpine grazing, specific biodiversity and the obtaining of milk and it's products. This study was conducted mainly aiming at the requirements of Regulation (EC) No 852/2004 as amended and updated, which refers to a high level of consumer protection in the field of food safety. Foodtraceabilityis an essential element in ensuring food safety and hygiene requirements for structures that are covered by this Directive were also followed when analyzing the criteria that must be met to obtain raw milk for dairy products. Laboratory test results were compared with EC Regulation No 852/2004 and the results obtained from milk samples from industrial farms for processing. The samples were analyzed for udder hygiene before milking, milking time elapsed until the milkthermal processing, hygiene conditions in the milk processingroom, sanitation conditions used for milk processing machinery, materials used for manufacture of containers for milk processing compliance of temperature, of preventing the bacterial growth during transport and processing of milk. Another direction was tracking healthyexploited animals. Animal health requirements mainly refer to contagious diseases that can be transmitted to humans through consumption of milk and milk products. These measures mainly refer to udder health and the general health of the animal. The anthrax

vaccination followsimmunization of all animals, bovine tuberculin of the entire population by TCS. Serology for brucellosis and enzootic bovine leukosis, carried to its full and effective control of bovine paratuberculosis in 12% of the herd, the animals were randomly selected. For herd of sheep were effectively controlled serological 5% for Brucellaovis and Melitensis.



OFICIUL DE STAT PENTRU INVENȚII ȘI MĂRCI

DECIZIE Nr. 243033

Examinând depozitul nr. M 2006 12837, din 15.11.2006, depus de către:
**CRAITA VALEA DOFTANEI SOCIETATE COOPERATIVA DE VALORIFICARE,** Sat Teșila, nr. 823, Jud. Prahova, **, COMUNA DOFTANEI ROMANIA , pentru înregistrarea mărcii înfățișate, combinată constând din denumirea **CASCAVEA DE DOFTANA,** cu element figurativ.

Culori revendicate: verde

Constatând că sunt îndeplinite condițiile legale pentru înregistrarea mărcii, în temeiul art. 22, al.1 al Legii nr. 84/1998, emitem următoarea

**DECIZIE**

Se admite înregistrarea mărcii având numărul de depozit **M 2006 12837** pentru următoarele clase de produse și/sau servicii:

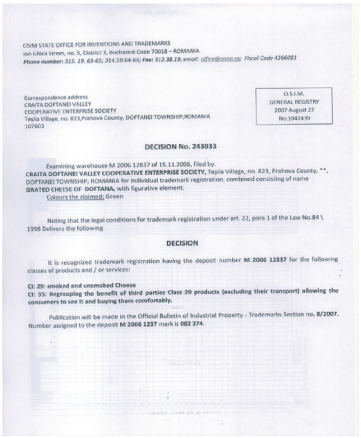
**Cl:29: Caşcaval afumat şi nefumat.**
**Cl:35: Regruparea în avantajul terților a produselor din clasa 29 (cu excepția transportului lor) permițând consumatorilor să le vadă și să le cumpere comod.**

Publicarea se va face în Buletinul Oficial de Proprietate Industrială - Secțiunea Mărci nr. **8/2007.**
Numărul de marcă atribuit depozitului **M 2006 12837** este **082374.**
După împlinirea termenului de 3 luni de la data apariției buletinului în care s-a publicat marca și dacă în acest termen nu s-au depus opoziții la publicarea mărcii, titularul trebuie să transmită la OSIM documentul care atestă achitarea sumei de **180 RON** în contul **RO 89TREZ 7005025XXX000278** DIRECȚIA DE TREZORERIE ȘI CONTABILITATE PUBLICĂ A MUNICIPIULUI BUCUREȘTI, sau la casieria OSIM, pentru eliberarea certificatului de înregistrare al mărcii (conform Legii nr. 383 pentru aprobarea OG nr. 41/1998). Codul fiscal al OSIM este 4266081.

Solicitantul (mandatarul) este rugat să precizeze pe chitanță (sau O.P.) cu care face dovada achitării taxelor legale, **numărul de depozit** pentru care se efectuează acestă plată, precum și **denumirea mărcii (mărcilor)** respective.

Prin aceasta se sporește identificarea depozitelor care au taxele achitate și sunteți sfătuiți de eventualele erori care pot apărea prin repartizarea acestor sume altor depozite (invenții, DMI) constituite de către dumneavoastră.

EXAMINATOR,
Voicu Raluca



OSIM STATE OFFICE FOR INVENTIONS AND TRADEMARKS

DECISION No. 243033

Examining warehouse M 2006 12837 of 15.11.2006, filed by:
**CRAITA DOFTANEI VALLEY COOPERATIVE ENTERPRISE SOCIETY,** Teșila Village, no. 823, Prahova County, **,
DOFTANEI TOWNSHIP, ROMANIA for individual trademark registration, combined consisting of name
**GRATED CHEESE OF DOFTANA,** with figurative element.
Colours the claimed: Green

Noting that the legal conditions for trademark registration under art. 22, para 1 of the Law No.84 \ 1998 Delivers the following

**DECISION**

It is recognized trademark registration having the deposit number **M 2006 12837** for the following classes of products and / or services:

**Cl: 29: smoked and unsmoked Cheese**
**Cl: 35: Regrouping the benefit of third parties Class 29 products (excluding their transport) allowing the consumers to see it and buying them comfortably.**

Publication will be made in the Official Bulletin of Industrial Property - Trademarks Section no. **8/2007.**
Number assigned to the deposit **M 2006 1237** mark is **082 374.**

## RESULTS AND DISCUSSION

ObviouslyCSVDoftanei Valley, which plans to vaccinate against anthrax the entire cattle and sheep populationin the territory of the constituency, it follows that in 2014 all cattle aged over 2 monthswere vaccinated and all bovine aged over 6 weeks tuberculinatedand those over 24 monthswere checked serologically for brucelloza and LEB and for 12% of the actual paratuberculosis. The entire flock of sheep with a 2 months of age were vaccinated against anthrax, campaign at the beginning of the second quarter (April-May) before departure to alpine meadows. Also 5% of the actual sheepsite over 12 months of age were blood sampled for brucellosis species Ovis and melitensis. We mention that in CSV records that matched records Doftanei Valley Record DSV epidemiological Prahova Valley CSV across Doftanei, not in the last 10 years there have been no cases of Anthrax, Brucelloza, Tuberculosis and LEB.

From the above, it is found that the animals are free of diseases communicable to humans, so the milk can be used without restriction for consumption and processing in agreement with Directive 64/432 / EEC (1) and Directive 91/68 / EEC (2) . Regarding tuberculosis, the TCS results correspond Directive 64/432 / EEC.

Milking animals is done manually in aluminum containers, observing the conditions of the udder before milking Zoo-hygiene by washing with water; transport of milk over a distance of between 20-50 meters from the animal and to the storage location is the same container and lasts about 2-4 minutes and milk storage space is located in a room protected by natural weathering in a container that is located above a heat source that provides the required temperature for heating (pasteurization) milk. The heat treatment is within the Regulation (EC) No 852/2004 Annex II, Chapter XI and was controlled at harvest samples by thermometry and was registered as a temperature between 60 and 70 degrees Celsius on a time interval 8-18 minutes.

Milk samples were taken before storing the container for pasteurization for TNG's determination and transport temperatures of 4 degrees Celsius and a time delay to an approved laboratory (the time required to transport the two hours ).

In all 5 samples collected NTG was as follows: the sample 1, 3 and 5 was 50.000, the sample 2 and 4 was 45.000.

It was the thermometer outdoor air temperature at the time of milking, finding a value of 8.5 degrees Celsius.

Milk is processed further washed with water collected in gauze temperatures exceeding 90 degrees Celsius and dried in natural conditions, then transferred in spaces specially arranged for the complete separation of the serum and the formation of the final product.

Containers used for pasteurization and getting cheese are made of metal and wood, with the possibility of proper sanitation.

Compared to the European Community Directives and Regulations 852/2004 and 853/2004, getting milk products in alpine grazing conditions meet all requirements and biosecurity products is achieved due to ambient temperature at milking (8.5 degrees Celsius) in August, short time needed milking and milk transport (about 5-10 minutes), pasteurization immediate health of the animals together not allow multiplication of bacteria in milk even if some links of the technological process are certain glitches regarding observance of hygiene conditions during milking, milk storage and processing. Sanitation samples also show that at least in terms of milk NTG, this is below the maximum allowed by almost 50%. Samples of the equipment used in the processing of milk were also below the maximum limits, absolute value of 18/cm$^2$ without coliform bacteria of 10/cm$^2$ and no bacteria were detected sampling species belonging to the genus Escherichia coli or Salmonella.

Even if the EC Directive No. 852/2004 as specified in point 3this Regulation shall not apply in the following circumstances :

a) primary production for private domestic use ;

b ) preparation, handling and storage of food (particularly dairy products ) in order private domestic consumption ;

c ) the direct supply by the producer to the final consumer or to local retail establishments that provide products directly to the end consumer with small quantities of primary products

However, the present experimental points out that dairy farming in alpine grazing conditions meet in a proportion of 95 % European Community directives .

**CONCLUSIONS:**

Biosecurity milk products processed in alpine grazing conditions in the village Doftanei Valley,Prahova County is at a maximum.

Biosecurity milk products in terms of obtaining them at an altitude of over 1,400 meters is provided by the absence of chemical treatments of alpine grassland, lack of pollution sources of any kind, the health of farm animals, animals allowance against communicable diseases, low ambient temperatures at the time of milking and the short time needed to process milk.

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Ministerul Agriculturii si Dezvoltarii Rurale; Oficiul national al produselor traditionale si ecologice romanesti. Promovarea produselor traditionale romanesti la nivel national si in U.E.

Directiva 64/432/CEE

Directiva 91/68/CEE

Directiva 98/8/CE 16.02.1998

Regulamentul CE 852 /2004

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Ordinul nr. 976/16.12.1998

Ordinul nr. 724/1082/360/2013 privind atestarea produselor tradiţionale

Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# ECOSANOGENESIS AND ECOPATOLOGY IN RELATION OF ANTHROPOZOONOTIC AGENTS IN CONTEXT OF BIOSAFETY

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***Abstract***
*An anthropozoonotic agent whose circulation is mainly determined affects the food complex managerial concerns. In the last 10 years Romania reported over 788,000 foods borne illness associated. The cost of medical therapies has increased considerably as determined by laboratory investigations for accurate etiological required. New rules were adopted security strategy taking into account the movement of aggressive agents and their potential contaminating the food. The pragmatic complex factors of ecosanogenesis contributing to the interdisciplinary vision in relation: human - product – nature. Redesigning techniques and technology, and management will lead to gradual replacement of the current economic guidelines with other levers that converge for example by minimal pollution, but also to the prices of raw materials and stimulating savings, reduction of energy consumption etc. Meeting the nutritional and sanitary quality requirements must adapt to new regulations to ensure consumer prerequisite – compliance. Enhancing food movement - national, regional, global - through trade creates new opportunities contaminant level amplification and diversification of pathogens.*

***Key words****: consumer, ecopathology, agents, trends*

## INTRODUCTION

The eco-pathology is a broad concept that describes the connection between disease and the environment. The need for such a concept appears in the current situation of over-exploited environment where the main concern is not only production, but also the effects of processes which reach the final result. These effects are referred eco-pathology it directly studying the link between environmental change and disease. The discovery that some pathogens emerging or re-occurring originate environment made it necessary to understanding how the environment influences. To understand the relationship between environmental changes and pathogenesis of various diseases is necessary to put forward several concepts from many different disciplines. It is important to understand the three main components of this process: environmental change manifests itself as a complex network of social and environmental issues that ultimately have an impact on the disease; the dynamics of infectious disease transmission is altered in many cases the environment; disease manifestations are often the result of interactions between environmental changes and transmission cycle of the pathogen. Public health experts have increasingly more arguments from studies that environmental changes that include social changes such as urbanization, development of transport infrastructure, and ecological processes such as the use of arable land, water and biodiversity decline by the disappearance of certain species and climate change are closely related with individual and population health. These results are alarming, considering that these phenomena are often anthropogenic interconnected and accelerated. Although about a century prevent and treat most often successful infectious diseases, they remain an important public health problem overall, accounting for over 13 million deaths every year. As already mentioned, changes in society, technology and the microorganisms itself contributes to the emergence of new

diseases, the re-emergence of diseases eradicated and not least the appearance of germs resistant to treatment.

## MATERIALS AND METHODS

The data, collected from INS and EUROSTAT, have been statistically processed and interpreted, building the trend line. Were used as statistical methods and methods casuistic. Data collection method will be particularly quantitative because it is an objective method, deductive and generalized. These quantitative approaches will be made in the methods concerned. It will use both sequential methods, each method (quantitative or qualitative) research will be addressed at the same time, as well as theoretical and methodological triangulation method for determining the indices. Numerous bibliographical sources were analyzed by experts in the field, FAO expert reports, scientific papers and documents of the Official Monitor.

## RESULTS AND DISCUSSIONS

The route of transmission is important in a changing environment. An example are both helminthes parasites and protozoa, which are very important for the transmission characteristics of water, soil and food. Both the potential to produce large numbers of transmissible stages and their stability in a wet environment, for example, makes them a threat to the public health and veterinary field. Due to the growing need to exploit the environment, increase the likelihood of exposure to these parasites. This can cause outbreaks in developed countries. It is therefore necessary to refine methods for the detection and isolation of these pathogens. Unlike helminthes parasites whose development cycle we know better, still doubts hanging over some protozoa, which led to intense study and improve detection techniques. Cryptosporidium is an example, where there are new immunological methods, microscopic and molecular screening and diagnostic. PCR- polymerase chain reaction methods such as increased sensitivity and specificity of these methods and standardization of techniques have been widely used.Another very important in ecosanogenesis is the climate. People have to understand the impact of climate change on disease long before discovering the role of infectious agents at the end of the 19th century.

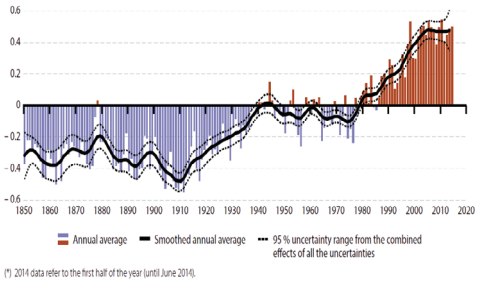
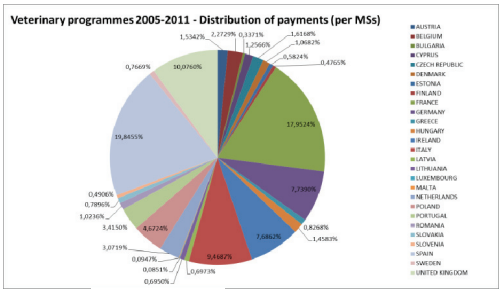



Figure 1.Global mean temperature deviation, 1850-2014
Sources: Eurostat statistics

There are three categories of studies on the link between climatic conditions and infectious disease transmission. The first type of study is studying the implications of climate change on their old, the second examines new links between both and third draw possible patterns that may appear in future relationship between the two. Conversely, the presence of certain species in the environment, can itself be an important indicator of environmental quality. When it comes to water, for example, the presence of certain parasites can provide valuable information. Reasons which are relevant for these species may represent a quality criterion. First, there are more species than parasitic species of its own. In the second place, for example the helminthes parasites have a complex life cycle with the different needs of each stage, each stage thus can provide data about the sensitive and various environmental quality. Third, the host parasite interaction itself can give valuable information. If, for example, the parasite is very sensitive to pollutants, then the incidence of infection with this host will fall. If, however, the host parasite itself is more sensitive than the average, it will decrease resistance and parasite prevalence and severity of infection increases. Assuming that current trends continue, global warming through the greenhouse effect becomes

unavoidable imbalances between ecosystems will emerge, and as these processes will be accelerated so it will be harder for people to adapt without major consequences. Climate change has always occurred, but what is different now is the speed with which changes occur because of human intervention. The potential impact that these changes have on the transmission of pathogens can be summarized in 3 main ideas: change the ecology of vectors (mainly arthropods) that link high incidence of infections in tropical and subtropical areas; the impact of risk factors such as reducing water availability, ultra-refined foods, ultra-efficient farming techniques; increasing incidence of diseases transmitted by water, soil and air as a consequence of socio-economic changes.

The main objectives of veterinary programs are to ensure a high level of protection of animal health and public health, encouraging livestock sector productivity growth and economic viability of sectors directly or indirectly affected by an outbreak of animal disease.



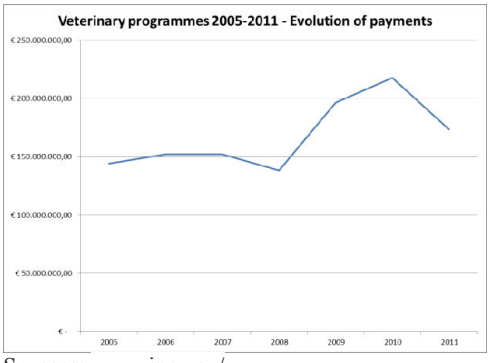
Sources: www.ipex.eu/

The EU contribution to the programs of eradication, control and monitoring of animal diseases are by far the largest expenditure in the EU budget for food safety. This contribution aims to help the gradual abolition of animal diseases and the implementation of disease surveillance measures in the Member States and the EU as a whole. It is also the EU Strategy in animal health, which aim to ensure a high level of animal health, public health and consumer protection.

*Changes in the distribution of parasitic diseases*. Infections dependent vector It depends on parasite survival and geographical distribution of the vector. Thus, according to new climatic trends, insect-borne infections will increase in intensity. An example is the Plasmodium of extrinsic incubation period which is inversely proportional to the ambient temperature. Vector-borne infections are uncommon in cold climates. The incidence of vector-borne diseases is determined by: the number of intermediate hosts or vectors and reservoirs present, environmental conditions (especially humidity and temperature);behavior and customs of society that are in dynamic equilibrium with vector-borne pathogens. Until recently, Plasmodium was present in the southern parts of Europe and North America. Withdrawal of this pest in southern areas has not yet sure why, but it is believed that was a contribution efficient sewerage systems and improve itself socio-economic conditions. This dynamic clearly illustrates the impact it has on socio-economic development pathogen transmission. There are still assuming that the temperature rise would favor the re-emergence of Plasmodium return of favorable conditions of life of the vector mosquito - anopheles.

There are parasites which are dependent on the vector to be transmitted. Two examples of Taenia Solium and are Strongyloides Stercoralis which eggs are laid in moist soil and whose development cycle is temperature dependent. In the event of rising global temperature environment they will be able to develop more effective. Another element that changes the dynamics of infectious diseases is the ultra-violet radiation type B, which is known to be immunosuppressive. If the population is exposed to this radiation, the percentage of suppressor T lymphocytes and helper lymphocytes will decrease, leading to immunosuppression population. Thus, the incidence of infectious disease increases apparently healthy patients and some opportunistic infections in immunosuppressed patients will have a high degree of lethality (such as in patients with HIV, tuberculosis). Global warming has an effect on the nutritional status of the population. This is already visible in underdeveloped countries like those in Africa, where drought are already visible evident when it is not able to

irrigate by modern methods. And global food production will decrease when the ocean levels will rise at the expense of arable areas. The same increase in intensity UV type B that I mentioned above is responsible for the decrease in photosynthesis and to hinder agriculture and food production. Malnutrition will in turn predisposes to tuberculosis and leprosy patients unimmunized. Despite this picture, there is a possible beneficial effect of the overall increase in environmental temperature. Multiple sclerosis is considered an aberrant immune response to a virus has not been isolated yet. The infectious agent is not associated with warm environments, multiple sclerosis manifested especially in cold climates. It is possible that with global warming, decrease the incidence of this disease to them.
In conclusion, the human impact on the environment will be increasingly faster, complicated and severe, and with it the transmission of infectious agents, especially parasites will increase.



Sources: www.ipex.eu/

For the whole period considered, two Member States, namely France and Spain, have absorbed nearly 38% of total EU contribution. The other major beneficiaries of the funds with an aggregate rate of absorption representing a further 35% payments made by the EU were Italy (9.5%), the UK (10.1%), Germany (7.7%) and Ireland (7.7%).

Antrophozoonosis are common diseases of man and domestic and wild animals. We also know of over 90 entities worldwide of which 20 are present and recognized in our geographical area. Tank, the most important source is represented by: wild and domestic birds (listeriosis, yersiniosis, salmonellosis, toxoplasmosis,), rodents (viruses, bacteria, fungi), carnivores and grazing livestock (sheep, cattle, goats, etc.). Anthropozoonosis have a professional nature (veterinarians , animal caretakers, foresters, forestry workers) are natural focal disease, the presence of pathogens being provided through a continuous cycle that occurs in a particular ecosystem, a specific biotypes. Clearly, the transmission can be done in both directions. The emergence of disease depends on: the introduction of the agent population, diffusion and persistence to the new host; factors that favor natural evolution and are considered less important than behavioral ones. Andy Fenton (2005) classifies anthropozoonotic agents in connection with the emergence of: pathogens which have a low transmission rate, both the host population is endemic and in the second one, after crossing the species barrier; interspecies transmission occurs, but it is rare, transient phenomenon is generated (for example, West-Nile virus with a transmission rate supported in the bird population, in excess of sending the species barrier to humans by vectors, while the transmission interpersonal, does not occur; pathogens with low transmission rate in the host population endemic, but can easily move frequently and species barrier. It is maintained by transmission between the two species, which is equivalent to the statement that 'apparent persistence (multi-host pathogen apparently) because there is no transmission interspecies in the second population. In the absence of host population endemic pathogen disappears from circulation. An example is the rabies virus in Africa, which has the only endemic host dog ( rabies in foxes being persistent because of their low density, with the reservoir epizootic domestic dog (C. Ciufecu, 2008) multi-host pathogens, showing a high rate of intra and inter transmission. The pathogen remains in either of the two populations in the absence of the other. For example: the presence of brucellosis around Yellowstone Park, the infection is endemic in domestic animals (especially cattle) with high potential emerging pathogens with a high transmission rate in the second population and a low transmission rate between the two

species. Crossing the species barrier, time and again, is rare, but once exceeded, interspecies transmission is high. If the home is zoonotic pathogens HIV and HIV 2 passing to humans from monkeys and transmitted interspecies effective enough to become persistent and generate pandemic. Most of zoonotic agents are multi-host, showing their pathogenicity for man (in over 60% of cases), wild primates (over 68% of cases), or domestic animals (90% of cases). Attention should be focused on specialist ecological and evolutionary characteristics of emerging agent, depending on the degree of interspecies transmission and after crossing interspecies endemic species barrier. Emergencies related to episodes of disease in animals, especially those that are subject to the phenomenon of "spill-over" (route) to the human host, requires an integrated approach to control. The likelihood of such diseases are continuing, as well as potential impairment of human health, surveillance and response systems / control for these emerging zoonoses re-emerging undoubtedly be improved and strengthened and expanded nationally internationally. Applied research, extended horizontally, based on modern diagnostic methods, including human health sectors, domestic and wild animals is existential to ensure planning and development based on scientific evidence of early prevention and control programs.

Biosafety is the concept which prevent the possible effect *of infectious and biochemical factors on the health of the* individual. It has many meanings in several disciplines. The first definition is the set of preventive measures that reduce the risk of transmission of infectious diseases among domestic animals and, in outbreaks involving quarantine, and living modified organisms. The term is first used in agricultural and environmental communities. After 1990, the concept appears bioterrorism prevention joins the idea of alienation biological materials in laboratories profile. This is the most complete definition published in 2010 (Koblentz) in the National Academy of Sciences, including security against any malevolent actions or use of any hazardous biological agents and especially against the possible development of biological weapons or biotechnologies such. Finally biosafety refers to protect against the development of a new outbreak of any kind. Due to the trends of globalization of food sources in the public health systems need to develop global surveillance networks and the establishment of early warning and communication networks at national, European and global levels.

## CONCLUSIONS

Confrontation between quantitative and qualitative requirements of the food is a balance of increasingly difficult to meet in the context of population (about 7 billion inhabitants) which requires a considerable increase of food. Geographically limited potential sources led to the acceptance and promotion of amplification solutions necessary food for animal consumption. Studies UN-FAO estimates that by 2050, agricultural production must increase by 70% to ensure a decent needed food. The trend of centralization of food production, increased food consumption raw, unprocessed or, on the contrary, foods that are not cooked in their own household and the trend of globalization of food sources increase the risk of transmission of disease through food and water. Interventions aimed at preventing and controlling this type of infection involves the collaboration of several factors responsible for providing conditions that avoid entering or interrupt their transmission chain, such as livestock best practices, best practices in food and related inspection procedures for promoting food safety, food microbiological monitoring procedures in the storage and marketing of food and related inspection, consumer education, food-borne disease surveillance - including microbiological surveillance by molecular typing of pathogens, investigating outbreaks, measures to limit transmission, resolution problems and prevent future unwanted events. The European Commission has reacted to new trends by adopting a comprehensive legislative package aimed at increasing food safety and consumer confidence, whose application is mandatory in all Member States. These measures based on scientific evidence include: a comprehensive and

holistic approach to food hygiene at all levels of the food chain; monitoring of zoonosis agents in the food chain and animal feed sources; establish control programs for salmonellosis and other food-borne zoonosis diseases to reduce the public health risk control measures and grounding; food safety and quality evaluation of microbiological criteria based on clear, applicable both at the manufacturing plant and products on the market; control of Spongiform Encephalopathy harmonizing measures in member countries and the adoption of clear rules on imports from third countries.

Prestigious international or European organizations such as the Council of the Codex Alimentations, WHO (World Health Organization), FAO (United Nations Food and Agriculture), EFSA (European Food Safety Agency) etc. and enrolled among the priority objectives and evaluation activities on Biosafety regulation of food. ECDC and EFSA developed a joint report on trends and sources of zoonoses and zoonotic agents and food-borne disease outbreaks in the European Union in 2011, which states that the European level is the most widespread zoonosis campylobacteriosis, salmonellosis while, as listeriosis have recognized a decreasing trend. Infections with E. coli verotoxigen recognized an increase of 159.4% compared to 2010, following the outbreak recorded in 2011 in Europe. Research attention must now turn to the particular zoonoses pathogens, given the growing contacts of human and animal populations in terms of zoonosis biodiversity pathogens, their prevalence in animals of environmental change and increasing contacts with human populations. Performance measures implemented by EU co-financing in the period 2005-2010 was assessed both internally and through external studies conducted in the last few years, based on tangible results of EU action to support Member States in eradicating, controlling and monitoring of certain animal diseases. Those studies showed an overall success veterinary programs, but revealed some weaknesses in the implementation problems have adversely affected the results of the program. It is expected by addressing the deficiencies, continuous modernization of financial management tools and optimization activities of the Task Force.

## ACKNOWLEDGEMENT

This paper has been financially supported within the project entitled ***„SOCERT. Knowledge society, dynamism through research",*** contract number POSDRU/159/1.5/S/132406. This project is co-financed by European Social Fund through Sectoral Operational Programme for Human Resources Development 2007-2013. **Investing in people!**‖ and contract number POSDRU/159/1.5/S/141086. This project is co-financed by European Social Fund through Sectoral Operational Programme for Human Resources Development 2007-2013. **Investing in people!** and Research Theme Romanian Academy-INCE-CSCBA- 6.2.9.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# ONE-YEAR FOLLOW UP STUDY FOR THE DETECTION OF STEC IN FOOD OF ANIMAL ORIGIN-THE PRESENCE OF THE MAIN VIRULENCE GENES, PRESENT AND FUTURE POTENTIAL RISK FOR CONSUMERS

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***Abstract***

*STEC are very important as emergent food-borne pathogens, being implicated in large outbreaks as well as in sporadic cases of hemorrhagic colitis and haemolytic uraemic syndrome (HUS). Following the largest HUS outbreak reported in Europe in May 2011 (3255 confirmed cases and 33 deaths), in Romania was performed during 2012 a study for detection and identification of Shiga toxin/Verotoxin-producing Escherichia coli (STEC/VTEC) from food of animal origin. According ISO 13136/2011, an E coli strain is considered STEC if its genome contains simultaneously both stx (stx1 and/or stx2) and eae genes.*

*STEC are widespread in animals but ruminants are thought to be their natural reservoir. Taking into account the high prevalence of STEC in the gastrointestinal tract of ruminants and the possibility to contaminate the meat during the slaughter process, there were investigated carcass swabs and meat subsequently processed. The techniques used included both molecular and microbiological methods. The molecular methods applied was based on the detection by real- time PCR of the major virulence genes of STEC, stx1, stx2 and eae, and as well of the serogroup associated genes O157, O145, O111, O103 and O26 from bacterial enrichment broths and subsequent isolated colonies. From 445 samples tested in this study, the STEC isolation was not achieved, but single and combinations of the target virulence genes stx1, stx2 and eae were detected in 128 samples. The presence of the major toxigenic genes in 28.76 % of the samples infer that the probable origin of target genes that we detected by PCR could be the free-Stx phages from outside bacteria cells that can be present in food samples. Knowing from literature that the Stx phages can propagate in E. coli becoming potentially able to transduce stx genes indicates that STEC food-borne outbreaks can occur anytime.*

***Key words****: Shiga toxin-producing Escherichia coli (STEC), real time PCR, Romania, stx.*

## INTRODUCTION

*Escherichia coli* is a Gram negative rod (bacillus) from the Enterobacteriaceae family. Most *E. coli* are normal commensals found in the human and animal intestine. Pathogenic strains of this organism are distinguished from normal flora by their possession of virulence factors such as exotoxins. Verocytotoxigenic (or verotoxigenic) *E. coli* (VTEC) produce a toxin that is lethal for continous Vero cell line, but not to some other cultured cell types. There are two major families of verocytotoxins, Vt1 and Vt2. A VTEC isolate may produce one or both toxins. Because verocytotoxin is homologous to the shiga toxins of *Shigella dysenteriae*, VTEC are also called shiga toxin-producing *E. coli* (STEC). STEC bacteria are in an increasing extent recognized as important food-borne pathogens worldwide with more than 265,000 illnesses each year in the United States and 13545 confirmed STEC infections and 777 HUS cases reported in the EU between 2007 and 2010 (EFSA, 2013, Da Silva Felicio et al., 2014). Most important effects of STEC infections are bloody diarrhea or hemolytic uremic syndrome (HUS), with renal failure, hemolytic anemia, and thrombocytopenia that can often leads to a fatal outcome or to haemolytic chronic renal insufficiency, chronic arthritis, irritable bowel syndrome and Guillain-Barré syndrome (Silvestro et al., 2004, European Centre for Disease Prevention and Control and European Food Safety Authority, 2011; **Askar, 2011**). Food-borne outbreaks are often caused by eating animal products undercooked or unpasteurized (STEC O157:H7 can survive for at least nine months in ground beef stored at -20°C) or contaminated vegetables (STEC surface contamination or

internalized in the tissues of some plants like lettuce). The reported survival time for STEC O157:H7 in contaminated soil varies from a month to more than 7 months. In marine water can survive for two weeks. In slurry can survive up to three months. Although it's a harmless bacteria, *E. coli* can „borrow, preserve or exchange" toxin's encoding genes from/by Stx phages, becoming a dreaded pathogen (Usein et al., 2008; Muniesa, 2013).

In 2011, a STEC O104:H4 caused a large outbreak with more than 800 HUS and 33 deaths in Germany. In EFSA Jurnal 2013, Romania reported for 2010 2 cases of human STEC, one belonged to O157 serogroup and the other one to O26.

In 2012, in accordance with the Directive 2003/99/EC and EFSA Technical Report-2009 and taking into account that the ruminants are being recognized as main animal reservoir of STEC (Caprioli et al., 2005), the Institute for Hygiene and Veterinary Public Health (IHVPH) has conducted one-year follow up study on detection of VTEC from carcass swab samples collected from cattle slaughterhouses and from beef/mutton and products thereof from retailers. This paper aims to assert the results from the study and to assess the risk of emerging STEC food-borne outbreaks that could occur in Romania in the future.

## MATERIALS AND METHODS

The food matrices used in the study were composed of minced beef meat, mixed minced beef with mutton or pork, beef meat preparations alone or mixed with mutton or pork and cattle carcass swabs from slaughterhouses.

According ISO 13136:2011, the method used for STEC detection comprises five sequential steps: microbial enrichment, nucleic acid extraction, detection of virulence genes (*vtx*1, *vtx*2*, eae)*, detection of serogroup-associated genes (*rfbE* (*O157*), *ihp1* (*O145*), *wzx* (*O103*), *wbdl* (*O111*) and *wzx* (*O26*)) and *E. coli* isolation from positive samples followed by target genes PCR detection from isolated colonies. In this study, samples were tested using ISO, but 30 positive samples for *stx*1/2-*eae* combinations genes without bacterial isolation were tested also by PCR from enrichment broth after filtration by using Millex-GV Syringe Filter Unit, 0.22 µm, gamma sterilized from Merck Millipore.

Meat sample preparation was done by homogenisation in modified tryptone soya broth supplemented with 10 mg/l of the antimicrobial novobiocin. Typically 25 g of sample is homogenised with 225 ml of enrichment medium and incubated at 41.5°C for 18 - 24 hours. For carcass swab samples, the enrichment step was done by immersion of the swabs in buffered peptone water followed by incubation at 37°C for 18 – 24 hours.

The PCR screening step consisted at first step by detection of *stx*1 and *stx*2 genes from DNA extraction of 1 ml enrichment broth, followed, in case of a positive result, by the *eae* gene detection from the same extract. If the two conditions were fulfilled, the next step was to detect the five serogroups of the presumptive present VTEC in the enrichment broth. When all results were positive until this step, it was intended to isolate a single bacterial colony which possess the genes previously detected by PCR from the enrichment broth. After 20 - 24 hours of cultivation at 37°C on agar plates with selective Oxoid mediums like TBX agar (Tryptone Bile X-glucuronide Agar) or SMAC agar (sorbitol MacCONKEY), up to fifty colonies have been isolated from every sample, followed by PCR *stx* and *eae* genes detection from pools of ten isolated colonies. Finally it was intended to isolate a single bacterial cell that included both *stx* and *eae* genes amongst those five serogroups, O157, O145, O111, O103 sau O26 (with PCR confirmation). This combination of virulence genes is often associated with the most severe forms of STEC-induced disease. Before streaking on agar plates, an immuno-magnetic separation (IMS) procedure was carried out using Dynal magnetic beads coated with antibodies specific to the five *E. coli* serogroups. The *E. coli* colonies from agar plates were identified by the indole formation assay before making pools of ten colonies for PCR detection. Presumptive colonies confirmation of VTEC O157, O145, O111, O103 and O26 was done serologically using a slide agglutination test from Statens Serum Institut from Denmark, but identification of the toxin-coding genes (*vtx* and *eae*) was done using Real-time PCR method. Amplification of every target gene was performed separately on Applied Biosystems

7900 HT Fast Real time PCR intrument (Figure 1) within a mixture of 20 µl containing 2 µl of the prepared DNA sample extracted with Instagene Matrix from Biorad-USA (from 1 ml enrichment broth or from one colony), 0.5 µM (each) primer, 0.20 µM probe (Life Technologies, USA), 10 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and PCR grade water to final volume. The real-time PCR conditions consisted of initial denaturation of DNA and Taq polimerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing&extension at 60°C for 1 min. Negative control *E. coli* K12 and positive control strains of STEC O157 (*stx*1+, *stx*2+,*eae*+), O26 (*stx*2+,*eae*+), O111 (*stx*1+, *eae*+), O103 (*stx*1+,*eae*+) and O145 (*stx*2+,*eae*+) were received from the European Reference Laboratory from ISS Rome, Italy.

Figure 1. ABI 7900 HT Fast Real time PCR System

## RESULTS AND DISCUSSIONS

According ISO 13136:2011, a STEC is a single *E. coli* bacterial cell that contains simultaneously *vtx*1 or *vtx*2 genes in combination with the intimin-coding *eae* gene (this gene is responsible for the attaching and effacing mechanism of adhesion of the bacteria to enterocytes). The results of real-time PCR for 445 food samples collected from different counties of Romania are given in Figures 2, 3 and 4.

Over 28% of all *samples* tested contain *virulence* genes on PCR screening step. In meat samples, the proportion of all target genes was 37 %, whereas the proportion of *vtx-eae* genes combination (relevant for STEC) was 12 %.

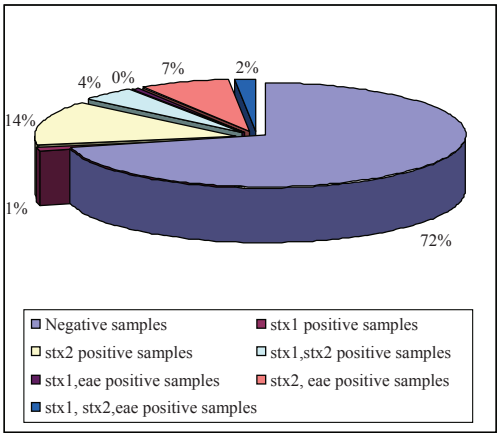


Figure 2. Virulence genes proportion in all analysed samples

Regarding the target genes presence per type of matrix, all matrices have approximately the same level of *vtx-eae* genes (~12%).

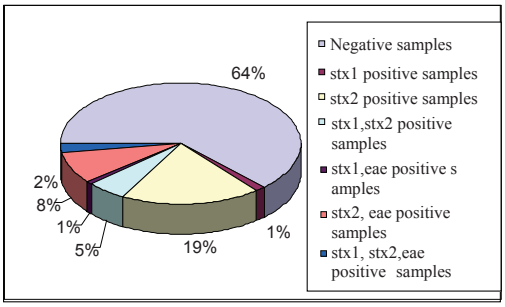


Figure 3. Virulence genes proportion in meat samples

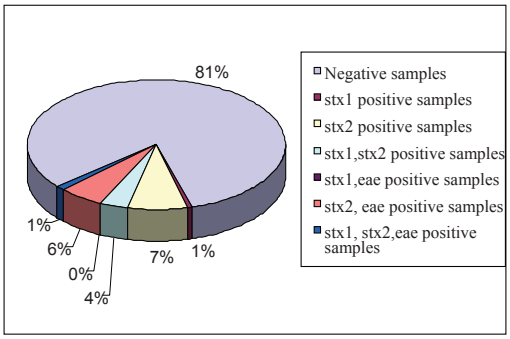


Figure 4. Virulence genes proportion in carcass swab samples

Regarding carcass swabs, all target genes were detected in 19% of samples, whereas the *vtx-eae* genes combination was found in 7%.

For both types of samples (meat and carcass swabs), the proportion of *vtx-eae* genes

combination was about one third from the positive samples for all target genes.
In Figures 5 and 6 it is shown the distribution of the serogroup associated genes detected in PCR screening step in 42 enrichment broths of samples that were firstly positive for *vtx-eae* genes combination.

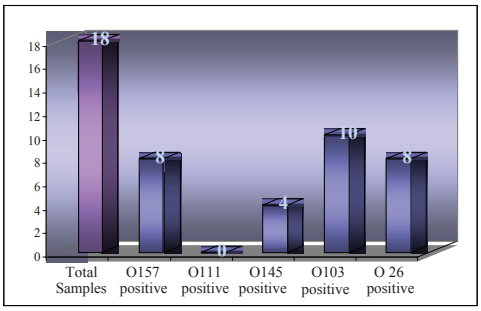


Figure 5. Serogroup associated genes tested by PCR in meat matrix (one sample may contain one or more of the five serogroups associated target genes)

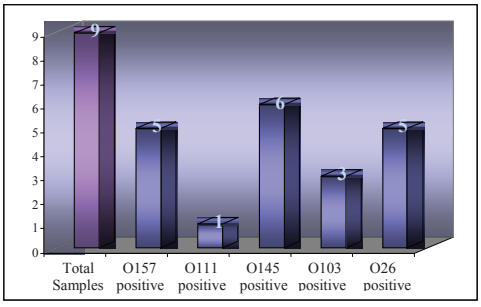


Figure 6. Serogroup associated genes tested by PCR in carcass swabs matrix (one sample may contain one or more of the five serogroups associated target genes)

During the 9th Annual Workshop of the National Reference Laboratories (NRLs) for *E. coli* in the EU (2014), several European countries claimed poor isolation results after they obtain positive results to the virulence genes PCR detection in the screening step. The conclusions were that the isolation should be achieved when the PCR show Ct values (PCR cycle threshold values) under 25. When PCR shows Ct values > 25, denotes that the isolation could not be accomplished. Our results presented at workshop can be seen in table 1. Amongst 445 samples tested only 9 are under Ct 25, but even so, because these samples have the Ct values comprised between 24 and 25 the isolation of the target genes couldn't be accomplished (Table 1 and figures 7 and 8).

Allison HE mentioned in 2007 that the bacteriophages (viruses that infect only bacteria) are the most abundant lifeforms on the globe and, referring to *E. coli*, they can carry the genes encoding Shiga toxin leading to emergence of Shiga toxin-producing pathogens.

Table 1. Detection of VTEC in food: the problem of low isolation rates from samples positive for *vtx* genes at the PCR screening step

| Matrix | PCR+ samples with Ct <25 (or = to 25) | | PCR+ samples with Ct > 25 | | Serogroup of the isolated strains |
|---|---|---|---|---|---|
| | No. of PCR+ samples | No. (%) with VTEC isolation | No. of PCR+ samples | No. (%) with VTEC isolation | |
| Beef minced meat (163 samples) | 2 | No isolation | 58 | No isolation | No isolation |
| Beef/ sheep prepared meat (79 samples) | 6 | No isolation | 25 | No isolation | No isolation |
| Carcass hide swabs (203 samples) | 1 | No isolation | 36 | No isolation | No isolation |

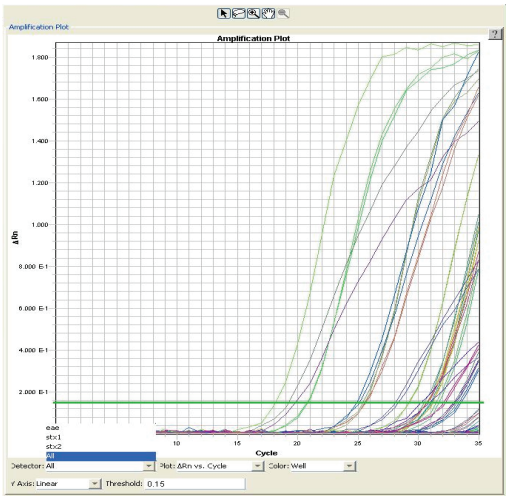


Figure 7. Virulent *stx/eae* target genes PCR detection from enrichment broths. Most of the samples gave Ct values over 25. The controls Ct values are under 25 from suspensions with 100 cfu/ ml

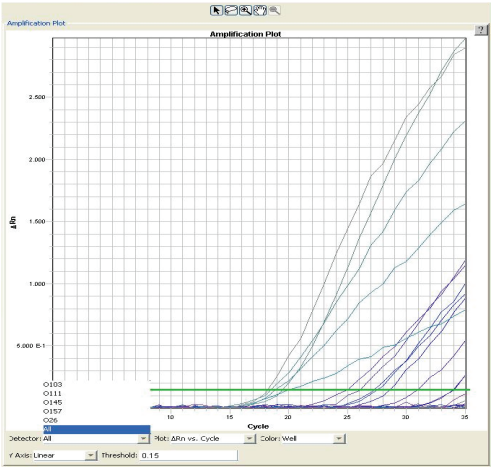


Figure 8. Serogroup - associated genes PCR detection from enrichment broths. Most of the samples gave Ct values over 25. The controls Ct values are under 25 from suspensions with 100 cfu/ ml

Taking into account this information, we performed PCR on enrichment broths filtered from samples which gave positive results to PCR in ISO screening step. After filtration through 0.22 µm, almost of 73 % of meat samples and 58 % of carcass swab samples still remain positive to PCR *vtx* and *eae* genes detection (the tests were done after 2 to 4 days from the first PCR, meanwhile the samples were kept refrigerated). This means that the PCR screening gave these results because of the existence of phages in the samples tested. This theory is also advanced by other researchers who reported that the detection of *stx* genes is not always an indicative of STEC because *stx* can be located in the genome of bacteriophages found in the samples as free particles. This explains the numerous reports of positive *stx* detection without successful STEC isolation (Quirós et al., 2015).

The existence of the Stx-phages in environment, humans, animals, food and water causes variability in STEC by *transduction* (horizontal transmission of the gene), phenomena demonstrated in the lab (Schmidt et al., 1999), in the intestinal tract (Toth et al., 2003), in biofilm (Solheim et al., 2013) and in food and water (Muniesa, 2013). Emergence of new STEC with different virulent genes configurations can occure anytime because Shiga toxin-converting bacteriophages (Stx phages) can carry the *stx* gene and convert nonpathogenic bacterial strains into Shiga toxin-producing bacteria (Brabban et al., 2005).

## CONCLUSIONS

From 445 samples tested in this study, the STEC isolation was not achieved, but one, two or all of the target genes *stx*1, *stx*2 and *eae* were detected in 128 samples.

The presence of the major toxigenic genes in more than 28% of the samples point out that the probable origin of target genes that we detected by PCR could be free-Stx phages from outside bacteria cells that can be present in food samples.

Genetic exchanges between phages in the same STEC genome indicate that are not two identical phages after passing through a new host (Muniesa, 2013). The criteria regarding pathogenicity assessment by molecular techniques approach (at least for *stx*1, *stx*2 and the *eae* intimin-coding gene) must be included, because only the classical serotyping with specific antisera is just not enough to know that an isolated *E. coli* is pathogenic or not, rendering unreal the classification of STEC serotypes into seropathotypes by Karmali in 2003.

Even though the prevalence of the virulence genes in tested food samples was relatively low and knowing from literature that the Stx phages can propagate in *E. coli*, becoming able to transduce *stx* genes, indicates that STEC food-borne outbreaks can occur anytime.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# THE IMPORTANCE OF CONTAMINATION WITH SALMONELLA SPP. IN ORDER TO OBTAIN SAFE PRODUCTS

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***Abstract***

*This paper aims to present the prevention and control of the bacteria from the Salmonella spp. genus in an intensive farm type, with growing ground technology for laying hens. The work is based on studies made at the farm S.C. Avicola Găeşti S.R.L. The bacterias belonging to the Salmonella genus cause various symptoms, from asymptomatic to "typhoid-like" syndromes in children or animals with high susceptibility. In adults Salmonella spp. are mostly responsible for food-borne diseases. For this reason salmonellosis are considered specific zoonoses. The World Health Organization (WHO) considers salmonellosis as one of the most important diseases caused by consumption of contaminated food. Regarding the species, Salmonella typhimurium and Salmonella enteridis are incriminated as the most important causes of food-borne diseases with serious risks in terms of human health. The basic characteristic of Salmonella contamination is that there are no organoleptic changes in order to draw the attention at the possible presence of germs, the eggs have the appearance color, smell and taste unchanged. In this study was used the official RENAR method, more specific the Horizontal method for the detection of bacteria of the Salmonella spp. genus for food offered for public consumption - SR EN ISO 6579: 2003 / AC 2009. Measurements were done for both shell and egg contents, loads being formed of 10 consumption eggs A category, Class L (63-73 g). The results revealed no bacteria of the Salmonella spp. genus regarding both determinations carried out on the shell as well as those that incurred the content (Absent / 25 g shell Absent / 25 g content). Therefore, the compliance of biosecurity measures in farm, bacteriological control of feed, manure and eggs are fundamental requirements in preventing the occurrence of bacteria of the Salmonella spp. genus which may jeopardize the safety and wholesomeness of the products obtained in the poultry sector.*

***Key words:*** *biosecurity, sanitation, safety, food-borne disease, zoonosis.*

## INTRODUCTION

Salmonellosis are infectious diseases caused by bacteria of the *Salmonella* genus, which produce human and animal illness, most commonly encountered being enteric disease and septicemia, abortion, arthritis and respiratory infections, sometimes resulting a state of carriers and excreted over a long period of time. Salmonellosis have a universal diffusion and are specific zoonoses, some strains are ubiquitous, and others having regional character. Most strains are pathogenic, requiring only 15-20 cells to cause infection, but this is influenced by the age of the person concerned, the physiological state, some pre-existing infections and the strain involved. In terms of food poisoning, caused by Salmonella, we can say that, in terms of frequency and implications hygienic - health, they occupy first place in most countries (CDC, 2007). Food poisoning caused by Salmonella, occure more frequently in warmer seasons (spring - autumn) when there are more human and animal carriers, the temperature being one of the predisposing factors for the development and multiplication of bacteria. The possibility of human infection is increased in the context of the circulation through contaminated food or raw materials during the technological flow, storage or distribution (CDC, 2010). Due to the high resistance of Salmonella in the environment and food, the disease can be triggered even by eating food in the form of powder with shelf life of years. Also,

increased incidence of Salmonella infections in gallinaceous due to the ubiquitous presence of germs and the existence of the carriers, but also in humans. Salmonella is one of the most important veterinary problems due to economic losses and their implications in human health by triggering food poisoning from eating contaminated food (CDC, 2008**).**

## MATERIALS AND METHODS

Research has been carried out during 2014, the determinations being carried out on a total of 5 lots. Each lot has corresponded to a total of 10 samples that were analyzed in a total number of 50 samples. A sample was represented by egg consumption from the A category (63-73 g). The determinations were both on the content and the shell of the eggs. Studies of this scientific work were held in the holding S.C. Avicola Găeşti S.R.L., a farm with intensive technology type of growth for laying hens. Sampling was an important step for laboratory examination results are largely contingent on how to harvest and transport them. Sampling was done in strict aseptic conditions, avoiding possible external contamination. Samples corresponding to each lot, harvested and individualized by serial number, were quickly dispatched to the laboratory where they were subjected to bacteriological investigations regarding the isolation, identification and serotyping germs, according to standards. The samples were analyzed using the methodology in *Horizontal method for the detection of bacteria of the genus Salmonella spp. for food offered to public consumption - SR EN ISO 6579: 2003 / AC 2009 and accredited RENAR official method*. The principle of the method consists in the isolation and identification of bacteria, on the basis of cultural, morphological, biochemical, serological, and in the case of widespread and important serovars in relation to epidemiology, phage sensitivity is set, bacteriocinotipia and antibiotipia. Important is the initial stage, the isolation and identification of the genus, which involves the differentiation of other members from the *Enterobacteriaceae* family. Examination methods include, firstly, bacteriological exam, which involves bacterioscopic examination, cultivation, isolation and identification of Salmonella. Bacteriological methods ensure the insolation of bacteria and identification of different morphological and cultural characters on which the classification may be done in strains of species and genera (CDC, 2011). In general, Salmonella can be isolated by a variety of techniques that can use or not pre-enrichmed media to revive Salmonella with reduced viability, enrichment media that contain inhibitory substances for contaminating germs. Selective media is used for differential diagnosis allowing their differentiation from other enteric bacteria. Because Enterobacteriaceae are Gram - negative and medium size like many other bacteria of other genera and families, direct microscopy is not usually useful in identifying salmonella. However, an examination of cultures obtained from samples investigated, germs with cocobacillary and bacillary form can be observed, Gram-negative, non-encapsulated, unsporulated but with cilia, which can be divided into Salmonella group. Bacteriological examination is aimed at isolating and respectively identifying species that has the highest diagnostic value, which is why it was done in the shortest time after the receipt of samples. Thus, for the isolation of Salmonella were used at the same time simple and usual media, pre-enrichmed and enrichmed media, then the culture was transferred to selective media for the isolation and purification of cultures. Bacteriological methods ensure the insulation of bacteria, and explore the different features by which it can be stated with certainty the nature of the contamination and to properly orient measures against possible pathological states (Chai et al., 2012). Salmonella can be isolated by a variety of methods that use pre-enrichment or not to revive bacteria with reduced viability, enrichment media that contain inhibitory substances contaminating germs and selective media for differential diagnosis that can be distinguished from other enteric bacteria. Cultural aspects of strains of *Salmonella spp*., isolated on different media, can take various expressions. On the surface of *nutrient agar, Salmonella spp.* develop

colonies of small, smooth or rough, semi-transparent. On blood agar, the majority of the colonies developed are species of the Enterobacteriaceae family. They are usually quite large, 2-3 mm respectively, after 24 hours in the thermostat, non-hemolytic, shiny, round and gray. Among the pre-enrichment media, buffered peptone water (BPW) is a vital medium for the isolation of Salmonella in eggs. The presence of bacteria, regardless of serovar seeded in this environment, increases the turbidity of the environment that acquires an opalescent appearance (Clarkson et al., 2010). The *Rappaport – Vasiliadis broth* is one of the commonly used enrichment media for the isolation of Salmonella in food of animal origin respectively eggs. If the presence and development of germs after 48 hours of incubation, the media changed in color from dark blue to light blue. Sowing on *agar with red phenol and brilliant green (Edel and Kampelmacher)* strains of *Salmonella spp*. cause transfer of the media color to red and colonies are large, white with transparent edges. Instead, *Escherichia coli* turns the environment yellow-green and the colonies are small, yellowish-white, smooth and glossy. Regarding the development of salmonella in *selective media and differentiation*, most of salmonella do not ferment lactose and produce pale colonies on *MacConkey agar* and an alkaline reaction in the environment (Clogher et al., 2012). However, it should be noted that some strains of *S. arizonae* are lactozo-positive and some strains of *S. typhimurium* were reported plasmids with genes encoding fermentation of lactose. Most Salmonella however give an alkaline reaction in the *brilliant green agar* and develop red colonies. It is worth mentioning that on the environment *Mac Conkey Salmonella spp*. grow translucent colonies, colored, medium size and smooth or rough compared to colonies of *Escherichia coli* which have a white color (Henao et al., 2010). On the *Levine environment*, Salmonella genus forms medium-sized, semi-transparent, tinted colonies, unlike *Escherichia coli* that formes metallic tint, black colonies. On the *Istrati-Meitert environment*, a moderately selective medium for enterobacteriaceae, *Salmonella spp*. develop blue-green colored colonies with dark blue to black center (due to $H_2S$ production), while Escherichia coli colony forming is small, pink-reddish (Clogher et al., 2012). *XLD medium (xylose-lysine-deoxycholate)* is a moderately selective medium for *Enterobacteriaceae*, which was used in all samples studied. On this medium the strains of Salmonella grows $H_2S$ positive colonies with black center and pink edges and transparent. The negative $H_2S$ colonies are translucent reddish pink, and the E. coli colonies are yellow. On *XLD*, most Salmonella serotypes produce hydrogen sulfide and pink-red colonies with a black center. Note that, on *XLD environment*, some strains of *Proteus* can mimic Salmonella colonies with a black center (production of H2S), but the colony periphery tends to have a yellowish tint (Cronquist et al., 2012). *The Rambach medium is a solid medium, which we used to differentiate between Salmonella spp. and other members of the Enterobacteriaceae family. This environment provides the research of a new character phenotype of the Salmonella spp., respectively the formation of the acid from propylene glycol, feature that can be used in combination with a color indicator for beta-galactosidase (the use of lactose L+), in order to distinguish Proteus spp. and other members of the Enterobacteriaceae family. As inhibitors of Gram positive environment we included the deoxycholate.* Except for *S. typhi* which appears particularly bright red colonies on this medium allowing an easy differentiation of *Salmonella* species, from those of the genus *Proteus*. On this medium, cultures of *Salmonella spp*. colonies formed a bright red color. A visual examination of *Rambach agar* plates and observing red colonies provide unambiguous detection of *Salmonella* strains (Crump et al., 2008). Making an appropriate selection to ensure specificity close to 100% is possible by combining two characteristics: distinctive red coloring colonies that metabolize propylene (local acidification causing precipitation of neutral red in the colony) and an intense blue staining of galactosidase producting colonies. Examination of a single colony is sufficient,

in contrast to conventional media which needs five colonies, because among colonies of Salmonella there are colonies from the species *Citrobacter* and *Proteus*. This simple selection reduces the number of examinations by excluding those who ought to be made for false positive colonies (Henao et al., 2012).

## RESULTS AND DISCUSSIONS

The results of tests for Salmonella spp. *Horizontal method for the detection performed by bacteria of the genus Salmonella spp. for food offered to public consumption - SR EN ISO 6579: 2003 / AC 2009* is provided in the following five tables.

As can be observed, no positive samples were detected for either of the 5 groups analyzed respectively did not reveal *Salmonella spp*. by tests carried out on 50 samples collected, 10 samples for each batch of analysis.

Table 1. The results for Lot 1

| Sample number | Content | Shell |
|---|---|---|
| Sample 1 | Negative | Negative |
| Sample 2 | Negative | Negative |
| Sample 3 | Negative | Negative |
| Sample 4 | Negative | Negative |
| Sample 5 | Negative | Negative |
| Sample 6 | Negative | Negative |
| Sample 7 | Negative | Negative |
| Sample 8 | Negative | Negative |
| Sample 9 | Negative | Negative |
| Sample 10 | Negative | Negative |

Table 2. The results for Lot 2

| Sample number | Content | Shell |
|---|---|---|
| Sample 1 | Negative | Negative |
| Sample 2 | Negative | Negative |
| Sample 3 | Negative | Negative |
| Sample 4 | Negative | Negative |
| Sample 5 | Negative | Negative |
| Sample 6 | Negative | Negative |
| Sample 7 | Negative | Negative |
| Sample 8 | Negative | Negative |
| Sample 9 | Negative | Negative |
| Sample 10 | Negative | Negative |

Table 3. The results for Lot 3

| Sample number | Content | Shell |
|---|---|---|
| Sample 1 | Negative | Negative |
| Sample 2 | Negative | Negative |
| Sample 3 | Negative | Negative |
| Sample 4 | Negative | Negative |
| Sample 5 | Negative | Negative |
| Sample 6 | Negative | Negative |
| Sample 7 | Negative | Negative |
| Sample 8 | Negative | Negative |
| Sample 9 | Negative | Negative |
| Sample 10 | Negative | Negative |

Table 4. The results for Lot 4

| Sample number | Content | Shell |
|---|---|---|
| Sample 1 | Negative | Negative |
| Sample 2 | Negative | Negative |
| Sample 3 | Negative | Negative |
| Sample 4 | Negative | Negative |
| Sample 5 | Negative | Negative |
| Sample 6 | Negative | Negative |
| Sample 7 | Negative | Negative |
| Sample 8 | Negative | Negative |
| Sample 9 | Negative | Negative |
| Sample 10 | Negative | Negative |

Table 5. The results for Lot 5

| Sample number | Content | Shell |
|---|---|---|
| Sample 1 | Negative | Negative |
| Sample 2 | Negative | Negative |
| Sample 3 | Negative | Negative |
| Sample 4 | Negative | Negative |
| Sample 5 | Negative | Negative |
| Sample 6 | Negative | Negative |
| Sample 7 | Negative | Negative |
| Sample 8 | Negative | Negative |
| Sample 9 | Negative | Negative |
| Sample 10 | Negative | Negative |

Negative results were recorded both through content analysis and by analyzing the shell, the components belonging to the samples considered in this study.

## CONCLUSIONS

Isolation of bacterial species, belonging to the genus Salmonella may be achieved by a

variety of methods that may use the pre-enrichment for reviving bacteria with reduced viability or with an enrichment media containing inhibitory substances for contaminated seeds, as well as a number of selective media allowing differential diagnosis of other enteric bacteria.

Bacteriological examination of egg samples was done by conventional methods derived from national and international standards, which have as a principle the isolation and identification of germs.

Laboratory examinations targeting Salmonella deceleration from samples studied were carried out according to international standardized reference method (*SR EN ISO 6579: 2003 / AC 2009*), with the compulsory pre-enrichment and enrichment stages, preliminary isolation of Salmonella.

Growing on media enrichment is an important step in detecting Salmonella that contaminate food. Choosing appropriate enrichment conditions allow for greater specificity and sensitivity of detection, while the use of inappropriate media may lead to a total failure and thus to increased risks to consumers.

Growing in two stages, which include non-selective broth recovery and selective cultivation under strict conditions, is considered today as the most feasible procedure for Salmonella enrichment. Duration of the procedure can theoretically be reduced by shortening the periods of cultivation in enrichment environments, which ensure faster and more efficient recovery and accelerating growth of target bacteria while suppressing background microbial development.

Concomitant use and comparison of a wide range of environments specific to Salmonella isolation proved superior environmental performance of *Rambach*, a chromogenic medium that ensures unambiguous detection of *Salmonella* strains, which greatly reduced the time and volume of investigations, goal extremely important for diagnosis promptness so necessary for the detection and prevention of animal and human salmonellosis episodes.

*Rambach environment* proved to be extremely useful, allowing exclusion of false positive colonies that before, needed biochemical confirmations; examining a single colony is sufficient, in contrast to conventional media from which was necessary to repick suspect colonies, as among colonies of *Salmonella* may also be colonies of *Citrobacter* and *Proteus*. The results revealed no bacteria of the genus Salmonella spp., both determinations carried out on the shell as well as those incurred when on content as shown in the results presented in the previous section 5 tables of contents. Following the determinations it proves that on the farm biosecurity measures are kept.

Bacteriological control of feed, manure and eggs are fundamental requirements for prophylaxis occurrence of bacteria of the Salmonella spp. genus which may jeopardize the safety and wholesomeness of the products obtained in the poultry sector.

## AKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132 765.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# THE EFFECT OF SIRE SELECTION ON PRODUCTIVITY IN HOLSTEIN HEIFERS

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***Abstract***

*This study aimed to determine the effects of sire selection on survival rate of calf, milk yield and reproductive parameters in high-yield dairy heifers. In the study, milk yield and reproduction parameters of 293 Holstein heifers from 7 bulls were determined. It was obtained that the first insemination age was higher in the daughter of number 1 bull. This statistical difference outcame from the daughter of number 1 bull (P<0.05). When calved age was determined, the daughter of the number 1 bull were higher than those of 2, 5 and 7 bulls (P<0.05). There was no effect of sire on the conception length of heifers, however there was significant effect of sire on live weights of hiefer during insemination period (P<0.05). The effect of bull on birth weight of calves was significant (P<0.05). Birth weights of calves were highest in the daughters of number 2 and 7 bulls, were lowest in the daughters of number 1 and 5 bulls. The effect of sire on lactation milk yield, lactation length and lactaion peak duration of the daughter were not significant, however was significant on peak yield (P<0.05). The birth type was significant on survival rate of calves. Survival rate of single born was 3.5 time higher than twins. In conclusion, the selection of bulls was found effective on reproductive parameters and birth weights of calves, however was ineffective on the lactation milk yield, lactation length and lactation peak duration except peak milk yield.*

***Key words:*** *sire selection, heifer, milk yield, reproduction, calf.*

## INTRODUCTION

High milk production and efficiency of reproductive yield are economically important traits in dairy cows. Profitableness of a dairy cow business can only be achieved by obtaining maximum milk production to the extent permitted by genotypic structure and high reproductive yield (Ozcelik et al., 2000). Reproductive yield is a comprehensive term which includes many parameters such as offspring yield. Reproductive yield is a parameter including some features such as gestation period, calving interval, age at first calving, number of inseminations per gestation, service period and number of straggler cows(Bayril et al., 2010a; Berry et al., 2014). Milk yield is a parameter which includes lactation milk yield, lactation period, peak yield and peak period(Bayril et al, 2010b).

Income of a dairy cow business is mostly generated by milk and calves(Wathes et al, 2014). In addition, a significant income is earned by selling breeding heifers. It is fundamental to obtain a calf from a cow every year for lifelong high milk production and calving. Thus, the ability of fertility must be high(Bach, 2011). Therefore, it is important to impregnate a heifer and obtain calves at the possible earliest age which does not negatively affect the growth, development, constitution and future yields(Wathes et al., 2014).

Since the age of first insemination and calving is the time at which the business begins to obtain economic benefit, it is very important in cattle breeding. This feature also plays role in productivity. Growth capacity of dairy heifers has been increasing in parallel with the selections to increase milk production yield. Decrease in the age of first calving has provided

advantage in herd management and decrease in herd renewal costs(Pirlo et al., 2000: Van Amburgh et al., 1998).
This study was conducted to determine the effect of sire selection on milk and fertility parameters of Holstein dairy cows.

## MATERIALS AND METHODS

### Management, feeding of the cows and data recording

In the study, 293 Holstein heifers of seven bulls of a private business were used as material (as frozen sperma). The heifers were housed in free movement paddocks as groups containing 25-30 heifers and fed by ration composed of corn silage, alfalfa hay, wheat straw and concentrate feed. Feeding was performed twice daily with 12 hours interval. Water was provided by automatic drinking bowls ad libitum. Heifers aged 14 months were inseminated by frozen sperma of the bulls (Anadolu Hayvancılık, Istanbul, Turkey) selected by herd management programme of the business. The heifers were taken to individual birth places 5 days before calving. 5 days after calving, heifers were taken to paddocks having automatic drinking bowls, free range, rubber beds, automatic enriching, cooling system with water spraying and fan. They were fed by mixed ration composed of corn silage, dry alfalfa hay, wheat straw and concentrate feed during lactation period. Feeding was performed by automatic feed mixing machine three times a day with 8 hours intervals (Unifit, Tekniktürk, Izmir, Turkey). Milking was done by automatic milking rotary system (Rotary Magnum 90, Westfalia Surge, Izmir Turkey) three times a day with 8 hours intervals. Individual milk yield parameters and live weights of the cows (Taxatron, Westfalia Surge, Izmir, Turkey) were measured by electronic systems in every milking and automatically recorded via computerized herd programme (DairyPlan C21 herd management programme, Westfalia surge, Izmir, Turkey). Estrus was detected by increased activity of the cows determined by a pedometer (Activity meters, Westfalia Surge, Izmir, Turkey), information given by zookeepers, increased tonus of uterine horns in rectal examination and presence of graafian follicle in ovarium. Fertility records of the heifers and the cows in the first lactation period were obtained from computerized herd management programme.

### Statistical analysis

Descriptive statistics of continuous variables were expressed as mean, standard error, minimum and maximum values, while categorical variables were expressed as numbers and percentages. In terms of continuous variables, single direction variance analysis was performed to compare the mean values of the groups. In addition, logistic regression analysis was performed to determine the effects of other variables on the viability of the calves. 5% was accepted as statistically significant level in the calculations and SPSS statistics package programme was used for calculations.

## RESULTS AND DISCUSSION

The data on the first insemination age, calving age, period of gestation and live weights of breeding bulls were presented in Table 1. The table shows that daughters of number 1 breeding bull had the highest age for the first insemination and statistical difference was due to daughters of this bull ($P<0.05$). In the evaluation of calving age, it was seen that the daughters of number 1 bull had higher calving ages when compared to the daughters of number 2, 5 and 7 bulls ($P<0.05$). It was also seen that sire had no effect on the gestation period of the heifers used in breeding, while the effect of sire was important in live weights during insemination period ($P<0.05$).
In farming business, it is important to impregnate the animals at the possible earliest age in order to make heifers the participants of the production process, while taking care not to negatively affect the growth, productivity and constitutions (Daşkaya 2005; Hamşa 2002). In the study,

the mean value for the first insemination age was found to be highest for the daughters of number 1 bull (516,06±11,16 days) and this created the difference. In the studies conducted by Sehar et al.(2005) and Erdem et al.(1997) the mean values for the first insemination age were 572,43±9,13 and 538,4±50 days, respectively. These values were higher than the values detected in our study. Given that the optimal first insemination age is 15-16 months in modern business (Akman, 1999; Şekerden et al. 1997), the proximity of our results to optimum value (15-17 months) may indicate that herd management had been properly applied in the private business in which the study was conducted.

The studies of Holstein herds have shown that the highest performance and economic return would be obtained from the heifers which give birth at the age of 23-24 months (Pirlo et al., 2000). In the study, the mean value for first calving age related to first insemination age was found to be highest in the daughters of number 1 bull (804,43± 11,3 days). It is seen that this value was higher than the optimal first calving age, but the mean values of first calving ages of other bulls were more close to the optimal value. In Holstein studies, the first calving ages were 936.7±33.2 days in the study of Kopuzlu et al. (2008) and 830,6±5.0 days in the study of Sehar et al. (2005). Atashi et al. (2006) reported that the first calving age was 801,5 ±93,2 days, which was consistent with the study.

Growth capacity of the dairy heifers has continuously increased with the selections made so in order to increase the milk production. Decrease in the first calving age, however, has provided advantage in the herd management and reduced the herd replacement costs. To provide the low age of the first calving in heifers, a suitable live weight and body size are required in the calving period. This can be achieved by the heifers having sufficient growth rate genetically and a good heifer management program (Wathes et al., 2014). That the daily live weight between the birth and the first conception age increases from 0.68 to 0.82 kg shortens the first pregnancy age by 32 days ((Bar-Peled et al., 1997). Some researchers have reported that the high live weight in the calving period has a positive effect on the first lactation milk production (Davis Rincker et al., 2011; Soberon et al., 2012). In the study conducted, it was detected that female bulls no. 4 and 5 had a higher live weight, but this did not have any effect of the milk production.

**Table 1.** Age at first insemination (day), calving age (day), gestation period (day) and body weight (kg) of Heifer.

| Item | Bulls | Heifer(n) | $\bar{X}$ | $S_{\bar{X}}$ | Min. | Max. | P |
|---|---|---|---|---|---|---|---|
| AFI | 1 | 35 | 516,1 a | 11,2 | 458 | 733 | * |
| | 2 | 39 | 476,7 b | 4,6 | 445 | 552 | |
| | 3 | 57 | 485,2 b | 3,8 | 445 | 575 | |
| | 4 | 40 | 481,2 b | 5,9 | 438 | 590 | |
| | 5 | 41 | 474,6 b | 2,8 | 450 | 544 | |
| | 6 | 43 | 491,6 b | 3,8 | 450 | 571 | |
| | 7 | 38 | 477,7 b | 4,8 | 445 | 559 | |
| AFC | 1 | 35 | 804,4a | 11,3 | 729 | 1020 | * |
| | 2 | 39 | 774,5 b | 7,5 | 723 | 912 | |
| | 3 | 57 | 781,7 ab | 5,7 | 718 | 914 | |
| | 4 | 40 | 790,1 ab | 14,3 | 719 | 1215 | |
| | 5 | 41 | 772,6b | 7,6 | 707 | 960 | |
| | 6 | 43 | 793,1 ab | 7,7 | 727 | 984 | |
| | 7 | 38 | 769,1 b | 6,9 | 723 | 912 | |
| GP | 1 | 35 | 276,7 | ,89 | 266 | 287 | - |
| | 2 | 39 | 277,6 | ,72 | 270 | 290 | |
| | 3 | 57 | 276,6 | ,59 | 261 | 283 | |
| | 4 | 40 | 275,4 | ,83 | 261 | 286 | |
| | 5 | 41 | 276,3 | ,81 | 261 | 289 | |
| | 6 | 43 | 275,4 | ,78 | 261 | 286 | |
| | 7 | 38 | 277,2 | ,71 | 270 | 290 | |
| BW | 1 | 35 | 499,7 ab | 5,1 | 431 | 554 | * |
| | 2 | 39 | 481,3 b | 7,1 | 398 | 588 | |
| | 3 | 56 | 495,7 ab | 6,1 | 376 | 622 | |
| | 4 | 40 | 511,13 a | 6,9 | 379 | 595 | |
| | 5 | 41 | 505,7 a | 7,8 | 399 | 647 | |
| | 6 | 43 | 495,7 ab | 7,6 | 385 | 650 | |
| | 7 | 38 | 480,1 b | 7,0 | 398 | 588 | |

ITY: Age at First Insemination, AFC: Age at First Calving, GP: Gestation Period, BW: Body Weight.

Table 2 shows lactation milk yield, lactation period, peak milk yield and peak period (days) of the daughters of breeding bulls. Table 2 reveals that the effect of sire on lactation milk yield, lactation period and peak lactation period of the cows was insignificant, but the effect of sire on lactation peak yield of the cows was found to be significant (P<0.05).

High milk production is one of the economically important features in the dairy cattle. Profit from a dairy cow business can be achieved only by obtaining maximum milk production allowed by genotypic structure(Lohakare et al., 2012). Therefore, the main object in the dairy cattle raising enterprises is to increase the milk production per cow by accommodating highly productive cows. In the study conducted, lactation milk production was similar among all the groups. Milk production levels in the studies made by some researchers and our levels show similarity(Cady, 1991;Castillo-Juarez, 2000;Costa et al., 2000). However, it was observed that the findings of some other researchers were lower than the levels reported in the study(Haile-Mariam, 2003;Bayram, 2006).

The period starting from the secretion of milk until the cessation of milk is called lactation. Lactation period varies between the races, the herds and the cows(Alpan, 1998). In our study, no difference was observed in the levels of the lactation among the groups. When compared to the other studies,(Ojongo et al., 2002; Haile-Mariam, 2003;Bayril et al., 2010b) the lactation period was longer. It can be said that this difference was based on the high milk productions of the heifers.

Subsequent to calving, the milk production continues by increasing and is maximized within 3-8 weeks (Etgen et al., 1987). High lactation milk production is associated with high peak milk production. The higher the peak milk production and the longer the peak milk production period, the higher the lactation milk production will be(Schmidt et al., 1998). In the study conducted, the peak milk production was higher in daughters of sires no. 1 and 4 and the difference was based thereon. In the current study, it was observed that the average peak milk productions of all the daugther of sires were higher than the other studies (Bayril et al., 2010b; Oneil et al., 2013). Thus, it is seen that the dairy cow business has approached to the expected target in relation to the milk production feature.

**Table 2.** Lactation milk yield (kg), lactation period (day), peak milk yield (kg) and peak duration(day) of Heifer.

| | Bulls | n | $\overline{X}$ | $S_{\overline{x}}$ | Min. | Max. | P |
|---|---|---|---|---|---|---|---|
| LMY | 1 | 34 | 10010,8 | 346,35 | 7040 | 15009 | - |
| | 2 | 32 | 10713,5 | 318,84 | 7371 | 15329 | |
| | 3 | 38 | 10794,2 | 390,78 | 7653 | 18176 | |
| | 4 | 36 | 10620,4 | 255,46 | 7329 | 14247 | |
| | 5 | 36 | 10710,3 | 334,11 | 7260 | 16869 | |
| | 6 | 35 | 10540,5 | 223,45 | 8674 | 15024 | |
| | 7 | 30 | 10481,9 | 452,93 | 7200 | 16188 | |
| LD | 1 | 34 | 334,2 | 9,47 | 268 | 485 | - |
| | 2 | 32 | 351,8 | 11,60 | 276 | 552 | |
| | 3 | 38 | 356,9 | 11,91 | 281 | 553 | |
| | 4 | 36 | 341,4 | 5,91 | 278 | 392 | |
| | 5 | 36 | 365,8 | 11,88 | 272 | 575 | |
| | 6 | 35 | 336,8 | 6,89 | 289 | 456 | |
| | 7 | 30 | 366,2 | 15,59 | 273 | 593 | |
| pMY | 1 | 34 | 38,7a | 1,1373 | 29,42 | 53,34 | * |
| | 2 | 32 | 37,3 ab | 1,0164 | 27,16 | 50,10 | |
| | 3 | 38 | 36,2 b | ,5798 | 27,15 | 43,97 | |
| | 4 | 36 | 38,9 a | ,6632 | 29,07 | 44,91 | |
| | 5 | 36 | 35,3 b | ,5936 | 26,91 | 43,02 | |
| | 6 | 35 | 35,9 b | ,6484 | 30,64 | 41,36 | |
| | 7 | 27 | 35,2 b | ,8110 | 29,09 | 46,46 | |
| PD | 1 | 34 | 69,1 | 4,19 | 29 | 141 | - |
| | 2 | 32 | 69,2 | 4,32 | 36 | 134 | |
| | 3 | 38 | 69,6 | 3,33 | 29 | 120 | |
| | 4 | 36 | 56,4 | 2,79 | 29 | 79 | |
| | 5 | 36 | 69,5 | 4,02 | 22 | 134 | |
| | 6 | 35 | 64,7 | 3,95 | 36 | 122 | |
| | 7 | 30 | 72,9 | 4,55 | 36 | 134 | |

LMY: Lactation Milk Yield, LD: Lactation Duration, pMY:Peak Milk Yield, PY: Peak Yield, PD: Peak Duration

Birth weights of the daughters of breeding bulls were presented in Table 3. These data shows that the effect of the bulls on birth weights of the calves was significant (P<0.05). The daughters of number 2 and 7 bulls had the highest birth weights, while the daughters of number 1 and 5 bulls had the lowest birth weights. One of the most important factors affecting the profitability in the dairy cow enterprises is the calf obtained from a cow annually. The main target in the dairy cattle raising is to obtain

a calf annually (Yüceer, 2008). To overcome the loss of calf, the calves are required to exhibit growth and development according to their races. The first parameter of the growth is the birth weight. To be more precise, the birth weight is the easiest and the most reliable parameter of the growth. Moreover, the birth weight is an important factor affecting the postnatal growth and development (Akbulut et al., 2001). The birth weight is affected by the genetic and environmental factors. In the study, when the calf birth weights were compared, significant differences were observed. This difference was based on the higher calf birth weights of the sires no. 2 (42.86kg) and 7 (42.86kg). Some researchers have found lower calf birth weights than ours in their studies (Kertz et al., 1997; Sparks et al., 2003; Bayril et al., 2010c). However, Arrayet et al.(2002) have reported higher calf birth weight.

**Table 3.** The birth weights of calves (kg).

| Item | Bulls | Calf (n) | $\bar{x}$ | $S_{\bar{x}}$ | Min. | Max. | P |
|---|---|---|---|---|---|---|---|
| CW | 1 | 75 | 41,2 b | ,503 | 30 | 52 | * |
| | 2 | 101 | 42,8 a | ,469 | 32 | 55 | |
| | 3 | 135 | 42,2 ab | ,449 | 32 | 55 | |
| | 4 | 93 | 41,5 ab | ,483 | 31 | 58 | |
| | 5 | 87 | 40,9 b | ,483 | 32 | 54 | |
| | 6 | 100 | 42,3 ab | ,500 | 28 | 53 | |
| | 7 | 101 | 42,9 a | ,469 | 32 | 55 | |

CW: Calf Weight

Effects of some factors on the viability of the calves were presented in Table 4. It shows that the effect of the type of delivery was important in viability and viability of twins was 3,5 fold lower when compared to single births.

Twin birth is an undesirable situation in the dairy cow enterprises. The cow pregnant with twins decreases the vitality, increases the mortality rates and reduces the reproductive performance. High birth weight increases the vitality( Bendixen et al., 1989;Nielen et al., 1989). That the twin calves have a lower birth weight than the single-born calves causes decrease in the vitality. In the study, it was observed that the vitality of the twin calves was 3.5 times lower than the single-born calves.

Calf breeding is an important source for the breeders due to the replacement of the herd and the income obtained from the sale of thereof. Increase in the calf deaths further increases the business expenses such as insemination, treatment of the sick animals, and the feed costs. Calf deaths are one of the most important indicators of the health condition in the dairy cow enterprises. Numerous factors such as management, calving season, colostrum quality, diseases and sex of the calf, play a role in the calf deaths(Holland et al., 1992). Some researchers have reported that sex of the calf has no effect on the vitality (Ertugrul et al., 2000; Bayril et al., 2010c). Our findings are in conformity with these results.

**Table 4.** The effect of some factors on the survival rate of calf

| | | | | | | 95% CI for odds | |
|---|---|---|---|---|---|---|---|
| | B | SE | Wald | Sig. | Odss | Lower | Upper |
| Sex of calf (1) | ,068 | ,275 | ,062 | ,804 | 1,071 | ,625 | 1,835 |
| Birth weight | ,049 | ,031 | 2,566 | ,109 | 1,050 | ,989 | 1,115 |
| Birth type (1) | 1,278 | ,426 | 8,980 | ,003 | 3,588 | 1,556 | 8,275 |
| Bull No | | | 3,523 | ,741 | | | |
| 1 | ,019 | ,563 | ,001 | ,973 | 1,020 | ,338 | 3,074 |
| 2 | ,000 | ,502 | ,000 | 1,000 | 1,000 | ,374 | 2,674 |
| 3 | ,209 | ,488 | ,184 | ,668 | 1,233 | ,473 | 3,209 |
| 4 | -,484 | ,478 | 1,025 | ,311 | ,616 | ,241 | 1,573 |
| 5 | -,435 | ,488 | ,792 | ,373 | ,648 | ,249 | 1,686 |
| 6 | -,190 | ,484 | ,154 | ,695 | ,827 | ,320 | 2,137 |
| Constant | -,895 | 1,336 | ,449 | ,503 | ,409 | | |

The effect of birty type on survival rate of calves is significan (P<0.01)

## CONCLUSION

In conclusion, the selection of sires was found effective on reproductive parameters and birth weights of calves, however was ineffective on the lactation milk yield, lactation length and lactation peak duration except peak milk yield.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# INFLUENCE OF PROBIOTICS CLOSTAT® AND LAKTINA® ON THE QUALITY OF MEAT OF PHEASANTS

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***Abstract***

*Probiotics are widely accepted as an alternative to the nutritive antibiotics in poultry production as opposed to farm breeding pheasants.The aim of the study was to investigate the influence of probiotics CloSTAT® and Laktina® on meat quality of 90 day-old pheasants. The experiment was conducted with 90 newly hatched pheasants (Phasianus colchicus colchicus), divided into 3 groups of 30 birds in each group, floor breeding with free access to food and water for 90 days. The three groups were fed with a standard compound feed for pheasants ad libitum, for the experimental groups as follows:for the second group (group B) probiotic CloSTAT® (0,5 g/kg feed) was added; and for the third group (group C) probiotic Laktina® (0,5 g/l of drinking water) was added. After completion of the experiment from each group were slaughtered 5 pheasants for meat sampling of the breast and leg. The following indicators were analysed: pH 24h post mortem, the water holding capacity, colour of the meat, content of myoglobin, protein and ash content. The results of the experiment showed that the use of the probiotics CloSTAT® and Laktina® increases the pH of the breast muscle, lightens the colour and decreases the myoglobin content in the leg and breast muscle, increases the water and mineral content in the leg muscle. The probiotics impact the protein metabolism in leg and breast muscle in different ways. The use of probiotic Laktina ® leads to the accumulation of a larger amount of proteins in the breast muscle, and the use of CloSTAT® - reduces their amount in the leg muscle.*

***Keywords:*** *pheasants, meat quality, probiotics.*

## INTRODUCTION

The widespread use of antibiotics in livestock farms to stimulate the growth, the increase of the efficiency of nutrition and the prevention of intestinal infections in recent years have led to the development of resistance in certain species of bacteria in the gastro-intestinal tract and the accumulation of residues in meat.

The use of probiotics as an alternative to the nutrition antibiotics is widespread in poultry, especially since some countries have banned certain antibiotics that are often included in the rations as growth promoters.

Probiotics are defined as viable microorganisms (bacteria or yeast) that competitively exclude colonization of intestinal pathogens and demonstrate a beneficial effect on the health of the host when ingested (Salminen et al., 1998).

Probiotics are widely used in poultry farming as opposed to farm bred pheasants. Many authors study the impact of probiotics on meat quality in broilers (Kabir, 2009; Ivanović et al., 2012; Hossain et al., 2012; Maiorano et al. 2012).

There are a number of studies on yield, chemical composition and quality of meat of wild and farm bred pheasants (Petkov 1984, Richter et al. 1992, Tucak et al. 2008, Hofbauer et al. 2010).

However, there are no studies on the quality of meat of pheasants, which orally ingest probiotics with feed and drinking water.

That is why our aim, in this study is to determine the impact some probiotics have on the quality of meat of pheasants.

## MATERIALS AND METHODS

The experiment included 90 newly hatched pheasants (*Phasianus colchicus colchicus*), divided into 3 groups of 30 birds. They were raised under controlled microclimatic conditions, extended light period (24 h / day) and free access to food and water for 90 days. The pheasants received identical in composition and nutritional value standard compound feed for pheasants, balanced in protein, energy, amino acids, etc. as required by the National Research Council (NRC) (1994). Composition and nutritional value of the feed mixture are presented in Table 1.

Table 1.Ingredients and analyzed composition of feed mixtures

| Ingredients, % | Starter (0-28 day) | Grower (29-90day) |
|---|---|---|
| Wheat+enzyme (10.5% crude protein) | 49,45 | 61,16 |
| Soybean meal (46% crude protein) | 38 | 30 |
| Fish meal (66% crude protein) | 9 | 5 |
| Sunflower oil | 1,2 | 1 |
| Synthetic L–lysine | -- | 0,1 |
| Synthetic methionine | 0,1 | 0,15 |
| Synthetic treonine | -- | 0,06 |
| Salt | 0,1 | 0,18 |
| Limestone | 1,2 | 1,1 |
| Dicalcium phosphate | 0,4 | 0,8 |
| Sodium bicarbonate | 0,2 | 0,1 |
| Aviax* | 0,05 | 0,05 |
| Micotox | 0,1 | 0,1 |
| Rovimix 11–C RonoP starter | 0,2 | 0,2 |
| Nutritive value | Starter (0-28 day) | Grower (29-90day) |
| Moisture,% | 11,1 | 11,8 |
| ME, (Kcal/kg) | 2872 | 2912 |
| ME (MJ/kg) | 12 | 12,2 |
| Crude Protein,% | 28 | 24,1 |
| Crude Fats,% | 3,6 | 3,3 |
| Linoleic acid,% | 1,6 | 1,4 |
| Crude Fiber,% | 3,8 | 3,6 |
| Crude ash,% | 5,8 | 5,5 |
| Ca,% | 1,07 | 0,98 |
| Available phosphorus,% | 0,54 | 0,51 |
| Phosphorus,% | 0,84 | 0,8 |
| Sodium,% | 0,21 | 0,18 |
| Chlorine,% | 0,21 | 0,22 |
| Chlorides,% | 0,3 | 0,33 |
| Lysine,% | 1,7 | 1,41 |
| Methionine,% | 0,54 | 0,5 |
| Methionine + Cysteine,% | 1 | 0,93 |
| Treonine,% | 1,05 | 0,92 |
| Tryptophane,% | 0,35 | 0,3 |
| Arginine,% | 1,85 | --- |

*to the combined forages of the control group is added Aviax 500g.kg-1 - which contains semduramicin sodium.

The experiment was conducted according to the following scheme (Table 2):

Group "A" (positive control) with antibiotic growth promoter Enrofloxacin and Colistin as a commercial product QUINOCOL (CEVA SANTE ANIMALE, France) in water (1 ml / 2 l of water); Second experimental group "B" - with the addition of probiotic CloSTAT® (Kemin, Inc., USA) in the feed (0,5 g / kg feed); third test group "C" - with the addition of probiotic Laktina® (Lactina, Bulgaria) in water (0,5 g / l of water).

Table 2. Scheme of experiment

| Indexes | Control group (A) | Experimental group (B) | Experimental group (C) |
|---|---|---|---|
| **Starter (0-28 day)** | Combined forages for pheasants + prevention | Combined forages for pheasants + probiotic CloSTAT® in dose 0.5 g/kg* | Combined forages for pheasants + probiotic Laktina® – 0.5 g /l drinking water ** |
| **Grower (29-90day)** | Combined forages for pheasants + prevention | Combined forages for pheasants + probiotic CloSTAT® in dose 0.5 g/kg* | Combined forages for pheasants + probiotic Laktina® – 0.5 g /l drinking water ** |

*-dosage of probiotic **CloSTAT®**- 0,5 g/kg (0,5 kg/t) is recommended by producer Kemin Industries, U.S.A.
**- dosage of probiotic **Laktina®** - 0,5 g/l drinking water ) is recommended by producer Lactina, Bulgaria

Prevention:

• Antibiotic QUINOCOL® in drinking water at a dose of approximately 1 ml/2l of water per day (on the recommendation of the manufacturer) -from 1st to 5th day

• Vaccination against Newcastle vaccine CEVAC BI L® by instillation into the eye on the instructions for use of the vaccine, the 7th, 28th, 64th and 80th day

• Vaccination against Gumboro vaccine CEVAC GUMBO L® by drinking water on the instructions for use of the vaccine, the 14th and the 22nd day

• Vaccination against Avian Pox vaccine CEVAC FP L® by applying in the wing fold on the instructions for use of the vaccine-on the 56th day

Description of probiotics:

• Probiotic CloSTAT® (Kemin, Inc.) contains: spores *Bacillus subtilis* $2x10^7$ cfu/g, Maltodextrine, Calcium Carbonate

• Probiotic Laktina® (Lactina) contains: *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus casei*,

*Bifidobacterium longum*, *Lactobacillus acidofilus* tbc in 1g not less than 1 billion.

*Slaughtering technology*

The pheasants had been slaughtered under the provisions of Council Directive 93/119/EC (1993). The birds were stunned by a blow on the occipital region of the head and killed by subsequent bleeding.

*Physical and chemical tests*

Laboratory analysis to establish the quality of the meat of pheasants were performed separately for breast and leg muscles in all three experimental groups.

Samples were taken from the pectoral muscles (breast) and femoral muscles (leg).

The muscle was separated from the bones and the skin and subcutaneous fat were also removed.

For characterizing the meat quality the following physical and chemical parameters were examined: pH value, colour, water holding capacity (WHC), water content, myoglobin, total protein and ash content.

Measurement of the active acidity (pH) was performed 24 h post mortem, according to ISO 2917:1999. pH meter HANNA instrument HI 8314 equipped with a thermometer and electrodes was used.

The indicator colour (R/525nm) was determined using spectrophotometer Specol 11 (equipped with a plug-in colour). Water holding capacity (WHC) was determined by the method of Grau and Hamm (1953) and expressed as content of free water in percentages.

The moisture content was determined according to BDS 5712: 1974.

Myoglobin content (mg/kg) by Hornsey (1956).

The protein content was determined by BDS 9374: 1982, and ash content - BDS 9373: 1980. The data was processed statistically by the Program StatMost 3.6, Dataxiom Software, 2003. The results were calculated using five replicates (n = 5) and presented as the average (mean) with a standard deviation (SD) (the SD is calculated for the group of 5 replicates).

The significance was defined as low ($p \leq 0,05$); average ($p \leq 0,01$) and high ($p \leq 0,001$) degree.

## RESULTS AND DISCUSSIONS

The results of the studied physical and chemical parameters of breast and leg muscles of pheasants are presented in Table 3.

Average mean pH values in meat from the breasts vary between 5.55 and 5.74, the differences between groups are significant.

Franco et al. (2013) obtained identical average pH(5, 69) in breast muscle of pheasant grown extensively and Kokoszyn'ski et al. (2012)

Table 3
Physical and chemical characteristics of breast and leg meat of pheasants

| Physical and chemical characteristics of breast and leg meat of pheasants | | | | |
|---|---|---|---|---|
| **Parameter** (n=5) mean±SD | **Type of meat** | **Control group A** | **Experimental group B** | **Experimental group C** |
| pH | breast | 5,553$^{b}$** ±0,1008 | 5,607$^{a}$** ±0,1151 | 5,744$^{a}$* $^{b}$** ±0,0812 |
| | leg | 6,511 ±0,0970 | 6,464 ±0,1539 | 6,513 ±0,2097 |
| Color, 525nm/R | breast | 38,7043 ±0,8529 | 38,810 ±0,9938 | 39,490 ±0,9329 |
| | leg | 37,044$^{ab}$* ±0,3393 | 38,471$^{a}$** ±1,1087 | 38,606$^{b}$** ±1,0910 |
| WHC, % free water | breast | 29,524 ±1,9099 | 27,556 ±4,4274 | 27,441 ±2,0141 |
| | leg | 19,620 ±2,6846 | 20,571 ±2,1765 | 20,413 ±1,9630 |
| Moisture, % | breast | 72,243 ±0,6480 | 72,313** ±0,2843 | 71,904** ±0,1474 |
| | leg | 74,764* ±0,2017 | 75,057* ±0,2133 | 74,893 ±0,1475 |
| Myoglogin, mg/kg | breast | 0,971 ±0,1469 | 1,013* ±0,1525 | 0,856* ±0,0489 |
| | leg | 2,577* ±0,2597 | 2,380 ±0,3878 | 2,234* ±0,2342 |
| Protein, % of total mass | breast | 25,614 ±0,6989 | 25,533** ±0,3630 | 25,979** ±0,1671 |
| | leg | 22,680 ±0,5879 | 22,213** ±0,0881 | 22,624** ±0,3349 |
| Ash, % of total mass | breast | 1,244 ±0,0355 | 1,247 ±0,0269 | 1,217 ±0,0461 |
| | leg | 1,147$^{a}$** ±0,0248 | 1,156$^{b}$** ±0,0270 | 1,200$^{ab}$** ±0,0224 |

*- p≤0,05; **- p≤0,01; ***- p≤0,001

slightly higher pH (5.80) in the breasts of Mongolian x Versicolor cross-bred.

In the breast muscle of the groups receiving probiotics (B and C) higher pH values were obtained in comparison to the control group (A) whose feed contained antibiotic QUINOCOL.

Fatma (2010) received similar results, measured the pH of breast muscle of broilers 24 hours after slaughter, and demonstrated a statistically significant difference between pH of the meat samples from chickens which had received probiotics in the food and chickens which had not.

Aksu et al. (2005) in a similar experiment with the addition of probiotics (*S. cerevisiae*) in the broiler feed found that the use of probiotic leads to an increase of pH in the meat from the breasts and leg.

Different results were obtained by Ivanović et al. (2012) about pH in the leg and breast of broilers, measured 5 hours after slaughter. According to the authors the probiotic was added to the diet of a test group which led to lower pH, in contrast to probiotics added to the food of the other test group which showed increased pH values compared with the control group, with statistically significant difference.

In our results , as opposed to the breast muscle, in the leg muscle significant difference between groups was not observed, the pH was in the range 6.46 to 6.51.

In all groups of experiments, the level of pH at the leg muscle on average is 0.86 units higher than that measured in the breast muscles.

These results are in accordance with studies of Richter et al. 1992, Kuzniacka et al. 2007, Paulsen et al. 2008, Hofbauer et al. 2010.

This fact can be explained by the different type of myofibres in the studied muscles. Breasts muscles of pheasants are composed mostly (> 70%) of fast-twich, glycolytic fibres (IIB type), while the muscles of the legs are with a higher percentage of anoother glycolyticoxidative (IIA) or oxidative fibre types(Kiessling 1977). This difference is common to Gallinaceous birds and affects the ability to scramble at the expense of short-haul flight (Pyörnilä et al. 1998).

Stress from manipulations before slaughter may also have an impact, especially in the breast muscles which contain much more IIB fibers (Lawrie and Ledward 2006).

The absence of differences in the pH of the leg muscle and the higher values of pH, in comparison to the breast muscle may be due to smaller glycogen reserves in the leg muscles after the slaughter of pheasants (Franco et al., 2013), which in our opinion is a result of earlier puberty, altered behavioural responses and expressed aggressiveness.

No statistically significant differences in WHC between the experimental and control groups were observed.

No significant differences in WHC and cooking loss (CL) are the results of Pelicano et al. (2003) who tested three different types of probiotics in broilers chicken.

Kim et al. (2010) found a positive influence of probiotics used in fattening pigs on WHC of meat and improving its technological and cooking qualities (tenderness, flavour characteristics, etc.).

The higher percentage of free water in the breast muscles for all groups of our experiment compared to the leg muscles can be explained by the low pH of this type of muscle.

Loss of water is higher in muscles with a low pH as shared by other authors (Hofmann, 2004).

The tested probiotics have influenced the colour of breast meat, with higher values for the test group that received probiotic Laktina® in water) 39.49 at $\lambda = 525$ nm.

However, differences between groups are not proved, unlike the meat colour in the leg, where we have significant difference ($p \leq 0,01$) between the experimental and control group. The observed differences in colour correspond with the content of myoglobin in red meat - significantly at the lowest in the test group (2,234 mg / kg) where the meat colour is lighter.

The result was the same, in the breast muscles, where the lightest coloured birds stand out from the test group (0,856 mg/kg) ,although not significantly.

While testing the three different types of probiotics in broiler chickens Pelicano et al. (2003) found that the concomitant use of probiotics in feed and drinking water significantly reduces the values of lightness in the colour of breast muscles after slaughter, leading to "a little pale" meat and values of the red component where redness were higher in the treated with probiotics groups than in the control group., Meng et al. (2010) also obtained the same results for colour and

higher values of the red component (redness) in pigs fed with probiotics compared with pigs fed without probiotics.
The values obtained for water content in the breast muscle in all groups were lower than the values in the leg muscle.
This corresponds with the results of other authors (Severin et al. 2007, Hofbauer et al. 2010, Franco et al., 2013), who received approximately 71.8 to 73.09% of moisture for breast muscle, and from 74.2 to 75, 2% moisture for the leg muscle of pheasants.
There were significant differences in water content between treatment groups B and C (which received various probiotics) in samples of breast meat.
There was significant difference ($p \leq 0,05$) for the samples of leg meat between the experimental and the control group (C) that received CloSTAT® in feed.
There were no significant differences in water content in group (C) that received probiotics in the drinking water. Protein content of the meat from the breasts of all groups of pheasants was higher than the content in the leg muscle.
Same results were obtained by Hofbauer et al. (2010) and Franco et al. (2013), where the protein concentrations in breast muscles were significantly higher than those in the muscles of the legs.
Same are the conclusions of Tucak et al. (2008), although Severin et al. (2007) found no difference between the protein content of breast and leg muscle of pheasants.
A high protein content is associated, by most authors, with a low content of moisture and fat in this type of muscle.
It is also important to note that the protein content that we found for the breast meat of pheasants (25.5 to 25.9%) is higher than that found in broiler chickens (Ding et al., 1999; Qiao et al ., 2002), which varies between 22.6 and 24.7%.
The protein content in the leg is higher 22.2 -22.6%) compared to the findings of Ivanović et al. (2012) in broilers - 19.83%.
Both probiotics affected the protein content in meat from the legs and breasts differently.
This confirms the assumption that probiotics affect the protein metabolism.
The results show no significance compared to the control group, a slightly higher amount of protein in the breast muscle of test group (C) and slightly lower - in the leg muscle of test group (B).
The opinions of other scientists on this issue are different.
Higher protein synthesis is established by Ignatova (2004), in experiments with broiler chickens.
Ivanović et al. (2012) and Hossain et al. (2012) found higher levels of protein in breasts and less in the leg, using probiotics also in broilers. Furthermore, according to Hossain et al. (2012) the addition of probiotic increases the absolute and relative weight of the breasts.
Again after using probiotics for broilers, Sazedul et al. (2010) found higher protein content in the meat from the leg (respectively 23.89 and 21.94%).
In terms of mineral composition, no difference in samples of breast meat was observed.
Values in all groups are close to those established by Petkov (1999) 1,08 - 1,23%, Franco et al. (2013) 1,26%, Hofbauer et al. (2010) from 1.30 to 1.39% of mineral substances in breast muscle of pheasants.
In the leg meat there were significant differences ($p \leq 0,01$) in the mineral composition of group (C) compared to the control and also between the two experimental groups (B and C) to which a probiotic was given.
The data correlated with the measured values by Petkov (1999) from 1.02 to 1.18%, and Tucak et al. (2008) from 1.06 to 1.15% for mineral substances in the leg muscles of pheasants.

## CONCLUSIONS

The results obtained in these studies on antibiotic growth promoters and their ecological alternatives - probiotics, give us reason to form the following conclusions:

- The use of probiotics (CloSTAT® and Laktina®) orally in pheasants results in an increase of pH in the breast muscle, lighter

meat color and lower amount of myoglobin in the leg and breast muscles.

• The use of the probiotic CloSTAT® in the feed resulted in an increase of water content in the leg muscle of pheasants.

• The use of the probiotic Laktina® in drinking water results in an increase of the mineral substances in the leg muscle of pheasants.

• Probiotics (CloSTAT® and Laktina®) impact differently on protein metabolism in breast and leg muscles of pheasants. The use of Laktina® leads to the accumulation of large amount of proteins in the breast muscle, and the use of CloSTAT® - reduces their amount in the leg muscle.

## ACKNOWLEDGEMENTS

This document was supported by the grant No BG051PO001-3.3.06-0056, financed by the Human Resources Development Operational Programme (2007 – 2013) and co-financed jointly by the European Social Fund of the European Union and the Bulgarian Ministry of Education and Science.

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# MISCELLANEOUS

Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# GENERAL PRINCIPLES CONCERNING THE HARMONIZATION OF ROMANIAN LEGISLATION WITH THE EUROPEAN UNION IN THE FIELD OF PROTECTION OF ANIMALS USED FOR SCIENTIFIC SCOPE

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***Abstract***

*The introduction of alternative methods in the research, in the diagnosis of diseases and in the production of bio-preparations, led over time to a drastic reduction in the number of animals used for scientific scope in Romania. However, our country has always aligned legislation in this area with the European Union. In this regard, last year was transposed into national law Directive 63/2010 which refers to animal protection used for scientific scope, transposition materialized by Law no. 43/2014.Although the law is very complex, including a large number of issues, including general and special requirements on the units, care and housing of animals, animal species that can be used in procedures, there are a number of issues for which the law requires the development of a secondary legislation in areas such as: authorize breeders and users of laboratory animals, create a bank of organs and tissues of animal origin, able to reduce the number of animals used in experiments, authorization of projects, setting and punishing contraventions and others. This law, new for Romanian legislative landscape, will determine an increase in the level of consciousness in the use of this category of animals and conducting scientific research of the best quality.*

***Keywords:*** *animal, experiment, harmonization, protection.*

## INTRODUCTION

The idea of using animals in experiments concerning scientific research on the human body appeared at an early stage of medical research and the development of techniques and procedures regarding animal models was parallel to the development of medicine.

Since the 18th century experimental medicine started being regarded as a necessity in the increase of human welfare and living conditions. It became also clear that the development of medicine is dependent on the results of experimental medicine.

At the end of the nineteenth century, animal experimentation started exploding and becoming an integral part of biomedical research, and the factors that contributed to this were: the discovery of anesthetics in the first half of the nineteenth century and their use in animals exposed to painful experiments. The book published in 1859 by Charles Darwin ,,Origin of Species", showed a homology between humans and animals, thus providing a rational basis for the use of animals as a model for humans.

The book ,,Introduction to the study of experimental medicine" published in 1865 by Claude Bernard's describes the tools used in the design of experiments. It has become a ,,bible" of animal experimentation and also of development of microbiology, thus contributing to the production and testing of serums and vaccines.

The development of industry in the 20th century resulted in a rapid increase of the use of laboratory animals. Both the number and also the species of animals used increased. While, at the beginning, only domestic animals were used, in the early 20th century species like mice, rats and other mammals, reptiles, birds, amphibians and fish started being used.

Every year, 75-100 million animals are used throughout the world, of which 10-12 million in Europe.

Most animal experiments are performed in medical sciences, biological, veterinary medicine and agriculture. In biology, veterinary medicine and agriculture, experiments on animals are used to obtain information about the species that have been made experiments.
A large number of animals are still used for medical research and safety testing of drugs and vaccines. In these fields, the animals are almost exclusively used as a replacement or as a model for humans.

## MATERIALS AND METHODS

Animal experiments have become a political issue by the emergence of legislation in this area. The rules regarding the protection of animals used for experimental and other scientific purposes is to put under the control of governmental authorities the breeding and the use of laboratory animals to ensure the welfare of this category of animals.
The regulations have emerged as a result of public and non-governmental organizations' pressure that fight to reduce animal testing on one hand, and of scientific society pressure on the other hand, to create a single framework for the use of laboratory animals.
In the European Union, by 2010, the legislation regarding protection of animals resulted in two acts, namely the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe, 1986) and the Council Directive 86/609/EEC (The Council of the European Communities, 1986).
After signing the accession treaty to the European Union, Romania was required to adopt and implement the European Union legislation, including regulations related to animals used for scientific purposes (European Union, 2005).
In 2010 the European Union adopted a new directive for the protection of animals used for scientific purposes, Directive 2010/63/EU (European Parliament, 2010) which was transposed into the Romanian Law No. 43/2014 (of the Romanian Parliament, 2014).
In addition to the implementation itself, the law comprises the preparation, adoption and implementation of a complex secondary legislation.
Analysis on historical and legislative framework on the use of laboratory animals in our country is done in compliance with the European Union legislation and principles.

## RESULTS AND DISCUTIONS

*Brief history of the use of laboratory animals in Romania*
The use of animals in experiments is attested from the late nineteenth century and early twentieth century. Former students of Louis Pasteur and Robert Koch founded several institutes in Romania, in which animals are used in the production of serums and vaccines. The first experimental medicine department in Bucharest was founded in 1904. Animal experimentation in Romania began using animals in the production of vaccines and serums and biological control of these products.
Using the large number of animals in the production of therapeutic sera and vaccines began after a campaign of anti-cholera and anti-dysentery serumization among soldiers who fought in the Second Balkan War (1913).
In 1921, the government of the time established the first institute to use laboratory animals, the Cantacuzino Institute, which was similar to Pasteur Institute in France.
During the communist regime (1945-1989), a strong increase in the number of animals used for experimental purposes could be observed, the reasons being: to pay for war reparations by selling serums and vaccines, to produce all medicines, serums and vaccines locally and to develop and establish new experimental research institutes.
The effects of this development of animal experimentation were: an increase in the number of animals used, the peak being reached in 1975 with 2.6 million animals used (Potorac, 1989), a higher number of users of laboratory animals (in 1975 there were 352 institutions and 27 breeders), a diversification of species used: rodents, monkeys, dogs, cats, ferrets, birds, reptiles and a continuous improvement and growth conditions of accommodation.

The highest number of animals was used in the production of vaccines and serums, safety control drugs and biological products, as well as diagnostic.

After the revolution in 1989, a dramatic reduction in the use of animals in experiments, has taken place, the main reasons being: reorganization, dissolution or privatization of research institutes, privatization and dismantling of most drug plants and animal facilities in research and development divisions, purchase of imported drugs and vaccines and implementation of alternative methods to animal experiments.

This decrease in animal experimentation reduced the number of animals used in experiments, restricting the number of species used and animal research.

In the last year of the communist regime the number of animals used was approximately 900,000. The first statistics after that year was made in 2002, the year when Romania began his road to the European Union. In 2007, the year of entry into the European Union, the number of animals used was approximately equal to that of 2002, but it was the first year without the use of non-human primates and carnivores. 2011 is the year of the third report to the Council of Europe of Romania. As it can be seen in table 1 the number of animals used is half compared to 2007 (Gonciarov 2014). This year there were also non-reported, cold-blooded animals used for scientific purposes, together with carnivores and non-human primates. The main reason for this reduction is the introduction of alternative methods in diagnosing diseases and in the production of biological products.

The main areas in which animals are used for scientific purposes are, in order of their numbers: production of biological products and their quality control; diagnostic, research and education. Romania filed in 2011 the third report on the statistics of the use of laboratory animals, according to the 2010/63/EU Directive.

The total number of animals represented 0.5% of the number of animals used in the European Union. Research used 10% of the animals; about 40% was used in diagnosis, 35% in production and quality control of human biological products and 10 % in veterinary products and in safety studies (Figure 1).

Table 1 – The number of laboratory animals used in Romania

| Species | Years | | | |
|---|---|---|---|---|
| | 1989 | 2002 | 2007 | 2011 |
| Mice | 560.000 | 80.000 | 95.000 | 44537 |
| Rats | 150.000 | 15.000 | 9.000 | 5314 |
| Guinea-pigs | 130.000 | 12.000 | 12.000 | 6602 |
| Others rodents | 4.300 | 1.500 | 700 | 240 |
| Rabbits | 22.000 | 12.000 | 4.000 | 2166 |
| NHP | 17 | 2 | 0 | 0 |
| Carnivores | 286 | 57 | 0 | 0 |
| Birds | 4.500 | 3.000 | 2.000 | 1.203 |
| Artio + perissadactyla | 1.003 | 427 | 156 | 124 |
| Cold blooded animals | 237 | 136 | 54 | 0 |
| Total | 872.343 | 124.122 | 122.910 | 60.186 |

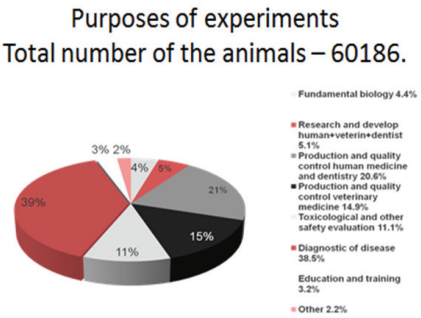


Figure 1 - Purposes of using laboratory animals in Romania

*Romanian legislation on laboratory animals in Romania*

Until signing the association agreement with the European Union, Romania had no specific legislation for animals used for scientific purposes. By signing the agreement for European Union membership Romania has assumed full adoption of the European Union legislation. In this respect, the European Union legislation on laboratory animals was adopted and then implemented (Gonciarov, 2011).

The first two laws transposing the 2010/63/EU Directive were the Government Ordinance No. 37/2002 adopted by Law No. 471/2002 on the protection of animals used for experimental and other scientific purposes. Other two laws (Law No. 205 / 2004 on the protection of animals, as amended by Law No. 9/2008 and the Order of the National Sanitary Veterinary and Food Safety Authority and Ministry of Interior and Administrative Reform No. 523/2008 approving the Methodological Norms for the application of Law No. 205/2004 on the protection of animals) contain specific provisions for the use of animals for scientific purposes in a comprehensive law for animal protection.

*The 2010/63/EU Directive and its implementation.*

Although the 2010/63/EU Directive had to be adopted by October 10, 2012 and be effective starting from 01.01.2013, Romania has transposed the directive quite late, by Law no. 43 of 11 April 2014 - Protection of animals used for scientific purposes, issuer: Romanian Parliament, published in Official Gazette no. 326 of May 6, 2014 – which entered into application starting with May 9, 2014.

Compared to the 471/2002 Law, this is much larger (if we look only at the number of pages, it is five times greater) and it is not like a law on national regulations, these regulations only give general principles and not content as does the present law. There was much talk about how to adopt, as government decision, a government ordinance.

The Directive was transposed by law (this is a rare procedure regarding Directives). The explanation was probably to avoid ordinances, as in ordinary laws; another reason is, in our opinion, that a legislation had to be replaced with another of the same or higher value to the first legislation.

Adoption of the 2010/63/EU Directive was made in a rush and Romania will enter in an infringement procedure (infringement of European Union law). As a result, the transposing law (43/2014 Law) is largely (approximately 75%) a simple translation of the directive, sometimes quite unclear. The competent authority on the protection of animals used for scientific purposes is the National Sanitary Veterinary and Food Safety Authority, having the role of initiation of normative acts and additional national legislation in this area. For joint projects to be elaborated within the law, other central state bodies (Ministry of Environment and Climate Change, Ministry of Health, Ministry of Education) join forces. The great majority (90%) of tasks such as implementation responsibility, creation of welfare and ethics committees, project evaluation, training of specialized staff, creation of a material and technical base, are the tasks of the National Sanitary Veterinary and Food Safety Authority.

In order for the law to be implemented, the legislation has to regulate the following aspects of the law:

*Authorization of breeders, suppliers and users of animals used for scientific purposes.*

While until now there was only one piece of legislation regulating veterinary authorization of all operators, the appearance of the 43/2010 Law led to the imposition of adopting a different and specific legislation to regulate the licensing of various fields. The same will happen for producers and users of laboratory animals. Until then, permits are suspended. In order to prepare for authorization, an evaluation form containing requirements related to the use of animals for scientific purposes, and also requirements on environmental protection, fire safety etc. will need to be completed. There will be a total of about 50 requirements, differently marked. In the case that one of the conditions are not met, the authorization will not be granted.

An important part of the licensing documentation will be the name and competence of persons responsible for the welfare of the animals. The law provides comprehensive staffing structure that will provide staff training, welfare, care and ongoing supervision of their.

In the case of large units, this structure requires active collaboration between those involved in animal monitoring and those who use the animals. In the case of small units, cooperation agreements with experts and specialists in the field, without the requirement of a fixed work schedule will be

accepted. Concerning staff competence, the requirements are set out in Annex V of 2010/63/EU Directive.

*Education and Training*

Until now, training in this area was not compulsory. Due to the small number of people involved in the growth and use of laboratory animals, the training has been made at the place of work. After joining the European Union a large number of students and researchers were trained in universities in Western countries. The competent authority will authorize courses organized on three levels: caretakers, technicians and experts. The main purpose of the success of organizing such courses is that diplomas will be recognized in all European Union countries.

*Authorization of projects*

So far, the only projects approved are research projects. In this regard, research ethics committees were established at the site of each user of research laboratory animals, as imposed by Law 206/2004.

For obtaining authorization for the use of animals in other areas, new licensing structures will be created. Competent authorities issue authorizations after receiving a positive report from a committee of ethics. Further rules concerning the election of the commission's evaluation, confidentiality, conflicts of interest, etc will need to be set. A simplified administrative procedure for animal use in our country is expected to ease the approval process of projects. This procedure will be established jointly by key ministries.

*Alternative methods to animal testing*

The competent authority will contribute to the development and validation of alternative methods that could provide the same level of information as those obtained in in vivo studies. A reference laboratory for coordination and promotion of the use of alternative methods to animal testing will be established. The Institute for Diagnosis and Animal Health will provide national promotion and will publicize information about alternative methods, and will also provide training in this area. Over the next two years, several alternative methods will be implemented in Romania, especially in terms of confirming diagnosis of disease, and some methods for testing the efficacy and safety of biological products. Also, in education, animals will be replaced with solid computer models, digital or similar.

*The use of animals from the wild*

Romania has a rich wildlife, and so far the use of wild animals for scientific purposes has not been subjected to any act regulations, protecting referring only to habitat protection and to avoid their hunting. The new law provides that this category of animals, when their use is for scientific purposes to be subject to authorization. The birds for the migratory bird communities are taken to monitor their blood for disease control, main transmitted by birds and through birds (meningoencephalitis, West Nile fever, avian flu, etc.). The huge number of wild mammals required monitoring in terms of disease incidence possible to be transmitted to domestic animals and humans.

We believe that implementation of this law requires the application of offenses which are currently not included in the law. The transposition will generate some costs, especially with the improvement of animal accommodation.

## CONCLUSION

Most European countries already have a strong culture for caring of animals used for scientific purposes and operate with high standards with consideration on animal suffering. The 2010/63/EU Directive, the new regulatory landscape for Romania, will increase the level of consciousness in the use of this category of animals.

Even if the number of animal users is small, implementation of the Directive is challenging, and we hope that the implementation will be done properly without subsequent necessary corrections.

Correct implementation of the Directive is very important especially for making high-quality scientific research, but also to reduce the suffering of these categories of animals. We estimate that the entire adoption of secondary legislation and its implementation at the user level will be completed by the end of 2017.

The new Directive changes substantially the legal system for regulating animal experiments in Romania and the implementation of this Directive in Romania will introduce a unified legal framework, which will benefit definitely animals and professionals involved.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# ARTIFICIALLY FORCED FERTILITY IN DAIRY CATTLE EMBRYO TRANSFER

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***Abstract***

*Forced increased fertility in cattle can be obtained by multiple ovulation embryo transfer (MOET). This paper presents results of a trial to shorten interval between two pollyovulation treatments to the term determined by the natural estrus shown by donors after the previous embryos' collection. Pollyovulation was provoked using FSH. Experiment started with 4 Romanian Black and White heifers, 17 month aged, but only two of them answered to the treatment for estrus synchronization. Both these heifers have shown heat in the same day, at 11 days after uterine washing. A total number of 27 yellow bodies were counted (14 units in one heifer and 13 in the other), but only 12 embryos were collected (11 embryos from the first heifer and 1embryo from the second). There is no explanation of the fact. The resulted interval between pollyovulation treatments was 53 days, 7 days less than the 60 days interval recommended by literature. More interesting was the presence of a total 26 atretic follicles in the 4 days (diestrus state) of embryo collection. They must pertain to the first wave of follicles starting maturation after prostaglandin injection given at embryo collection to prevent pregnancy. The fact suggests the possibility to stimulate these waves of follicles to have shorter interval between pollyovulation treatments. It is counted that the interval could be shortened up to 25 days. If that is possible MOET could be applied in heifers to increase selection precision of mother of sire dams by progeny testing them. New idea is to use MOET in dams of known genetic merit to discover better compatibility of parental pairs to obtaining the wanted type of the breed. Experiments are needed also to establish if more frequent uterine washings cause or don't cause alteration of uterine mucosa functions.*

***Key words****: dairy cattle, embryo transfer, poliovulations, cow fertility.*

## INTRODUCTION

Berge S. says "*fertility is the property of mammals to give birth to viable progeny*" (Berge S., 1965).That means fertility (F) is a trait of organisms like anyone else which can be measured for individual animals or for groups of animals. Fertility quantification is done by the number of progenies given in determined time, usually per year or per life. Paraschivescu M proposes to measure the fertility of cows also by the calving interval (CI) **(**Oțel V., Paraschivescu M., Mihăilescu C., 1967).

Artificial Insemination in dairy cattle has given the possibility to tremendously increase the fertility of bulls. Par consequence that has permeated selecting sires for artificial insemination with great intensity and precision (vertical genetic improvement) and also a large dissemination of their traits within the breeds (horizontal genetic improvement). Results are already wonderful. Theoretically nothing could be added in testing bulls.

But people wants more and try to get it by a higher precision in selecting sires dams. They might get it if would induce sufficiently high fertility in heifers tested to become mother of sires and in cows with known genetic merit already selected as breed dams. Such hopes are related to increased performances of Multiple Ovulation Embryo Transfer (MOET) especially by shortening the interval between embryo collections.

As it is known cow fertility is low because ovogenesis is stopped in the embryonic stage of ontogenesis and the number of emitted gametes is limited at the number of the primary follicles in the ovary. In addition, naturally, only one ovary follicle is dehiscent in one estrus cycle, even more of them entered maturation. More than that after the ovum fertilization a long gestation has to be run in order to allow the development and growth of one new born calf. Pregnancies with twins are not well sustained by dairy cows and the trait is not wanted in the commercial dairy farms. Then it is necessary to call for receptor foster (or surrogate) mothers after pollyovulation by hormonal treatments has been induced in donor mothers.

The present experiment is limiting its goal to start investigations concerning the shortest interval between ovulations keeping acceptable pollyovulation rate and normally formatted embryos. Questions as having good conception rate in receptors after embryo transfer or of normal fertility in donors after embryos' collections are not covered by the present paper. Nevertheless the experiment was put on donor heifers, having in view future research on this theme.

## MATERIALS AND METHODS

For the initial estrus synchronization of donors we disposed of 4 heifers aged 17 months. They were clinically inspected for the normal development of the ovaries and of the genital tract. Nevertheless only 2 of them had well formatted yellow body and responded to the normal standard of diestrus after prostaglandin treatment for estrus synchronization. Treatment has been done at 8 hour in the afternoon and the two of them answering to the treatment showed heat after in the third day after 72 hours from the prostaglandin injection.

That was the 0 day of the pollyovulation trearment. Maturation of ovary follicles started in the $11^{th}$ day. (E. Robertson 1999).

Both heifers received the same treatment by intramuscularly injection following the next schedule (Tobă G. et al., 2011):

-$11^{th}$ day at 7 hour a.m. 4 ml FSH,
-$11^{th}$ day at 7 hour p. m. 4 ml FSH;
-$12^{th}$ day at 7 hour a.m. 3 ml FSH,
-$12^{th}$ day at 7 hour p.m. 3ml FSH;
-$13^{th}$ day at 7 hour a.m. 3ml FSH, $13^{th}$ day7 hour p.m. 3ml FSH + 2ml $PGF_{2\alpha}$.

In the $14^{th}$ day from the treatment supervision for proestrus signs was provided. No sign was noticed.

In the $15^{th}$ day at 17 o'clock 3 ml of GnRH (Receptal) were inoculated and after 2 hours (at 19 o'clock) heifers have been inseminated. Insemination was repeated after 12 hours at 7 o'clock in the next morning.

Embryo collection was provided by uterus washing with buffered solution in the $7^{th}$ day after insemination. It started with the counting of number of yellow bodies and of atretic follicles on the ovaries. Inoculation of 2 ml of $PGF_{2\alpha}$ and 3ml of GnRH was done after washing in order to avoid occasional pregnancy.

The next follicular stimulation was decided to take place after the first not induced estrus by repeating the same procedure for pollyovulation as before.
The entire experiment protocol with the two accepted heifers is presented in table 1. There is a perfect synchronization of the ovarian activity in both heifers and in both cycles

Table 1 Treatment timing in the two heifers

| Donor heifer | Order of treatment | The 0 Day | Folicular stimulation | Insemination | Washing |
|---|---|---|---|---|---|
| 3618 | I | 19.05 | 30.05-01.06 | 04.06 | 11,06 |
| | II | 11.07 | 22.07-25.07 | 28.07 | 03.08 |
| 1092 | I | 19.05 | 30.05-01.06 | 04.06 | 11.06 |
| | II | 11.07 | 22.07-25.07 | 28.07 | 03.08 |

Collected embryos were classified for the biological quality and were frozen in yellow straws when ethylene glycol crioprotector was used (first collected embryos) and in white straws when glycerol as cryoprotective agent was used (second embryo collection.

## RESULTS AND DISCUSSIONS

Two heifers have out of four shown well developed ovaries after the first estrus synchronization. The 50% of not answering to the treatment heifers at 17 month of age show unfair life conditions of the replacement heifers of the farm.
The 0 day interval between the two embryo collections was 53 days, 1 week less than the minimum interval recommended by literature. It includes 11 days after the naturally expressed estrus by the heifers, what happened to take place in the same day. The term of 11 days is the middle of diestrus when the prostaglandin injection for new synchronization was done.
Trying to understand how the 53 days interval is formatted we will find: the above mentioned 11 days, other 11 days + 3 days for the next pollyovulation treatment, 3 days up to heifers' insemination and 7 days passed up to the second embryos' collection. That means a total of 35 days. There is a difference of 18 days that remains for the natural estrus cycle proposed by the experiment protocol to have place between the two induced heats. This assertion has to be accepted since it respects the variability limits of normal ovary cycle (21±3 days) in cattle.
We can conclude that the applied hormonal treatment did not impaired normal function of the ovary. We can consider, also, that the second estrus synchronization wasn't necessary since the natural estrus was synchronized in the two heifers. If that's true the inter pollyovulation treatments' interval might be reduced to 39 days (the actual 53 days interval – (11 + 3 days for the second induced estrus synchronization).
The ovaries' answers to poliovulations stimulation are presented in table 2.

Table 2 Ovary answer to the poliovulations stimulations

| Donor heifer | Order of treatment | Ovary answer | | |
|---|---|---|---|---|
| | | Yellow bodies | Antral follicles | Collected embryo |
| 3618 | I | 3 L + 4 R | 3 L + 3 R | 4 |
| | II | 4 L + 3 R | 1 L + 1 R | 7 |
| 1092 | I | 2 L + 2 R | 6 L + 5 R | 0 |
| | II | 5 L + 4 R | 3 L + 4 R | 1 |

Looking figures in Table 2 first thing to be noticed is the big difference between heifers concerning the number of collected embryos. After the first induced poliovulations 4 embryos have been collected from the 3618 heifer and 0 embryos were collected from the heifer 1092. At the second attempt the difference was even greater (7 to 1). Since the embryo washing was done by the same operator using the same procedure the difference must have a biological explanation. Which one it might be we can't say.
No unfertilized ova were found 7 days after follicular dehiscence.
Remarkable thing is the number of antral follicles in the 7$^{th}$ day after insemination what

means in the 8th day after the previous estrus. Totally in 4 diestrus states 26 antral follicles were detected. They must pertain to the first wave of follicles entering maturation of the cycle induced by the prostaglandin injection that has commanded estrus. Then why not try to stimulate maturation of this wave of follicles, too?

There is a hypothesis that has to be verified. Using the same presented schedule this stimulation can start 13 days after the prostaglandin injection. Then 5 days later the donor heifer can be inseminated. If maturation had success embryos should be collected after 7 days. Thus the interval between two successive washings is reduced to 25 days (the above 13 + 5 + 7 days). That makes possible to have 5 embryo collections within 101 days. If 5 embryos were collected by one washing we would dispose of 25 embryos to be transferred. With the 50% sex rate in embryos and 60% pregnancy success obtained using E. Robertson's procedures of freezing embryos under ethylene glycol protection, about 7 – 8 daughters might be got from one donor heifer (Paraschivescu, M. Th., 2010). If the number of successive pollyovulation stimulations is increased the donors' fertility is larger.

Now, what benefit could be obtained increasing females' fertility in cattle breeding?

The question has particular response in case of heifers and in case of adult cows.

Since in dairy cattle the genetic merit of heifers for milk production can be estimated only by ancestors' performances and the hereditability coefficient is low ($h^2$=0, 2) there is no interest to increase their fertility. Nevertheless it could be of interest if heifer would receive such a high fertility as to be progeny tested for milk production (Paraschivescu M. et al. 1989). In this case dams' selection to produce sires for artificial insemination will become much precise. But the breeder will be confronted with some difficulties. The first one is the future dam must have enough number of daughters to be tested on progeny (in bull progeny test at least 30 daughters are required for a 70% repeatability of the result) (Paraschivescu M. et al. 1989). The second one is the desire to have the result of the test as early as possible so that she would get in calf at least 4 -5 times after the result of her progeny test is known. Other way the procedure is too costly to be practically applied (Paraschivescu, M. Th., 2010). To reduce costs sexing embryos for female become of interest.

If the donor is a dam of known genetic merit increasing her fertility is always desired. Progeny of any sex are wanted.

The forced fertility could bring a new idea in using the breeding stock. That is to find out the genetic combinability of partners. The idea is used in poultry breeding when industrial hybrids are obtained. In cattle when a registered cow receives the merit of dam after her first parturition she can be included in an individual test program for compatibility with bulls of different families. The program can start with the first follicular stimulation for poliovulations in the 13th day from day of heat. Then the treatment schedule mentioned in the present paper could be applied.

## CONCLUSION

Efficient poliovulations and embryo collection can be applied using the treatment schedule and uterus washing terms presented in this paper. Individual differences in the in the heifers answer to the treatment can't be excluded. Further ovary function is not affected by the treatment.

The presence of many atretic follicles on ovaries in the day of embryo collection suggests that the 53 days interval between treatments for pollyovulation could be shortened up to 25 days if a new stimulation of ovary follicles stimulation is promoted 15 days after embryo collection and prostaglandin injection has to avoid pregnancy.

Experiments to estimate the effects of more frequent uterine mucosa must include longer chains MOET components. Success of these procedures opens new ways to use MOET in genetic improvement of breeds.

With heifers increased precision in selecting dams as mother of bulls for AI could be obtained testing them by progeny.

With cows of known genetic merit the compatibility of dam and sire pair could be discovered if insemination of donors cows at

repeated embryo collections is done using semen of different bulls.

he program gives the possibility to elect among the best made combination of parental pairs.

In order to reduce cost of such a program cheep receptor heifers of beef breeds. Using receptor heifers there is chance to have better embryo transfer pregnancy rates than using receptor cows (Paraschivescu M.Th. et al.,2003).

The main risk of forced increased fertility results from infiltration of uterine tissues during washing embryos. Less danger is apart Prostaglandin or FSH hormones since they have a great division speed.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXI (1)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

# AN EXPLORATORY RESEARCH REGARDING ROMANIAN ORGANIC FARMING SECTOR

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***Abstract***

*The paper is aimed to identify how large is the sector of organic farming in Romania and why this sector should be developed. Because of the changes in the social structure, the economic crisis and the ageing of population, Romania must identify other segments and industries for an economic prosperity. This study focuses on a market niche represented by organic farming. In order to present how large is the sector of organic farming in Romania, statistical data regarding agricultural areas and livestock under organic farming were gathered and analyzed. Results shows that organic farming has a low impact in Romanian agriculture, and in agro-food system, but because of accelerated growth of indicators, Romania has a big potential and interest in developing the organic sector. As a conclusion, it is a very interesting and attractive subject, because people are more and more focused on consuming natural products for a healthy life.*

***Key words****: agriculture, organic farming, evolution, sustainable development, Romania.*

## INTRODUCTION

According to the Codex Committee on Food, organic farming is a production system management that promotes and also enhances the agro-system health, biodiversity, biological cycles and soil biological activity. On the other hand, this is a form of agriculture that is based on techniques like green manure, crop rotation, biological pest control and compost. Organic food also includes fertilizers and pesticides which include insecticides, herbicides, but all of these are natural, like bone meal for animals or pyrethrin for flowers. In this process, the use of other methods, which includes the synthetic petrochemical fertilizers and pesticides, is excluded.

Organic farming is one of the most important sectors of agriculture and of Romanian economy, because it can bring significant contribution to a sustainable development, increasing the economic activities, thanks to the significant added value of the organic products. The premium price of organic products is paid by people from countries where there is a sizeable middleclass in the population, and where consumers are more educated and informed of food issues, and they incline to buy organic products, whether for food safety, concern over the environment or health reasons (Voilcilas Dan Marius, 2009).

The concept of sustainability, which is incorporated into the definition of organic farming, has a wider sense, not only to underline the conversion of non-renewable resources (soil, minerals and energy), but also in the social sustainability (Radev et al., 2012). Organic farming also increases the interest in rural areas; the methods and materials that organic farmers use in order to keep and build soil structure and fertility are: crop rotation, the right soil cultivation at the right time, composted and recycled crop wastes and animal manures, mulching on the soil surface and green manures and legumes. In order to control pests, diseases and weeds, the methods and materials are: a good cultivation practice, encouraging useful predators that eat pests, careful planning and crop choice, increasing genetic diversity, crop rotation, using natural pesticides, and the use of resistant crops. A good practice of organic farming also involves

a careful use of water resources and a good animal husbandry.

Moreover, the main objective of ecological food system is to produce cleaner food according to environmental conservation and development, using safe methods in correlation with nature and its systems (Manole, 2006). Organic farming has a small share in the Romanian agriculture, in terms of agricultural area and livestock production, however Romania has a high potential for developing the organic farming sector (Ion Andreea-Raluca, 2012).

This paper aims at answering the question how large is the organic farming sector in Romanian agriculture. In pursuing this, statistical data regarding agricultural areas and livestock under organic farming were gathered and analyzed.

## MATERIALS AND METHODS

In order to analyze the organic farming sector in Romania, the following indicators were used: area under organic farming (hectares), area under organic farming, by category of use (percentage), area under organic farming, by main plantations and crops (hectares), evolution of livestock under organic farming (heads). For analyzing how large the organic farming sector is, we used data collected from the Ministry of Agriculture and Rural Development (MARD) and the European Commission (Eurostat), and the period analyzed was 2002-2012. The data collected has been statistically processed and interpreted, building the trend line and setting up the forecast for the next years.

According to Eurostat in 2011, the EU27 had a total area of 9.6 million hectares cultivated in accordance with the organic farming rules, up to 5.7 million in 2002. It increased by 1.6%, during the last decade. Although the whole organic area represents only 5.5% of the total utilized agriculture area in EU, we can say that is a significant increase. The European Commission provided Eurostat data which shows that in 2012 the area under organic farming is 5.7% of utilized agriculture area in EU27, which increased by 0.2% than 5.5% in 2011.

In Romania, agriculture is now one of the major branches of the Romanian economy. The contribution of agriculture, forestry, fisheries in gross domestic product, stands around 6% of GDP and the EU Member States stands at about 1.7%, according to INS Bucharest.

In Romania, in 2013, according to MARD, the total area cultivated in accordance with the organic farming rules, was of 301,148 hectares. In 2003 the surface was 56.800 hectares, which means that it increased 5 times between 2003-2013 (Table 1).

Table 1. Evolution of land under organic farming, in Romania, 2003-2013 (hectares)

| 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|---|---|---|---|---|---|
| 56,800 | 73,300 | 92,770 | 107,582 | 131,401 | 140,132 | 168,288 | 182,706 | 229,946 | 288,261 | 301,148 |

Figure 1. View from a land under organic farming

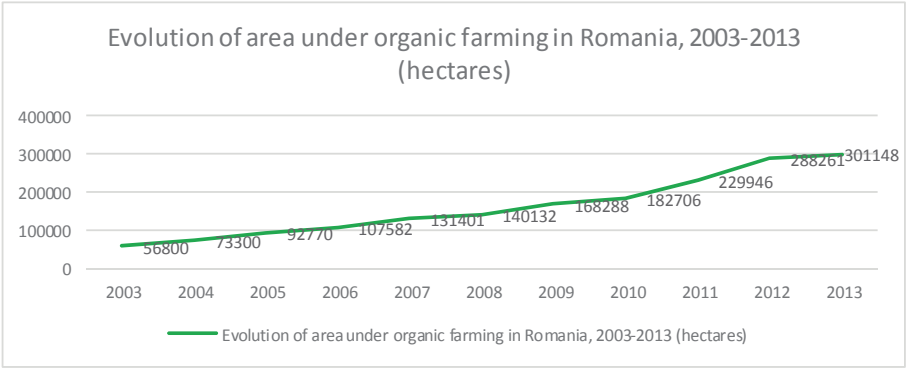


Figure 2. Evolution of area under organic farming in Romania, 2003-2013 (hectares)

In Figure 2, we can see that during 2003-2013, the evolution of area under organic farming in Romania has increased, mainly because the farmers were attracted to invest in organic farming, due to the satisfactions they can later obtain.

In 2012 Romanian agriculture area was 14,615.1 thousand hectares, of which 64.26% arable land, 22.38% pasture, 10.57% meadows, 1.44% vineyards and nurseries, and 1.35% orchards and nurseries, according to INS Bucharest (Figure 3).

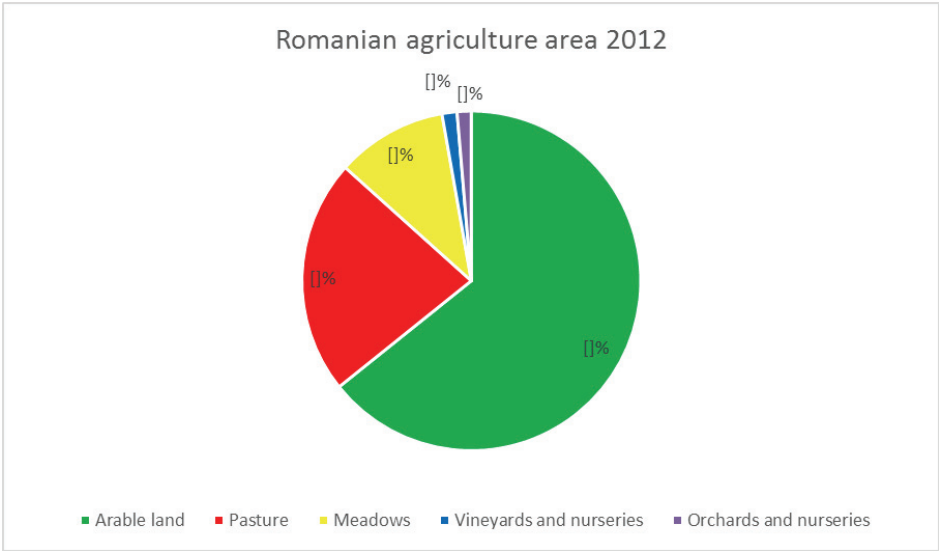


Figure 3. The Romanian agriculture area under organic farming, by category of use, 2012

In Table 2 surfaces of cereals under organic farming increased almost seven times during 2006-2013. Wheat is grown on more than half of the area occupied by cereals. Areas planted with roots, permanent orchards and vineyards become larger almost every year. Surfaces planted with dried pulses, fresh vegetables and permanent pastures, vary from year to year.

Table 2. Evolution of land under organic farming, in Romania, 2003-2013 (hectares)

| Plantation/Crop | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|---|---|---|---|
| Cereals | 16,310 | 32,222 | 56,337 | 63,446 | 72,298 | 79,167 | 105,149 | 109,105 |
| - Wheat | 11,965 | 18,418 | 36,137 | 38979 | 39,159 | 40,529 | 56151 | - |
| Dried pulses | 7,777 | 1,394 | 870 | 6,088 | 5,560 | 3,147 | 2,764 | 2,397 |
| Roots | 29 | 45 | 407 | 435 | 504 | 1,075 | 1,125 | 741 |
| Fresh vegetables | 727 | 310 | 259 | 344 | 734 | 914 | 896 | 1,068 |
| Permanent pastures | 51,200 | 57,611 | 46,007 | 39,233 | 31,579 | 78,198 | 105,836 | 103,702 |
| Permanent orchards and vineyards | 294 | 862 | 1,551 | 1,870 | 3,093 | 4,167 | 7,781 | 9,400 |

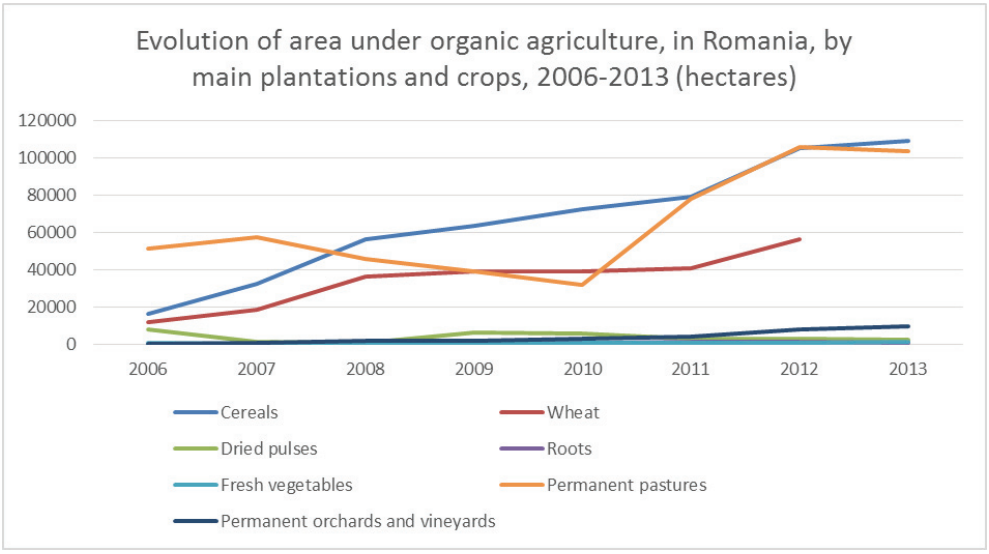


Figure 4. Evolution of area under organic agriculture, in Romania, by main plantations and crops, 2006-2013

In Figure 4 we can see the evolution of the area under organic agriculture, in Romania, by main plantations and crops. The highest increase was registered by cereals, especially wheat. In 2006, the wheat crop was 11,965 hectares and in 2012 it was 56,151 hectares. It means that wheat crop increased almost five times during 2006-2013.

In Table 3 we can see the evolution of livestock under organic farming in 2006-2012. As we can see in this table, the livestock under organic system is depends on the species. The number of sheep decreased with 60% during 2006-2012, but also had one of the largest oscillations from this sector. The number of cattle, swine and goats, vary from year to year. The number of bees and poultry are the ones which increased significant during the analyzed period.

Table 3. Evolution of livestock under organic farming, in Romania, 2006-2012 (heads)

| Species | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 |
|---|---|---|---|---|---|---|---|
| Sheep | 86,180 | 59,680 | 121,175 | 51,470 | 18,883 | 27,389 | 51,722 |
| Bees | 30,796 | 37,260 | 52,599 | 59,414 | 64,836 | 77,994 | 85,225 |
| Cattle | 11,365 | 6,985 | 7,567 | 8,145 | 5,358 | 6,894 | 7,044 |
| Poultry | 4,300 | 4,320 | 6,080 | 9,400 | 21,580 | 46,506 | 60,121 |
| Swine | 1,652 | 1,174 | 416 | 603 | 320 | 414 | 344 |
| Goats | 117 | 215 | 4,296 | 4,738 | 1,093 | 801 | 1,212 |

## RESULTS AND DISCUSSIONS

Currently, Romanian agriculture is one of the most important sectors of the Romanian economy. The contribution of agriculture, forestry, gross domestic product in Fisheries, stands around 6% of GDP. Sector represented agriculture occupies a small share of Romanian agriculture.

The data supplied from European Commission and MAPDR reveals the increasing importance of the organic sector and the positive evolution of areas under organic farming like cereals (especially wheat)

Areas planted with roots, permanent orchards and vineyards become larger almost every year. The number of bees and poultry are the ones which increased significant during the analyzed period. All of this are proves that the organic This shows potential and initiative of development in Romania. Both land areas under organic farming and the number of animals under organic farming increased in the investigated period (with minor exceptions). The trend is increasing and the perception of this sector as an alternative activity and income source is positive. Every farmer knows that his organic products will bring an added value.

In Romania, in 2013, there were 15,194 operators practicing organic agriculture. In 2010, their number was 3,155, which means that in 2010-2013, the number of operators increased almost five times. This increase is due to increasing awareness and information on the notion of agriculture and organic products.

Table 4. Evolution operators under organic farming, in Romania, 2010-2013 (heads)

| | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|
| **Operators under organic farming** | 3,155 | 9,703 | 15,544 | 15,194 |

## CONCLUSIONS

The organic sector in Romania is highly increasing from year to year, and is characterized by diversity. This sector has an insignificant weight in agro-food system, regarding agricultural area and livestock production. However, it has a high potential of development due to accelerated growth of indicators. This sector can bring significant contribution to a sustainable development, and can increase the economic activities, thanks to the significant added value of the organic products. The premium price of organic products is paid by people from countries where there is a sizeable middleclass in the population, and where consumers are more educated and informed of food issues, and they incline to buy organic products, whether for food safety, concern over the environment or health reasons. The organic market is increasing, and is characterized by diversity from year to year and the supply of products on the market. The demand for certified organic products is growing in Europe, because the consumers are more educated. However, consumption of organic products in Romania still remains at a low level compared with other European countries - which is determined mainly by the low purchasing power of the population and additional price difference of about 20-40% compared to conventional products (eco products consumption in Romania, representing about 1% on total consumption of products, while the European average is 3-5%). Even in these circumstances, with the appropriate information and pro-motion, designed to increase public awareness regarding organic products, Romania could significantly increase market share and attractiveness of the organic products.

## ACKNOWLEDGEMENTS

This research work was carried out with the support of Association of Organic Agriculture Operators - BIO ROMANIA and Ministry of Agriculture and Rural Development.

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