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# RESULTS OBTAINED AFTER USE OF TREATMENTS FOR INDUCING AND SYNCHRONIZING OESTRUS IN COWS

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**Key words:** cows, oestrus, ovulation, $PGF_2$ alfa, GnRH.

## SUMMARY

The authors induced and synchronized the oestrus and ovulation at a number of 157 Romanian spotted dairy cows, using in association preparations of $PGF_{2\alpha}$ (Proliz) and GnRH (Receptal), according to four therapeutic schemes.
The lowest levels of oestrus manifestation (80,09) were shown in cows treated according to scheme I and the highest levels (93,61) appeared at scheme IV.
Most of the cows and heifers synchronized after the scheme II manifested oestrus between 60 and 72 hours.
The lowest rates of pregnancy (46,15%) were observed at scheme III and the highest (53,12%) at scheme II.
In the experimental group, cows resumed the postpartum sexual activity in a natural manner, without hormonal intervention, the rate of gestation reaching 58,06%.
In USA, in dairy cattle farms with medium production, 50% of the cows pass the oestrus period unobserved (Stevenson 2005). Due to secondary effects generated by the application of several hormonal protocols (using progesterone, estrogen), the practice became oriented towards the use of hormonal protocols based only on $PGF_{2\alpha}$ or the association of GnRH and $PGF_{2\alpha}$.
The administration of GnRH is followed by:

- The atresia of the dominant follicle;
- Ovulation (if the intervention was made during the luteal phase of the cycle);
- Stimulation of follicular maturation and ovulation of the dominant follicle by GnRH;
- Recruitment of a new follicular wave less then 4 days after treatment (Dolezel et al, 2002).

The Ovsynch procedure got its name because the GnRH- $PGF_{2\alpha}$-GnRH sequence assures synchronized ovulation (Pursley et al, 1995).
The Presynch procedure was proposed in 1998 and it is a modification of the Osynch procedure by completing its lacks (Thatcher et al., 1998). The rate of gestation is superior in the case of Presynch utilization (43%), comparing the results obtained ulterior of the Ovsynch use (29%) (Moriera et al., 2000).

## 1. MATHERIALS AND METHODS

Out of the 308 cows examined in the two units, 157 cows pursuant to the gynecological diagnose in accordance to the protocols were treated conform the 4 therapeutic hormonal schemes.

For the recovering of these cows we used 4 therapeutic protocols of induction, synchronizing of oestrus and ovulation, as it shows below:

- ***Scheme I*** consisted in the administration in day 0 of a dose of $PGF_{2\alpha}$ (Proliz), followed by the administration in day 3 of a dose of GnRH (Receptal), then, followed by the A.I. at 8 hours from administration of Receptal. Conform this scheme 42 cows have been treated in the 2 farms.
- ***Scheme II*** consisted in the administration of a $PGF_{2\alpha}$ (Proliz) dose, followed by GnRH (Receptal) dose in day 17 and A.I. at 8 hours after the Receptal. Conform this scheme 37 cows were treated in the 2 farms.
- ***Scheme III*** or ***OVSYNCH protocol*** consisted in the administration of GnRH (Receptal) in day 0, a $PGF_{2\alpha}$ dose in day 7, one more GnRH (Receptal) dose in day 9 and A.I. at 16 hours following this. Conform this protocol 31 cows have been treated in the 2 farms.
- ***Scheme IV*** or ***PRESYNCH + OVSYNCH scheme*** consisted in the administration of a $PGF_{2\alpha}$ dose (Poliz) in day 0, then the administration of GnRH (Receptal) in day 4, then $PGF_{2\alpha}$ again in day 12 and repeated GnRH (Proliz) in day 14, followed by A.I. at 16 hours after last administration. Conform this protocol 47 cows have been treated.
- ***Scheme V*** or ***control group*** consists of 62 cows in the 2 farms that manifested normal birth, with physiological puerperium. These cows manifested estrus in the first 60 days postpartum, being able to be inseminated.

The results obtained after A.I. of the cows synchronized with hormonal methods have been compared with the cows that had natural oestrus.

For the females synchronized with $PGF_{2\alpha}$ we used the Romanian Proliz pharmaceutical product, which contains an active substance named Cloprostenol, a synthetic analog of $PGF_{2\alpha}$. The cloprostenol was administrated in 2 doses of 0,500 mg each, at an interval of 11-14 days.

Like a source of GnRH we used the pharmaceutical product Receptal, in 2 ml dose.

For a better precision in knowledge of the oestrus and ovulation inception moment, the females were observed 3 times a day (early morning, noon and late night), moments when we appreciated the modifications that appeared at the genital tract by trans-rectal examination and by observing the females behavior.

To establish the moment of the ovulation, trans-rectal examinations were done twice a day, at an interval of 12 hours (morning and night). The females found in heat, synchronized with synthetic analogs of $PGF_{2\alpha,}$ were artificially inseminated, conform the protocol antepartum/postpartum and those synchronized with the Ovsynch method, scheme I, were inseminated first time at exactly 60 hours after administration of Cloprostenol.

## 2. RESULTS AND DISCUTIONS

The experiments were done in years 2007-2009, on 308 dairy cows and heifers.

The cows that had to resume the reproductive cycle postpartum were submitted to gynecological investigation by the veterinary doctor.

Females that presented functional corpus luteum (CL) on one of the ovaries and that were not diagnosed with genital affections, were considered having normal cyclic ovarian activity and were synchronized with $PGF_{2\alpha}$ .

The two doses of $PGF_{2\alpha}$ were administered at an interval of 14 days in cows and 11 days in heifers.

Any of the dominant follicles has the capacity to ovulate. $PGF_{2\alpha}$ has no effect over the normal development of the follicular waves, but it has the capacity to destroy the CL. The stage of follicular development in the moment of $PGF_{2\alpha}$ administration, will influence the period of time from the injection to the first oestrus. The animals injected at the time of dominant follicle growth will get in heat in 2-3 days, while the animals with dominant follicle in regression need 4-6 days until a new follicle will come to ovulation. A new “synchronized” follicular wave is initiated in 2-3 days. Because the dominant follicle will develop a luteal tissue, due to GnRH stimulation, a larger percent of cows will show better results to the $PGF_{2\alpha}$ injection, 7 days later. This gives better results in comparison to $PGF_{2\alpha}$ used alone.

Even if GnRH is synchronizing the follicular development in the majority of cows, some cows are not responding at the first GnRH injection.

If the GnRH injection is not determining the follicular luteinization of the animals that should naturally enter in heat after the $PGF_{2\alpha}$ injection, the treatment fails.

We have to report that a quarter of the cows with prolonged anoestrus showed ovarian hypoplasia, maybe due to the foraging and maintenance of these cows, that were not the best ones.

The obtained results have been appreciated, based on: the grouping of heat, the reproductive function stimulation, the repeated oestrus and A.I. at induced estrus and on the number of females that remained pregnant after the A.I. (Table 1).

***Table 1*.**

**SUMMARIZER of the results obtained in the 2 farms regarding the inducing and synchronizing of oestrus and ovulations, applying the 4 therapeutic hormonal schemes**

| **Crt. Nr.** | **S[1]** | **Ex. cows[2]** | **Tr. Cows [3]** | **Cows in heat** | | **Cows A.I.** | | **Pregnant cows** | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **N** | **%** | **N** | **%** | **N** | **%** |
| 1 | Scheme I | 57 | 42 | 37 | 80,09 | 34 | 91,89 | 18 | 52,94 |
| 2 | Scheme II | 59 | 37 | 34 | 91,89 | 32 | 94,11 | 17 | 53,12 |
| 3 | Scheme III | 57 | 31 | 28 | 90,32 | 26 | 92,85 | 12 | 46,15 |
| 4 | Scheme IV | 73 | 47 | 44 | 93,61 | 43 | 97,72 | 20 | 46,51 |
| 5 | Control group | 62 | - | 62 | 100,00 | 62 | 100,0 | 36 | 58,06 |
| | **TOTAL** | **308** | **157** | **205** | **-** | **197** | **96,09** | **103** | **52,28** |

1. Synchronization
2. Examined cows
3. Treated cows

In the experiment realized in the two zootechnical units, 308 cows were taken in study, of which 157 cows were treated conform the four therapeutic schemes.

Of all the examined cows, 205 manifested oestrus, 197 have been inseminated artificially, obtaining a rate of pregnancy of medium 52,28%.

In case of scheme I, the estrus was manifested at 37 cows (80,09%), 34 cows got A.I. (91,89%), obtaining a medium rate of gestation 52,94%.

The scheme II used 37 animals to treatment out of which 91,89% (34 cows) manifested oestrus. Out of these, 32 cows have been inseminated, the rate of gestation was 53,12%.

Conform scheme III OVSYNCH, in the two units were treated 31 cows, 90,32% (28 cows) manifested oestrus, 26 were inseminated. The rate of gestation was 46,15% (12 animals).

In scheme IV we treated 47 cows, 93,61% (44 cows) manifested oestrus and 43 cows were inseminated. After applying this protocol we obtained 46,51% the rate of gestation (20 cows).

Out of 62 inseminated cows from the control group, 36 cows were diagnosed pregnant (58,06%).

Concluding the number of cows taken in study from the total 308, 157 got treatment and 205 presented oestrus; counting the natural oestrus of the control group, 197 cows were inseminated (96,09%) and the gestation was present in 103 cows (52,28%).

Using the OVSYNCH method in inducing and synchronizing the heat and ovulation in cows, gives good results, producing a grouping of ovulation on a short time, between 60 and 70 hours after the treatment finished, allowing the A.I. on a fix time with high results, without need of tracking the heat.

In case of using method OVSYNCH – scheme I, no late ovulation was determined, nor anovulatory heat, due to GnRH that induces ovulations.

Even if this method of synchronizing is pretty costly due to the high cost of the pharmaceutical substances based on gonadotropin releasing hypothalamic hormones (GnRH), it is justified the use of this method regarding to the advantages brought up by no need to track down the heat, use of a single A.I. at fix therm.

The biotechnological view of oestrus synchronization is different from the ovarian activity of the females. At cyclic females, which present ovarian activity, with or without heat manifestation (silent heat), the time control of the CL function is done by luteolitic hormones ($PGF_2\alpha$).

In females with anoestrus due to ovarian inactivity, the ovulation must be induced with the help of GnRH.

These cases are frequently met in lactating cows and in those cows that doesn't get the maintenance comfort, in which the endogen progesterone doesn't imply uptake of the estrus cycle nor the synchronizing treatment does.

Tracking the females in heat puts lots of problems, especially in farms in which cows are grassing all summer season.

STEVENSON and col. (27) observed that nearly 50% of the manifested estrus cycles are not being detected, which means big economy loss.

As well, 30% of cows present shorter estrus then 12 hours, this needs more observation per day for tracking as many females as possible. These all ideas impose upon the use of biotechnology of reproduction in the management of reproduction of dairy cattle farms, inducing and synchronizing the oestrus makes the work easier and reduces the costs of tracking heat and reducing the number of estrus cycles unobserved.

HOLMANN, (1984) cited by (27) have shown that the optimal time of interval between birth (C.I.-calving interval) in dairy cows is 12-13 month, with an uterine rest of 85 days. Because all the cows are acyclic on a variable duration after birth, and the fecundity is almost 50%, it is important that the cycles are resumed as fast as possible after birth.

Inducing and synchronizing the oestrus in cows with prolonged anoestrus as well as synchronizing the oestrus in cyclic females, consists in a biotechnological method of upgrading the principal indicators of reproduction (rate of gestation, uterine rest, C.I.).

## 3. CONCLUSIONS

3.1.The lowest rates of oestrus manifestation (80,09%) appears in cows treated conform scheme I ($PGF_{2\alpha}$ + GnRH), the highest rates (93,61%)consisting in scheme IV (Presynch + Ovsynch);

3.2.The majority of cows and heifers synchronized with Ovsynch method, scheme II (one dose of GnRH and two doses of Cloprostenol), manifests heat between 60-72 hours.

3.3.Cows react better then heifers at hormonal treatment for synchronizing heat, no matter what method had been used. Heifers show a tendency to reduce the rates of mainfesting heat with 9-12% in the first two schemes, comparing to cows.

3.4.The lowest rates of pregnancy (46,15%)are registered in scheme III (Ovsynch) and the highest in scheme II (53,12%).

3.5.Females synchronized by Ovsynch method, scheme I showed grouped ovulation at 12 hours interval between 60-72 hours after treatment.

3.6.Using the scheme II ($PGF_{2\alpha}$ + $PGF_{2\alpha}$ + Gn-RH) of ovarian stimulation in postpartum cows has the advantage of being less costly and permits A.I. in fix time.

3.7.Cows which resumed the postpartum sexual activities after hormonal treatment or medications a rate of 58,06% was obtained, little more superior of that obtained in control groups.

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# CYTOMORPHOLOGIC ASPECTS OF THE MALIGNANT LYMPHOMAS IN DOGS AND CATS

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**Key words:** The malignant lymphoma, malignant oncopathy, Hodgkin lymphoma, non-Hodgkin lymphoma

**SUMMARY**

On the one hand, the authors draw a conclusion based on the statistic comparison of the malignant lymphoma in the two species, and, on the other hand, they present statistical data referring to the occurrence of the malignant lymphoma in the general oncologic context, as well as in the context of malignant oncopathy.
We will present the following cell forms of malignant lymphomas:
-Hodgkin lymphoma
- non-Hodgkin lymphoma : - B - cell - centrocytic
- centroblastic
- immunoblastoma
- plastocytoma
- Waldenstrom disease
-T-cell - Mycosis fongoides
- Sezary
- N.K.-cell
- histiocitary

## INTRODUCTION

For a long time now dogs and cats have been used as models for discovery and research on some medicines; this is possible because there are several similarities between their anatomy and physiology and the human one, especially concerning the nervous, cardiovascular, urogenital, muscular and bone system.

The recent completion of the canine genome opens the way to the development of some resources that will allow the integration of canine cancers in the main domain in cancer research.

Cancers in dogs and cats are characterized by growth throughout a long period of time, if the immune system is intact and if there is the case of a interindividual and intratumoral heterogeneity; development of resistant and relapsing diseases and metastases at long distances of time. In comparison with other big animals frequently used in biomedical research, such as dogs and non-human primates, the supplementary

advantage offered by dogs and cats is that they are taken care of at ages that are frequently associated with the highest risk of cancer. The risk, associated with the big size of their population, results from a cancer rate that is sufficient enough for the capacity of clinical trials.

According to basic estimations of cancer occurrence in the USA only, there are almost 4 million new cancer diagnoses in dogs every year. Such examples are: non-Hodgkin lymphoma, osteosarcoma, melanoma, prostate carcinoma, lung carcinoma, head and neck carcinomas, breast carcinoma and soft tissues sarcomas. There is less research on cancer in cats, that is why we will present some aspects related to the canine species.

Many of these cancers present strong similarities with human cancers, including histological aspects, tumoral genetics, biologic behaviour and the answer to conventional therapies. The condensed course of cancer development in dogs allow one to establish new therapies in due time.

Indeed, preliminary observations on the canine genome suggests that there are more similarities between canine and human chromosomes than between human and rats chromosomes, in terms of reshuffling and rearrangement of nucleotides.

For as start, CCOGC (*Canine Comparative Oncology Genomics Consortium*) intended to use the advantages put forth by these opportunities by undertaking the following actions:

1. developing big, well-supplied, acceptable bio—warehouses of canine cancers and tissues-but it is difficult to find this in the existing conditions;
2. improving opportunities to relate efforts made by veterinarians and specialists in compared oncology with fundamental oncologic research and clinicians' work;
3. initiating preclinical trials using dogs with cancer that are integrated within the research of new medicines for cancer therapy
4. mechanisms for the analysis of these preclinical trials through regulation bodies should be developed so that the information on these studies should be useful for reaching the goal in emerging human clinical trials.

Non-clinical studies on dogs and cats with cancer answered questions to which it would have been difficult or impossible to answer in the case of rats or humans.

## MATERIAL AND METHODOLOGY

The investigation method in the veterinary oncologic clinic for the elaboration of the positive and differential diagnosis of malignant lymphomas is based on:

1. Clinical examination-insistence on palpation of the whole external lymphonodal link.
2. Biochemical blood and urine test
3. Hematological test, including leukocyto-concentrate test
4. Radiological examination of the thoracic cavity
5. Echographic examination of the abdominal cavity
6. If the oncologist requires it, bone punction for the investigation of hematopoietic spine
7. If the oncologist requires it, punction of a lymph node with an increased size for the investigation of lymphonodal cytology.

Current cytological examinations can be also done through:

- urine
- the liquid of pleural, pericardic or peritoneal cavities

On demand, the ordinary or the special histopathological biopsical examination can be done.

## RESULTS AND DISCUSSIONS

In the diverse range of cancer occurrences in dogs and cats, the malignant lymphoma has a special place. These malignant lympho-proliferations can be with or without cytemic discharge. Our statistics show that the highest frequency is that of the non-Hodgkin malignant lymphoma without cytemic discharge, ¾ of the total of cases hold the first place. It is obvious that the cell base of the malignant proliferation is dominated by non-Hodgkin malignant lymphomas (91,2%), while the rest of 8,8% is represented by Hodgkin lymphomas. Out of the total of 50 cases of malignant lymphomas, 90% are in dogs, while 10 % are in cats.

The predominant cell form is ensured by B-cell malignant lymphoma (80 %), while the other proliferations of T lymphocytes, NK cells and histiocytes represent only a minor par

The following forms predominate in B-cell malignant lymphoma:

- centrocytic malignant lymphoma- 13 cases;
- centroblastic malignant lymphoma-5 cases ;
- immunoblastoma- 5 cases ;
- plastocytoma -4 cases;

-Waldenstrom lymphoma-3 cases.

Proliferations with a reduced frequency are those whose cell base is either T-lymphocyte (Mycosis fongoides) and NK-cell –each with two cases.

A special situation is represented by malignant histiocitary lymphoma which has a high frequency (5 cases) and has the highest degree of malignity, therefore aggression.

What is extremely important and worth mentioning is the fact that malignant lymphomas are very different in their evolution and answer to therapy, according to each individual and the cell base of malignant proliferation. In the case of a proper therapy survival within the same cell form can vary between 6 months and 4 years.

## CONCLUSIONS

1. The canine species furnishes much more cases of malignant lymphomas as compared with the feline species.
2. Non-Hodgkin malignant lymphomas are more frequent than Hodgkin ones.
3. Within non-Hodgkin malignant lymphomas centrocytic cell form is predominant
4. Histiocitary malignant lymphomas are the most aggressive with a high degree of malignity.
5. The Waldenstrom malignant lymphoma is the least aggressive and answers well to proper therapy with a long evolution.

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# PRRSV AND PRV CONTROL PROGRAMS IN PROFESSIONAL SWINE HERDS

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**Key words**: swine pathology, PRRS, Aujeszky's disease, disease control measures evaluation

## SUMMARY

Usually, the control measures for PRRSV include the implementation of bio-security rules, the management of replacement gilts and the vaccination. These measures are helpful to reduce the risk of PRRSV spread within and between Romanian herds. According to the EU and OIE recommendations, the control strategies below was proposed: (a) in infected establishment: 30 days after removal of infected animals, all breeding animals will be tested using the ELISA PRV test (the results mast be PRV negative on two successively tests, ruled 2 months later); (b) in establishments located in the 5-kilometre radius zone: a significant number of pigs from each establishment mast be subjected to ELISA PRV test and all results have to be negative. Our initial investigations was carried out in a swine population about 4000 animals, divided in five groups: Suckling pigs, Gilts, Sows, Growing-finishing pigs (115 days) and Nursing/weaned pigs, 20 dead pigs (suckling pigs, young animals) were examined post mortem using the necropsy protocol. To asses the exposure to the PRRSV and PRV the following ELISA tests were used: HerdChek PRRSV-Ab Test Kit (IDEXX Lab, Inc., USA) and HerdChek Pseudorabies Virus gB Antibody Test Kit (IDEXX Laboratories, Inc., USA). The primary evaluation of swine herd was carried out on serum specimens, as fallow: suckling pigs - 5 samples (samples 1-5), gilts - 4 samples (samples 6-9), sows - 6 samples (samples 10-15), growing-finishing pigs - 5 samples (samples 16-20) and from nursing/weaned pigs - 10 samples (samples 21-30). This paper presents the 12 month evaluation of SRRPV and PRV control programs implemented in a professional swine herd. The results are as expected and control programs are running in the farm.

In farms, PRRS suspicion is based on reproductive failure and increased levels of neonatal mortality [2], but also in PRV infected sows in middle pregnancy experience abortion with mummified fetuses, while the ones infected in late pregnancy often give birth to weak or stillborn pigs [4]. In Aujeszky's disease the clinical features are strongly related to the age of pigs, to the route of infection, to the virulence of the strain and to the immunological status of the animal, latter hiding both, disease and virus. The most susceptible are young piglets. Mortality rates reach 100% in pigs under 2 weeks of age and decrease as the age of infected swine increases. These animals experience severe neurological signs:

muscular trembling, incoordination, ataxia, posterior paresis, nystagmus, opisthotonus, severe epileptiform seizures and fever. In weaned pigs clinical signs are similar to those in neonatal pigs, but less severe. In grower-finisher pigs, respiratory signs, flu-like, are most common: sneezing, coughing, nasal and ocular discharge, dyspnoea [4].

The presence of PRRSV or PRV can be certified by several methods of antigen/antibody identification. The control measures for PRRS are based on the implementation of biosecurity protocols, on the management of replacement gilts and on the vaccination. Using those, the risk of PRRSV spread within and between herds can be reduced [3]. PRV vaccination can be performed with live or killed vaccines. Also, some modified attenuated live-vaccines (deletion of PRV *UL23* gene or deletion of *gE* gene) and DNA vaccines are on the market.

The surveillance and monitoring of some infectious disease by ELISA technique improve the programs of health control in investigated farms, identify the prevalence of some infectious agents in herds, and measure the quality of the vaccination. The results of the investigations carried out in swine on different ages made and show the business profitable [16, 17].

## 1. MATERIALS AND METHODS

The swine population submitted in this study, counting almost 4000 pigs, integrated in the same production chain, included: Suckling pigs, Gilts, Sows, Growing-finishing pigs (115 days) and Nursing/weaned pigs. Casualties - 20 suckling pigs, young animals - were investigated using the described necropsy protocol [11]. Investigated serum specimens were sampled from: suckling pigs - 5 serum samples (samples 1-5), gilts - 4 serum samples (samples 6-9), sows - 6 serum samples (samples 10-15), growing-finishing pigs - 5 samples (samples 16-20) and from nursing/weaned pigs - 10 samples (samples 21-30). To assess the exposure to the field PRRSV and PRV we used Porcine HerdChek Reproductive and Respiratory Syndrome Virus Antibody Test Kit (IDEXX Laboratories, Inc., USA) and HerdChek Pseudorabies Virus gB Antibody Test Kit (IDEXX Laboratories, Inc., USA).

## 2. RESULTS AND DISCUSSIONS

PRRS and Aujeszky's disease suspicion in the studied farm was based on the characteristic reproductive signs experienced by most of the gilts and sows associated with respiratory signs showed in growing

pigs, as well as the increased mortality in suckling pigs [7, 9, 10, 11, 13, 17]. Reproductive disorder is also described in Aujeszky's disease, classical swine fever, African swine fever, porcine parvovirus, porcine enterovirus, haemagglutinating encephalomyelitis virus, and leptospirosis. Post-weaning pathology with increased levels of neonatal mortality can be associated with swine influenza, enzootic pneumonia, proliferative and necrotising pneumonia, *Haemophilus parasuis* infection, haemagglutinating encephalomyelitis virus, porcine respiratory coronavirus, syncitial pneumonia and myocarditis, porcine circovirus-associated disease, or Nipah virus infection [3].

Laboratory diagnostic revealed the presence of PRRSV and PRV antibodies, considering no PRRS or PRV vaccination applied. The ELISA diagnostic results are presented in following chart (fig.1.).

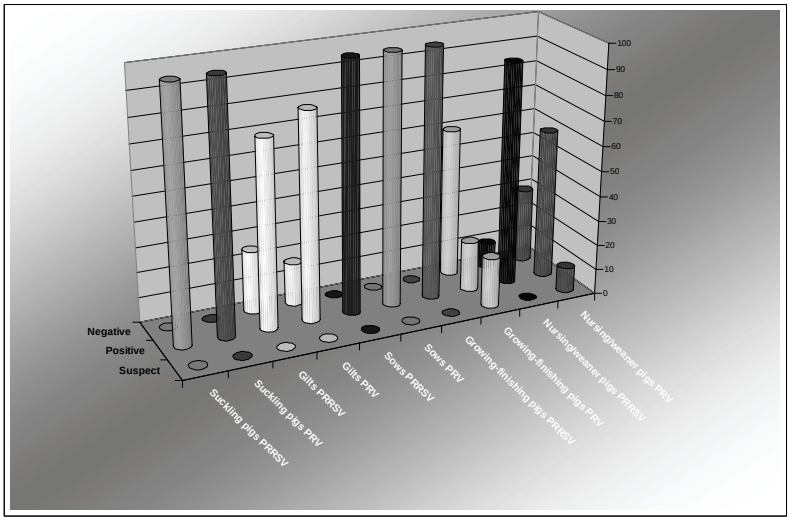


**Fig. 1. ELISA PRRSV-Ab and PRV-Ab Test Kit results**

The following actions for PRRSV and PRV control programs was performed:

- vaccination of the sows prior to freshening and before 90 days of gestation against Pseudorabies and PRRS;
- vaccination of piglets at weaning against both PRRS (previously diagnosticated) and Pseudorabies diseases;
- Pseudorabies and PRRS emergency vaccination of the growing pigs, after weaning, will keep going at least during two reproductive cycles or until the infected gilts and sows are replaced.

After that, vaccination will be limited to reproduction swines, only if the all in/all out protocol is followed. Emergency PRRS vaccination of the growing pigs, after weaning, will keep going at least during two reproductive cycles or until the infected gilts and sows are replaced. Depending on the respiratory signs prevalence and it's etiology, piglets ware emergency vaccinated against the specific agents. The gilts, sows and boars were primary vaccinated for PRV and after one day for PRRSV. The protocol of PRV immunization consisted in 3 doses inoculated at 14 days interval, and for PRRSV in two doses inoculated also al 14 days. The first doses of PRV vaccine was used at 7 days age piglets (intranasal vaccine), the second doses was inoculated at 10 weeks, and the last dose at 13 weeks.

Once sated up this vaccination protocol the casualties of piglets and growing pigs decreased: in February the mortality was 241 piglets from 417 newborn and 93 growing pigs, in March 245 piglets from 384 newborn and 180 growing pigs, and starting to April the mortality decreased at 24 piglets from 370 newborn and 15 growing pigs. In May the mortality was lower, practically into technologically accepted limits. The following 6 month of surveillance showed a good herd management. The control implemented programs are still setting up in the farm.

## 3. CONCLUSION

3.1. The lesions in respiratory pathology of piglets and growing pigs are highly of polymorphic, and for this reason the surveillance based on serological screening is the best solution. In our study the surveillance and monitoring of PRV and PRRSV by ELISA technique improved the programs of health control in investigated farm, identified the prevalence of some infectious agents in herds, and evaluated the quality of vaccination. Short time after the identification of PRV and PRRSV in farm, the control programs based on general measures of control and vaccination were introduced, minimising the economical impact of those diseases.

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# IMMUNOLOGICAL DIAGNOSTIC IN SURVEILLANCE OF FELINE RETROVIRUSES IN ROMANIA

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**Key words:** feline retroviruses, FIV, FeLV, in-clinical immunological tests, retroviruses surveillance

**SUMMARY**

Feline retroviruses study remains one of the major items for worldwide researcher teams. The interest for tumor diseases with biotic etiology and feline immunodeficiency is continuous by their utility as a model for human oncology and AIDS studies. The diagnostic of FeLV and FIV infections is based on immunological methods, due to their Ab-Ag specificity, and from this group of methods the most used are in-clinical tests. Our previously study suggests a low incidence of FIV and FeLV in Romania, but more cats from catteries need to be submitted to immunological investigations, if contamination is suspected or unknown. The use of rapid and low-cost in-clinical tests, with a great sensibility and specificity is an objective approach in epidemiological management of catteries, hospitals, shelters, or other similar houses. This paper describes the significance of feline retroviruses surveillance and the opportunity of in-clinical immunological test use for all feline population in Romania.

*FeLV* has been studied over 30 years, both to evaluate the incidence and the prevalence in cat populations as well as animal model for some human diseases. The researches identified a few key characteristics of *FeLV*: it is contagious, it is the direct cause of some tumour and non-tumour diseases, it can exist "asleep" in bone marrow for a long period of time and the animal can be protected against it through vaccination.

*FeLV* can not be transmitted to humans or other animal species except those belonging to feline family [3]. The management of *FeLV* infection is based on testing and identifying infected cats and this measure cannot be replaced by vaccination. The „*FeLV* free" status requests a national schedule for screening [2].

Identification of feline retroviruses (*FIV* and *FeLV*) in some Romanian areas was started by us several years ago. The Faculty of Veterinary Medicine Bucharest use in clinical laboratory service both rapid diagnostic tests and classical lab methods.

For *FeLV/FIV* diagnostic the most used methods are the immunological methods, based by Ab-Ag specificity, and into this group the most used are in-clinical tests: immunomigration test, rapid immunoenzimatic test and sometimes IFA. Correlating the

epidemiological status of feline origin area (free or non-free *FeLV/FIV*), the clinical status of feline, with the result of in-clinical rapid test, we scheduled the surveillance programs previously proposed. We tested feline serum sample for *FeLV* diagnostic by FeLV IC (AGROLABO, Italy), SNAP™ p27-FeLVAg test (IDEXX Inc., USA), ELISA p27-FeLVAg (IDEXX Inc. USA), IFA p27-FeLVAg (VMRD Inc. SUA), and for *FIV* diagnostic: FIV IC (AGROLABO, Italy), SNAP™ FIVAb test (IDEXX Inc. USA), ELISA FIVAb test (IDEXX Inc., USA), Ingezim FIV-Vet [1, 6, 7, 8].

Our previously study suggests a low incidence of FIV and FeLV in Romania [2, 4, 5, 6], but more cats from catteries should be submitted to immunological investigations, if contamination is suspected or unknown. The use of rapid and low-cost in-clinical tests, showing the requested sensibility and specificity is a justified goal in epidemiological management of catteries, hospitals, shelters, or other similar houses.

One of the last introduced test in our screening activities is FeLV/FIV IC (AGROLABO, Italy). The FeLV IC test for the detection of p27 antigen against the Feline Leukaemia Virus hold on the immunochromatographic technique, as whole IC test line, including the FIV IC test.

The first in-clinical test we used was also a twin test, SNAP® FIV/FeLV Combo Plus Test (IDEXX Laboratories Inc.) for the some viruses mentioned before: this one is detecting FeLV antigen and FIV antibodies in serum, plasma or whole blood of felines revealing them by immunoenzymatic principles. The presences of p27 FeLV antigen confirm the FeLV infection, the presence of specific antibodies to FIV indicating the exposure to FIV of the cat and even an active FIV infection. This kit is designed with monoclonal antibodies against p27FeLV, positive and negative controls.

In-clinical tests could be the first choice in the *FeLV* surveillance/monitoring programs for Romania, whose positive results can be submitted to confirmation by IFA and ELISA; the two last methods for feline retroviruses should be implemented in Veterinary Laboratories. Both choused tests need to have high values of sensibility and specificity, but different principles of work. The programs for *FIV* surveillance need to relate epidemiological status of feline origin area (free or non-free *FIV*) and the clinical status of feline with the result of the in-clinical rapid test: if it raises FLV/FIV suspicion, the immune status of feline mast evaluate by a different method for *FIV* diagnostic, preferable in Veterinary Laboratory.

Cats could be tested at any age as maternal immunity does not interfere with the post infection immunity. In order to identify all infection sources, life style of cats being free range, all cats from the area need to be tested [2].

A cat exposed to FeLV risk which tested negative to the first in-clinical test need to be tested twofold, in order to avoid the negative results still showed by cats in incubation time. Even if the result to the second test, performed a few weeks later, is positive, the final test has to be done 90 days after exposure looking to the pathogenesis of this infection and to the four status possible for un exposed cat.

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# STUDY REGARDING CERTIFICATION OF SOME WILD RUMINANTS POPULATIONS FROM NEAMȚ COUNTY AS FREE OF BLUETONGUE

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**Key words:** blutongue, virus, vectors

**SUMMARY**

Bluetongue is un infectious disease that affect domestic and wild animals from many countries, but Romania is not one of them. That is why is very important to control animals that are imported from areas with restriction and not only. For these reason animals were serological and virusological tested in order to descover any potential risk. In the same time there were tested insects from *Culicoides* family because there are vectors for the bluetongue virus. After tests were made is was revealed the fact that all the samples tested were negative in all tests.

Bluetongue disease spread in subtropical and tropical areas became a regular visitor in South Europe in the last decade (Manuel terestre de l 'OIE, 2005). The economical increasing of the global comerce in animal efectives was followed by the increasing of the frequency that exotic viruses are introduced in Europe, by strains that persist because of the climaterical changes on this continent (Perianu, T. et al, 2005, Perianu T., 2006).

Bluetongue virus (BTV) reached Europa in 2006, affecting 2000 exploitations and expansion was finished in January 2007. The focuses reappeared few months later made that virus duffuse in other exploitations in August and Septembre 2007. The virus is present in many countries, the extention being between 40ºN and 35ºS. By Mellor PS, BoormanJ, Baylis, 2000of culicoides in diseases epidemiology demonstrate that the prevalence is reglemented by ecological factors that favourise insect surveillance, such as temperature, humidity and soil characteristics (Lysyk TJ, Danyk T, 2005).

## MATERIAL AND METHOD

Wild ruminants surveillance was realised in the Research programe ROPATOSILVA by harvesting samples for laboratory serological and virusological tests.

The serological surveillance was realised in all ruminants that came in Romania following intracomunitar comerce, unvaccinated or without passing through natural infection and all the ruminants imported from third countries during waiting periode.

Serological exams were realised using competition ELISA to detect specific antibodies for VP7 protein of Bluetongue virus (Institut Pourquier), ELISA kit to detect specific antibodies for Bluetongue (VMRD INC, SUA), ELISA kit to detect specific antibodies for Bluetongue (Ingezim BTV) (Afshar, Ahmad et al, 1995).

Virusological surveillance was realised on wild ruminants dead or hunted in normal conditions or hunted because they presented clinical signs that can be conected to the virus (Manual de standarde de diagnostic teste şi vaccinuri, 2000, Sohn R, Yuill T, 1991).

Colaboration with Romsilva Neamț branch begun once with starting the hunting season on wild ruminants with the occasion of taken trofee, selection or other actions preview in the Hunting and protection of the cinegetic stock Law nr. 407/2006, published in Romania Official Monitor, Part I, no. 944 from 22 November 2006, with the later modifications.

For the virusological exam were taken organs (bone marrow, limphonods, lung fragments, spleen fragments, kidney fragments and blood from the heart) from roebucks hunted in the season.

In order to isolate the virus inoculations on embrionated eggs (first passage), inoculation on cell culture VERO or BHK21 (later passages) were realised. A more efficient way to isolate the virus was the inoculation on hen embrions. The blood taken from wild animals on EDTA was washed 3 times with PBS, resuspended in PBS and kept on 4 $^{o}$C. The limphonods and spleen tritutated in PBS were inoculated on hen embrions and the ones that died between second and seventh days were kept in the freezer. The embrions had to express vizible hemoragies and after the head removal they were triturated and the eventual virus from the supernatant could be identified by ELISA , immunofluorescence or immunoperoxidase.

Also there were taken blood samples on EDTA from vaccinated animals came from countries with restriction and were tested using RT-PCR.

Vectors monitorisation was used in order to obtain datas regarding their distribution in a certain area to establish seasoning distribution of the vector insects to isolate and identify certain viruses they carry and transmit.

## RESULTS AND DISCUTIONS

Because in this moment Romania is free of bluetongue infection, the most important mesure is tu assure a surveillance of the animals thet enter in Romania, of the vectors that can transmit the diseases and the animals that can came in contact with the vectors.

A passive surveillance consist in monitorisation of the documents that can give relevante datas, specially sanitary-veterinary, and of some other papers that accompanie wild and domestic animals transports that came from member state of European Union before debarcation to the destination.

An active surveillance consist in the inspection of the receptive domestic and wild animals existent in Neamț county such as animals found in target regions, all ruminants that enter in Romania (intracomunitar comerce) and all ruminants imported from third contries in the waiting periode, wild ruminants with the occasion of takening trofee, selection and other actions preview in the Hunting and protection of the cinegetic fond Law nr. 407/2006, published in Romania Official Monitor, Partea I, no. 944 from 2006, November 22, with the later modifications.

Serological investigations began in July 2009 on 4 aurochs imported from a country of Europene Union free of bluetongue. The animals were brought with the aim of protecting Cinegetic stock by reintegration in nature, in Vânători Neamț National Park.

The surveillance continued with the monitorization in conformity with the legislation about domestic ruminants (ovines and bovines) and were taken 100 sampes of bovine and 16 samples of ovine hemoser from target locations around Vânători National Park.

For a complete monitorization of bluetongue in the specific area, more preciselly Dumbrava stallion warehouse where a bright trap was placed in order to colect competent vectors in bluetongue transmision.

The serological examinations using ELISA of the wild ruminants imported from areas free of BTV showed that during carantine periode the results were negatives.

The periodical clinical inspection of the imported effectives revealed the fact that animals hadn’t presented clinical signs or modifications of

the general state that could be associated with the infection produced by bluetongue virus.

The virusological exams to detect viral antigen in blood samples taken on EDTA from vaccinated animals imported from restriction area for BTV gave negative results on RT-PCR.

The serological exams to detect viral antigen in blood samples taken on EDTA from roebucks during authorised hunting are not finished yet.

There were examined 5367 insects and 2379 of them were a fart of *Culicoidae* family. 2300 (96,68%) of them were morphological identified as *C. obsoletus* and 79 (3,32) as *C. pulicaris.*

Because of the relief configuration, vegetation and clime, vectors specie distribution in Romania is the following:

a. In the south area of the country are prevalent insectes from *Pulicaris* complex.
b. In the centre and in the north (Vâlcea, Braşov, Neamţ) are prevalent insectes from *Obsoletus* complex, because of the coniferous forest.

The insectes abundancy (number of insects in a area in a certain periode of time) is bigger for *C. obsoletus* comparativ with *C. pulicaris.*

These aspects presented above brought to the modification of legislation regarding serological surveillance strategy, so begining with 2007 and continuing with 2008 and 2009 the serological surveillance be extended in conformity with european legislation.

## CONCLUSIONS

1. The surveillance of the receptive animals and of the vectors represented by insects of the *Culicoides* family is the only way to prevent the entrance of the virus in Romania.
2. The serological examinations using ELISA of the wild ruminants imported from areas free of BTV showed that during carantine periode the results were negatives.
3. The periodical clinical inspection of the imported effectives revealed the fact that animals hadn’t presented clinical signs or modifications of the general state that could be associated with the infection produced by bluetongue virus.
4. The virusological exams of vaccinated animals imported from restriction area for BTV gave negative results on RT-PCR.
5. In the south area of the country are prevalent insectes from *Pulicaris* complex and in the centre and in the north (Vâlcea,

Braşov, Neamț) are prevalent insectes from *Obsoletus* complex, because of the coniferous forest.

6. Using those datas obtained during 2004 – 2009 can be continued the proposed objectives being the serological surveillance of the ovines in transhumancy and virusological surveillance of wild ruminants in Ropatosilva research programe. The results can be used also to make epidemiological and risk analyses.

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# ASPECTS REGARDING SEROLOGICAL AND VIRUSOLOGICAL SURVEILLANCE OF THE MOVING BOVINES AND OVINES FLOCKS FROM TCE 3 BRAZI FARM

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**Key words:** blutongue, virus, vectors

**SUMMARY**

Domestic and wild ruminants can be infected by the bluetongue virus, but Romania is declared free of this infection. So, in order to maintain this state the animals were serological and virusological tested in order to descover any potential risk. In the same time there were tested insects from *Culicoides* family because there are vectors for the bluetongue virus. At TCE 3 BRAZI FARM was placed a trap to collect vectors because the location had all the condition to attract insects. After tests were made is was revealed the fact that all the samples tested were negative in all tests.

Blue tongue known as well as the ovine cataral fever or "paintfull mouth" is un acute uncontagious infectious disease transmitted by insects (arbovirosis), specific to ovines and rarelly affecting goats, bovines, cervides and the great majority of african antilops and various species of artiodactiles.

The disease is produces by a RNA virus (Carp-Cărare M, 2001) transmitted and mantained by artropodes (insectes from *Culicoides* genre) and is a part of *Reoviridae* family (Tenner, 1976, cited by Perianu, (OIE Codul animalelor terestre /17.07.2008), *Orbivirus* genre. Those viruses can be differentiated and identified by the structure of the viral genome, the categories of host animals, serological proprieties, proteines composition, symptoms and, more recently, by analyzing and comparing of genome sequencies by PCR (Maan S, 2004).

The insectes from *Culicoides* genre transmits the bluetongue virus to receptive animals after they get infected from a viremic animal (Lysyk TJ, Danyk T, 2005).

## MATERIAL AND METHOD

The surveillance programe for bluetongue was realized during 2004 - 2009 and was implemented by ANSVSA / DSVSA.

To accomplish the proposed aimes biological samples were taken from alive animals (ovines) for serological (Afshar, Ahmad et al, 2001) and virusological exams (Manual de standarde de diagnostic teste şi vaccinuri, 2000).

Serological investigations were executed on 3203 samples, 2352 of them taken from bovines, 839 from ovines and 12 from goats for the entire Neamț County. Referring to TCE 3 Brazi SRL Farm, using ELISA, in 2009 68 samples from bovines and 21 from ovines were investigated. In 2008 43 samples from bovines, 15 from ovines were investigated by ELISA. In 2007 57 samples from bovines, 65 from ovines were investigated by ELISA.

For serological investigations venous blood samples were taken in sterile vacutainers in order to obtain the serum and were tested using competition ELISA to detect specific antibodies for VP7 protein of bluetongue virus (Institut Pourquier), ELISA kit to detect specific antibodies for bluetongue (VMRD INC, SUA), ELISA kit to detect specific antibodies for bluetongue (Ingezim BTV).

The entomological monitorisation was realised installing traps in order to capture insectes during the surveillance programes, followed by the identification of culicoides and structuring a entomological card for every capture.

Identification of *Culicoides* genre species was realised in 2004 during August – October, on 15 gross insect captures conserved, captured using mobile traps in different locations. In the same time were taken every 10 hemoser samples from ovines and 10 from bovines from sanitar veterinary circumscriptions where the insect were captured.

For virusological investigations venous blood samples were taken in sterile vacutainers with anticoagulant (EDTA).

Vectors monitorisation was used to obtain datas regarding their distribution in the teritory, to establish sezoning abundancy of insects species in a certain area, in order to isolate and identify certain viruses they carry and transmit.

For vectors virusological exams they were taken using bright traps in PBS pH 7,2-7,4, transfered in sterile containers, ermetic closed and identified. After morfological exam in containers and with sterile instruments, the culicoides from capture were plased in different

containers in function of the specie, in sterile PBS, and frozed at –72$^{o}$C minimum.

## RESULTS AND DISCUSSIONS

Even if till 2004 when the surveillance programe for bluetongue virus (BTV) was implemented in Neamt, respectivelly Sanitar Veterinary and Food Safety Laboratory Neamț – pilot laboratory for BTV, Romania was declared free of bluetongue, the periodes extremely dry and heat were identified as helpfull for masive development of vector populations. So, the vectors found in 2004 in Neamț County (similar with the ones found in Italy) are suited between 46 and 47$^{o}$ north latitudin, which came in contradiction with datas from speciality literature.

The results obtained in the serological surveillance using ELISAwere all negatives.

The citerias for choosing TCE 3 Brazi SRL Farm, ovine Girov Farm to fix a trap for capture vectors were: close location of running waters with segments of puddles, swampy areas, coniferous forest, garbage platformes, irrigated grounds, horses which are atractive species for culicoides, place protected of powerfull winds and chemical pollution and there weren't made dezinsections by aspersation.

Identification of the species from *Culicoides genre* was realised begining with 2004, when in Neamț County were found vectors of bluetongue virus, placing mobile bright traps at Dumbrava Timişeşti stallions warehouse and TCE 3 Brazi SRL Farm, ovine Girov Farm.

The studies realised during April – May 2004 demonstrated that in Neamț county predomins *C. obsoletus*, comparativ with *C. pulicaris*, and this is important because *C.obsoletus* is a more complex vector for bluetongue virus, comparativ with *C. pulicaris*.

## CONCLUSIONS

1. In TCE 3 Brazi SRL Farm, using ELISA, in 2009 68 samples from bovines and 21 from ovines were investigated. In 2008 43 samples from bovines, 15 from ovines were investigated by ELISA. In 2007 57 samples from bovines, 65 from ovines were investigated by ELISA.
2. The results obtained in the serological surveillance using ELISAwere all negatives.

3. The trap for the vectors was place in this farm because there are all the conditions to atract the insects.
4. The conclusion that came out is reffering to the fact that the strategy which considers that the area of risk for bluetongue virus is limited only in the south region of the country must be modified. So it was taken in consideration that centre and north regions of Romania are equaly exposed to the risk of infection.
5. The serological monitorisation continued at TCE 3 Brazi SRL monthlly conform to strategic programe in 2009 in function of the periode conected with the active periode of the vectors and continue with the transhumancy for ovine.

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# RESEARCH ON THE I.C. AND S.P. IN RELATION WITH NUTRIENT PROFILE, GLUCOSE LEVELS AND CHOLESTEROL IN A COW FARM IN THE NORTH-EASTERN ROMANIA

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**Key words:** dairy cows, glucosis, cholesterol, service period, calving interval

**SUMMARY**

Research has been conducted on a lot of 15 cows chosen randomly from a farm of 100 cows in the North - Eastern Romania.The presence of elevated glycemia (76 to 89.8 mg / dl) and cholesterol (187.9 to 278.5 mg / dl) in 6 cows of all the 15 (reference group) during the maximum production 34-35 liters milk / day coincided with the observation of S.P. between 46-87 days, the other cows that had values of these biochemical compounds within the normal limits, but lower than the previous ones, had an S.P. of 90 days.

Energy profile depends on the gross and net energy levels ingested by cows, on its metabolism by fermentation and on the amount of various volatile fatty acids from stomach. Glucose content of propionic acid and fat content of acetic acid and butyric acid are means of regulating the insurance of increased production (KRANFELD şi col., 1982).

In screening actions for determining metabolic health and productive potential, intermediary metabolism can hardly be studied, for which in practice is examined only some stages of intermediary metabolism (intermediary stages). For the same reasons are used the final metabolites, which are important parameters of energy metabolism normal or pathological, such as glucose, lactic acid, cholesterol, total lipids, pyruvate acid and ketones corpus (PÂRVU GH., 1992).

Changes of energy metabolism may lead to a series of conditions like:

- Decrease in milk production, fat content and milk protein;
- Reproductive disorders (low fertility) and parturitions with nonviable calves;
- Some illnesses such as: ketosis, metabolic acidosis, endocrine dysfunction, etc.

Study of energy profile parameters revealed that for the cows in advanced pregnancy and for the dairy cows, daily glucose formation

varies between 1500 and 2000 g, of which about 25% is used by the fetus, and 60% makes glucose from blood (GVOZDIC D. şi col., 2007).

Fat metabolism depends on age, level of feeding, pregnancy and lactation status. It is evaluated by determining total lipids and cholesterol.

In the literature, dosage of cholesterol is shown to be effectuated in cows with advanced pregnancy, in order to detect early specimens that are predisposed to post-partum disorders. Currently, it is considered that lower cholesterol and increased GOT are associated with the risk that those cows to suffer parturition syndrome. Decrease of cholesterol may also occur during the peak of lactation and liver diseases (BUTLER W.R. and SMITH. 1989).

Hypercholesterolemia is encountered during feeding with pasture.

Infertility problems (ovary problems, genital tract problems) may occur when administered to cows of rations rich in gross protein with a high digestibility level and a low percentage of energy from that portion (DE KRUIF A., P. MIJTEN, 1992).

## 1. MATERIAL AND METHOD

Nutritional-metabolic surveillance carried out by making metabolic profile tests was performed in a farm located in north-eastern Romania.

The researched material is represented by cows, Holstein breed and Romanian Black Pond (BNR), the farm population consists of 100 animals.

Exploitation system is the permanent stabulation, placing animals in the stands together, head by head and the system type of binding is Grabner.

Research has been conducted on a lot of 15 cows from the herd queen selected randomized, aged 2,5 - 3 years, clinically healthy, weighing approximately 500 to 550 kg.

Ration fodder used in farm corresponds to specific standards, which is chosen according to physiological needs of cows, milk production (6000 - 6600 liters / lactation), but also the physiological state of females (lactation, pregnancy, breast rest).

Females were maintained under the same conditions as the herd queen, separated from other cows only during the peak of lactation, when were performed sampling (bleeding).

Blood samples collected by coccygian vein puncture were stored in tubes with coagulation activator for biochemical determinations and were centrifuged at 3000 rotations per minute for 15 minutes for a clear

expression of serum. Serum was stored in Eppendorf tubes with a lid and refrigerated until biochemical determinations.

## 2. RESULTS AND DISCUSSIONS

Nutritional profile was made by determining the energy and nutritional values of each component of the existing feed ration from the farm, then calculating its total concentration determined by summing the results. Reporting the nutritional profile is made in accordance with the type of digestion in ruminants, with breed, milk production (25-30 l / day), weight (600 kg) and physiological status of females

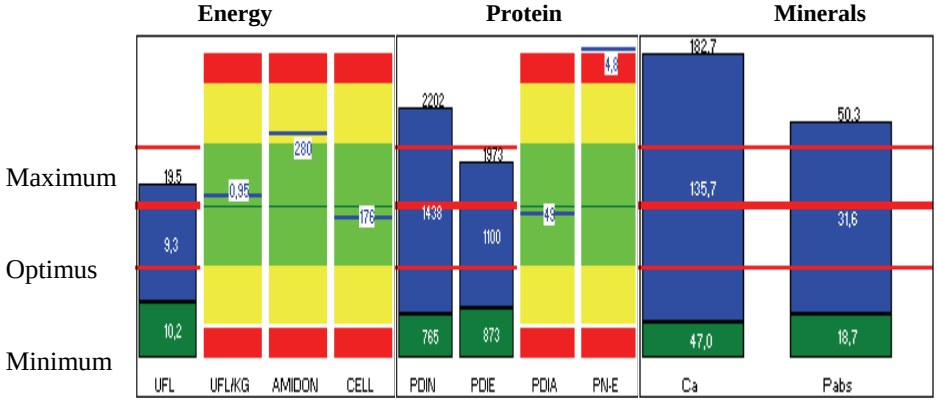
***Table 1***

**NUTRITIONAL AND ENERGY VALUE OF FODDER RATION**

| Product | S.U. (Kg) | Cantitate (Kg) | UNL | PDIN (g) | PDIE (g) | P (g) | Ca (g) | Cell. |
|---|---|---|---|---|---|---|---|---|
| Lucerne | 2,55 | 3 | 0,67 | 115 | 90 | 2,2 | 11 | 361 |
| Maize silage | 9,45 | 27 | 0,90 | 50 | 68 | 2 | 2 | 187 |
| Ground oil sunflower | 1,79 | 2 | 0,72 | 219 | 115 | 9,2 | 2,8 | 204 |
| Corn | 2,59 | 3 | 1,05 | 64 | 84 | 2,6 | 0,4 | 22 |
| Wheat bran | 1,31 | 1,5 | 0,82 | 94 | 79 | 9,9 | 1,4 | 91 |
| Proteic Concentrate | 1,76 | 2 | 0,86 | 310 | 230 | 6 | 7 | 130 |
| Energetical concentrate | 0,39 | 0,4 | 3,24 | 0 | 0 | 0 | 80 | 0 |
| Brewers grains | 0,82 | 4 | 0,19 | 46 | 39 | 1,2 | 0,7 | 31 |
| Ca Carbonat | 0,1 | 0,1 | 0 | 0 | 0 | 0 | 400 | 0 |
| **Total intake** | **20,76** | **43** | **19,82** | **2339** | **2089** | **82,3** | **144,8** | **3682** |
| **Coverage** | | | **110%** | **136%** | **121%** | **114%** | **113%** | |
| **Concentration** | | | **0,95** | **113** | **101** | **4** | **7** | **177** |

Values are calculated according to INRA verification system of feed rations, this pointed out that the ration of forage have increased levels of the available energy above the average level, slightly above the upper limit of amidine and cellulose level decreased slightly below the accepted average. Protein feed recorded a stable trend between average and maximum permissible limt. Absorbable calcium and phosphorus had a higher development, above maximum limit permitted (Graphic No. 1.)

*Graphic 1*




To track the effectiveness of farm feed rations, in addition to the nutrient profile as part of the metabolic profile were carried out determinations of glucose and cholesterol in female-intensive application periods (peak lactation), seeking the productive and breeding activity.

Thus, of all females, the reference group (15 cows) was observed the service period (SP) between 46 and 118 days, with an average of 87, 55 days and a standard deviation of 20.26 against the average SP of the farm, which was assessed at 102 days, with a standard deviation of about 12.3 days.

The presence of elevated glucose blood levels (76 to 89.8 mg / dl) and high cholesterol (187.9 to 278.5 mg / dl) in 6 cows of all the 15 (reference lot) during the maximum production 34-35 (liters milk / day), coincided with the observation of SP between 46-87 days, the other cows that had values of these biochemical compounds within the normal limits, but lower than the previous ones, had an SP of over 90 days.

Calving interval (C.I.) is influenced by the service period, because in the same time with increase its value, it produces a proportional increase in the interval between two calving. Thus, C.I. values varied from 321 to 396 days with an average of 365.46 days.

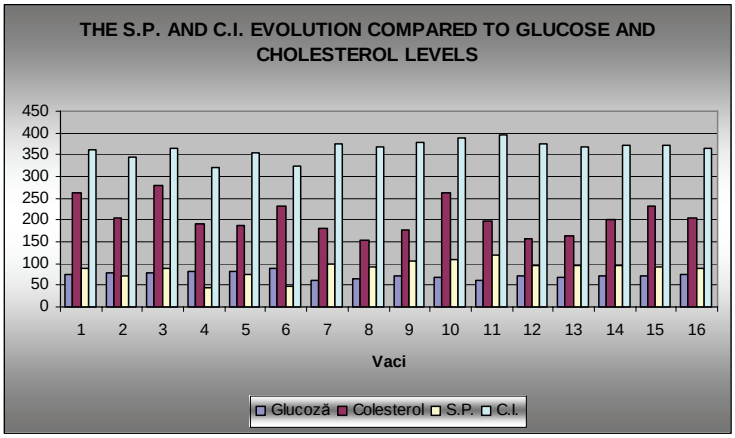
*Table 2*

**THE S.P. AND C.I. EVOLUTION COMPARED TO GLUCOSE AND CHOLESTEROL LEVELS**

| No. Of cow | Glucose ( mg/dl) | Cholesterol (mg/dl) | S.P. (days) | C.I. (days) |
|---|---|---|---|---|
| 64733 | 76 | 262,7 | 87 | 366 |
| 52996 | 78,66 | 206,2 | 70 | 351 |
| 64332 | 80 | 278,5 | 87 | 366 |

| | | | | |
|---|---|---|---|---|
| **10092** | **82,55** | **190,6** | **46** | **321** |
| **10080** | **82,85** | **187,9** | **74** | **359** |
| **64748** | **89,85** | **232,3** | **49** | **331** |
| **04931** | 62,74 | 182,2 | 99 | 375 |
| **91859** | 65,19 | 153,5 | 92 | 367 |
| **49477** | 72,9 | 176,2 | 104 | 378 |
| **64725** | 67,99 | 262,2 | 110 | 387 |
| **06182** | 61,98 | 198,0 | 118 | 396 |
| **84006** | 70,92 | 157,1 | 97 | 374 |
| **83507** | 66,62 | 163 | 94 | 369 |
| **64735** | 72,13 | 199,5 | 94 | 371 |
| **52996** | 72,9 | 230,6 | 92 | 371 |
| **Average** | 73,55 | 205.36 | 87,55 | 365,46 |
| **Standard deviation** | 8 | 39,6 | 20,26 | 20,78 |

***Graphic 2***




## 3. CONCLUSIONS

3.1. Research has been conducted on a lot of 15 cows chosen randomly from a farm of 100 cows in the North - Eastern Romania.

3.2. Reporting the nutritional profile is made in accordance with the type of digestion in ruminants, with breed, milk production (25-30 l / day), weight (600 kg) and physiological status of females.

3.3. The parameters determined were concerned to the energy and nutrients of feed ration, glucose and blood cholesterol levels, correlated with development of service-period and calving interval

3.4. From the total number of females in the farm, at the reference group (15 cows) was observed a service period (S.P.) between 46 and 118 days, with an average of 87, 55 days and a standard deviation of 20.26 against the average S.P. of the firm, which was assessed at 102 days, with a standard deviation of about 12.3 days.

3.5. The presence of elevated glycemia (76 to 89.8 mg / dl) and cholesterol (187.9 to 278.5 mg / dl) in 6 cows of all the 15 (reference group) during the maximum production 34-35 ( liters milk / day) coincided with the observation of S.P. between 46-87 days, the other cows that had values of these biochemical compounds within the normal limits, but lower than the previous ones, had an S.P. of 90 days.

3.6. Service period influences the calving interval; once its value rises, we can see a bigger interval between two births, values of CI varying between 321-396 days, with an average of 365,46 days.

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# STUDY ON THE MICROBIOLOGICAL CHARGE OF COLOSTRUM IN PRIMIPAROUS COWS

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**Keywords:** cow, mammary gland, primiparous, colostrum, bacteria.

**SUMMARY**

Colostrum represents the secretion of mammary gland in the first days after parturition. It is essential for the survival of new-born and for its adaptation to extrauterine life.
Theoretically, colostrum must be microbiologically sterile, especially at primiparous cows, females in which mammary gland is virgin; practically, it is admitted a microbial charge of 100,000 colony formation units (CFU)/ml.
There are data demonstrating that colostrum from primiparous cows contains high bacterial charges and they are predisposed to develop mastitis in the first days postpartum.
In this study, colostrum was sampled from primiparous cows in order to appreciate its sanitation, which is an important criterion of quality.
The obtained results confirmed the existence of a high microbial charge, beyond admitted limits. Thus, from all 40 examined samples, 5% mammary quarters were unfunctional, 17.5% mammary quarters were sterile and 77.5% mammary quarters were not sterile, with values of CFU ranging between $2.6x10^5$ and $1.56x10^6$ CFU/ml.
The existence of microbial pollution over the admitted limits in colostrum sampled from primiparous cows denotes the infectious pressure in the studied farm and the high risk of intramammary infections on all categories of lactating cows.

Sanitation represents an important feature of qualitative colostrum due to the fact that, in the last years, food industry provides colostrum-based products for human consumption; besides that, colostrum is the only way for transferring maternal immunity to new-born calves (Johnson *et al.*, 2007; Serieys, 1993; Swan *et al.*, 2007). On the other hand, colostrum can assure the first exposure of new-born calves to pathogen germs, some of them being extremely aggressive (*Escherichia coli, Salmonella, Mycobacterium*) (Fecteau *et al.*, 2002).

A part of these bacteria are present in the exterior, on the mammary skin, and they be controlled by the breeder through feeding hygiene, but it also exists a second category of germs, present in colostrum, even at primiparous cows, females in which mammary gland is virgin.

Starting from literature data, which affirm that colostrum from primiparous cows contains high bacterial charges and they are

predisposed to develop mastitis in the first days postpartum (Swan *et al.*, 2007), it was performed the present study on a group of 10 primiparous cows from a farm located in the south of Romania.

## 1. MATERIALS AND METHODS

Mammary epidemiological unit is represented by mammary quarter. There were taken colostrum samples from 10 primiparous cows, from each mammary quarter. The sampling was realized immediately after parturition, before the first feeding of new-born calves.

It is well-known the fact that, right before parturition, can act a series of factors that predispose the contamination of mammary secretion. For instance, antepartum mammary edema determines the increase of papillary duct diameter and the elimination of keratin stopper, permitting the microorganisms to get into the mammary gland. Therefore, in order to avoid confusions concerning the origin of pathogens, the first jets of colostrum were removed and the mamelons were disinfected with alcohol.

The sampling technique was applied in aseptic conditions; the tubes were identified and sent to laboratory, where they were subdued to usual analyze procedures.

## 2. RESULTS AND DISCUSSIONS

The results obtained for the 10 studied primiparous cows (40 samples – 40 mammary quarters) are presented in Fig. 1 and table 1.

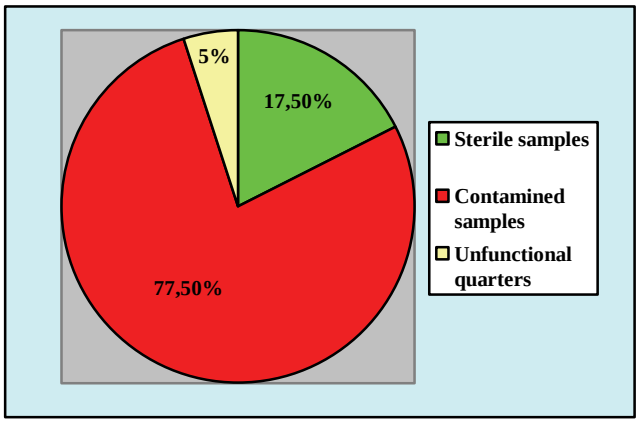


**Fig. 1. Percentual distribution of the results obtained after microbiological exams of 40 colostrum samples**

*Table 1*

**The values of colony formation units (CFU) from the analyzed colostrum samples**

| Case nr. | Identification nr. | Date of parturition | Mammary quarter | CFU Nr./ml |
|---|---|---|---|---|
| 1 | 7942 | 5 July 2009 | FRQ | 1,500,000 |
| | | | FLQ | 1,200,000 |
| | | | BRQ | 950,000 |
| | | | BLQ | 340,000 |
| 2 | 7951 | 9 July 2009 | FRQ | Sterile |
| | | | FLQ | Sterile |
| | | | BRQ | 260,000 |
| | | | BLQ | Sterile |
| 3 | 7971 | 11 July 2009 | FRQ | 1,560,000 |
| | | | FLQ | 700,000 |
| | | | BRQ | 1,420,000 |
| | | | BLQ | Unfunctional |
| 4 | 7985 | 29 June 2009 | FRQ | Sterile |
| | | | FLQ | Sterile |
| | | | BRQ | Sterile |
| | | | BLQ | 850,000 |
| 5 | 7993 | 8 July 2009 | FRQ | 1,450,000 |
| | | | FLQ | 1,200,000 |
| | | | BRQ | Sterile |
| | | | BLQ | 1,500,000 |
| 6 | 7994 | 3 July 2009 | FRQ | 1,500,000 |
| | | | FLQ | 1,100,000 |
| | | | BRQ | 640,000 |
| | | | BLQ | 340,000 |
| 7 | 3245 | 5 July 2009 | FRQ | Unfunctional |
| | | | FLQ | 950,000 |
| | | | BRQ | 1,100,000 |
| | | | BLQ | 320,000 |
| 8 | 3176 | 7 July 2009 | FRQ | 1,200,000 |
| | | | FLQ | 1,500,000 |
| | | | BRQ | 750,000 |
| | | | BLQ | 350,000 |
| 9 | 3107 | 10 July 2009 | FRQ | 1,240,000 |
| | | | FLQ | 680,000 |
| | | | BRQ | 970,000 |
| | | | BLQ | 260,000 |
| 10 | 3536 | 30 June 2009 | FRQ | 1,450,000 |
| | | | FLQ | 1,520,000 |
| | | | BRQ | 650,000 |
| | | | BLQ | 740,000 |

***Legend:***

FRQ – Front Right Quarter; BRQ – Back Right Quarter;

FLQ – Front Left Quarter; BLQ – Back Left Quarter.

Analyzing the data in table 1, there can be presented the following comments:

- 31 colostrum samples were microbiological contaminated, which represents 77,50% from total number of samples;
- 7 colostrum samples were sterile (17.5%), while 2 mammary quarters (5%) were unfunctional;
- the microbial charge in the analyzed samples ranged between 260,000 CFU/ml and 1,560,000 CFU/ml;
- none of the contaminated colostrum samples presented values of CFU/ml under the admitted limits (100,000 CFU/ml).

## 3. CONCLUSIONS

3.1. In the conditions of cow breeding in farms from south of Romania, the colostrum produced by primiparous cows can represent a source of infection for the new-born calves starting even from the first feeding.

3.2. The high proportion of primiparous cows whose colostrum contains values of colony formation units/ml (CFU/ml) higher than the admitted limits (100,000 CFU/ml) demonstrates that this category is neglected from the mammary hygiene's point of view.

3.3. The high bacterial charge of colostrum in primiparous cows emphasizes the infectious pressure in the studied farm, which represents the cause of intra-mammary infections in the first days post-partum.

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# COMPATIBILITY ASSESSMENT IN XENOTRANSPLANT AFTER ATTEMPT OF IMMUNOTOLERANCE INDUCTION IN EMBRIONARY STAGE

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**Key words:** compatibility, xenotransplant, immunotolerance, poultry

**SUMMARY**

The aim of the present work was to establish the compatibility of the donor and recipient birds (muscovy ducks, respectively Cobb 500 hybrids) after immunotolerance induction by *in ovo* inoculation of bone marrow mononuclear cells obtained from donor birds in fifth days recipient embryo and before xenotransplant of the skin. We used 15 immunotolerized Cobb 500 poultry (experimental group) and 15 intact poultry (control group). The compatibility was assessed by mixed lymphocyte reaction and by flow cytometry. The results showed that 86,66% of the experimental group individuals were compatible with the donor birds and absolute incompatibility of the control group birds. Also, flow cytometry showed a significant differences in lymphocyte T subsets proportion between experimental and control group birds.

According to Burnet MacFarlane's theory, the contact with antigenic information in the so called "immunological window" (in embrionary or fetal life) would ensure the recognition as self of all the components of the organism from which the information has come. This phenomenon is known as *immunotolerance* and can be explained through the fact that the immune system is immature, i.e. its effecting cells haven't gained the surface markers that transform them in active, immunocompetent cells (Burnet, 1959).

Thus, the grafts (cells, tisues or organs) derived from the donor organism of antigenic material, transplanted to the recipient organism that has already become mature, should be assimilated to the autografts and shouldn't require any supplementary measures for the prevention of the rejection (immunosuppression).

Thus, in this study we try to establish the compatibility of the donor and recipient birds (muscovy ducks – *Carinia moscata*, respectively Cobb 500 hybrids) after immunotolerance induction by *in ovo* inoculation of bone marrow mononuclear cells obtained from donor birds in fifth days recipient embryo.

## 1. MATERIALS AND METHODS

***Biologic material***: 15 immunotolerized Cobb 500 poultry (experimental group) and 15 intact poultry (control group). The donor birds for antigenic material were three muscovy ducks (*Cairina moscata*).

***Immunotolerance induction.*** The antigenic material, mononuclear cells from the bone marrow, was obtained by three centrifugations of the diluted samples gathered from donor birds, after Ficoll-Paque solution addition (Fernandez-Botran and Větvička, 2000). The resulting antigenic material was inoculated *in ovo* in the fifth days embryos from experimental group.

***Mixed lymphocyte reaction.*** We made 30 mixed lymphocyte cultures (15 for experimental group and 15 for control group) using Sigma PK Linker modified protocol, respectively a bidirectional reaction in which proliferative response of both cell populations (donor and recipient T cells) is measured. The blood samples (source of T lymphocytes) were gather from donor birds and three weeks age recipient and intact poultry.

***Evaluation of the lymphocyte T subsets.*** At the age of three weeks, from both experimental and control individuals peripheral blood was gathered for obtaining the T lymphocytes using the Ficoll-Paque solution for separation. T lymphocytes were labeled with monoclonal antibodies as follows: antibodies anti-CD3 for label T lymphocytes, antibodies anti-CD4 for label T helper lymphocytes, antibodies anti-CD8 for label cytotoxic T cells, antibodies anti-CD45RO for differentiating between memory and naive T cells, antibodies anti-CD28 for differentiating between memory and effector T lymphocytes, and antibodies anti-CD25 for label the eventual activate subset of T cells from subpopulation Treg - regulatory T cells. This labeling served in determining the lymphocytes T profile in experimental and control groups.

By *flow cytometry*, the cells population labeled in four colors (CD3FITC, CD4PE CD45RAPerCP, and CD28APC for determining the T helper subsets and CD3FITC, CD8PE, CD45ROPE, and CD28APC for determining the T cytotoxic subsets) were quantitative analyzed in both experimental and control groups. The lymphocytes subsets were defined as follows: naive $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^-CD28^+$; memory $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^+CD28^+$; effector $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^+CD28^-$; effector $CD4^+$ helper T

cells with phenotype $CD3^+CD4^+CD45RO^-CD28^-$; naive $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^-CD28^+$; memory $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^+CD28^+$; effector $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^+CD28^-$; and effector $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^-CD28^-$.

For data acquisition and analysis, Cell Quest and Win MDI2.9 softwares were used.

## 2. RESULTS AND DISCUSSIONS

We appeal to the inoculation of antigenic material in the fifth day of the embrionary development knowing that a tardily intervention (i.e. inoculation in allantoidal vessels of the eight days embryos) is insufficient for immunotolerance induction (Şereş et al., 2008).

Assessment of the compatibility using mixed lymphocyte reaction showed that 86,66% of the experimental group individuals were compatible with their correspondent donor birds. In these cultures we don't observed neither a proliferative response of the two lymphocyte populations (donor and recipient T cells) nor a lysis of the donor T cells (fig. 1 a). As opposed to experimental group, cultures corresponding to control group showed an intense fluorescence PK27/PK67 (FL1/FL2) due the cell-mediated immune response (proliferation of the recipient T lymphocytes and lysis of the donor cells). These differences are statistically significant ($P < 0.05$).

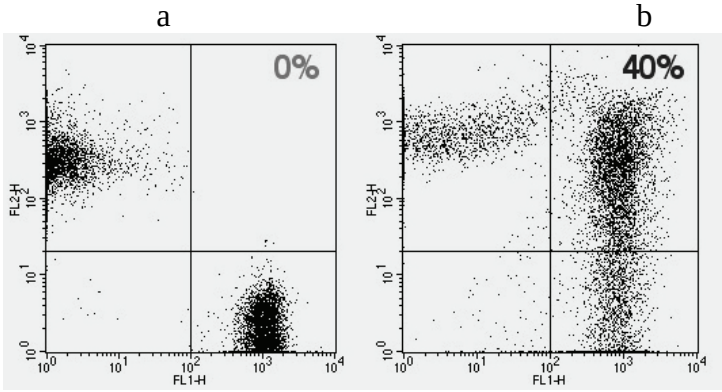


**Fig. 1. Proliferative and lytic cells` response, : a) experimental group, b) control group**

Flow cytometry shows a significant difference between representation of the lymphocyte T subsets recorded in experimental and control group (fig. 2 and 3). Naive T cells ($CD3^+CD45RO^-CD28^+$) were

represented in a superior proportion in the individuals from the control group (62.15 ± 9.86%) comparing with the experimental group (34.16 ± 13,75%). Also, the proportion of Treg cells was higher in 86,66% of the experimental group individuals (10.60 ± 5.20%) comparing with control group (3,15 ± 1.90%). All these aspect suggest that inoculation of the xenogenic mononuclear cells in the embrionary stage has important effects upon the immune repertoire.

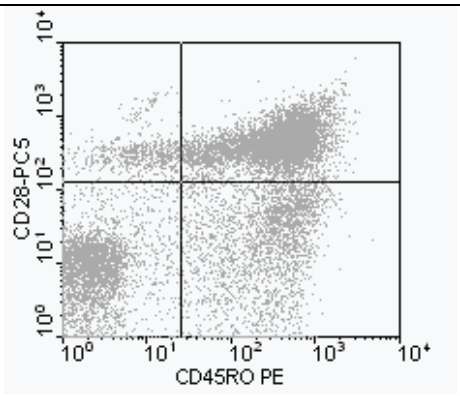


**Fig. 2. Distribution of the T cell subsets – experimental group**

Multiple Document Interface for Flow Cytometry
WinMDI Version 2.9 - Windows 3.95/DOS 5.0
Gates: R1*R2
Project: Experiment: 3
Date: 20-July-08 Parameters: 6
Total Events 100000
System: Log Parameter Means: Geometric
Quad Stats
CD45RO PE(Log) vs CD28-PC5(Log)
Quadrant x,y: 360,540

| Quad | X-Mean | Y-Mean | %Gated |
|---|---|---|---|
| 1 UL | 8.4 | 323.2 | **5.63** |
| 2 UR | 374.6 | 462.4 | **48.15** |
| 3 LL | 2.2 | 7.8 | **27.34** |
| 4 LR | 256.1 | 15.0 | **18.88** |

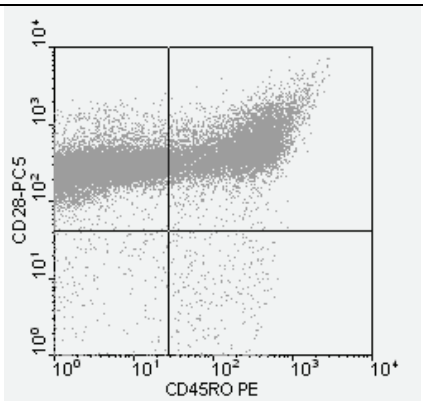


**Fig. 3. Distribution of the T cell subsets – control group**

Multiple Document Interface for Flow Cytometry
WinMDI Version 2.9 - Windows 3.95/DOS 5.0
Gates: R1*R2
Project: Experiment: 3
Date: 20-July-08 Parameters: 6
Total Events 100000
System: Log Parameter Means: Geometric
Quad Stats
CD45RO PE(Log) vs CD28-PC5(Log)
Quadrant x,y: 360,540

| Quad | X-Mean | Y-Mean | %Gated |
|---|---|---|---|
| 1 UL | 3.0 | 226.4 | **61.25** |
| 2 UR | 217.3 | 520.7 | **35.75** |
| 3 LL | 4.1 | 6.7 | **1.63** |
| 4 LR | 146.1 | 8.8 | **1.36** |

## 3. CONCLUSIONS

Mixed lymphocyte reaction, sustained by the flow cytometry results, showed to be a reliable method for compatibility assessment in xenotransplant.

*In ovo* inoculation in the fifth days embryos of the mononuclear cells obtained from the donor birds bone marrow determine immunotolerance at least for the subsequent xenotransplat of the same type of cells.

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# CLINICAL AND HISTOPATHOLOGICAL ASSESSMENT OF SKIN XENOGRAFT REJECTION IN POULTRY

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**Key words:** concordant xenografts, skin, reject, poultry

**SUMMARY**

The aim of the present study was to establish the clinical and histopathological features associated with skin xenografts rejection in poultry. There have been used 15 Cobb 500 hybrids in which full-thickness concordant xenograts (from muscovy ducks) were engrafted at four weeks age. The subjects have been examined daily, paying attention to the 21 macroscopic characteristics of the transplanted skin. In the purpose of histopathological exam, tisular samples (grafts and 1-2mm adjacent skin) were excised at three days after transplant. Clinical and histological findings sugest that skin xenografts rejection in poultry is an acute one - acute vascular rejection, the primary type of rejection seen in concordant tissue xenografts applied in mammal recipients.

For nearly a century, xenotransplantation (the transplantation of cells, tissues or organs between individual of different species) has been seen as a potential approach to replacing organs and tissues damaged by disease. Until recently, the mechanisms responsible for xenografts rejection and accommodation were unknown even in mammals. The aim of the present study was to establish the clinical and histopathological features associated with skin xenografts rejection in poultry.

## 1. MATERIALS AND METHODS

***Biologic material***: 15 four weeks age Cobb 500 poultry – recipient birds and three muscovy ducks (*Cairina moscata*) – donor birds.

***Skin transplant.*** Transplantation of the full-thickness xenogenic skin graft was made at the age of four weeks of recipient birds and surgical procedure followed the principles suggested by Swaim (2003) and Shannon (2002): sampling of the skin free-flaps from the donor birds, removal of the subcutaneous connective tissue, segmentation of each free-flaps in five 1.5x1.5cm skin grafts, preparation of recipient bed – surgical wound, and fixation of the skin grafts at the sides of the

wound. The full-thickness skin free-flaps were taken from the donor birds under neuroleptanalgesia (Xylazine 2mg/kg and Ketamine 10mg/kg), from axillary region. The acceptor bed for the full-thickness skin grafts was represented by surgical wounds created in the axillary region. This election place, both for donor and recipient birds, was preferred because the region presents few feather follicles and the low degree of covering allowed feathers removal without hurting the skin too much.

The surgical technique was verified by autotransplantation of skin segment from one axillary region to other one in five subjects.

***Clinical monitorization.*** The subjects were examined daily, paying attention to the 21 macroscopic characteristics of the skin grafts among the most important being: color, aspect and adherence of the grafts to the recipientbed, as well as the aspect of the sides of the wound, making different measurements and taking pictures.

***Skin sample processing for histopathological exam.*** The skin samples (entire grafts and 1-2mm adjacent skin) were detached at day three after transplant. After fixing in ethanol $80^{o}$, tissue samples were washed, dehydrated, embedded in paraffin, and cutted in 5μm thick sections which were stained by Mallory Trichrome method.

## 2. RESULTS AND DISCUSSIONS

The transplant has been made at the age of four weeks knowing that an earlier intervention, especially during the first week of life, could have led to the acceptance of an incompatible graft or to delaying the reject (Billingham et al., 1961).

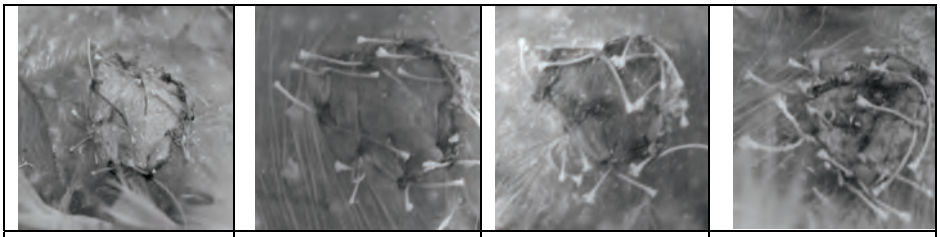
Results of the clinical exam

*In the first day after transplant* the xenografts were ischemic, slightly dehydrated and slightly adherent to the acceptor bed (fig. 1A). Additionally, it was registered a congestive reaction of the recipient bed sides, with different degrees of intensity.

*Two days after the surgical intervention*, all the xonografts were cyanotic, dehydrated, and unadhesive to the recipeint bed, some of them being slightly prominent in comparison to the side of the wound. Also, the congestion of the sides of the wound was completed with their endeme and intense cyanosis (fig. 1B).

*In the third day* all the grafts were cyanotic, dehydrated, unadhesive to the acceptor bed, having tendencies to transform into a crust (fig. 1C).

*In the fourth day after transplant* all the grafts were transformed into crusts (fig. 1D). We considered that skin xenografts were rejected.



| A | B | C | D |
|---|---|---|---|

**Fig. 1. Macroscopical aspects of skin xenografts: A) first day, B) second day, C) third day, D) forth day after transplant**

The complete acceptance of the skin autografts validated the applied surgical technique for transplantation.

Results of histopathological exam

The histopathological images show endothelial injury and swelling (fig. 2D), ischemia and diffuse thrombosis (fig. 2B). Also, it could be seen an infiltrate consisting of mononuclear leukocites and rare neutrophils (fig. 2C and 3C), enlargement of the dermal nodules (fig. 2A) and fragmentation of the collagen fibres (fig. 3B). The presence of some normal blood vessels (fig. 3A and D) and viability of the epiderm (fig. 3D) rule out various non-imunnological factors of rejection, like primary non-function, failure of neovascularization or failure of the microenviroment to support the survival and function of the foreign tissue (Saadi and Platt, 1998).

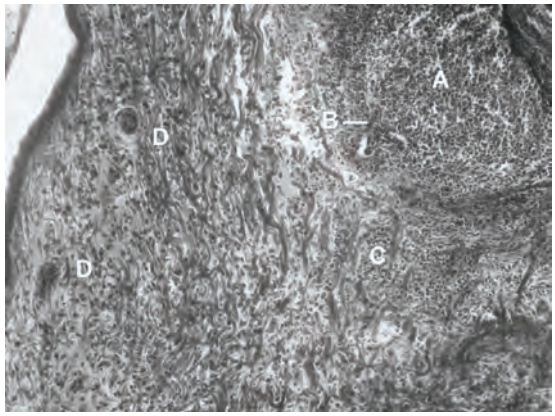



**Fig. 2. Histopathological findings in rejected skin xenograft: A) enlargement of a dermal nodule, B) diffuse thrombosis, C) mononuclear cells` infiltrate, D) endothelial injury and swelling. Mallory Trichrome stain, x20**

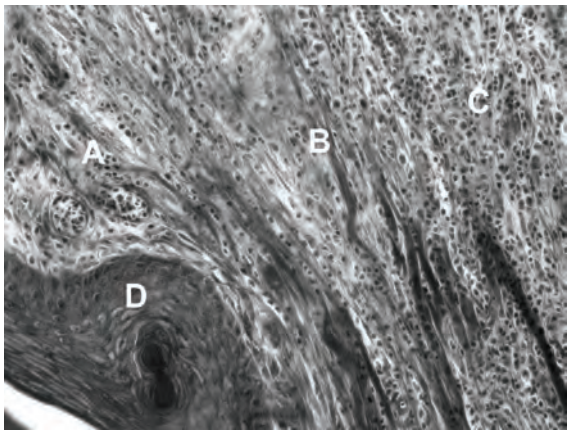



**Fig. 3. Histopathological findings in rejected skin xenograft: A) normal blood vessels, B) fragmentation of the collagen fibres, C) mononuclear cells` infiltrate, D) viable epiderm and follicular vessels. Mallory Trichrome stain, x40**

All these patholological changes suggest an "acute vascular rejection", similar the primary type of rejection seen in concordant tissue xenografts applied in mammal recipients (Parker et al., 1998, Saadi and Platt, 1998).

## 3. CONCLUSIONS

The mechanisms involved in the rejection of the skin xenografts in poultry are of an immunological order.

The rejection of skin xenografts in poultry is an acute one - acute vascular xenograft rejection, aspect suggested by the results of clinical and histopathological exams.

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# INCIDENCE OF SUBCLINICAL MASTITIS IN COWS ACCORDING TO PRODUCTION AND LACTATION

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**Key words**: raw milk, mastitis, somatic cells, milk electrical conductibility

**SUMMARY**

Milk production is influenced by the health of the cows mammary gland. Worldwide, the most spread mammary gland diseases are mastitis, inflammatory reaction of the mammary gland, often caused by bacterial infection (Hocquette J.F., S. Gigli, 2005). Hygiene of whole technological process is necessary to prevent subclinical mastitis (Reneau, J.K., 2005).
Subclinical mastitis are desirable to be detected, so that milk should maintain its organoleptic properties (Bondoc I, E Şindrilar, 2002). The increased number of somatic cells and the high milk conductibility are supporting an early mastitis detection (Drugociu D., 2005).
The aim of this paper is to find the correlation between the number of somatic cells and milk electrical conductibility in cows mastitis detection.

## 1. MATERIALS AND METHODS

To respect E.U. standards concerning milk quality, it must fulfill certain conditions, including the maximum number admitted of somatic cells 400.000/ml. (Directive 92/46/EC, Decision 95/342/CEE).

Studied material was represented by cows of breed Romanian Black Pond (NBR). The incidence of subclinical mastitis was determined in dairy cows of different ages and at different stages of lactation. Obervation was conducted in a farm located in Iassy County, with a herd of 290 lactating females.

Research has been conducted on a lot of 17 randomly selected cows from the herd, housed in permanent stabulation system. Studied females were clinically healthy, aged between 1 and 7 years and maintained under the same conditions as the rest of the cows from the herd.

Milk samples were collected from examined cows in sterile containers and afterwards analyzed at the Ekoscope device, available at Reproduction discipline of FMV Iassy, in order to determine the number of somatic cells for each sample.

From all high producing cows in the farm a group of 5 cows was formed, to whom was examined also the electrical conductivity of milk. The examination was performed at the time of milking, using hand Draminski detector.

## RESULTS AND DISCUSSIONS

In September 2009 an inspection was conducted on a lot represented by 17 cows to determine the incidence of subclinical mastitis in the unit. Three experimental lots were chosen: A-cows with daily milk production over 30 liters, B- cows with daily milk production between 20-30 liters, and lot C, with daily milk production under 20 liters. Lot A consisted of 5 cows, lot B and C consisted of 6 cows, each lot of different ages.

***Table 1***

**Somatic cell count values, correlated with electrical conductivity of milk harvested from cows examined in the lot A**

| No. of cow | **Anterior left quarter** | | **Anterior right quarter** | | **Posterior left quarter** | | **Posterior right quarter** | |
|---|---|---|---|---|---|---|---|---|
| | N.C.S | Val. C.E. | N.C.S | Val. C.E. | N.C.S | Val. C.E.. | N.C.S | Val. C.E.. |
| 522 | 137.956 | 610 | 451.832 | 430 | 295.621 | 550 | 187.810 | 580 |
| 716 | 295.621 | 500 | 407.518 | 400 | 296.722 | 540 | 187.226 | 450 |
| 9770 | 857.297 | 450 | 364.322 | 310 | 492.701 | 380 | 657.204 | 280 |
| 9420 | 218.248 | 730 | 452.712 | 370 | 519.708 | 310 | 398.541 | 420 |
| 460 | 492.701 | 480 | 197.081 | 440 | 652.251 | 380 | 365.981 | 410 |

From all the 15 cows with the highest yields in the farm, 5 cows were selected to form the experience lot A. They were aged between 3 and 7 years, being in different months of lactation. For lot A milk samples were collected from each quarter of the examined cows, so that 20 samples were analyzed to determine the number of somatic cells (NCS).

In the same time was determined the electrical conductivity (E.C.) of milk for each quarter, when milking, with Draminski detector. Electrical resistance of milk decreases in case of infection of the mammary gland (Norberg, E, *et al.*, 2004). Using Draminski device there can be detected electrical resistance values of each quarter. Thus,

in Table 1 are highlighted those quarters that have low electrical resistance of milk. They correspond to samples with high number of somatic cells.

After examining the samples by both methods, it was reached to the same conclusion: all females examined in the lot A have subclinical mastitis. All 5 examied cows, at the time of study, were at the beginning or at the end of lactation, with a daily milk production of over 30 liters per day.

At 60% of examined cows in group A was noted that the hindquarters are more prone to subclinical mastitis occurrence.

In Table 2 are observed somatic cells values obtained for each cow from experimental lots B and C.

***Table 2***

**Somatic cells values for cows from experimental lot B and C**

| LOT | No. Of cow | Age of cow (Years) | No. Of lactation | Period of lactation (months) | No. Of somatic cells |
|---|---|---|---|---|---|
| B | 51 | 4 | 3 | 5 | 548.164 |
| | 97 | 4 | 2 | 5 | 298.540 |
| | 615 | 3 | 2 | 5 | 177.372 |
| | 9766 | 5 | 2 | 11 | 197.080 |
| | 9664 | 5 | 3 | 10 | 147.810 |
| | 3899 | 2 | 1 | 2 | 434.215 |
| C | 598 | 4 | 2 | 4 | 349.705 |
| | 697 | 3 | 2 | 3 | 146.305 |
| | 9438 | 7 | 4 | 5 | 857.297 |
| | 136 | 4 | 2 | 5 | 491.987 |
| | 9753 | 5 | 3 | 3 | 424.751 |
| | 862 | 3 | 1 | 11 | 171.898 |

Thus, the maximum permissible value of somatic cell is exceeded in lot B. High number of somatic cells have been detected in samples from young cows, aged from 2 years to 4 years.

They have been recorded with a daily production between 20 and 30 liters of milk. Regarding the experimental lot C, the examined cows had a daily production of milk under 20 liters and somatic cell count was higher in cows with advanced age (7 and 5 years).

Females who had high somatic cell counts above the maximum allowed limit, were at the beginning of lactation, from month 2 up to the 5-th month of lactation.

In fig.1 is highlighted the increasing somatic cells in experimental lot B, the first cow examined (4 years) had 548,164 somatic cells / ml milk. The last female examined in experimental lot B was 2 years old and had 434.215 somatic cells /ml of milk.

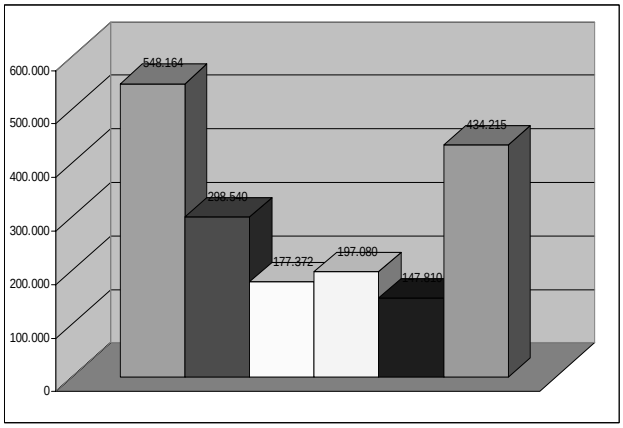


**Fig. 1., Number of somatic cells highlighted at experimental lot B**

In experimental lot C, cows had a daily production of less milk, less than 20 liters milk per day, but in this experimental lot, samples have been inappropiate for the standards, with a high number of somatic cells.

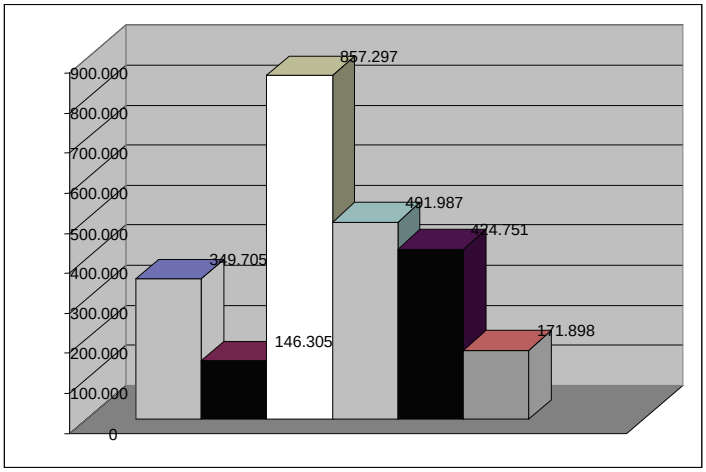


**Fig. 2., Number of somatic cells highlighted at experimental lot C**

The incidence of subclinical mastitis in cows from experimental lot C is higher than in lot B. Thus, half of the examined cows had values above the maximum allowed limit, registering 857,297, 491,987 respectively 424,751 somatic cells / ml milk. The highest value was

registered in a milk sample from a 7 years old female, currently in the 5-th month of lactation (857,297 somatic cells / ml milk).

## 3. CONCLUSIONS

3.1. Milk samples were examined from 17 cows in a farm from Iassy County, with different ages and lactations, in order to achieve a correlation between these parameters and the occurrence of subclinical mastitis.

3.2. Subclinical mastitis was diagnosed by somatic cell counts and by detecting electrical resistance of milk (for experimental lot A only). In lot A were analyzed 20 samples from each quarter of each 5 examinated cows. It was noted that the quarters that had low values for electrical resistance of milk corresponded with the samples with high number of somatic cells.

3.3. The examined females were divided into 3 groups, A, B and C, according to daily milk production. It was observed an increase of somatic cells and decrease of electrical resistance of milk from all cows examined in group A, with daily production of over 30 liters milk.

3.4. Samples of milk which had an increased number of somatic cells were from females at the beginning or the end of lactation. They had subclinical mastitis. The elderly examined female was 7 years old, belonged to experimental lot C and was in the 4-th lactation. Samples taken from this female registered the highest value of somatic cell count - 857,297/ ml milk.

3.5. The incidence of subclinical mastitis in cows in experimental lot C was higher than in experimental lot B. In lot C, the cows average age was higher, the daily milk production was lower and the number of somatic cells was increased in most analyzed samples.

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# NEW ASPECTS IN MYXOMATOSIS EVOLUTION

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**Key words:** myxomatosis, rabbit, epidemiology, morpho-clinical aspects

**SUMMARY**

Is well-known that the classic form of myxomatosis is evolving in non vaccinated rabbits or, if the disease occur in vaccinated rabbits, the disease is usually less severe. In the last nine years, myxomatosis in rabbits population from Bucharest and Ilfov county area show new morpho-clinical and epidemiological aspects of disease. In the last years, in pet rabbits the disease often progresses more slowly and death occurred in less than 50% of clinical cases.

## 1. MATERIAL AND METHODS

In the clinic of infectious disease and preventive medicine, between 2000 to 2009 have been investigated 28 rabbits with myxomatosis: 16 from 2000 to 2002 and 12 from 2003 to September 2009.

The two groups were divided based on cases incidence and prevalence and on the clinical features.

Up to the location, 16 cases belong to Ilfov County from 6 back-yards in the rural area and 12 belong from 10 Bucharest sites: the county cases are from familial farm, 10 to 50 rabbits, all Bucharest cases, 7 to 10, being raised alone, as pet.

We gathered data about the flock, the animal origin, disease history in the outbreak and in the neighborhood area, we estimate the morbidity and mortality rate in outbreaks, the specific clinical findings, the post mortem lesions and samples were taken for histopathological examinations.

## 2. RESULTS AND DISCUSSIONS

The geographical distributions of submitted cases show the highest disease frequency in the north, west and south of the county.

The remark may be subjective, due to the number and distribution of the examined cases did not reflect the objective reality on the ground.

Disease prevalence registered 57% in the first stage from 2000-2002, is higher than the prevalence registered in the second stage, over five years, and meaning 43% (2003-2009).

The clinical and pathological features exhibit significant differences between the first and second stage cases:

- the first stage cases was acute, abrupt, pushing the owner to ask the vet;the myxomes occurred and raised quickly (Figure 1, 2 and 3), covering in 2-3 days the whole body skin and secondary signs succeeded 2-3 days later (dyspnea, blindness, milky ocular discharge, listless and anorectic, invariably purulent nasal discharge and wasting).

Up to anamnesis collected data, the estimated morbidity was 50 to100% in rabbits from the same familial farms, the mortality, after 7 to 10 days of clinical evolution (shorter in youngest), reached 100%.

The extent of cutaneous lesions interested nose, lips, and ears who become edematous and gives a swollen appearance to the head. In females, the vulva was inflamed and edematous; in males, the scrotum swelled. The lesion was firm, myxomas being mobile and the conjunctive tissue is slightly hemorrhagic. On dead rabbits we registered pulmonary/hepatic congestion.

The histopathological findings: the derma was edematous and cells are harboring cytoplasm-vesicles (Figure 4), the nuclei of infected cells exhibit apoptotic bodies in the cytoplasm, fibrinoid necrosis in small dermal blood vessel and mononuclear dermal infiltration, lungs hyperemia with edema and mononuclear invasion in connective tissue, hepatic centro lobular congestion (Figure 5) surrounded by dystrophic areas.

In 2003-2009, disease prevalence decreased markedly (from 10 cases/year in 2000 to 1-2 cases/year in 2003-2009), but also the severity of clinical findings. All features was slightely, the size and number of cutaneous nodules decreased (Figure 6 and 7), and rabbit recovered frequently (6 from 12), keeping as sequels alopecia in area of healed skin, or discoloration of fur (Figure 8 and 9).

Just for once we recommended to apply vaccination, but disease still occur one week later, proving that infection take place before vaccination.

The decrease of prevalence cases in registered rabbits (2000-2009) was mentioned in the neighborhood countries (Table 1), suggesting a global change of epidemiological features of myxomatosis, caused either by the virus virulence decrease either by a highest specific resistance in domestic rabbits.

## 3. CONCLUSIONS

3.1. Mixomatosis evolved bouth in farm and in pet rabbits;

3.2. The disease spreaded randomly in investigated area;

3.3. The vaccination after the vector resurrection don't protect subjects previously infected;

3.4. The vaccination should be applied before the outside temperature reach the $10^0$C average;

3.5. The same epidemiological pattern of myxomatosis – decreased number of cases and less severe(2000-2009) is mentioned in neighborhood countries.

***Table 1***

**Multiannual situation of myxomatosis in neighboring Romania**

| **Country** | **1999** | **2000** | **2001** | **2002** | **2003** | **2004** | **2005** | **2006** | **2007** | **2008** |
|---|---|---|---|---|---|---|---|---|---|---|
| Bulgaria | 0000 | 0000 | + | (2001) | (2001) | (2001) | (2001) | (2001) | (2001) | (2001) |
| Hungary | +() | +() | +() | +() | +() | +() | +() | +() | +() | +() |
| Moldova | (1985) | (1985) | (1985) | +() | +() | (2003) | (2003) | (2003) | (2003) | + |
| Poland | + | + | + | +() | + | + | + | + | + | + |
| **Romania** | **-** | **+()** | **+()** | **+()** | **(2002)** | **(2002)** | **+** | **+** | **(2006)** | **(2006)** |
| Slovenia | + | + | + | + | (2002) | + | + | + | + | (2007) |
| Ukraine | (09/1998) | +() | (07/2000) | (07/2000) | +() | (10/2003) | (10/2003) | (10/2003) | (10/2003) | (10/2003) |

**Figure 1 - Rapid and massive expansion of myxomas**

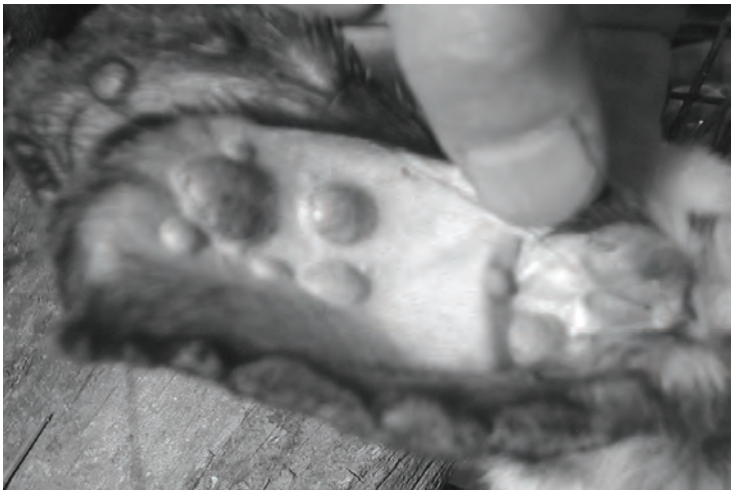

**Figure 2 - Myxomas covering auricular area**

**Figure 3 - Myxomas covering genital area**

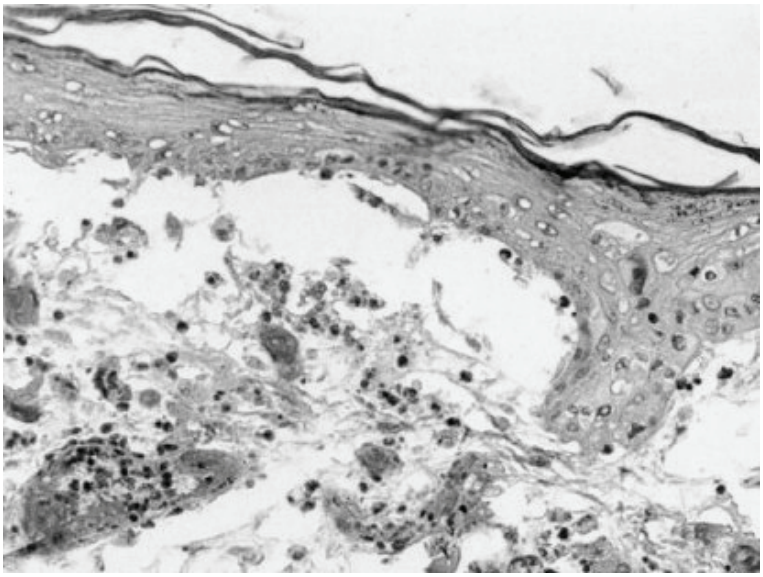

**Figure 4 - Dermal edema with vesicled cells on the surface of the skin**

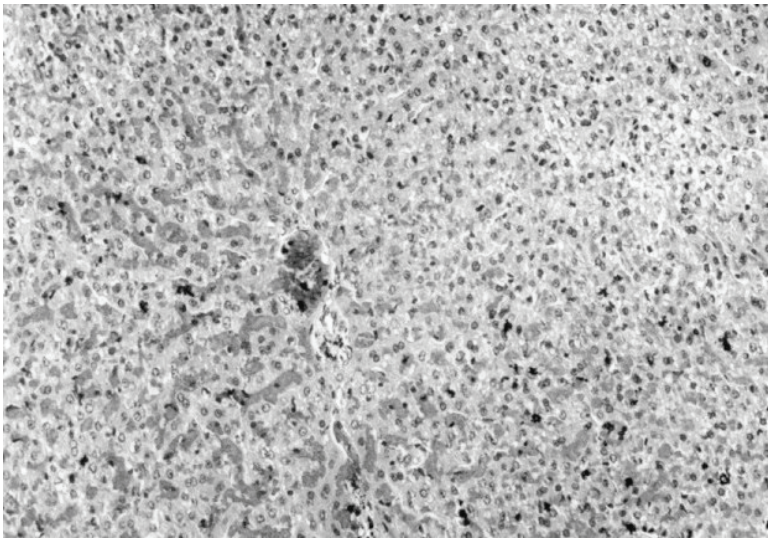

**Figure 5 - Hepatic centro lobular congestion surrounded by dystrophic areas**

**Figure 6 - Moderate evolution of myxomatosis**

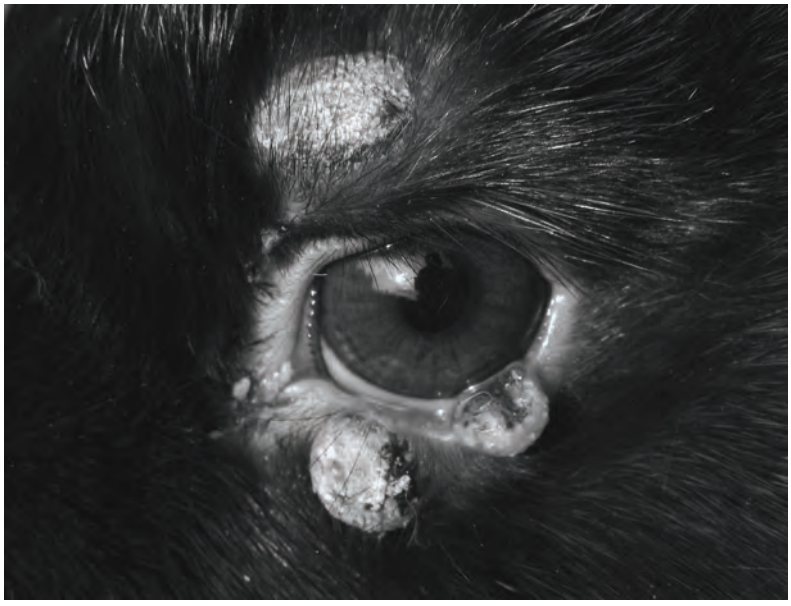

**Figure 7 - The size and number of cutaneous nodules decreased**

**Figure 8 - Rabbit in recovering phase**

**Figure 9 - Alopecia in area of healed skin**

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# *PSEUDOMONAS SPP.* INDUCED LESIONS IN NON-VENOMOUS SNAKES

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**Key words:** *Pseudomonas spp*., fat liver, snakes

**SUMMARY**

*Pseudomonas spp.* infection of captive snakes is considered opportunistic, meaning that it usually induces disease in compromised organism due to immune suppression, cool environment, malnutrition or viral infection.
Two young snakes (male of *Python molurus bivittatus* with 5-month-old and male of *Boa constrictor* with 18-month-old) were submitted for necropsy. Both individuals presented prior death restless movements, anorexia, obvious changes of skin colours turned into dark nuances. Death occurred 3 days after disease debut. The liver, kidneys, heart, small intestine and lungs were sampled for bacteriological, cytological and histological investigations.
The most relevant lesions observed in gross investigation were necrotizing enteritis, fat liver and discrete pulmonary acute edema. Cytological and histological findings revealed necrotic pneumonia and pulmonary edema, acute tubulary necrosis with hyaline droplet degeneration of nephrocytes and into the tubular lumen and diffuse hepatic lipidosis.
There were no clinical or morphological evidence for Paramyxovirus infection or inclusion body disease (IBD). Pulmonary lesions did not exhibit hyperplasia and hypertrophy of septal and faveolar epithelial cells (specific for Paramyxovirus infection). Even IBD is specific for pythons and boids, cytological and histological findings did not revealed oxiphilic or amphophilic inclusions into the epithelial cells (liver, pancreas and kidneys), smooth muscle cells or neurons. It seems that the metabolic disturbances induced bad condition of snakes, generating opportunistic *Pseudomonas* infection.

Scientific research activity based on experimental models uses a great variety of animals, each species being strongly correlated with subjects the scientists are focused on. Same diversity of species is recorded in pet commercial activity. Considering these two directions, breeding of snakes in captivity, mainly of non-poisonous species, take an interest in considerably, both for people fond of animals and scientific research purpose. Once with these activities, the “know how” for breeding technology, management of reproduction and pathology of captive non-venomous snakes became more than necessary. The most solid reasons concerning the necessity of having good knowledge about diseases of snakes are the risk of spreading in humans who have direct contact with these animals and loses by mortality due to infection or

breeding deficiencies. The most encountered captive species of snakes commercialized in Romania are *Pythonidae* şi *Boidae.* Previous studies focused on normal bacterial flora or potential pathogenic revealed a broad variety of aerobic species in snakes: *Staphylococcus spp, Salmonella spp, Shigella spp, Klebsiella spp., Pseudomonas spp., Mycobacterium spp, Leptospira spp.*, and many [2, 4, 5, 11]. *Pseudomonas spp.* infection in captive snakes is considered an opportunistic event, meaning that it usually induces disease in compromised organism due to immune suppression, cool environment, malnutrition or viral infection [7]. Genetic analyses of *Pseudomonas aeruginosa* isolates sampled from healthy snakes, owners, water and prey evidenced various sources of contamination and cross-contamination between snakes and owners [5].

Previous studies proved that the most frequently encountered episodes of *Pseudomonas sp.* infections are linked with viral infection as Paramyxovirus or Retrovirus infection [7].

This paper presents some of the morphological and bacteriological findings in two captive snakes diagnosed with *Pseudomonas spp.* infection, the viral infection being not recorded.

## 1. MATERIAL AND METHODS

Two young snakes (male of *Python molurus bivittatus* with 5-month-old and male of *Boa constrictor* with 18-month-old) were submitted for necropsy. The owner described an unspecific clinical behaviour: both individuals presented prior death restless movements, anorexia, obvious changes of skin colours turned into dark nuances. The death of the snakes occurred 3 days after the disease debut, without any medication. The organs (liver, kidneys, heart, intestine, lungs and muscle) were sampled for bacteriological, cytological and histological investigations. Bacterial cultures were obtained using brain heart infusion broth (BHI broth), brain heart agar and cetrimide agar. Liquid medium was incubated in aerobic atmosphere at 37°C and solid media in 30°C for 24 hours respectively. Isolated colonies from solid media were submitted for evaluation of morphological and biological features (Gram stained and optic microscopy). Subcultures were obtained subsequently using BHI broth. These bacterial subcultures were transferred in API 20E galleries for species identification.

Liver, lungs, gut and kidney were used for cytology (May Grunwald Giemsa stained imprints). All organs submitted for histology were fixed

in 10% formaldehyde solution. Histological section were Masson trichromic stained.

## 2. RESULTS AND DISCUSSIONS

The results of bacteriological investigations are presented in table 1

***Table 1***

**Result of bacteriological investigation**

| | Liver | Kidney | Intestine |
|---|---|---|---|
| *Python molurus bivittatus* | *Pseudomonas fluorescens* | *Pseudomonas fluorescens* | *Yersinia spp.* |
| *Boa constrictor* | *Pseudomonas aeruginosa* | *Pseudomonas aeruginosa* | *Shigella spp.* |

Gross findings were almost the same in both snakes. Watery faeces clogged the cloacae orifice. Pulmonary lesions were expressed as acute oedema and congestion. Liver exhibited different yellowish discoloration of parenchyma and friable consistency, specific for the fat liver. Enlargement of volume and fine, mineral, whitish casts were seen in kidneys (fig 1). Diffuse necrotizing enteritis was the most serious lesions, being severely expressed in boa and moderately in python (fig. 2).

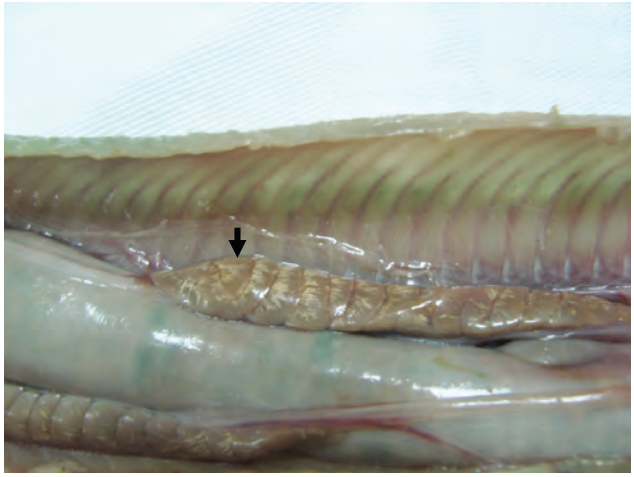

**Fig. 1, *Boa constrictor*, kidneys with mineral, whitish casts (arrow)**

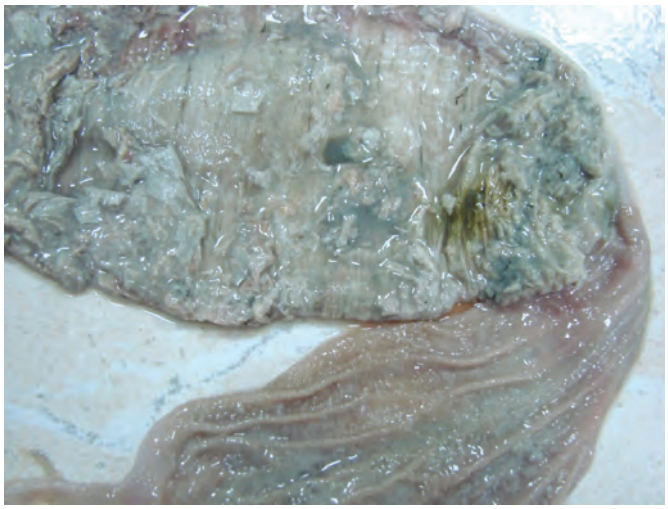

**Fig. 2, *Boa constrictor*, necrotizing enteritis (notice the normal aspect of gastric mucosa)**

Cytological imprints of liver in boa revealed clearly foamy cytoplasm of hepatocyte, with small, eccentric nuclei in boa. The liver of python presented mild cytological features of fat liver and numerous heterophils. Bowl imprints did not point out any parasite organisms.

Histological findings in liver were consistent with diagnosis of hepatic lipidosis, the lesions being severe in boa and mild in python (fig. 3). Numerous foci of lytic necrosis of hepatocytes, surrounded by scattered heterophils into the peripheral sinusoids were observed only in liver of python. Acute tubular necrosis was the most prominent lesion of the kidneys, associated with the presence of the hyaline droplets and mineral storage into the tubular lumen.

***Table 2***

**Pathological findings in *Python molurus bivittatus* and *Boa constrictor***

| Species | Liver | Kidney | Intestine | Lungs |
|---|---|---|---|---|
| *Python molurus bivittatus* | Hepatic lipidosis (mild) Hepatic necrosis | Acute tubular necrosis, tubular mineral storage and hyaline droplets | Necrotizing enteritis (mild) | Necrotic pneumonia |
| *Boa constrictor* | Hepatic lipidosis (severe) | Acute tubular necrosis, tubular mineral storage and hyaline droplets | Necrotizing enteritis (severe) | Acute pulmonary oedema and congestion |

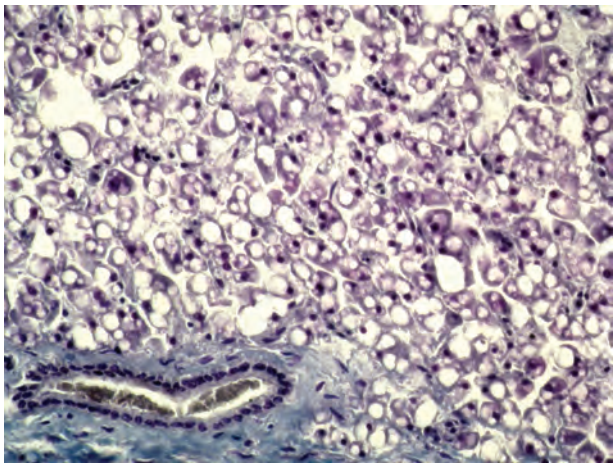

**Fig. 3, *Boa constrictor*, hepatic lipidosis. Masson trichromic, x400**

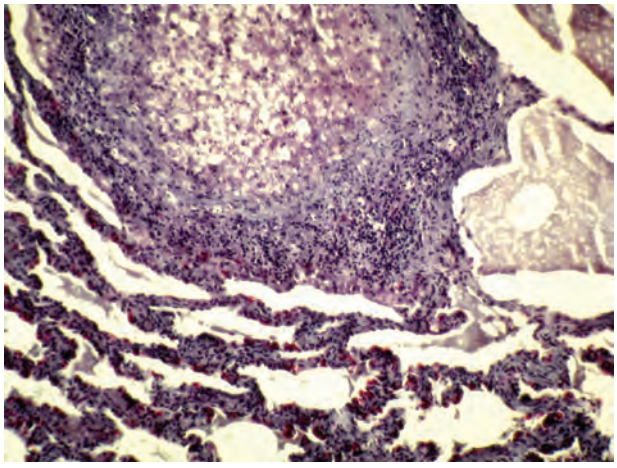

**Fig. 4, *Python molurus bivittatus*, necrotic pneumonia. Masson trichromic, x100**

The lungs of boa presented only acute oedema, associated with congestion of faveolar capillaries. Necrotic pneumonia was expressed as lytic foci surrounded by a heterogeneous inflammatory cell population in python (fig. 4). Pathological findings in boa and python are presented in table 2.

As we mentioned before, *Pseudomonas spp.* is one of the bacterial species isolated from normal, healthy snakes from mouth and faeces, bacterial carriage being higher in captive snakes than the wild one [5]. Bad conditions of environment, bacterial contamination from food and water, viral infection or immune suppression generate the perfect conditions for these bacteria to become pathogenic [7].

The snakes considered in our study proved that there were no clinical or morphological evidence for the most common viral diseases described in ophidians, being known that a virus contamination can

create perfect conditions for immune suppression and subsequent bacterial infection. Paramyxovirus or Retrovirus infection (inclusion body disease - IBD) presents different clinical signs and lesions, with specific histological features. Boids with Paramyxovirus infection seems to be uncoordinated and disorientated, regurgitation and paresis being observed often. The most relevant histological features are interstitial pneumonia, associated with hyperplasia and hypertrophy of septal and faveolar epithelial cells or pancreatic hyperplasia [6, 8]. Even IBD is specific for pythons and boids, cytological and histological findings did not revealed oxiphilic or amphophilic inclusions into the epithelial cells (hepatocytes, bile duct epithelial cells, pancreatic epithelial cells, renal tubular epithelium) [3, 10, 12, 13].

Previous studies in tropical non-venomous snakes emphasized that *Pseudomonas* was isolated from mouth and lungs, lesions being identified in mouth as mouth rot (necrotic stomatitis), lungs (necrotic pneumonia), vessels (degenerative injuries), epithelial cells and connective tissue of internal organs [1, 14].

Considering the references, none of the previous studies have mentioned gut injuries induced by *Pseudomonas spp.* infection. These two cases we have been focused on did not exhibit characteristic lesions for this infection. It is difficult to be absolutely sure about the etiology of lesions in bowl. As we mentioned before, bowl bacterial isolates were represented by *Yersinia spp*. in python and *Shigella spp.* in boa. None of these two species proved to have virulence associated features. The first species is very frequently encountered in gut isolates in domestic, wild and zoo, most of the serotypes being non-pathogenic [9]. *Shigella spp.* infection is correlated with bowl lesions and clinical signs of dysentery. Most of the references describe these aspects in humans and correlate the episodes of illness with house-lizard and other pet reptiles contact.

Considering these findings, we cannot make a strong correlation between necrotizing enteritis and *Pseudomonas spp*. etiology.

We estimate that the most important pathogenic causes associated with *Pseudomonas spp.* infection were metabolic disturbances (proved by skin colours turned into dark nuances and specific features of fat liver)

## 3. CONCLUSIONS

1.1 Two young boids were submitted for necropsy. Gross lesions were consistent with necrotizing enteritis, fat liver and discrete pulmonary acute edema.

1.2 Cytological and histological findings revealed necrotic pneumonia and pulmonary edema, acute tubulary necrosis with hyaline droplet degeneration of nephrocytes and into the tubular lumen, diffuse hepatic lipidosis and hepatic necrotizing foci.

1.3 The most important pathogenic causes associated with *Pseudomonas spp.* infection were metabolic disturbances proved by skin colours turned into dark nuances and specific features of fat liver, viral infection being excluded.

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# DERMATOPHYTE SURVEILLANCE IN COMPANION ANIMALS: HORSES, DOGS AND CATS

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**Keywords:** dermatophytosis, skin diseases, ringworm, in-house screening tests , dermatophyte culture medium

**SUMMARY**

Dermatophytosis is a highly contagious zoonotic skin disease produced by different genera of fungi (e.g. Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes). The importance of those infections increase with regard of pets as cats, dogs or horses. Usually, dermatophytosis, generally referred to as tinea or ringworm, cannot be diagnosed only by the presence of skin lesions, and skin lesions of ringworm can mimic other diseases. The standard procedure for dermatophyte infection diagnosis is based on clinical exam suited by direct examination of hair and skin using a microscope, and, if necessary, isolation of the dermatophyte "in vitro" on appropriate medium. Dermatophytosis can only be confirmed based on the findings of a fungal culture and examination. Veterinary practitioners prefer simple and easy tests, able to confirm the diagnosis of dermatophyte infections. In this paper we present several strategies of diagnostic using in-house screening tests for the rapid identification of dermatophyte infections in dogs, cats and horses. These in-clinical tests consist in selective and differential mediums for dermatophytes, that contain an easy-to-interpret color indicator that changes from yellow to red when dermatophyte fungi are present in the patient sample. Usually, positive result evaluation is supplied in circa 72 hours.

Dermatophytosis is a highly contagious zoonotic skin disease produced by different genera of fungi (e.g. *Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes*). The importance of those infections is most increased when in discussions are involved companion animals like cats, dogs or horses. Usually, dermatophytosis (tinea or ringworm) cannot be diagnosed based solely on whether or not the animal has skin lesions, and skin lesions of ringworm can mimic other diseases.

*Microsporum canis* is the most common cause of feline dermatophytosis, cats being considered the natural reservoir for this organism. This well-adapted dermatophyte induce minimal host response in cat, this being currently inapparent carriers (usually the Persian ant other cat breeds with long-haire). Also, *Microsporum*

*gypseum* and *Trichophyton mentagrophytes* can occasionally cause feline dermatophytosis. Classically, clinicaly features of dermatophytosis in cat are ring lesions characterized by expending circular patches of alopecia with central healing and peripheral small papules [4].

Like cats, dermatophytosis in dogs is produced usually by *Microsporum canis*. The lesions are similar with those expressed by cats [4].

In horse ringworm is typical and often can be diagnosed by clinical appearance. In horse lesions dermatophytosis are usually on the face, neck, chest, shoulder or on the girth area but can affect any part of the body. Those are small hairless lesions that may be red and sometimes look like hives and scaly or crusty areas. Lesions may or may not be itchy or painful. Symptoms in horses usually start with patches of raised hairs in a circular (ring) pattern. The lesions are mainly itching in the early stages of the disease but can remain sensitive to the touch for longer periods [5].

Diagnosis of dermatophytosis are frecvently given by clinical apparence alone, but the most reliable diagnostic tool is fungal culture.

## 1. MATERIALS AND METHODS

Samples need to be obtained before any local or systemic antifungal treatment. Results of the mycological diagnosis may vary according to the quality and quantity of submitted material. The diagnosis of dermatophytosis require samples that are properly collected. For isolation of dermatophytes can be used hair, skin and nail. Some authors recommend cleaning the lesion area with alcohol before sampling to remove contaminants such as bacteria [7].

If the clinical exam doesn’t identify the classical ringworm lesion, the veterinarian can use a Wood’s lamp for identifying the infected area of the skin. A light green fluorescence will attest of a microsporic tinea [6]. Additionally, human or animal asymptomatic carriers may be detected by rubbing the whole scalp or hair with a sterile piece of carpet, a sterile swab humidified with distilled water or a toothbrush [8]. In veterinary practice, toothbrush fungal culturing is preferred in the USA, whereas Denman brushes or sterile carpet squares are favored in the United Kingdom and in France, respectively [6].

Several companies supply original or modified Dermatophyte Test Medium, but those are usually dehydrated products: BBL™ Dermatophyte Test Medium Base (Becton Dickinson & Co., USA) and

Company , BBL™ Dermatophyte Test Medium, Modified with Chloramphenicol (Becton Dickinson & Co., USA), Delasco - Dermatophyte Test Medium Base (Delasco, USA) etc. The dehydrated products can be used only in laboratory with specific equipments, and the results are provided in several days. Also, several laboratories are ready-to-use Sabouraud's agar plates commercially available under various names (such as bacti-lab, Himedia, bioMérieux, Bio-Rad, AES, Oxoid or Becton-Dickinson).

It described several strategies of diagnostic using in-house screening tests for the rapid identification of dermatophyte infections in dogs, cats and horses. These in-clinical tests consist in selective and differential mediums for dermatophytes, that contain an easy-to-interpret color indicator that changes from yellow to red when dermatophyte fungi are present in the patient sample. Usually, positive result evaluation is supplied in 72 hours.

The dermatophyte test medium (DTM) represents an alternative for the isolation. Using an in-house test, like Dermakit RapidVet-D (DMS Laboratories Inc, Switzerland), InTray dermatophyte test (BIOMED Diagnostics, USA) or Dermatophytes kit (Biovet, USA), as opposed to sending it out to a diagnostic laboratory, has several advantages.

RapidVet-D is not a fungal growth media, even though it looks like one. It is a diagnostic test for dermatophytes in dogs, cats, rabbits and horses employing 1990's technology which relies solely on a color change, or lack thereof, within 24-72 hours [2]. The veterinarians are thankful for two major advantages of such a diagnostic test: treatment can be initiated much more quickly in case of positive result (unlike a decade ago, the preferred antifungal treatment is now essentially the same regardless of which veterinary dermatophyte is involved), in the case of a negative result, other tests (such as for allergy, etc.) can be ordered and the real problem identified more quickly (fig 1).

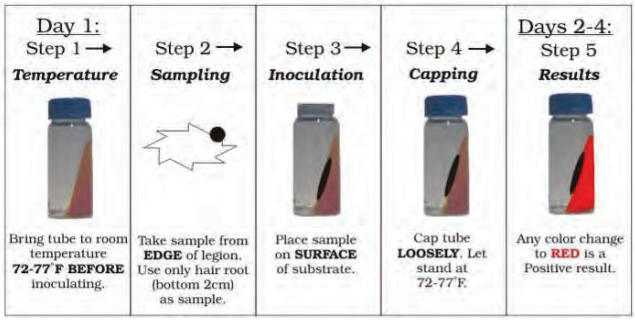



**Figure 1. The protocol of in-house dermatophyte screening test Rapivet-D (DMS Laboratories Inc, Switzerland) [2]**

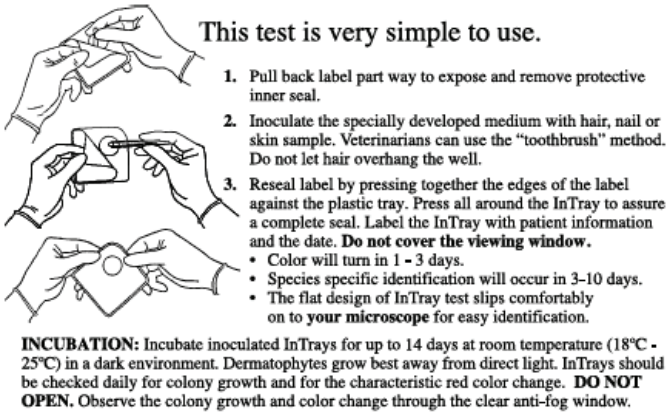



**Figure 2. The protocol of in-house InTray dermatophyte test (BIOMED Diagnostics, USA) [3]**

## 2. RESULTS AND DISCUSSIONS

The standard procedure for dermatophyte infection diagnosis is based on clinical exam, direct examination of hair and skin using a microscope, and if necessary isolation of the dermatophyte on a culture medium. Dermatophytosis can only be diagnosed based upon the findings of a fungal culture and examination. At this moment veterinary practitioners prefer simple and easy tests, which are able to confirm the diagnosis of dermatophyte infections.

Accurate dermatophyte infection diagnosis is very important. In case of false negative result the consequences may be severe, as it may allow an infected individual to spread a pathogen dermatophyte within a veterinary hospital or to a foster or adoptive home.

Clinical appearance, Wood's lamp exam end/or direct microscopic examination can be use by veterinarians in clinical diagnostic but the only truly reliable way to diagnose ringworm is via fungal culture.

Direct examination is essential, as it allows the clinician to start treatment, pending culture, but microscopic examination requires very thin fragments. Using thin samples prevents the presence of air bubbles that could interfere on examination and therefore facilitates the investigation [6]. Correct visualization of the fungal elements in direct microscopic examination requires the dissociation of collected material. This objective is covered by keratin digestion. One of those reagents is 10–20% potassium hydroxide (KOH) with or without dimethyl sulfoxide (DMSO) [9]. Other dissociating agents have also been proposed including Amann's chloral-lactophenol, 10% sodium hydroxide (NaOH)

and detergents. But visualization of fungal elements at direct examination is sometimes difficult. For this reason were proposed several stain methods. Various stains which can be associated to clearing agents may be used:

- Chlorazol black E (CBE) (Sigma-Aldrich): stains only the fungal structures and excludes many artifacts;
- Blue–Black Ink permanent (Parker Quink®): stains fungal elements in deep blue;
- Cotton blue C4B (Bacti-lab inc., R.A.L. or Bio-Rad), associated with lactic acid and phenol: stains fungal elements in blue;
- PAS coloration: stains polysaccharides such as glycosaminoglycans, and an be adapted to direct examination of epidermal scales;
- Congo red: binds to polysaccharides of the fungal cell wall, particularly β-D-glucans, and therefore facilitates the detection of fungal elements [10]
- Calcofluor white (Fluorescent brightener 28®, Sigma-Aldrich): fluorochrome than binds to chitin, a polymer of *N*-acetyl-D-glucosamine which is one of the main polysaccharides of the fungal cell wall - may be used in KOH 20% (1:1, v/v);
- Blankophor P Flüssig® (Bayer): fluorochrome than binds to chitin, a polymer of *N*-acetyl-D-glucosamine which is one of the main polysaccharides of the fungal cell wall - may be used in KOH 20% (1:1, v/v);
- Uvitex 2B (Fungiqual A®, Ciba Corning): fluorochrome than binds to chitin, a polymer of *N*-acetyl-D-glucosamine which is one of the main polysaccharides of the fungal cell wall [11].

Calcofluor white is the most convenient fluorochrome allowing a rapid and accurate diagnosis of dermatomycosis. With this fluorochrome the fungal elements appear blue when using a fluorescence microscope equipped with a 330–380-nm excitation filter and an emission filter of >420 nm.

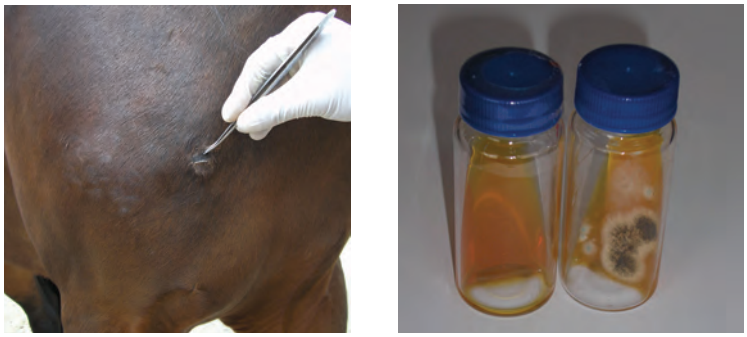
Today are available commercially like Mycetcolor® and Mycetfluo® from SR2B, both containing sodium dodecyl sulfate, allowing the digestion of skin/hair specimens and the staining of fungal elements for direct microscopic examination [6].

In most cases, cultivation and isolation of dermatophytes is required for the diagnosis of dermatophytosis. Technicians and biologists may have some difficulties when using conventional methods. The dermatophyte test medium (DTM) represents an alternative for the isolation at classical mycological methods. Due to the alkaline by-

products generated during growth of dermatophytes, the colour of this medium changes to deep red. some non-pathogenic fungi give false-positive results and false-negative results may be obtained with some *Microsporum* species such as *Microsporum persicolor* or in case of contamination by bacteria. In our study some hair lesions suspected to be produced by pathogenic dermatophytes were isolated saprophytic fungi (fig. 3). Similar results were obtained by Aho R (1998), then isolated from domestic companion animals the members of the genera Penicillium, Cladosporium, Aspergillus, Mucor, Aureobasidium, Alternaria, Scopulariopsis, Trichoderma and Trichothecium [1].

Typical strains of dermatophytes can be identified directly from primary cultures, but subcultures on specific media are often necessary. Usually, the following specific media are used;

- Borelli's lactrimel agar (BLA): enhances sporulation for the majority of dermatophytes, but also the production of pigments (wine-red for *T. rubrum*, ochraceous-yellow for *Microsporum canis*) [12]
- Potato dextrose agar (PDA): *M. canis* develops its yellow pigment and therefore can be easily distinguished from *Microsporum audouinii*.



a. b.

**Fig. 3. Dermatophyte diagnostic using in-house dermatophyte screening test Rapivet-D (DMS Laboratories Inc, Switzerland). a. skin lesion posible dermatophyte asociate. b. negative result: contamination with non-pathogenic fungi and bacteria.**

## 3. CONCLUSIONS

1. Sensitivity of direct examination may be low due to the clinical sample or to the technique used.
2. The lack of commercially available specific culture media may be hampered for the isolation and identification of some dermatophytes at the species level.

3. Using an in-house test, like Dermakit RapidVet-D (DMS Laboratories Inc, Switzerland) the time of etiological diagnostic is reduced and the terapy can be rapid aplied.

## ACKNOWLEDGEMENTS

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# IDENTIFICATION OF CANINE BLOOD GROUPS USING IN-CLINICAL MEHODS

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**Keywoeds:** pet bood groups, in-clinical tests, canine blood immunology, blood bank

**SUMMARY**

The modern concept of transfusion protocols in pets veterinary medicine is based on blood group determination at all young animals and development of the blood bank facilities. The internationally accepted canine blood group system is the DEA (Dog Erythrocyte Antigen). There are 8 major blood groups in the dog, labeled as DEA 1 to 8. DEA 1.1 and DEA 1.2 are the most significant blood factors in the dogs. They are both antigenic, but DEA 1.1 is the most important in transfusion. In this paper we use an assay based on the agglutination reaction that occurs when an erythrocyte which contains a DEA 1.1 antigen on its surface membrane interacts with a murine monoclonal antibody proven specific to DEA 1.1 which is lyophilized on the test card (RapidVet®-H. Canine DEA 1.1, **dms**laboratories, inc. USA). It is a general opinion that acute hemolytic transfusion reactions only occur in dogs with DEA 1.1 and DEA 1.2 negative blood. These dogs, without naturally antibodies, will express a reaction only after sensitization through exposure to DEA 1.1 or 1.2 positive blood (antibody production takes 7-10 days after exposure). Because a number of dogs auto-agglutinate and because a very anemic dog may give equivocal results, typing prior to an urgent need for the information is indicated. The main objective of our study was to classify dogs as DEA 1.1 positive or negative as prevention facing a possible transfusion.

The modern concept of transfusion protocols in companion animal veterinary medicine is based on blood group determination at all young animals and development of the blood bank facilities. The internationally accepted canine blood group system is the Dog Erythrocyte Antigen (DEA) [Vriesendorp, 1976, Giger U, 2005]. RBCs have antigenic markers on their surface, genetically determined by a set of 2 to several alleles at 1 gene locus, which are specific for a blood type. There are 8 major blood groups in the dog, labeled as DEA 1 to 8. DEA 1.1 and DEA 1.2 are the most significant blood factors in the dogs. They are both antigenic, but DEA 1.1 is the most important in transfusion. The identification of these antigens is done by the hemagglutination reaction used in several methods (the binding of RBC surface antigen with monoclonal or polyclonal antibodies).

Previous studies evaluated and demonstrated the quality of some methods used in canine blood-typing: CARD, GEL, MSU, and TUBE tests [Giger U, 2005]. After the mid 1990's blood typing CARD tests were available in commercially kits and some brands are supplied in Romania by different companies. This opportunity can be used by several veterinary doctors in scope of hemolytic transfusion reactions prevention.

## 1. MATERIALS AND METHODS

For this study, were used 110 blood samples from 100 dogs (table 1). The samples (drown from cephalic vein) were collected in the 2-ml tubes with EDTA as anticoagulant, and were tested short time as fresh samples, or were stored overnight at 4ºC before. 10 dogs were included in group of the reproducibility assay evaluation, and they supplied double samples of blood collected in same day (1 dog), at 7 days (3 dogs), at 14 days (3 dogs) and at 21 days (3 dogs) interval.

To prevent the equivocal results the blood samples recruited for this study were supplied by healthy animals, without haematological disorders history (e.g. anaemia, haemotropic organisms).

We used an assay based on the agglutination reaction that occurs when an erythrocyte which contains a DEA 1.1 antigen on its surface membrane interacts with a mouse monoclonal antibody specific to DEA 1.1 which is lyophilized on the CARD test (RapidVet®-H. Canine DEA 1.1, **dms**laboratories, inc. USA). The test kit includes all the materials required for the assay (the test cards, reagents, pipettes and stirrers). The test card contains 3 blood typing wells: "Auto-Agglutination Saline Screen", "DEA 1.1 Positive Control" and "Patient Test" (fig. 1).

After dispensing one drop of diluent (40µl) into the well marked „Auto-Agglutination Saline Screen", using the pipette to aspirate a small amount of sample and the stirrer to mix the sample (in order to assist reconstitution of the lyophilized DEA 1.1 antibodies), one drop of patient sample (50µl) was released into the same well. Then, we dispensed one drop of diluent (40 µl) into each of the two remaining wells, one drop (40 µl) of Positive Control into the well marked „DEA 1.1 Positive Control" and one drop (50 µl) into the well marked "Patient Test".

***Table 1.***

**Blood-typing results for the dog erythrocyte antigen (DEA) 1 system by use of CARD test (RapidVet®-H. Canine DEA 1.1, dmslaboratories, inc. USA).**

| *Breed* | | *No. DEA 1.1. positive* | *No. DEA 1.1. negative* |
|---|---|---|---|
| German Shepherd Dog | Male | 0 | 4 |
| | Female | 3 | 8 |
| Caniche | Male | 2 | 6 |
| | Female | 1 | 5 |
| Cocker Spaniel | Male | 1 | 7 |
| | Female | 3 | 5 |
| Rottweiler | Male | 0 | 6 |
| | Female | 0 | 4 |
| Boxer | Male | 1 | 5 |
| | Female | 2 | 3 |
| Various breeds | Male | 2 | 16 |
| | Female | 2 | 14 |
| **Total** | | 17 | 83 |

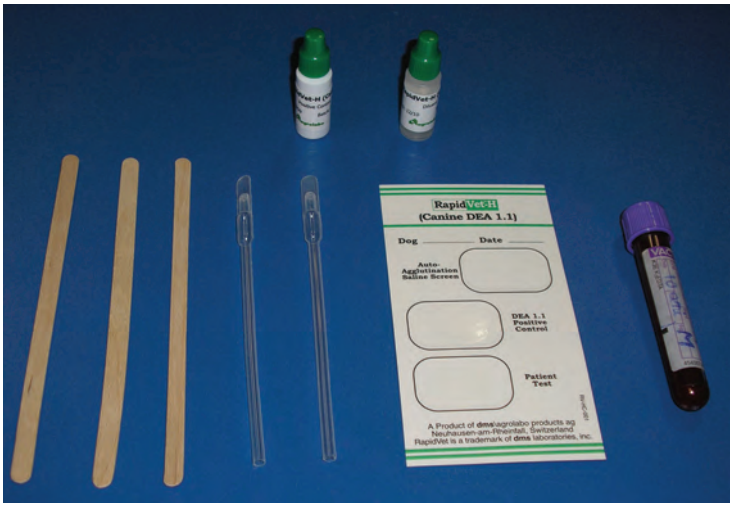


**Fig 1. Reagents and materials used in a canine blood groups identification based on the agglutination methodology**

## 2. RESULTS AND DISCUSSIONS

In veterinary medicine practice blood compatibility testing is recommended to assure safe and effective transfusions. Because DEA 1.1 is the most clinically important type, it is critical to accurately detect this antigen at the donor and recipient to assure blood type-compatible transfusions [Giger U & Blais MC, 2005]. It is a general opinion that acute haemolytic transfusion reactions only occur in dogs with DEA 1.1 and DEA 1.2 negative blood when this re-received a DEA 1.1 or 1.2 positive blood (usually antibody production takes 7-10 days after first exposure). Because a number of dogs auto-agglutinate and because a very anaemic dog may give equivocal results, typing prior to an urgent need for the information is indicated [1, 2, 3].

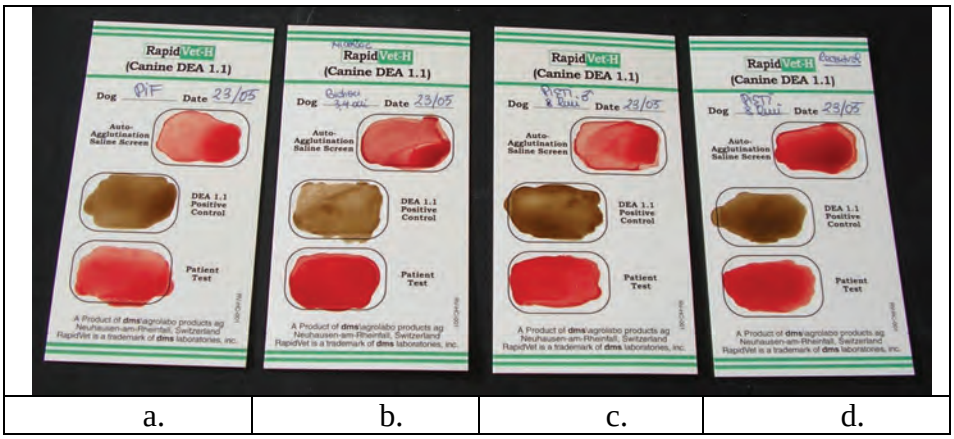


**Fig 2. Canine blood groups identification based on the agglutination methodology. All results are DEA1.1.-negative. One of the samples was tested multiple times in same day (c and d), and the results were used for the reproducibility of the assay evaluation.**

The main objective of our study was to classify dogs as DEA 1.1 positive or negative in scope of haemolytic transfusion reactions prevention.

In this study 17% of all tested dogs were DEA 1.1 positive, and 83% DEA 1.1 negative. The majority of dogs, in all type of dog breeds were DEA 1.1 negative. In contrast, Ejima et al. described in Japan a higher incidence of DEA 1 positive dogs (82%) among mongrel dogs, when compared to Beagles (55%), and Hale described a prevalence of 63.5% for DEA 1.1 positive mongrel dogs, while 1.2% was DEA 1.2 positive. Also, they found that 43.5% of German Shepherd dogs were DEA 1.1 positive and only 4% were DEA 1.2 positive. The obtained results

showed a general prevalence of 91.3% for the DEA 1 system, counting 51.3% of DEA 1.1 type dogs, while 40% of the animals were positive for DEA 1.2 type. Only 8.7% of tested dogs were negative for DEA 1 system.

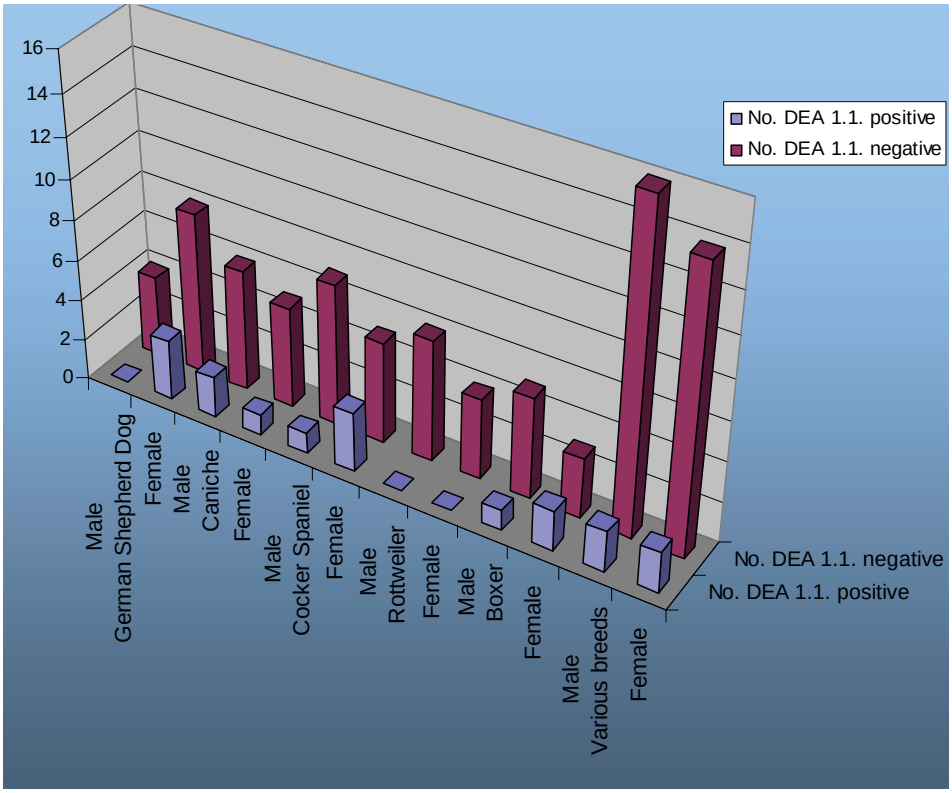


**Fig 3. Racial and sexual distribution of the tested canine population in absolute values**

## 3. CONCLUSION

The heterogeneous data obtained in our preliminary study recommend further studies to establish blood type frequencies or peculiarities among canine breeds.


## ACKNOWLEDGEMENTS

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# DIAGNOSIS OF PYODERMA IN DOG

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**Key words:** dog pyoderma, cytological examination, histological examination

**SUMMARY**

Pyoderma is the purulent inflammation of skin and adnexa. The clinic and lesional variability can stand for a starting point of numerous misdiagnosis and inadequate treatment. The aim of this study was to highlight the cytological and histological aspects of the pyoderma in dog and to establish the incidence of pyoderma in correlation with certain factors. The study was performed on 75 dogs pathologically diagnosed with different types of primary and secondary pyoderma. The sampling for the cytological investigation used fine needle aspiration. Imprints and smears were May Grünwald Giemsa stained. The 4-6 microns thick histological sections were stained by trichromic Masson. Acute primary pyoderma was characterised by the presence of normal and degenerated neutrophils, activated macrophages, pyocites and numerous degenerated keratinocites. A long-term evolution of pyoderma was featured by a pyogranuloma-type cell population. Data offerred by the histological sections made possible the classification of the pyoderma as primary or secundary. Pyoderma evolves mainly as foliculitis (6.6%), involving dominantly the males (68%) and the adult dogs (48%); deep pyoderma dominated the majority of evolution forms (99.33%) being often secundary to other lesions (72%).

The term pyoderma is used to denote the inflammation of skin and adnexa, which is characterised by the presence of purulent exudate in the affected areas. This exudate can infiltrate in the structure of multiple layers of the skin or it can confine to one of the skin's layer. There are a few diseases which encompass such a multitude of clinical manifestations. Thus, the lesions vary from trivial to life threatening (4, 7).

The potential factors for dog's high susceptibility are: comparatively thin canine stratum corneum, the relative paucity of intercellular lipid-rich material of the canine stratum corneum, relatively high pH of canine skin, the lack of lipid-squamous epithelium plug in the ostia of canine hair follicles (2, 3, 4, 7, 9, 10).

Superficial pyoderma involves only the epidermis and its invaginations (hair follicle). Deep pyoderma develops when the inflammation spreads to the dermis and panniculus. (3,7,9).

Although is isolated from more than 80% of the cases of pyoderma, *Staphylococcus intermedius* does not have the necessary virulence to be

considered a pathogen agent for the healthy skin. The proliferation of *S. intermedius* occurs as the result of a number of underlying primary diseases and host factors like: allergies (flea allergy dermatitis, atopy, food allergies), endocrinopathies (hypothyroidism, hyperadrenocorticism), seborrhea, ectoparasitism (dermatophytes, demodicosis) (1, 3, 4, 5, 6, 8, 10).

## 1. MATERIALS AND METHODES

The study was conducted on a number of 75 dogs with a clinical suspicion of pyoderma, based on the cutaneous purulent lesions. The diagnosis was confirmed due to the investigatory activity of the Pathological Anatomy Department of the Faculty of Veterinary Medicine Bucharest, between 2003-2007.

One of the aims of the study was to establish the following epidemiological features of the pyoderma: age related incidence of pyoderma, breed related incidence of pyoderma, sex related incidence of pyoderma, the incidence of pyoderma related to the depth of the affected skin layer, the incidence of different types of pyoderma, the incidence of pyoderma related to the affected body region.

Pathological diagnosis was performed by gross, cytologic and histological examinations.

The sampling for the cytological investigation was performed by fine needle aspiration. Imprints and smears were May Grünwald Giemsa stained.

The 4-6 microns thick histological sections were stained by Trichromic Masson.

The microscopic examination took place with a Carl Zeiss Axio Imager A1 microscope, supplied with an automatic sistem designed for making photographies.

## 2. RESULTS AND DISCUTIONS

The cases included in the present study showed single or multiple cutaneous purulent lesions.

The group of study included 51 males and 24 females, with ages ranging from 1 to 14 years (with an average age of 7,7 years). The most affected breeds were: German Shepherd (19 cases), crossbreed (9 cases), Cocker Spaniel (7 cases), Caniche-toy variety (5 cases), Rottweiler (5 cases), Doberman (5 cases), Boxer (4 cases), Mioritic Shepherd Dog(3 cases), Schnautzer, Irish Setter, Pitbull, Amstaff, Pekinese (2 cases of

each breed), while Dog de Bordeaux, Husky, Airdale Terrier, Golden Retriever, Brack, Great Dane, Carpathian Shepherd Dog, Belgian Shepherd were represented by one individual for each breed.

Pyoderma has a higher incidence in males, with a 68%, in comparison with females (32%). Mostly affected were the adult dogs, the age categories included in this study being: 9-14 years (48%), 5-8 years (28%), 1-4 (24%).

The gross aspects of the lesions inflicted by pyoderma asociated, also, with the extension of the inflammatory process can be found in table 1.

***Table 1***

**The gross aspects of the lesions inflicted by pyoderma**

| Lesion | Number of cases | Superficial pyoderma | Deep pyoderma |
|---|---|---|---|
| Ulceration of the epidermis | 16 | 3 | 13 |
| Diffuse swelling w/o fistulisation | 29 | 8 | 21 |
| Diffuse swelling w fistulisation | 7 | - | 7 |
| Lesion | Number of cases | Superficial pyoderma | Deep pyoderma |
| Focal swelling | 18 | 12 | 6 |
| Hiperkeratosis affected skin area | 1 | 1 | - |
| Subcutaneous cyst | 4 | 1 | 3 |
| Total number of cases | 75 | 25 | 50 |

35 cases were submitted only for the cytologic investigation and 3 cases, only for the hystologic examination. Regarding the other 37 cases, the diagnosis was established through both methods of investigation.

The incidence of pyoderma, related to the depth of the affected skin layer was found to be higher with deep pyoderma (70 cases – 99.33%) in comparison with superficial pyoderma (5 cases – 0.67%).

Pyoderma evolves secondary to other diseases in most of the cases (54 cases – 72%); it has been registered as a primary disease with a much less frequency (21 cases – 28%).

The types of pyoderma cited in the references are: pyotraumatic dermatitis, intertrigo, impetigo (superficial pyoderma), folliculitis, furunculosis, cellulitis, pododermatitis (deep pyoderma) (2, 4, 7).

In this study folliculitis, pododermatitis and furunculosis had the highest frequency (6.6%, 5.33%, 4%). The anal furunculosis showed the smallest percentage (1.33%). The type of pyoderma remained undetermined in the rest of the cases (77.41%).

Secondary pyoderma appeared to be associated to a diversity of primary lesions. In most of the cases, pyoderma evolved secondary to benign tumors (hepatoid glands adenoma, papiloma, epidermal cyst, sebaceous gland adenoma, hamartoma). Pyoderma associated to the malign tumors, parasitary diseases and traumatisms had a smaller incidence (2.66%, 3.70%, 1.85%). The rest of 56% of cases had been secondary to an unknown primary disease.

Pyoderma located in the limb region had a high chance of evolving as response to trauma. This supposition is based on the nodular aspect of the interdigital or plantar lesions and on the fact that this region is the subject of persistent trauma (5, 7).

The most affected body regions were the ones of the limbs and head (37.33%, 12%). Pyoderma evolves rarely on the cervical and preputial regions (4%, 1.33%). The rest of 45.34% reffers to the folowing body regions: thighs, thorax, cervical region, perianal region, abdomen and rump.

Cytologic examination is one of the easiest, most cost-effective, and potentially most beneficial diagnostic tests for the documentation of the bacterial involvement in the canine skin disease.

In the early stages of primary acute of pyoderma, the cytologic population included normal and degenerated neutrophils, activated macrophages which presented phagocytized detritus in the cytoplasm and numerous degenerated cells (shed keratinocytes as a result of acantholysis) (figure1).

A long-term evolution of the lesions, sustained by the anamnesis data, was featured by a pyogranuloma-type cell population. This type of population includes a larger number of activated macrophages, most of them presenting characteristics of epithelioid cells, binucleated or multinucleated cells, lymfocytes and plasmocytes (figure 2).

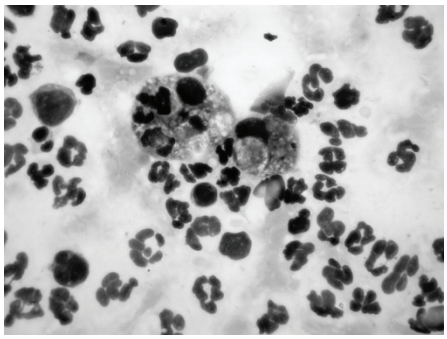

**Fig. 1 German Shepherd, acute deep dermatitis, neutrophils, lymphocytes, activated macrophages. MGG stain, x1000**

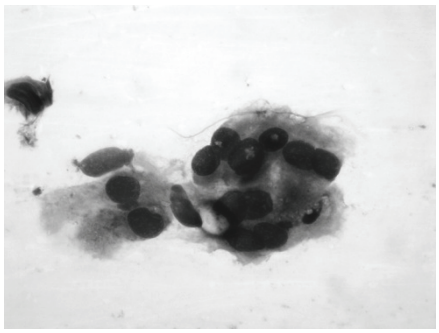

**Fig. 2 German Shepherd, chronic deep dermatitis, multinucleate cell. MGG stain, x1000**

Chronic lesions are much harder to be diagnosed, due to the paucity of relevant cells present in the smear. In this cases, the population is represented mainly by stromal cells (fibrocytes, fibroblasts) and rare lymfocytes.

Diagnosing a secondary pyoderma associated to benign or malign tumors can turn up to be a delicate problem. In these situations, the main goal was to establish the characteristics of the inflammatory cells population and the way it mixes with the epithelial or mesenchymal cells, found in different stages of dysplasia or anaplasia.

The histopathological examination established the actual involvement of different layers of the skin and its adnexa (glands and hair follicles).

Similar to the cytologic examination, the histological examination featured certain aspects related to the evolution course of the lesions. The acute lesions involved superficial layers of the skin (epidermis and papilar dermis), in contrast to subacute and chronic pyoderma confined to reticular dermis and panniculus.

According to the references, the extension of the inflammatory process from the superficial layers to the deeper ones depends on a longer period of time, which is provided by the chronic evolution of pyoderma (2,4).

In the present study, the average duration of the chronic pyoderma, which permitted the extension of the inflammation from epidermis and dermis to the panniculus, was of 7.37 months.

Deep pyoderma can develop *de novo* without evidence of prior superficial pyoderma. The mechanisms by which pyoderma can develop from the early stages as a deep inflammation remain unclear (2, 4).

Table 2 shows data regarding the connection between the type of pyoderma and the duration of evolution.

***Table 2***

**The correlation between the type and evolution of pyoderma**

| | Acute pyoderma | Chronic pyoderma | Undetermined course of evolution |
|---|---|---|---|
| Superficial pyoderma | 5 cases | 4 cases | 24 cases |
| Deep pyoderma | 21 cases | 21 cases | |

The examination of the histologic sections confirmed the presence of the same type of cell population as in the cytologic examination.

The histologic examination of surgical removals and samples yielded by biopsic procedure which had ended with unconclusive or uncertain results after cytologic examination (28 cases) brought crucial data for the specification of the type of pyoderma (primary pyoderma or secondary pyoderma). Thus, the primary lesions were accurately diagnosed and classified; following this pyoderma was established as a dominant or auxiliary process.

The microscopic lesion of superficial pyoderma consist of superficial epidermal pustule, composed predominantly of neutrophils, accompanied by lifting of the overlying stratum corneum from the epidermal surface. The lifted stratum corneum is generally of basketweave appeareance.

The microscopic appereance of superficial pyoderma can be seen in figure 3, as it had spread from the superficial layers of the skin, the final diagnosis being anal furunculosis.

The typical histopathological findings in deep pyoderma reffer to the presence of neutrophils, lymphocytes, macrophages, eosinophils and plasma cells in dermis and panniculus. The epidermis is usually acanthotic. These features can be seen partially in figure 4.

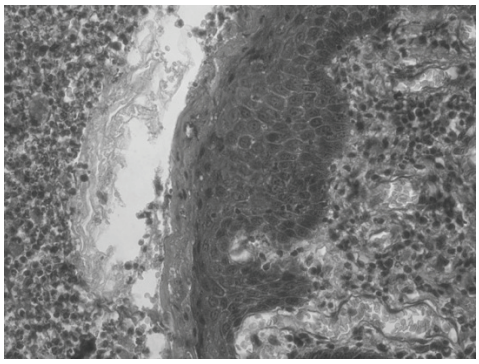

**Fig. 3 German Shepherd, anal furunculosis, intraepidermic purulent exudate, the congestion of the dermic capillaries, mononuclear infiltrates. TM stain, x400**

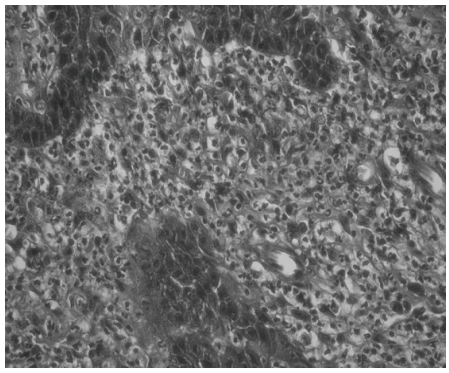

**Fig. 4 German Shepherd , deep pyoderma, pododermatitis, neutrophils and acantholysis. TM stain, x400**

## 3. CONCLUSIONS

3.1 The incidence of pyoderma is the highest in adults dogs, the most affected individuals being included in the 9-14 years category (48%). The most affected breed is German Shepherd (25.33%). Pyoderma involves dominantly the males (68%).

3.2 The most affected body region is the one of the limbs (37.33%), followed by the one of the head (12%) and groin (9.33%).

3.3 Secondary pyoderma occurs more often (72%) than primary pyoderma (28%).

3.4 Deep pyoderma has a higher incidence (99.33%) than superficial pyoderma (0.67%); the most frequent types of deep pyoderma are: folliculitis (6.66%), cellulitis (5.33%), pododermatitis (5.33%), furunculosis (4%) and anal furunculosis (1.33%).

3.5 The cytologic examination revealed cell populations specific for acute/chronic pyoderma and also for primary/secondary pyoderma.

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# THE EFFECT OF AMBIENTAL TEMPERATURES, DURING THE SUMMER SEASON, ON THE MILK FAT GLOBULE SIZE, IN DAIRY COWS

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**Key words:** fat globule size, milk yield, milk fat

**SUMMARY**

The aim of the present study was to study the effect of ambiental temperatures, during the summer season, on the milk fat globule size, in dairy cows. An experiment was performed during 20 days, during the summer season, using 10 cows. Milk yield was measured and milk samples was analyzed for fat content, mean fat globule numbers and mean fat globule size. The results of our experiment show that milk yield was 13.9% greater (significantly) and the fat milk content was 9.37% higher (significantly), in the days with ambiental temperature lower 25$^{o}$ C, then the same parameters, measured in the days with higher temperatures. In the same time our experiment show that mean milk fat globule number was 2,8% higher (nonsignificantly) and the mean milk fat globule volume was 12,9% higher (significantly), in the days with ambiental temperature lower 25$^{o}$ C, then the same parameters, measured in the days with higher temperatures.

The MFG membrane (**MFGM**) is composed of phopholipids, cholesterol, lipoproteins, glycoproteins, and proteins (e.g., xantine oxidase, butyrophillin, and -glutamyl trans-peptidase; Evers, 2004).

Milk fat is synthesized as globules in the secretory cells of the mammary glands. At the surface of endoplasmic reticulum, the lipid is synthesized. These microlipid droplets are released into the cytoplasm with a surface of protein and polar lipids (Mather and Keenan, 1998). On their way to the apical cytoplasm, they fuse and, thereby, increase in size. The MFG are released from the secretory cells into milk through the apical plasma membrane. There, they are covered by an outer bilayer membrane.

The objectives of the present study were to evaluate the impact of ambiental temperatures, during the summer season, on the milk fat globule size.

## 1.MATERIAL AN METHODS

In total, 10 cows from the experimental herd of Romanian Holsteain cows were used in the study. Cows were between 10 and 45

wk of lactation. They were housed in tie stalls and milked with milking machine. The teats were dried with a wet paper towel, and control milk was removed from each individual quarter before the milking claw was attached. Prestimulation lasted for an average of 30 to 60 s. Once milked, the milking claw was detached manually. The teats were dipped in disinfectant solution after milking. The cows were fed 4 times daily at 0600, 1000, 1200, and 1600 h. They were fed silage ad libitum, 1 kg of hay, and concentrate according to milk yield to meet 60 MJ of ME for maintenance and 5 MJ of ME/kg of ECM.

The experimental period lasted 20 d. During this period, each udder half was milked twice daily at 06.00 and 18.00 h. Milk yield was measured, and milk samples for analyses of fat content and fat globule number, were collected at early milking on each days of experiment.

The ambiental temperature in stalls, was determined daily, using a termomether at 1200 h. The obtained values were used to calculate a main value of daily temperature.

The milk yield was measured daily.

Milk fat was analyzed by using the mid infrared spectroscopy method.

The number of milk fat globules/ml, was calculated using a Turk chamber. The fresh milk samples was diluted 1:100 with distilled wateradded to an Acridine Orange solution 0,1% in phosphate buffer pH 6,8. The main volume of milk fat globules was calculated using the next formulas:

V=G/N

All data were statistically evaluated using a paired *t*-test.

## 2.RESULTS AND DISCUSSION

The mean milk yield and mean fat percentages in the experimental period, are summarized in Table 2. Milk yield was 13.9% greater ($P < 0.05$) in the days with ambiental temperature < $25^{o}$ C.

The fat milk content was 9.37% higher in the days with ambiental temperature < $25^{o}$ C.

The mean milk fat globule numbers and mean milk fat globule volume in the experimental period, are summarized in Table 3.

Mean milk fat globule number was 2,8% higher in in the days with ambiental temperature < $25^{o}$ C.

Mean milk fat globule volume was 12,9% higher in the days with ambiental temperature < $25^{o}$ C.

*Table 1*

**The mean ambiental temeperature in the experimental period**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days with temperature >26°C | 26 | 28 | 29 | 31 | 29 | 33 | 28 | 31 | 27 | - | - | - | - | 26 | - | 26 | - | - | 27 | - |
| Days with temperature <26°C | - | - | - | - | - | - | - | - | - | 24 | 22 | 25 | 22 | - | 24 | - | 23 | 24 | - | 24 |

*Table 2*

**The mean milk yield and mean fat percentages in the experimental period**

| | Mean milk yield (l) | Mean milk fat content (%) |
|---|---|---|
| Days with temperature >26°C | 10,5* | 2,9* |
| Days with temperature <26°C | 12,2 | 3,2 |

* P<0,05

*Table 3*

**The mean milk fat globule numbers and mean milk fat globule volume in the experimental period**

| | Mean milk fat globule number ($10^9$/mL) | Mean milk fat globule volume ($\mu m^3$) |
|---|---|---|
| Days with temperature >26°C | 14,53 | 27* |
| Days with temperature <26°C | 14,95 | 31 (13-85) |

* P<0,05

## 3.CONCLUSIONS

The results of our experiment show that milk yield was 13.9% greater (significantly) and the fat milk content was 9.37% higher (significantly), in the days with ambiental temperature lower 25° C, then the same parameters, measured in the days with higher temperatures. In the same time our experiment show that mean milk fat globule number was 2,8% higher (nonsignificantly) and the mean milk fat globule

volume was 12,9% higher (significantly), in the days with ambiental temperature lower 25$^{o}$ C, then the same parameters, measured in the days with higher temperatures.

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# HYSTOLOGICAL RESEARCHES OF THE TESTES AT THE COCKS 30-60 DAYS OLD

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**Key words**: seminiferous tubules, gonocytes, Sertoli cells

**SUMMARY**

At the age of 30 days, the seminal epithelium is simple, and the dominated cells are the Sertoli cells and the spermatogoniums.
In some specimens were observed some lymphid nodules with different sizes and located immediately below the albuginea or in the depth of the testicular parenchyma.
At this age it have been highlighted the myofibroblastes, the circular lumen of seminiferous tubules and the nucleus of interstitial endocrinocytes are spherical, euchromatic, central willing, with obvious nucleolus.
At the age of 60 days in the testicular parenchyma ascertained seminiferous tubes which present simple epithelium and in individuals older than 30 days, and Sertoli cells are very obvious.
The albuginea and the basal membrane of seminiferous tubules are PAS positive. At the albuginea surface are arranged numerous blood vessels, capillary, arterial and small veins. There are not conjunctive septs only fine strands of connective tissue that separates the seminiferous tubes.
The microscopic investigations undertaken on permanent histological preparations made from rooster testicles aged 30 and 60 days, confirmed that at the birds, the testicle is covered by a fine fibro-elastic capsule, the albuginea tunic, which, unlike mammals, does not issue conjunctive septs so that no testicular lobules are identified and there was no mediastinal testis. The testicle is permanently placed into the abdominal cavity.

## 1. MATERIAL AND METHOD

The researches were conducted on testes harvested from cock, race-variety white Leghorn 30 and 60 days, normally developed, clinically healthy, vaccinated, macroscopically and microscopically examined.

It made an experiment which included two lots of cocks, each lot consisting of 4 copies each, from each individual were collected pieces of testicular parenchyma from skull pole, caudal, medial edge and lateral edge.

The inspection of histological preparations were made on permanent histology, processed by usual histological techniques and colored by hematoxylin-eozine method, Giemsa slow sections, Tanzer-Unna and P.A.S histochemical techniques.

## 2. REZULTS AND DISCUSSIONS

It was observed the evolution of primary cellular elements.

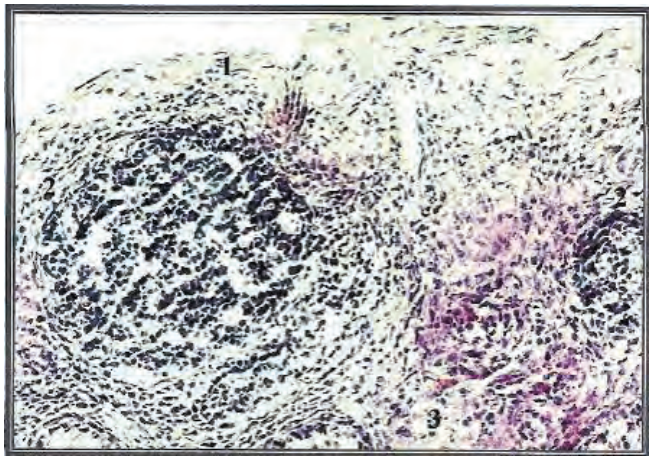


**Fig. 1 Cocks testicle by 30 days. H.E., ob 10X**
**1- Albuginea; 2- Nodules limfoids; 3-Interstitial gland**

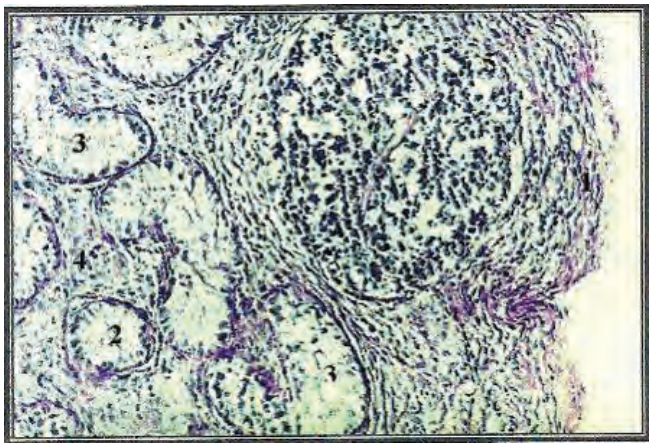


**Fig. 2. Detail of previous image. Cock by 30 days.H.E., ob. 20X**
**1-Albuginea; 2-Limfoid nodule; 3- Interstitial gland**

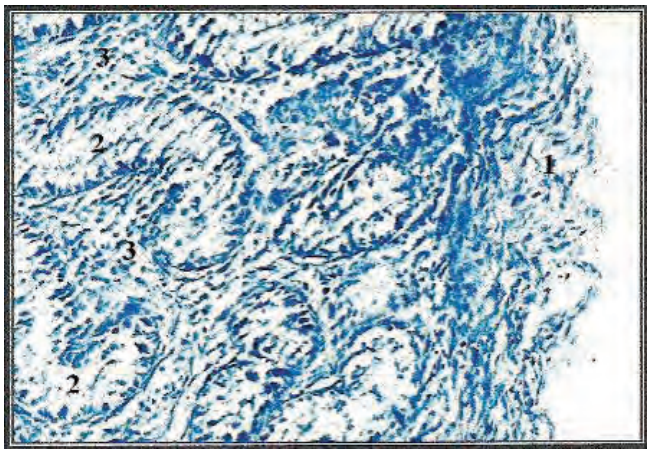


**Fig. 3 Cocks testicle by 30 days. P.A.S., ob. 20X**
**1-Albiginea; 2- Seminiferous tube cut transversely; 3- Seminiferous tube obliquely cut; 4-Nodules limfoids; 5-Interstitial gland**

Most of thickness of albuginea is composed of a dense layer of fine collagen fibers, elastic fibers and numerous fibroblasts.

Tunica albuginea is not organized in clear layers.

In the sections made in the testicular parenchyma have seen that seminiferous tubes lumen is bounded by a germinative epithelium located on a basal membrane. Histologically, seminiferous tubes have three components:

The basal membrane, the Sertoli cells and the line seminal cells.

The basal membrane is shape the seminiferous tube.

The internal layer is the basal lamina.

This is connected to some fibers of collagen and elastic fibers, peritubulary cells (myofibroblasts) that forms a blanket layer consists of 1-5.

At hatching, these cells resembles with the mesenchymal cells, which then gradually differentiate into contractile cells.

The peritubulary cells resembles with smooth muscle cells and are involved in providing tubular contraction.

In this way they participate in the transport of tubular content and at the spermiation process.

The most external layer of molding tubular basement contains fibrocim and collagen fibers.

Free mononuclear cells (lymphocytes and monocytes) invades the lamina propria, but never invades the seminal epithelium.

Seminal epithelium is simple, consisting of Sertoli cells and spermatogonium. The myofibroblasts are evident.

The lumen of the seminiferous tube is circular, the interstitial endocrinocytes nucleus are spherical, euchromatic, central willing, with obvious nucleolus.

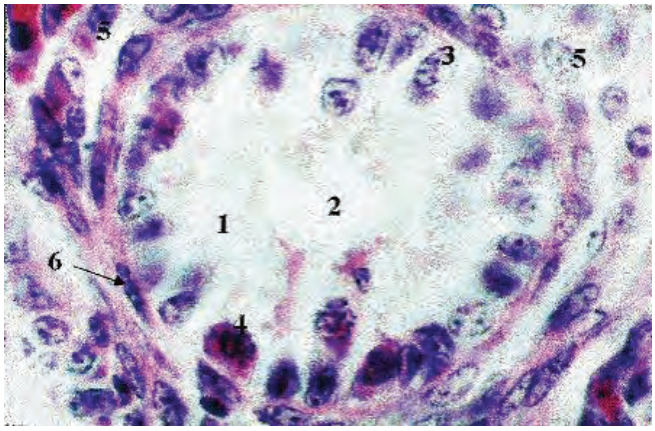


**Fig. 4 Cocks testicle by 30 days. P.A.S., ob. 100X**
**1- Seminal epithelium; 2- Lumen; 3- Spermatogoniums; 4- Sertoli cells; 5-Leydig cells 6- Myofibroblasts**

At the age of 30 days in some individuals stand out formations, few circumscribed with different sizes, located in the depth of testicular parenchyma.

Inside of the nodular formations is observed a heterocellular population, characterized by cellular polymorphism with different types of cells, monocytes, macrophages, lymphocytes, plasma cells, with powdery chromatin.

Morphometric measurements were carried out in outer diameter, seminiferous tubule lumen, diameter and height of the seminal epithelium. They were taken into account 10 seminiferous tubes.

In the following table are given values obtained from measurements of the tubes.

The outer diameter of seminiferous tubules at the age of 30 days was an average of 53, 176 μm while the lumen diameter was on average 13, 352 μm.

In the specimens older than 60 days the testicular parenchyma consists of seminiferous tubes epithelium presents simple as in individuals older than 30 days and consists of Sterol cells and spermatogenesis. The myofibroblasts are clear and peritubulary located. Peritubulary connective tissue in the testicular parenchyma is

quantitatively reduced. Following morphometric determinations of the outer diameter of seminiferous tubules in rooster, at the age of 60 days, ware found that the lumen diameter and height of the seminal epithelial increased comparative with the values obtained at the age of 30 days.

Thus, the outer diameter reached an average of 66, 336 micrometers and the lumen diameter value was 24.146 micrometers and the seminal epithelium height was 21.097 μm.

## 3. CONCLUSION

3.1. The hystostructure of the parenchyma of the the testicles at the age of 30 days is characterized by reduced diameter of seminiferous tubules and well represented intertubular space occupied by a mass of connective tissue in which endocrynocites interstitial are desiminate.

3.2. At age of 30 days seminiferous tubes epithelium is wallpapered semen monostratification and consists mainly of two types of cells: which are the spermatogoniums elements, spermatogenetic line, primary Sertoli cells.

3.3. The basal membranes of seminiferous tubules are PAS positive and peritubular are localized many myofibroblasts.

3.4. At age of 30 days, in some individuals were observed limfoids nodules of different sizes within the testicular parenchyma in the space of tubes disorders, these formations are characterized by the presence of different types of lymphoid cells. In terms of dynamic morphometric found that the thickness is reduced as albuginea advancement in age.

3.5. At age of 60 days npt appear major differences in comparison with previous age regarding the appearance and structure of seminiferous tubules.

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# THE MORPHOLOGY OF THE PARIETAL LYMPHNODS IN THE ABDOMINAL AND PELVIC CAVITY

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**Key words:** lomboaortic lymphcenter, ileopelvin lymphcenter, iliac (sacral) lymphnodes

**SUMMARY**

There were sacrificed and dissected a total number of 14 rabbits, there were identified and studied the mural lymphnodes of abdominal and pelvic cavity, represented by the lomboaortic lymphcenter and the epigastric lymphnodes along with the ileopelvin lymphcenter which are fickled.
The lomboaortic lymphnodes are represented by the lomboaortic renal lymphnodes, located between the aorta and the caudal cava vein.
The renal lymphnodes are very small, and are situated on both sides of the pelvis.
The epigastric lymphonod is located immediately above of the rim of the xifoidian appendix. This lymphonode have ovalar form and presents a hilus at one pole.
The ileopelvin lymphcenter is systematized into 3 limfonodale groups located between the terminal branches of the aorta and the deep iliac circumflex artery: the lateral iliac lymphnodes, the media iliac lymphnodes and the middle (sacral) iliac lymphnodes.
The iliac lymphnodes are located side by side and across the terminal bifurcation of the descending aorta.
The iliac lymphnodes are represented averages of 1-3 small lymphnodes and situated between the internal and external iliac arteries.
The middle (sacral) iliac lymphnodes are the well-represented separation angle in both iliac arteries (common) median arranged.
When it was injected at a single pelvis member, it was performed most often a bilateral staining of the lymphnodes, which advocates for a intense anastomoza of the lymphatic vessels.

## 1. MATERIAL AND METHOD

In a total of 14 rabbits were sacrifice and dissected lymph nodes identified and studied mural of abdominal and pelvic cavity

He proceeded to inject Evens blue areas and China ink solution of 40%.

Lymph nodes for evidence of the abdominal cavity was injected intraperitoneally Evens blue solution 0.1-0.2 ml in small doses and repeated every 12 hours, obtaining a clear staining of lymph nodes and lymphatic vessels. For evidenceIleopelvine lymph nodes were made

injections with Evens blue solution and China ink to the planting of the pelvic limb.

## 2. REZULTS AND DISCUSSIONS

The parietal lymph nodes in the abdomen and pelvis.

They have identified and studied the parietal lymph nodes in the abdomen and pelvis of the rabbits were sacrificed and dissected. This are represented by the lumbar aortic lymphocenter and the lymphocenter of the pelvic limb who are joining the epigastric lymph nodes that are are unsteady.

The lumbar lymph nodes (Lyrnphonodi lumbales aortici)

This are the lumbar lymph nodes, around the renal vessels.

There were found over the abdominal aorta a number of 2-10 lymph nodes of very different sizes, oblong or multiform, located in the tissues between the aorta and vena cava, starting from the origin of the deep iliac circumflex artery to the left kidney.

The afferent lymphatic vessels in the area collect lymph from the abdominal cavity, from the aorta, vena cava, testicles, testicular cord and from the vas deferens. The efferent lymph vessels converge to the cisterna.

Their coloring was made more difficult and they were highlighted only several days after injection with Evans blue at the pads of the hind limbs but parietal lymph nodes of abdomen were colored very easy in 24 hours.

The renal lymph nodes (Lymphonodi renales)

They have highlighted the corpse of a rabbit two small lymph nodes by repeated injections of Evans blue, situated on both sides of the basin, on the path of renal artery compared with the renal hilum.

Sometimes these renal lymph nodes may be confused with the lombo-aortic lymph nodes.

The renal lymph nodes were located quite difficult because of the large adipose tissue surrounding the kidney.

Afferent lymphatic vessels collect lymph from kidney, adrenal glands, ureters, regional perirenal tissue.

The efferent lymphatic vessels depends lymph nodes or may drains directly into the cisterna.

The epigastric lymph nodes (Lymphonodi phrenicoabdominali)

It has highlighted in the third part of dissected rabbit an odd lymph node located just below the peritoneum and above the xifoidian

appendix. This limph node has ovular form and presents a hilus at one pole. It has sizes between 4 and 6 mm long and 3-4 mm wide.

Their highlight was possible after a intraperitoneal injection of Evans blue.

The efferent lymphatic vessels of the lumbar lymph center form the lymphatic lumbar trunks that open in cisterna.

The parietal lymph nodes in the abdomen and pelvis

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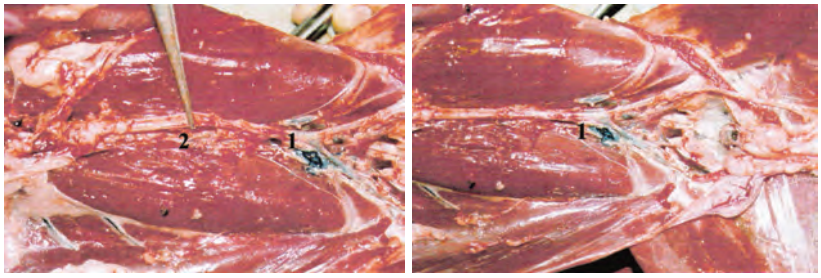


**Fig. 1 Macroscopic aspects of the iliac lymph nodes (1) and lomboaortics (2), highlighted by Evens blue injection method, at rabbit, LEPUS EUROPEUS**

The afferent lymphatic vessels in the area collect lymph from the abdominal cavity, from the aorta, vena cava, testicles, testicular cord and from the vas deferens. The efferent lymph vessels converge to the cisterna.

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The efferent lymphatic vessels of the lumbar lymph center form the lymphatic lumbar trunks that open in cisterna.

The iliosacral lympho center (Lymphocentru m iliosacrale)

This lymph nodes are summarized in 3 lymph node groups due to topography, located between terminal branches of the deep iliac circumflex artery.

The lateral iliac lymph nodes (Lymphonodi iliaci laterales)

It was located and highlighted a group of lymph nodes. They were well colored with Evans blue and were situated at the terminal bifurcation of the aorta, between this one and caudal vena cava.

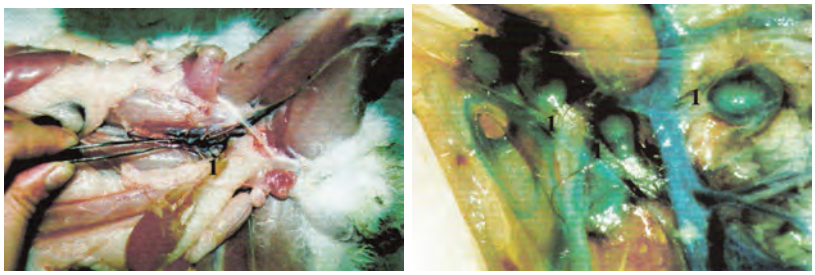


**Fig.2 Macroscopic aspects of the iliac lymph nodes from the terminal branches of the aorta (right-marry with Lupei Nikon stereo-microscope), highlighted by Evens blue injection method, the pets rabbit - Oryctolagus cuniculus**

Were generally observed one or two lymph nodes, oval ranging, in size from 4-6 mm long and 2-4 mm wide, around the aorta and between common iliac trunk and deep circumflex iliac vessels. They isolated a lymph node between iliac vein and iliac artery.

The medial iliac lymph nodes (Lymphonodi ilaci mediates)

They are constant and represented by 1-3small lymph nodes. They are located between the internal and external iliac arteries.

It is required a prior injection of China ink or Evans blue for their disclosure, because they are difficult to observe.

The sacral lymph nodes ((Lymphonodi sacrales)

This lymph nodal package is best represented, it is the largest one of the ilio pelvic lymph nodes.

The lymph nodes that form this group looks very well with the lymph nodes of the headland, described in the hominids and cattles.

## 3. CONCLUSIONS

3.1. This are represented by the lumbar aortic lymphocenter and the lymphocenter of the pelvic limb who are joining the epigastric lymph nodes that are are unsteady.

3.2. Sometimes these renal lymph nodes may be confused with the lombo-aortic lymph nodes

3.3. There were found over the abdominal aorta a number of 2-10 lymph nodes of very different sizes, oblong or multiform, located in the tissues between the aorta and vena cava, starting from the origin of the deep iliac circumflex artery to the left kidney.

3.4. The renal lymph nodes were located quite difficult because of the large adipose tissue surrounding the kidney.

3.5. The medial iliac lymph nodes – is constant and represented by 1-3 small lymph nodes. They are located between the internal and external iliac arteries.

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# DETECTION OF BOVINE PESTIVIRUS INFECTION (BVDV) USING DIRECT IMMUNOFLUORESCENCE METHOD

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**Key words**: bovine, BVDV, direct immunofluorescence

**SUMMARY**

Bovine viral diarrhea virus (BVDV) infection of cattle is one of the most significant diseases affecting dairy herds.
Animals can have signs that range from subclinical to chronic debilitating disease with high mortality. Fetal infections in utero may produce immunotolerant persistently infected (PI) calves that continually shed virus. Circulation of BVDV within the herd due to the presence of PI animals results in poor reproductive performance from early fetal loss, abortion, and sporadic clinical disease in susceptible animals.
The aim of this study was to determine if BVDV is present in young unvaccinated bovines, without any clinical signs. The tissues samples were collected from cattle slaughtered from the farm and household system. From the 27 animals tested using direct immunofluorescence, were found positive 15 bovines, all from the household system.

Infections with bovine virus diarrhoea virus (BVDV) are widespread throughout the world. Although the prevalence of infection varies among surveys, the infection tends to be endemic in many populations, reaching a maximum level of 1-2% of the cattle being persistently infected (PI) and 60-85% of the cattle being antibody positive (2, 3). This unique ability gives BVDV a selective advantage allowing continual mutation and antigenic variation within cattle populations. Persistently infected cattle are the main source for transmission of the virus. However, acutely infected cattle as well as other ruminants, either acutely or persistently infected, may transmit the virus. Transmission is most efficient by direct contact.(7)

Differences in BVDV prevalence among regions or introduction of virus in herds previously free of BVDV are often associated with particular epidemiological determinants such as cattle population density, animal trade and pasturing practices. Estimates of economic losses due to BVDV infection vary depending on the immune status of the population and the pathogenicity of the infecting virus strains.

## MATERIAL AND METHOD

The study was conducted over a period of six months (May-October 2009) on a total of 27 clinically healthy animals from Iaşi County. Tissue samples were harvested in the slaughterhouse and were represented by: lung, trachea, tracheo-bronchial lymph nodes, thymus, mesenteric lymph nodes and small intestine.

Tissue samples were collected individually in sterile containers and were transported at refrigeration temperature. After selection of tissue for examination by direct immunofluorescence (direct FA), countersamples were stored at $-80^0$C.

Staining procedure for direct immunofluorescence (direct FA): tissue sections were aired for at least 30 minutes at room temperature and were fixed on slides for 20 minutes in acetone-methanol (3:1). To verify the method we used positive and negative control slides for BVDV (VMRD-USA).

Slides were stained with 50µl conjugate (bovine viral diarrhea virus - direct FA Conjugate, VMRD-USA) for 30 minutes at 37°C in humid chamber. Slides were gently rinse briefly in FA Rinse Buffer, pH 9.0 (VMRD-USA) and then soak for 10 minutes in FA Rinse Buffer, pH 9.0. After that slides were drained and dry back and edges with a paper towel.

Final slides were mounted with FA Mounting Fluid (VMRD-USA) and viewed with fluorescence microscope Olympus IX-51 at 100X-200X. Confirmation was made at 400X.

## RESULTS AND DISCUSSIONS

From 27 animals tested, six of them came from three farms (2 bovines from each farm) and the remaining 21 came from the household system. The tested animals were young males, not vaccinated, with any clinical signs characteristic for BVD, which were slaughtered in the slaughterhouse.

***Table no. 1***

**The origin of the tested animals and the result of BVDV direct immunofluorescence**

| No. | Origin | Tested animals | Direct FA | |
|---|---|---|---|---|
| | | | Positive results | Negative results |
| 1. | Farms | 6 | 0 | 6 |
| 2. | Household system | 21 | 15 | 6 |
| Total | | 27 | 15 | 12 |

From the slaughtered animals were collected tissue samples such as: lung, trachea, tracheo-bronchial lymph nodes, thymus, mesenteric lymph nodes and small intestine. The age of the tested animals was between 10 and 16 months. We found positive result only in bovines from household system.

***Table no. 2***

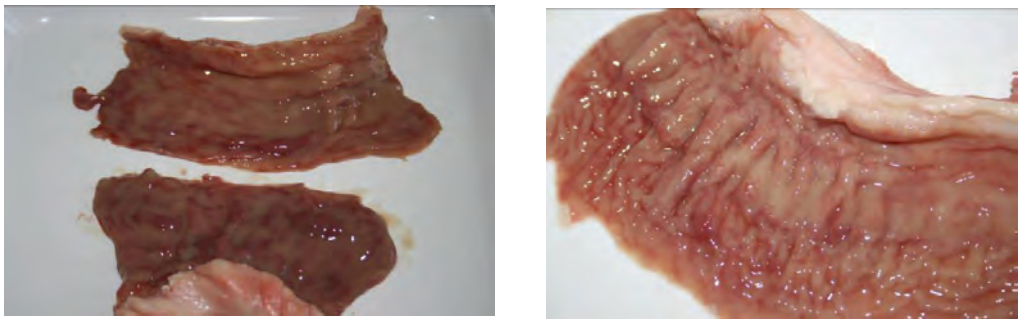
**Direct FA results depending on the type of tissue samples collected from animals of household system**

| Animal number | Age | Trachea | Lung | Tracheo-bronchial lymph nodes | Thymus | Mesenteric lymph nodes | Small intestine |
|---|---|---|---|---|---|---|---|
| 1 | 14 months | - | - | - | 0 | 0 | + |
| 2 | 16 months | 0 | - | - | 0 | 0 | + |
| 3 | 17 months | - | - | - | 0 | 0 | + |
| 4 | 14 months | - | - | - | 0 | - | - |
| 5 | 14 months | - | - | + | 0 | 0 | + |
| 6 | 14 months | - | - | + | 0 | + | + |
| 7 | 15 months | - | - | + | 0 | + | + |
| 8 | 14 months | - | - | - | 0 | 0 | - |
| 9 | 12 months | - | - | + | + | 0 | + |
| 10 | 13 months | 0 | - | - | 0 | 0 | - |
| 11 | 12 months | - | - | + | + | + | + |
| 12 | 12 months | - | - | - | - | - | - |
| 13 | 14 months | 0 | - | - | 0 | 0 | + |
| 14 | 10 months | 0 | - | + | + | 0 | + |
| 15 | 14 months | - | - | - | 0 | + | + |
| 16 | 14 months | - | - | + | 0 | 0 | + |
| 17 | 12 months | - | - | - | 0 | - | - |
| 18 | 12 months | - | - | + | 0 | 0 | + |
| 19 | 12 months | - | - | - | 0 | - | - |
| 20 | 16 months | - | - | - | 0 | + | + |
| 21 | 14 months | 0 | - | - | 0 | + | + |

**Legend**

- **direct FA positive results: +**
- **direct FA negative results: -**
- **not tested sample: 0**

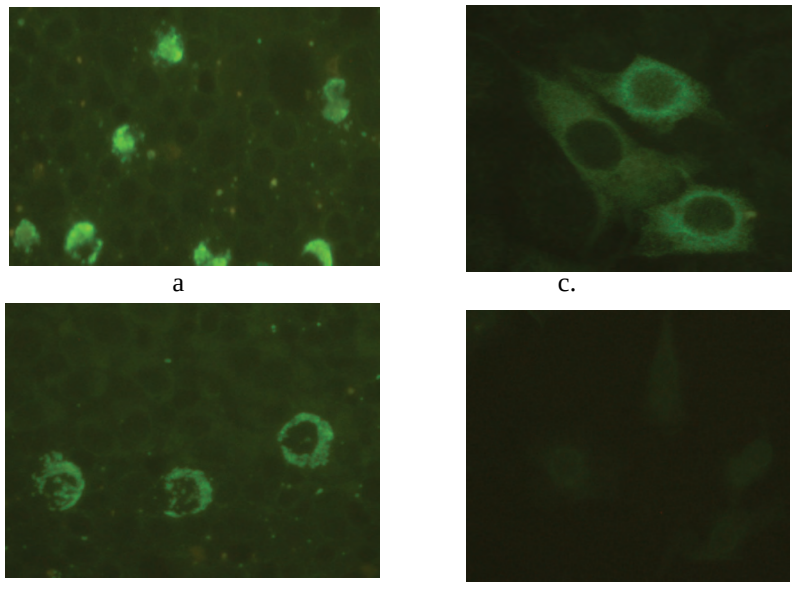
In four cases (animal 6,7,11 and 13), changes were observed at morphopathological exam of small intestine. These were represented by small hemorrhages on the intestinal mucosa (figure no.1).



A B

**Figure no.1 The presence of hemorrhages on intestinal mucosa.**
**A- in the case of animal no.6; B-in the case of animal no.11**

In all animal tested we didn't obtain any positive results for BVDV using direct FA in lung and trachea tissue samples. On the contrary, from all animals we have tested tracheo-bronchial lymph nodes detecting 8 positive samples. The most positive samples were identified in slides made from the section of the small intestine mucosa (15 out of 21 tested). In all cases to check the results of direct FA we used positive and negative control slides for BVDV (figure no.2).



a c.

b. d.

**Figure no.2 Aspects from direct FA test**
**a. positive mesenteric lymph node, 400x; b. positive small intestine, 400x**
**c. BVDV positive control, 400x; d. BVDV negative control, 400x**

## CONCLUSIONS

1. From the 27 tested animals, we detected positive response to Direct FA against BVDV in 15 animals, the most positive sample being the mucosa of the small intestine (55,5%).
2. The interaction of bovine viral diarrhea virus (BVD virus) with its host has several unique features, most notably the capacity to infect its host either transiently or persistently.
3. The present results indicate that cattle persistently infected with BVD virus are present in household system and they don't have any clinical signs characteristic for this disease. But in four cases we founded hemorrhagic lesions on small intestine mucosa.

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# THE INFLUENCE OF REFRIGERATION TIME ON COMMERCIALY FRESH AND MATURATED BEEF AND POULTRY MEAT

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**Key words:** meat refrigeration, proteolysis, electrophoresis, calcium, phosphorus

**SUMMARY**

Maturated and fresh poultry (breast) and beef meat samples were purchased from different food stores. There was not recorded information about maturation process and technology. Samples (5 samples of each, maturated poultry breast and beef, and also 5 samples of fresh poultry breast and beef) were preserved at 4°C and aliquots assayed every 24 hours for a period of four days. Crude extracts obtained by homogenization in PBS were assayed for the total soluble protein content, calcium and inorganic phosphorus. Agarose and polyacrylamide gel electhophoresis was performed for proteins polymorphism changes evaluation. The obtained results revealed a decrease of soluble protein content of both fresh and maturated poultry meat during refrigeration, while in extracts of maturated beef meat, at the end of the experiment, soluble proteins concentration was 120% higher than at the beginning. Electrophoretic profiles were in accordance with the variation of protein content, emphasizing an increase of the number of fractions during refrigeration, for the beef samples, and a smaller number of fractions in the poultry meat extracts in the last samples. Both calcium and phosphorus levels increase during refrigeration.

Meat palatability characteristics, such as tenderness, juiciness, flavour, are influenced by many factors, including carcass maturity and animal age (Li *et al.*, 2007) feeding regime of the animals (Gallo *et al.*, 2009), but also the post-mortem changes that occur in the meat during storage and maturation. A great number of physico-chemical processes occure during post-mortem storage. Considerable stability of proteasome activities, despite the pH decrease (Lamare *et al.*, 2002) in syergy with other proteolytic systems, like calpains (Pomponio *et al.*, 2008), play an important role in meat tenderdization and maturation.

The aim of the present paper was to investigate the influence of refrigeration time on some biochemical parameters of commercially fresh and maturated poultry and beef meat.

## 1. MATERIALS AND METHODS

Biological samples consisted of fresh chicken breast, maturated chicken breast, fresh veal and maturated veal, purchased from the market. The experiment was done over a 4 days period, in which samples were refrigerated at $4^{o}C$. During the whole period of the preservation, refrigeration temperature was surveyed every 12 hours.

Reagents used were of analytical purity (purchased from specialised companies).

**Extraction:** aliquots of every sample were weighed, homogenized with phosphate buffer saline, pH 7.2, (1:3 w:v ratio), extracted for three hours at refrigeration temperature, crude extracts separated by centrifugation at 12000 rotations per minute, for 20 minutes.

**Protein assay:** The total protein assay was performed using Bradford Test.

**Serum proteins electrophoresis** was done in agarose gel and in polyacrylamide gel, proteins stained with Coomassie Blue R250. The proteins fractions were evaluated using a DVSE electrophoresis system.

**Calcium, phosphorus** assays were performed with reagents from Cromatest.

## 2. RESULTS AND DISCUSSIONS

The obtained results emphasized that in chicken breast, because of the lower content of collagen, elastin and other insoluble structural proteins, the decrease of the concentration along the maturation period is associated with a faster degradation of the proteins in amino acids. In veal muscle it was noticed the growth of the soluble proteins concentration along the refrigeration period probably due to the transformation of collagen and elastin in peptides and soluble peptides. The process is more pronounced with maturated meat than with the fresh and refrigerated one. (Fig.1)

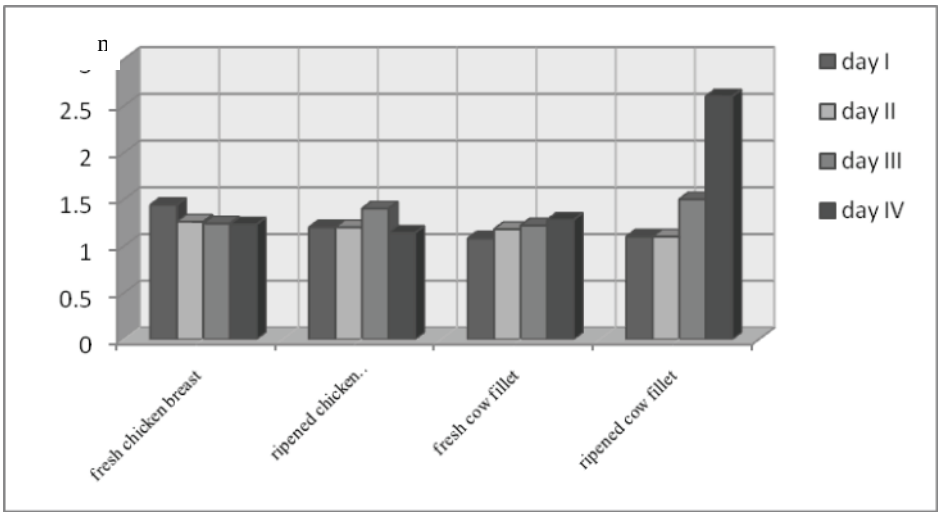


**Fig. 1 – The variation of soluble proteins in fresh and maturated chicken breast and fresh and maturated veal muscle extracts (mg/g muscle)**

Electrophoresis in polyacrylamide gel highlights the presence of a similar number of proteic bands in the fresh and maturated chicken breast extract afferent to each day of evaluation, with the difference that in the maturated chicken breast extract the intensity of the bands is smaller than the one in the fresh chicken extract (Fig. 2).

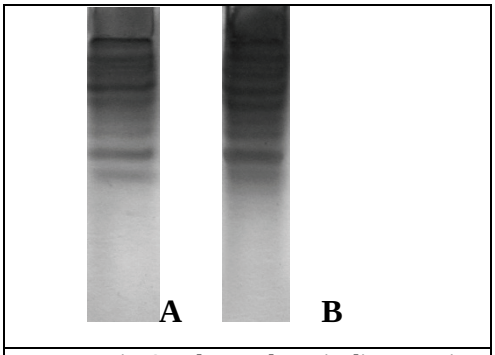


**Fig. 2– Electrophoretic diagrams in polyacrylamide gel of soluble proteins from th extract of fresh chicken breast (A) and maturated (B) (I day )**

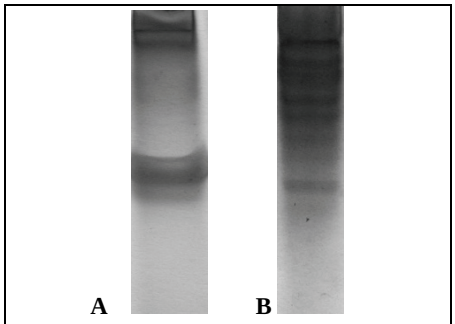


**Fig. 3 - Electrophoretic diagrams in polyacrylamide gel of soluble proteins from the extract of fresh veal muscle (A) and maturated (B) (I day)**

The polyacrylamide gel electrophoresis in non-denaturating conditions of the soluble proteins from fresh veal extracts reflects the deployment of processes that affect the proteins from the veal muscular

tissue, different from the ones which occur in the chicken muscle. Thus, electrophoresis highlights the increase of the number of proteic bands with maturation (Fig.3).

The integration profile of the electrophoretic diagrams obtained in agarose gel shows that during refrigeration, the number of fractions with large molecular weight increases, and that can result from the degradation of the proteic macromolecules from the conjunctive tissue (Fig. 4) .

The integration of electrophoretic diagrams in agarose gel reflected the same type of behaviour for the extracts from the maturated veal muscle tissue ( Fig. 5).

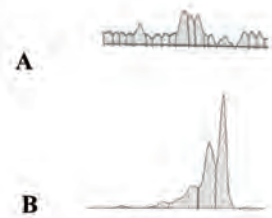


**Fig. 4** Spectrophotometric integration of electrophoretic diagrams in agarose gel of soluble proteins from the extract of fresh chicken breast (A – day I, B- day IV)

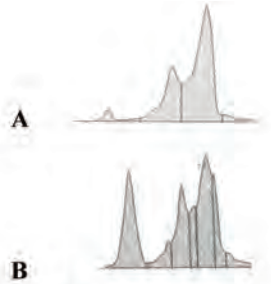


**Fig. 5** – Spectrophotometric integration of electrophoretic diagrams in agarose gel of soluble proteins from the extract of fresh veal muscle (A – day I, B- day IV)

*Phosphorus and calcium assays*

Calcium concentration in chicken breast and veal muscle extracts, both fresh and maturated, reflects a constant growth during the refrigeration period.

The ripening process may be associated with a better preservation of muscular fibres integrity, both of chicken breast and veal muscle, as the extracts from these tissues showed lower values for calcium concentration when compared with fresh samples (Fig.6).

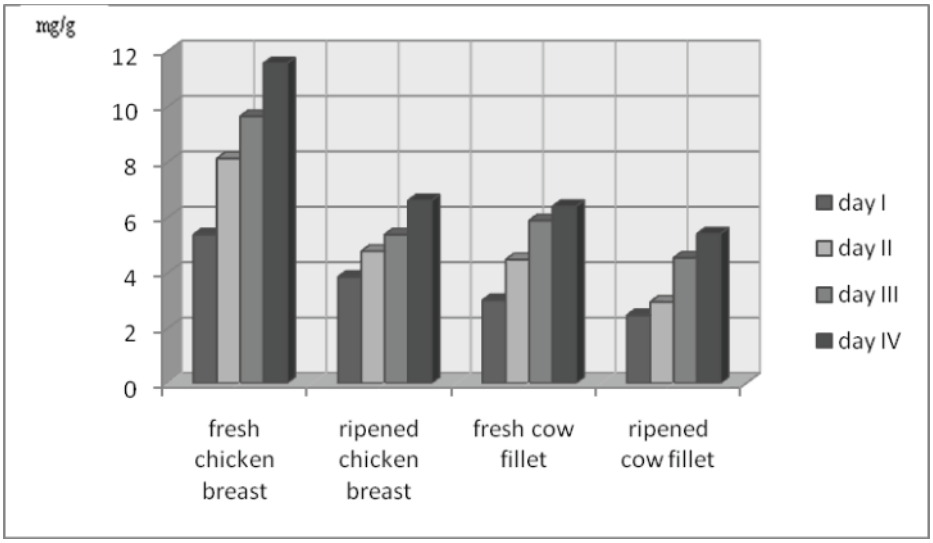


**Fig. 6 – Calcium determination in extracts of fresh and maturated chicken breast and in fresh and maturated veal muscle (mg/g muscle)**

The evaluation of phosphate ions content shows the decreasing of it in fresh tissue extracts, both of veal muscle and chicken breast in the second day of refrigeration, whilst, in both maturated meat extracts it increses. The values obtained in the experiment are graphically illustrated in fig.7

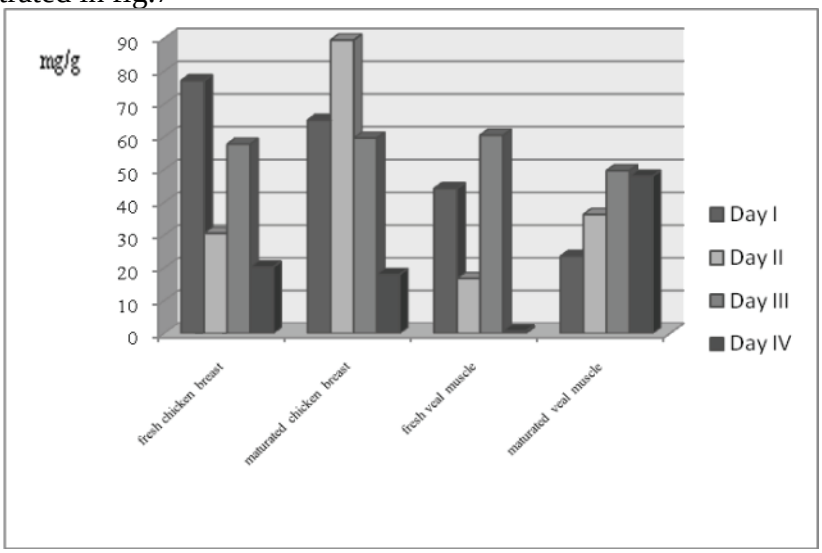


**Fig.7 - Phosphorus determination in extracts of fresh and maturated chicken breast and in fresh and maturated veal muscle (mg/g tissue)**

These investigations were aimed to evaluate the influence of refrigeration time on meat samples about there were not recorded information about the animal's age, the time of slaughter, the technology used for maturation. The main observation was that important changes take place and these are reflected by the variation in biochemical compounds studied. These changes occur due to the survival of some enzymes, mainly the proteolytic ones. The appearance of proteins with high molecular weight after a longer refrigeration time is also associated with the partial degradation of the miofibrils during the refrigeration process and may be indicators of the age of the samples.

## 3.CONCLUSIONS

3.1. The electrophoretic assays, especially in agarose gels, could be used to evaluate the age of refrigerated samples of unknown origin.

3.2. The increase of cacium levels could be associated with proteinases activation.

3.3. The inorganic phosphorus is an indicator of the maturation, registering significant increase with the refrigeration time.

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# CONSIDERATIONS ON INTESTINAL SUTURES AND ENTEROANASTOMOSIS IN ORDER TO PREVENT EXTERNAL POSTOPERATIVE DIGESTIVE FISTULAE

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**Key words:** enterotomy, enteroanastomoses, digestive fistulae, postoperative

**SUMMARY**

The digestive fistulae is one of the most serious complications that might appear following different types of resectional digestive surgery. The postoperative fistulas, due to their physiopathological and clinical complexity, induce the disturbance of the biological equilibrium. Optimal intestinal healing depends on a good supply in blood, accurate mucosal apposition and minimal surgical trauma. Thus, considering the techniques used for suturing the intestine, the approximationg suture patterns facilitate rapid healing. Though the intestine heals rapidly, healing can be delayed by local and systemic factors, such as: shock, hypovolemia, hypoproteinemia, concurrent infections.

External digestive fistulae are among the earliest complications of intestinal surgery in animals. A possible definition of external postoperative fistula consists in a leakage of digestive liquid between two epithelial-covered surfaces, through a newly-formed and maintained tract.

The definiton of a fistula is limited to the immediate loss of digestive tract seal and to loose intestinal sutures.

Optimal intestinal healing depends on a good blood supply, accurate mucosal apposition, and minimal surgical trauma. Approximating suture patterns facilitate rapid healing. Everted and inverting suture patterns retard inestinal healing, and result in greater stricture formation. Healing is facilitated by adjacent serosal surfaces and omentum, which help seal wounds and contribute to blood supply.

The intestine ussualy heals rapidly, but healing can be delayed by local and systemic factors, such as: hypovolemia, shock, hypoproteinemia, debilitation and concurrent infections.

## 1. MATERIAL AND METHOD

Researches have been made on pacients brought in for consultations at the Surgical Clinic from the Faculty of Veterinary Medicine in Iasi, during the year of 2009.

There have been performed surgical techniques, such as enterotomy and intestinal resection and anastomosis, at different intestinal sites, precedeed by ventral median laparatomy (fig. 1). As methods for ensuring hemostasis, we used clamping and simple ligatures, or double ones for larger vessels, and electrocoagulation, for vessels less than 1,5 – 2 mm in diameter.

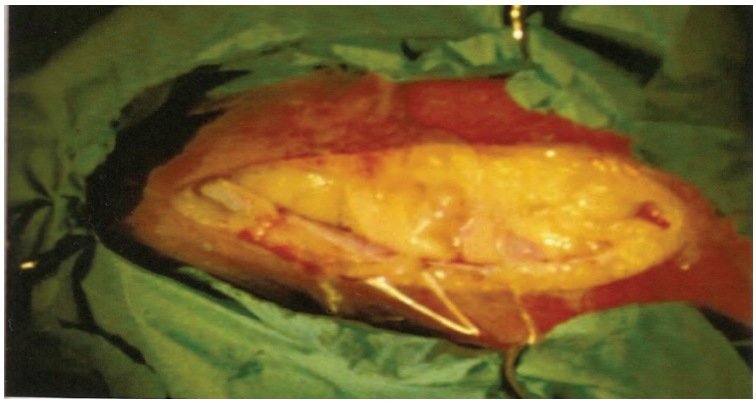

**Fig. 1. Ventral median laparatomy**

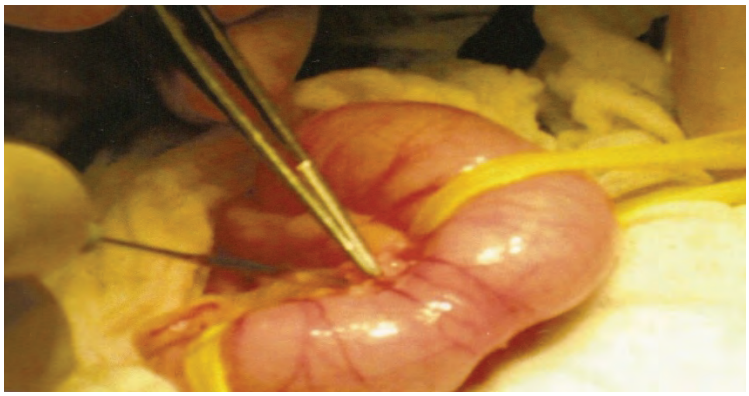

**Fig. 2. Electrocoagulation for obtaining hemostasis**

## 2. RESULTS AND DISCUSSIONS

Causes of digestive external fistulae may be grouped into surgical and local factors. Among the surgical factors we can find suture dehiscence, use of wrong instruments (needles in particular), suture materials with high leakage potential, cutting off the boold supply of that intestinal portion by making a much too close continuous suture pattern. The local factors are inflamed, friable, swelled tissues, local ischemia, local contamination, prolonged intestinal paresis (atony), high capacity for local autolisis.

Depending on the type of tract, fistulae can be:

- Exposed fistulae, when the organ is open to the abdominal wall;
- Fistulae with tract, when between the organ and the abdominal wall we can find a communication.

Depending on its position to the intestinal segment:

- Lateral fistulae, following enterotomy or anastomosis;
- Terminal fistulae, after end-to-end or side-to-side anastomosis.

Depending on the organ:

- Gastric fistulae (gastrotomy, pyloroplasty, gastro-jejunostomy, gastro-duodenostomy);
- Duodenal fistulae (on a side);
- Intestinal fistulae;

Depending on their moment of apparition:

- Immediate fistulae, due to major techique errors,
- Precociuos fistuale, within the 5th and 12th day after surgery,
- Belated fistulae, 15 days after surgery.

Symptoms which indicate us of the presence of a fistula are anorexy, lack in appetite for water, intestines distended by the presence of gas, abdominl pain, fever, dehidration, peritoneal irritation. During the fistulae formation, we find:

- A short period in which the general status of the animal is better, by the dropping of fever, and resuming of bowel movement for a short period of time,
- External leakage of irritant digestive content,

In order to prevent the apparition of external digestive postoperative fistulae, the perfect contact between all intestinal layers must be ensured by appropiate suture patterns, therefore a quick and resistent scarr will be formed (BOJRAB, M.J., et al, 1990, COOLMAN, BR. et al, 2000).

The most frequently used suture patterns are those based on aposition or enverting the intestine(FOSSUM WELCH et al, 2002). The single layer suture has been considered, for a long time, as a useless technique, but as the time passed it has been proven that the mucosal layer rapidly heals and the inflammation disappears quickly, in fewer days, following this suture pattern (IGNA, C., 1996, WEISMAN, DL. et al, 1999 ).

Suture patterns can consist in simple continuous or crushing points and simple interrupted sutures, all of these may be used to close the enterotomy (fig. 3).

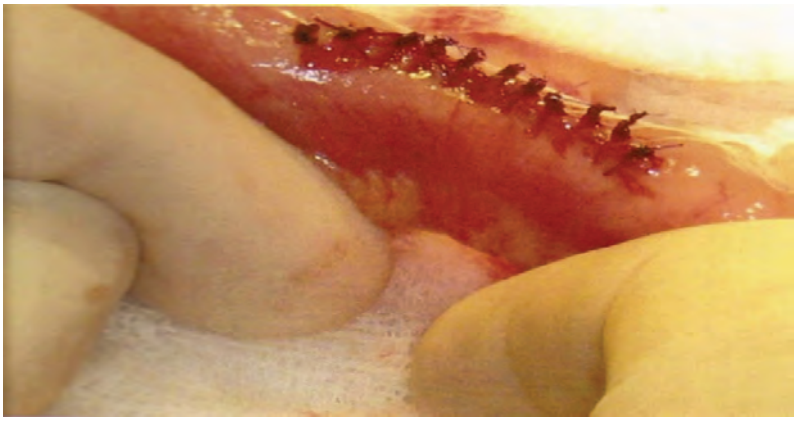

**Fig.3. Closed incision with simple interrupted suture**

Choosing the appropiate technique for intestinal anastomosis following the resection of the affected area is made after considering the intestinal site on which we operated. End-to-end anastomoses are recommended by their closely respect of bowel anatomy, making sure that intestinal flow is well kept (fig.4, fig.5). Nevertheless, authors recommend side-to-side anastomosis as being the most accurate when it comes to prevention of digestive fistulae (ALLEN, DA. et al, 1992, COOLMAN, BR. et al, 2000).

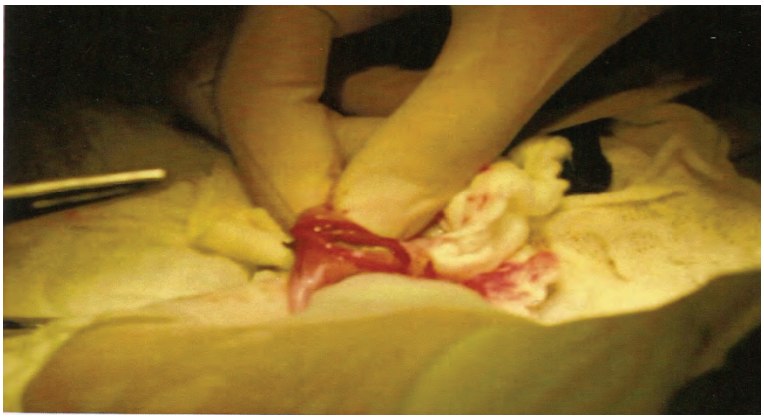

**Fig. 4. End-to-end anastomosis**

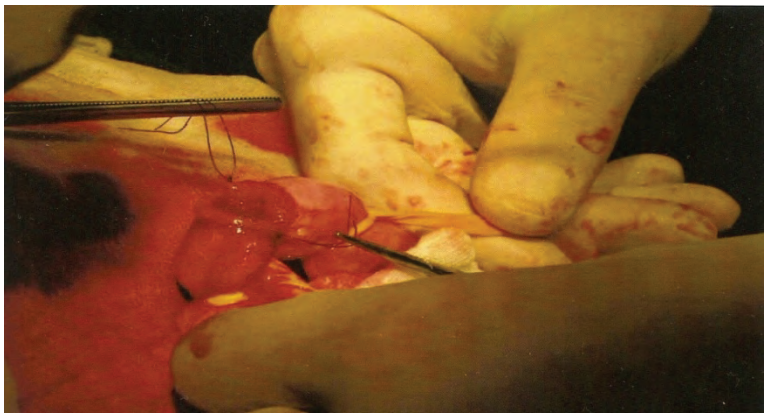

**Fig. 5. End-to-end anastomosis using appositional sutures**

## 3. CONCLUSIONS

3.1. Although intestinal suture techniques are very well defined, they can sometimes become responsible for postoperative external digestive fisutale.

3.2. Suture patterns can consist in simple continuous or crushing points and simple interrupted sutures, all of these may be used to close the enterotomy

3.3. Sutures are performed beginning from inside out, each layer being sutured by placing and tying appositional sutures at the antimesenteric and mesenteric borders.

3.4. End-to-end anastomoses are recommended because they respect bowel anatomy, making sure that intestinal flow is well kept, but for preventing postoperative digestive fistulae, side-to-side anastomoses are recommended as being the most accurate.

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# RESEARCH ON THE INFLUENCE OF ENVIROMENTAL FACTORS ON THE HEALTH OF RUMINANTS IN THE LOWER AREA OF THE OLT RIVER

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**Keywords:** dessertisation, ruminal geosedimentosis

**SUMMARY**

After abandonment of the facilities for plantations of rice in the lower Olt-Siiul-Danube meadow, in this area has established and extensive dessertisation, prevailing wind blew sand, depositen on pasture and very poorly developed vegetation, sand is ingested in grazing, especially in the rumen while making deposits, 30-60 kilo, causing paresis and even atonic of prestomach, especially at large ruminants.
For the prevention and removal phenomenon of geosedimentosis is recommended the elaboration an implementation a plan of an improvement agro-pedologycs area.

In the past 50 years, at the confluence with the river Siiul-Danube, suffered major geo-pedo-climatics change produced by the human intervention of socio-economic interests, as between the years 1959-1989 applying optimal programs use of soil were arranged 8-9000 hectares of plantations of rice with the result at improvet hydro situation and increasing humidity of the area. Amind these changes increased the incidence of soil diseases like parasitological etiology: fasciolosis, dicroceliosis, scabies, with a share of 70-80% of the total pathology areas.

After 1989 until the present crop type of the plantations of rice abandoned holdings, appreciate that unprofitable and requiring huge investment around the 8-9000 hectares, area has suffered from gradually draining a real desertisations.This phenomenon of sometimes desertisations manifested itself in the form of “loops” of quicksand. Very fine dry sand swept away by the wind and was filed on pasture and vegetable crops (fodder) adversely, influencing the development of vegetation, invaded increasingly frustrated over sand, uploading plants with sandy deposits. Many and large areas remained uncultivated because very low productions and do not justify the investment made for the planting, rountime maintenance works, there are no designs and facilities for irrigation in the whole area.

Underdeveloped and overwhelmed sandy grass was a new risk factor, changing the overall character of animal pathology area.

Ingested with grass “stunted”, sand, gravel, earth Malos, was filed on time in prestomach compartments of ruminants, mainly in the rumen and the previous portion of intestines, causing sablosis, geosedimentosis, sometimes accumulated 30-60 kilo geosediments on the ventral compartment of stomach, which by the action of physical and chemical factors decoy digestive tract mucosa, causing in finally atonal (paralysis) rumen, degradation functions of the digestive absortion of nutrients, degradation of the condition of the body, decreased production, low concentration, chectisations animals and death-exitus.

Diagnosis of this great suffering to the animal in life is difficult and the symptoms are incharacteristics: reduction of the production, dehydration, weight loss, continous cahectisations of animals leads to the death-exitus. The diagnosis is established in certain cases at the necropsy examination.

The situation found the only way to solve this pain is removing the causes that provoke the creation of a comprehensive program to stabilize the process of desertisations area, protection curtains, re-entry into the economic circuit of the area throught irrigation, fertilization and other works for stimulation of vegetations in general, with priority on pasture by creating a modern irrigation system from the storage tanks of the 4 hydropowers plants, removing themselves entirely to the negative effects on animals, also winning record and good quality productions per hectars.

## MATERIALS AND METHODS

The study was made mainly on large ruminants, sick, presented to veterinary medical consultations at the 7 Olt county veterinary clinics and the 7 Teleorman county veterinary clinics.

Accuracy early correct diagnosis, a history fair, accurate and fast medical measures, together with appropriate preventive measures leads to a rapid recovery and any negative effects worse injuries and lowering production, dehydration, weight loss and cahectisation continous leads to the death-exitus.


**Pig. 1 Pasture-desert area**

**Fig.2. Cow with paresis of prestomach-sablosis suffering**

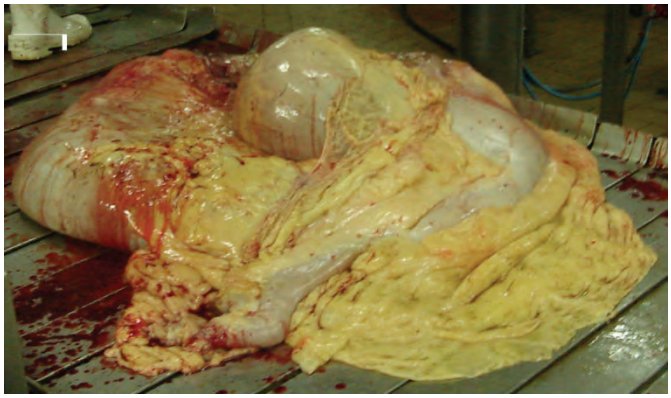

**Fig.3. Prestomach of cow**


**Fig.4. Sections of prestomach of cow, with accumulation of sand, geosediments**


**Fig.5. Sections of prestomach, mucous membrane lesions of various objects accumulated over time (nails, wire, sand, geosediments)**


**Fig.6. Sand, geosediments, 30-60 kilo**

## CONCLUSIONS

1. Excessive dessertisation by the effects shown above leads to geosedimentosis, which if diagnosed in early stage has a good prognosis provided that medical measures to be quickly put into practice, and prevention and preventive measures to be observed.

2. Prevention dessertisation area and avoid geosedimentosis, will be remedied by measures to improve soil and plant the crops of pasture feed.

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# RESEARCHES CONCERNING HEAVY METALS IN MILK

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**Key words:** heavy metals, milk, ICP-OES

**SUMMARY**

This study presents a short characterization of the heavy metals contamination in different milk samples. The concentration of aluminum, nickel, strontium, cadmium, barium and lead was determinate in 12 different milk samples by inductively coupled plasma-optical emission spectrometry (ICP-OES) after microwave digestion.
The results were subjected to ANOVA and Tukey Kramer statistical analysis.
The highest concentration was found for aluminum (0,056- 0,125 ppm) and the lowest for cadmium (0,002-0,021 ppm ).
Milk is one of the most important foodstuff with a high nutritional value and it is considered to be a complete nourishment because of the equilibrate reports between its elements.
The contamination with heavy metals of the milk represents a complex and actual issue, because milk is a product that accumulates an important quantity of toxic metals.
Heavy metals are chemical elements with high toxicity for plants, animals and humans. The toxic effect appears after a certain level under which some are essential components of proteins. [1,6].
In the first place, heavy metals are dangerous because of their bioaccumulation process at the tissues level by minimizing or blocking the intracellular biochemical processes. Their toxicity does not manifest immediately, only after a certain period when it is reached the toxic dose for the organism.
Secondly, in preparing food heavy metals do not decompose, contrary the concentration on mass unit increase.
Thirdly, the majority of heavy metals have mutagenic and carcinogenic properties.

## 1. MATERIAL AND METHODS

1.1. Sampling

For heavy metal analyse 12 samples of whole milk have been chosen and collected from different shops in Bucharest during March-May 2009.

Milk samples were kept in original packaging at a temperature of 4°C until analyze was completed.

Samples were analyzed independently with 3 measurements for each sample.

1.2. Samples analyse

In a 70 ml quartz vessel, 5 ml milk have been blent with 2 ml of $HNO_3$ and samples have been introduced in a digestion procedure using a microwave oven.[3]

Parameters for sample digestion are presented in Table 1[4]

***Table 1***

**Parameters for sample digestion**

| Heating stages | Initial temperature | Time | Final temperature |
|---|---|---|---|
| 1 | 40°C | 30 min | 110°C |
| 2 | 110°C | 30 min | 110°C |
| 3 | 110°C | 60 min | 250°C |
| 4 | 250°C | 90 min | 250°C |

After preparing the samples in microwave oven, they have been introduced in iCAP 6000 ICP- OES spectrometer.

ICP-OES operating conditions are presented in Table 2. [2]

***Table 2***

**ICP-OES operating conditions**

| Parameter | Operation condition |
|---|---|
| Generator Free-running at | 27.12 MHz |
| RF power | Kw 1.5 |
| Spray chamber | cyclonic |
| Outer gas/L min21 12 | 14 L/min |

Standard solution ICP-OES MERCK (1000 mg/ l) containing aluminum, boron, barium, cadmium, chromium, copper, iron, lithium, magnesium, manganese, nickel, lead, selenium, strontium, zinc was used to draw the calibration curve of the spectrometer.

## 2. RESULTS AND DISCUSSIONS

Each sample was subjected to three measurements.

In Table 3, there are presented the mean value obtained for each sample.

*Table 3*

**Main concentration (ppm) of Al, Ni, Sr, Cd, Ba, Ld in each milk sample and their standard deviation**

| Sample | **Al** | **Ni** | **Sr** | **Cd** | **Ba** | **Ld** |
|---|---|---|---|---|---|---|
| 1 | 0,101± 0.002 | 0,005± 0,0001 | 0,017± 0,001 | 0,009± 0.001 | 0,077± 0,001 | 0,084± 0.001 |
| 2 | 0,056± 0.001 | 0,009± 0,0001 | 0,018± 0,001 | 0,002± 0.0005 | 0,077± 0,001 | 0,044± 0.001 |
| 3 | 0,075± 0.001 | 0,010± 0,0001 | 0,036± 0.001 | 0,013± 0.0005 | 0,082± 0,001 | 0,033± 0.008 |
| 4 | 0,064± 0.002 | 0,023± 0,001 | 0,021± 0,001 | 0,021± 0.001 | 0,078± 0,003 | 0,026± 0.001 |
| 5 | 0,116± 0.007 | 0,037± 0,001 | 0,009± 0,001 | 0,021± 0.001 | 0,090± 0,005 | 0,062± 0.003 |
| 6 | 0,106± 0.002 | 0,036± 0,001 | 0,025± 0,001 | 0,009± 0.0005 | 0,046± 0,001 | 0,091± 0.002 |
| 7 | 0,125± 0.003 | 0,018± 0,0005 | 0,026± 0,001 | 0,007± 0,0001 | 0,100± 0,002 | 0,057± 0.001 |
| 8 | 0,075± 0.002 | 0,039± 0,001 | 0,036± 0,002 | 0,008± 0,0001 | 0,053± 0,001 | 0,027± 0.007 |
| 9 | 0,061± 0.001 | 0,029± 0,010 | 0,020± 0,006 | 0,016± 0,0005 | 0,046± 0,006 | 0,040± 0.001 |
| 10 | 0,068± 0.013 | 0,019± 0,011 | 0,030± 0,002 | 0,020± 0,002 | 0.051± 0,010 | 0,045± 0.001 |
| 11 | 0,093± 0.004 | 0,031± 0,006 | 0,027± 0,011 | 0,018± 0,010 | 0,041± 0,0005 | 0,051± 0.003 |
| 12 | 0,098± 0.002 | 0,017± 0,010 | 0,014± 0,001 | 0,006± 0,001 | 0,052± 0,019 | 0,037± 0.005 |

A statistical analysis was performed using JMP 6.0 software which is a part of SAS program.

The method used to identify differences between the three measurements for each milk sample was ANOVA analysis.

Tukey-Kramer analysis was used to compare the mean concentration of the elements for each sample and to determinate significative difference between them.

**Aluminium**

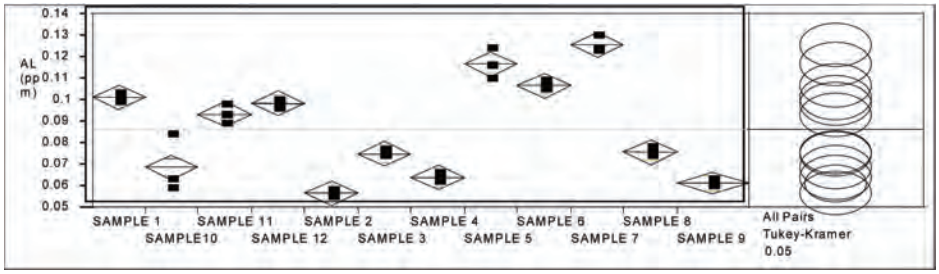


**Figure 1 Graphical representation of ANOVA and Tukey Kramer analysis for aluminum**

The statistical analysis has found that:

-samples 7 and 5 are not statistically different

-samples 1, 12 and 11 are not statistically different

-samples 8, 3, 10 and 4 are not statistically different

- samples 9 and 2 are not statistically different

The decreasing order of the concentrations of aluminum in milk samples is:

sample 7> sample 5> sample 6 > sample 1> sample 12> sample 11> sample 8> sample 3> sample 10> sample 4> sample 9> sample 2.

It was found that the maximum concentration of aluminum can vary in the range of 0.123 and 0.130 (sample 7), and minimum values may vary within 0.055 and 0.058 (sample 2).

**Nickel**

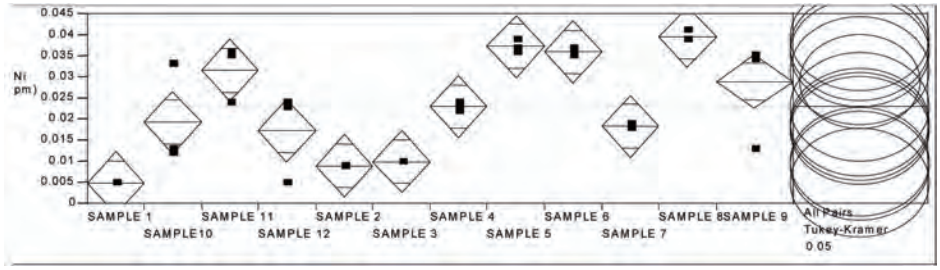


**Figure 2 Graphical representation of ANOVA and Tukey Kramer analysis for nickel**

The statistical analysis has found that:

- samples 8, 5, 11, 9 and 4 are not statistically different

- samples 3, 2 and 1 are not statistically different

The decreasing concentrations of nickel in milk samples is:

sample 8> sample 5> sample 6> sample 11> sample 9> sample 4> sample 10> sample 7> sample 12> sample 3> sample 2> sample 1.

It was found that the maximum concentration of nickel may vary in the range 0.039 and 0.041 (sample 8), and minimum values may vary in the range 0.049 and 0.050 (sample 1).

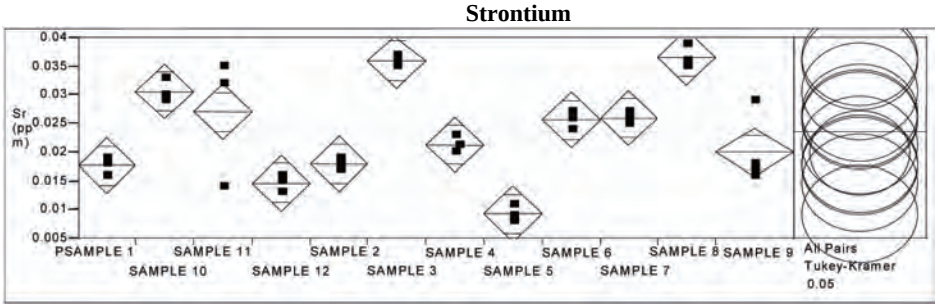


**Figure 3 Graphical representation of ANOVA and Tukey Kramer analysis for strontium**

The statistical analysis has found that:

- samples 8, 3, 10, 11, 7 and 6 are not statistically different
- samples 4 and 9 are not statistically different
- samples 2 and 1 are not statistically different

The decreasing concentrations of strontium in milk samples is:

sample 8> sample 3> sample 10> sample 11> sample 7> sample 6> sample 4> sample 9> sample 2> sample 1> sample 12> sample 5.

It was found that the maximum concentration of strontium may vary in the range 0.035 and 0.039 (sample 8), and minimum values may vary in the range 0.008 and 0.011 (sample 5).

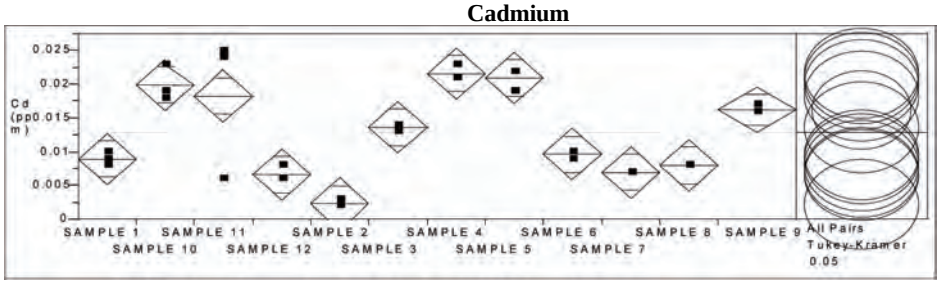


**Figure 4 Graphical representation of ANOVA and Tukey Kramer analysis for cadmium**

The statistical analysis has found that:

- samples 4, 5 and 10 are not statistically different
- samples 9, 3, 6, 1 and 8 are not statistically different
- samples 7 and 12 are not statistically different

The decreasing concentrations of cadmium in milk samples is:

sample 4> sample 5> sample 10> sample 11> sample 9> sample 3> sample 6> sample 1> sample 8> sample 7> sample 12> sample 2.

It was found that the maximum concentration of cadmium may vary in the range 0.021 and 0.023 (sample 4), and minimum values may vary in the range 0.002 and 0.003 (sample 2).

**Barium**

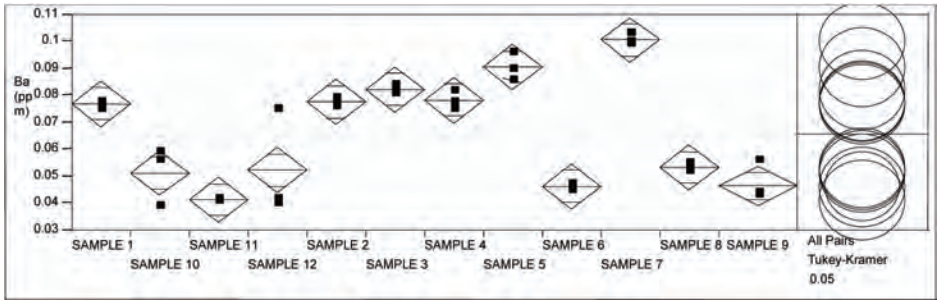


**Figure 5 Graphical representation of ANOVA and Tukey Kramer analysis for barium**

The statistical analysis has found that:

- samples 5, 3, 4, 2 and 1 are not statistically different
- samples 8, 12, 10, 9, 6 and 11 are not statistically different

The decreasing concentrations of barium in milk samples is:

sample 7> sample 5> sample 3> sample 4> sample 2> sample 1> sample 8> sample 12> sample 10> sample 9> sample 6> sample 11.

It was found that the maximum concentration of barium may vary in the range 0.099 and 0.103 (sample 7), and minimum values may vary in the range 0.039 and 0.042 (sample 11).

**Lead**

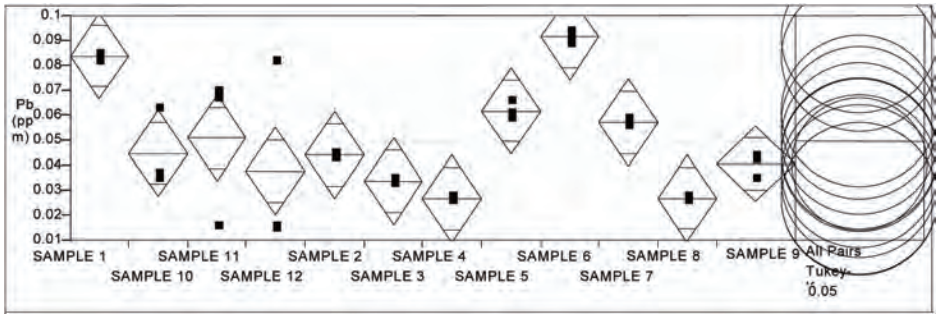


**Figure 6 Graphical representation of ANOVA and Tukey Kramer analysis for lead**

The statistical analysis has found that:

- samples 6 and 1 are not statistically different
- samples 5, 7, 10, 11 and 2 are not statistically different
- samples 9, 12, 3, 8 and 4 are not statistically different

The decreasing concentrations of lead in milk samples is:

sample 6> sample 1> sample 5> sample 7> sample 11> sample 10> sample 2> sample 9> sample 12> sample 3> sample 8> sample 4.

It was found that the maximum concentration of lead may vary in the range 0.089 and 0.092 (sample 6), and minimum values may vary in the range 0.026 and 0.028 (sample 4).

Regulation (EC) No 1881/2006 established the maximum permissible limit of lead in milk at the value of 0,020 ppm [5].

The results show that all samples have values above the Regulation (EC) No 1881/2006 of 0,020 ppm.

## 3. CONCLUSIONS

3.1. The heavy metals from milk could not be evaluated in comparison to the maximum permissible limits, with the exception of lead, because there are no standards for milk concerning these dangerous metallic elements.

3.2. The results show that there are significant differences between the heavy metals concentrations in the samples.

3.3. The levels of heavy metals may be reduced by taking in account the management of quality in handling practices and processing of raw materials.

3.4. Because of the complexity of interactions in human body it is hard to evaluate the risk of heavy metals for health.

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# THE VARIANCE OF SOME MICROELEMENTS IN MILK DETERMINED BY ICP-OES

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**Key words:** microelements, milk, ICP-OES

**SUMMARY**

The objective of this research was to determinate the concentration of some microelements in different milk samples. The concentration of chromium, manganese, iron, zinc and selenium was evaluated in 12 different milk samples by inductively coupled plasma-optical emission spectrometry (ICP-OES) after microwave digestion.
The results were subjected to ANOVA and Tukey Kramer statistical analysis.
The highest concentration was found for zinc (0.147-0.442 ppm) and the lowest for manganese (0.009-0.019 ppm ).
Milk is considered to be an important source of essential microelements for human nutrition.
The studies performed in the last years determinate the researchers to establish recommendable concentrations of microelements in milk. [1,7]. These concentrations are: 0,3 ppm for iron; 0,03 ppm for selenium; 0,03 ppm for manganese; 0.02 ppm for chromium. [7]
These microelements have vital functions in human body such as:
chromium is a cofactor of enzymes involved in protein, carbohydrate and lipid metabolism; manganese participates in bone mineralization; deficiencies in zinc metabolism is the main cause of appearance of diabetes.
[1,3,6].

## 1. MATERIAL AND METHODS

1.1. Sampling

For microelements analyse 12 samples of whole milk have been chosen and collected from different shops in Bucharest during March-May 2009.

Milk samples were kept in original packaging at a temperature of 4°C until analyze was completed.

Samples were analyzed independently with 3 measurements for each sample.

1.2. Samples analyse

In a 70 ml quartz vessel, 5 ml milk have been blent with 2 ml of $HNO_3$ and samples have been introduced in a digestion procedure using a microwave oven.[4].

Parameters for sample digestion are presented in Table 1[5].

*Table 1*

**Parameters for sample digestion**

| Heating stages | Initial temperature | Time | Final temperature |
|---|---|---|---|
| 1 | 40°C | 30 min | 110°C |
| 2 | 110°C | 30 min | 110°C |
| 3 | 110°C | 60 min | 250°C |
| 4 | 250°C | 90 min | 250°C |

After preparing the samples in microwave oven, they have been introduced in iCAP 6000 ICP- OES spectrometer.

ICP-OES operating conditions are presented in Table 2. [2]

Table 2

ICP-OES operating conditions

| Parameter | Operation condition |
|---|---|
| Generator Free-running at | 27.12 MHz |
| RF power | Kw 1.5 |
| Spray chamber | cyclonic |
| Outer gas/L min21 12 | 14 L/min |

Standard solution ICP-OES MERCK (1000 mg/ l) containing aluminum, boron, barium, cadmium, chromium, copper, iron, lithium, magnesium, manganese, nickel, lead, selenium, strontium, zinc was used to draw the calibration curve of the spectrometer.

## 1. RESULTS AND DISCUSSIONS

Each sample was subjected to three measurements.

In Table 3, there are presented the mean value obtained for each sample.

*Table 3*

**Main concentration (ppm) of Cr, Mn, Fe, Zn and Se in each milk sample and their standard deviation**

| Sample | **Cr** | **Mn** | **Fe** | **Zn** | **Se** |
|---|---|---|---|---|---|
| 1 | 0.008± 0.0005 | 0.014± 0.0005 | 0.057± 0.0005 | 0.147± 0.004 | 0.006± 0.001 |
| 2 | 0.007± 0.0005 | 0.010± 0.0001 | 0.034± 0.001 | 0.302± 0.007 | 0.005± 0.0005 |
| 3 | 0.008± 0.0001 | 0.011± 0.0005 | 0.058± 0.002 | 0.204± 0.005 | 0.005± 0.0005 |
| 4 | 0.023± | 0.012± | 0.056± | 0.164± | 0.005± |

| | | | | | |
|---|---|---|---|---|---|
| | 0.0005 | 0.0005 | 0.002 | 0.007 | 0.0005 |
| 5 | 0.020± 0.001 | 0.017± 0.001 | 0.070± 0.004 | 0.221± 0.011 | 0.004± 0.0005 |
| 6 | 0.021± 0.0001 | 0.016± 0.0005 | 0.076± 0.001 | 0.278± 0.005 | 0.005± 0.0005 |
| 7 | 0.018± 0.0005 | 0.019± 0.0005 | 0.101± 0.002 | 0.442± 0.010 | 0.005± 0.0005 |
| 8 | 0.017± 0.0005 | 0.009± 0.0005 | 0.054± 0.002 | 0.244± 0.007 | 0.004± 0.0005 |
| 9 | 0.022± 0.003 | 0.013± 0.0005 | 0.064± 0.007 | 0.241± 0.006 | 0.005± 0.0005 |
| 10 | 0.017± 0.0005 | 0.012± 0.002 | 0.058± 0.006 | 0.230± 0.027 | 0.005± 0.0005 |
| 11 | 0.019± 0.001 | 0.011± 0.0005 | 0.066± 0.007 | 0.216± 0.005 | 0.236± 0.400 |
| 12 | 0.015± 0.006 | 0.012± 0.001 | 0.056± 0.001 | 0.188± 0.040 | 0.434± 0.374 |

A statistical analysis was performed using JMP 6.0 software which is a part of SAS program. The method used to identify differences between the three measure-ments for each milk sample was ANOVA analysis.

Tukey-Kramer analysis was used to compare the mean concentration of the elements for each sample and to determinate significative difference between them.

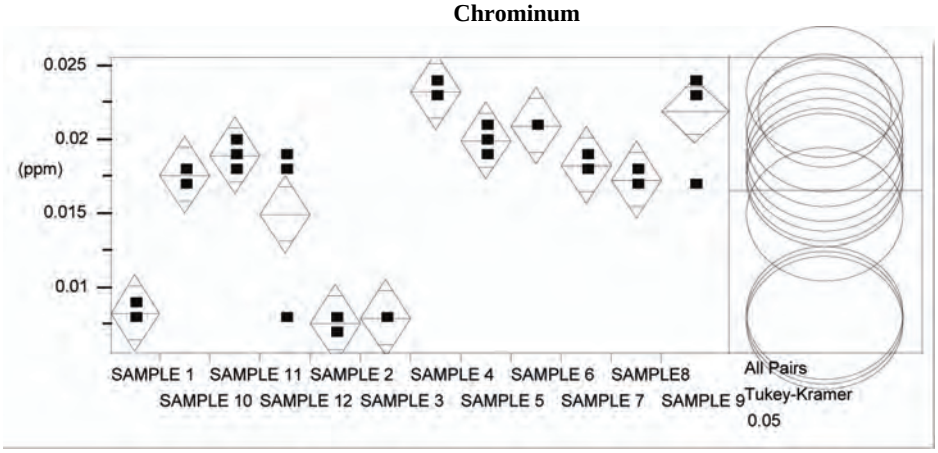


**Figure 1 Graphical representation of ANOVA and Tukey Kramer analysis for chromium**

The statistical analysis has found that:

-samples 4 and 9 are not statistically different

-samples 5, 6, 11, 7, 10, 8 and 12 are not statistically different

-samples 1, 2 and 3 are not statistically different

The decreasing order of the concentrations of chromium in milk samples is:sample 4> sample 9> sample 6 > sample 5> sample 11> sample 7> sample 10> sample 8> sample 12> sample 1> sample 3> sample 2. It was found that the maximum concentration of chromium can vary in the range of 0.021 and 0.024 (sample 4), and minimum values may vary within 0.006 and 0.009 (sample 2).

The standard value for chromium is 0,02 ppm [7]; the analyzes show that all samples 1, 2, 3, 5, 7, 8, 10, 11 and 12 are under this value.

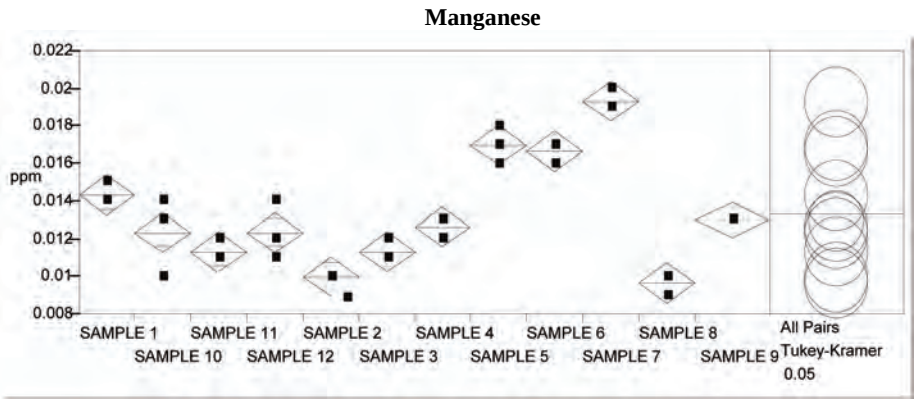


**Figure 2 Graphical representation of ANOVA and Tukey Kramer analysis for manganese**

The statistical analysis has found that:

- samples 7 and 5 are not statistically different
- samples 4, 9 , 10 and 12 are not statistically different
- samples 11 and 3 are not statistically different

The decreasing concentrations of manganese in milk samples is:sample 7> sample 5> sample 6> sample 1> sample 9> sample 4> sample 10> sample 12> sample 11> sample 3> sample 2> sample 8.

It was found that the maximum concentration of manganese may vary in the range 0.017 and 0.020 (sample 7), and minimum values may vary in the range 0.008 and 0.011 (sample 8).

The standard value for manganese is 0,03ppm [7]; the analyzes show that all samples are under this value.

**Iron**

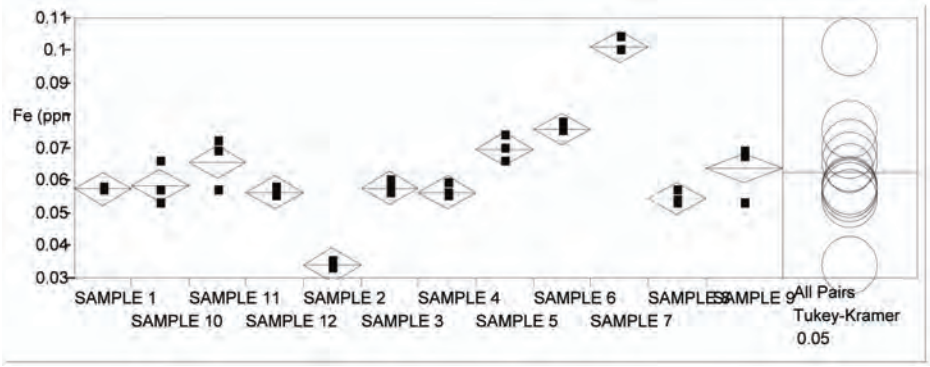


**Figure 3 Graphical representation of ANOVA and Tukey Kramer analysis for iron**

The statistical analysis has found that:

- samples 6, 5 and 11 are not statistically different
- samples 9, 10, 3 and 1 are not statistically different
- samples 12, 4 and 8 are not statistically different

The decreasing concentrations of iron in milk samples is:

sample 7> sample 6> sample 5> sample 11> sample 9> sample 10> sample 3> sample 1> sample 12> sample 4> sample 8> sample 2.

It was found that the maximum concentration of iron may vary in the range 0.095 and 0.107 (sample 7), and minimum values may vary in the range 0.031 and 0.037 (sample 2).

The standard value for iron is 0,3 ppm [7]; the analyzes show that all samples are under this value.

**Zinc**

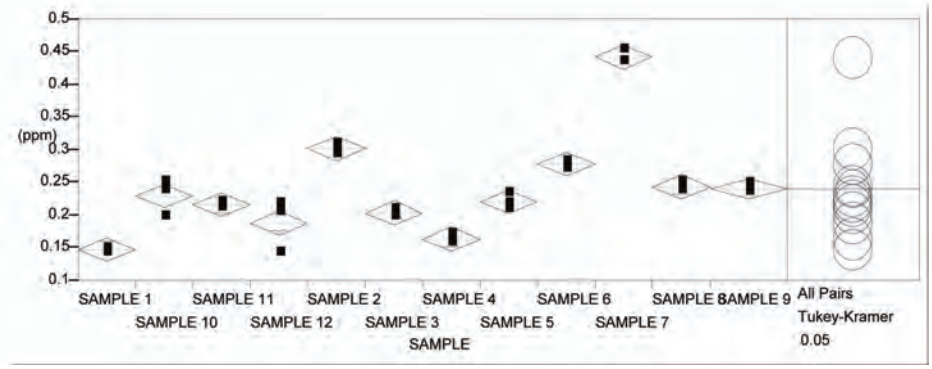


**Figure 4 Graphical representation of ANOVA and Tukey Kramer analysis for zinc**

The statistical analysis has found that:

- samples 2, 6 and 8 are not statistically different

- samples 10, 5, 11 and 3 are not statistically different
- samples 4 and 1 are not statistically different

The decreasing concentrations of zinc in milk samples is:

sample 7> sample 2> sample 6> sample 8> sample 9> sample 10> sample 5> sample 11> sample 3> sample 12> sample 4> sample 1.

It was found that the maximum concentration of zinc may vary in the range 0.417 and 0.467 (sample 7), and minimum values may vary in the range 0.137 and 0.157 (sample 1).

**Selenium**

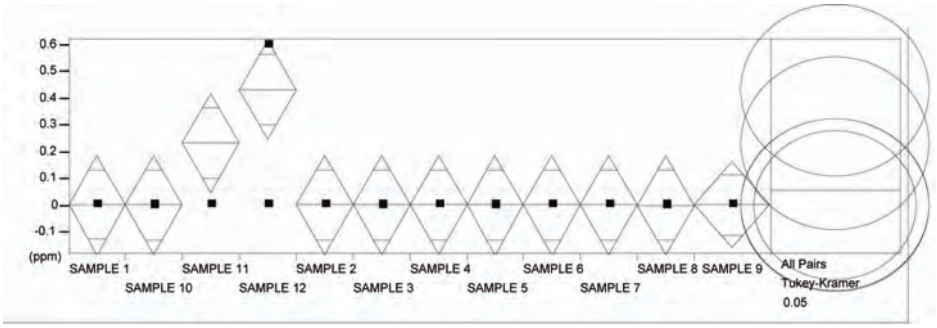


**Figure 5 Graphical representation of ANOVA and Tukey Kramer analysis for selenium**

The statistical analysis has found that:

- samples 2, 6 and 8 are not statistically different
- samples 10, 5, 11 and 3 are not statistically different
- samples 4 and 1 are not statistically different

The decreasing concentrations of selenium in milk samples is:

sample 12> sample 11> sample 1> sample 2> sample 4> sample 7> sample 9> sample 10> sample 6> sample 3> sample 5> sample 8.

It was found that the maximum concentration of barium may vary in the range 0.417 and 0.451 (sample 12), and minimum values may vary in the range 0.0029 and 0.0058 (sample 8).

The standard value for selenium is 0,03 ppm [7]; the analyzes show that only samples 11 and 12 are over this value.

## 3. CONCLUSIONS

3.1. The microelements concentration, with the exception of zinc, could be evaluated in comparison with the standard values recommended by USDA (United States Department of Agriculture).

3.2. The results show that there are significant differences between the microelements concentrations in the samples.

3.3. Chromium, manganese, iron, zinc and selenium are important elements for human metabolism; without them occur serious disturbance of protein, lipid or carbohydrate metabolism.

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# DETERMINATION OF HEAVY METALS CONTENT OF MEAT AND MEAT BY-PRODUCTS BY USING NEUTRON ACTIVATION ANALYSIS AND ATOMIC ABSORPTION SPECTROMETRY

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**Key-words:** heavy metals, meat, meat products, atomic spectroscopy

**SUMMARY**

By using neutron activation analysis and atomic absorption spectrometry, there were determined the following elements: As, Cd, Co, Cr, Fe, Hg, Ni, Pb, Sb and Zn from meat, intestine and liver of cow and goat, as well as in broiler and local breed chicken. Mercury was first separated by radiochemical techniques. The results revealed that the essential elements studied (i.e. Cr, Cu, Fe, Zn, Co and Ni) had a higher concentration in liver and intestine than in the meat, but these levels were in normal ranges. Meanwhile, the toxic elements, As, Cd and Pb were impossible to detect in all the prelevated samples.

The metallic elements play various roles in the living organisms, being implied in the structure of the tissues, being part of the control mechanisms (as in nerves or muscles), and mostly as an activator of the enzymes, or a part of the redox systems. Several of the metals, like Co, Cr, Cu, Fe, Ni and Zn are considered essential. In the interim,others, like As, Cd, Hg and Pb are non-essential. The essential elements' deficiency provokes deterioration of the biological functions, but at the same time, an excess of those elements brings about a toxic status, as the main route of access in the organism of these elements is through nourishment. The primary sources of metals in the environment are considered to be: the mining industries, the waste discarding, the household sewage and the combustion of the fossil fuels. Other sources are included also, but with a minor importance, like farming and forestry, through fertilizers and pesticides. The metals content of meat (pig, beef), intestines, liver and chicken is due to the feed that they receive and consume.

The main purpose of this study is to establish wether the levels of heavy metals in meat, liver, intestine, broiler and local breed chicken destined for human consumption surmounts the maximum allowed level comprised in the international rules.

The elements taken into consideration in this research were: As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Sb, Se and Zn. The atomic absorption

spectrometry was used to determine the level of Cd, Cu, Ni and Pb, while neutron activation analysis was used for the other metallic elements. Mercury was first separated from selenium by radiochemical techniques.

## 1. MATERIALS AND METHODS

The samples, prelevated from pork and beef (meat, intestine), broiler and local breed chicken, were obtained from local markets, in a number of 63, each weighing about 1-2 kg. They were introduced in plastic bags and transferred to the laboratory, where they were cleaned and washed using demineralized water, before cooking, cut into small pieces using a stainless steel knife and exposed to drying in an oven, at 65$^{o}$C, for 48 hours, using a discontinuous method of drying, until obtaining a constant weight (see Table 1 for the moisture content of the samples). After drying, the samples were transformed into powder, and a quantity of 200-500 mg of each sample was distributed into polyethylene vials. The standard solution was prepared using 5 μl of fresh standard solution (1000 ppm) of each element, on a Whatman filter paper, and subsequently, all the vials (samples, standard and reference standard solutions) were put in an aluminium container.

***Table 1***

**Samples' moisture contents**

| ***Samples*** | **Beef** | | | **Pork** | | | **Poultry** | |
|---|---|---|---|---|---|---|---|---|
| | *Meat* | *Liver* | *Intestine* | *Meat* | *Liver* | *Intestine* | *Broiler* | *Local Breed Chicken* |
| ***Moisture contents (%)*** | 78 | 72 | 80 | 78 | 73 | 81 | 75 | 79 |

### Instrumental Neutron Activation Analysis (INAA) step

The containers with samples and standard solutions were exposed to irradiation for 36 hours in Triga Mark II Reactor, with a flow of $10^{11}$ n $cm^{-2}$ $sec^{-1}$. Following the irradiation, the containers were cooled for a period of 2 days, for short half life radionuclide and 2 weeks for long half life radionuclide. The counting of the samples and standards was performed using a Nuclear Data 62 Multi Channel Analyser, containing a high purity Germanium detector. The resolution of the system is 3.75 keV at 1332 keV of $^{60}$Co, and the time consumed for counting was of

900 seconds. The nuclear data of the elements Se, Hg, Cr, As, Sb, Zn and Co is shown in Table 2.

**Radiochemical Separation step**

The irradiated samples were subjected to digestion using $HNO_3$ in a flask, and heated until the apparition of white fumes. Because the samples were formed by organic materials, a quantity of $H_2O_2$ was added (30%), followed by a cooling step, reaching the room temperature, and dilution to 20 ml, attaining a pH value of 2-4. As a carrier, Hg was added to the solution formed from the samples, and also a quantity of 4 ml of $8x\ 10^{-4}$ M $Pb(DDC)^2$, shaking it afterwards for 10 minutes. Using a Multi Channel Analyzer, at a gamma ray energy of 279 keV for $^{203}Hg$, the organic phase, formed by $Hg(DDC)_2$ was separated and counted.

***Table 2***

**Nuclear data for the investigated elements**

| **Elements** | **Se** | **Hg** | **Cr** | **As** | **Sb** | **Zn** | **Co** |
|---|---|---|---|---|---|---|---|
| **Nuclear reaction** | $^{74}Se$ (n, γ) $^{74}Se$ | $^{202}Hg$ (n, γ) $^{202}Hg$ | $^{50}Cr$ (n, γ) $^{50}Cr$ | $^{75}As$ (n, γ) $^{75}As$ | $^{121}Sb$ (n, γ) $^{121}Sb$ | $^{64}Zn$ (n, γ) $^{64}Zn$ | $^{59}Co$ (n, γ) $^{59}Co$ |
| **Energy keV** | 264 | 279 | 320 | 559 | 564 | 1115 | 1173 |
| **t ½** | 128.4 days | 46 days | 27.8 days | 26.8 hours | 2.76 days | 245 days | 5.2 years |

**Determination by Atomic Absorption Spectrometry step**

In a distilling flask, a quantity of 5-10 g from the dried samples was digested along with a mixture of concentrated $H_2SO_4$ and $HNO_3$ (1:3 v/v). In order to eliminate nitrogen dioxides, there was added a small amount of $H_2O_2$ (30%), and the digestion procedure continued until the solution became clear. Subsequently, the samples were transferred into a volumetric flask and diluted to 50 ml, with demineralized water. Elements like lead, cadmium and copper were ignited usign an oxygen actehylene flame. The absorption of the studied elements was compared with the standard one.

## 2. RESULTS AND DISCUSSION

The results obtained following this research are included in Table 3. The value of photopeaks for $^{51}$Cr at 20 keV, $^{75}$Se at 264 keV, $^{76}$As at 559 keV and $^{60}$Co at 1173 keV were used in order to avoid any interference from other isotopes. The photopeak for $^{203}$Hg was determined at 279 keV, without any interference, after being separated from selenium, using radiochemical separation methods. Elements as As, Cd and Pb were impossible to detect, due to the fact that their concentration was lower than the value of the detection limit (see Table 4), albeit Ni was detected only in several samples, as cow liver and intestine and local breed chicken. The other elements (Co, Cr, Cu, Fe, Hg, Sb, Se and Zn) were able to be detected in all the analyzed samples.

In liver, intestine and local breed chicken, Co, Cu, Cr, Fe and Zn presented higher concentrations. These metallic elements accumulate in liver, kidney, muscle, intestine and other organs, because liver has the function of transferring these elements to the whole body. Chromium has also considered an essential element, along with Zn and Cu, as it has a physiological role in the body, being a cofactor for insulin, in the insuline responsive cell membrane. The concentration of Cr in various food is generally 0.5 μg/g wet weight, while the concentration of Cr in the analyzed samples was 0.035-0.45 μg/g wet weight. Copper, another important element stored in the liver, kidney and muscle molecular weight protein and ceroplasmin, produced in the liver, functions also as an oxidase. Its concentration in food is established at 20-50 μg/g, while the concentration value obtained in this study was 0.09-26.85 μg/g. In kidney, liver and ham, the concentration value of iron is of 30-150 mg/kg, compared to the value obtained in this study, by analyzing the samples, of 5.33-37.24 μg/g.

Essential for the function of several mammalian enzymes, zinc has its concentration in food of 10-50 μg/g, while the concentration value obtained in this study was about 0.86-27.64 μg/g.

Included in vitamin B12, cobalt is broadly distributed in the animal organs, with a high value of concentration (0.10-0.25 μg/g) in liver, kidney, bones, spleen and other glandular tissue. The concentration of Co in the analyzed samples were still in the normal range.

Nickel is considered essential for poultry and pigs, in experimental conditions. Ni is weakly absorbed from usual diets, but when this happens, it is accumulated in liver, kidney and lungs.

Sheep, cattle and poultry need selenium, as the deficiency of this element causes serious problems. The highest concentrations of Se are

found in liver, kidney, brain and muscle. The concentration of Se in food products is determined bu its origin and the processing method. The analyzed samples revealed a concentration of Se less than 2 μg/g.

Antimony is not an essential metallic element, usually found as deposits in kidney and liver. After performing the analysis of the samples, the concentration values of Sb were higher in liver and intestine, as compared with meat concentration value.

***Table 3***

**Concentration of heavy metals in meat, intestine, liver of beef, pork, broiler and local breed chicken (μg/g)**

| ***Samples/ Elements*** | **Beef** | | | **Pork** | | | **Poultry** | |
|---|---|---|---|---|---|---|---|---|
| | ***Meat*** | ***Liver*** | ***Intestine*** | ***Meat*** | ***Liver*** | ***Intestine*** | ***Broiler*** | *Local Breed Chicken* |
| **Co** | 0,12 ± 0,03 | 0,13 ± 0,03 | 0,10 ± 0,03 | 0,15 ± 0,04 | 0,16 ± 0,04 | 0,13 ± 0,04 | 0,86 ± 0,03 | 0,12 ± 0,02 |
| **Cr** | 0,16 ± 0,14 | 0,25 ± 0,08 | 0,10 ± 0,06 | 0,08 ± 0,03 | 0,13 ± 0,05 | 0,45 ± 0,02 | 0,04 ± 0,01 | 0,13 ± 0,03 |
| **Cu** | 0,44 ± 0,33 | 10,64 ± 6,46 | 0,25 ± 0,08 | 0,31 ± 0,18 | 26,85 ± 9,77 | 0,27 ± 0,19 | 0,13 ± 0,07 | 0,17 ± 0,07 |
| **Fe** | 7,59 ± 4,70 | 16,64 ± 6,46 | 25,82 ± 6,14 | 23,11 ± 5,11 | 37,24 ± 3,29 | 17,72 ± 1,58 | 5,33 ± 1,71 | 18,84 ± 5,30 |
| **Hg** | 0,001 ± 0,0005 | 0,002 ± 0,0006 | 0,0004 ± 0,0002 | 0,00 ± 0,00 | ND | 0,0012 ± 0,0006 | 0,0010 ± 0,0002 | 0,0006 ± 0,0001 |
| **Ni** | ND | 0,16 ± 0,13 | 2,96 ± 1,04 | ND | ND | ND | ND | 0,10 ± 0,06 |
| **Sb** | 0,02 ± 0,01 | 0,03 ± 0,01 | 0,04 ± 0,01 | 0,03 ± 0,01 | 0,53 ± 0,30 | 0,01 ± 0,00 | 0,10 ± 0,06 | 0,01 ± 0,00 |
| **Se** | 0,09 ± 0,03 | 0,09 ± 0,04 | 0,11 ± 0,06 | 0,88 ± 0,03 | 0,06 ± 0,02 | 0,05 ± 0,03 | 0,05 ± 0,03 | 0,06 ± 0,03 |
| **Zn** | 10,35 ± 2,53 | 12,83 ± 5,88 | 2,97 ± 1,04 | 9,81 ± 5,43 | 12,67 ± 6,63 | 1,97 ± 0,41 | 0,86 ± 0,40 | 2,17 ± 0,55 |

*Table 4*

**Limit of detection for the analysed elements**

| ***Elements*** | **As** | **Cd** | **Co** | **Cu** | **Cr** | **Fe** | **Hg** | **Ni** | **Pb** | **Sb** | **Se** | **Zn** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***LOD (µg/g)*** | 0,8 | 0,2 | 0,2 | 0,5 | 0,1 | 1,0 | 0,05 | 1,0 | 2,0 | 0,1 | 0,1 | 0,2 |
| ***Method*** | NAA | AAS | NAA | AAS | NAA | NAA | NAA | AAS | AAS | NAA | NAA | NAA |

## 3. CONCLUSION

1. The concentration values of toxic and essential elements in the samples analyzed were lower than the values of maximum bearing capacities (MBC).
2. The final conclusion is that the samples were not contaminated with toxic and essential metallic elements.
3. The concentration values of Co, Cr, Cu, Hg, Sb, Se and Zn were comprised in the natural normal range.

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# THE COMPARATIVE STUDY OF SOME ISOLATION METHODS OF PERIOSTEAL CANINE CELLS

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**Key words**: isolation, subculture, periosteal cells, dog

**SUMMARY**

This study has as objective the determination of the most efficient methods for the isolation of the periosteal cells because there is little information in this direction. The research compares two methods for isolateing the cells from the periosteum fragments: the first is the explant technique and the second is the enzymatic digestion of the periosteum fragment. The explant technique supposes a period of four days of subculture in the DMEM medium before the cells can be isolated, while the digestion technique is more rapider the isolation the morphological characters of the cells are the same fibroblast like cells, but after a few days of cultivation of the isolated cells in DMEM medium,the density is higher in the wells with cells isolated from explant.

The periosteum is a specialised connective tissue forming the outer lining of long bones and is intimately involved in driving the cell differentiation processes of bone development and repair (Orwoll, 2003). A number of studies have aimed to utilize this potential, using either periosteal explants or isolated cells to generate bone (Hutmacher and Sittinger, 2003). We still lack a well-defined protocol for the isolation and expansion of canine periosteal cells to make these techniques available for clinical purposes. Thus, the aim of this study was to isolate periosteal cells derived from adult dog donors using two techniques: the explant technique and the enzymatic digestion for obtaining a monolayer culture of periosteal cells and to observe the morphology of cell populations during the subculture and culture periods.

## 1. MATERIAL AND METHOD

The study was carried out on four dogs (table 1), of common breed, from which, under general anesthesia (acepromasine – ketamine - isofluran), the periosteum was harvested. The periosteal explants were detached from the middle femoral diaphysis, from both legs, the scraps

having approximatel 10x20 mm in dimension. After the harvesting, the scraps were washed with Ringer solution containing 0.5% penicillin and streptomycin, and then introduced in plates with dishes, with Ringer solution and antibiotic, and shipped for processing to the Imunophysiology and Biotechnologies Centre of the University of Medicine and Pharmacy „Victor Babeş" Timişoara.

***Table 1.***

**Physical parameters of the animals from which the periosteumwas harvest**

| Crt. no. | Weight (kg) | Age (years) | Sex |
|---|---|---|---|
| 1 | 23,5 | 3 | male |
| 2 | 19 | 3½ | male |
| 3 | 17 | 3 | female |
| 4 | 25 | 4 | male |

Half the number of the periosteal scraps were cut in fragments of 10x5mm (0.5cm$^2$) and used subsequently,unmodified, being considered explants, while the others were minced and underwent the artificial digestion.

1. The isolation technique of the periosteal cells from the explants

The explants were washed with PBS and placed in Petri dishes, with the osteogenic belt in contact with the dish. They were maintained for 10 minutes at 37$^0$C in the incubator, for assuring the initial adherence. The adherent scraps were covered with a sufficient DMEM nutritive medium, so their floating may not be possible.

The isolation of cells from the explants means the subcultivation for four days in a DMEM nutritive medium.

After the proliferation of the cells limit of the periosteal scrap, in all the space available, the explant was removed and the cellular layer was tryipsinized and neutralised with fetal bovine serum and centrifugated (1000 rpm, 10 minutes), thus obtaining the isolation of the periosteal cells in day five, day in which there was also performed the morphological examination of the cells.

2. The method of periosteal cells isolation through digestion

The pieces of minced periosteum were treated with 0.1% EDTA and 0.2% trypsin for one hour at 37$^0$C. The mixture was washed three times with PBS and subjected to digestion with 0.2% collagenase (type 1, of bacterial origin *"Clostridium histolyticum"*, SIGMA) at 37$^0$C for three

hours in digestion rooms. The resulted fragments were put into in sterile test tubes of 5 ml and covered with approximately 1 ml of collagenase.

Each vial with collagenase was reconstituted with 5 ml. of PBS, and the resulting solution was filtered (0.22 μ filter). The use of this type of filter led to the sterilization of the solution.

After the completion of the digestion, the resulting cell suspensions were filtered (70 μ filter), put into tubes and supplemented up to 20 ml with PBS (first wash).

The diluted cellular suspensions were centrifuged at 1500 rpm for 10 minutes. After this most of the supernatant (approximately 19 ml) was removed. The resulted button from cell sedimentation was homogenized mechanically with the retained supernatant, and on this cellular suspension being added PBS up to 20 ml (second wash).

Cellular suspensions were subjected to a new centrifugation at 1500 rpm for 10 minutes. After the centrifugation all the supernatant was removed and the sedimented cells were PBS washed three times and were mechanically homogenized with a reduced amount of culture medium (DMEM, approx. 1-2 ml). After isolation the cells were examined under a microscope.

## 2. RESULTS AND DISCUSSIONS

Following the explants' processing four days after their detachment, the isolation of the periosteal cells was achieved (fig. 1a and 1b), which subsequent, to explant removal, remained in the same culture medium in order for the next activity (cultivation) to be performed.

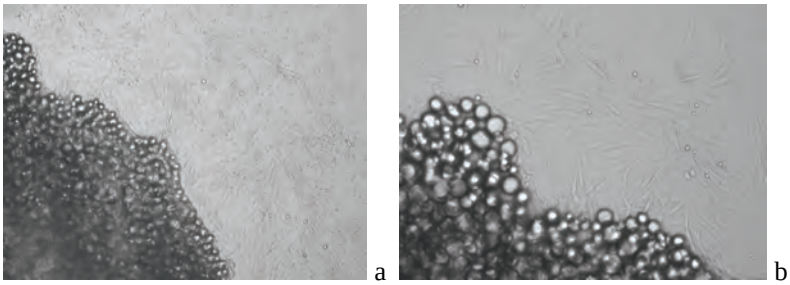


**Fig. 1. Explant appearance after 4 days from placing into the growing medium a) image with 10X objective, b) image with 20X objective**

The analysis of the morphological characteristics of the cells isolated from explant, after four days of subcultivation with the purpose

of isolating them (fig. 2), allowed the assessment of their viability and multiplication rate.

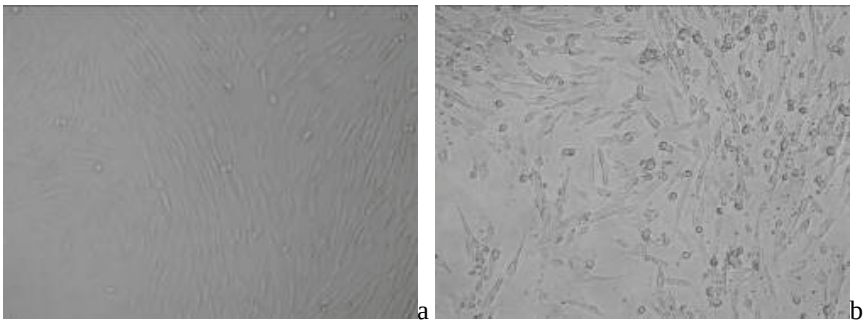

**Fig. 2. The appearance of cells isolated from the explant in the first day (a) and after 8 days (b) from introduction into the culture medium, image with 20X objective**

Since the canine periosteal tissue is a dense connective tissue composed mainly of collagen and reticular fibers, cell isolation required the use of bacterial collagenases to digest the extra-cellular tissue. Digestion of fragments of the initial periosteal fragment through the method described above allowed the isolation of periosteal cells (fig. 3), which then were passed in the DMEM medium for cultivation.

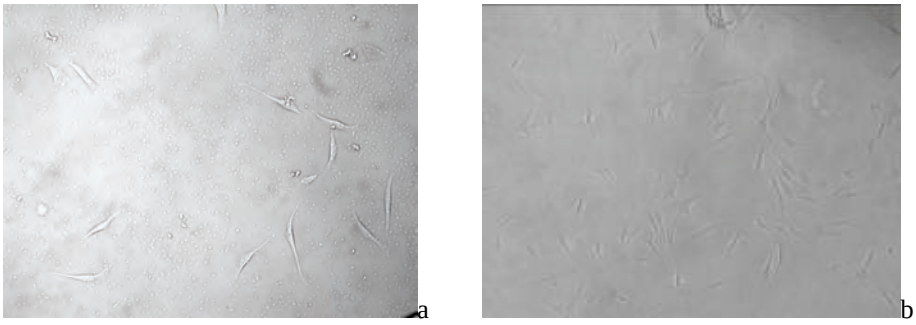

**Fig. 3. Periosteal cells isolated by digestion in 4 (a) and 8 (b) days of cultivation, image with 20X objective**

After microscopic examination it was observed that also the isolated cells from the subculture were grown into a monolayer pattern. The cell proliferation rate, evaluated through extension in the growing medium, was in all cases closed, the cells occupying the entire surface of the well in eight days. In terms of cellular density there were found differences, wells containing cells isolated through digestion presented a lower density than in the case of the cells from the explant.

For cell cultures resulted both from explant and from digestion, during the cultivation period, the cell morphology was similar to fibroblasts. The cells are filiform and present eddy arrangements. Similar issues in the same growing time interval after isolation were reported by Declercq et al, 2005, Ito et al, 1999, who isolated *muridae* and *leporidae* periosteal cells.

In the wells there have been observed clusters of cells similar to epithelial cells, which are composed of grouped independent fibroblast cells . These cell clusters are present in higher numbers in cell cultures obtained from periosteal explants. These facts were also recorded by Declercq et al, 2005.

## 3. CONCLUSIONS

3.1. Both methods of isolation of periosteal cells proved valid. Obtaining the cells through the digestion technique has the advantage of achievement in a shorter period of, i.e. time about four-five hours, compared with the explant method, which involves four days of subcultivation in order of isolation.

3.2. After eight days of maintenance of the isolated cells within a culture medium, the cell density is higher than in the case of cells obtained from explant.

## ACKNOWLEDGEMENTS

This paper was realized in the base of ID program 130 “Guiding periosteal bone regeneration” obtained by Prof. Dr. Igna C.

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# HISTOPATHOLOGICAL ASPECTS FOUND IN SUBCLINICAL PARASITARY ENTEROPATHY OF FOXES FOR FUR

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**Key words:** subclinical parasitary aggression, small intestine, foxes for fur

**SUMMARY**

Investigations were conducted on 50 dead bodies of foxes for fur, slaughtered for economic valorisation, in order to point out the parasitary aggression on the mucous of the small intestine in subclinical parasitary enteropathy. We have taken samples from the small intestine (duodenum, jejun, ileon) and mesenteric lymphonodes that were preserved in formaldehyde 10% and have been processed for the histopathological examination. Fragments were cut at 5 μm and coloured by HEA and MGG methods. Examination and taking microphotographs were done at microscope, using 10 x ob. 10, 20, 40.
The histopathological examination of the intestinal mucous membrane has shown a variable intensity aggression on the intestinal structures: epithelial desquamation, basal membrane discoloration, villosity apex decapitation, progressive atrophy of mucous membrane till the disappearance of villosities and pointing out glandular crypts, inflammatory infiltrate in *lamina propria*, hypertrophy of intestinal glands. In the glandular epithelium, they found schizogonic stages (trophosoits and schizonts), as well as mature disporic, tetrazoic oocysts belonging to *Sarcocystis* genus. The subclinical evolution has shown a varied aggression of different intensity on the mucous membrane of the small intestine, capable to start clinical episodes.

Foxes for fur that were raised in an intensive system on a closed circuit benefit from a raising technology through which the contact with animals is avoided. In such conditions the main source of contamination with parasitic elements of the foxes is the food consisting of slaughterhouse leftovers that were not thermally treated. The reduced intensiveness of the parasitic elements that were paraclinically identified (*Cryptosporidium, Cystoisospora (Isospora), Ancylostoma*, *Uncinaria*, *Toxocara*), lead to minimum level infestations and a long term subclinic evolution. Subclinic intestinal infestations generate intestinal mucosa lesions that have an effect on the local digestive processes and upon the general metabolic processes, lacking a clinical expression. Subclinic infections do not draw the specialists' attention, but constitute a single source of contamination for the life environment with parasitic elements, having a major zoonotic risk (Iacob, Olimpia 2002, 2006).

The purpose of this research is to reveal the parasitic aggression on the intestinal mucosa on foxes for fur in subclinic parasitic infestations

with species belonging to the *Cryptosporidium, Cystoisospora (Isospora), Ancylostoma, Uncinaria,Toxocara),* genus that were paraclinically identified.

## 1. MATERIAL AND METHODS

The research took place between 2005-2007 on 50 dead bodies of foxes for fur, on a state property, that were grown in order to be slaughtered for economic valorisation. After the general examination of the abdominal cavity, fragments of the  The fragments were fixed in watery solution of formaldehyde 10% for 2-7 days and then they were processed; they were re-included in a fresh formaldehyde solution, and then they were inserted in a Bouin solution for 72 hours; they were washed in regular water, they were dehydrated, cleared, impregnated and included in paraffin; the section was made at 5 µm and thee product was displayed on slide; the products have been colored using the HEA and MGG methods. The examination and the microphotography has been made using the Motic, oc. 10, ob. 10, 20, 40, 63 Imm.

## 2. RESULTS AND DISCUSSION

The morphopatological examination was performed on the bodies of the foxes that have been sacrificed for their fur, which presented a good caring state.

On a necropsy the most frequent modifications were observed on the small intestine segments in the shape of extended anse filled with gasses (fig. 1), the watery intestinal contents with feces of a yellow color; the mesenteric lymphonodes slightly increased in volume.

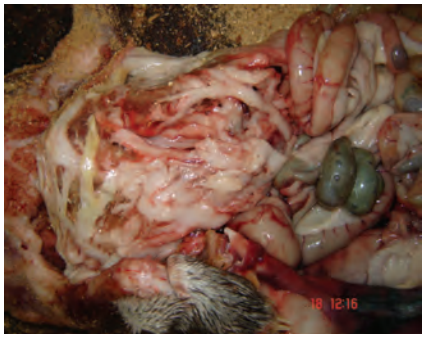

**Fig. 1. The necropsy of the abdominal cavity in a young fox. There can be observed segments of the small intestine with extended areas filled with gasses or with watery contents (nov. 2006).**

The histopathologic examination underlined variable modifications of the intestinal mucosa and in some cases schizogonic stages of some protozoan species. Zones with a progressive atrophy were present from

the denudation of the villositary epithelium (fig. 2.), the decapitation and the reduction to one half of the villosities (fig.3), up to the severe atrophy (fig. 4.), or a total atrophy of the mucosa (fig. 5). These aspects suggest also the intervention of the associated microflora along with the aggression of the parasites that acted on a local basis according to the intestinal segment that they occupied.

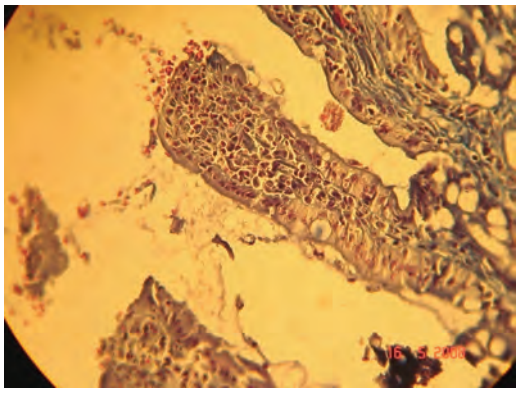

**Fig. 2. Young silver fox. Small intestine. Denudation of the villositary epithelium . Col. HEA. Oc. 10 x ob.20**

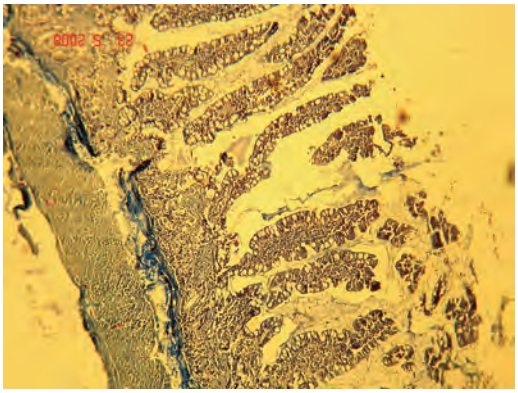

**Fig. 3. Young silver fox. Small intestine. Moderate atrophy of the mucosa. Inflamatory infiltrate. Col. HEA 10 x 10**.

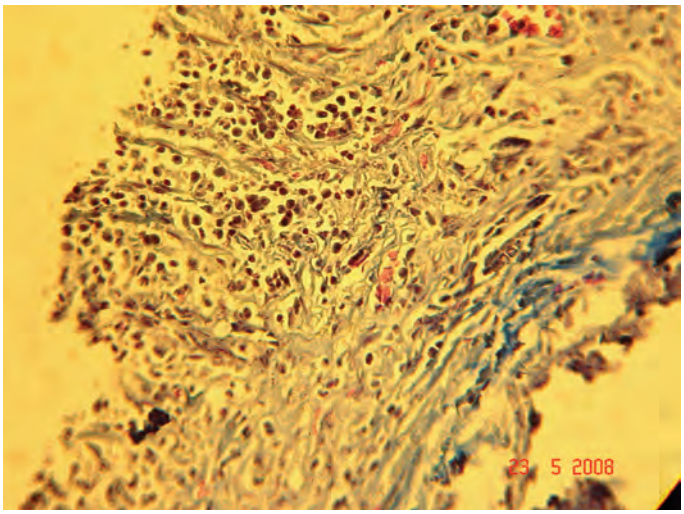


**Fig. 4. Young silver fox. Small intestine. Severe atrophy of the mucosa. Col. HEA 10 x 10.**

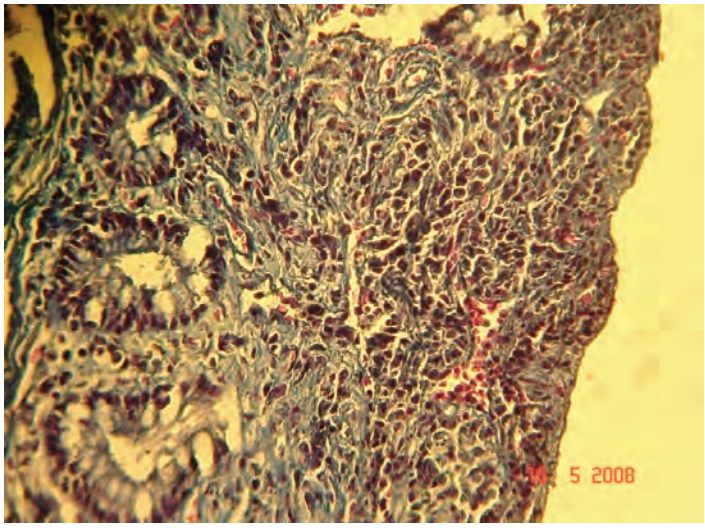


**Fig. 5. Young silver fox. Small intestine. Total atrophy of the mucosa and glandular ectazy. Col. MGG 10 x 20.**

The schizogonic stages, trophosits (fig. 6, 7), and schizonts (fig. 8) belonging to the *Cystoisospora* (*Isospora)* or *Eimeria* genus were identified in the glandular epithelium cells of the small intestine.

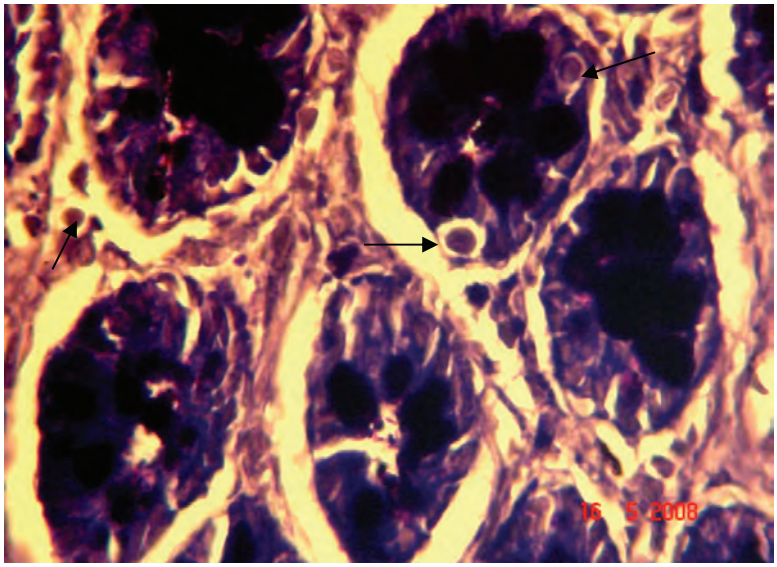


**Fig. 6. Young silver fox. Small intestine. In the glandular epithelium there can be found trophosites, incipient stages of schizogonia on the *Isospora* and *Eimeria* genus. MGG, 10 x 40.**

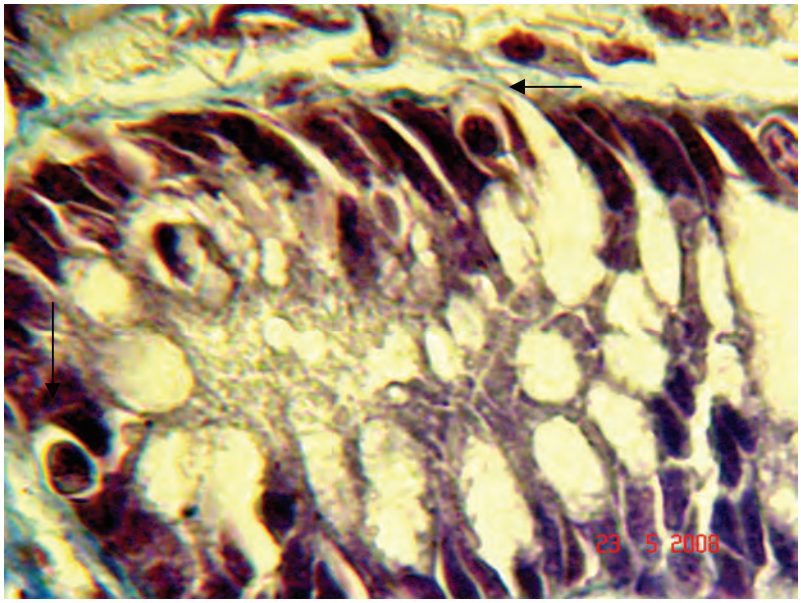


**Fig. 7. Young silver fox. Small intestine. Trophosoites, incipient stages of schizogonia on the *Isospora* and *Eimeria* genus. MGG, 10 x 63. Imm.**

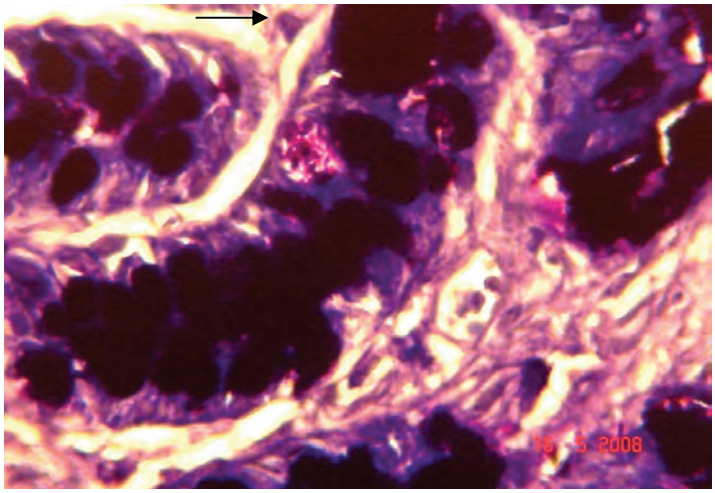

**Fig. 8. Young silver fox. Small intestine. Schizonts stages of schizogonia on the *Isospora* and *Eimeria* genus. MGG, 10 x 63. Imm.**

In some sections there were identified in the *lamina propria* of the mucosa in the final stages of the biological cycle similar to the *Sarcocysts* genus, more precisely mature oocysts that contain two sporocysts each having four sporozoites. The oocyst is characteristic, having a very fine membrane that is moulded on sporocysts (fig. 9).

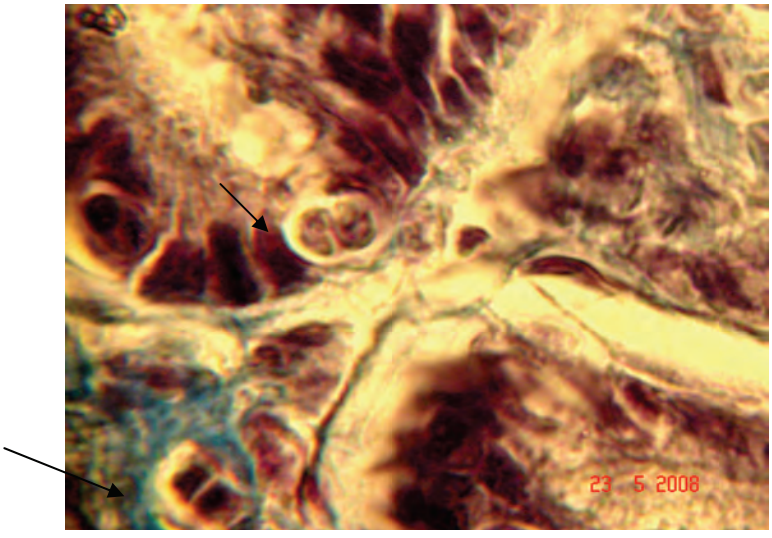


**Fig. 9. Young silver fox. Small intestine. In *lamina propria* there can be observed sporulated oocysts each having two sporocysts, each having four sporosites similar to the *Sarcocystis* genus. MGG 10 x 63 Imm.**

The histological examination of the mesenteric lymphonodes show an increased activity through the germination centers and active lymphocytes; there can be observed edema areas (fig. 10, 11)

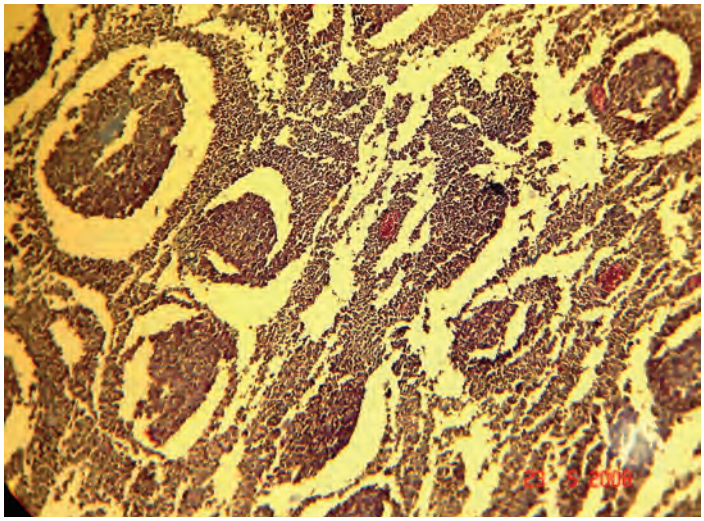

**Fig. 10. Young silver fox. The mesenteric lymphonode: evidence and limits of the germinal ceters MGG. 10.x 10.**

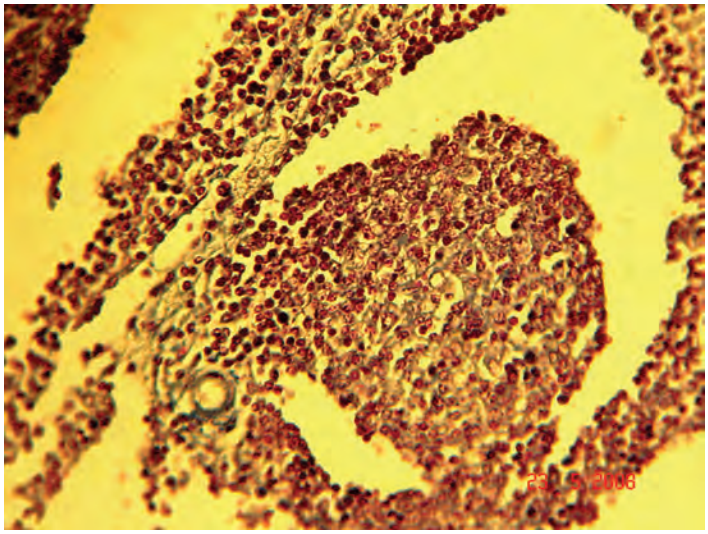

**Fig. 11. Silver fox. Mesenteric lymphonode: germinal center with active lymphocytes. MGG, 10 x 20.**

The presence of similar oocysts of the *Sarcocysts* in *lamina propria* of the small intestine on foxes for fur suggest the administration as food

of thermally untreated slaughterhouse leftovers, ensuring thus the continuation of the biological cycle of the *Sarcocystis* genus: *S. ovicanis*, *S. bovicanis*, *S. ovifelis*, *S. bovifelis* etc. The progressive atrophy of the intestinal mucosa was generated by protozoans of the *Cryptosporidium*, *Cystoisosopora* (*Isospora)* or *Eimeria* type in association with the nematodes of the *Ancylostoma, Uncinaria* and *Toxocara* genus that are frequently localised in the small intestine of the carnivores. The results of the research are in concordance with the literature (Paul, I, 2001; Şuteu, I., Cozma, V., 2004).

## 3. CONCLUSIONS,

3.1 The study aimed at evidencing the tissular and cellular modifications induced by the parasitic aggression in subclinic infestations with protozoans belonging to the identified genuses: *Cryptosporidium*, *Cystoisospora* (*Isospora)* and *Sarcocystis* in association with nematodes belonging to the *Ancylostoma, Uncinaria, Toxocara* genuses with minimum intensivity.

3.2. Morphopathologically, the infestation diagnostic has been confirmed with Cystoisospora (*Isospora*) and *Sarcocystis*, through the deceleration of the characteristic parasitic stages, trophosites, schizonts, oocysts in the examined histological products.

3.3. The administration in the food of the foxes for fur of thermally untreated slaughterhouse leftovers allow the infestation of foxes and the continuation of the biologic cycle in the small intestine mucosa, the apparition of the parasitic elements through which the environment is polluted ensuring the perpetuation of the parasitic species.

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# RESEARCH ON POLYMERIC MATERIALS BIOCOMPATIBILITY

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**Key words:** polymeric, biocompatibility, biomaterial, immunohistochemical, electrono-microscopic

**SUMMARY**

We were carried out research in experimental models watching the reaction of tissue to contact with materials polymer impregnated with different substances of protein nature. Histopathological, electronmicroscopy and immunohistochemistry tests were performed.
The research has shown the evolution of the tisular tissue in contact with polymeric materials with possible applications into the prosthesis and implants manufacturing. Was used 4 rats batch and performed comparative analyses achieving hypodermically implants. In vivo testing of the tissue response has demonstrated the appearance of a conjunctive capsule with variable dimensions which is correlated with the hypodermically tissue reactivity.
Polymeric materials biocompatibility, serves medical devices in contact with normal tissue. Experiments were done on animals in order to demonstrate in vivo the degree of biocompatibility. The study was limited to biomaterials interaction with blood and tissue synthesis.

## 1. MATERIALS AND METHODS

**M**aterial-tissue interactions analysis, tissue response in vivo testing and evaluation thrombogenic evaluation was performed on polymer materials presented in Table 1.

***Table 1***

**Types of polymeric materials tested**

| No. | POLYMER TYPE | Abbreviation |
|---|---|---|
| 1 | Polyurethane | PU |
| 2 | Polyurethane blended with hydrolyzed collagen | PU-COL |
| 3 | Polyurethane blended with hydrolyzed collagen and K-elastin | PU-HC-EL |
| 4 | Polyurethane blended with hydrolyzed collagen, K-elastin and chondroitin sulfate | PU-HC-EL-CS |

In vivo biocompatibility testing of the polymeric material, has been realized by implanting all of these materials, subcutaneously, in rats. Rats have been sacrifyed at 2, 4 and 6 weeks after the polymers implantation.

Histological analysis

For biocompatibility analyze, were taken subcutaneous tissue which contained the implant, and fixed in Bouin Catchers, in 4% paraformaldehyde and in phosphate buffered saline (PBS). After fixation, tissue fragments were dehydrated in increasing concentrations of ethanol, clarified in toluene and included in paraffin. 5 mm sections were stained with hematoxyline-eozyne (H & E) or fluorescent dye 4 ' 6-diamidino-2-phenylindole (DAPI). The examination has been performed in an optical microscope, equipped with fluorescence (Zeiss Axiostar Plus), and the obtained results have been shown at the Faculty Veterinary Medicine Symposium in 2008.

Electronmicroscopy analysis

Tissue fragments were included in 0.2 M sodium cacodilat buffer and were fixed into a 4% osmium dinitrogene (prepared in buffer cacodilat sodium) solution, at 4 $^{0}$C, overnight. After fixation, samples were washed, dehydrated with three concentrations of alcohol series (50 %, 60%, 96 %), with absolute alcohol, absolute alcohol-acetone mixture, then with acetone and were included in Epon 812. After these, the samples have been cut by using the Leica EMUC-6 ultramicrotome. Ultra thin sections, were obtained from 7 µm, which were stained with uranyl acetate and lead citrate. Contrasting sections were fixed on microscopic grid and were examined on an Philips EM 208S electronmicroscope.

## 2. RESULTS AND DISCUSSION

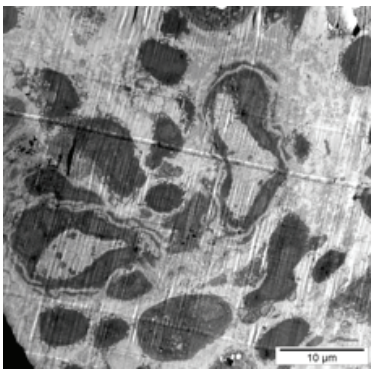


**Fig. 1. Capillaries Electronmicroscope image around the PU, 6 weeks after subcutaneous implantation.**

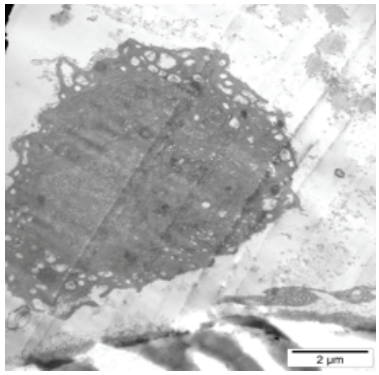


**Fig. 2 Macrophage electronmicroscope image near PU polymer 2 weeks after subcutaneous implantation.**

Macrophages surrounding the implant, were identified as isolated elements (Fig. 1) or as multinucleated giant cells. Isolated macrophages

could been identified by electronmicroscopy, around the implants or in contact with them.

Macrophages fused to form multinucleated giant cells were identified in contact with all polymer variants but their proportion is significantly increased for polyurethane and PU-COL polymer blend. The presence of these cells is associated with polymer degradation, which is greater in PU-COL variant.

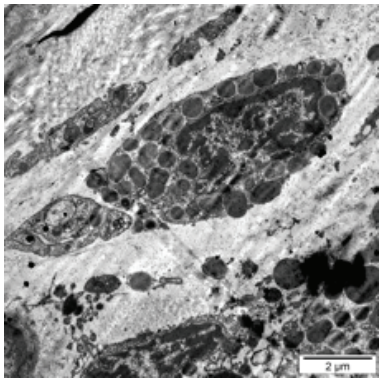


**Fig. 3. Mastocyte electronomicroscopy image present into the connective tissue surrounding the PU-COL polymer, at 4 weeks after the subcutaneous implantation**.

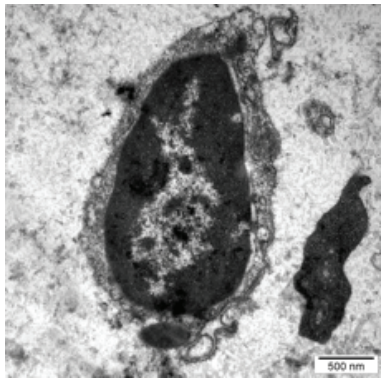


**Fig. 4. Lymphocyte electronmicroscopy image, present into the connective tissue surrounding the PU polymer, at 4 weeks after subcutaneous implantation.**

In vivo testing local tissue response to biomaterials by detect biochemical markers using immunohystochemical methods.

Tissue fragments for immunohystochemical analysis, were fixed in 4% formaldehyde in PBS, ethanol dehydrated, toluene clarified and included in paraffin. 5 mm sections were processed by indirect immunoperoxidase method which consisted of the following stages:

1. endogenous peroxidase blocking with 3% $H_2O_2$;
2. blocking nonspecific binding with 2% bovine serum albumine;
3. incubation overnight at $40^0$C with primary antibodies, diluted in phosphate buffer saline with 2% bovine serum albumine;
4. washing with phosphate buffered saline;
5. incubation with secondary antibodies coupled with peroxidase, 1 hour at room temperature;
6. washing with phosphate buffered saline;

**7.** developing immune complex in the presence of 0.05% 3,3 '-diaminobenzydine and 0.003% $H_2O_2$ in phosphate buffered saline

Cell nucleus was contrasted with hematoxyline. Were used the following primary antibodies: (1) rabbit antibodies against interleukin-1β (USBiologicals), diluted 1:50; (2) goat antibodies anti-interleukin 6 (Santa Cruz Biotechnology), diluted 1:100; as secondary antibodies were used: (1) goat antibodies against rabbit IgG coupled with peroxidase (Rockland), diluted 1:1250.

Testing in vivo of the local tissue response in the presence of biomaterials has been achieved through immunohystochemical analysis, which aimed at assessing the capacity of the polymers tested by highlighting inflammatory cytokines : interleukin-1β (IL-1β) and interleukin 6 (IL-6).

In the control subcutaneous tissue were not identified interleukins IL-1β by immunohistochemistry.

Immunohistochemical reaction for IL-1β was present around implants in all the intervals but the reaction intensity was more pronounced in cells around the polyurethane implant and around capillaries in the connective sheath PU-HC (Fig. 5).

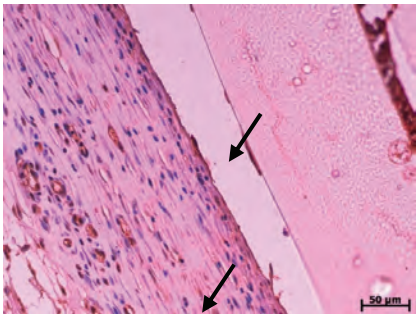


**Fig. 5. IL-1β immunohistochemistry reaction in the capillaries around PU-COL (arrows). Contrast with hematoxyline.**

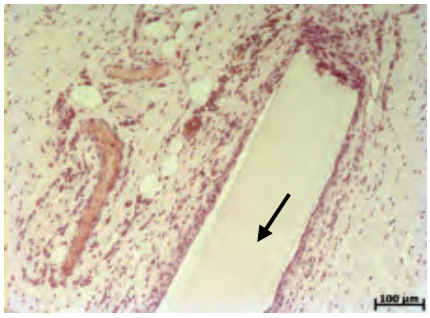


**Fig. 6. IL-6 immunohistochemistry reaction in blood vessels (arrow) around the PU to 2.4 and 6 weeks of implantation. Contrast with hematoxyline.**

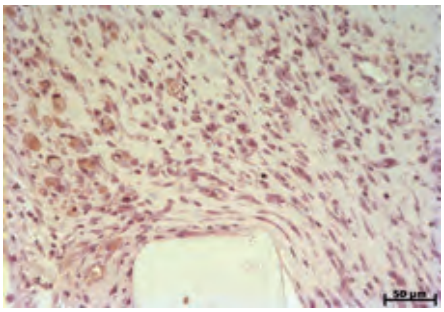

**Fig. 8A. Lack of IL-6 immunohistochemical reaction in blood vessels (arrow) around the PU-COL, after 2 weeks of implantation. Contrast with hematoxyline.**

Immunohistochemical reaction for IL-6 was present in the blood vessels, always the case polyurethane implants. The most intense reaction in this case, was identified at 4 weeks after implantation.

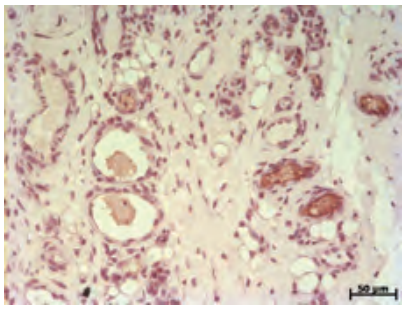

**Fig. 8 B IL-6 immunohistochemical reactions in the blood vessels (arrow) around the PU-COL-EL after 4 weeks. Contrast with hematoxyline**.

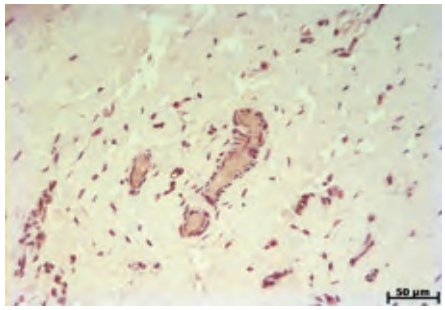

**Fig. 9 Lack of IL-6 immunohistochemical reaction in blood vessels (arrow) around the PU-COL-EL-CS, after 2 weeks. Contrast with hematoxyline**.

After macrophages activation they produce a wide range of cytokines such as IL-1, IL-6, IL-10, IL-12, IL-18, TNF-α. IL-1 production is generally related to inflammation and plays an important role in regulating the immune response and inflammatory process by activating of T lymphocytes, B lymphocytes and NK (natural killer) cells. IL-1 lead to B lymphocytes proliferation and maturation and immunoglobulin synthesis. L-6 is a multifunctional protein that plays an important role in immune response and haematopoiesis. Constitution is expressed by epidermal Langerhans cells. Pro-inflammatory cytokines secretion such as IL-1β and IL-6 by activated macrophages was reported for a wide range of biomaterials (Anderson and col., 2007). These studies have shown that activation of macrophages can be modulated by biomaterials surface properties and chemical composition.

## 3. CONCLUSIONS

3.2. In vivo studies on animals have shown that bio-materials based on polyurethane polymer made by mixing polyurethane with extracellular matrix molecules collagen, elastine, glycosaminoglycans) were found to be biocompatible, for short and medium term.

3.3. The best stability of polymers in the tissue was made by mixing polyurethane-collagen-elastine, followed by version polyurethane-collagen-elastine-chondroitin sulfate and polyurethane-collagen variant.

3.4. Analysis of immunohistochemical expression of inflammatory cytokines IL-1β and IL-6 showed that all variants of Biopolymers have induced tissue inflammation in your low to moderate at time of implantation studied. It was found that at times greater than implantation, inflammatory reaction decrease.

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# RESEARCH ON UNILATERAL HYDRONEPHROSIS SURGICAL TREATMENT OF THE DOG

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**Key words:** hydronephrosis, surgery, dog, decapsul, uremia

**SUMMARY**

Five dogs were studied after unilateral hydronephrosis diagnosed by clinical examination and supplementary in some cases, with ultrasound. Biochemical examinations of blood and urine did not reveal significant changes. On the opening of the abdominal cavity, were found large structures, containing blood serum or purulent, bold walls, compressing the other viscera, peritoneal reaction without detectable macroscopical peritoneal reaction. Affected kidney cortex was strongly thickened, presenting itself as a fibrous membrane, with dilatated blood vessels. And one case has been diagnosed with bilateral hydronephrosis, which present serious uremia (Urea 420 mg / dl, creatinine 3.9 mg / dl). It shown a history of serious urinary transit disturbances and it couldn't be saved.
Unilateral hydronephrosis is often a surprise diagnosis in dogs, because clinical signs are discreet because of the compensatory activity of the congener kidney. In chronic hydronephrosis, appears the disproportion of the abdomen, obvious discomfort, no reliable diagnostic elements suggests the disease. The appearance of batracian abdomen requires differential diagnosis by special tests for ascites, Cushing's syndrome, piometru, etc..

## 1. MATERIALS AND METHODS

Research has been conducted on five dogs (four males and one female), aged 4 to 14 years. Following clinical examination and / or ultrasound, were diagnosed large formations, cystic appearance, with cystic wall with cataral - sangvinolent or purulent containing. Formations present in the abdominal cavity had slope, signaling the very least ascites fluid, bloody. To explore the abdominal cavity manually, was done on partial disposal of contents, which was taken for making macroscopic and laboratory examinations. Under general anesthesia was performed removal of pathological formations after progressive decompression of abdominal cavity and ensure hemostasis.

Postoperative treatment: sought fluid, electrolyte, nutrient-energy balance administration.

## 2. RESULTS AND DISCUSSION

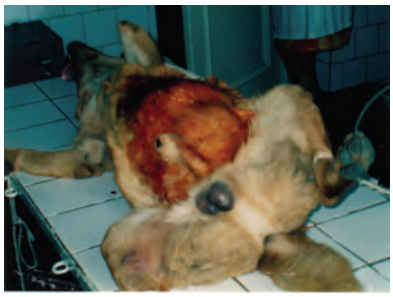

**Fig. 1. Dog, 12 years, with massive hydronephrosis, ready for operation**

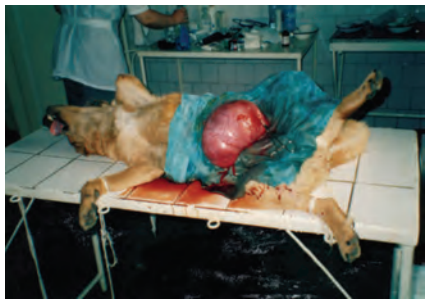

**Fig. 2. Large hidronephrrotical formation (10 kg) protrudes partly**

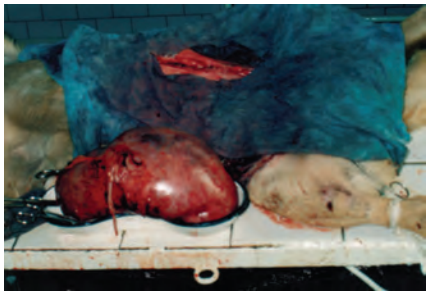

**Fig. 3. Hydronephrosis in dogs, the postoperative appearance. Observe ureter, bold, but waterproof.**

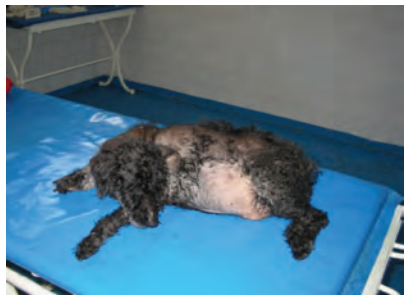

**Fig. 4. Dog, 7 years, diagnosed with unilateral hydronephrosis, preparation for surgery.**

In unilateral hydronephrosis in dogs in early stages of disease, clinical signs are few and homeostasis is very little changed, making early diagnosis difficult. Regardless of the diagnosis method and evolutionary phase, treatment is only surgical. In huge unilateral hydronephrosis, clinical signs that highlightened (deformation of the abdomen, abnormal bowel movements, changes in frequency, patterns and amplitude of breath, etc). Affected kidney is present with completely destroyed parenchyma, the capsule is thickened, with rich vascularization. Removal of pathological formations was performed in all cases with gradual decompression by cutting the capsule. Content was bloody or foul and on the macroscopic examination were not reported evidence that suggested urinary origin. In all cases, postoperative evolution was favorable.

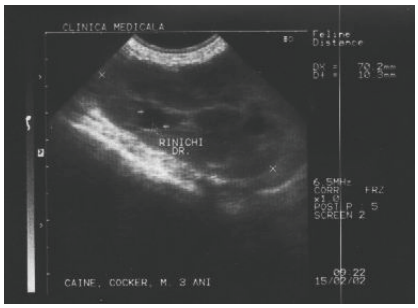


**Fig. 5 . Hydronephrosis in dog. Ultrasound image**

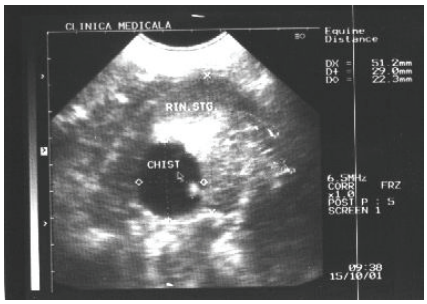


**Fig. 6 . Hydronephrosis in dog. Ultrasound image**

## 4. CONCLUSIONS

4.2. Unilateral hydronephrosis, sometimes raises diagnostic problems, because of the compensatory function.

4.3. The least traumatic method used for pathological formation extraction was after decapsul and ensure hemostasis by ligature in a block.

4.4. Prevent shock by abdominal decompression and postoperative complications were achieved by partial and progressive discharge and severe aseptic conditions.

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# RESEARCH AND OBSERVATIONS ON THE INTERFERENTIAL CURRENTS STIMULATION FOR THE TREATMENT OF INFLAMATORY JOINT PAIN IN DOG

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**Key words:** interferential therapy, pain control, dog.

**SUMMARY**

Interferential therapy in fighting acute and chronic pain of the locomotors apparatus is a well-known and explored domain in human medicine, which is why we try putting it in practice and in veterinary medicine. Interferential therapy is a method that involves overlapping two currents of medium frequency slightly delayed (up to 100 Hz). In this way results a low frequency current (interferential) whose amplitude modulation occurs with a frequency of 0 to 100 Hz.
Observations were made on a number of 5 dogs with different acute or chronic inflammatory joint diseases (spondylitis, arthritis, osteoarthritis). Treatment was performed with the electrotherapy device Med-Mode System Interferenz 3, mark BOSCH, with circular suction electrodes.
After setting the diagnosis and the seat of pain by clinical and paraclinical methods, has been established a therapeutic program for each individual, which has further been modulating depending on the response of each individual part.
Patients haven't expressed any discomfort conditions during treatment application, and to the skin were not observed any injury due to currents shift. Recovery was good, being a temporary remission of symptoms (after applying the treatment for 1-3 hours) during the first week, for towards the end of the third week to be permanent.

Stimulation of the body's natural physiological healing processes with physical methods of treatment is an old therapeutic principle. Interferential stimulation (IFS), also known as interferential therapy (IFT), is a type of electrical stimulation where two slightly different (ex: one with a frequency of 4000 Hz and the other with a frequency of 3900 Hz), medium frequency alternating currents are simultaneously applied to the affected area through electrodes. Superposition or interference between the two currents causes the combined electrical current to rise and fall at a slower frequency, often referred to as the "beat" frequency (Low *et al*., 2000).

This method combines the favorable effects and removes the unfavorable ones of those 2 types of currents, low frequency and medium frequency: crosses the skin with greater ease and with less

stimulation (skin resistance is low => no skin irritation), reaches greater depths and over a larger volume of tissue than other forms of electrotherapy. So, IFT uses the beneficial effects of low frequency currents to a greater depth (G C Goats, 1990).

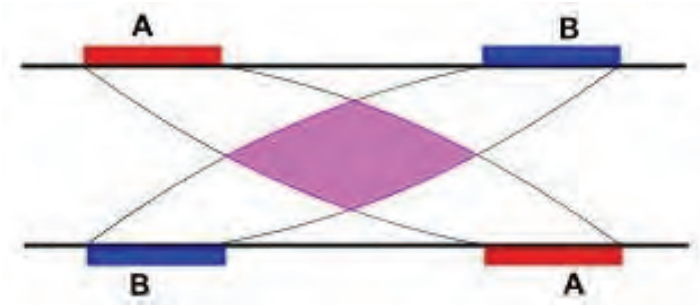


**Fig. 1. Interference current, "beat frequence", generated in the central zone.**

Physiological and therapeutic effects of interferential currents are expressed by:

- control of pain (“pain gate” theory);
- motor stimulation (muscle contractions production);
- edema and inflammation reduction (improving the local blood and lymphatic flow);
- muscular spasms reduction an relief

Therefore, the therapeutic indications of interferential currents are states such as:

- posttraumatic states, bruises injuries (fractures, sprains, luxations, bruises without bone lesions, hematoma);
- joints sufferings (arthritis, periarthritis, arthrosis);
- painful affections of the spine (spondylosis, spondylitis, neuromuscular pain, bruises);
- neuralgia and neuritis;
- paresis sequels of limbs, in remission.

The aim of the study was to work for implementation of the physiotherapy procedures in the treatment of painful diseases of the locomotors apparatus in dog and highlighting the benefits of interferential current therapy in acute and chronic pain release.

## 1. MATERIALS AND METHODS

The study was conducted in the Surgery Clinic of the Faculty of Veterinary Medicine from Cluj-Napoca, during 2008-2009, on a number

of 5 animals of canina species, which were submitted in consultation with various painful disorders of the locomotors apparatus (Tab. 1).

To induce the interferential currents stimulation we used the electrotherapy device Med-Mode System Interferenz 3, mark BOSCH, with circular suction electrodes (Fig. 2). A vacuum suction unit provides the vacuum.

***Tab. 1***

**Casuistry taken in the study**

| Breed | Age | Clinical signs | Diagnosis |
|---|---|---|---|
| Labrador | 9 years | Lumbar region stiffness<br>Prefers lying position | Lumbar spondylitis |
| Metis | 11,5 years | Lumbar region stiffness<br>Refuses to descend stairs | Lumbosacral spondylitis |
| Metis | 8 years | First degree lameness on the left hint limb<br>Femorotibiopatellar joint stiffness | Posterior knee arthritis |
| German shepard | 8 years | Right hint limb lameness<br>Coxofemoral joint stiffness | Osteoarthritis |
| Rottveiler | 7,5 years | Hint limbs stiffness | Coxofemoral joint arthritis |


**Fig.2. Med-Module System Interferenz 3 electrotherapy device, BOSCH brand.**

For the group of dogs taken in observation, depending on the severity and the nature of injury, we established a protocol of therapy that includes: the site of electrodes application, method establishment, with two or one pair of electrodes, the currents parameters (intensity, frequency, duration) and the number sessions.

Depending on the seat of disease is established the electrodes application spot (Millis *et al.*, 2004). Electrodes application spot is prepared by hair cutting and shaving (Fig. 3) in order to perform the vacuum needed for electrodes fastening on the skin.

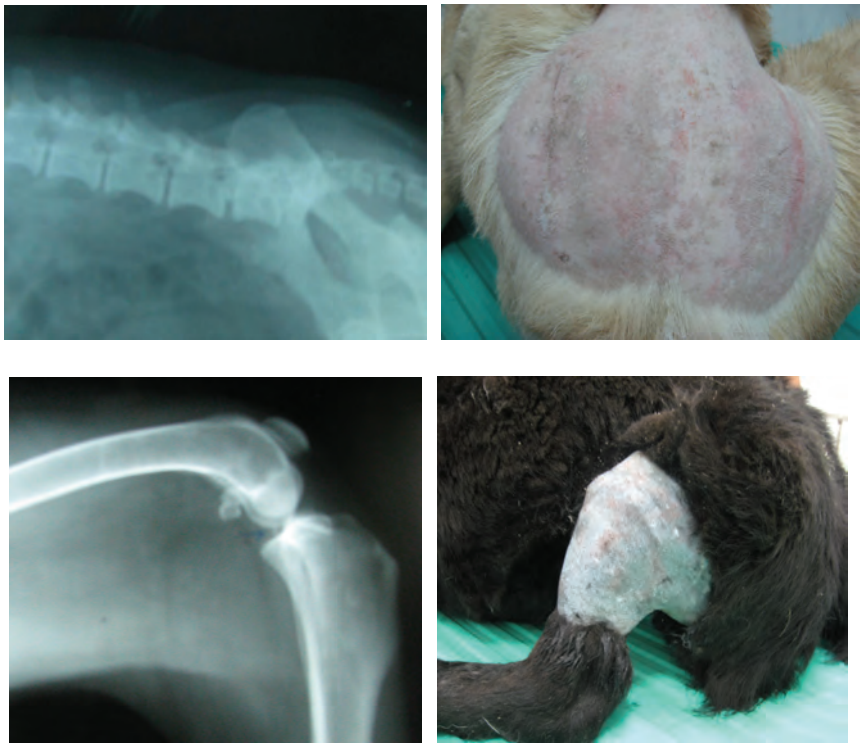

**Fig.3. Electrodes application spot establishments on the basis of the disease seat.**

In the deeper located conditions, covered with large muscle mass, we used the interferential current application with 4 electrodes (Fig. 4), to have an area of greater coverage and to penetrate deeper. Thus, in the two dogs diagnosed with spondylitis, electrodes were placed in the lumbar area, on the one side and the other of the spine, at a distance of approximately 3 cm between them, in a the right angle.

In more superficial conditions, where the musculature is not very rich and does not allow multiple electrodes application, we used the premodulated interferential current implementation through 2 electrodes (Fig. 5). This method involves mixing the two medium frequency currents inside the machine to produce the low frequency output, the "beat frequency". Thus, in the dog diagnosed with posterior knee arthritis, the electrodes were placed on the medial and lateral sides of the

femorotibiopatellar joint, so the articulation to be in the middle of the currents action.

In the case of the 8 years old German Shepard, diagnosed with osteoarthritis, the electrodes were place so as to include both coxofemoral and femorotibiopatellar joint. In this patient we used a combined treatment, conducting sessions with 4 electrodes, respectively 2 electrodes. Regardless of the method of electrodes application, they must be placed so that the maximum field of currents action includes in the center the lesions of tissues to be treated.

In our case, the parameters used were the following:

- intensity of 6-8 mA;
- frequency of 80-100 Hz;
- duration: sessions of 10 - 15 minutes, depending on the seriousness and the acute or chronic disease evolution.

A meeting itself of electrotherapy involves performing the following steps:

- positioning the electrodes on the skin;
- conducting the vacuum under electrodes by the aerator switch torsion to right until reaching a value of approx. 0.4 kg/m2;
- duration of session setting;
- frequency setting;
- setting the intensity on 0;
- startup the current application;
- increasing the intensity gradually until we reach the fixed intensity, and before the end of the session, reductions slowly, gradually, till 0.

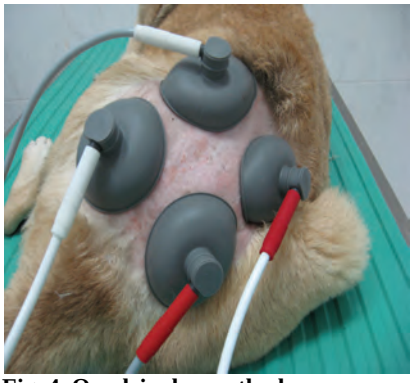

**Fig. 4. Quadripolar method.**

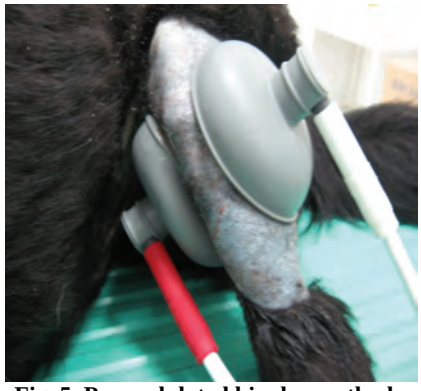

**Fig. 5. Premodulated bipolar method.**

Treatment was performed during 3 weeks, as follows: in the first week, one session per day, and the 2 weeks following one session at 2 days.

## 2. RESULTS AND DISCUSSION

Patients showed no side discomfort during treatment application, and no lesions occurred on the skin (burns or congestion) due to current application. During treatment patients showed a state of comfort due to physical sensation of massage carried out with vacuum electrodes.

The use of suction electrodes adds another form of sensory stimulation to complement that produced by the interferential treatments. The (rhythmically varying) negative pressure produces a "massage-like" effect that gives a state of physical comfort (G C Goats, 1990). The aspiration deep massage reduces the electrical resistance of the tissue, increasing the tissue conductibility of the interferential currents through better liquidity allocation under the electrodes (Radulescu, A., 1991).

At the local level during treatment application, there are mild rhythmic contractions of the muscles due to waves of electrodes with suction vacuum. One of the effects of these contractions is to improve the local circulation that leads to speeding up the processes and the metabolism of local repair and healing.

In the first week after the treatment application, we noticed a gradual remission of the symptoms and a greater mobility of the affected limbs for a short but upward period of time (in the beginning for about 30 minutes - one hour, for during the therapy progress to increase this period).

Towards the end of the two weeks we have seen an increasing remission of symptoms and an improved mobility throughout the limbs concerned. An almost total remission we observed only towards the end of the third week. Indeed, the remission of symptoms and the recovery of the limbs mobility were not total, but we can say with certainty that the quality of life of these animals was much improved.

Using the combination of intensity of 6 mA, frequency of 80-100Hz and sessions of 10 minutes, we had the best analgesic effect. Regarding the intensity, it is known that the intensity under 8mA is used for sensory stimulation, and the intensity over 8mA for motor stimulation.

## 3. CONCLUSIONS

After conducting the first study on the electrotherapy with interferential currents effect in the patients with various painful conditions of the locomotor's apparatus recovery, we reached several key conclusions:

3.1. Electrotherapy is a non-pharmacological and non-invasive treatment of various painful conditions of soft and hard tissues of the locomotive, and not only;

3.2. The electrotherapy variant with interferential currents is advantageous because it can handle very deep areas with very little damage to the skin;

3.3. Interferential currents application with vacuum electrodes is preferably at least for 2 reasons: - gives a feeling of physical comfort through the massage performed;

- reduces the electrical skin resistance;

3.4. Interferential therapy gives good results in treating various painful conditions, but for best results it should be associated with the administration of small doses of anti-inflammatory drugs.

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# IN VITRO DIFERENTIATION OF PERIOSTEAL CANINE CELLS

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**Key words**: diferentiation, periosteal cells, dog

**SUMMARY**

Isolated periosteal cells, approximately 30.000 cells in suspension, were placed in the differentiation chambers with osteogenic culture medium. In order to analyse osteogenic differentiation of cell cultures there was established an alkaline phosphatase activity, type I collagen, and extracellular matrix mineralization. A storage for 10 days in the differentiation chambers provide sufficient time for the osteogenic capacity development. A positive alkaline phosphatase activity in the case of differentiated cells to osteoblasts suggests an intense osteogenic activity. Also, the morphology of these cells,the polyhedric and cubical appearance, as well as a vertical growth in the differentiation medium are characteristics of the osteoblasts. Confirmation of results obtained from the review of alkaline phosphatase activity was obtained through the Von Kossa reaction, which has revealed the presence of calcium deposits in the cells membrane and in their vicinity. Identification of type I collagen needs a specific anticollagen antibody to react primary with the canine collagen.

The present study is in accordance with world researches objectives which have as main aim the development of novel therapies for the repair and regeneration of musculoskeletal tissues. Periosteum and periosteum-derived cells are known to play an intimate role in the formation of bone and cartilage tissue during bone tissue development and repair, and have demonstrated multilineage potential *in-vitro*. As such, the periosteum represents an interesting new candidate for osteochondral tissue engineering strategies (Orwoll, 2003, O'Driscoll and Fitzsimmons, 2001). To date, however, only a few groups of researchers have sought to exploit this potential from periosteum tissue derived from human donors (Bonzani, 2008). Consequently, there are no standardized methods for the culture of adult human periosteum *in-vitro* and little information is available on the behaviour and characteristics of periosteal cells on animals, especially dogs.

## 1. MATERIAL AND METHOD

Isolated periosteal cells obtained using two different techniques, from explants and through digestion, grown for 6 weeks, were cryopreserved for 4 months and then defrosted. Qfter they were placed in the differentiation chambers; on each slade four chambers of differentiation are sealed with silicone (fig. 1). In the first two chambers there was introduced cell suspension with osteogenic culture medium – experimental, and in the other two - control chambers there was placed cell suspension treated with DMEM nutritive medium. The medium was refreshed three times throughout 10 days. In each differentiation chamber there were introduced approximately 30.000 cells in suspension.

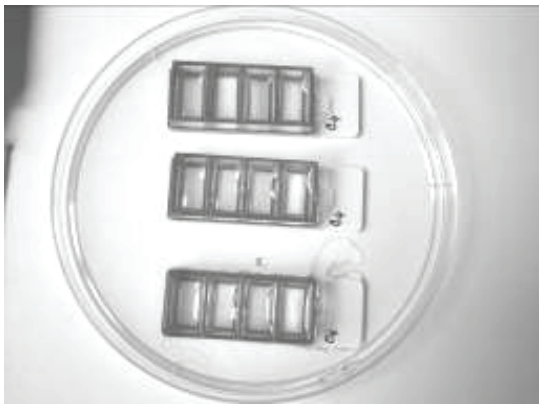

**Fig. 1. Slide with differentiation chambers containing cell cultures in suspension with osteogenic medium (experimental) and DMEM medium (control)**

In order to analyze the osteogenic differentiation of cell cultures, there was established an alkaline phosphatase activity, type I collagen, and an extracellular matrix mineralization through the following protocol:

1. The histochemical determination of the alkaline phosphatase activity (Wergedal and Baylink, 1969, Miao and Scuttle, 2002).

After removing the culture medium with Ringer solution at $4^0$C, the cells were fixed in acetone at $-20^0$C for 5 minutes; after flowing the acetone from the differentiation chamber the cultures were washed with cold distilled water and left to dry for 30 minutes. The cultures were incubated for 10 minutes with BCIP/NBT (5 bromo– 4 chloro-3 indole phosphate/ blue tetrazolium nitrate), a substrate liquid, at room temperature and continuous stirring. The reaction was stopped by

removing the substrate solution and washing with distilled water. The cultivation chambers were removed from theslide, it being fixed with a fixing medium over which another slide was added.

2. The imunne localisation of type I collagen (Declercq 2005, Ueno 2007).

After the removal of the culture medium and the rinse of the differentiation chambers with Ringer solution at $4^0$C, the cells were fixed in 4% buffer formaldehyde with 10mM phosphate solution (the pH of the solution was 6.9) at $4^0$C for 10 minutes. The fixed cultures were washed with cold distilled water and let to dry. The cultures were incubated for 30 minutes with the blocking serum (COL1A1 (D-13): sc-25974 Santa Cruz Biotechnology Inc.). After a blocking serum washing with PBS the second non-specific blocking serum was added. The cells were incubated for another 30 minutes, then the cultures were washed with PBS and conjugate peroxidase streptavidin was added. It followed another period of 30 minutes of incubation and then other 10 minute of incubation in DAB peroxidase substrate.

After washing with tap water and colouring with hematoxylin, the slide was fixed with fixing medium over which another slide was added.

3. Extracellular matrix mineralization (Sheehan, 1980, Rungby, 1993)

The presence of the phosphate deposit was analysed through the histochemical Von Kossa reaction, the detected calcium phosphate deposits appearing in black colour. The cell cultures were rinsed of the serum culture medium with Ringer solution at $4^0$C and were fixed with 4% buffered formaldehyde with 10 mM phosphate ( the solution pH was 6.9) at $4^0$C for 10 minutes.

After removing the fixative substance, the cells were washed with cold distilled water and covered with 5% silver nitrate and kept in the dark for 30 minutes. After removal of silver nitrate cells were rinsed with distilled water, then for 2 minutes there was added, a solution of sodium carbonate and formaldehyde, (5 g $Na_2CO_3$ in 75 ml distilled water and 25 ml of 36 % formaldehyde).

The cultures were rinsed with distilled water for 10 minutes, then following a period of 20 minutes of incubation with a solution containing one part water based solution of 10% potassium ferrocyanide and nine parts of 10% sodium thiosulphate solution.

After the incubation there followed a new rinsing of the cell cultures with distilled water. The cells were left to dry, after which cultivation chambers were removed from slide and fixed with a fixining

medium over which another slide was fixed for the final microscopic examination.

## 2. RESULTS AND DISCUSSIONS

The microscopic examination (objective 10X, 20X and 40X)of the cells from the explant made after the period of differentiation, during which the cells were maintained in suspension with an osteogenic medium, it was observed that these became polyhedric in appearance and their multiplication within the culture medium was more on the vertical. After the reaction for the determination of the alkaline phosphatase activity, the cells were intensly coloured which shows a high positive activity of the alkaline phosphatase (fig. 2), a phenomenon found in all individuals.

In the case of cells obtained from the explant and differentiated in control chambers, it came out that they had round or slightly fusiform shape, morphological characters typical of fibroblasts, features that these cells had also before entering into the differentiation chambers. After the reaction for the determination of alkaline phosphatase activity, the cells were not stained, remains of stain being observed stain in the spaces between cells, which shows the lack of a phosphatasic activity.

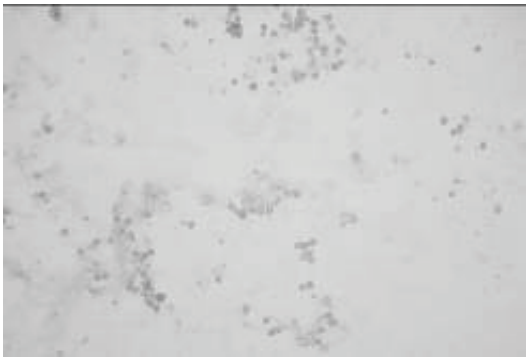

**Fig. 2. The positive reaction of cells from explant grown in the differentiation chambers with osteogenic medium, image with 20X objective**

On microscopic examination of the slides on which were fixed periosteal cells obtained through digestion and differentiated in experimental chambers, it was observed that these cells, shape was polyhedric, roughly cubical. After the reaction for the determination of the alkaline phosphatase activity, the cells displayed a positive

phosphatasic activity but were less intensely stained than the cells obtained from explant and differentiated in the same environment.

The examination of slides with cells from the control differentiation chambers obtained through digestion, were round in shape, slightly fusiform, characteristics specific of fibroblasts. The reaction for the identification of the alkaline phosphatase activity did not result in any change of colour in the cell structure, which corresponds to the lack of the phosphatasic activity.

The detection of type I collagen, based on the immunohistochemical antibody antigen reaction, was negative both for the cells obtained through explant and these resulted through digestion from the experimental and control chambers. It was found that no cell had a fixed hematoxylin which would have had a significance of a positive reaction. On the slides there were observed the cells with different morphology according to the type of environment in which they were maintained during the differentiation period as well as traces of stain.

Only one slide, on which explant cells were grown in the differentiation chambers with DMEM medium (control), from subject three, had a false positive response. The reaction was considered false positive as it occurred only at one end of the cell culture field, the field centre of the field not stained (fig. 3).

**Fig. 3. False positive reaction of cells from explant grown in differentiation chambers with DMEM medium - control, subject 3, objective 20X.**

Through Von Kossa reaction there are highlighted $Ca^{2+}$, the calcium carbonates or phosphates, in the form of intra- and extracellular deposits, deposits that appear stained in brown or black, the cell nucleis in red. This type of deposits, intra and extracellular, being specific to the cells. When the slides with cells obtained from explant in experimental differentiation chambers,when examined uniform coloured was

observed in the entire cell mass, the cells being intense in colour with reddish nucleis the cell membrane and the adjacent zone stained in black. Mineralized calcium deposits inside cells and around them that already gained a polyhedric appearance shows a positive osteogenic activity (fig. 4a). The examination of the slides with cells obtained through digestion in experimental differentiation chambers showed intense stained cells and the presence of calcium deposits in the cell membrane and around the cells, staining characterizing an intense osteogenic activity (fig. 4b). The same as in the case of the slides with cells obtained from explant, here too there slides were observed cells with polyhedric appearance, typical for osteogenic cells.

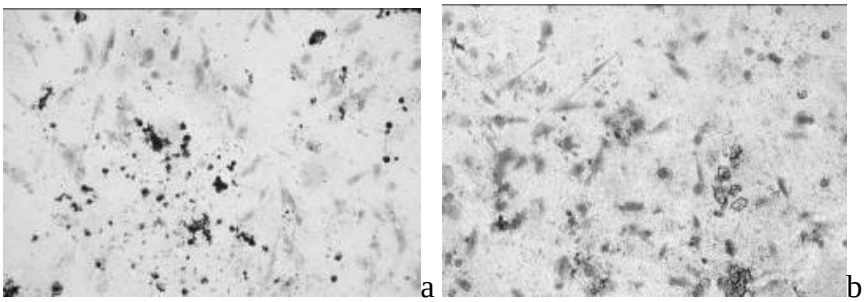
a b

**Fig. 4. Positive reaction of cells obtained through explant (a) and through digestion (b) - experimental differentiation chambers, 20X objective**

On the slides with cells from explant and obtained through digestion in the differentiation control chambers there was observed only a brown-reddish intense staining of the cells, without calcium deposits, the characteristic appearance of the lack of osteogenic activity. Morphologically, these cells have specific fibroblast fusiform appearance.

The storage, for 10 days, in the differentiation chambers in an osteogenic medium, medium containing ascorbic acid, β-glycerophosphate and dexamethasone, represents an interval of time sufficient to the direct development towards the osteogenic line. Similar data was obtained by Gröger et al, 2005, who carried out a research on small breed pigs, and by Uneo et al, 2007, who used guinea pig as the animal experimental model. Both research teams used the same medium for differentiation but the differentiation period was longer, about 14 days.

The positive activitz of the alkaline phosphatase in the case of cells differentiated to osteoblasts suggests an intense osteogenic activity.

Also, the morphology of these cells, the polyhedric and cubical appearance, as well as the vertical growth in the differentiation medium are characteristics of the osteoblasts. The confirmation of the results obtained from the analysis of the alkaline phosphatase activity was also obtained through the Von Kossa reaction, which revealed the presence of calcium deposits in the cells membrane and in their vicinity.

The lack of positive reactions, in all subjects, tested for immunohistochemical of type antigen antibody can be attributed to the lack of reaction of the anticollagen I antibody used with the collagen of canine origin. This antibody (COL1A1 (D-13): sc-25974 Santa Cruz Biotechnology Inc.) primary reacts to collagen of goat origin, human and *muridae*, being also considered reactive with other mammals including the dog.

## 3. CONCLUSIONS

3.1. Storage for 10 days in the differentiation chambers provides sufficient time for osteogenic capacity development.

3.2. The osteogenic capacity of differentiated cells is sufficiently demonstrated by corroborating the morphological analysis with the histochemical test results, more precisely with the phosphatase activity and calcium deposits presence.

3.3. For the identification of collagen type I it is necessary a specific anticollagen antibody to react primarily with the canine collagen.

## ACKNOWLEDGEMENTS

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# A STUDY OF BRAINSTEM AUDITORY EVOKED POTENTIALS (BAER) IN CATS

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**Key words:** brainstem auditory evoked potentials, latency, cat

**SUMMARY**

The passing of an acoustic stimulus from the ear to various structures within the nervous system generates a series of electrical signals with latencies of several milliseconds to hundreds of milliseconds. The aim of this study is to establish reference values for the latencies of waves I, III and V, as well as for intervals I–III, I–V and III–V on a group of five cats. The data analysis shows that the latencies of wave I as well as III and V increase with lower stimulus intensity ($p< 0,05$). In the case of binaural stimulation, the latency of waves III and V was greater as compared to monaural stimulation (left or right) - $p < 0,01$, and relatively unchanged for wave I ($p>0,05$). The statistical analysis of the BAER interwave latencies according to the intensity ot the applied stimulus showed that regardless of the intensity, the interwave latency was constant, with insignificant differences between the results ($p > 0,05$).

Hearing disorders are difficult to diagnose in veterinary medicine because of the limited ways of clinical investigation. Unlike bilateral deafness, where the owner can assess the animal's response to acoustic stimuli in the environment, in the case of unilateral deafness or of a decreased hearing acuity, clinical diagnosis becomes subjective. Breeding-related issues (at least 48 breeds of dogs are known to have hereditary congenital deafness - Dalmatians, Old English Sheep Dogs, etc.), impose an early diagnosis of dogs suffering from hearing disorders (Wilson J.W., 2005).

The passing of an acoustic stimulus from the ear to various structures within the nervous system generates a series of electrical signals with latencies of several milliseconds to hundreds of milliseconds. These auditory evoked potentials (BAER) are led through the tissues and can be collected by electrodes placed on the skin, thus evaluating the functioning of the ear and of the structures in the central nervous system that are activated by acoustic stimuli (Legatt A.D, 2005). Unlike clinical examination, BAER offer a high degree of objectiveness in the interpretation of results. The advantages of this method are: relatively easy to perform, noninvasive and safe for the patient and the results are sensitive, anatomically specific, independent

of the level of consciousness, rarely influenced by the administering of drugs. Furthermore, the analysis of the resulting waveforms can prove it's usefulness for the diagnosis of various conditions of the central and peripheral nervous system.

The existing studies on BAER in cats offer a wide range for the latencies and intervals (often grouped with the corresponding values in dogs). This study aims to establish reference values for the latencies of waves I, III and V, as well as for interwave latencies I–III, I–V and III–V.

## 1. MATERIALS AND METHODS

The study was made at the Internal Medicine Clinic of the Faculty of Veterinary Medicine in Iasi.

*Animals*: the study included five clinically healthy cats (three females– P01, P03, P05 and two males – P02, P04), aged between two and five years (an average of 3,2 years) and weighting between 1,8 and 3,6 kg (an average of 2,54 kg). After a clinical examination (NI), the cats were chemically restrained with Medetomidine at a dose of 0,05 mg/kg inj. IM., and placed in sternal recumbency.

*Equipment and electrode positioning:* the waves were recorded with the Neuropack S, MEB 9400K Electrodiagnostic system (NIHON KOHDEN) in the ABR program. Surface electrodes were placed as follows: the active electrode on the vertex, reference electrodes at the base of each ear and the grounding electrode on the median line, retrooccipitally. The area on which the electrodes were placed was trimmed, degreased, and covered with special adhesive paste.

*Procedure*: An impedance check was performed before each test, so as for it to be < 5Ω. Alternating click stimuli of 0,1 ms were applied through earphones inserted into the auditory canal. We performed individual tests on each ear, as well as binaural stimulation, with stimulus intensity decreasing from 90 to 40 dBSPL (decibells sound pressure level), using steps of 10 dBSPL (the nontested ear was masked with white noise of an intensity under 40 dB). Each waveform was the average of 500 stimulations, using a High-cut filter of 100 Hz and a Low-cut filter of 3000 Hz. Artefacts were automatically rejected. When rejected waveforms represented more than 5% of the averaged waveforms, the testing was repeated. The waves were manually labeled by the same examiner (MM), each positive peak receiving a roman numeral from I through V, and latencies of waves I, III, and V were observed, as well as the intervals I-III, III-V and I-V. (Fig. 1)

*Data processing*: the statistic significance of the results was tested with the t Student test, with a significance threshold of < 0,05.

## 2. RESULTS AND DISCUSSIONS

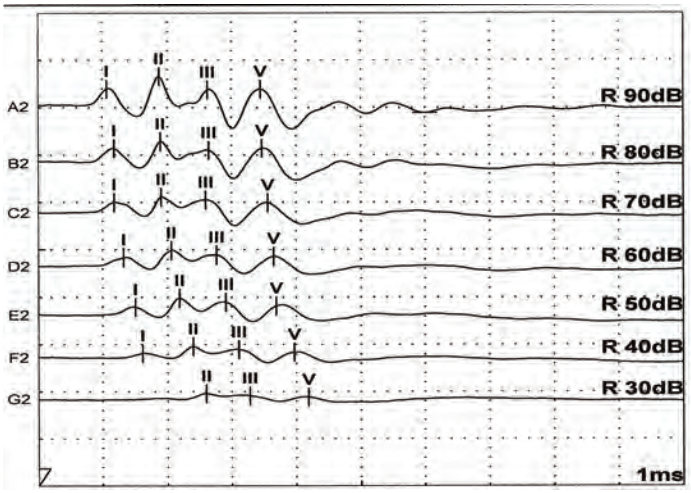


**Fig. 1 – BAER recorded at the stimulation of the right ear of P03 cat with intensities between 90 and 30 dBSPL.**

We noticed an increase in the latency of waves I, III and V and a decrease in wave amplitude with relatively constant interwave latencies. According to Wilson J.W. (2005) each wave is attributed to a different anatomical structure: wave I is generated by the cranial nerve VIII, wave II by the cochlear nucleus, wave III by the superior olivary complex, wave V by the caudal colliculus. Wave IV, which is not present in Fig. 1 originates in the nucleus of the lateral lemniscus.

The data obtained were reported as an average, with standard deviation. Average values for wave I, III and V at different stimulus intensity are shown in Table 1.

After examining the results, we didn't notice any statistically significant differences between the values recorded for each of the cats in the latencies of waves I, III and V ($p > 0,05$), the group having a high degree of homogeneity.

The data analysis shows that the latencies of wave I as well as III and V increase with lower stimulus intensity ($p< 0,05$).

*Table 1*

**Average values for the latencies of waves I, III and V (in ms) at different stimulus intensities (dBSPL)**

| | | 90 dBSPL | 80 dBSPL | 70 dBSPL | 60 dBSPL | 50 dBSPL | 40 dBSPL |
|---|---|---|---|---|---|---|---|
| **latency I** | average L | 1,03 | 1,07 | 1,11 | 1,23 | 1,31 | 1,44 |
| | average R | 1,01 | 1,08 | 1,14 | 1,24 | 1,4 | 1,58 |
| | average B | 0,98 | 1,07 | 1,13 | 1,24 | 1,41 | 1,61 |
| | **average** | **1,00 ± 0,025** | **1,07 ± 0,005** | **1,12± 0,015** | **1,23± 0,005** | **1,37± 0,055** | **1,54± 0,090** |
| **latency III** | average L | 2,6 | 2,59 | 2,63 | 2,73 | 2,77 | 2,94 |
| | average R | 2,57 | 2,63 | 2,63 | 2,77 | 2,89 | 3,03 |
| | average B | 2,62 | 2,66 | 2,71 | 2,83 | 3 | 3,03 |
| | **average** | **2,59± 0,025** | **2,62± 0,035** | **2,65± 0,046** | **2,77± 0,050** | **2,88± 0,115** | **3,00 ± 0,051** |
| **latency V** | average L | 3,33 | 3,38 | 3,38 | 3,56 | 3,64 | 3,77 |
| | average R | 3,34 | 3,38 | 3,45 | 3,63 | 3,71 | 3,91 |
| | average B | 3,42 | 3,47 | 3,55 | 3,67 | 3,82 | 3,91 |
| | **average** | **3,37± 0,049** | **3,42± 0,051** | **3,48± 0,083** | **3,63± 0,051** | **3,74± 0,088** | **3,87± 0,070** |

At an intensity of the stimulus of 90 dBSPL, the latency of wave I was 1,00 ± 0,025 ms, of wave III - 2,59 ± 0,025 ms and wave V 3,37 ± 0,049 ms, the values increasing up to 1,54 ± 0,09 ms for wave I, 3,00 ± 0,051 ms for wave III and 3,87 ± 0,07 ms for wave V at 40 dBSPL.

The comparative results of individual stimulation of the right and left ears of each cat didn't show statistically significant differences for waves I and III ($p > 0,05$). Significant differences ($p = 0,03$) were only noticed for the results obtained at monaural stimulation for wave V.

As for binaural stimulation, the latency of waves III and V was greater as compared to monaural stimulation (left or right) - $p < 0,01$, and relatively unchanged for wave I ($p>0,05$).

The statistical analysis of the BAER interwave latencies according to the intensity ot the applied stimulus (Table 2) showed that regardless of the intensity, the interwave latency was constant, with insignificant differences between the results ($p > 0,05$).

*Table 2*

**Average values of interwave latencies I-III, III-V and I-V (in ms) at different stimulus intensities (dBSPL)**

| | | 90 dBSPL | 80 dBSPL | 70 dBSPL | 60 dBSPL | 50 dBSPL | 40 dBSPL |
|---|---|---|---|---|---|---|---|
| **interval I-III** | Average L | 1,53 | 1,48 | 1,46 | 1,43 | 1,42 | 1,52 |
| | Average R | 1,55 | 1,52 | 1,45 | 1,48 | 1,41 | 1,49 |
| | Average B | 1,61 | 1,56 | 1,54 | 1,55 | 1,5 | |
| | **average** | **1,56 ± 0,025** | **1,52 ± 0,005** | **1,48 ± 0,015** | **1,48 ± 0,005** | **1,44 ± 0,055** | **1,50 ± 0,090** |
| **interval III-V** | Average L | 0,76 | 0,77 | 0,76 | 0,83 | 0,83 | 0,79 |
| | Average R | 0,77 | 0,75 | 0,85 | 0,87 | 0,83 | 0,87 |
| | Average B | 0,79 | 0,8 | 0,83 | 0,82 | 0,81 | 0,87 |
| | **average** | **0,77 ± 0,025** | **0,77 ± 0,035** | **0,81 ± 0,046** | **0,84 ± 0,050** | **0,82 ± 0,115** | **0,84 ± 0,05** |
| **interval I-V** | Average L | 2,3 | 2,27 | 2,23 | 2,3 | 2,28 | 2,31 |
| | Average R | 2,33 | 2,26 | 2,3 | 2,33 | 2,24 | 2,37 |
| | Average B | 2,4 | 2,35 | 2,37 | 2,37 | 2,28 | 2,39 |
| | **average** | **2,34 ± 0,049** | **2,29 ± 0,051** | **2,3 ± 0,083** | **2,33 ± 0,051** | **2,26 ± 0,088** | **2,35 ± 0,070** |

At an intensity of 90 dBSPL (the examination standard), the values obtained for wave latencies are lower than those reported by Cauzinille L. (1997) but the examination technique was different (his study was made with subcutaneous needle electrodes).

The morphological analysis of waveforms showed that a decrease of the intensity of the stimulus led to a decrease in the amplitude of the wave, up to the loss of individuality of some waves. Thus, at a stimulus intensity of 90 dBSPL we could identify all the waves (I, II, III, V and sometimes IV). At 40 dBSPL we could easily identify only waves I, III and V, whereas wave II was only present in one individual.

Concerning binaural stimulation, the differences between the latencies of waves III and V as compared to monaural stimulation showed the process of binaural interaction. It was observed in both humans and animals and reffers to the activation of some inhibitory mechanisms that cause a prolongation of the latency of waves of central origin (McPherson D.L., 1993). Studying the influence of the ventral nucleus of the lateral lemniscus, on the processing of mono and binaural auditory excitation in rabbits, Batra R. (2002) showed that this nucleus has a filter effect on auditory information (by eliminating the disparities caused by the arrival of impulses). Furthermore, between the two caudal colliculi (which generate wave V) there are morphological and

physiological connections that allow the summed analysis of the information. Caird D.M. (1987) showed that a lesion located in one caudal colliculus in cats will cause a delay or absence of wave V, after the stimulation of the controlateral ear. Therefore, there is a posibility that the caudal colliculi didn't have an inhibiting effect capable of increasing the latency of wave V as compared to monaural stimulation. The prolongation of wave V latency could be the result of the active involvement of the nuclei of the lateral lemniscus alone. Regarding the morpho-physiological substrate of wave IV (formerly located in the lateral lemniscal pathways) in dogs, Wilson J.W (2005) states that it must be considered in conjunction with wave V ( the wave IV – V complex) because of the small size of the nuclei and the reduced likelihood of the simultaneous neuronal firing, in light of the innervation from many different pathways.

Considering these informations, it is highly probable that the differences in waves III and V between monaural and binaural stimulation were influenced by lateral lemniscal pathways alone rather than by lateral lemniscal pathways and caudal colliculi together.

## 3. CONCLUSIONS

3.1. The decrease of the stimulus intensity determined a prolongation of waves I, III and V latencies ($p< 0,05$) with relatively constant I-III, III-V and I-V interwave latencies ($p>0,05$).

3.2. In the case of binaural stimulation, wave III an V latencies were greater as compared to monaural stimulation (left or right) - $p < 0,01$, and relatively unchanged for wave I ($p>0,05$).

3.3. The differences in waves III and V between monaural and binaural stimulation (binaural interaction phenomenon) could be influenced by lateral lemniscal pathways alone rather than by lateral lemniscal pathways and caudal colliculi together.

## ACKNOWLEDGMENTS

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# DEUTERIUM DEPLETED WATER EFFECT ON CROMIUM, CALCIUM AND MAGNESIUM LIVER LEVELS IN CHROMIUM (VI) INTOXICATED RATS

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**Key words**: cromium(VI), calcium, magnesium, deuterium depleted water, rats

**SUMMARY**

The present paper deals with the study of the deuterium depleted water (DDW) treatment effect on the liver levels of calcium and magnesium, after cromium(VI) intoxication**.**
The effect of the DDW (30 ppm) treatment on rats intoxicated with $K_2Cr_2O_7$ in single dose (20 ppm Cr(VI)/kg b.w.) has been observed.
Cromium(VI) administration in experimental groups (six groups of 12 female rats each) showed a strong augmentation of this toxic metal content in liver (24 times as control), suggesting it's high bioavailability. Calcium liver average values were 2.8 time higher at Cr intoxicated group as control. Similar values of magnesium were registered in all tested groups; DDW (30 ppm) treatment mantained both calcium and magnesium average values at the control (L1) levels after 60 days of *ad libitum* administration.
DDW performed an important Cr (VI) scavenger role in the rats` liver.

The deuterium depleted water (DDW) have special influence on the whole animal organism respectively on cells and tissues development (Somlyai,1998); a decreasing of the deuterium concentration in tissues or bodies, slow down the proliferation of a lot of types of cancer (Manolescu *et al*, 2006).

The chromium toxicity depends on the oxidation stage and its solubility. Cr(VI) is more toxic than Cr(III). Hexavalent chromium is used for the production of textile dyes, wood preservation, stainless steel, leather tanning and for its` anti-corrosion properties. Its`compounds are genotoxic carcinogens via inhalation; the major risk is of lung cancer kidneys and intestine. Cr(VI) reduction to inferior oxidation stages causes ADN lesions, thus rendering the Cr (VI) compounds carcinogenic. Cr (VI) induce an oxidative stress that results in oxidative deterioration of biological macromolecules; its` mecanism undergoes redox cycling. Workers who handle chromate-containing products are more exposed to Cr (VI).

The ability of carcinogenic chromium(VI) compounds to damage DNA depends on the presence of cellular redox components that reduce chromium(VI) to reactive species capable of interacting with DNA /chromatin. It is possible as the hydroxyl radical may be the ultimate carcinogenic species in cells and systems exposed to Cr(VI).

A number of mechanisms have been reported by which Cr (VI) is reduced to Cr (III). *In vitro* and underphysiological conditions, ascorbic acid, thiols, glutathione, cysteine, cysteamine, lipoic acid coenzyme A, and coenzyme M reduce Cr (VI) at a significant rate. The *in vitro* reaction of Cr (VI) with glutathione results in the formation of a Cr (V) intermediate that is possibly the form that interacts with cellular macromolecules. (Molyneux, Davies, 1995)

It is transported into cells via the sulfate transport mechanisms, because of the similarity in structure and charge of sulfate and chromate. Trivalent chromium, which is the more common variety of chromium compounds, is not transported into cells.

## 1. MATERIAL AND METHOD

The experiment was carried on 72 adult female Wistar rats, with a body weight of 220-240 g, maintained in good physiological conditions. They were divided in six groups. Each group included 12 rats.

L1- control, received tap water *ad libitum* during 61 days; L2 – received DDW (with a deuterium content of 30 ppm/l) *ad libitum* during 61 days; L3- received tap water during 30 days, in the 31 day, 20ppm Cr /kg b.w (as $K_2Cr_2O_7$) single dose was administrated by gastric tubing and after 24 hours L3 rats were sacrificed; L 4 – were pretreated with DDW *ad libitum* during 30 days, in the 31 day 20 ppm Cr/kg b.w in single dose was administrated by gastric tubing and after 24 hours L4 rats were sacrificed and L5 – were pretreated with DDW *ad libitum* during 30 days, in the 31 day 20 ppm Cr/kg b.w in single dose was administrated and more 30 days treated with DDW *ad libitum* and L6 – were pretreated with DDW *ad libitum* during 30 days, in the 31 day 20 ppm Cr/kg b.w in single dose was administrated and more 60 days treated with DDW *ad libitum.*

After 31 days from the beginning of the experiment (respectively 24 hours after Cr intoxication) blood was collected (on heparine), by cardiac punction and than sacrificed (L1, L2, L3 and L4); a second respectively a third sampling took place after 61 days (L5) and at the end of the experiment (91 days) (L6). Blood and tissue samples were collected under general narcosis.

Chromium content in liver was determined by atomic absorbtion spectrometry (AAS-Shimadzu 6200). Liver was digested in teflon containers in a microwave oven closed system (MARS X CEM).

The investigations were carried out with the approval of the Local Ethics Committee according to the Romanian law 205 /2004, art.7, 18, 22 and the regulations no. 143/400/2002 and 37/2002, concerning with the protection of vertebrate animals used for experimental and other scientific purposes.

The data are presented as means ± S.D. values. ANOVA, TTest, MINITAB and the nonparametric test Mann-Whitney were used to analyze mean differences between experimental groups for each parameter separately and between groups

## 2. RESULTS AND DISCUSSION

Chromium(VI) administration in experimental groups showed a strong augmentation of this toxic metal content in liver (24 times as control), suggesting it's high bioavailability.

Chromium average values at the pretreated and Cr intoxicated group (L4) were 7,59 times higher as at L2 (DDW) in comparison with L3 ($H_2O$+Cr) where there were registered average values 24,14 times higher as at L1. After 30 respectively 60 days DDW treatment and 30 days DDW pretreatment, the Cr average values were similar with the control group. In the same time, calcium liver average values were 2,8 time higher at Cr intoxicated group (L3) as control (L1) respectively 2,54 times higher at DDW pretreated and Cr intoxicated group (L4) as at DDW treated group (L2).

Similar values of magnesium were registered in all tested groups; DDW (30 ppm) pretreatment and treatment mantained both calcium and magnesium average values at the control (L1) levels after 60 days of *ad libitum* administration.

The results are presented in figure 1.

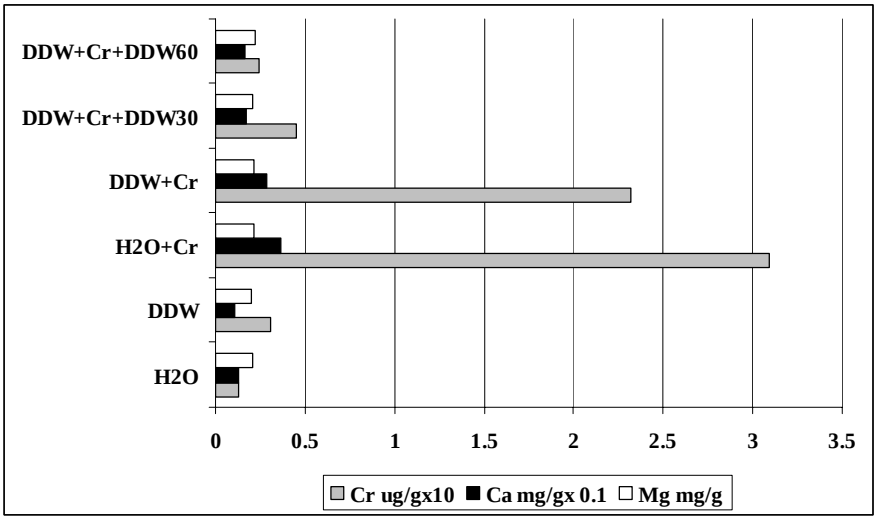


**Fig.1. Liver average values of Cr(VI), Ca and Mg in DDW treated rats**

## 3. CONCLUSION

3.1. DDW has an important scavenger role; after 90 days of DDW pretreatment and treatment the Cr liver registered 9,4 times lower average values at (L6) as at the intoxicated group (L3).

3.2. DDW pretreatment has a liver protective role in Cr (VI) intoxication

3.3.Liver calcium absorbtion increases in Cr (VI) intoxicated groups, but after 60 days of DDW treatment the average Ca values were similar as controls.

3.4.Chromium intoxication had no influence on the liver magnesium average values in rats

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# STUDIES CONCERINING THE BIOLOGICAL EVOLUTION OF ITALIAN BEES IN REPLACEMENT, GROWTH AND SWARMING PERIODS

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**Key words:** bees, biological evolution, swarming

**SUMMARY**

The study was conducted on Italian bee families comparative with the Carpathian bees. The bees were housed in multi-storey hives. The following parameters were studied: the queen bee prolificacy, the flight intensity during harvesting and during bad weather, the irascibility, the behaviour of the bees during the survey and the predisposition to swarming. Queen bee prolificacy and the rate of old bee's replacement were significantly higher in Italian bees. On the other hand, flight intensity during bad weather was 22.4% higher which caused high losses during the overcast periods. This breed didn't display the swarming instinct.

The Italian bee race (*APIS MELLIFERA LIGUSTICA*) is originated from Italy and has several varieties, including Sicilian and Iberian. It is the most widespread race of the specie *APIS MELLIFERA*, beekeepers are appreciated because it adapts well to all climates (temperate, mediterranean, subtropical), particularly in areas with warmer climate (Badino *et al*, 2006). The queens are very prolific and beekeepers can work without using the smoke. The study was conducted at the request of the many beekeepers to Romania who wants to grow this race to capitalize the early harvest.

## 1. MATERIALS AND METHODS

The experiment involved two bee breeds: the Italian and Carpathian Foti races. The bees were housed in multi-storey hives. The study was took place from February to May 2009.

The following parameters were studied: the queen bee prolificacy, the flight intensity during harvesting and during bad weather, the irascibility, the behaviour of the bees during the survey and the predisposition to swarming.

The queen bee prolificacy was established by the quantity of eggs deposited by the queens in 3 months (15 February-15 May 2009).

The flight intensity during harvesting and during bad weather were appreciated by number of bees (during a minute) which return from harvest and enter the hive.

The irascibility was evaluated by research without smoke.

The predisposition to swarming was established between 20-31 May, based on specific characteristics .

## 2. RESULTS AND DISCUSSION

The queens prolificacy was appreciated until the early harvesting of acacia, data are presented in figure 1.

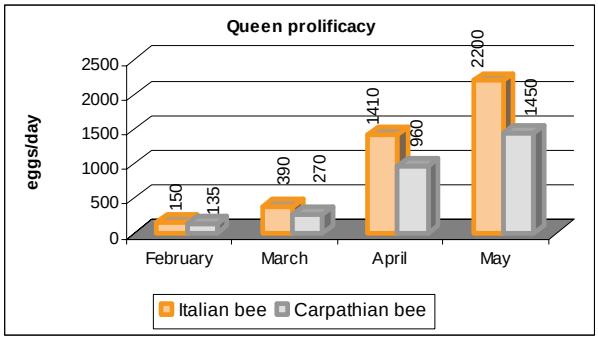


**Fig.1. Queen prolificacy**

At the Italian bee was found that prolificacy was higher, with 11% in February, 44.4% in March, 46.8% in April and 51.7% in May. In the conditions of our country, the results from the Italian bee are similar to those in the literature, which states that prolificacy is a feature race.

The data concerning the rate of bee's replacement during spring are shown in figure 2.

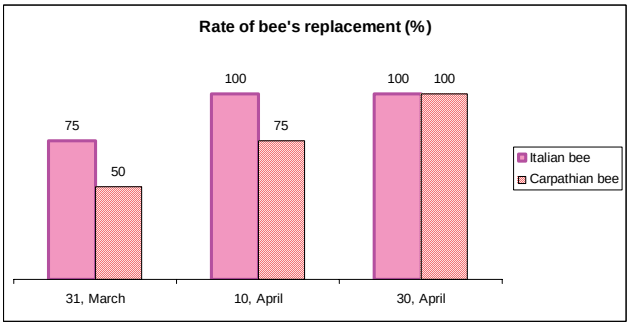


**Fig. 2. Rate of bee's replacement**

On March 31 the share of young bees was 75% at the Italian and 50% at the Carpathian race. Differences were represented by older bees. At the Carpathian bee, data entered in the values cited in literature (Bura *et al.*, 2005), indicating that the end of March half of the population is the young bees.

On April 10 the share of young bees was 100% at the Italian and 75% at the Carpathian race. In early April, the Italian bee, the entire population was replaced. In late April at the Carpathian bee, the entire population was replaced by young bees.

Between the two races there was a disparity of 20 days, to the Italian race advantage, which influenced the biological evolution of families, because only after replacing all old bees, a population start to development grows (Bura *et al.*, 2005).

The data concerning the flight intensity during harvesting are shown in table 1.

***Table 1***

**The flight intensity during harvesting**

| | Italian bee | Carpathian bee |
|---|---|---|
| Nr. Bees/minut | 151 | 124 |
| Bees amount/family, kg | 2,5 | 2,6 |
| Flight intensity | 60 | 49 |

It was noted that the intensity of the flight was 60 units of Italian bee and 49 units of Carpathian bee. The Italian race had a higher activity during the harvest, thus ensuring a higher recovery.

The data from literature shows that Italian bee is characterized by a high intensity of harvesting on over 55 units, which ensures high production of honey per family. Because of intense exploitation, bee longevity is less than in other races, but this is offset by negative feature high prolificacy queens (Cho *et al.,2005*, Jensen *et al.*, 2005).

Data concerning the flight intensity during bad weather are shown in table 2.

***Table 2***

**The flight intensity during bad weather**

| | Italian bee | Carpathian bee |
|---|---|---|
| Nr. bees/minut | 149 | 57 |
| Bees amount/family, kg | 2,5 | 2,6 |
| Flight intensity | 60 | 22 |

The flight intensity during the bad weather was 60 units of the Italian bee and 22 units of the Carpathian bee.

The literature shows that at the Italian bee the hydrants and thermal analyzers apparently are missing, therefore bees do not anticipate bad weather, registering heavy losses (Franck *et* al., 2000, Cho *et al.,2005*, Jensen *et al.*, 2005).

Table 3 shows the results concerning the bee's gentleness.

***Table 3***

**The bee's gentleness**

| | Italian bee | Carpathian bee |
|---|---|---|
| Score | 4 | 2-3 |

The Italian bees were received 4 points, corresponding to a very gentle behaviour, being able to work without a mask and smoke-free.

The Carpathian bees were received 2-3 points, corresponding to a peaceful behaviour, being able to work with less smoke, although sometimes the bees were nervous.

The data concerning the predisposition to swarming are shown in table 4.

***Table 4***

**The predisposition to swarming**

| | Italian bee | Carpathian bee |
|---|---|---|
| Score | 4 | 2-3 |

At the Italian bees swarming instinct was 4 points, which means that was absent.

The Carpathian bees were received 2-3 points, swarming instinct was reduced or was present but it could be mastered, because the queens were not to be replaced with other more young.

## 3. CONCLUSIONS

3.1. In conditions of Romanian breeding, at the Italian bees, the queen prolificacy and the replacement rate are higher than 47.3%.

3.2. The total bee's replacement has made in early April, with 20 days before the Carpathian bee.

3.3. At the Italian bees, the flight intensity during bad weather was higher, resulting significant losses in cloudy weather.

3.4. The behaviour is harmless and the swarming instinct wasn't present.

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# POTASSIUM DICHROMATE IMPACT ON SOME MARKERS OF FEMALE REPRODUCTIVE SYSTEM PERFORMANCES (LITTER SIZE, SEX RATIO) AND PHYSICAL DEVELOPMENT (VAGINAL OPENING) IN FEMALE RATS EXPOSED *IN UTERO*

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**Kek words:** female, rat, Cr VI, vaginal opening, sex ratio.

**SUMMARY**

Hexavalent chromium administration in drinking water during gestational period in female rats revealed significant decrease of alive pups number at birth, increase of dead rat pups, sex ratio perturbation in female favor; delayed puberty – dose dependent - over aged female offspring at sexual maturity (the vaginal opening moment), tardily reaching the necessary body weight for this physiological process.

Chromium is a heavy metal used in various industrial sectors. Improper handling and storage of chromium-laden effluents or wastes could lead to environmental pollution. The most toxic form is the more mobile one: hexavalent chromium (CrVI) (1, 7, 8), important reproductive toxicant (8, 15).

## MATHERIAL AND METHODS

The study was carried out on 20 pregnant female rats (females and males were not exposed to potassium dichromate before mating) divided in three experimental groups (E) and one control group (C). The E groups received potassium dichromate in drinking water as follows: $E_1$: 25mg Cr (VI) - LOAEL (15), $E_2$: 50 mg Cr (VI) (2 x LOAEL), $E_3$: 75 mg Cr (VI) ) (3 x LOAEL), C: tap water not containing chromium. In female offspring, at birth, alive and dead pups number, the age, weight (by technical balance) at vaginal opening, and sex ratio were evaluated.

The results were statistically analized by Anova method and Student test.

Food and water were *ad libitum.*

All assays with animals were conduced in accordance with present laws regarding animal welfare and ethics in animal experiments (9, 10, 11, 12, 13, 14).

## RESULTS AND DISSCUTIONS

The results regarding alive and dead pups number are presented in table 1, figure 1.

***Table 1.***

**Alive and dead (mean) number of rat pups at birth**

| | Alive pups | | | Dead pups | | |
|---|---|---|---|---|---|---|
| **Group** | **X±Sx** | **S.D.** | **C.L.95 %** | **X±Sx** | **S.D.** | **C.L.95%** |
| **C** | 13.8±0.37 | 0.84 | 0.70 | 0.00±0.00 | 0.00 | 0.01 |
| **$E_1$** | 7.60±0.24 | 0.55 | 0.70 | 2.30±0.01 | 0.01 | 0.01 |
| **$E_2$** | 6.80±0.2 | 0.45 | 0.70 | 3.80±0.01 | 0.01 | 0.01 |
| **$E_3$** | 6.00±0.45 | 1.00 | 0.70 | 4.80±0.01 | 0.01 | 0.01 |

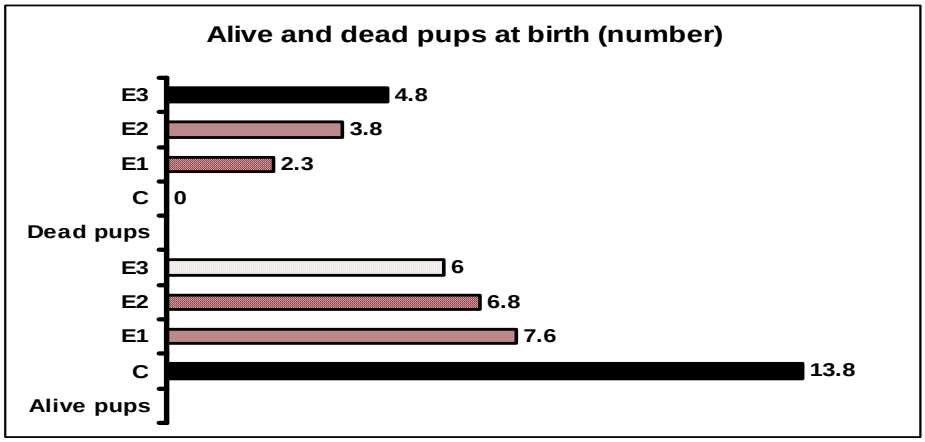


**Fig. 1.Alive and dead pup number dynamics**

Chromium exposure determined the birth of a significant, decreased number of alive rat pups in E groups comparative to C group ($E_1$/C:-44.92%, $E_2$/C:-50.72%, $E_3$/C:-56.52%), inversely corelated, with different degrees of significance, to the exposure level ($E_2/E_1$:-10.52%, $p<0.05$, $E_3/E_2$:-11.76%, $p>0.05$, $E_3/E_1$:-21.05%, $p<0.05$).

The pups dead number at birth increased significantly in E groups comparative to C group ($p<0,01$) ($E_1$/C:2.30/0, $E_2$/C:3.80/0, $E_3$/C:4.80/0)

and directly, significantly, ($p<0.01$), correlated to the exposure level ($E_2/E_1$:+65.21%, $E_3/E_2$:+26.31%, $E_3/E_1$:+108.69%).

Similar results regarding decrease of alive pups number and increase of dead pups number consecutive exposure to potassium dichromate were obtaind by Junaid et al., 2005, after gestational exposure, by Kanojia et al., 1996, after preconceptional exposure, and by Kanojia et al., 1998, after three months exposure.

Hexavalent chromium impact on sex ratio is presented in table 2, figure 2.

***Table 2.***

**Sex ratio in offspring exposed *in utero* to potassium dichromate**

| Group | Sex | Number | % | Sex ratio M/M+F |
|---|---|---|---|---|
| C | ♀ | 6.9 | **50%** | **0.50** |
| | ♂ | 6.9 | **50%** | |
| | **Total/group** | **13.8** | **100** | |
| $E_1$ | ♀ | 6 | **60.60%** | **0.39** |
| | ♂ | 3.9 | **39.39%** | |
| | **Total/group** | **9.9** | **100** | |
| $E_2$ | ♀ | 6.80 | **64.15%** | **0.35** |
| | ♂ | 3.8 | **35.84%** | |
| | **Total/group** | **10.6** | **100** | |
| $E_3$ | ♀ | 7 | **64.81%** | **0.35** |
| | ♂ | 3.8 | **35.18%** | |
| | **Total/group** | **10.8** | **100** | |

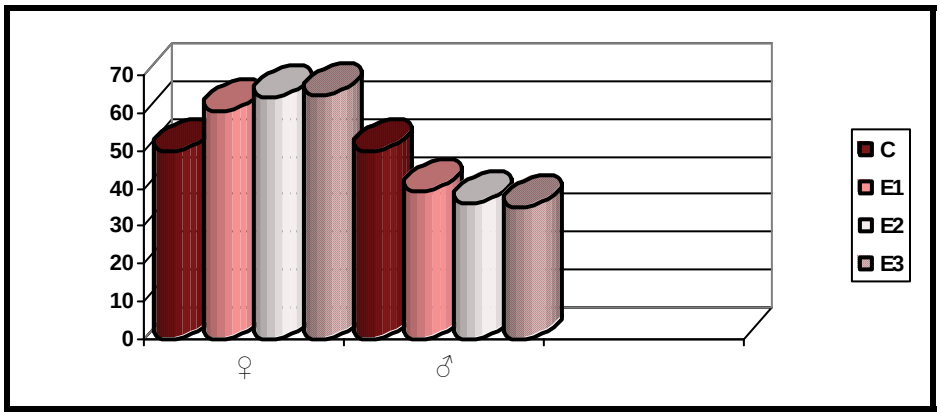


**Fig. 2. Sexual dynamics of male (♂) and female (♂) percentage at sexual maturity**

In E groups, increase of female pups percentage and decrease of male pups percentage comparative to control group were recorded (C: 50%/50%, $E_1$: 60.60%/39.39%, $E_2$: 64.15%/35.84%, $E_3$: 64.81%/35.18%).

Exposure level significantly influenced the female and male pups percentage dynamics, in direct correlation in the females: $E_2/E_1$:+5.85%, $E_3/E_2$:%+1.02%, $E_3/E_1$:+6.94%, and inversely correlated in males: $E_2/E_1$:-9.01%, $E_3/E_2$:-1.84%, $E_3/E_1$:-10.68%.

Sex ratio was modified in E groups comparative to C group in female advantage: $E_1$/C: +21.2%, $E_2$/C:+28.3%, $E_3$/C:+29.62%, $p<0.01$.

Few literature data quote hexavalent chromium impact on sex ratio. Only one reference mentioned that Cr VI does not modify sex ratio (Kanojia et al., 1998).

The consequences of exposure to potassium dichromate on vaginal opening in female rats exposed *in utero* are presented in table 3, figure 3.

***Table 3.***

**Age and weight (mean values) at vaginal opening in female rats exposed *in utero***

| | Age (days) | | | Body weight (g) | | |
|---|---|---|---|---|---|---|
| **Group** | **$X \pm S_x$** | **S.D.** | **C.L. 95%** | **$X \pm S_x$** | **S.D.** | **C.L. 95%** |
| **C** | 40.50±0.34 | 1.08 | 0.71 | 101.60±0.37 | 1.17 | 0.68 |
| **$E_1$** | 69.70±0.42 | 1.34 | 0.71 | 78.00±0.26 | 0.82 | 0.68 |
| **$E_2$** | 79.40±0.34 | 1.07 | 0.71 | 74.50±0.34 | 1.08 | 0.68 |
| **$E_3$** | 86.00±0.26 | 0.82 | 0.71 | 64.50±0.34 | 1.08 | 0.68 |

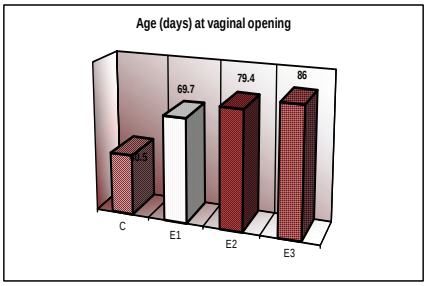


**Fig. 3. Age of female rats at sexual maturity**

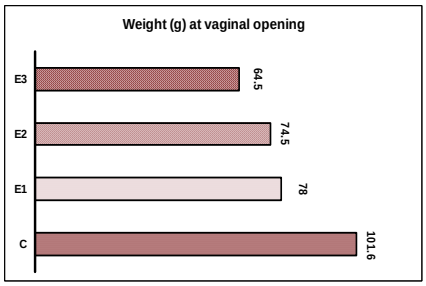


**Fig. 4. Weight of female rats at sexual maturity**

In E groups, the age at vaginal opening was significantly ($p<0,01$) delayed comparative to C group and over physiological limits (33-43 days, 5) ($E_1$/C:+72.09%, $E_2$/C:+96.04%, $E_3$/C:+112.34%). The body weight at the puberty onset was under 100g (considered phisiological, 5)

(64,5 – 78 g), and significantly ($p<0.01$) under the weight of C group individuals ($E_1$/C:-23.22%, $E_2$/C:-26.67%, $E_3$/C:-36.51%).

The delay of the vaginal opening was directly, significantly ($p<0,01$), correlated to the exposure level ($E_2/E_1$:+13.91%, $E_3/E_2$:+8.31%, $E_3/E_1$:+23.38%).

The weight at the vaginal opening and the exposure level were inverselly corelated ($p<0.01$) ($E_2/E_1$:-4.48%, $E_3/E_2$:-13.42%, $E_3/E_1$:-17.30%).

The results are in accord to those obtained by KEI-ICHIRO et al., 2000 underlinying the importance of the corelation between body weight and the moment of the vaginal opening and the fact that it depends more of the body weight than of the age.

The delay of sexual maturity consecutive potassium dichromate administration was recorded by other authors too (Sakhila et al., 2008).

## CONCLUSIONS

3.1. Significant decrease of alive pups number in the exposed groups comparing to control group and inversely, significantly correlated to the exposure level, excepting the increase of the exposure level from 50 to 75 ppm;

3.2. Significant increase of dead pups number at birth in experimental groups, comparative to control group and in dirrect, siginificant correlation with the exposure level;

3.3. Significant and dose dependent sex ratio modification in female favor,

3.4. Significant increase, with over passing the physiological limits, of the age at the vaginal opening moment, comparative to the control group and in direct, significant correlation, with the exposure level;

3.5. Significant decrease, under physiological limits, of the body weight, at the vaginal opening moment, comparative to control group and inversely, significantly correlated to the exposure level.

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# THE STUDY OF SOME DETAILS REGARDING THE MASETER MUSCLE IN SHEEP

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**Key words**: dissection, masseter, sheep.

**SUMMARY**

Research has been performed on five head of sheep. Masseter muscle was dissected carefully, noting the three parts of its relations with vasculo-nervous formations from the region. The main purpose of this study was to observed the development of three parts in connection with the role which they carry out.

Small ruminants are an excellent biological material for experiments and research in biology and medicine compared. In literature there are physiological and histochemical comparative studies on masseter muscle in domestic animals (3,7). Also there are studies of experimental surgery in animals and functional anatomy studies in humans masseterin region (6). However, the literature does not describe in detail the three parts of the muscle in sheep (1,2,4,5). Sometime there are even confusion. For this reason we broached this subject.

## 1. MATERIALS AND METHODS

The dissection was realized on five adult sheep heads. The skin was carefully, with a view to notice the relationships of all superficial anatomical formations with masseter muscle. The last was dissected in anatomical layers, taking photographs of the most important aspects. The identification, description and homologation of the anatomical formations was made according to Nomina Anatomica veterinaria - 2005.

## 2. RESULTS AND DISCUSSIONS

The masseter muscle can be separated in three parts. The superficial part has its origin on the facial tubercule, through a tendon, which is

widened in lower part, changing itself into a aponevrosis, whose oral border get under the middle part of masseter muscle (Fig. 1).

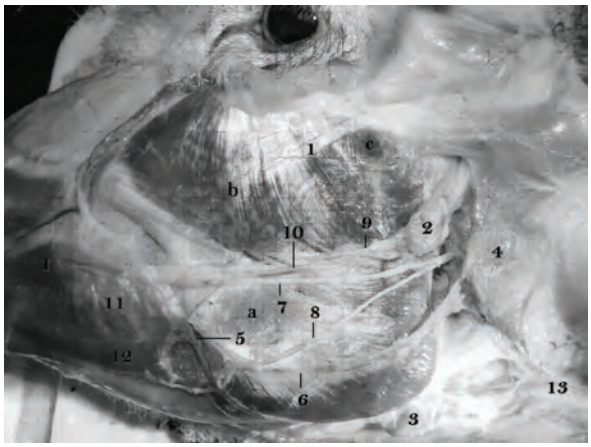


**Fig. 1 The topography of anatomical formations in the masseter region (superficial plane after removal of zygomatic muscle and parotid gland)**
**a- masseter muscle, superficial part, b-masseter muscle, middle part, c-masseter muscle, deep part; 1-m. zygomatic; 2-parotid ln; 3-mandibular ln; 4-parotid gland; 5-facial vein; 6-parotidian duct;7- dorsal buccal n.; 8- ventral buccal n.; 10- transverse facial artery and vein; 11- buccinator m.; 12-depressor labii inferioris m.; 13- jugular vein.**

The lateral face of this part is covered by a thin and transparent aponevrosis, wich is attaching to facial crest (the last marks the external lacrimal fossa) and to lower edge of orbit, up to the zygomatic arch.

The muscle fibres of superficial part are inserting to the external face of middle part, which to a great extent is aponevrotic, and to the lower edge of the mandibular branch, from the vascular notch to the angle of mandible.

The middle part has its origin on maxilla, ventral from facial crest, and to lower part of zygomatic arch (Fig. 2). The lateral face of this part is doubled by a white-mother of pearl aponevrosis which receives the insertion of muscle fibres belong-ing to the superficial part. The cranial border of the middle part is sourounded by a fold of superficial aponevrosis. The insertion is achieved on the lateral face of distal half of vertical part of mandible branch.


**Fig. 2 Masseter muscle - middle plane**
**a- masseter, superficial part; b- masseter, middle part, c- masseter, deep part; 1-facial vein; 2- mandibular ln.; 3- linguofacial vein; 4- jugular vein; 5- jaw, 6-orbicularis oculi; 7-zygomaticoauricularis; 8- dorsal buccal n.; 9 - transverse facial artery and vein; 10-superficial temporal n.**


**Fig. 2 Masseter muscle - middle plane**
**a- masseter, superficial part; b- masseter, middle part, c- masseter, deep part; 1-facial vein; 2- mandibular ln.; 3- linguofacial vein; 4- jugular vein; 5- jaw, 6-orbicularis oculi; 7-zygomaticoauricularis; 8- dorsal buccal n.; 9 - transverse facial artery and vein; 10-superficial temporal n.**

The deep part is the smallest; it has vertical fibres with theirs origin on the ventral side of zygomatic arch, behind the middle part (Fig. 3). Their insertion is on the vertical part of the mandible branch, in the

upper half. It comes in contact with the temporomandibular joint capsule and is rostro-ventraly by the masseterin nerve endings destined to superficial and middle parts.

## 3. CONCLUSIONS

3.1. The superficial part of masseter muscle have a small fleshy part, principally beeing responsible for mandibular propulsion.

3.2. The middle part is the best represented, plainy a proeminent part in raising and propulsion of the mandible.

3.3. The deep part is the smallest, and it is responsible for lateral and backward mouvements of the mandible.

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# HISTOLOGICAL ASPECTS OF THE ESOPHAGUS IN STRUTHIO CAMELUS

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**Key words:** ostrich, esophagus, histostructure.

**SUMMARY**

In the literature, the information on the digestive system histology of ostrich (Struthio camelus) is rare, most data referring to the species *Gallus domesticus*.
Histological structure of the esophagus to the ostrich is largely similar to the species *Gallus domesticus*. Esophageal wall is composed of four layers: mucous membrane, submucous membrane, muscular, adventitious or serous.
The organs mucous are covered by a pluristartified epithelium that presents numerous longitudinal folds, and between them appear displaced esophageal glands, which are a type mucous aspect saciform.
The sub mucous membrane is poorly represented.
The muscular membrane is highly developed and presents striated muscle fibres in the third superior part and in the rest of the esophagus there are smooth muscle fibres arranged in two levels: internal circular and external longitudinal.
The adventives is non-uniformly developed, is composed of lax subjunctive tissue, blood vessels and nerve formation.
Knowing in detail the elements of the cells, tissue or organ at certain ages, allows a proper explanation and interpretation of various physiological and pathological aspects related to the studied structures.

The existing data in the literature on the histology of esophagus to birds are relatively rare and refers particularly to the species Gallus domesticus. Retrieving some histological data from specie and their transposing to specie is inadequate, being known that the structure and function of organs show marked features of species.

Esophagus is relatively narrow and has a very thin wall. Only the initial and the final segment of the esophagus have a wider lumen. Crossing the esophagus into the stomach occurs gradually, without a visible border between the two organs.

A characteristic of the ostrich esophagus is the absence of the crop and a high extensibility of the organ in width, which allows to the ostrich objects large enough to swallow.

Cervical portion of the esophagus is deviated to the left, but in the average third of the neck is placed on its dorsal side, forming a furniture dowser.

In the cranial portion of dorsal thoracic side, the esophagus passes dorsally towards the bronchi and further between the heart and lungs. Ventral cord is enclosed by auriferous clavicle bags. At the level of the sixth cervical rib, the esophagus distends, opening itself inside the pro ventricle.

The histological structure of the esophagus to the ostrich is largely similar to the structure of hens.

The esophageal wall is composed of four layers: mucous membrane, sub mucous membrane, muscular, adventitious or serous.

This paper work will present histological aspects of the esophagus to Struthio camelus species at the age of 17 days.

## 1. METHODS AND DOCUMENTATION

Researches have followed the morphological study of the esophagus to the ostriches aged 17 days. The study was conducted on a total of 5 subjects, experimental killed, coming from a farm in Constanta County.

The pieces collected, represented by fragments of esophagus, fixed in neutral saline formol, were processed by the histological technique based on inclusion in paraffin. The paraffin blocks were sectioned at 6 microns, the coloration being performed by haematoxylin methods.

The examination of the obtained histological preparations was done using optical microscope NIKON-LABOPHOT 2, equipped with a BG-33 filter light, Spot Meter Nikon AFX-DX, a Canon 640 digital camera and the images were processed with the help of the computer using Adobe Photoshop 6.0.

## 2. RESULTS AND DISCUSSIONS

The histological structure of the esophagus to the ostrich is largely similar to the structure of hens.[1,2,3]

The esophageal wall is composed of four layers: mucous membrane, sub mucous membrane, muscular, adventitious or serous. [4,5,6]

The mucous membrane of the organ is covered by a stratified epithelium. The lamina of the esophageal mucous membrane is poor in elastic fibres and lymphoid tissue, and presents many growths and papilla such as esophageal mucous glands. According to their histological structure, these glands are sacciforme. From the gland cavity, is leaving an excretory and large duct, in which are opened some glandular lobules. The last glandular lobe ends with a hole that rises to the surface lining of the esophagus. The glands are placed as a package,

each of them having 2-4 lobules. The ostrich glands are arranged along the length of esophagus in the form of a necklace in one row, their concentration being non - uniform. The glands present a simple prismatic epithelium, and the ducts represent a simple cubic epithelium. The muscular mucous is represented by smooth muscle tissue, the muscle fibres being oriented longitudinally. (Fig.1 and Fig.2)

The sub - mucous membrane is poorly represented, containing elastic fibres, vascular and nerve plexus.

The muscular membrane is represented by striped muscular fibres on the third superior part and in the rest of the esophagus there are smooth muscle fibres arranged in two levels: internal circular which is highly developed, poorly developed at the external longitudinal of the initial part and most developed in the final portion of the esophagus, among which there are integrated neurons belonging to Auerbach nerve plexus.(Fig.3 and Fig.4)

The outer layer is in contact with lax connective tissue, covered by a simple pavement epithelium, forming together serum membrane, where vascular and nerve formations are numerous.(Fig.5)

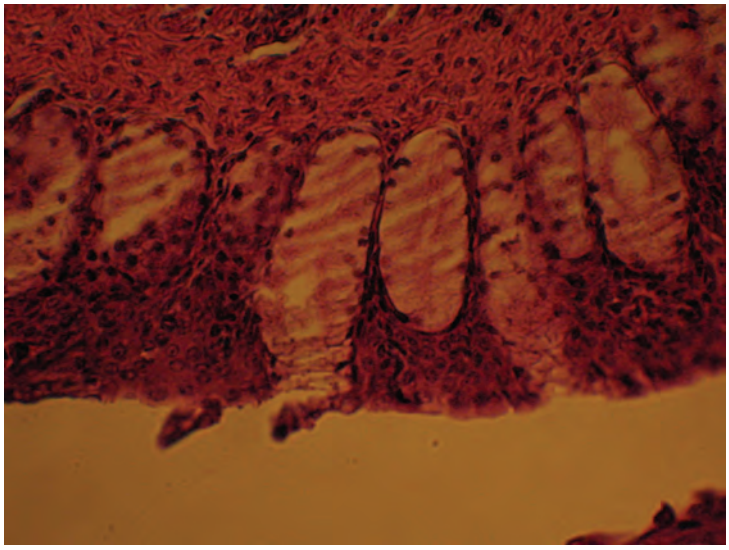

**Fig. 1. Detail – esophagus membrane, col. H.E. ob.40x**

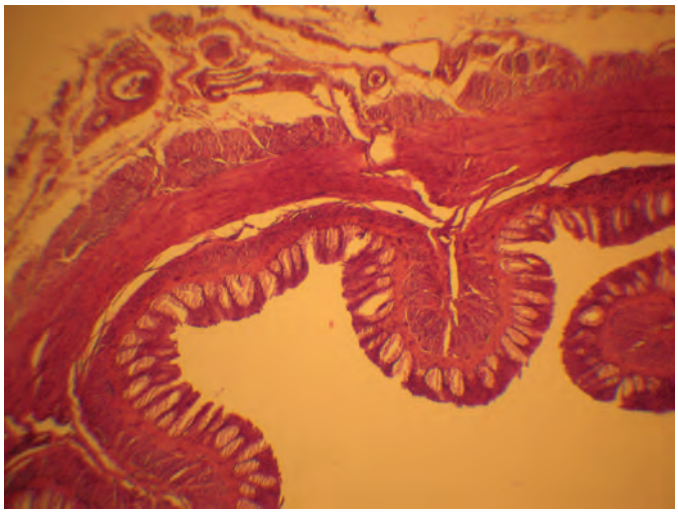

**Fig. 2. Esophageal wall – General View, col H.E, ob. 10x**

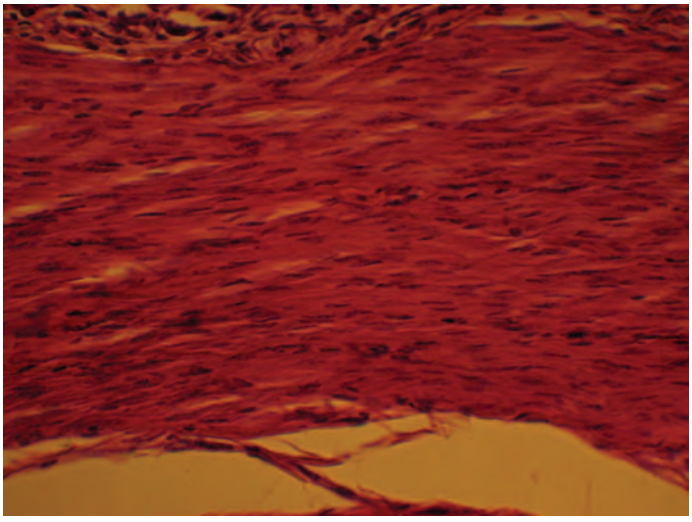

**Fig. 3. Detail – muscular membrane - internal circular layer, col.H.E, ob.40x**

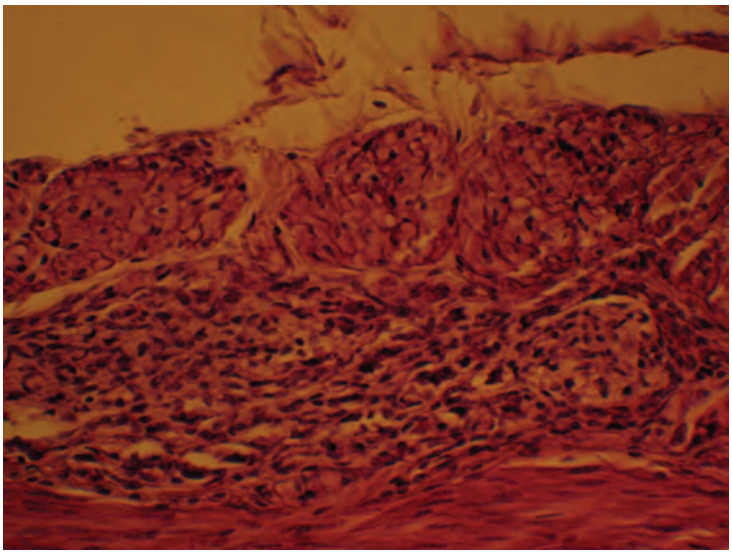

**Fig. 4. Detail – muscular membrane - external longitudinal layer, col. H.E, ob.40x**

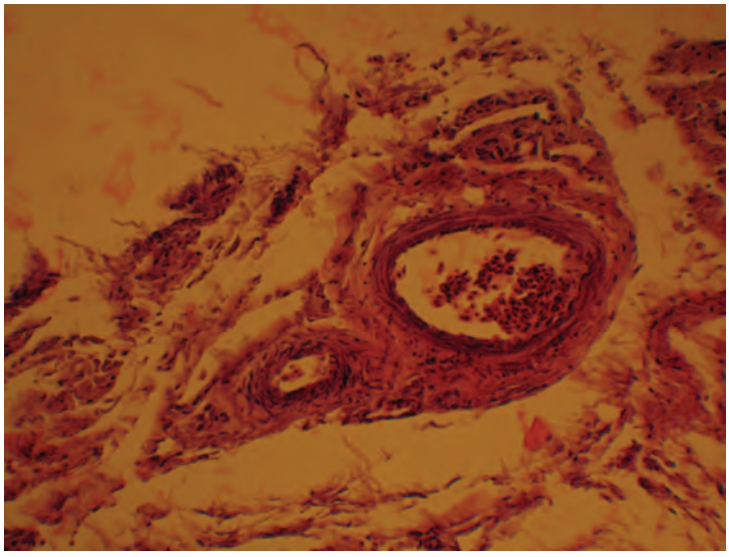

**Fig. 5. Detail – serous membrane – vascular formations, col. H.E, ob.40x**

## 3. CONCLUSIONS

3.1. A characteristic of the ostrich's oesophagus is the absence goiter and the extensibility in the width of the organ.

3.2. The mucous membrane presents longitudinal folds in which the oesophageal glands are deployed.

3.3. The oesophageal glands are mucous type aciform, placed in a single row, rarely in two. The degree of concentration and form on the stretched organ are inconsistent.

3.4. The muscular membrane is highly developed.

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# COMPANION ANIMALS ROMANIAN EPIDEMIOLOGICAL SURVEILLANCE NETWORK: PETEPINETVET

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PetEpiNetVet, companion animals, veterinary epidemiology, epidemiological network, surveillance

**SUMMARY**

In clinical practice, there is a daily need for valid, up-to-date, information about diagnosis, therapy and prevention. The epidemiological studies using large clinical and laboratory data allow vets to estimate the risk of infection for pets in their area and to perform individualized risk assessments for vaccination or therapy. The current epidemiological concepts explore and assimilate the new information technology services, and some of those resources have already been integrated in companion animal medicine. In this paper we propose a classical and easy to use network designed for companion animal veterinary practitioners whose acronym is PetEpiNetVet (**C**ompanion **A**nimals **R**omanian **E**pidemiological **S**urveillance **Net**work**).** The main objective of PetEpiNetVet is to collect and share in real-time the epidemiological data of infectious and parasitic diseases in companion animals: dogs, cats, horses, and exotic animals. The network is designed to provide information, support the utilization of national and regional resources, to improve communication between veterinarians, to contribute to the search for partners, to support the experience exchange and to improve the research in veterinary and biomedical disciplines. PetEpiNetVet is open to field veterinarians, university teachers, research institute, staff members, laboratory diagnosticians and advanced students.

In clinical practice, there is a daily need for valid, up-to-date information about diagnosis, therapy and prevention. This situation is complicated by the inadequacy of traditional sources for this type of information, since they may be out-of-date (text books), they are frequently wrong (experts), ineffective, too overwhelming in volume, or too variable in validity [2].

Epidemiologic large data bases can produce the evidence required to address important veterinary clinical and public health issues. The epidemiological studies using large clinical and laboratory data allow veterinarians to determine the risk of infection for pets in their area and to perform individualized risk assessments for vaccination or therapy.

The current epidemiological concepts explore and assimilate the new information technology services, and some of those resources have already been integrated in companion animal medicine. If we want a

betterment and health of all species our actions need to be integrated into concept "one medicine-one health". This means more sharing of surveillance data and greater cooperation among organizations involved in surveillance [4].

In this paper we propose a classical and simple network designed for companion animal veterinary practitioners with acronym PetEpiNetVet**.** The main objective of PetEpiNetVet is to collect and share in real-time the epidemiological data of infectious and parasitic diseases in companion animals: dogs, cats, horses, and exotic animals. Also, this network will be able to: provide information about syndrome and disease events in companion animals, conduct statistical analyses to identify space-time clusters of events and risk factors (host, environment) for diseases, alert to the occurrence of potential acts of bioterrorism and emerging zoonoses and characterize safety and efficacy of veterinary drugs and vaccines (pharmacoepidemiology).

The PetEpiNetVet goals are to help veterinary doctors to discover new introduced disease into the area, to prevent disease dissemination or to adopt the best therapeutical protocol.

The goal is to help veterinarians and pet owners develop practical preventive measures to reduce the incidence of these diseases.

In most countries, including Romania, the information on zoonoses and other diseases with national importance are managed by governmental agencies (e.g. Health Ministry, Romanian Veterinary and Food Safety Agency). Also, the funds are usually available for research on diseases of economically important livestock.

However, investigation of companion animals' diseases, especially if they are not demonstrably of public health significance, relies on the limited financial support available from welfare societies and charities. Lack of funds therefore can restrict companion animals data collection [1].

In spite of the lack of financial support, there are a few networks that refer to companion animal infectious diseases. For example, a National Companion Animal Surveillance Program (NCASP) was established at Purdue University to monitor clinical syndromes and diseases using the electronic medical records of >80,000 companion animals visiting >500 Banfield hospitals weekly in 44 states. NCASP recieved funding from the Centers for Disease Control and Prevention, and was initially developed for syndromic surveillance of Category A agents of bioterrorism. Surveillance was expanded through inclusion of electronic reports from Antech Diagnostics, a nationwide network of integrated veterinary diagnostic laboratories serving >18,000 private

veterinary practices. NCASP characterizes and displays temporal and spatial patterns of diseases in dogs, cats, and other companion animals. It detects unusual clusters of potential emerging/zoonotic infections and monitors flea and tick activity. Data is processed and analyzed using SAS and ESRI software products. The NCASP can be used by veterinarians to enhance their practice of evidence-based medicine by providing information needed to individualize vaccine protocols for animals in specific geographic areas [2].

For example, NCASP carried a study to evaluate the prevalence of and risk factors for canine tick infestation. Knowledge of the geographic range and seasonal activity of vector ticks is important for determining which people or animals are at risk of acquiring tick-borne infections. Several methods require large-scale organization and are used to map geographic and seasonal variations in tick distribution. Prevalence of canine tick infestation in 40 states was estimated by analyzing electronic medical records of more than 8 million dog visits to Banfield veterinary hospitals in 2002-2004. In multiple logistic regressions, younger dogs, male dogs, and sexually intact dogs, were at increased risk of tick infestation. Toy breeds were least likely to be infested, but no linear pattern of risk was evident with body weight. Identified risk factors should enable veterinarians to prevent tick infestation in pet dogs [3].

Similarly, Companion Animals Romanian Epidemiological Surveillance Network is designed to provide information, support the utilization of national and regional resources, to improve communication between veterinarians, to contribute to the search for partners, support the exchange of experience and to improve the research in veterinary and biomedical disciplines. PetEpiNetVet is addressed to field veterinarians, university teachers, research institute staff members, laboratory diagnosticians and advanced students.

At this moment PetEpiNetVet uses the web resources of our partner www.magazinveterinar.ro. The network website uses the Romanian language and to facilitate the registration the veterinary doctors will complete a simple form with contact information: name, city, office address, telephone number and e-mail address. The main tools used in order to improve communication and collect valuable information are the PetEpiNetVet section of www.magazinveterinar.ro webpage, instantly distributed e-mail messages, e-bulletins distributed to the registered members and e-workshops. The webpage is designed to help pet owners as well as veterinarians using the „search for a veterinarian“ tool which helps users find all registered veterinarians in a specific area (Fig 1).

All registered veterinary doctors will report to PetEpiNetVet any companion animal disease, regardless of the case status: suspect (clinical signs or laboratory results pending) or laboratory confirmed. The information collected (veterinary identity, location, reported disease) will be inserted into a database and then statistically processed (maps and charts). Also, veterinary doctors will be able to access all information posted: epidemiology, clinical signs, diagnosis, standardized confirmation methods, profilaxy (Fig 2).

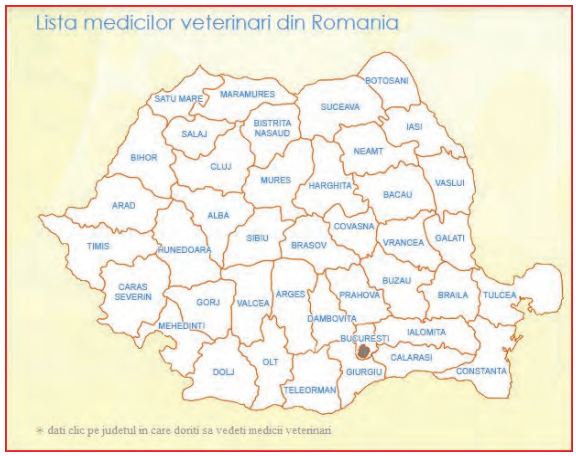


**Fig 1 The „search for a veterinarian" tool on www.magazinveterinar.ro website**

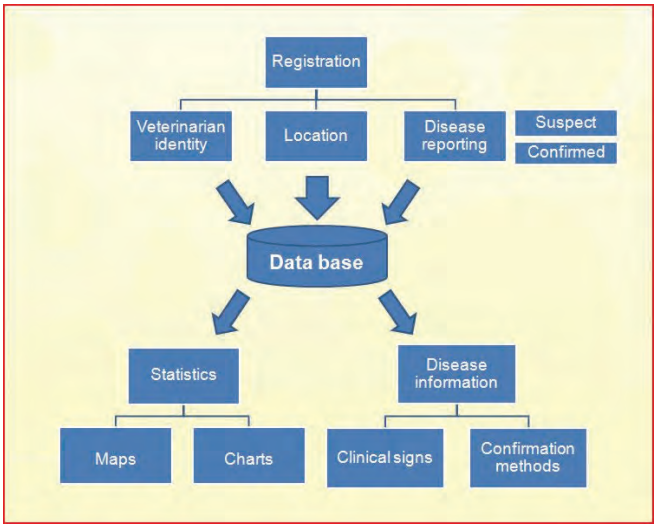


**Fig 2 PetEpiNetVet data flow scheme**

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# POTASSIUM DICHROMATE CHRONIC EXPOSURE IMPACT ON RAT SPERM QUALITY

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**Key words**: chromium VI, toxicity, rat, sperm

**SUMMARY**

The aim of the study was the evaluation of potassium dichromate cumulative and differentiate intake on integrity and performances of male reproductive system biomarkers. All assays with animals were conduced in accordance with present law regarding animal welfare and ethics in animal experiments (143.400/2002; 471/2002; 205/2004; 206/2004; 9/2008; 86/609/CEE). The study carried out on 28 white Wistar male rats divided in three experimental groups which received potassium dichromate in drinking water for six months as followed $E_1$: 25 ppm (LOAEL); $E_2$: 50 ppm (2 x LOAEL); $E_3$: 75 ppm (3 x LOAEL) and one control group which received tap water, not containing chromium, pointed out: significant decrease of sperm count comparative to control group and in inverse correlation to exposure level; significant decrease of total and progresive motility comparative to control group and in inverse correlation to exposure level; significant increase of sperm anomalies comparative to control group and in direct correlation to exposure level. Both, primary (detached head, flex head) and secondary anomalies (bent tail, broken tail, curl tail, tail without head, tail disintegration and fragmentation) were found with predominance of the secondary one.

Chromium is the 24$^{th}$ element of the Periodic table. The most important forms are trivalent (Cr III) and hexavalent (Cr VI) chromium. Chromium III is an essential element, low toxic, poorly absorbed and little crosses cell membrane (EPA, 2001).

Chromium VI is strong oxidizing agent being considered toxic and carcinogenic. In this state (hexavalent) chromium crosses cell membrane and exerts its toxic effect, which is 1000 times higher than that of Cr III (EPA, 2001). Chromium VI, metal used in more than 50 industries: leather tanning, chrome electroplating, welding, stainless steel factory, wood processing etc. (ATSDR, 2001), caused wide spread contamination of air, water, soil and food (Rizescu, 2007).

Manny heavy metals are known to affect testicular structure, spermatogenesis and steroidogenesis in man and animals, leading to infertility (Aruldhas *et al*., 2004). Testicular tissue is a major target for metal-induced oxidative damage because of its high content of polyunsaturated membrane lipids (Acharya *et al.*, 2004).

There are few studies in the literature concerning chromium as male reproductive toxicant. That's why the aim of this study was the evaluation of potassium dichromate cumulative and differentiate intake on integrity and performances of male reproductive system biomarkers. The objective of the study was the evaluation of the potassium dichromate impact on sperm quality.

## MATERIALS AND METHODS

Twenty eight adult white Wistar male rats were divided in three experimental and one control group. Experimental groups received potassium dichromate in drinking water for six months as followed $E_1$: 25 ppm (LOAEL) (EPA, 2001); $E_2$: 50 ppm (2 x LOAEL); $E_3$: 75 ppm (3 x LOAEL); control group received tap water (without chromium content).

Animals were provided free access to food and water *ad libitum*. All assays with animals were conduced in accordance with present law regarding animal welfare and ethics in animal experiments (143.400/2002; 471/2002; 205/2004; 206/2004; 9/2008; 86/609/CEE).

After six months of exposure, rats were sacrificed following protocols and ethical procedures and sperm quality markers were determined. Sperm samples were collected from cauda epididymis. The sperm number, total and progressive motility was determined using computer assisted semen analysis – CASA and percentage of abnormal sperm morphology on smear stained with eosine (400x magnification).

The results were statistically analyzed by Anova method and Student test.

## RESULTS AND DISCUTION

The results are summarized in tables 1, 2 and figures 1- 4.

***Table 1***

***Mean sperm count ($x10^6$) after six months of potassium dichromate exposure***

| Groups | x±Sx | S.D. | CI 95% |
|---|---|---|---|
| C | 751.56±0.29 | 0.77 | 0.50 |
| $E_1$ | 656.64±0.12 | 0.32 | 0.50 |
| $E_2$ | 643.21±0.33 | 0.87 | 0.50 |
| $E_3$ | 637.74±0.17 | 0.45 | 0.50 |

In experimental groups sperm count was significantly ($p<0.0001$) lower as compared to control group ($E_1$/C: -12.62%; $E_2$/C: -14.41%; $E_3$/C: -15.14), in inverse correlation to exposure level also significantly ($p<0.0001$) ($E_2/E_1$: -2.04%; $E_3/E_2$: -0.85%; $E_3/E_1$: -2.87%).

Total sperm motility decreased significantly ($p<0.0001$) after chromium treatment in comparison to control group ($E_1$/C:-20.18%; $E_2$/C:-32.45%; $E_3$/C:-43.20%), in indirect, significant ($p<0.0001$) correlation to chromium exposure level ($E_2/E_1$:-15.37%; $E_3/E_2$:-15.91%; $E_3/E_1$:-28.84%).

Comparative to control, in all experimental groups was observed significant ($p<0.0001$) decrease of progressive motility, indirectly, significantly ($p<0.0001$) correlated to exposure level ($E_1$/C:-53.60%; $E_2$/C:-68.46%; $E_3$/C:-77.92% $E_2/E_1$:-32.04%; $E_3/E_2$:-30.00%; $E_3/E_1$:-52.42%).

Percentage of abnormal sperm was significantly ($p<0.0001$) higher in experimental groups comparative to control ($E_1$/C:+246.54%; $E_2$/C:+328.30%; $E_3$/M: +412.82%). Increase of spermatozoa with abnormal morphology was directly, significantly ($p<0.0001$), correlated to exposure level ($E_2/E_1$:+23.59%; $E_3/E_2$:+19.73%; $E_3/E_1$:+47.97%).

**Table 2**

***Total, progressive motility and sperm with anomalies percentage***

| Groups | Total motility | | | Progressive motility | | | % sperm anomalies | | |
|---|---|---|---|---|---|---|---|---|---|
| | x±Sx | S.D. | CI 95% | x±Sx | S.D. | CI 95% | x±Sx | S.D. | CI 95% |
| C | 75.71±0.29 | 0.76 | 0.64 | 63.43±0.30 | 0.79 | 0.63 | 10.14±0.34 | 0.90 | 0.63 |
| $E_1$ | 60.43±0.30 | 0.79 | 0.64 | 29.43±0.30 | 0.79 | 0.63 | 35.14±0.26 | 0.69 | 0.63 |
| $E_2$ | 51.14±0.34 | 0.90 | 0.64 | 20.00±0.31 | 0.82 | 0.63 | 43.43±0.30 | 0.79 | 0.63 |
| $E_3$ | 43.00±0.31 | 0.82 | 0.64 | 14.00±0.31 | 0.82 | 0.63 | 52.00±0.31 | 0.82 | 0.63 |

Main abnormalities found were primary: detached head, flex head; secondary: bent tail, broken tail, curl tail – in terminal piece of tail, tail without head and the predominant one tail disintegration and fragmentation.

Regarding anomaly type it was observed that primary anomalies were less represented but the secondary one were the most frequent especially at maximum level of exposure. Fragments of tail, results of spermatozoa disintegration, are the evidence of increased sperm fragility.

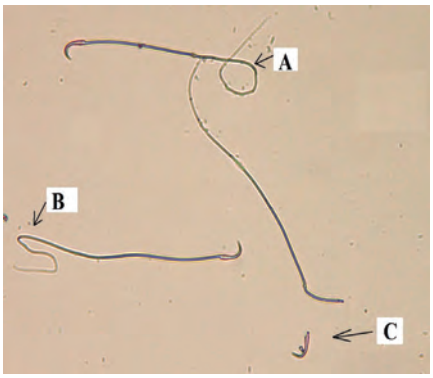


**Fig. 1 A – curl tail, B – bent tail, C – detached head**

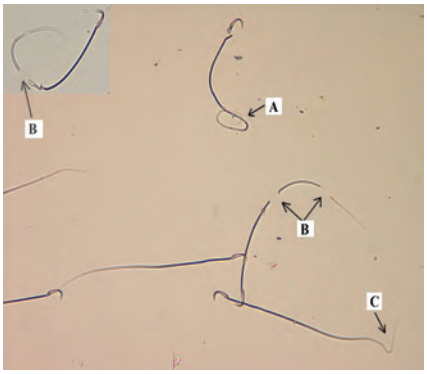


**Fig. 2 A – curl tail, B – tail fragmentation, C – bent tail**

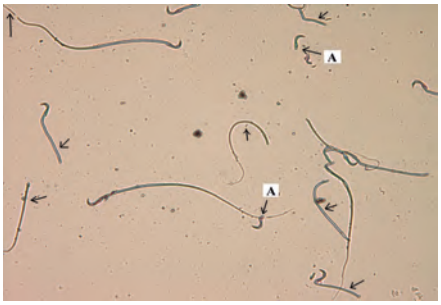


**Fig. 3 A – detached head, fragmentation of spermatozoa**

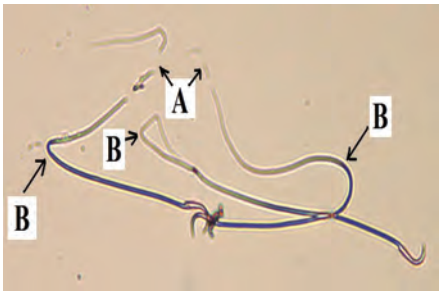


**Fig. 4 A – bent tail, B – sperm fragmentation**

The results obtained regarding sperm count after chromium exposure are in agreement to those obtained by the other authors (Ernst, 1990; Li *et al*., 1999; Li *et al*., 2001; Afonne *et al*., 2002; Danadevi *et al*., 2003; Pereira *et al*., 2005; Acharya *et al*., 2006; Subramanian *et al*., 2006; Yousef *et al*., 2006; Chandra *et al*., 2007) but are in contradiction with Bonde and Ernst, 1992, and Kumar *et al*., 2005, which studied industrial workers occupationally exposed to chromium and haven't observed alteration in sperm count.

Regarding total motility, results coincide with some authors (Ernst and Bonde, 1992; Li *et al*., 2001; Danadevi *et al*., 2003; Pereira *et al*., 2005; Yousef *et al*., 2006) and are contradictory to others (Bonde and Ernest, 1992, Kumar *et al.*, 2005), which found no modification in total sperm motility after chromium treatment.

The decrease of progressive motility was also observed by Subramanian *et al*., 2006 and Yousef *et al*., 2006. However Bonde and

Ernst, 1992. Kumar *et al*., 2005, observed that men, occupationally exposed to chromium had no reduced sperm progressive motility.

The increase of abnormal sperm percentage after chromium exposure was emphasized also by Li *et al*., 2001; Kumar, 2004; Acharya *et al*., 2004; Kumar *et al*., 2005; Acharya *et al*., 2006, and are contradictory to those obtained by Bonde and Ernst, 1992. They studied sperm of occupationally exposed workers and observed no increase of sperm abnormalities.

The results of our study indicate that Cr VI is a male reproductive toxicant, affecting sperm quality in adult Wistar rats.

## CONCLUSIONS

The exposure of adult male rats to potassium dichromate for six months determined:

1.1. Significant decrease of sperm count comparative to control group and in inverse correlation to exposure level;

1.2. Significant decrease of total and progressive motility comparative to control group and in inverse correlation to exposure level;

1.3. Significant increase of sperm anomalies percentage comparative to control group and in direct correlation to exposure level. Both, primary and secondary anomalies were found with predominance of the secondary one.

## ACKNOWLEDGMENTS

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# CONCENTRATION OF SERUM PROTEIN AND PROTEIN FRACTIONS IN HOLSTEIN CATTLE IMPORTED FROM EUROPEAN UNION IN A COMMERCIAL DAIRY FARM DOLJ DISTRICT

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**Key words:** primiparous cows, serum protein, serum protein fractions.

**SUMMARY**

The aim of this research was to assess total protein and its fractions concentrations in the blood serum, to monitor adaptation stress in Holstein cattle imported from European Union, in a commercial dairy farm. Three groups of animals each consisting of ten animals were studied in march 2008. The animals were from different countries in different physiological stage and after different time of adaptation to the new environment. Group I (n=10) pregnant heifers, 30-21 days (d) before parturition [7 from Germany (DE) and 3 from Czech Republic (Cz)], group II (n=10) fresh primiparous cows 0-21 days post partum(p.p.)[5 from DE, 4 from Cz, 1 from France(F)], and group III lactation primiparous 21-40 days post partum (p.p.) ( 5 from DE, 3 from Cz and 2 from F). The mean content of total serum protein was 6,73g/dl in group I, 6,75g/dl in group II and 6,90g/dl in group III. With regard to serum protein fractions the mean content of albumin was 3,24g/dl in group I, 2,73 g/dl in group II and 2,79 in group III and globulines were 3,49 g/dl in group I, 4,03 g/dl in group II and 4,10 g/dl in group III. During this research period the law content of total protein and albumin in blood serum in case of cows after calving may reflect an inadequate protein intake relative to requirement in p.p. period. Also lower albumin: globulin ratio in p.p. primiparous cows suggests inadequate protein intake or the protein is not digested or absorbed properly.

Reactions of animals to stress are not uniform and therefore they are difficult to interpret. The answer to stress is relating to timing of stress and its necessity as well as to previous experience of animals, their physiological stage and environmental effects combined with animal's genotype, age and sex.

To score animal's adaptation to local environment and welfare, behavioral, hematological, biochemical, physiological as well as neurohormonal tests are performed.

Blood tests from individual animals are routinely used to diagnose disease problems in dairy cattle. Veterinarians, producers and nutrition consultants are interested in extracting pertinent information relative to hard nutrition, health and adaptation status from blood tests (Metabolic Profile).

The Metabolic Profile (M.P.) is intent to: 1) monitor metabolic health of the herd ; 2)help diagnose metabolic problems and production diseases and 3) indentify metabolically uperior cows.

Being familiar with the internal environment of the cows we can easly detect any dangers to the health of the animal.

The aim of this research was to assess some blood indices to monitor adaptation stress in Holstein cattle imported from European Union in a new comercial dairy farm from Dolj district. In this paper we will present serum protein and protein fractions.

## MATERIALS AND METHODS

Three groups of animals were studied after relocation to a new wnvironment in a commercial dairy farm in Dolj district. Group I (n=10) pregnant haifers , 30-21 days So fore postnutrition(7 from DE, and 3 from Cz), droup II (n=10) fresh primiparious cows 0-21d.p.p. (5from DE, 4FROM Cz and 1 from F) and group III lactation primiparious 21-40 d.p.p. (5 from DE, 3 from Cz and 2 from F). The groups of animals were studied in march 2008 and were from different countrys, in different physiological stage and after different time of adaptation to the new environment.

All animals were examined and blood samples were obtained from coccygeal vein. All blood samples were tasted at Diagnostic and Animal Health Institut (I.D.S.A.) Bucharest, Romania, using classical methods.

Data were analysed statistically.

## RESULTS AND DISCUSSION

Mean values of serum protein and protein fractions are presented in table 1

***Table 1***

**Mean values of serum protein and protein fractions -g/dl-**

| Group | Protein × ± S d | Albumin × ± S D | Globulin × ± S D | Albumin: Globulin A/G ratio |
|---|---|---|---|---|
| 1(n=10) | 6,73±0,12 | 3,24±0,25 | 3,49±0,31 | 0,92 |
| 2(n=10) | 6,75±0,13 | 2,73±0,26 | 4,03±0,27 | 0,67 |
| 3(n=10) | 6,90±0,11 | 2,79±0,38 | 4,10±0,35 | 0,68 |
| Total average | | | | |

Mean values of serum protein was in group I 6,73 g/dl, in group II 6,75 g/dl and in group III 6,90 g/dl . Cows in group I were in the range

of reference values (table 2) and cows in group II and III were lower than reference values (table 2).

**Table 2**

**Reference intervals for stages of lactation, devised from Holstein dairy herd in Ontario (Brent Haff et al 2003)**

| Parameter | Close –up cows | Fresh cows | Mid-lactation cows |
|---|---|---|---|
| Protein g/dl | 6,7-8,8 | 6,9-8,7 | 7,3-8,6 |
| Albumin g/dl | 3,2-4,1 | 3,5-4,2 | 3,1-4,3 |
| Globulins g/dl | 3,2-5,4 | 3,0-5,0 | 3,6-5,4 |

Kupezynski et al 2002 reported 7,67 g/dl of serum protein in cows antepartum 7,67 g/dl, 7,48 g/dl in the first 8 d p.p. and 7,89 g/dl at 28 d p.p.

The blood protein levels declined sharply during late gestation of Makuii ewes, when the nutriment demands of the fetus were maximal (Betavani et al 2006).

Malnutrition decreases the concentration of total protein in the blood by absent 10% (Chadly et al 1992).

Mean values of serum protein fraction are presented in table 1.Mean values of albumin of prepartum primiparous cows is 3,24 g/dl and was found to be within the normal reference range (table 2) . In p.p. cows serum albumin concentration is lower than reference range. Mean values of serum globulins in all three groups were found to be within the normal reference range (table 2).

Albumin to globulin ratio was 0, 92 in group I, 0,67 in group II, and 0,68 in group III. Globulins were higher than albumin fraction in p.p. cows, which is almost equal in normal cattle.

Magnus et al. 2009 reported total serum protein of 6, 1 g/dl, 2, 8 g/dl of albumin and 3, 3 g/dl of globulins in p.p. metritis cows. Albumin to globulins ratio was 0, 85.

Low serum total protein levels can suggest a liver disorder, a Kidney disorder, or a disorder in which protein ingested is not digested or absorbed properly. Low levels may be seen in severe malnutrition and with conditions that cause malabsorption .

High serum total protein levels may be seen with chronic inflammation.

Normally, there is a little more albumin than globulins, giving a normal A/G ratio of slightly over 1. Disease statuses affect the relative

changes in albumin and globulins in different ways. A low A/G ratio may select over- production of globulins of underproduction of albumin, such as occurs with Cirrhosis or selective loss of albumin such as occurs with Kidney disease.

A high A/G ratio suggests underproduction of immunoglobulin.

## CONCLUSIONS

- The last part of gestation did not influence serum protein and protein fractions in cattle imported from European Union.
- Calving an p.p. period did influence serum protein and protein fractions indicating an inadequate protein intake or the protein is not digested or absorbed properly( during the research period).
- Being familiar with the internal environment of the animal enables early detection of any dangers to the health of the primiparous dairy caw.

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# THE CRYOPRESERVATION OF THE PERIOSTEAL CANINE CELLS

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**Key words**: cryopreservation, periosteal cells, dog

**SUMMARY**

The main goal of this study was to select and design the method to preserve the periosteal cells without compromising their viability because there is a lack of studies regarding the cryopreservation of the canine periosteal cells. The researches were carried out on isolated cells using two methods, and cultivated for six weeks in the nutritiveent medium DMEM. Cells morphology and cells viability were assessed. The cultured cells were cryopreservated for four months. After thawing the cells viability was assessed again. The data obtained in this study demonstrates that the method of cultivation in DMEM medium permitted the obtaining of an optimal cell density for cryopreservation and later to maintain the number of cells at a sufficient level - 1 million/ml – for the stage of differentiation in 75% of the samples included in the work.

There a lot of reasons for mammalian cells cryopreservention, for example to avoid loss by contamination, to minimize genetic change in continuous cell lines or to avoid ageing and transformation in finite cell lines (Liu et al, 2008).

Most mammalian cells can be stored at temperatures below –130°C for many years (after Kielberg Consult ApS Techiques, 1995), and the freezing of single cells is considered the simplest, since only the physicochemical characteristics must be taken into account in predictions of the response to freezing (Özkavukcu and Erdemli, 2003). The viability of the cells after cryopreservation depends on their ability to cope with the variety of stresses imposed on them during the freezing and thawing procedures. There is a lac of in vitro studies which investigate the canine periosteum for proving viable periosteal cells after the preservation period. The main goal of this research was to select and design the method to preserve the periosteal cells without compromising the viability and/or their therapeutic potential.

## 1. MATERIAL AND METHOD

From four dogs there was prelevated the periosteum and the periosteal cells were isolated using two methods. The cells obtained following the isolation from the periosteal explants were grown for six weeks, the nutritive medium (DMEM) being changed every 48 hours, during which time three passages were made. Cell morphology was assessed using a phase-contrast microscope before cryopreservation.

From these suspensions there were taken samples which were transferred to a plate with wells and were stained with blue tripan (10 µl cell suspension and 50 µl blue tripan). The colour blender, blue tripan, was transferred into the haemocytometer for the cells count and for the determination of their viability (through microscopy) before cryopreservation.

The cultured cells were cryopreservated for four months using the protocols developed by Kielberg Consult ApS Techiques, 1995 and TCF, 2007. A cryogenic mixture was prepared from one part DMSO and four parts FCS, to which was added 1% penicillin. In a cryotube of 1.8 ml there were introduced ½ cryogenic mixture and ½ cell suspension in DMEM culture medium. The cryotubes were introduced in a cryobox containing isopropyl alcohol. This cryobox was introduced into the freezing room at $-80^0$C for 24 hours, and then into the N liquid at $-180^0$C. Thawing was achieved by immersing the cryotubes in water at $37^0$C. After thawing, the cryogenic mixture was washed with PBS and afterwards the cell viability was assessed .

## 2. RESULTS AND DISCUSSIONS

To consider periosteal cells as a viable cell source for cell and tissue therapies, they need to possess the ability to be expanded in culture, relatively quickly, under conditions in which they retain their potential to proliferate and differentiate. The cultivation surface available to the cells obtained through both isolation procedures was fully occupied by the eighth day of cultivation. Under identical cultivating conditions, this capability was demonstrated also by periosteal cells of human and mouse origin (Bozani, 2008).

The analysis of the morphological characteristics of the cells isolated from the explant (fig.1a) and through digestion (fig.1b), before cryopreservation, made possible the assessment of their viability.

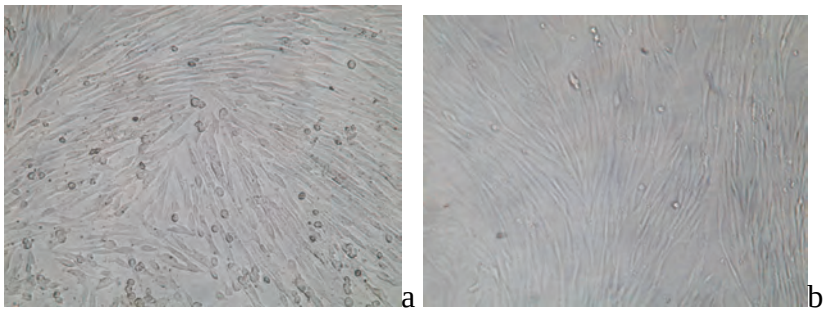

**Fig. 1. The appearance of cells isolated from explant (a) and through digestion (b) before cryopreservation; image with 20X objective**

Comparing the microscopy images, it was observed that in the fields with periosteal cells isolated as a result of digestion there was a lower cell density (fig. 1) the values recorded being between 2.8 and 4.5 million of cells/ml. The variation limits in the number of cells were much greater in the case of those from explants, with extremes between 10.5 and 1.8 millions/ml. For all the cell cultures resulted from the explant and also through digestion, the cell morphology was similar to that of fibroblasts. The cell viability in all the examinated fields was more than 95%.

After thawing the number of cells had very large limits depending on the individual and on the number of cells placed in the cryotubes, these data being given in table1 and fig. 2.

***Table 1.***

**The viable cells number before and after cryopreservation**

| Subject | Method of cell isolation | Cell number/ml before cryopreservation | Cell number/ml after cryopreservation |
|---|---|---|---|
| 1 | Explant | 4,5 millions | 2,4 millions |
| 2 | | 1,8 millions | 375.000 |
| 3 | | 10,5 millions | 4 millions |
| 4 | | 3,2 millions | 1,2 millions |
| 1 | Digestion | 4,5 millions | 3,5 millions |
| 2 | | 3 millions | 870.000 |
| 3 | | 3,2 millions | 2,5 millions |
| 4 | | 2,8 millions | 1,1 millions |

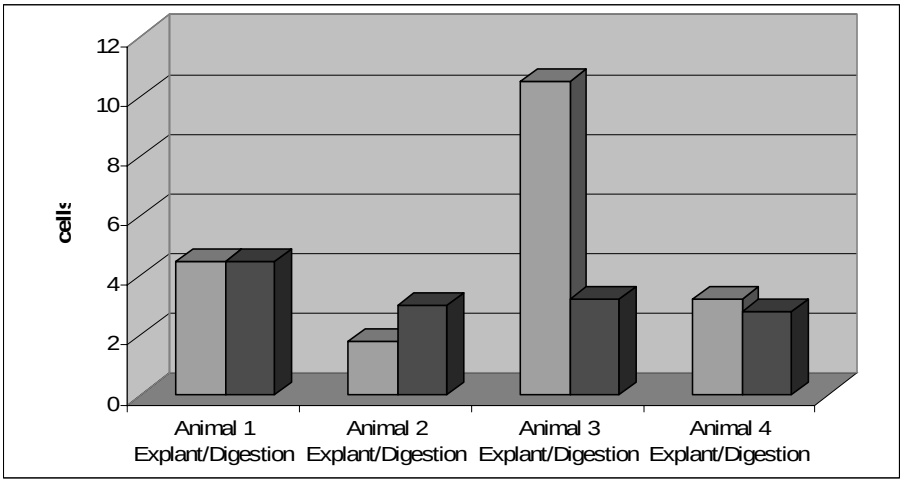


**Fig. 2. Graphic representation of cells number obtained through cultivation before cryopreservation**

Following the analysis of the data summarized in table 1 it was found a decrease by 47-80% in the number of viable cells obtained from the explant (fig. 3) and in case of the cells obtained through digestion of 22-81% (fig. 4). This decrease in the number of cells after thawing is directly connected with the harmful effects of the negative temperatures to the cell cytoplasm but can be correlated at the same time with the initial cell density into cryotubes. In this study, the freezing temperature was by $50^0$C higher than the usually used temperatures. It is alsoknown the influence of the cryogenic mixture on cell viability after thawing. The cryopreservation medium generally consists of a base medium, cryopreservative, and a protein source. The cryopreservative and the protein protect the cells from the stress of the freeze-thaw process (Kielberg Consult ApS Techiques, 1995). The cryogenic mixture used in this study meets the requirements generally accepted and therefore we can say that the influence on the cell viability is almost insignificant compared to the other two influence factors, the effects of temperature and the initial cell density.

Successful cryopreservation of human mesenchymal stem cells is well documented and it has been proven that the process of cryopreserving and thawing had no effect on either their growth or on the osteogenic differentiation in vitro (Bruder et al, 1997, Kotobuki et al, 2005, Rust et al, 2006). The data obtained in this study demonstrate that the method of cultivation in a DMEM medium permitted the obtaining of an optimal cell density for cryopreservation and later to maintain the

number of cells at a sufficient level - 1 million/ml – for the stage of differentiation in 75% of the samples taken in the work. The smaller number of cells, under one million, obtained in two of the samples afterwards, gave the possibility for deployment of the differentiation phase, but a larger number of cells provided a greater margin of safety in the case of unexpected cell loss.

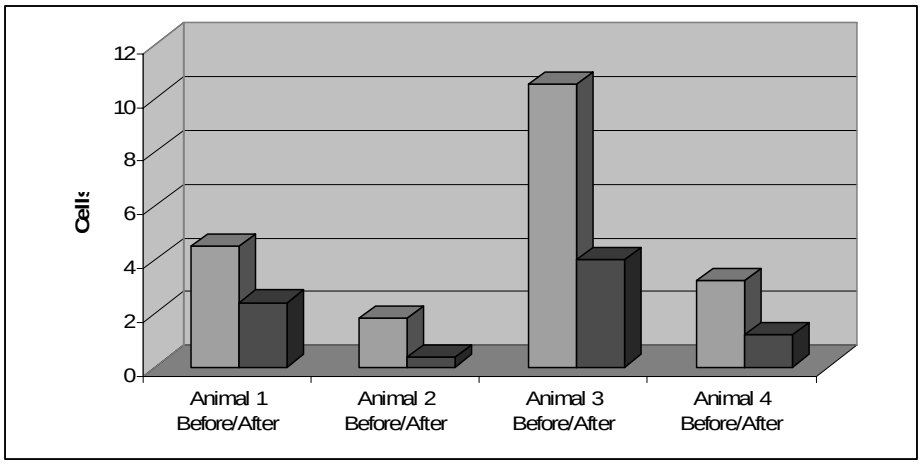


**Fig. 3. The ratio between the number of viable cells from explants - before and after thawing**

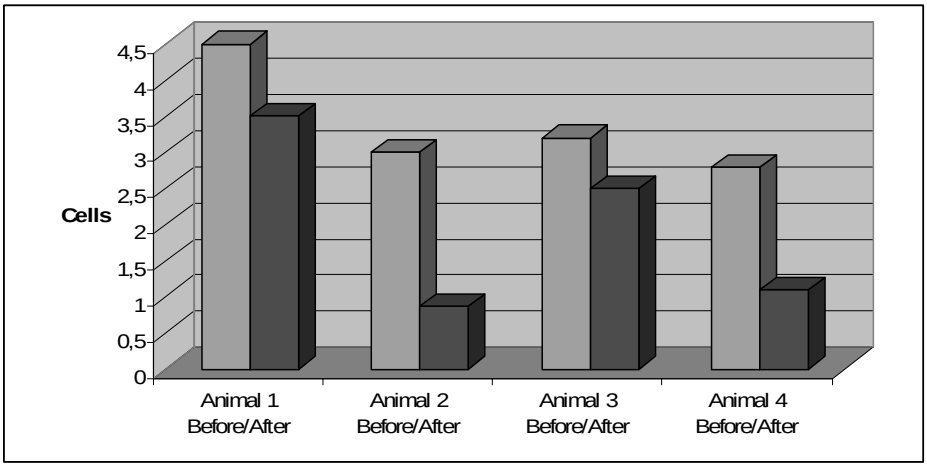


**Fig. 4. The ratio between the number of viable cells obtained throug digestion - before and after thawing**

## 3. CONCLUSIONS

3.1. The method of cultivation in the DMEM medium allows us to obtain an optimum cell density for cryopreservation.

3.2. Cryopreservation at -180$^0$C permitted to maintain the number of cells at a sufficient level - 1 million/ml – for the stage of differentiation in 75% of the samples taken in the work.

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# COMPARING ASPECTS CONCERNING THE DIAGNOSE VALUE OF CT SCAN AND MRA TEHNIC IN A CASE OF DOG TETRAPLEGY.

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**Key words**: dog CT, dog MRA, dog tetraplegy, spondylosis, osteophytes.

**SUMMARY**

In this study is presented a clinical case of cervical syndrome displayed by tetraplegy in which there were used the CT scan and MRA in order to determine an accurate diagnose. The investigation performed showed osteophytes developed in the cervical spinal canal that put pressure in the spinal cord, the illness being called spondylosis deformans.

## 1. MATERIAL AND METHOD

Investigations were performed on a 4 years male Pekinese. Clinical examination revealed a motor deficit sindrome, manifested by tetraplegy installed suddenly, according to the anamnesis. After complete examination, we concluded that the affection was localised at a cervical level. In order to establish the cause of this syndrome, we performed radiologic, computer tomograph and nuclear magnetic resonance investigations. We used a Röntgen Basic, CT Toshiba Aquilion 16 and RMN Tesla Vantage in order to perform our investigations.

## 2. RESULTS AND DISCUSSIONS

Clinical examination of the patient revealed a flat paralysis of the four limbs, severely amplified profound reflexes and urinary incontinence.

Radiologic examination was not conclusive due to the position of the osteophytes in the vertebral channel. Myelography with contrast substances was not recommended, being considered an invasive and high-risk procedure.

CT examination of the cervical vertebrae evidentiated, in the vertebral channel, at C2, C3, C4, C5, the presence of proeminent bone

formations (osteophytes), developed from the vertebral body. These bone structures had a compressive action on the cervical spine marrow that justified the clinical symptoms of the animal.

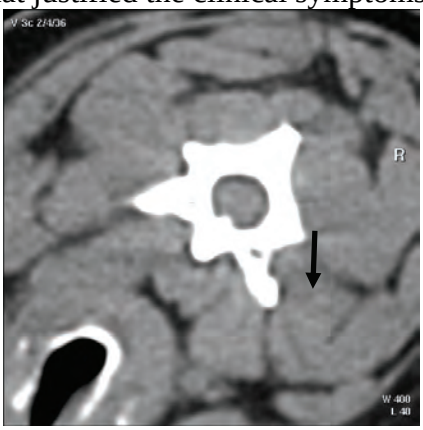


**Fig.1. CT of C2 vertebra with osteophyte in the vertebral channel**

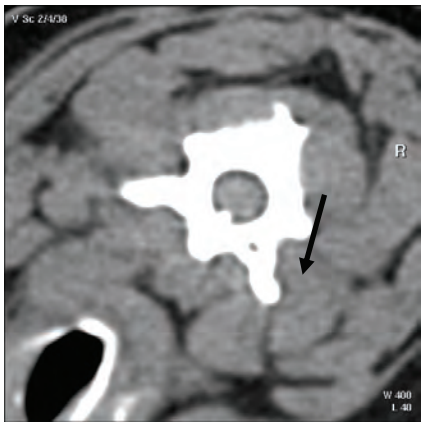


**Fig.2. CT of C2 vertebra with osteophyte in the vertebral channel**

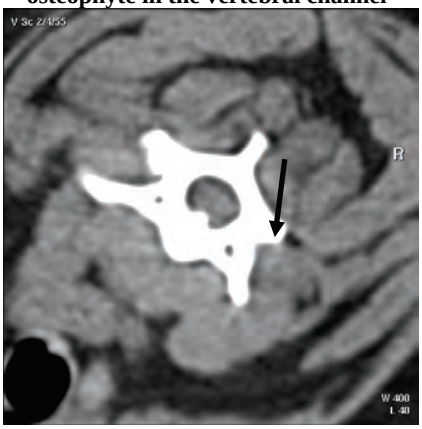


**Fig.3. CT of C3 vertebra with osteophyte in the vertebral channel**

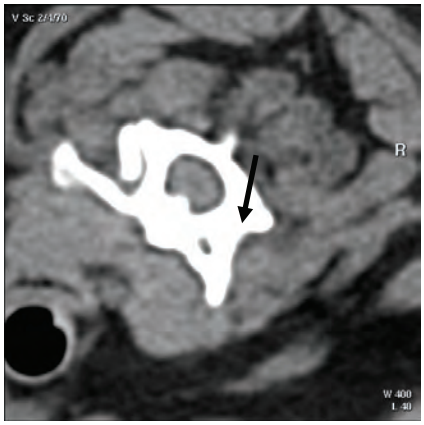
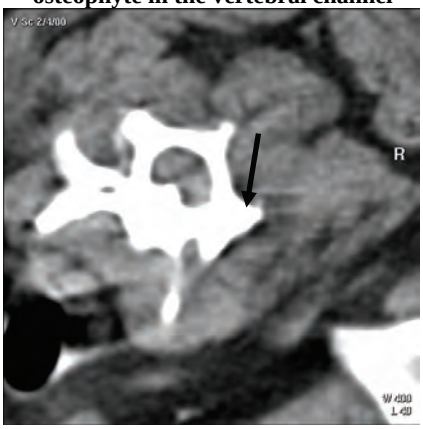


**Fig.4. CT of C3vertebra with osteophyte in the vertebral channel**



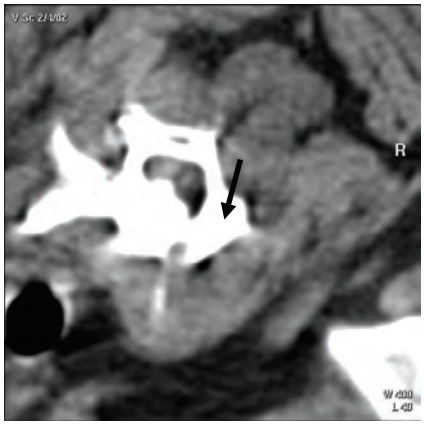


**Fig.5-6. CT of C5 vertebra with osteophyte in the vertebral channel**

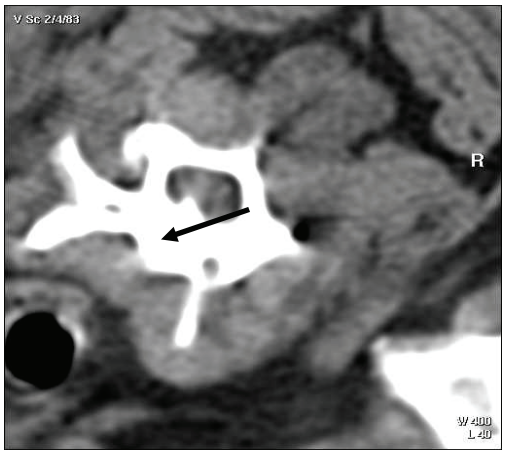



**Fig.7. CT of C5 vertebra with osteophyte in the vertebral channel**

We also performed a MRI examination, by scanning the spine in longitudinal sections. The MRI examination evidentiated the precise position of the osteophytes and also the compression areas of the spine at this level. The maximum amplitude in the vertebral channel was reached at C4, were the osteophyte occupied almost 1/3 of the vertebral channel.

Both imagistic methods combined with clinical results identified tha cause of the symptoms.

Osteophytes development on C2-C5 vertebral bodies, in the medular is a bioplastic disorder of the bone tissue – *spondylosis deformans*- with a low frequency among dogs, the most frequent localisation being an external one.

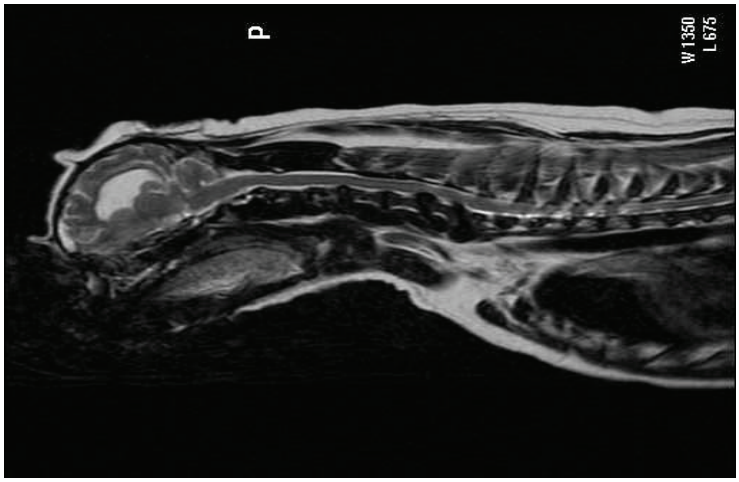



**Fig. 8. RMN of the spine. Stenosis of the rhachidien channel at C2-C5**

## 3. CONCLUSIONS

3.1. In this type of disorders, clinical examination is purely orientative and can not establish the certain etiology of the disease.

3.2. Due to the localisation inside the vertebral channel, radiologic examination does not offer conclusive diagnostic

3.3. CT and MRI examination the patient was diagnosed *Spondylosis deformans*, disease with a low incidence of appearance inside the vertebral channel, compared to classic localisations on the ventral and lateral sides of the vertebrae.

3.4. CT images identified the presence of the osteophytes inside the vertebral channel, but RMN images identified the size of the multiple areas of medular compression caused by these formations.

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# THE URINAR APPARATUS HISTOSTRUCTURE IN 6 MONHTS OLD PHASIANUS PHASIANUS

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**Key words:** pheasant, urinary apparatus, six months, histostructure.

**SUMMARY**

The authors have studied the histological structure of the kidney and the ureter in six months old Phasianus phasianus. The harvested histological pieces have been fixed in saline neutral formalin, included in paraffin and sliced into 6 microns thick sections. These sections were colored using the Hematoxylin-Eosin and Giemsa methods.
This study concerning the normal microscopical aspects of the pheasant's kidney and ureter has been considered to be necessary in order to bring basic theoretical and practical information in finding out and interpreting the pathological alterations that may occur at this level, because illiteracy may create confusions in the diagnosis of some lesions.
The kidney presents three types of nephrons: cortical, medullar and intermediary nephrons, all the morphostructural elements being differentiated.
Concerning the ureter, there are three components: the mucosa, the muscularis and the adventitia. The epithelial cells of the mucosa (the cubic basal cells and the mucus secretory tall cells) are present, the mucosecretory activity of the epithelium is betrayed by distinguishing the secretory granules at the apical pole of the tall cells.
The mechanism of secretion in the epithelial cells found in the ureter opposed to the mechanism of mucus secretion in the collecting ducts seen in the medulla of the kidney are debatable.

There are three types of nephrons found in the renal lobule in pheasants, as well as in all the birds: *the cortical type*, *the medullary type* and *the intermediary type* (Kurihara, H., M. Yasuda, 1975). Each nephron is composed of renals corpuscles and renal tubules.

The nephron is the functional unit of the kidneys, as it is in mammals. Structurally, there are no differences from the nephron found in mammals, excepting the reptilian type, which lacks the Henle's loop.

The studies conducted by Morild, I. and Co. (1985), concerning the aspect of the juxtaglomerulary apparatus in cortical and medullar nephrons have led to noticing some small histostructural differences (Morild, I. et al., 1985).

Therefore, the medullary nephrons have in the structure of the renal corpuscle the mesangial cells of the lacis found between the arterioles, the glomerule and the macula densa, much more crowded and numerous than in the case of the cortical nephron of reptilian type (Morild, I. et al., 1985).

Also, ocasionally, the granular epithelioid cells found next to the afferent arteriole in the corpuscle of the medullary nephron in mammals has been evidentiated. The mesangial cells found in the structure of the same type of corpuscle are crossed by the afferent arteriole in the center of the cellular mass, unlike the reptilian nephron, where this layout is imprecise (Morild, I. et al., 1985).

It has been concluded that the strong relation between the afferent arteriole and the mesangial cells of the lacis established on a wide are suggested an implication of these cells in the adjusting of theglomerular filtration (Hughes, M.R., 2003).

The studies conducted by Boykin S.L. and Braun E.J. (1993) in the dessert quail have revealed that both types of nephrons open in a medullary cone after previously merging (Boykin, S.L., E.J. Braun, 1995).

The researches conducted by Radu C.N. (1978) have demonstrated the existence of a rich, well structered network of reticulin fibers in the medullar cones (Radu, C.N., 1978).

Casotti, G. and Co. have evidentiated, after researches done on american quails, a remarkable variability of the medullar cones concerning the basal diameter, the length and the number of the Henle's loops and of the renal tubules (Casotti, G., E.J. Braun, 1995).

The ureters are made of a *renal component* (inside the kidneys) and of a *pelvian component* (which connects the kidneys to the cloaca) (Predoi, G. and Co., 1997).

In birds, the lack of renal pelvis, the urinary bladder and the urethra, determines a communication at the cloacal ending between the digestive and the urinary apparatus, the result being the reabsorbtion of some electrolites, water and macromolecules in the cecum and the colon (Inoue, H., 1953; Phalen, D.N. and Co., 1990).

Each kidney is drained by a ureter, that starts from the anterior margin of the kidney to the cloaca (urodeum) (Predoi, G. and Co., 1997).

Histostructural, the uretral wall is formed by three components: the mucosa, the muscularis and the adventitia.

## 1. MATERIAL AND METHOD

The researches were interested in the histostructural study of the kidney and of the ureter in six months old studies. The study was done on five subjects, experimentally killed. The pieces of ureter were then harvested and fixed in saline formalin.

The working methodology has been the clasical one, the pieces were processed in order to be included in paraffin. The blocks of paraffin were sectioned in six microns slices and colored using the Haematoxylin-Eosin and Giemsa stains.

The slides have been examined using a Nikon microscope, microphotografied and the images were then digitally enhanced using the Corel Photo Paint software.

## 2. RESULTS AND DISCUSSIONS

At this age, the kidney is limited on the exterior by a fine renal capsule, rich in collagen fibers, and the renal parenchyma is formed of two areas: the cortical and the medullar. The limit between the two areas is imperceptible, not as obvious as in mammals.

The nephrons are made of renal corpuscles and renal tubules in the cortex (fig. 1). The renal ducts and Henle's loops of the medullary nephrons are seen in the medulla (fig. 1).

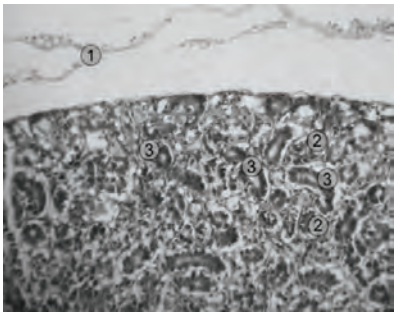


**Fig. 1 – Kidney; Giemsa stain 10x**
**1. Capsule; 2. Glomerulus; 3. Kidney tubules.**

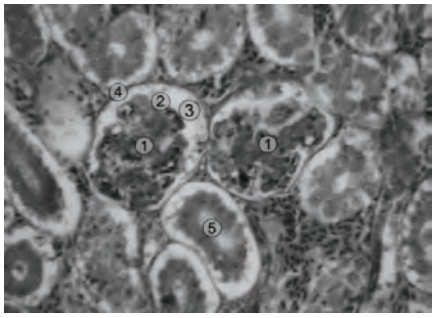


**Fig. 2 – Kidney; Giemsa stain 20x**
**1. Glomerulus; 2. Visceral layer of Bowman's capsule; 3. Filtration slits; 4.Parietal layer of Bowman's capsule; 5.Proximal convoluted tubule.**

The renal corpuscles are evident, having in their structure an admirable rete of fenestrated capillaries, along with the mesenchimal connective tissue of the renal mesangium with numerous basofile mesangial cells (fig. 2).

The capsule of the renal corpuscle is composed of a visceral layer formed with podocytes, and a parietal layer, limiting between them the filtering space (fig. 2).

The podocytes of the visceral layer have globular nuclei, whereas the parietal layer presents epithelial cells with elongated nuclei.

The juxtaglomerular complex is evident at the vascualar pole of the renal corpuscle, all the morphological element being present, except for the cells of the macula densa. The faint difference of the components makes the juxtaglomerular complex to be less evident, in this specie, as opposed to mammals (fig.3).

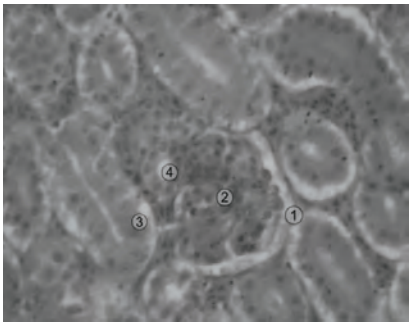


**Fig. 3 – Kidney; HE stain 40x**
**1. Filtration slits; 2. Glomerulus; 3. Distal convoluted tubule; 4. Proximal convoluted tubule.**

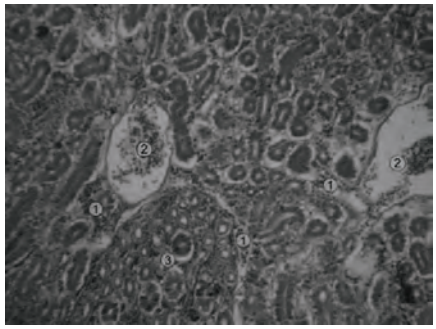


**Fig. 4 – Kidney; Giemsa stain 10x**
**1. Stroma; 2. Interlobular artery; 3. Kidney tubules.**

In the connective-reticular stroma limiting two renal lobules, the interlobular artery with tipically structured wall and an endothelium formed of scuamous simple epithelium, muscularis and adventitia, is evident (fig. 4).

At the periphery of the lobules, the collector ducts formed of a simple cubic epithelium are obvious (fig. 4). The overall aspect of the renal parenchyma in the medular area of the renal lobules includes reticular connective tissue and portions of longitudinaly and cross sectioned nephrons.

In cross sections in the vicinity of the coprodeum of the ureter, this presents the three typical layers: the mucosa, the muscularis and the adventitia (fig. 5). The mucosa is formed of a pseudostratified epithelium and *lamina propria* (fig. 6).

The muscularis is formed of two layers of smooth muscular fibers: the internal longitudinal layer under the mucosa and the external circular layer covered by adventitia, with which it adheres to the adjacent structures.

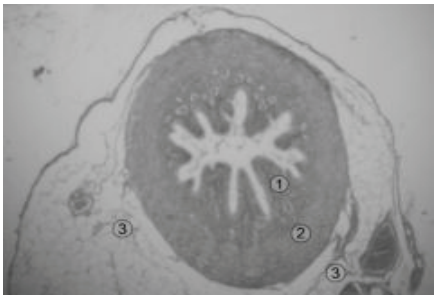


**Fig. 5 – Ureter; HE stain 6x**
**1. Mucosa; 2. Muscularis; 3. Adventitia.**

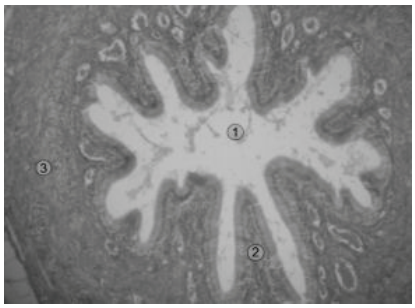


**Fig. 6 – Ureter; HE stain 10x**
**1. Lumen; 2. Mucosa fold; 3. Muscularis.**

This way of arrangement creates the general aspect of the cross sectioned ureter especially scalloped, the mucosa layering a narrow lumen (fig. 6).

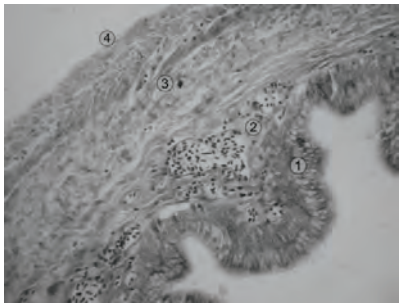


**Fig. 7 - Ureter; Giemsa stain 20x**
**1. Epithelium; 2. Lymphoid infiltration in lamina propria; 3. Muscularis; 4.Adventitia.**

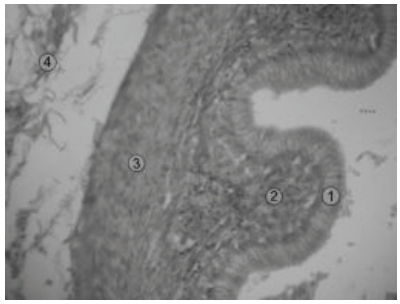


**Fig. 8 – Ureter; HE stain 40x**
**1. Epithelium; 2. Lamina propria; 3.Muscularis; 4. Adventitia.**

At this age the mucosal epithelium includes areas of simple tall epithlium. The component cells are distinguished by the shape of the nuclei disposed on the basal membrane (fig. 7). The undifferenciated basal cells, with round nuclei and poorly acidophile cytoplasm form a generator layer,

The cuboidal epithelial cells form a generator layer with round nuclei and poorly acidophile cytoplasm, and the tall cells have the oval nucleus in the basal third of the cells and present secretion vacuoles with mucoid substance in the apical pole (fig. 8).

As a result of this distribution of cells, the nuclei appear to be arranged at different heights, although the cells are arranged as a single layer.

The *lamina propria* is formed of highly vascularised loose connective tissue. At this age, lymphocytes are diseminated in the *lamina propria*, along with connective cells, between the connective fibers (fig. 7,8).

## 3. CONCLUSIONS

3.1. In pheasant, the kidney presents all the structural elements of the nephron, the Malpighi corpuscle is evident and the flattened cells of the parietal layer of the Bowmann capsule limits, along with podocytes of the visceral layer, the filtering space.

3.2. The juxtaglomerular complex is poorly differentiated, being less obvious compared to the mammalian one.

3.3. At this age, the ureter has all the three tunics well differentiated: the mucosa is extremely folded, the muscularis is organised on two muscular layers and the connective adventitia is vascularised and innervated.

3.4. The epithelium of the uretral mucosa is differentiated, and it is formed of cuboidal and tall cells that have their nuclei situated at different heights, the tall cells having secretory activity.

3.5. *The lamina propria* that constitutes the mucosa contains numerous lymphocytes disseminated between connective fibers.

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# LH AND TESTOSTERONE SERIC LEVEL DYNAMICS IN MALE RATS CONSECUTIVE THREE MONTHS POTASSIUM DICHROMATE (CR VI) INTAKE

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**Key words:** chromium VI, male, rats, LH, testosterone

**SUMMARY**

The aim of this study was the biochemical biomarkers of testicular function evaluation: seric LH and testosterone level. The study was carried out on 28 white Wistar male rats divided in three experimental and one control group: $E_1$: 25 ppm Cr (LOAEL); $E_2$: 50 ppm Cr (2 x LOAEL); $E_3$: 75 ppm Cr (3 x LOAEL); control group received tap water without chromium content. Experimental groups received potassium dichromate (Cr VI) in drinking water for three months. All assays with animals were conduced in accordance with present laws regarding animal welfare and ethics in animal experiments (143/400/2002; 471/2002; 205/2004; 206/2004; 9/2008; 86/609/CEE). Exposure to Cr VI determined: significant increase of seric LH level comparative to control group and in direct, significant correlation to exposure level, excepting at 1.5 times increased dose; significantly decrease of seric testosterone level comparative to control group and in inverse correlation to exposure level, significantly only when exposure level increased from minimum to maximum.

Chromium is a naturally occurring element found in rocks, animals, plants, soil and in volcanic dust and gases. It is hard steel-gray metal that is highly resistant to oxidation, even at high temperatures. It is the sixth most abundant transitional, metal in the earth's crust (ATSDR, 2000).

Among heavy metals, chromium was less studied than Cd, Zn, Cu and Pb. The chemistry of chromium is complicated by its existance in six major oxidation states (Acharya *et al*., 2004). Biologically, trivalent and hexavalent chromium are the most important. Chromium compounds have no taste or odor. Trivalent chromium is an essential nutrient for human diet, but only in small amounts. Chromium VI is considered 1000 times more toxic to living beings than trivalent form (ATSDR, 2000).

Hexavalent chromium (Cr VI) is an important heavy metal pollutant used in more than 50 industries (Barceloux,1999): refractory, pigment, stainless steel factory, leather tannery, wood processing, cement manufacturing factory etc. Cr VI from fossil fuel combustion and steel

production plant comprises two-thirds of the chromium in the air urban areas (Chandra *et al*., 2007). Like all metals, chromium is non-biodegradable, persisting in the environment (Costa, 2003).

The metal seems to act directly on reproductive organs, it could also affect pituitary hormones secretion (Quinteros *et al*., 2007).

The aim of this study was the biochemical biomarkers of testes function evaluation: seric LH and testosterone level.

## MATERIALS AND METHODS

The study was carried out on 28 white Wistar male rats divided in three experimental and one control group. Experimental groups received potassium dichromate in drinking water for three months as followed: $E_1$: 25 ppm (LOAEL) (EPA, 2001); $E_2$: 50 ppm (2 x LOAEL); $E_3$: 75 ppm (3 x LOAEL); control group received tap water (without chromium content).

Animals were provided free access to food and water.

All assays with animals were conduced in accordance with present laws regarding animal welfare and ethics in animal experiments (143/400/2002; 471/2002; 205/2004; 206/2004; 9/2008; 86/609/CEE).

After three months of exposure all animals were sacrificed following protocols and ethical procedures and biochemical markers were determined. Seric testosterone and LH level was determined by Tody Laboratories using chemiluminicence method.

The results were statistically analyzed by Anova method and Student test.

## RESULTS AND DISCUSSIONS

The results are presented in table 1 and figure 1.

***Table 1***

**Seric LH and testosterone (ng/ml) dynamics in control and experimental groups**

| Groups | Testosterone | | | LH | | |
|---|---|---|---|---|---|---|
| | x±Sx | SD | CI 95% | x±Sx | SD | CI 95% |
| C | 3.55±0.52 | 1.36 | 0.75 | 4.96±0.10 | 0.27 | 0.45 |
| $E_1$ | 1.85±0.23 | 0.61 | 0.75 | 5.88±0.14 | 0.36 | 0.45 |
| $E_2$ | 1.12±0.44 | 1.17 | 0.75 | 6.75±0.27 | 0.72 | 0.45 |
| $E_3$ | 0.56±0.08 | 0.22 | 0.75 | 7.07±0.29 | 0.78 | 0.45 |

Comparative to C group, in all exposed individuals significantly increase ($p<0.0001$) of seric LH concentration was recorded ($E_1$/C: +18.54%; $E_2$/C: +36.08%; $E_3$/C: +42.54%), in direct and significant correlation to exposure level, excepting, not significantly ($p>0.05$), at 1.5 times increased dose ($E_2/E_1$: +14.79%, $p<0.05$; $E_3/E_2$: +4.74%, $p>0.05$; $E_3/E_1$: +20.23%, $p<0.01$).

LH seric level was higher than the physiological limit (0.5 ng/ml – Krinke, 2000) in both C and E groups but more evident in E groups.

Seric testosteron level decreased significantly in all individuals exposed to potassium dichromate, comparative to control, being high significant ($p<0.0001$) at 75 ppm Cr VI ($E_1$/C: -47.88%, $p<0.05$; $E_2$/C: -68.45%, $p<0.01$; $E_3$/C: -84.22%) and in inverse correlation to exposure level ($E_2/E_1$: -39.45%, p 0.05; $E_3/E_2$: -50%, p 0.05), significantly ($p<0.05$) only when exposure level increased from 25 to 75 ppm Cr VI ($E_3/E_1$: -69.72%, $p<0.05$).

Testosterone seric level was slightly higher (C/Ph: +42%) than the maximum physiological limit (2-3 ng/ml – Krinke, 2000) in C group but evidently lower in E groups ($E_1$/Ph: -26%; $E_2$/Ph: -55.2%; $E_3$/Ph: -77.6%).

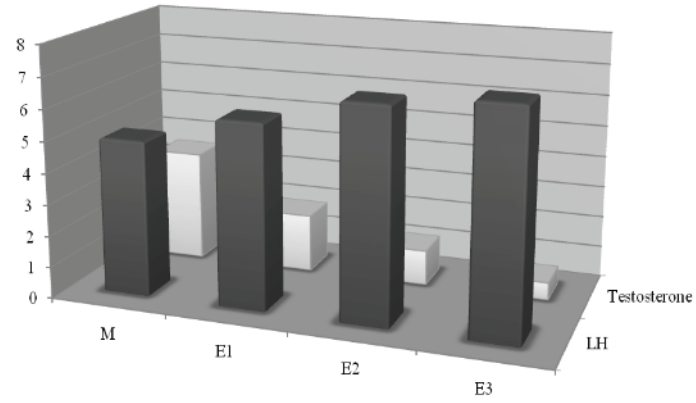


**Fig. 1** ***Seric Testosterone and LH level dynamics after potassium dichromate exposure***

Results regarding LH seric level after three months exposure to potassium dichromate are in accordance with those obtained by other authors: Ernst and Bonde, 1992, in rat ; Li *et al*., 1999, in men but are controversal to Chandra *et al*., 2007, in rat results.

Results related to seric testosterone level increase are similar to those obtained by Ernst and Bonde 1992 in rat; Yousef *et al*., 2006 in rabbit and Chandra *et al*., 2007 in rat.

Decrease of testosterone seric level could be explained by the impairment of Leydig cells which plays a pivotal role in steroidogenesis (Yousef *et al*., 2006).

LH level is regulated by negative feed-back by testosterone. That explains the increasing dynamics of LH levels. Also, the increase of seric LH emphasized that pituitary gland is still functional, though there are experiments that pointed out the negative impact of Cr VI on pituitary gland structure and functionality, respectively on hormones secretion (Quinteros *et al*., 2007).

Testosterone dymanics was determined by structural changes in testes, specially of Leydig cells. Structural changes in testes after Cr VI exposure were observed by some other authors (Saxena *et al*., 1999; Pereira *et al*., 2002; Aruldhas *et al*., 2005; Muselin *et al*., 2007) and by team researches too (unpublished data).

## CONCLUSIONS

The exposure of adult male rats for three months to potassium dichromate (Cr VI) in drinking water (25, 50 and 75 ppm Cr) determined:

1.1. significant increase of seric LH level comparative to control group and in direct, significant correlation to exposure level, excepting at 1.5 times increased exposure level;

1.2. significantly decrease of seric testosterone level comparative to control group and in inverse correlation to exposure level, significantly only when exposure level increased from minimum to maximum level (25 to 75 ppm Cr VI).

## ACKNOWLEDGEMENTS

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# POTASSIUM DICHROMATE IMPACT ON SOME MARKERS OF FEMALE REPRODUCTIVE SYSTEM PERFORMANCES (LITTER SIZE, SEX RATIO) AND PHYSICAL DEVELOPMENT (VAGINAL OPENING) IN RATS ($F_1$ GENERATION)

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**Key words:** female rats, Cr VI, vaginal opening, sex ratio, litter size

**SUMMARY**

The study was carried out on $F_1$ generation white Wistar female rats derived from 20 white Wistar adult female rats (exposed three months before mate and during pregnancy and lactation) to 25 (LOAEL), 50 and 75 ppm Cr VI), mated with white Wistar male rats exposed to same chromium levels along three months before mating.
$F_1$ generation was further exposed to the same chromium levels until sexual maturity.
The study pointed out: significant decrease of alive pups number/increase of dead pups number, impaired sex ratio in male favor, significant delay of puberty onset: late vaginal opening and decreased body weight.

Hexavalent chromium, important reproductive and developmental toxicant (1, 2) has major side effects in humans and animals. After short and long term exposure, there are developing important irreversible perturbances, covering both structure and function of the organism (1, 7, 9). The aim of this study was to evaluate potassium dichromate (Cr VI) impact on litter size, sex ratio, female age and weight at vaginal opening.

## MATHERIAL AND METHODS

The study was carried out on $F_1$ generation white Wistar female rats derived from mothers exposed for three months to potassium dichromate in drinking water as follows: $E_1$: 25mg Cr (VI)-LOAEL (16), $E_2$:50 mg Cr (VI) (2xLOAEL), $E_3$:75 mg Cr (VI) (3xLOAEL), C: tap water not containing chromium, mated with males exposed also three months at the same potassium dichromate levels. The exposure at the same potassium dichromate levels continued during pregnancy period and further, the pups from $F_1$ generation being exposed *in utero*, during suckling periods and until sexual maturity.

In female offspring, at birth, alive and dead pup number was evaluated. Also sex ratio, the age and weight (by technical balance) at vaginal opening were recorded.

Food and water were *ad libitum.*

All results were statistically analized by Anova method and Student test.

The experiment was conduced in accordance with present laws regarding animal welfare and ethics in animal experiments (10, 11, 12, 13, 14, 15).

## RESULTS AND DISSCUTIONS

Litter size (alive and dead pups at birth) consecutive potassium dichromate intake in $F_1$ generation is presented in table 1, figure 1.

***Table 1.***

**Alive and dead (mean) number of rat pups at birth in $F_1$ generation**

| | Alive pups | | | Dead pups | | |
|---|---|---|---|---|---|---|
| **Group** | **X±Sx** | **S.D.** | **C.L.95 %** | **X±Sx** | **S.D.** | **C.L.95%** |
| **C** | 12.60±0,24 | 0.55 | 0.56 | 0.00±0.00 | 0.00 | 0.01 |
| **$E_1$** | 11.40±0,24 | 0.55 | 0.56 | 2.01±0.01 | 0.02 | 0.01 |
| **$E_2$** | 10.40±0,24 | 0.55 | 0.56 | 3.01±0.01 | 0.02 | 0.01 |
| **$E_3$** | 9.00±0,32 | 0.71 | 0.56 | 4.04±0.01 | 0.01 | 0.01 |

Chromium exposure determined the birth of a significant, ($p<0.01$), lower number of alive pups in E groups comparative to C group: $E_1$/C:-9.52%, $E_2$/C:-17.46%, $E_3$/C:-28.57%.

The pups number decreased significantly ($p<0.01$), being inversely correlated to the exposure level: $E_2/E_1$:-8.77%, $E_3/E_2$:-13.46%, $E_3/E_1$:-21.05%.

The dead pups number at birth increased significantly ($p<0,01$) in exposed groups comparative to control group, ($E_1$/C:2.01/0, $E_2$/C:3.01/0, $E_3$/C:4.04/0) and in direct correlation, significantly, ($p<0.01$), with the exposure level: $E_2/E_1$:+49.75%, $E_3/E_2$:+34.21%, $E_3/E_1$:+100.99%.

These results are similar to those obtained by other authors after potassium dichromate exposure: Junaid et al., 2005 (during pregnancy), Kanojia et al., 1996 (preconceptional exposure), and Kanojia et al., 1998 (after three months exposure).

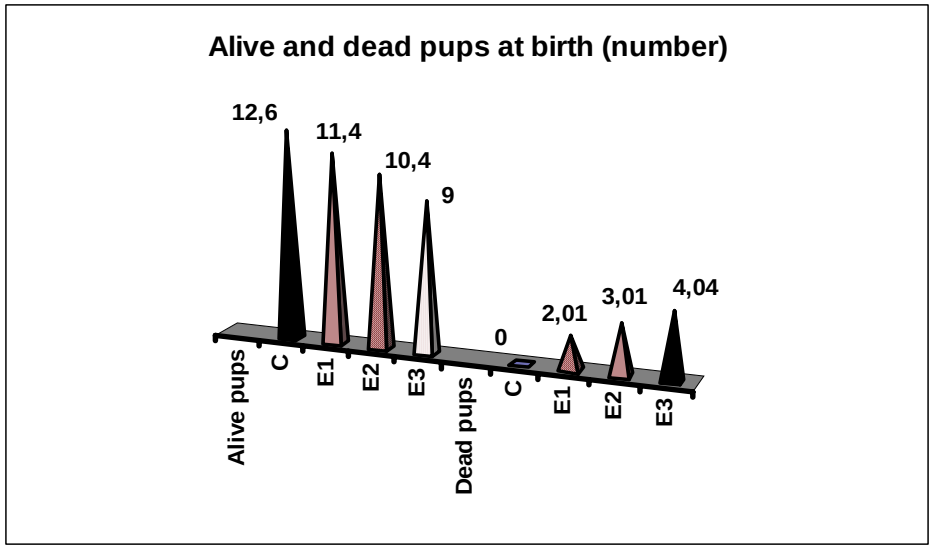


**Fig. 1. Alive and dead pups number dynamics at birth, in F1 generation**

Sex ratio is presented in table 2, figure 2.

***Table 2.***

***Sex ratio in $F_1$ generation***

| Group | Sex | Number | % | Sex ratio M/M+F |
|---|---|---|---|---|
| C | ♀ | 6.6 | 52.38% | 0.47 |
| | ♂ | 6 | 47.61% | |
| | Total/group | 12.6 | 100 | |
| E1 | ♀ | 6 | 44.74% | 0.55 |
| | ♂ | 7.41 | 55.26% | |
| | Total/group | 13.41 | 100 | |
| E2 | ♀ | 5.2 | 38.77% | 0.61 |
| | ♂ | 8.21 | 61.22% | |
| | Total/group | 13.41 | 100 | |
| E3 | ♀ | 4 | 30.67% | 0.69 |
| | ♂ | 9.04 | 69.32% | |
| | Total/group | 13.04 | 100 | |

In experimental groups, decrease of female pups procentage and increase of male pups percentage comparative to control group, C, was observed: 52.38%/47.61%, $E_1$: 44.74%/55.26%, $E_2$: 38.77%/61.22%, $E_3$: 30.67 %/69.32%.

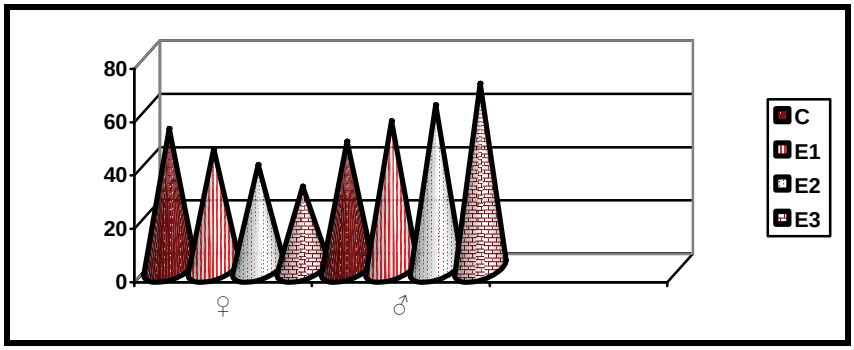


***Fig.2. Percentage dynamics of the sexes in $F_1$ generation***

The exposure level significantly influenced the female and male pups procentage dynamics, being inverselly correlated in the case of females: $E_2/E_1$:-13.34%, $E_3/E_2$:-20.89%, $E_3/E_1$:-31.44% and directly correlated in the case of males: $E_2/E_1$:+10.78%, $E_3/E_2$:+13.23%, $E_3/E_1$:+25.44%.

Sex ratio was modified in E groups comparative to C group in male favour: $E_1/C$:+16.06%, $E_2/C$:+28.58%, $E_3/C$:+45.59% ($p<0,01$).

Data regarding potassium dichromate impact on sex ratio are few and contradictive, some authors sustaining that it has no impact (Kanojia et. al., 1998).

The age and the body weight at sexual maturity (vaginal opening moment) are presented in table 3, figure 4, 5.

***Table 3.***

**Age and weight (mean values) at vaginal opening in $F_1$ generation**

| | Age | | | Body weight | | |
|---|---|---|---|---|---|---|
| **Group** | **$X \pm S_x$** | **S.D.** | **C.L.95%** | **$X \pm S_x$** | **S.D.** | **C.L.95%** |
| **C** | 41.00±0.33 | 0.93 | 0.70 | 90.84±0.54 | 1.53 | 1.12 |
| **$E_1$** | 70.25±0.45 | 1.28 | 0.70 | 79.35±0.58 | 1.64 | 1.12 |
| **$E_2$** | 80.12±0.30 | 0.83 | 0.70 | 70.03±0.39 | 1.11 | 1.12 |
| **$E_3$** | 88.00±0.27 | 0.76 | 0.70 | 61.11±0.64 | 1.81 | 1.12 |

In the E individuals, the vaginal opening age was significantly delayed ($p<0.01$) comparative to C group and over physiological limit (33-43days) (6) ($E_1/C$: +71.34%, $E_2/C$: +95.41%, $E_3/C$: +114.63%). The body weight at puberty onset was under 100 g (phisiological weight, 6) (61.11 – 79.35 g) and under the weight of C group ($E_1/C$: -12.64%, $E_2/C$: -22.90%, $E_3/C$: -32.72%), ($p<0.01$).

The delay of the vaginal opening was in direct corelation, significantly, ($p<0.01$) with the exposure level: $E_2/E_1$:+14.04%, $E_3/E_2$: +9.83%, $E_3/E_1$: +25.26%.

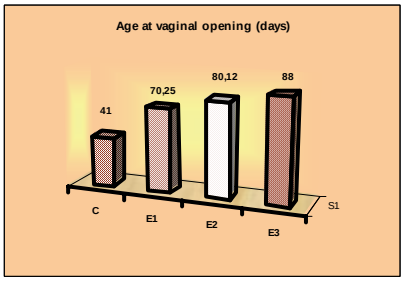


**Fig.4. Age of $F_1$ generation at vaginal opening**

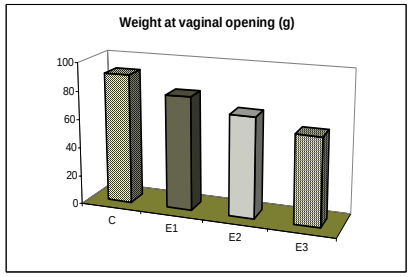


**Fig.5. $F_1$ generation body weight at the vaginal opening**

Between the weight at vaginal opening moment and the exposure level an inverse correlation was established ($p<0.01$) ($E_2/E_1$:-11.74%, $E_3/E_2$: -12.73%, $E_3/E_1$: -22.98%).

Delay of sexual maturity onset consecutive potassium dichromate exposure was recorded by other authors too (Sakhila et al., 2008).

KEI-ICHIRO et al., 2000 pointed out the corelation between body weight and the moment of the vaginal opening, its importance as markers for this phisiological moment, and the fact that it depends more of the body weight than of the age.

## CONCLUSIONS

3. 1. Significant decrease of alive pups number at birth, inverselly, significantly correlated to the exposure level;

3. 2. Significant increase of dead pups number, directly correlated to the exposure level;

3. 3. Impaired sex ratio, in male favor;

3. 4. Significant increase, with overlap of the physiological limits, of the age at vaginal opening moment, comparative to control group and directly, significantly correlated to exposure level;

3. 5. Significant decrease, under optimum limit, of body weight at vaginal opening moment, comparative to control group and inversely, significantly correlated to the exposure level.

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# ECTOPARASITES INFESTATION STUDY IN DOGS FROM BUCHAREST AREA

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**Key words:** prevalence, ectoparasites, dog

**SUMMARY**

This study investigated ectoparasites species and their prevalence in dogs from Bucharest and neared areas. Along year 2008 there have been 134 dogs diagnosed with ectoparasites (71 males and 63 females), aged between one month and 12 years. There have been identified 5 species of ectoparasites: *Ctenocephalides canis*, *Demodex canis*, *Otodectes cynotis*, *Sarcoptes scabiei* and *Trichodectes canis* with an infestation rate of 45.52%, 35.82%, 20.15%, 14.18% and 6.72% respectively. From all the dogs examined 76.12% were infested with a single ectoparasites specie and 23.88% with two ore more species of ectoparasites. 35.82% have been aged less than 6 months, 26.12% were between 6 and 12 months, 20.90% were between 12 and 24 months, 11.94% were between 24 and 36 months and 5.22% had more than 36 months old. Regarding the consequences of these parasites presence in dogs, both to veterinary medicine and human medicine, we recommend periodic checkups over the animal health and also informing the owners about the zoonotic risk.

Domestic animals and man can be hosts for some species of ectopararasites arthropods. Some of them don't have a strict specificity, so they infestate multiple hosts. So, can pass easily from one host to another, which is an opportunity for transmission of various pathogens (Cosoroabă, 2005). They can cause sever skin lesions, manifested by itching, dermatitis, and hair loss of different degrees. Their action is in direct line with the maintenance status and immunological status of the host and the parasite intensity (Gonzales et. al., 2004).

Some species of ectoparasites arthropods normally found in animals can pass to humans, at least temporarily, they cause some serious cases of dermatoses and/or allergic dermatitis (Cosoroabă, 2005; Aldemir, 2007). In other situations may act as a catalyst in the streaming of important human diseases (Nithikathkul, 2005; Chee et al., 2008).

This study investigated ectoparasites species and their prevalence in dogs from Bucharest and neared areas.

## 1. MATERIALS AND METHODS

Between January and December 2008, a total of 134 (71 males, 63 females) dogs with an age range of 1 month to 12 years were diagnosed with ectoparasites. Information on age, feeding and keeping place were obtained by questioning the owners. No animal has received treatment against ectoparasites before examination. Each animal has been examined by inspecting every corporal regions and analising every skin lesions. There have been taken samples. The samples were used to obtain native probes for microscop exam (using lactophenol for clarification).

## 2. RESULTS AND DISCUSSION

In this study were identified 5 species of ectoparasites, represented by *Ctenocephalides canis*, *Demodex canis*, *Otodectes cynotis*, *Scarcoptes scabiei* and *Trichodectes canis*, which have been registered with different prevalence (Table 1). Ectoparasitosis diagnosis was established to identify consecutive etiologic agents. Studies conducted in different parts of the world have shown the presence of various species of ectoparasites in dogs, with prevalence and intensity variables (Gonzales et al., 2004; Aldemir, 2007; Chee et al., 2008; Agbolade et al., 2008; Xhaxhiu et al., 2009). The differences can be attributed to emerging geographical characteristics, climatic and epidemiological areas studied.

***Table 1***

**Prevalence of ectoparasitic infestations in dogs in Bucharest area**

| Parasites | Infested Dogs (Number) | Prevalence (%) |
|---|---|---|
| *Ctenocephalides canis* | 61 | 45.52 |
| *Demodex canis* | 48 | 35.82 |
| *Otodectes cynotis* | 27 | 20.15 |
| *Sarcoptes scabiei* | 19 | 14.18 |
| *Trichodectes canis* | 9 | 6.72 |

From all the dogs examined 76.12% were infested with a single ectoparasites specie and 23.88% with two ore more species of ectoparasites.

35.82% have been aged less than 6 months, 26.12% were between 6 and 12 months, 20.90% were between 12 and 24 months, 11.94% were

between 24 and 36 months and 5.22% had more than 36 months old. Most of the cases were registered in the hot season in comparison with the cold season.

In this study it was found that the highest prevalence was recorded for the species *C. canis*. These results are consistent with data obtained by Aldemir (2005) in Turkey and Xhaxhiu et al. (2009), in Albania. The explosive multiplication of genus *Siphonaptera* insects is due to poor sanitation and environmental conditions, the information provided by owners pointing out that all animals examined were held in court. Prevalence increased, accompanied by low host specificity of fleas, requires more attention to this ectoparasitosis. Fleas have both a direct and an indirect pathogenicity. As hematophagous insects can cause anemia, and the sting causes obvious discomfort. Stings cause inflammatory phenomena accompanied by itching of variable intensity, so that the dog has injured scratches (Niculescu, 1998). In other news, these insects can play an important role of vectors and is responsible for transmission of pathogens and cestoda *Dipylidiu caninum* in dogs and humans (Cosoroabă, 2005).

*D. canis* in the present study showed the highest prevalence of scabies. Previous studies conducted in different geographical areas showed infestation with *D. canis* as the most common in dogs (Rabdea et al., 2002; Rodriguez-Vivas et al., 2003). *D. canis* is found in the skin of healthy dogs becoming pathogen especially when the immunological status of the individual is reducted. Demodicosis evolve as localized and/or widespread, affecting in particular young canine, without a predisposition related to sex exist. Solanki et al. (2007) consisted presence of demodicosis in proportion of 47.62% in dogs younger than 1 year.

*O. cynotis* and *S. scabiei* have value of 20.15% and 14.18%, having a lower frequency compared with scabies produced by *D. canis*. Unlike the results of this study, Chee et al. (2008), established that *O. cynotis*, followed by *S. scabiei*, has the highest values of scab diagnosis in dogs in the street. The differences can be attributed to the epidemiological factors, and especially geographical and climatic differences.

With regard to infestation by lice, that *T. canis*, it has the lowest prevalence of all species of ectoparasites diagnosed in dogs in this study, similar results were observed by Chee et al. (2008). Besides the discomfort they cause infected dogs, the importance of this parasitoze consists in the fact that *T. canis* is the host for *Dipylidium* Cestoda, which may infect other dogs and humans (Niculescu, 1998).

Our results reveal the presence of simple infestations over 2/3 of the dogs examined against poliparasites infestation. From the analysis results, we found out that most arthropods prevalence of ectoparasites was recorded in animals younger than one year. This could be due to a low immune status, which can add poor hygiene and prolonged contact with infected mothers, where cubs. Similar results were reported and Chee et al. (2008), which identified the presence of ectoparasites in 66.70% of dogs aged up to one year.

The existence of ectoparasites in pets is important for both the veterinarian, but also for human doctor. By their action, ectoparasites arthropods cause a wide range of pathological processes in the hair, skin, subcutaneous tissue and blood, and some species act as vectors for different pathogens by participating in the transmission of serious diseases in humans.

**In conclusion,** it requires regular epidemiological surveillance of ectoparasites infestation pets and owners information about the risk of zoonosis posed by these parasites. It also recommends the application of appropriate treatments to limit infestations.

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# EPIDEMIOLOGICAL AND CLINICAL FEATURES REGARDING DIROFILARIOSIS IN DOG

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**Key words**: dirofilariasis, dogs, clinical features

**SUMMARY**

The aim of the paper was to present the epidemiological data and clinical features of dirofilariasis in dogs in Bucharest area. The study was carried out from Mai 2008 to April 2009. The anamnesis revealed that all dogs lived in gardens or yards, most of them being used as guard dogs. *D. immitis* infection diagnosis was established corroborating the clinical signs with the radiographic data and the microscopic identification of microfilaria. For detection of microfilariae in the canine peripheral blood, a modified Knott's test was performed. Identification of *D. immitis* microfilariae was performed according to their morphological characteristics. 35 cases were confirmed as positive, 62.86% were males and 37.14% were females. Within the positive dogs, 37.14% (13/35) were 1-3 years and 62.86% (22/35) were aged more than 3 years. Clinical signs detected on physical examination of 35 dogs with dirofilariasis were: tachypnoea (45.71%, 16/35), fast tiredness (31.43%, 11/35), dry cough (25.71%, 9/35), dyspneea (25.71%, 9/35), weakness (20.0%, 7/35). The radiographic dates detected of positive dogs were represented by enlargement of the pulmonary vessels (42.86%, 15/35) and right heart dilatation (22.86%, 8/35).

*Dirofilaria immitis*, roundworm of the genus Dirofilaria, spreaded all over, has been identified especially in tropical, subtropical and temperate regions. In Europe, the dilofilariosis produced by the *D. immitis has* been usually reported in the Mediterranean countries, but recent findings have shown an increased incidence of this disease also in the northern and eastern European countries (Genchi et al., 2005a). With dog being the main host, the disease has been diagnosed in coyote, fox, wolf, polecat, rabbit, deer, bear, horse, cat, monkey and humans (Duran-Struuck et al., 2005; Manfredi et al., 2007). The adults are located in the right ventricle, the pulmonary artery, the right atrium and the vena cava (Cosoroabă, 2005). As it is a vivipar roundworm, the females of *D. immitis* are eliminating the larvas directly in the blood stream. In order to evolve further, they need an intermediary host, represented by different species of mosquitos. The larvas ingested by mosquitos during blood feeding process from the infested animals are experiencing several successive sheddings, getting to the phase III (L3) and thus becoming

infester (Niculescu et al., 1998). During the feeding process, the mosquito can transmit the L3 larvas to a new final host. Once inside the new host, the larvas migrate from the point of sting through the conjunctive tissue, muscles or fat tissue, suffer two more sheddings and then get into the blood stream, inside the heart, where they become adults (Cosoroabă, 2005).

In dogs, the clinical evolution of cardio-pulmonary dirofilariasis is usually cronical. Most infected dogs do not show any symptoms of the disease for a long time, months or years, depending on infestor number, individual reactivity and exercise, as arterial damages are more severe in dogs with intensive exercise than in dogs at rest (Dillon et al., 1995; Venco, 2008). Signs of the disease develop gradually (Venco, 2008). The initial signs of infection are restricted movements and weight loss followed by coughing, respiratory difficulties and chest pain. Pulmonary edema, right heart insufficiency and ascites worsen the clinical situation (Saritaş et al., 2005). Typical radiographic changes of this disease include right ventricle enlargement, enlargement of the main, lobar and peripheral pulmonary arteries and perivascular parenchimal disease (Bolio-Gonzalez et al., 2007).

The aim of this study was to present the epidemiological data and clinical features of dirophilariosis in dogs in Bucharest area.

## 1. MATERIALS AND METHODS

The study was carried out from Mai 2008 to April 2009. The anamnesis revealed that all dogs lived in gardens or yards, most of them being used as guard dogs. Therefore, the thoraco-pulmonary radiography and blood parasitic exams were made, as the dirofilaria infection was suspected. The animals included in the study were differentiated by age and sex. *D. immitis* infection diagnosis was established corroborating the clinical signs with the radiographic data and the microscopic identification of microfilariae. For detection of microfilariae in the canine peripheral blood, a modified Knott's test was performed. Identification of *D. immitis* microfilariae was performed according to their morphological characteristics.

## 2. RESULTS AND DISCUSSION

Following the parasitological exam, 35 dogs, aged between 1 - 13 years, turned out positive. 22 dogs (62.86%) were males and 13

(37.14%) were females. From the positive dogs, 13 (37.14%) were 1-3 years old and 22 (62.86%) were aged more than 3 years.

The subjects that turned out positive at the parasitological exam have been placed in two groups: animals with clinical signs of the disease, and asymptomatic animals. A number of 29 animals (82.86%) have been included in the first group; they displayed tachypnea (45.71%, 16 subjects out of 35), rapid fatigue (31.43%, 11/35), dry coughing (25.71%, 9/35), dyspnea (25.71%, 9/35), loss weight (20.0%, 7/35). A number of only 6 animals (17.14%) have been placed in the second group. These subjects had in common an asymptomatic evolution of the disease, being diagnosed following the parasitological exam. The subjects presented different clinical signs depending on each case - some more severe, some less severe. The Rx exam was neccessary in order to establish the diagnosis.

The radiographic data collected from positive dogs showed enlargement of the pulmonary vessels (42.86%, 15/35) (Fig. 1) and right heart dilatation (22.86%, 8/35) (Fig. 2).

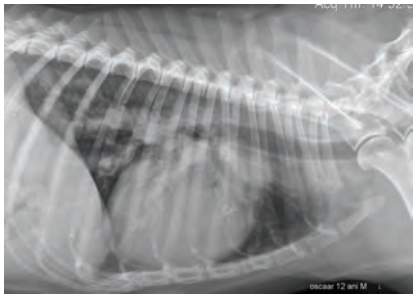

**Fig 1 – A 12-year-old male mixed breed dog. The pulmonary arteries are enlarged and tortous.**

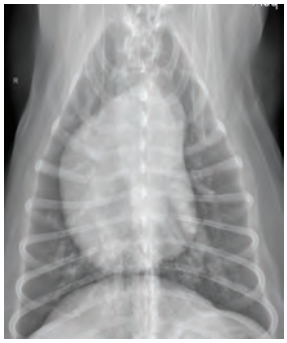

**Fig. 2 – A 4-year-old female mixed breed dog - right-side cardiomegaly with rounding of the right side of the cardiac silhouette**

The anamnesis showed that most dogs have been kept in open air, being used as guardian dogs in the owner's courtyards and gardens. Most locations were placed nearby lakes and places with abundant vegetation. Together with age and environment, the animal's lifestyle is an important risk factor in the emergence of the disease. The results of this study show that most of the positive diagnosed dogs were more than 3 years old. Similar studies showed that adult animals are more affected by this disease than younger subjects, because older animals are being exposed a longer period of time to the mosquito stinging, therefore there

is a larger probability for them to contract the disease (Yildirim et al., 2007; Bolio-G. et al., 2007).

*D immitis* (roundworm) has a reasonable long lifespan, thus the cardiovascular dirofilariasis becomes cronical. A microfilariae carrying dog can rarely be diagnosed early in the evolution of the disease as being infested with this parasit, but only when he already displays clinical signs of the disease. The clinical picture of the disease can be determined both by the action of the adults in the heart and lungs, as well as by the action of the larvas stages (Grandi et al., 2007). The high percentage of subjects manifesting clinically the disease (82.86%) in this study can be caused by a massive parasitical infestation, by a low reactivity of the body or by an intense effort of these animals, considering these issues are favourable to the patological changes at the cardio-pulmonary level.

The radiographic exam showed cardio-pulmonary changes in a large number of animals, as well as a corelation between the clinical signs and the Rx results. Similar results have been reported also by Polizopoulou et al. (2000), who have stated that radiographic cardio-pulmonary changes have been positively correlated with the clinical stages of the disease. An Rx of the thoracic cavity is an useful exam for determining the diagnosis of cardio-pulmonary dirofilariasis, emphasizing cardio-pulmonary lesions, and identifying asymptomatic cases (Cosoroabă, 2008).

Recent epidemiologic studies have shown a high prevalence of cardiopulmonary dirofilariasis (Montoya et al., 2007; Tudor et al., 2008; Meriem-Hind et Mohamed, 2009; Yaman et al., 2009). Although considered a veterinary parasitological disease, the dirofilariasis have been recognised as a zoonosis (Ulutas et al., 2007), as an increase of the prevalence of this disease in humans has been noticed (Genchi et al., 2005b, Fok, 2007). The profilaxy and early detection of the disease should play an important role as the clinical signs of the disease are tardive. It is well-known that infested animals are not a direct risk for other dogs or for humans, but they represent a permanent reservoir for mosquitos, thus enforcing measures against vectors is necessary.

## 3. CONCLUSION

The dirofilariasis produced by *D. immitis* has been diagnosed in a high percentage in animals older than 3 years and especially in animals living in open air.

Clinical signs of animals identified as positive have been correlated with the semiological radiographic aspects.

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# MOLECULAR DIAGNOSTIC OF FOOT AND MOUTH DISEASE

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**Key words:** Foot and Mouth Disease Virus, diagnostic, RT-PCR

**SUMMARY**

Epidemiologic importance of Foot and Mouth Disease (FMD) raised the necesity of establishing fast and accurate diagnostic of the disease in the initial phase, as well as the need for diagnostic techniques that can be further applied at a large scale, especially for virus identification in clinical specimens from the field. Among all the diagnostic tests that can be used for such cases, revers transcription polymerase chain reaction (RT-PCR) technique represent the method of choice mainly due to the sensitivity, specificity and rapidity.

Foot and mouth disease (FMD) is recognised to be the most contagious disease of mammals, causing exceptional economic losses through the species and number of animals that are affected, as well as severe restrictions that follows the diagnostic. It is caused by a virus of the genus *Aphthovirus*, family *Picornaviridae*. There are seven serotypes of FMD virus, namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven-hoofed animals (OIE Manual). The virus contains only one single-stranded, positive-sense RNA genome of approximately 8500 nucleotides surrounded by a coat composed of 60 copies of each of four structural proteins that give raise to an icosahedral symmetry. Like the majority of other RNA viruses, FMD has very high mutation rates, between $10^{-3}$ to $10^{-5}$ per nucleotide site per genome replication, owing to the lack of error correction mechanisms during RNA replication (Domingo *et al*., 2003, Xuan Huang *et al*, 2009).

Taking into consideration the actual status of Romania, as being free of FMD, as well as the potential threats given by the regional epidemiological situation (especially the outbreaks from Middle East and Turkey) and the character of the disease, the need for accurate and rapid response to a potential case of FMD leaded to the necessity of implementing molecular techniques to complete the diagnostic protocol.

## MATERIALS AND METHODS

1.1. Biological specimens

were represented by inactivated viral suspension originated from 2008 ring test trial organiseed by Comunity Refference Laboratory Pirbright, United Kingdom (Table 1).

1.2. RNA Extraction

was performed using commercially available kits (*RNeasy Mini Kit* - Qiagen) following manufacturer recommendations. The nucleic acid was eluted in a final volume of 50µl.

1.2. Revers transcription and amplification

Methods used for FMD genome detection consisted of two real time RT-PCR protocols (*TaqMan* assay protocols) and one nested PCR protocol. Real time PCR assays were designed to target two different conserved regions: segment (3D) of the FMDV RNA polymerase gene (Callahan *et al*, 2002, Paixao *et al*, 2008 ) and the internal ribosomal entry site located in the 5' untranslated region (5'UTR) (Reid *et al*, 2003). For the nested PCR protocol, the primer sequences were selected from the conserved genomic sequences of the viral RNA polymerase gene (Bahari *et al*, 2007).

Amplification for real time protocols was performed using commercially available kits – *OneStep RT-PCR kit* (Qiagen) for revers transcription and PCR amplification, briefly with 0.4μM final concentration of primers, 0.24 μM for probe, extra $MgCl_2$ with final concentration of 1.25mM (protocol available on request).

For the nested PCR, amplification was performed using *OneStep RT-PCR kit* (Qiagen) for revers transcription and first PCR amplification and *GoTaq Flexi DNA Polymerase* (Promega) for the second nested PCR – folowing the manufacturer reccomendations, generating amplification products of 898bp (base pairs) for the first PCR and 222bp for the second.

The samples were loaded into a real-time instrument (*Smart Cycler*, Cepheid) or a conventional thermocycler (*ICycler*, Bio Rad). Thermal profiles for the real time protocols were slightely modified by intruduction of an additional 72ºC extension step (*three step* PCR instead of *two step* PCR).

1.3. Agarose gel electroforesis.

For the nested RT-PCR, the products were loaded into an agarose 1,5% gel stained with ethidium bromide for UV visualisation. Migration was performed using 1X TBE buffer.

## RESULTS AND DISCUSSIONS

All three protocols used for FMD detection proved to be reliable enough for genome detection, with some advantages and disadvantages from the rest, as follow:

*2.1*. Callahan Protocol, targeting the 3D gene region, proved to be the most sensitive, with good Ct (*crossing treshold*) values and good reaction eficiency, as shown in Figure 1 and Table 1.

*2.2*. Reid protocol, targeting 5'NTR region, showed good sensitivity and reaction eficiency (Figure 2, Table 1), eventhough the Ct values were higher than the first protocol (less sensitive). However, a study performed by the CRL (Comunity Refference Laboratory) Pirbright, United kingdom, regarding the two protocol, on a significant number of samples, indicate the need for using both protocol in diagnostic, since sometimes either one can show false negative results (King *et al*, 2006).

*2.3*. The nested protocol showed good reaction sensitivity and specificity, with no cross-reaction especially with SVDV (*Swine vesicular disease virus*) (Figure 3, Table 1). However, since the 1:250 dilution of the SAT 2 serotype showed no specific bands for both amplification protocols, it is less sensitive that the above mentioned for this particular serotype. Also, for high viral load samples, it is recomended to subject both amplification products to electrophoresis, since unspecific bands appear with high rate for the nested PCR, due to the high amount of starting template and also for speeding up the results (Figure 3). On the other hand, care should be taken when handling the PCR products, since cross-contamination and consequently false positive results might appear with high frequency (as in any open system); for this reason, the protocol can be used as a „*back-up*" solution.

In order to compare the overall sensitivity of the RT-PCR test, all samples subjected to the study were analyzed with specific antigen ELISA kit. Results obtained showed good specificity of the antigen ELISA, but lower sensitivity than the RT-PCR (especially for the samples with high dilution rate) (Table 1). However, the method is capable of discriminate between serotypes, thing that cannot be done with neither of the RT-PCR protocols described (all three protocols are „generic" methods, *i.e.* they detect the FMD genome without discriminating the serotypes).

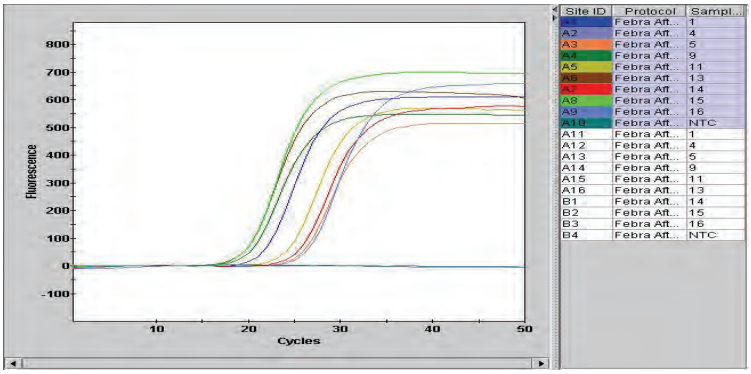


**Fig. 1**
**Amplification curves obtained with Callahan protocol**
**The shape of the amplification curves and the early Ct values show good protocol performance**

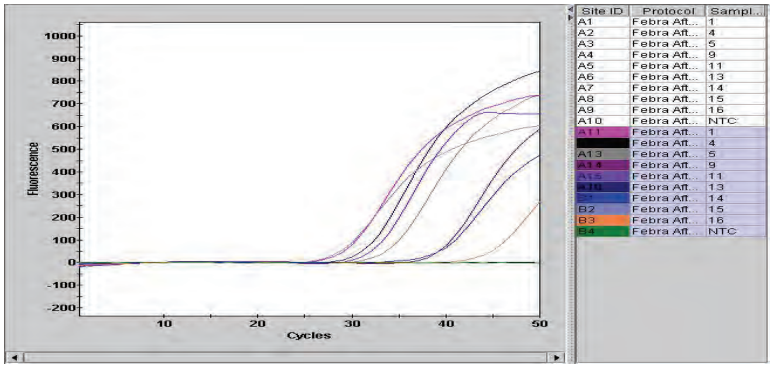


**Fig. 2**
**Amplification curves obtained with Reid protocol**
**Amplification curves show higher Ct values than Callahan protocol**

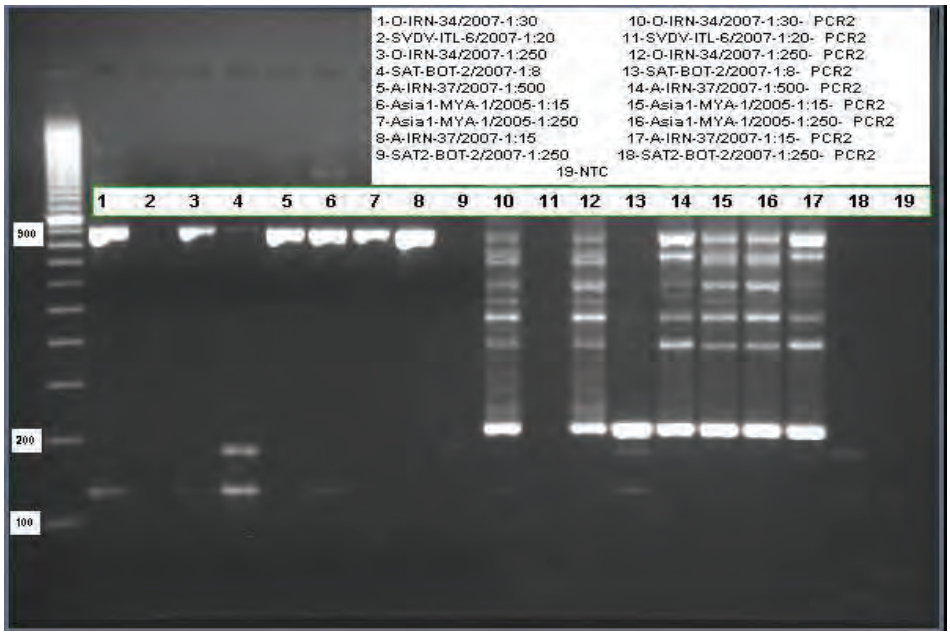


**Fig. 3**
**Electrophoresis results for the nested PCR**
**Lane 1 – molecular marker 100bp (base pairs); lanes 2 to 10 (numbers 1 to 9 on the photo) – RT-PCR products; lanes 11 to 19 (numbers 10 to 18 on the photo) – nested PCR products;**
**lane 20 (number 19 on the photo) – No template control (NTC).**

***Table 1***

**Samples panel subjected to the study**

| **Sample detailes** | **Serotype** | **Dilution factor** | **Antigen ELISA results** | **RT – PCR Results** | | |
|---|---|---|---|---|---|---|
| | | | | **Callahan protocol** | **Reid protocol** | **Nested PCR protocol** |
| O-IRN-34/2007 | O | 1:30 | Positive | Positive Ct = 20.78 | Positive Ct = 28.18 | Positive |
| SVDV – ITL-6/2007 | Swine vesicular disease virus (SVDV) | 1:20 | SVDV | **Negative** | **Negative** | **Negative** |
| O-IRN-34/2007 | O | 1:250 | **Negative** | Positive Ct = 25.12 | Positive Ct = 32.68 | Positive |
| **Sample detailes** | **Serotype** | **Dilution factor** | **Antigen ELISA results** | **RT – PCR Results** | | |
| | | | | **Callahan protocol** | **Reid protocol** | **Nested PCR protocol** |

| | | | | | | |
|---|---|---|---|---|---|---|
| SAT2-BOT-2/2007 | SAT 2 | 1:8 | Positive | Positive Ct = 19.28 | Positive Ct = 38.44 | Positive |
| A-IRN-37/2007 | A | 1:500 | **Negative** | Positive Ct = 23.18 | Positive Ct = 32.20 | Positive |
| Asia1-MYA 1/2005 | Asia 1 | 1:15 | Positive | Positive Ct = 18.70 | Positive Ct = 30.18 | Positive |
| Asia1-MYA 1/2005 | Asia 1 | 1:250 | **Negative** | Positive Ct = 24.47 | Positive Ct = 38.17 | Positive |
| A-IRN-37/2007 | A | 1:15 | Positive | Positive Ct = 18.75 | Positive Ct = 27.77 | Positive |
| SAT2-BOT-2/2007 | SAT 2 | 1:250 | **Negative** | Positive Ct = 25.45 | Positive?? Ct = 43.89 | **Negative** |

## 3. CONCLUSIONS

*3.1* The National Refference Laboratory for vesicular diseases manage to succesfully implement the molecular biology tests for detection of FMD virus, therefore completing and updating the diagnostic and surveillance protocol.

*3.2* All three optimized tests showed good to adequate sensitivity for viral genome detection and good specificity.

*3.3* As expected, results obtained for the samples with low viral load showed superior sensitivity of the molecular biology methods, making them ideal as early diagnostic methods for antigen detection and also for screening in herds that can be suspected as being contaminated.

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# THE INFLUENCE OF THE FREEZING PROCESS ON THE STALLION SPERM HEAD CITOMOPHOMETRY

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**Key words:** morphometry, acrosome reaction, stallion.

**SUMMARY**

The evaluation of sperm quality is useful in predicting the fertility of sperm and is of great importance in maximizing reproductive efficency. This work used semen samples collected from 20 stallions and assessed for sperm morphometry (length, width, area, perimeter and ellipticity, rugosity, elongation, regularity) before and after cryopreservation, using the Sperm Class Analzyer Sistem (SCA®), with two freezing extenders and then compared. Cryopreservation succes was different between freezing methods. Sperm head dimensions were significantly ($p<0.05$) smaller in cryopreservated samples that in the fresh ones. These data suggest that changes in sperm head morphometry might reflect spermatozoa injury during cryopreservation.

The assessment of sperm quality is useful in predicting the male fertility (Colenbrander B. et all., 2003) and it is of a major importance in reproduction efficiency, both in natural conditions and within the assisted reproduction programmes (Rodriguez I. et all., 2001). Moreover it is a key tool in the clinical diagnosis of subfertile animals. Conventional evaluation techniques of the sperm quality have been based on the subjective assessment of semen parameters such as motility, morphology, semen volume or concentration (Vestergen J. et all., 2002). A high percentage of morphologic anomalies is an important indicator of fertility decrease. These assessment methods, based on visual appreciation has lead to widely varying results intra- and inter laboratories. The introduction of computer-assisted sperm morphometry analysis (ASMA) systems attempted to overcome the problem of the subjectivity of visually based methods of assessment. Although this technology was originally designed for human sperm (Davis R. et all., 1992;) it has been progressively adapted to some animal species, including the stallions. These systems are capable of detecting subtle differences that conventional methods were unable to identify, such as the relationship between sperm morphometry and fertility. The introduction in the andrologic practice of the assessment methods based

on fluorescence made possible a more accurate assessment of the functional integrity of plasmatic and acrosomial membranes (Casey P. et all., 1997).

The aim of the present study was to evaluate the effect of freezing/thawing process and of freezing extender on the stallion sperm head morphometry, with SCA® system, and the freezing influence on acrosomial integrity of the sperm cell.

## 1. MATERIALS AND METHOD

The researches were made on a batch of 20 Andalusian stallions. The stallions used for the study were clinically healthy, being used for semen recovery in view to artificial insemination or cryoconservation. We realized a recovery for each stallion. The sperm spermatozoa concentration has been determined by using the fotometer method, with SpermaCue® (Minitübe). Total and progressive motility was assessed objectively using the motility module of the Sperm Class Analyzer® (SCA) (Microptic S.L. Barcelona, Spania), 2002 version. The acrosome reaction was assessed using 2 staining methods : Spermac® and FITC-conjugated peanut agglutinin (*Arachis hypogaea*, PNA) after the method described by Cheng and coll., 1996.

After centrifugation, the sludge was resuspended in a 1:1 proportion in (1): A: M1 extender (Minitübe$^{tm}$), at which we added lactose (20%), yolk (20%) and glycerol (3%), marked with MO and (2) B : a commercial extender, that present the same componence, but in different proportions. Each ejaculate was divided in 2 parts, that were diluted in the two extenders and frozen using the slow freezing protocol. Initially, the ejaculates resuspended in the extenders were refrigerated for an hour at 5°C, and then packed in 0,5 ml straws and then frozen in nitrogen vapours. After thawing (30 seconds in 38°C water), after an hour in thermostat, the samples were assessed for morphometry, motility and acrosomial integrity assessment.

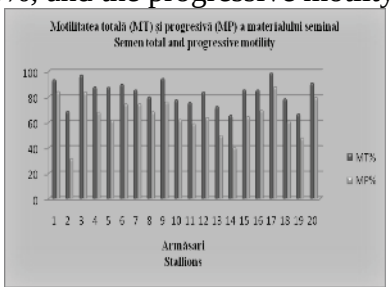
Three microscopic slides per sample were prepared, one for the fresh semen and two for the frozen/thawed one for each extender used, by extension on a slide of 7 μl semen. The staining used for the morphometric study was realized with Hematoxilina Harris (Papanicolau solution 1a, Merck Cat. No. 9253, Darmstad, Germania).

At least 100 digitised sperm heads were analysed per slide. The morphometric analysis of the sperm head was realized using the same computerized system Sperm Class Analyzer® (SCA). The results obtained after the measurements were stocked directly by the system in

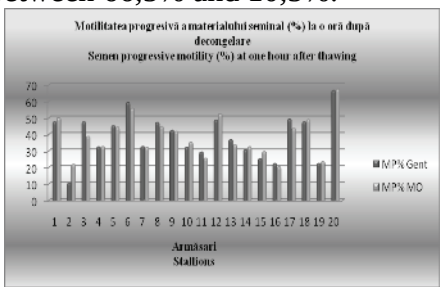
an Excel program for the ulterior analysis. The computer software calculated 8 morphometric parameters automatically : 4 for the sperm head size : length, width, area (in $\mu m^2$), perimeter (in µm) and 4 for the head form : ellipticity (defined as length/width ratio), rugosity, elongation and regularity (Hidalgo M. et all., 2006).The measurements for each individual spermatozoa were saved in Excel® (Microsoft). We realized the average of the obtained values for each stallion and staining, the standard deviation for morphometric and qualitative parameters. We also calculated the variability coefficient (CV%). The average values of the spermatic parameters were compared using the *t* Student test.

## 2. RESULTS AND DISCUSSIONS

The assessment of spermatic motility is considered to be an essential laboratory test for spermatozoa fertilizing capacity appreciation. Thus, the values obtained after total and progressive motility assessment enframed in the race standards. The total motility had an average value of 82,9 % ± 9,89, with a 11% CV, the limits being between 98,5% şi 65,3%. The progressive motility eas comprised between 87,6% and 31,2% , with an average of 65,14% ± 14,73, and The CV presentd a value of 22%. The values that presented interest were those of the total motility and especially of the progressive one, at an hour after the maintenance in thermostat, after the thawing. For the commercial medium, the total motility, after an hour, was between 88,6% and 37,3%, and the progressive motlity between 66,5% and 10,5%.




***Graphic 1. Graphic representation of total and progressive motility values for the stallions studied for the fresh semen***




***Graphic 2. Graphic representation of total and progressive motility values for the stallions studied for the frozen/thawed semen***

For the extender prepared in the laboratory, the total motility was between 78,5% and 39,5%, whereas the progressive motility varied between 67,3% and 20,2%. We observe an important decline of total and progressive motility for the frozen samples, in comparison with fresh semen, regardless of the freezing extender used. After comparing the

results recorded for progressive motility an hour after freezing for the two extenders used, the *p* value was statistically insignificant.

***Table 1.***
***Global average values of spermatic parameters obtained for the fresh and frozen/thawed semen by the 2 freezing extenders***

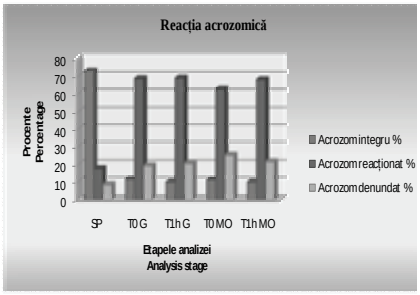
| Morphometric parameters | Fresh semen | Extenders | |
|---|---|---|---|
| | | Commercial extender | MO extender |
| **Length (µm)** | 5,6±0,20 | 5,55±0,2 | 5,53±0,2 |
| **Width (µm)** | 2,72±0,10 | 2,67±0,11 | 2,66±0,1 |
| **Area (µm$^2$)** | 12,68±0,43 | 12,45±0,50 | 12,44±0,49 |
| **Perimeter(µm)** | 14,50±0,33 | 14,41±0,36 | 14,39±0,35 |
| **Ellipticity** | 2,06±0,13 | 2,07±0,13 | 2,07±0,12 |
| **Rugosity** | 0,75±0,02 | 0,75±0,02 | 0,74±0,02 |
| **Elongation** | 0,34±0,26 | 0,34±0,02 | 0,33±0,03 |
| **Regularity** | 0,94±0,008 | 0,94±0,005 | 0,94±0,003 |

Analysing globally the averages of the calculated values for the sperm head morphometry before and after thawing using the two freezing extenders, we obtained statistically significant differences ($p<0,05$). The parameters recorded for the sperm head length, for the fresh semen, were between 6,01 µm and 5,29 µm, with an average of 5,6 ± 0,20 µm. The width was between 2,54 µm and 2,85 µm, with an average of 2,72 ± 0,1 µm. The freezing/thawing process had effects upon sperm head cell morphometry. All the spermatic parameters studied were smaller for the semen samples submitted to the freezing/thawing process, in comparison with the fresh ones. Comparing the results obtained for the two freezing extenders, we observed that all the dimensional and undimensional parameters were significantly smaller ($p<0,05$) in the case of the semen samples frozen with MO extender, than in the case of the commercial extender. Although both extenders lead to the head dimension reduction of the frozen spermatozoa, this effect was less considerable in the case of the commercial extender.

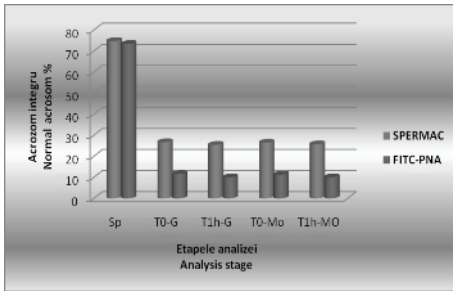
The freezing/thawing procedures lead to the reduction of sperm cell head dimensions, for the cryopreserved samples, in comparison with the fresh semen samples. These results correspond with those obtained in the case of other species such as the human specie (Thompson L. et all., 1994), bulls (Gravance C. et all., 1998), stags (Esteso C. et all., 2003), and stallions (Arruda R. et all., 2002), where the sperm cell head

dimensions in the case of cryopreserved samples were significantly smaller than in the case of fresh semen samples.

For the frozen/thawed semen we observed a drastic reduction of the intact acrosomes percentage, regardless of the staining method and the frozen extender used. We also remarked the raise of the percentage of reacted acrosome spermatozoa, and of the sperm cell population that have completely lost the acrosome. Thus, from the value of 74,75% ± 5,68 (Spermac staining) intact acrosomes in fresh semen, respectively 73,38% ± 5,63 (FITC-PNA staining), the perecntage of the spermatozoa with unreacted acrosome reached to 26,7% ± 7,08 (commercial extender) or 26,49% ±7,31 (MO extender), for Spermac staining and at 11,58% ± 3,21 (commercial extender) or 11,29% ± 3,05 (MO extender) for the fluorescence staining. The differences regarding the acrosomial interpretation between the two stainings are considerable, concerning the thawed semen.




***Graphic3. Acrosomial reaction variation according to the semen processing***




***Graphic 4. Graphic representation of intact acrosomes percentage obtained after Spermac and FITC-PNA staining***

Having in view the fact that the FITC-PNA fluorescent lectins bind only the outer acrosomial membrane, we considered that this staining is the most efficient for the acrosome status assessment.

Comparing the freezing extenders, from the point of view of the spermatic cells protection from the damage produced by freezing, we didn't obtain significant differences ($p$>0,05) for the intact acrosomes percentage. The differences appeared only in the case of the reacted acrosomes protection, at 5 minutes after thawing, the MO extender assuring a better protection (68,79% ± 5,47 for the commercial extender and 62,96% ± 9,46 for MO extender). But the situation inverses in the case of the sperm without acrosomes, these ones being present in a bigger percentage 25,49% ± 9,44 for MO extender than for the commercial extender 19,64% ± 5,29.

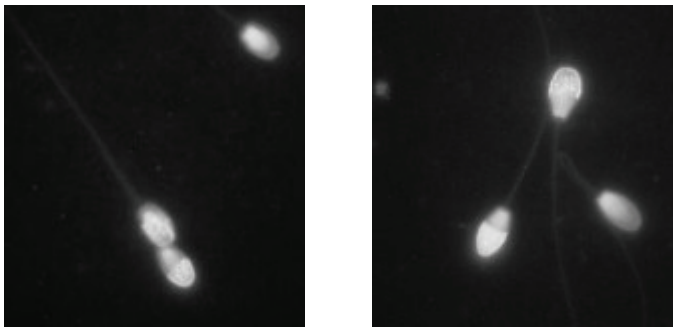


**Fig 1. FITC-PNA/PI stained spermatozoa; 100x**

The physiological reaction of the intact acrosome is an essential step in the fertilization process. Thus, the acrosomial status and acrosomic reaction assessment is important for the semen characterization regarding cryoconservation or artificial insemination. The percentage of the intact spermatozoa with the capacity to develop a normal acrosomial reaction represent a relevant information for the fertilization (Blottner S. et all., 1990). The cryopreservation process presents an important influence on acrosomial integrity. The dramatic growth of the percentage of spermatozoa with damaged head after the freezing/thawing process was considered as a reduction of the semen fertilization capacity. This fact was observed by the two staining methods used, and also after the freezing with the two extenders.

In this study we included two freezing extenders, one of them commercial, frequently used for cryopreservation, to test the protection capacity of the extender regarding the acrosomial membrane. But the results obtained demonstrate the fact that the extender composition has a weak cryoprotection effect.

## 3. CONCLUSIONS

3.1. the freezing/thawing process had effects on sperm cell head morphometry, all the studied spermatic parameters were smaller for the semen samples submitted to the freezing/thawing process, in comparison with the fresh ones;

3.2. comparing the results obtained for the two freezing extenders used in the experiment, we observed that all the dimensional and undimensional parameters were significantly smaller ($p<0,05$) in the case of the semen samples frozen with MO extender, than in the case of the commercial extender;

3.3. both freezing extenders lead to the dimension reduction of the spermatozoa head submitted to freezing, this effect being less considerable when we used the commercial extender;

3.4. for the frozen/thawed semen, we observed a drastic reduction of the intact acrosomes percentage, regardless of the staining method and of the freezing extender;

3.5. the cryopreservation process presents an important negative influence on the acrosomial integrity.

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# THE STATUS OF SELENIUM AND VITAMIN E IN THE SANGUIN SERUM FOLLOWING THE ADMINISTRATION OF DEFICIENCY RATION IN CASE OF CHICKENS BRED IN A SEMI – INTENSIVE SYSTEM

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**Key words**: vitamin E, selenium, zoofort, serological samples.

**SUMMARY**

The current study has proposed as an objective to determine the dosage of selenium and vitamin E in blood serum of chickens bred in a semi-intensive system and fed using fodder portions that were deficient in selenium and vitamin E.
The research was conducted on two batches of chicken, the control group was made up of 400 chickens that were fed using the standard 21 – 1 recipe and an experimental group that consisted of 600 chickens fed the same recipe from which microvitamins E and sodium selenite were taken off from the zoofort A3. (( Pârvu *et al*, 2003)
The dosages of vitamin E and selenium in serological tests were done by conventional methods, meaning the modified Hashin and collaborators method to set the dosage of vitamin E and the modified Cummings method to set the dosage of selenium. (Bruger, 1987; Sanders, 1986))
The results obtained show a close correlation between feeding a ration that has deficiencies in selenium and vitamin E and the serum levels of vitamin E and selenium that are much lower for the experimental group than those for the control batch.
Even clinically speaking, the experimental group presented starting from the 56th day of life ataxia, forced stretching of wings, muscular tremors, incapacity to walk and growing delays. (Rădoi, 2003)
After reintroducing in the fodder portions of the zoofort A3 with all its components (microvitamin E and sodium selenite), when the last biochemical determination was made at the age of 14 week old, there was noticed a spectacular increase of the serumal values of vitamin E and selenium, thing that proves once more that there is a close connection between the composition of the fodder ration and the status of vitamin E and selenium in blood serum. (Sklan and Donoghue, 1992)

The current work has established as an objective to realiye the dosage of selenium and vitamin E from the blood serum at chickens that were bred in a semi – intensive system an which were fed fodder rations that have deficiencies in vitamin E and selenium.

## 1. MATERIAL AND METHOD

To highlight the effects of experimental deficiencies in vitamin E and selenium at chickens it was made up a 400 chicken control group

that were fed according to the 21 to 1 recipe from the age of one day old to the fourteenth week of life.

At the same time it was created an experimental batch of 600 chickens that were grown under the same microclimate conditions as the control batch; the experimental group was fed according to the same recipe as the standard one, except that from the zoofortul A3 that supplemented the diet microvitamins E and sodium selenite were totally removed from the first day of life (Pârvu *et al*, 2003; Pârvu, 1992) .

From the first week of life of the chickens from the experimental group, there were randomly extracted 20 of them that were sent to the Institute for Diagnosis and Animal Health, where the chickens were slaughtered in order to collect blood samples. The measurements were made weekly until the age of 14 weeks (Bruger, 1987; Sanders, 1986).

The dosage of vitamin E and selenium for the biochemical profile determination were made using conventional methods, meaning the HASHIM and collaborators method that was modified, for the dosage of serumal vitamin E and the CUMMINGS method, which was modified in what the dosage of serumal selenium is concerned.

## 2 .RESULTS AND DISCUSSION

When the biochemical determinations were finished, there were obtained values for each sample of investigated serum. To improve the results for serum samples from each age it was taken as final value the average of 20 samples of serum from each lot.

***Table 1***

**The normal status of selenium and vitamin E for chickens. (Pârvu, 1992)**

| No | Age (weeks) | No. of serum samples | Selenium (gamma/ml) | | Vitamin E (gamma/ml) | |
|---|---|---|---|---|---|---|
| | | | Reference values | Control batch values | Reference values | Control batch values |
| 1 | One day | 20 | | 0,210 | | 8,068 |
| 2 | 1 | 20 | | 0,149 | | 5,20 |
| 3 | 2 | 20 | | 0,079 | | 5,20 |
| 4 | 3 | 20 | | 0,123 | | 6,75 |
| 5 | 4 | 20 | >0,07±0,03 | 0,081 | >5* | 6,20 |
| 6 | 5 | 20 | | 0,176 | | 5,47 |
| 7 | 6 | 20 | | 0,201 | | 5,40 |
| 8 | 7 | 20 | | 0,098 | | 18,40 |
| 9 | 8 | 20 | | 0,090 | | 16,80 |
| 10 | 9 | 20 | | 0,112 | | 5,84 |
| 11 | 10 | 20 | | 0,199 | | 8,39 |
| 12 | 11 | 20 | | 0,193 | | 8,17 |
| 13 | 12 | 20 | | 0,087 | | 9,41 |
| 14 | 13 | 20 | | 0,081 | | 10,11 |
| 15 | 14 | 20 | | 0,080 | | 9,34 |

As it can be noticed in the table, all the values obtained were normal, well above the reference values. It should be remembered that the feeding was done according to the standard recipe. In the first day of life the values were well above the benchmark, because selenium and vitamin E in blood serum were inherited from their mothers (Sanders, 1986). From the first week of life and up to week 6 the values of vitamin E began to decline.

From the 7 week the values started to increase getting to the value of 18.40 gamma / ml respectively 16.80 gamma / ml during the 7th and 8th week. Then these values gradually began to fall, noticing light oscillations of the values obtained each week.

The selenium values obtained were normal. The highest value obtained was on the first day of life, respectively 0.210 gamma / ml, after that there were registered slight oscillations of the obtained values.

***Table 2***

**TABLE 2. Selenium status in blood serum after the administration of deficient rations.**

| No. | Age (weeks) | No. of serum samples Experimental batch | Selenium gamma/ml Experimental batch |
|---|---|---|---|
| 1 | One day | 20 | - |
| 2 | 1 | 20 | 0,052 |
| 3 | 2 | 20 | 0,050 |
| 4 | 3 | 20 | 0,044 |
| 5 | 4 | 20 | 0,041 |
| 6 | 5 | 20 | 0,032 |
| 7 | 6 | 20 | 0,028 |
| 8 | 7 | 20 | 0,022 |
| 9 | 8 | 20 | 0,020 |
| 10 | 9 | 20 | 0,014 |
| 11 | 10 | 20 | 0,012 |
| 12 | 11 | 20 | 0,010 |
| 13 | 12 | 20 | 0,031 |
| 14 | 13 | 20 | 0,059 |
| 15 | 14 | 20 | 0,078 |

*Table 3*

**Vitamin E status in blood serum after the administration of deficient rations.**

| No. | Age (weeks) | No. of serum samples Experimental batch | Vitamin E gamma/ml Experimental batch |
|---|---|---|---|
| 1 | One day | 20 | - |
| 2 | 1 | 20 | 4,82 |
| 3 | 2 | 20 | 4,75 |
| 4 | 3 | 20 | 3,61 |
| 5 | 4 | 20 | 3,53 |
| 6 | 5 | 20 | 3,11 |
| 7 | 6 | 20 | 2,83 |
| 8 | 7 | 20 | 2,50 |
| No. | Age (weeks) | No. of serum samples Experimental batch | Vitamin E gamma/ml Experimental batch |
| 9 | 8 | 20 | 2,41 |
| 10 | 9 | 20 | 2,03 |
| 11 | 10 | 20 | 1,98 |
| 12 | 11 | 20 | 1,92 |
| 13 | 12 | 20 | 3,97 |
| 14 | 13 | 20 | 5,11 |
| 15 | 14 | 20 | 9,98 |

*Table 4*

**Values of selenium and vitamin E in serum of chickens in experimental and control group. (Sanders, 1986)**

| Age (weeks) | Selenium (gamma/ml) | | | Vitamin E (gamma/ml) | | |
|---|---|---|---|---|---|---|
| | Average reference values | Average values for the control batch | Average values for the experimental batch | Average reference values | Average values for the control batch | Average values for the experimental batch |
| 14 | >0,07± 0,03 | 0,08 | 0,079 | >5 | 9,34 | 9,98 |

## 3. CONCLUSIONS

When checking the obtained values in the table, there is a visible decrease in serum values of vitamin E depending on the age of the chickens. This shows that the values obtained from biochemical determinations of the control group were normal, well above the reference values. Recall that the feeding was done according to standard recipe (Pârvu *et al*, 2003; Pârvu, 1992).

Thus, if during the first week the value of serum vitamin E was 5.20 gamma / ml in the control group, the one for the experimental batch (fed by the same standard recipe from which there have been removed microvitamins E and sodium selenite) got to 4, 82 gamma / ml, reaching during the 11th week of life the value of 1.92 gamma / ml.

Dosing the serum selenium there can also be noticed a marked decrease of it compared to the control group. While during the first week of life it was recorded a value of 0.149 gamma / ml, for the experimental group the value was 0.052 and it got to 0.010 gamma / ml during the 11th week of life.

From a clinical point of view, the experimental batch presented from their 56th day of life: ataxia, forced stretches wings, incapacity of walking, delays in growth. A large number of these chickens had muscle trembling (Rădoi, 2003).

From their 11th week of life zoofortul A3 with all components (microvitamine E and sodium selenite) was reintroduced in the fodder recipe and, at the same time, they also received vitamin E and selenium as treatment doses.

With the last biochemical determination at the 14th week of life, there can be noticed a major, even spectacular, growth in serum vitamin E and selenium after returning to the normal feeding recipe and after the treatment with vitamin E and selenium (Table 4) (Sklan şi Donoghue, 1992).

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# TESTING GALLIMUNE 201 IBD + REO INACTIVATED VACCINE EFFECTIVENESS AGAINST INFECTIOUS BURSAL DISEASE AND AVIAN REOVIROSIS FLU

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**Key words:** gallimune, vaccine, infectious bursal disease, avian reovirus flu

**SUMMARY**

World Organisation for Animal Health (OIE) introduced avian infectious bursal disease in List B of diseases, being the object of annual information by Word Animal Health because of the economic loss that it produces to the poultry farming through its immunosuppressive effects, as well as the morbidity and mortality.
Even if a bird survives the initial acute infection, many problems can occur. The harmed immune system is much less able to defense against infections with normal pathogen agents and is likely that the bird suffers or dies because of any of a number of secondary infections. Herds that have experienced disease are usually underweight, lack of uniformity and have a higher mortality from various causes.
The infectious bursal disease virus can not be controlled with antibiotics and is almost impossible to be removed from the shelter with disinfectants.
By the state of immune suppression that it creates, the infectious bursal disease virus cause a negative effect on the development of antibodies that appear after prophylactic vaccination, against Newcastle disease, and dramatic reduction of nonspecific resistance to the conditional pathogen germs. This condition is very common at broilers and in the first phase at the replacement young chicken. These immune-depression states are favored by larger fluctuations which occur in the quality and quantity of feed.
Reovirus infections in birds are frequently associated with increased mortality, viral arthritis / tenosynovitis and an overall decrease in performance, including profit growth in weight, poor feed conversion, unequal growth rates, confiscation at the slaughterhouse and a poor recovery, at the market, for the affected birds.
The product tested is an inactivated vaccine against: avian infectious bursal disease (VMJO strain) and avian reovirosis flu (S1133 strain).The product is recommended for active immunization of laying hens against infectious bursal disease and avian reovirosis flu.

The vaccine is administered in laying birds with 2-4 weeks before entering the laying. Vaccine dose is 0.3 ml of vaccine administered IM (thigh muscle or breast) or SC (at the bottom of the neck).

To obtain an optimal “booster” effect, the birds will be vaccinated initially with a live vaccine against infectious bursal disease and avian reovirosis flu.

Best results are obtained if vaccination with inactivated vaccine is made after at least 4 weeks after administration of a live vaccine. It will be vaccinate only the healthy birds. Before using, the vaccine is brought at a temperature of 15 – 25 $^0$C and before and during use, it is homogenize. The vaccine will use clean and sterile equipment.

No equipment will be used for vaccination with the composition of rubber because the excipient can affect all types of rubber.

Do not mix with other vaccines. It will be given with 2-4 weeks before entering the lay.

## 1. CLINICAL TESTING

Clinical testing of immunological products GALLIMUNE 201 IBD + REO, regarding the active immunization of poultry against infectious bursal disease and avian reovirosis flu was made to farm flock of 42,000 birds, young breeding, heavy breed, hybrid Ross 308, aged 18 weeks.

Material and method: The vaccine was used GALLIMUNE 201 IBD + Reo, batch: F38589, vials x1000 doses, validity: 03/11/2007. The vaccine was administered by intramuscular: 0.3 ml / bird.

## 2. MATERIAL AND METHOD

The vaccine used was GALLIMUNE 201 IBD + REO, batch: F38589, vials x1000 dose, validity: 03/11/2007. The vaccine was administered by intramuscular: 0.3 ml / bird. Were collected a total of 50 blood samples: before vaccination (T0), at 28 days (T1) and at 56 days (T2) after vaccination, to determine the immune status of birds before and after vaccination (tab 1).

The flock was daily clinically examined throughout the period (56 days), by the specialists of SC Avicola Buzau, Farm 1, Săhăteni.

***Tab 1.***

**The experimental flock has undergone following vaccination program**

| Nr. Crt. | Age of birds | Biological product given | Given way |
|---|---|---|---|
| 1 | 1 day | Vaccine against Marek disease | Subcutaneous |
| 2 | 5 days | Vaccine against avian coccidiose | Drinking water |
| 3 | 8 days | Vaccine against avian salmonelossis and avian reovirosis flu | Subcutaneous |
| 4 | 9 days | Vaccine against Newcastle Disease (I) + vaccine against infectious bronchitis (II) | Drinking water |

| | | | |
|---|---|---|---|
| 5 | 11 days | Vaccine against infectious bursal disease | Drinking water |
| 6 | 21 days | Vaccine against Newcastle disease (II) + vaccine against infectious bronchitis (II) | Drinking water |
| 7 | 42 days | Vaccine rhinotracheitis disease | Drinking water |
| 8 | 48 days | Vaccine against avian salmonelossis + avian reovirosis flu | Subcutaneous |
| 9 | 54 days | Vaccine against Newcastle Disease (III) + vaccine against infectious bronchitis (III) | Drinking water |
| 10 | 73 days | Vaccine against avian pox and avian encephalomielitis | Stick |
| 11 | 89 days | Vaccine against Newcastle Disease (IV) + vaccine against infectious bronchitis (IV) | Drinking water |
| 12 | 109 days | Vaccine against avian salmonelossis | Drinking water |

## 3. RESULTS

At the ends of the test were found following:

- The vaccine was well tolerated, without general reactions attributing to the product
- Mortality rate was within the technology limits.
- The flock did not develop infectious and contagious diseases or other conditions that undercurrent technology loss.

The immune response induced subsequent administration of GALLIMUNE 201 IBD + REO product was determined by the immune-enzyme test - ELISA, using kits of Hipra Laboratorios SA company: “CIVTEST AVI IBD”, batch: CBD.1W4X, validity: 04.2007 (component IBD) and “Reo CIVTEST AVI”, batch: CRE.1V9C, validity: 04.2007.

Serum samples were processed as kits instructions, and the reaction reading after the cessation, was achieved at $\lambda = 405$ nm. Interpretation of results was done according to kit instructions, that the ELISA titers of more than 357 EU (for IBD), i.e. 913 EU (for REO) indicating a positive reaction.

Were obtained the following antibodies titers average:

| Vaccinated batch with GALLIMUNE 201 IBD+REO | T0 – before vaccination | T1 – 28 days after vaccination | T2 – 56 days after vaccination |
|---|---|---|---|
| IBD Component | 4250 E.U. | 5506 E.U. | 5469 E.U. |
| REO Component | 4133 E.U. | 6025 E.U. | 5724 E.U. |

Before and after vaccination antibody titers subsequent immunological product management, against infectious bursal disease and avian reovirosis flu:"Gallimune IBD + REO" are given in figure 1 and 2.

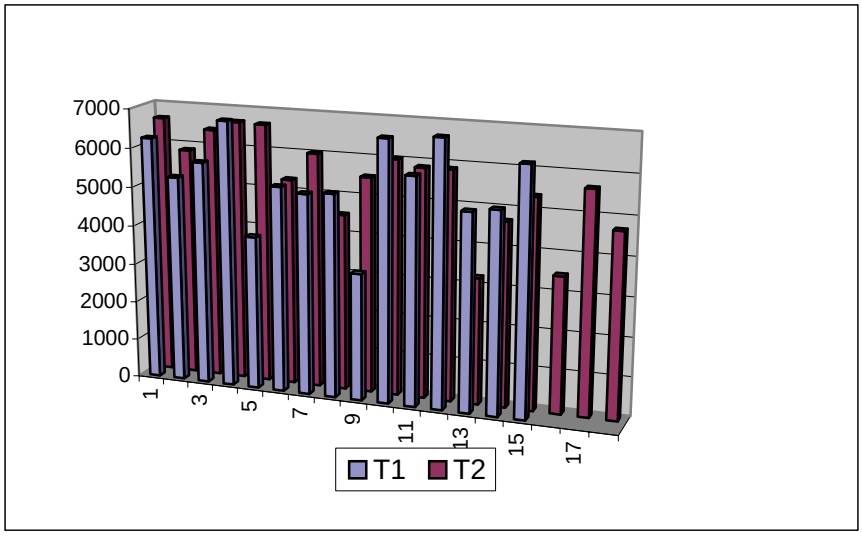


**Fig. 1. The immune response in 28 days, i.e. 56 days after vaccination with 201 IBD + REO Gallimune product, component IBD.**

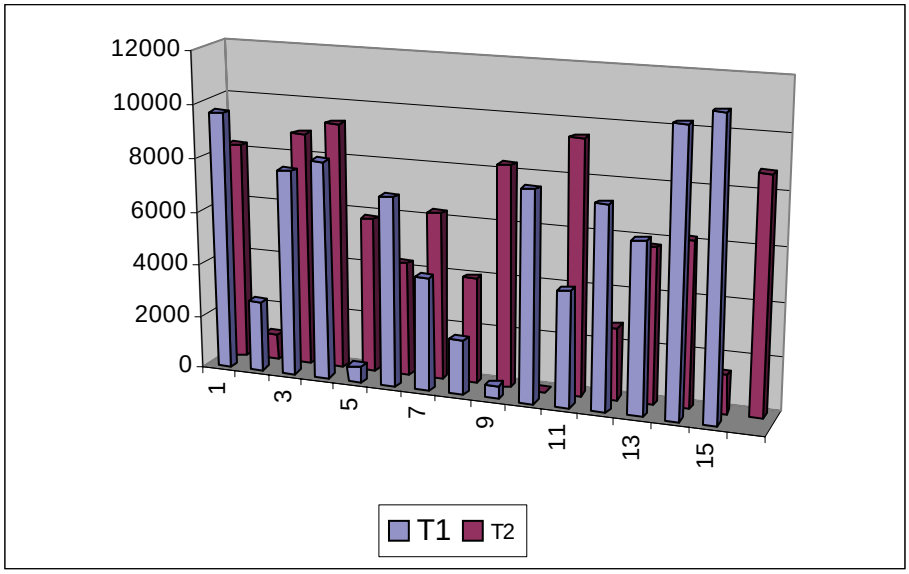


**Fig. 2. The immune response in 28 days, i.e. 56 days after vaccination with NDV + IB + EDS product, component REO.**

## 4. CONCLUSIONS

1. Immunological product Gallimune 201 IBD + REO has a good innocuity and local/or general reactions were not recorded;
2. The immune response induced by the vaccine was appropriate, registering higher values threshold of positivity (357 E.U. for IBD, respectively 913for Reo).
3. At 28 days after vaccination, the level of after vaccination antibodies was 5506 EU for IBD component and 6025 EU for REO component.
4. At the end of the monitoring period of the vaccineted batch (56 days), the antibody titer reached an average very high (IBD = 5469 EU, for REO=5724 EU).
5. During the testing, intercurrent illnesses were not recorded and the percentage of mortality was in the technological limits allowed for this category.

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