ANALELE
UNIVERSITATII
Spiru Haret

Seria Medicina Veterinara

Anul IX, nr.9
2008

Analele Universitatii Spiru Haret

Seria Medicina Veterinara

Anul X nr.10
2009

Redactia:
Bucuresti, str. Masina de Paine, nr. 47, sector 2, tel: 021.242.15.75

CLARIFICATIONS REGARDING THE TOPOGRAPHICAL LOCATION OF THE VASCULAR, LYMPHATIC AND NERVOUS FORMATIONS FROM THE THORAX APERTURE IN PIGS AND SHEEP

PRECIZĂRI PRIVIND SITUAȚIA TOPOGRAFICĂ A FORMAȚIUNILOR VASCULAR LIMFATICE ŞI NERVOASE DE LA NIVELUL APERTURII TORACALE LA PORC ŞI LA OAIE

BERGHES Carmen[1], **M. CUCOANEŞ** [1], **D. CUCA** [1], **C. COMAN** [2], **E. VLASE** [2]
[1]Faculty of Veterinary Medicine
Spiru Haret University
e-mail: carber63@yahoo.com
[2]Cantacuzino Institute

REZUMAT

Studiile întreprinse au rolul de a aduce unele precizări cu privire la situația topografică a formațiunilor vascular limfatice şi nervoase de la nivelul aperturii toracale la porc şi la oaie

Datele din literatura sunt puțin relevante deoarece surprind alte formațiuni de la nivelul mediastinului anterior fără să se facă o corelație între acestea.

Studiul a fost efectuat pe 20 cadavre provenite de la purcei din ferme de creştere care au prezentat în general afecțiuni digestive şi nu respiratorii pentru a nu fi afectata zona luată în studiu şi pe cadavre de oi folosite pentru disecția studenților. Formațiunile vasculare au fost injectate cu un amestec preparat în laboratorul de anatomie al disciplinei.

Lucrarea prezintă fotografic mai multe modele disecate stabilind cu cât mai multă precizie situația topografică a formațiunilor anatomice şi are un pronunțat caracter aplicativ în medicina umană, dacă se ține cont de faptul că specia prezinta cea mai mare asemanare cu morfologia umana.

Cuvinte cheie: *mediastin, conduct limfatic, vena cava craniala, ggl. cervical caudal*

ABSTRACT

The purpose of the studies is to contribute with some clarifications to the topographical location of the vascular, lymphatic and nervous formations from the thorax aperture in pigs and sheep.

The literature data is little relevant because they depict other formations from the anterior mediastinum, without making a correlation between them.

The study was conducted on 20 pig corpses from production farms, with digestive, not respiratory disorders in general, so as not to affect the studied area, and on sheep corpses used by students for dissection. The vascular formations were injected with a mixture prepared in the laboratory of anatomy.

The paper shows pictures from several dissections, determining as accurately as possible the topographical location of the anatomical formations, and it has a strong applicative character for human medicine, since the closest species to man as experimental morphological model is the pig.

Keywords: *mediastinum, lymphatic duct, cranial vena cava, caudal cervical ganglion*

INTRODUCTION

The fundamental research on the topography of the vascular nervous formations from the aperture of the thorax cavity in animals is approached by many researchers, but the data are presented separately, either for the vascular formations, or for the nervous formations, or for the lymphatic formations (1, 2, 4). These data are a real support to interpret he physiological phenomena and to clarify several aspects regarding the way of approaching the formations during surgery on the anterior mediastinum. The morphology of the species resembles that of the man, which recommends it as an experimental model, provided the European legislation of the experimental animals is observed (4, 5)

MATERIAL AND METHOD

The studies were conducted in the laboratory of anatomy of the Faculty of Veterinary Medicine, on 20 pig corpses from a production farm. Before dissecting, the aorta and the veins were injected with a mixture of substances prepared in the laboratory of anatomy. The nervous formations were treated with a solution of acetic acid 10%. The lymph formations were injected with methylene blue. The lymphatic anatomy of 5 pigs was studied and classified and a new technique for lymphatic cannulation was developed. The cannulation success rate was 55%.

RESULTS AND DISCUSSION

Formation anatomical approach is at chest level as having first milestone coast. It protects the right apical pleural recessive and dissect contained septal formations precardiac mediastinal. In relation to the first rib to show the skull mediastinal lymphonodes who are willing and medial to this axilar lymphonode of the first rib that is located in relation to the edge of the skull. Vegetative plexus is located between cervicotoracic formations located superficial venous and arterial located medial formations (fig.1).

Fig. 1 – mediastinal aperture approach

The right caudal cervical ganglion, joined in 15 animals with the thoracic paravertebral ganglion 1 and 2 forms a pericarional aglomeration located on the median face of the first rib in the dorsal side of the anterior mediastinum, being placed dorsally in relation to the long neck muscle, laterally in relation to the vertebral artery, ventrally in relation to the right subclavicular artery and on the right of the bicarotic trunk. In all studied cases we have identified the middle cervical ganglion which is attached to the caudal cervical ganglion through the subclavicular loop (fig.2).

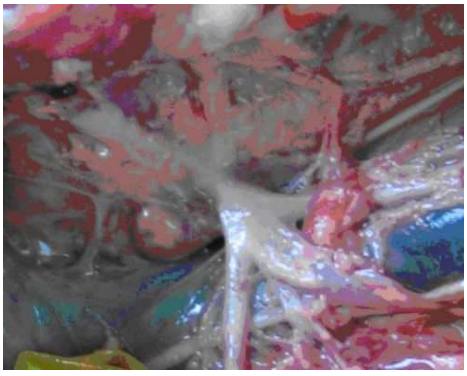


Fig. 2 – ggl. cervicotoracic

The right lymph duct passes at a distance of 2 cm ventrally from the cervical-thoracic plexus formed around the cervical-thoracic ganglia, running thereafter sideways vento-cranially, descending from the right side of the aorta towards the cranial vena cava into which it pours. Before pouring in the cranial vena cava, the duct displays a branching which, after passing the aorta-pulmonary ligament, joins again the main duct (fig. 3).

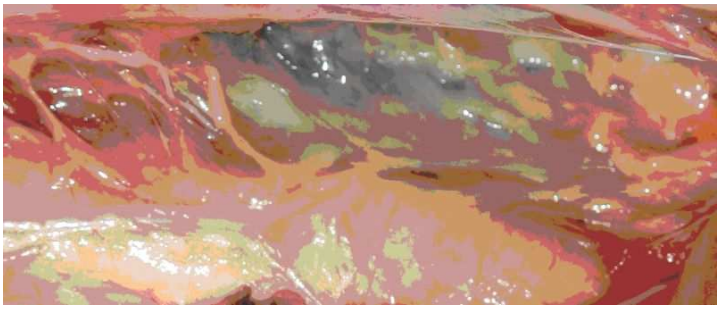


Fig. 3 – lymphatic duct

Cardiac lymph is the most direct medium for analyzing metabological changes in the myocardial cell. Currently, sheep are the animals used for investigation of myocardial lymphatic function. However, questions arise when comparing and interpreting the human system to the experimental model, since the sheep coronary anatomy is different from human anatomy and pulmonary lymph contamination is found in up to 81% of the cases. Swine, having similar coronary anatomy to humans, are a proven model for cardiovascular research. The purpose of this study was to investigate the cardiac lymphatic anatomy of the swine and to develop a reliable cannulation technique to collect the lymph (fig.4).

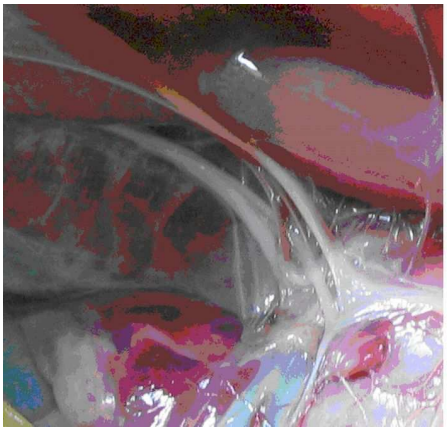


Fig. 3 – lymphatic duct cannulated

Conclusion: We conclude that porcine myocardial lymphatics can be successfully cannulated for the investigation of myocardial lymphatic function.

REFERENCES

1. Jaime F. Vazquez-Jimenez, Marie-Christine Seghaye Ma QingOliver J. Liakopoulos Marcia L. Rosenbaumand Bruno J. Messmer. Thoracic Cardiovascular Surgery, Universitätsklinikum RWTH Aachen, Pauwelsstrasse 30, 52057 Aachen, Germany

2. Braune S (2001) The role of cardiac metaiodobenzylguanidine uptake in the differential diagnosis of parkinsonian syndromes. Clin Auton Res 11:351–355

3. **Braune S, Reinhardt M, Schnitzer R, Riedel A, Lücking CH (1999)** Cardiac uptake of [^{123}I]MIBG separates Parkinson's disease from multiple system atrophy. Neurology 53:1020–1025

4. Daniel SE (1999) The neuropathology and neurochemistry of multiple system atrophy. In: Mathias CJ, Bannister SR (eds) Autonomic failure. Oxford University Press, Oxford p 321–328

ACTIVITY OF LOW-LEVEL LASER THERAPY (LLLT) ON SEPTIC WOUNDS INDUCED EXPERIMENTALLY IN RABBITS

ACTIVITATEA RADIAȚIILOR LASER DE MICĂ PUTERE (L.L.L.T.) ASUPRA PLĂGILOR SEPTICE PRODUSE EXPERIMENTAL LA IEPURI

COMAN T[1]., Gh. PÂRVU [1], N. BERCARU [1], A. MIHAI [2], T. P ETRUȚ [1], P. GRIGORESCU [1], A . SALLAY [1]

[1]Faculty of Veterinary Medicine
Spiru Haret University
e-mail: ushmv_coman.toma@spiruharet.ro
[2]DSVSA Ilfov

REZUMAT

Un număr de 15 iepuri au fost operați prin laparotomie, iar plăgile au fost infectate experimental cu o suspensie de 24 ore de Staphylococcus aureus, tulpină izolată și caracterizată la FMV, USH. După contaminare cu 0,5 ml suspensie bacteriană/plagă, plăgile au fost suturate. Un număr de 3 iepuri au constituit lotul martor neoperat.

Timp de 9 zile, un număr de 10 iepuri au fost supuși zilnic, începând din prima zi post operator, tratamentului cu radiații laser de mică intensitate (L.L.L.T.) la lungimea de undă de 635 nm, cu o sondă de emisie continuă la puterea de 15mW, timp de iradiere de 600 secunde, la distanța de 0,5 cm. Un număr de 5 iepuri au constituit lotul martor netratat. În perioada de observație clinică, iepurii au fost supuși examenului hematologic, biochimic și morfologic al sângelui, examenului histologic prin probe biopsice la sfârșitul experimentului și examenul parametrilor urinari.

După 9 zile de tratament cu L.L.L.T. iepurii au prezentat plăgi vindecate în proporție de 85% fără a folosi o medicație locală sau generală antiinfecțioasă. Lotul tratat a prezentat procese de cicatrizare a plăgilor aproape încheiate, dar examenul histologic a evidențiat că în profunzime țesutul conjunctiv de granulație era infiltrat cu limfocite și polimorfonucleare neutrofile. Lotul martor netratat a prezentat după 10 zile plăgi supurate, cu abcese profunde și fistule iar un animal a murit prin septicemie. Parametrii hematologici, biochimici sangvini, biochimici urinari nu au prezentat modificări față de lotul martor neoperat, cu excepția evoluției temperaturii în primele zile post-operatorii. Examenul morfologic al sângelui a evidențiat o puternică trombocitoză și monocitoză la lotul tratat și o accentuată neutrofilie la lotul netratat. Lotul tratat cu laser a prezentat, un nivel crescut al fosfatazei alcaline în perioada de tratament.

Cuvinte cheie: *L.L.L.T., iepuri, plăgi septice infectate experimental*

ABSTRACT

A number of 15 rabbits were operated by laparotomy and the wounds were infected experimentally with a 24h suspension of *Staphylococcus aureus*, strain isolated and characterized by FMV, USH. A group of 3 rabbits formed the unoperated, control group.

A number of 10 rabbits were treated daily for 9 days, starting with the first day after surgery, by low-level laser therapy (LLLT), on a wavelength of 635 nm, with a 15 mW power probe with continuous emission, irradiation time 600 seconds, from a distance of 0.5 cm. A number of 5 rabbits formed the untreated, control group. During the period of clinical observation, the rabbits underwent a haematological, biochemical and morphologic examination of the blood, a histological examination through biopsy samples at the end of the experiment and the examination of the urinary parameters.

After 9 days of treatment with LLLT, 85% of the rabbits displayed healed wounds. The histological examination showed that the wound healed superficially and that deep inside, the conjunctive granulation tissue was infiltrated with lymphocytes and polymorphonuclear neutrophils.

The untreated, control group displayed after 10 days festering wounds, with deeps abscesses and fistulae, and one animal died of sepsis. The haematological, blood biochemical, urine biochemical parameters showed no differences from the unoperated, control group, except for the thrombocytosis observed in the LLLT group.

The morphological examination of the blood revealed a strong monocytosis in the treated group during the final days of the treatment and neutrophilia in the untreated group. The group treated with laser displayed a high level of the alkaline phosphatase.

Keywords: LLLT, septic wounds, rabbits

INTRODUCTION

Low-level laser therapy is used in the human medicine and less in the veterinary medicine as non-polluting, uninvasive and atoxic therapy (3).

Low-level laser therapy is based on the photostimulating effect of the radiations with wavelength of 630 – 904 nm, emitted continuously or discontinuously, on the superficial tissues. Within the interval of 630 – 904 nm, the effects of the laser radiations are anti-inflammatory, of regeneration of the blood capillaries, alleviating oedema, stimulating collagen synthesis and also analgesic effects (4).

Alena R.A.P. et al. (2003), have shown that wound healing in the rats is stimulated by LLLT at an intensity of 4.0 J/cm^2, consecutively with the reduction of the local inflammatory processes, the quantitative increase of the collagen and myofibroblast multiplication (1). At higher intensities, the effects have been inhibitory. This explains partially some failures following the low-level laser therapy, by the lack of precise therapy protocols, determined by experiments, in order to produce the expected photostimulating effects.

The *in vitro* studies conducted on fibroblasts have revealed the role of mitochondria in the release of ATP to accelerate collagen synthesis metabolism and to stimulate the macrophages for the synthesis of the replication factors and for cell differentiation, for the stimulation of interleukin and interferon synthesis (2, 6, 9, 10).

The statistical study performed on 36 works done with LLLT in the field of traumatology, regarding the stimulation of wounds healing, has shown that 22 cases (67%) displayed positive results (5). This proves once more the necessity to determine the irradiation parameters (wavelength, energy density applied on the place of irradiation, the time of irradiation, the power density, frequency, etc.) which must be used constantly according to the treated affection and to the biological substrate which must be treated.

In the veterinary medicine, the surgical wounds contaminated with bacteria are frequently met in practice. The contamination can be the consequence of accidents or it may appear, as exceptions, post-surgery.

The healing of these wounds is mixed and it takes a long time, involving the surgical drainage of the fistula and abscesses that form, the local and general treatment with antibiotics, or chemotherapy, etc.

The healing is characterised by the stage of self-cleaning, when the inflammatory processes in the wound are acute, marked by infiltration with macrophages or polymorphonuclear neutrophils. The process ends with the formation of the granulation tissues and wound closing by healing. Healing usually is vicious. Petersen S.R. et al., using LLLT to treat aseptic wounds in horses only obtained results after a long-period treatment (6).

Studies conducted on the effect of LLLT in the treatment of aseptic wounds in rabbits have shown the stimulating action on the wounds *per primam intentionem,* a lower duration of the therapy and lower costs of the therapy (5).

The purpose of the paper was to determine a protocol for low-level laser therapy for the treatment of wound infected experimentally in rabbits (septic wounds), monitoring the haematological, biochemical, serologic, histopathological, urinary parameters for each individual case.

MATERIAL AND METHOD

Laser equipment. The laser used consisted of a command module and two laser probes: an impulse emitting probe (wavelength 830 nm and 30 mW power) and a multiple continuous wave emitting probe (wavelength 635 nm and 15 mW power).

Irradiation protocol: it was set based on the experiments on soft tissues aseptic wounds treatment in rabbits (5), observing the following parameters:

- star-shaped multiple probe with wavelength 635 nm
- continuous emission
- power 15 mW
- distance to the wound 0.5 cm
- frequency of treatment: daily
- period of irradiation: 600 seconds
- duration of treatment: 9 days.

Methods of evaluation: all animals were monitored for a period of 10 days, period in which besides the clinical examination and body temperature evaluation, the haematological, morphological and serological parameters were also evaluated as controls for possible changes of the parameters; biopsy histological examinations were performed in the end of the experiment.

The haematological examination was conducted by recording the erythrocytar parameters (total count, erythrocytar volume, CHEM, HEM, etc.) the total count of thrombocytes and leukocytes. The examinations were performed on a haematological analyzer ACT-5-DIF.

The morphological examination of the blood was performed by smears stained with May Grümvald Giemsa and by evaluating the leukocytar formula.

The biochemical examination of the blood was performed be investigating the following blood parameters: glucose, azotemia, transaminases, gammaglutamyltransferase, alkaline phosphatase, GPT and GOT.

The examination of urine parameters monitored the following parameters: density, glycosuria, pH, proteinuria, ketone bodies, bilirubin, haematuria, leukocituria, urobilinogenuria.

The histological examination was done by biopsy samples, fixed in alcohol-formalin, saline neutrally formalin and Bouin fixative. The samples were embedded in paraffin and 6 micron slices have been cut. The sliced samples were stained with Mallory's trichromic technique, HE, May Grümwald Giemsa on blade, alcian blue 0.2%.

Experimental model: 18 clinically healthy Large Belgian rabbits were selected, weighing 1.5 – 2 kg. Three rabbits, the control group, no surgical treatment, were reared in identical conditions.

A number of 15 rabbits were incised under narcosis in parallel with the median line on the sides of the flank, on a distance of 7 cm. The anatomic parts of interest were the skin, the subcutaneous conjunctive tissue and the skin muscles. Each animal received 0.5 ml of *Staphylococcus aureus* strain applied on the entire area of the wound. Betadine applications were done every day on the entire wounded area. No local or general antibiotics were used.

Ten rabbits were treated with laser, according to the selected parameters, starting with the first day after surgery, while 5 animals were not treated with laser.

The animals were monitored on a daily basis.

RESULTS AND DISCUSSION

Clinical examination. During the first 48 hours after surgery, the animals displayed a slight lack of appetite and polydipsia, accompanied by hyperthermia in the control group (Table 1).

Table 1

Average body temperature. Septic surgical wounds

Groups	Days in treatment								
	1	2	3	4	5	6	7	8	9
1	38.7	39.3	38.9	38.6	38.9	38.8	38.8	38.6	38.5

2	**39.2**	**39.5**	**39.4**	**39.2**	**39.1**	**39.4**	**39.3**	**39.2**	**39.5**

The laser treated group displayed a slight 48 hours after surgery, while hyperthermia was constant in the control group even on day 9 post surgery.

The irradiated septic wounds didn't fester after 7 days of therapy and the local oedema remitte (Fig. 1). On day 9 of therapy, the wounds display de suture lines but are partially healed. The resulting scar is tough at palpation and with no mobility.

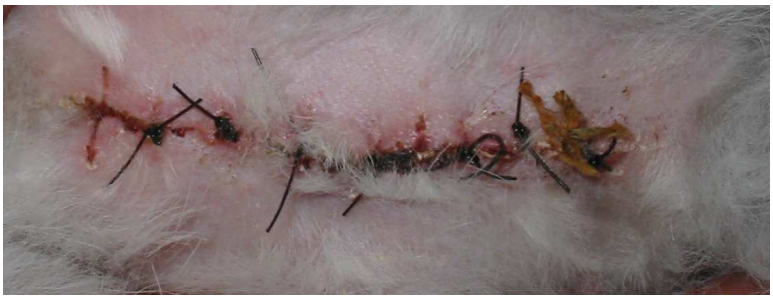


Fig. 1 – Septic surgical wound on day seven of irradiation

The control group healed slowly. On day 7 post surgery, the wounds were still festering and there were local oedema visible in the incision area, together with the suppuration of puss. Healing occurred partially at some extremities of the wound, 14 days after surgery, and on day 8 one rabbit died. The necropsy revealed sepsis wounds.

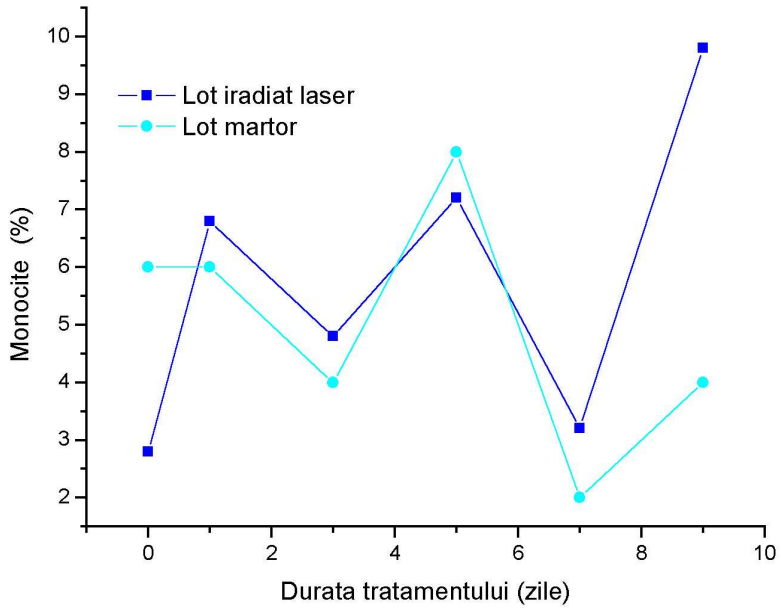
Haematological examination. The main erythrocytar indices are within the normal limits in the laser treated group (Table 2).

Table 2

Average values of the main haematological parameters. Septic surgical wounds

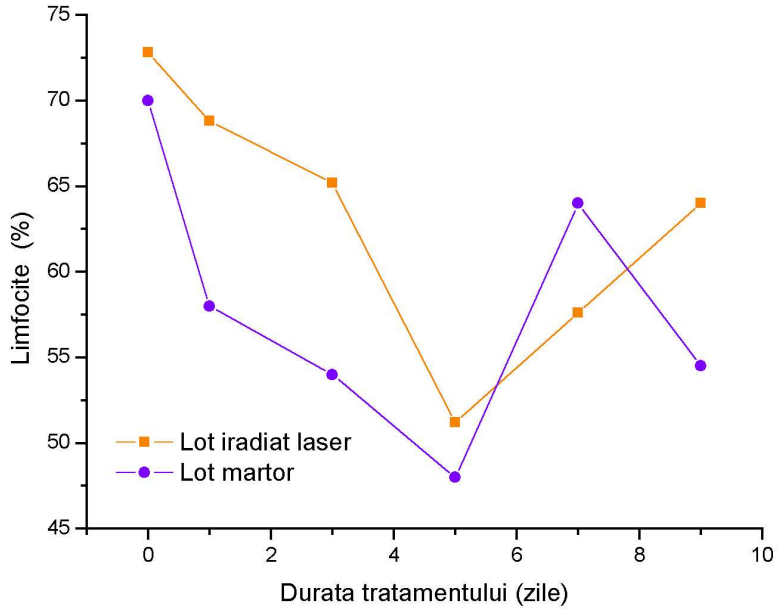
	Erythrocytes		**Haemoglobin**		**Average erythrocytar volume**		**Average erythrocytar haemoglobin**		**Concentration of average erythrocytar haemoglobin**		**Hematocrit**		**Leukocytes**		**Thrombocytes**	
Normal values	**5x10^6/µl**		**12.2 gr./dl**		**59/µ^3**		**18pg/E**		**33 g/dl**		**37 – 41 %**		**8x10^3/µl**		**120 – 500 x 10^3/µl**	
Days of therapy	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 1**	**Group 2**
0	5.57	5.40	12.92	12	67	68	23.17	23.8	34.57	33.5	37.37	35.70	9.6	10.1	881.2	206
1	5.39	5.33	11.5	10.25	65.8	71	21.4	23.2	32.46	32.7	35.48	37.95	10.5	10.3	958.7	198
3	4.77	5.49	10.46	12.7	65.6	70	21.98	23.1	33.62	32.9	31.14	38.55	12.2	9.1	1418.7	751.5
7	5.05	5.67	11.4	12.4	67.2	67	22.58	21.9	33.66	32.6	33.9	38.05	9.98	9.8	508.8	908
9	4.67	6.17	10.5	13.87	68	71	22.52	22.4	33.8	31.6	31.12	43.75	9.82	10.2	384.5	627

The thrombocytes and leukocytes counts in the treated group increased significantly. The average leukocyte count was increased both in the treated group and in the control group. The leukocytosis observed in both groups was determined by monocytosis (Graph 1).



Graph 1 – Dynamics of monocytes in the groups of rabbits with septic surgical wounds

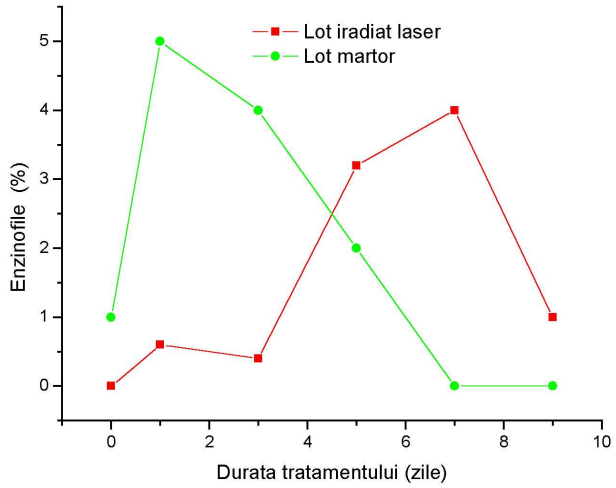
During the first 18 hours post treatment, a state of lymphocytosis was observed (Graph 2).



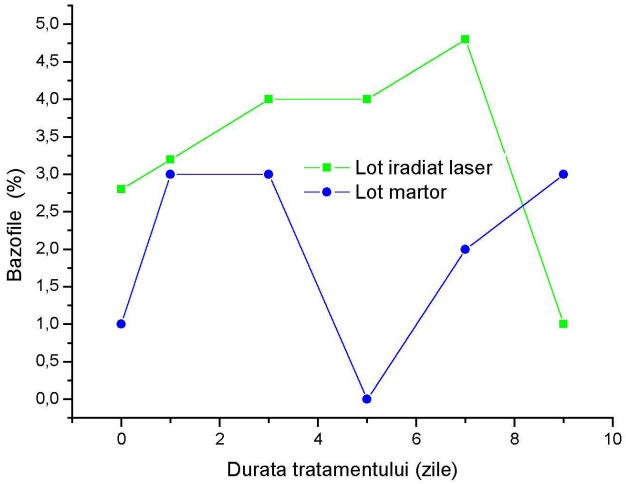
Graph 2 - Dynamics of lymphocytes in the groups of rabbits with septic surgical wounds

The increase of the lymphocytes was determined by the start of the immune processes; it is an established fact that the laser therapy stimulates the installation of the immune processes of

defence, including the increase of macrophages and neutrophils. Monocytosis was high in the treated group during the late period of treatment, a process which characterises healing. The monocytes that get to the tissues are transformed into macrophages, cells involved in the processes of phagocytosis and of transformation in antigen bearing cells. The process of eosinophilia and basophilia observed during late healing (Graphs 3, 4) presumes the participation of eosinophils and basophils by the elaboration of leukotriene, as well as the shaping of the inflammatory processes through the biogenic amines which the basophils elaborate.



Graph 3 - Dynamics of eosinophils in the groups of animals with septic wounds



Graph 4 - Dynamics of basophils in the groups of animals with septic wounds

Because of the high content of histamines which the basophils synthesise, they participate in the processes of tissue regeneration and healing by the synthesis of leukotriene and prostaglandins, products which speed up the local processes of extravasation and diapedesis of the derma capillaries.

In the control group, leukocytosis is accomplished through neutrophylia, when the values exceed the normal limits during the last 5 days of monitoring. The high values of the neutrophils suggest that the inflammatory processes are in full swing in the control group, while in the treated group monocytosis is predominant, these cells being involved in the processes of regeneration. This

shows that laser therapy stimulated the processes of tissue regeneration, compared to the control group, as also documented by the macroscopic evolution of the wounds.

The **histological examination** revealed in the treated group the existence of a mixed process of healing, consisting in the alternation of healing processes which ended after 9 days of treatment, with the existence of processes of lymphocyte and macrophage infiltration deep in the derma (Fig. 2).

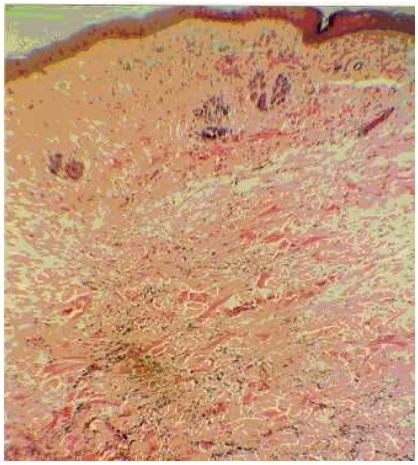


Fig. 2 – Septic surgical wound on day 12 post-surgery and day 9 of irradiation; H.E. staining technique: Ob. 10x

In the case of the mixed processes of healing, epithelisation runs deficiently and displays a strong acanthosis (Fig. 3).

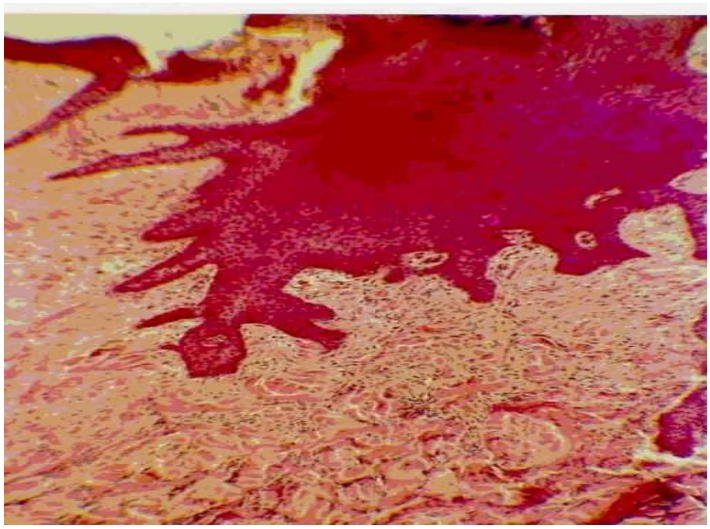


Fig. 3 – Acanthosis. Surgical septic wound – irradiated group. Deep on the derma processes of lymphohistocytar infiltration persist, as well as necrotic processes. H.E. staining technique; Ob. 10x

Even though in the superficial epidermal and dermal area the healing processes seem to be concluded, in the deep derma, inflammatory processes continue, characterised by lymphohistocytar and neutrophils infiltrations.

In the control group not treated with laser, after 9 days of treatment we observed both at the surface of the wound, which was in full process of removing the necrosed tissue, and in deep tissue the formation of micro abscesses (Fig. 4).

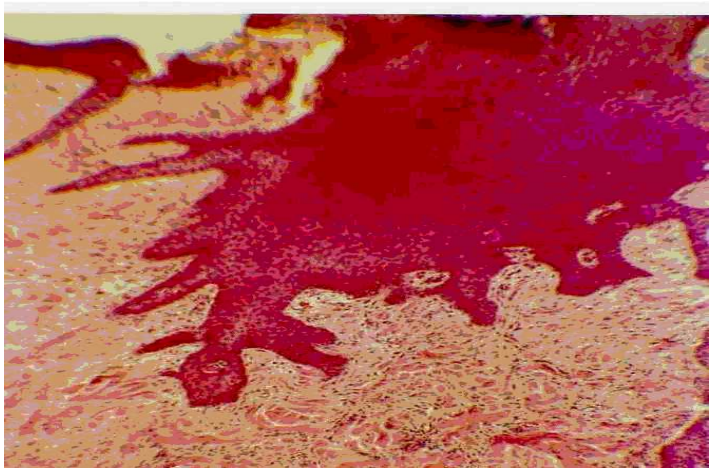


Fig. 4 - Surgical septic wound – control group. Micro abscesses in the derma, 9 days after surgery.
May – Grümvald – Giemsa staining technique; Ob. 20x.

Haemorrhagic areas, oedema and necrosis were observed in the area of the reticular derma and in the basal layer of keratinocytes (Fig. 5).

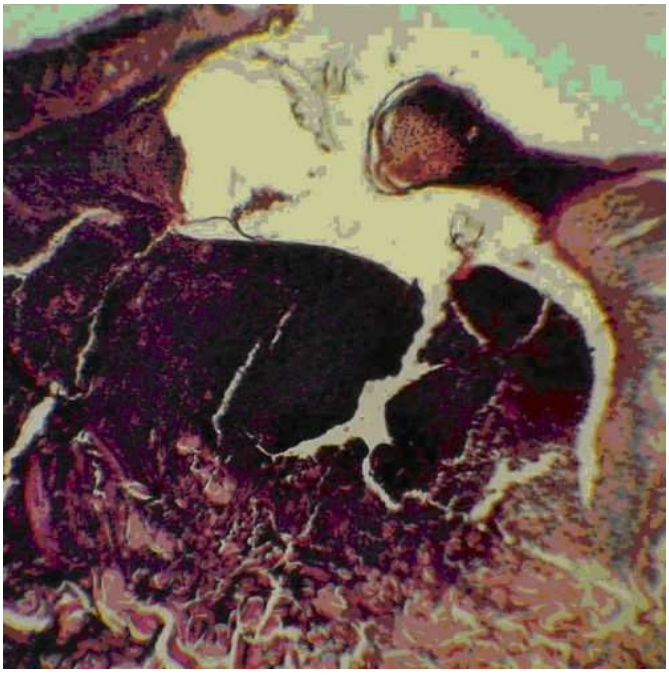


Fig. 5 - Surgical septic wound – control group. Necrosis in the derma, 9 days after surgery.
H.E. staining technique; ob. 10x

Biochemical examination of the blood; the vales range within the normal limits both in the treated group and in the control group (Table 3), except for the alkaline phosphatase, which is increased..

Table 3

Average values of the serum biochemical parameters. Septic surgical wounds

Parameters	Normal values	Days in treatment									
		0		1		3		7		9	
		Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Glucose (mg/dl)	112-160	83	93.95	118.4	68.2	136	75.6	67.85	70.4	117.8	143.5
Urea (mg/dl)	‹ 54	50.98	54.7	24.64	30.5	22.34	47.3	36.95	32.35	46.62	35.2
Alkaline phosphatase (mg/dl)	34-110	189.95	96.2	344.4	198.2	424.4	60.75	163.25	317.5	161.5	183
AST (GOT)	76-129	81	179	41.84	47.6	47.6	24.14	31.8	35.4	127	76.5
ALT (GPT)	75-119	29.95	43.9	55.4	49	49	45.7	40.87	75.05	71.9	70.05
ΓGT	0.15-18	5	5	14.94	18.4	18.4	18.3	8.25	15.05	7.21	20

We consider that the constantly higher level of the alkaline phosphatase was determined by the process of accelerated phagocytosis observed in the septic wounds and due to the build up of cell detritus and to the necrotic process started by the bacteria. The bacteriological examination performed after 7 days of laser treatment, using cotton swabs to collect material from the wound surface, showed a lower number of Gram-positive germs compared to the control group.

The **biochemical examination of the urine** didn't show changes of the urine parameters in the two groups.

After 9 days of laser therapy on the septic wound produced experimentally, we consider that phototherapy stimulated the healing processes and tissue regeneration at skin surface without, however, solving the deep inflammatory processes; the necrotic processes continued in deep derma, which may cause micro abscesses and fistula.

We consider that the process of superficial stimulation of tissue regeneration was determined by the limits of action of the radiation with a wavelength of 630 nm, which is limited to a few cm in deep, which could not determine in the short time of therapy the deep sterilization of the tissues.

CONCLUSIONS

1. The laser treatment of the septic wounds contaminated experimentally with the mentioned germs, for 9 days, stimulated the processes of superficial healing of the wounds.

2. Deep in the derma, inflammatory and lymphohistocytar infiltrative processes persisted, evolving towards micro abscesses.

3. The quantitative haematological examination and the leukogram showed a strong leukocytosis in both groups during the first post-operatory days, leukocytosis determined by the lymphocytosis in the LLLT treated group, followed by monocytosis, which proved the beneficial, not noxious, action of the laser radiations.

4. The serum biochemical parameters ranged within the normal limits in both groups, including in the control group which didn't have surgery, except for the alkaline phosphatase which was increased throughout the monitoring period in both operated groups; this supports the non toxic action of the treatment.

5. Regarding the septic wounds, we recommend the use of laser therapy after the application of local and general therapies, function of the antibiogram, followed by the stimulation if tissue regeneration and healing by laser therapy.

REFERENCES

1. Alena R.A.P., Livia Medrado, S. Pugliese, Sivia Regina, Z.A. Andrade, 2003, *Influence of Low Laser Therapy on Wound Healing its Biological Action upon Myofibroblasts*, Laser Surg. Med., 32, pg. 239 – 244.

2. Bolton P., S. Young, Mary Dyson, 1991, *Macrophage Responsiveness to Light Therapy with Varing Power and Energy Densities*, Laser Therapy, 3, pp. 105 – 111.

3. Coman T., T. Petruț, Adriana Pădure, Mihaela Antonina Călin, Nicoleta Herescu, Iuliana Gruia, 2004, *Terapia cu lasere de mică putere în practica medical-veterinară*, Analele Universității Spiru Haret, Seria Medicină Veterinară, Anul 5, pg. 103 – 113.

4. Coman T., T. Petruț, Adriana Pădure, Mihaela Antonina Călin, Nicoleta Herescu, 2004, *Mecanisme de acțiune ale aparatelor laser de mică putere asupra țesuturilor biologice*, Analele Universității Spiru Haret, Seria Medicină Veterinară, Anul 5, pg. 95 – 102.

5. Coman T., Al. Mihai, N. Bercaru, P. Grigorescu, T. Petruț, Adriana Pădure, Mihaela Antonina Călin, 2006, *Fototerapia laser în plăgile aseptice produse experimental la iepure*, Analele Universității Spiru Haret, Seria Medicină Veterinară, Anul 6-7, pg. 147 – 164.

6. Lubart R., H. Friedmann, L. Peled, N. Grossman, 1993, *Light Effect of Fibroblast Proliferation*, Laser Therapy, 5, pp. 55 – 57.

7. Lucas C., L. j. Criens-Publow, C.T. Cockrelli, R.J. de Haan, 2002, *Wound Healing in Cell Studies and Animal Model Experiments by Low Laser Therapy were Clinical Studies Justified A Asystematic Review*, Laser in Surgery and Medicine, 17 (2), pg. 110 – 134.

8. Petersen S.L., C. Botes, A. Olivier, A.J. Guthrie, 1999, *The Effect of LLLT on Wound Healing in Horses*, Equine Veterinary Journal, 31 (3), pp. 228 – 331.

9. Rigau J., C.H. Sun, M – A Trelles, M.W. Berns, 1996, *Effects of the 633 nm Laser on the Morphology of Primary Fibroblast Culture.* Procc. SPITE, vol. 2630, pp. 38 – 42.

10. Young S.R., M Dyson, P Bolton, 1991, *Efect of Light on Calcium uptake by Macrophages*, Laser Therapy, 3, pp. 55 – 41.

BACTERICIDAL AND ANTIFUNGAL ACTIVITY OF CATIOROM, PRODUCT PREPARED FROM QUATERNARY AMINES
ACTIVITATEA BACTERICIDĂ ŞI ANTIFUNGICĂ A PRODUSULUI CATIOROM

ŢOGOE I. [1], T. COMAN [2], C. CHIURCIU [3], Viorica CHIURCIU [3], P. ŞTIUBE [3]
[1] USAMV Bucharest
[2] Faculty of Veterinary Medicine
Spiru Haret University
e-mail: ushmv_coman.toma@spiruharet.ro
[3]Romvac S.A.

REZUMAT

A fost studiată activitatea bactericidă şi antimicotică, „in vitro" şi „in vivo" a soluţiei de 1% de Catiorom, biocid, pe bază de aminequaternare.

Testarea „in vitro" s-a efectuat faţă de un număr de 14 tulpini bacteriene şi de 4 tulpini de miceţi, izolate şi tipizate, din colecţia A.T.C.C. şi FMV. Tulpinile, cultură proaspătă de 24 ore, au fost puse în contact cu soluţia 1% Catiorom în raport de 1⁄10 şi lăsate timp de 10, 30 şi 60 minute. După acest interval fiecare soluţie a fost reînsămânţată pe mediu cu bulion şi agar nutritiv, bulion şi agar VF şi Sabouraud solid.

Activitatea antibacteriană şi antimicotică înregistrată după 7 zile şi respectiv 14 zile de la reînsămânţare a fost de 100% după un contact de 10 minute faţă de tulpinile: Enterococus fecalis, Enterococus faecium, Bacillus anthracis, Listeria monocitogenes, Erysipelatrix insidiosa, Escherichia coli 0 157:H7, Escherichia coli var. Bruxelles, Salmonella enteritidis, Salmonella typhimurium, Aspergilus niger, Penicilium glaucum. După 30 minute de contact, activitatea bactericidă şi antimicotică a fost de 100% şi faţă de Staphylococus aureus var Oxford, Staphylococcus intermedius, Baccilus cereus, Clostridium perfringens, Pseudomonas aeruginosa, Candida albicaus, Malassesia pachydermatis.

„In vivo", activitatea antibacteriană şi antimicotică a fost testată după decontaminarea cu soluţia 1% Catiorom, a unor spaţii de creştere şi de spitalizare a animalelor. Probele de sanitaţie recoltate la 2 ore şi, respectiv, 24 ore de la decontaminare au fost negative, NTG fiind sub 10 germeni ⁄ ml. În mediile însămânţate nu au fost evidenţiate bacterii coliforme sau streptococi.

Cuvinte cheie: *amine quaternare, activitate bactericidă şi antimicotică, catiorom – biocid*

ABSTRACT

The *in vivo* and *in vitro* bactericidal and antimycotic activity of 1% Catiorom solution, biocide based on quaternary amines was studied. Its efficacy for the decontamination of animal rearing and hospitalization areas has also been investigated.

The *in vitro* testing was conducted on 14 bacterial strains and 4 mycetes strains, isolated and typified, from FMV Bucharest collection.

The strains, fresh 18-24h culture, were brought into contact with the 1% Catiorom solution, in a 1/10 ratio, and left so for 10, 30 and 60 minutes. After contact, each solution was reseeded on tomato broth and nutritive agar, tomato broth and VF agar and solid Sabourand mediums.

The antibacterial and antimycotic activity recorded after 7 and 14 days from seeding on nutritive mediums and incubation at 37^{o}C and at 25^{o}C was 100% after 10 minutes of contact, against *Enterococus fecalis*, *Enterococus faecium*, *Bacillus anthracis*, *Listeria monocitogenes*, *Erysipelatrix insidiosa*, *Escherichia coli 0 157:H7*, *Escherichia coli var. Bruxelles*, *Salmonella enteritidis* var *Oxford*, *Salmonella typhimurium*, *Aspergilus niger*, *Penicilium glaucum*. After 30 minutes of contact the bactericidal and antimycotic activity was 100% against *Staphylococus aureus* var *Oxford*, *Staphylococcus intermedius*, *Baccilus cereum*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Candida albicans*, *Malassesia pachydermatis* too.

The antibacterial and antimycotic activity of the quaternary amines within the 1% Catiorom solution was tested by the decontamination and disinfection of areas used for animal rearing and hospitalisation. The samples collected 2 and 24 hours after decontamination were negative, TGC being below 10 germs/ml. The seeded mediums were free of coliform bacteria or streptococci.

Keywords: quaternary amines, bactericidal and antimycotic activity, Catiorom biocide

INTRODUCTION

The quaternary amines have been used in medicine starting since 1935 as antiseptic substances due to their bactericidal, algicide and antifungal properties (6). Due to the substitution of the organic radicals with 4 hydrogen atoms, the quaternary amines acquire important biological and physico-chemical properties, among which a very good solubility, electrolytic and emulsification power. Due to their properties, the quaternary amines are used as antiseptics, detergents, disinfectants, sanitation agents (6).

The bactericidal and bacteriostatic activity was studied in general, showing that the quaternary amines have bactericidal activity against Gram-positive germs, while their activity against the Gram-negative germs is less powerful (5). The quaternary amines have been used as disinfecting substances due to their low toxicity on the tegument and due to their lack of noxiousness against the environment (4). Due to their bactericidal, algicide and antimycotic activity, the quaternary amines are used in the human medicine as antiseptic substances for the disinfection of the surgery instruments and equipment or as disinfectants for the surgery rooms. The mechanism of action on the bacteria has yet to be elucidated.

The quaternary amines are supposed to act on the membrane of the microorganisms preventing the accomplishment of the metabolic processes by the inactivation of the enzymes from the structure of the cell membrane.

Catiorom is a commercial preparation produced on the basis of quaternary amines. The active substance is alkyl-dimethyl-benzyl-ammonium chloride. The active substance is used to disinfect the vehicles used for animal transportation, for the disinfection and decontamination of the animal farms, for the decontamination of the flooring from the food industry units, in meat processing units, slaughter houses, etc.

The virucide activity of the active substances has been proved by the inactivation of the Newcastle disease virus, avian infectious bursitis virus, avian influenza virus and avian infectious bronchitis virus after 30 minutes of contact (2).

The purpose of the paper is to show the *in vitro* bactericidal and antifungal activity of the alkyl-dimethyl-benzyl-ammonium chloride from the composition of 1% Catiorom solution, as well as the result of its action for the decontamination of areas used for animal rearing and exploitation.

MATERIAL AND METHODS

Strains. Fourteen Gram-positive and Gram-negative bacterial strains and four fungi strains have been selected, from FMV-USAMV Bucharest collection, as follows:

Bacteria

1. *Bacillus anthracis*
2. *Bacillus cereus*
3. *Clostridium perfringes – biotip A*
4. *Escherichia coli var Bruxelles*
5. *Escherichia coli 0 175: H_7*
6. *Enterococcus faecalis*
7. *Enterococcus faecium*
8. *Erysipelotrix insidiosa*
9. *Listeria monocytogenes*
10. *Pseudomonas aeruginosa*
11. *Salmonella tyhphimurium*
12. *Salmonella enteritidis var Oxford*
13. *Staphylococcus aureus var Oxford*
14. *Staphylococcus intermedius*

Fungi

1. *Aspergillus niger*
2. *Candida albicans*
3. *Malassezia pachidernatis*
4. *Penicilinium glaucum*

Culture medium. The following culture mediums have been used to test *in vitro* and *in vivo* the bactericidal activity:

- Tomato broth and nutritive agar;
- Tomato broth and VF agar;
- Sabouraud agar.

Test water. The *in vitro* fungicide and bactericidal activity was tested on hard water with 300 mg calcium/l, according to standard *SR EN 14675*.

Catiorom is a disinfecting solution with 1% alkyl-dimethyl-benzyl-ammonium chloride and contains 2% isopropyl alcohol as emulsifying agent. The product is recommended for use diluted 1%. At this dilution the product is not toxic, doesn't endanger life and work security, or the environment. The product is recommended for the prophylactic and required disinfection of the areas from animal houses, poultry farms, slaughterhouses, small slaughter units, meat processing units, dairy factories, sanitary facilities, vehicles transporting animals.

Experimental model

a) "*in vitro" testing*. The bactericidal and antifungal activity of the product was evaluated on fresh, 18-24h, cultures of the strains mentioned earlier. The bacterial and fungal suspensions were put into contact with the solution of 1% Catiorom in a ratio of 10/1 (9 parts Catiorom and 1 part bacterial or fungal suspension).

After a contact of 10, 30 and 60 minutes, each mixture (Catiorom and the bacterial or fungal suspension) were seeded with 0.1 ml in specific growth mediums, i.e., in plates with Sabourand agar, and incubated at 37^{o}C and 25^{o}C, respectively, for 15 days. The samples were examined on a daily basis.

b) *Decontamination testing*. This was done applying the 1% Catiorom solution on the flooring of an animal house and on the entire surface of a hospital-clinic for pets, after a rigorous mechanical cleaning, using 1 l/square meter of area.

The decontamination state was evaluated using the standard method i.e. areas of 100 cm × 100 cm were decontaminated and wiped with sterile cotton swabs 2 and 24 h after decontamination.

The samples were collected from 5 locations of each objective. The effect of the decontamination was determined using the total germ count (TGC) technique and the presence of coliform bacteria or of staphylococcus, using classical methods for each cotton swab.

RESULTS AND DISCUSSION

Table 1 shows the results of the bactericidal and antifungal activity of the alkyl-dimethyl-benzyl-ammonium chloride.

Table 1

Evaluation of the bactericidal and antifungal activity of Catiorom

	Bacterial strain	**Gram tinctoriality**	**Period of contact (minutes)**			**Final result**
			10	**30**	**60**	

1	*Staphylococcus aureus var Oxford ATCC*	+	+	-	-	inactivated
2	*Staphylococcus intermedius FMV 204*	+	+	-	-	inactivated
3	*Enterococcus faecalis FMV 12*	-	-	-	-	inactivated
4	*Enterococcus faecium FMV 30*	-	-	-	-	inactivated
5	*Bacillus cereus FMV 1240*	+	+	-	-	inactivated
6	*Bacillus anthracis FMV 1190 R*	+	-	-	-	inactivated
7	*Listeria monocytogenes FMV 51*	+	-	-	-	inactivated
8	*Erysipelotrix insidiosa FMV 412*	+	-	-	-	inactivated
9	*Clostridium perfringes A FMV 214*	+	+	-	-	inactivated
10	*Escherichia coli 0 175: H_7, FMV 302*	-	-	-	-	inactivated
11	*Escherichia coli var Bruxelles ATCC*	-	-	-	-	inactivated
12	*Salmonella enteritidis var Oxford ATCC*	-	-	-	-	inactivated
13	*Salmonella tyhphimurium FMV 53*	-	-	-	-	inactivated
14	*Pseudomonas aeruginosa FMV 1420*	-	+	-	-	inactivated
	Fungal strain					
1	*Candida albicans FMV 218*		+	-	-	inactivated
2	*Malassezia pachidernatis FMV 154*		+	-	-	inactivated
3	*Aspergillus niger FMV 307*		-	-	-	inactivated
4	*Penicilinium glaucum* FMV 402		-	-	-	inactivated

The experimental results show that the 1% alkyl-dimethyl-benzyl-ammonium chloride solution displayed an outstanding *in vitro* effect after 10 minutes of contact against most strains that were used (9/14), while after 30 minutes of contact, the bactericidal activity was 100% against all used strains (14/14). The strains of *Staphylococcus aureus, Staphylococcus intermedius, Bacillus cereus, Clostridium perfringes* and *Pseudomonas aeruginosa* are those which were not activated after a contact of 10 minutes with the disinfecting solution.

Most strains which were not inactivated during the first 10 minutes belong to the Gram-positive group. After 30 minutes of contact, these strains were eventually inactivated as well.

The antifungal activity of the disinfecting solution was 100% after 30 minutes of contact. The 2 tested yeasts, *Candida albicans* and *Malassezia pachidernatis*, remained viable after 10 minutes of contact, but were eventually inactivated after 30 minutes of contact.

The sanitation tests conducted in the animal stable and in the veterinary hospital-clinic, 2 and 24 h from decontamination, produced results within the admitted range, the total germ count (TGC) being lower than 10 germs/ml. The collected sanitation samples didn't show coliform bacteria or staphylococci.

CONCLUSIONS

The 1% alkyl-dimethyl-benzyl-ammonium chloride from the composition of Catiorom manifested a strong bactericidal and antifungal activity after a 10 minutes contact *in vitro*.

The quaternary amines diluted to 1%, in the composition of Catiorom product, acted on the Gram-positive bacteria after a period of 30 minutes.

The Gram-negative bacteria were inactivated after a contact of 10 minutes *in vitro*.

A contact of 2 hours was sufficient to demonstrate the bactericidal and antifungal effect of the 1% Catiorom solution for the decontamination of the areas from production units.

Some Gram-positive bacteria were more resistant to the bactericidal action of the quaternary amines, but were eventually destroyed after 30 minutes of contact.

REFERENCES

1. BâlbâieV., N. Pozsgi (1995). Bacteriologie medicală Vol. II. Ed. Medicală 1995, Bucureşti, pag. 7 – 14.
2. Coman T., I. Ţogoe, P. Ştiube, C. Chiurciu, Viorica Chiurciu. Virulicide activity of „*Catiorom*" biocide a preparation from quaternary ammines. Analele *Universităţii Spiru Haret* Seria Medicină Veterinară Vol. 8, Nr. 8, 2007, pag. 19 - 24.
3. Coman T., I. Ţogoe, P. Ştiube, C. Chiurciu, Viorica Chiurciu (2008). The virulicid activity of „*Decontaminol*" biocide. Analele *Universităţii Spiru Haret* Seria Medicină Veterinară Vol. 9, Nr. 9, 2008, pag. 11 - 16.
4. Kanerva L., T. Estlander, R. Jolanski (2001). Occupational Allergic Contact Dermatitis from Alkilammonium Benzoat. European Day of Dermatology (2001), Vol 11/3, pag. 240- 244.
5. SNACH A., J. GEIER, M. UTER, P.J. FROSH. Preservatives Antimicrobials and (1998) Industrial Biocides. British Dermatology 1998, Vol. 38, pag. 467 – 476.
6. * * * N. Alkil Dimethil Benzyl Ammonium Chloride. Monography. w.w.w. chemical land 21 com.

BACTERICIDAL AND ANTIFUNGAL ACTIVITY OF DECONTAMINOL
ACTIVITATEA BACTERICIDĂ ŞI ANTIFUNGICĂ A PRODUSULUI DECONTAMINOL

ŢOGOE I. [1], T.COMAN [2], C.CHIURCIU [3], Viorica CHIURCIU [3], P.ŞTIUBE [3]
[1]USAMV Bucharest
[2] Faculty of Veterinary Medicine
Spiru Haret University
e-mail**:** ushmv_coman.toma@spiruharet.ro
[3]Romvac S.A.

REZUMAT

A fost studiată activitatea bactericidă şi antimicotică, „in vitro" şi „in vivo" a soluţiei de 1% de Decontaminol, biocid, pe bază de amestec de aminequaternare şi 5% glutanaldehidă.

Testarea „in vitro" s-a efectuat faţă de un număr de 14 tulpini bacteriene şi de 4 tulpini de miceţi, izolate şi tipizate, din colecţia A.T.C.C. şi FMV. Tulpinile, cultură proaspătă de 24 ore, au fost puse în contact cu soluţia 1% Decontaminol în raport de 1⁄10 şi lăsate timp de 10, 30 şi 60 minute. După contact soluţiile au fost reînsămânţate pe mediu cu bulion şi agar nutritiv, bulion şi agar VF şi Sabouraud solid.

Activitatea antibacteriană şi antimicotică înregistrată după 7 zile şi respectiv 14 zile de la reînsămânţare a fost de 100% după un contact de 10 minute faţă de tulpinile: Enterococus fecalis, Enterococus faecium, Bacillus anthracis, Listeria monocitogenes, Erysipelatrix insidiosa, Escherichia coli 0 157:H7, Escherichia coli var. Bruxelles, Salmonella enteritidis, Salmonella typhimurium, precum şi faţă de Candida albicaus, Malassesia pachydermatis, Aspergilus niger, Penicilium glaucum. După 30 minute de contact activitatea bactericidă şi antimicotică a fost de 100% şi faţă de Staphylococus aureus var Oxford, Staphylococcus intermedius, Pseudomonas aeruginosa.

„In vivo", activitatea antibacteriană şi antimicotică a fost testată după decontaminarea cu soluţia 1% Decontaminol, a unor spaţii de creştere şi de spitalizare a animalelor. Probele de sanitaţie recoltate la 2 ore şi respectiv 24 ore de la decontaminare au fost negative, NTG fiind sub 10 germeni⁄ml.În mediile însămânţate nu au fost evidenţiate bacterii coliforme sau streptococi.

Cuvinte cheie: *amine quaternare şi glutanaldehidă, activitate bactericidă şi antimicotică, decontaminol – biocid*

ABSTRACT

The *in vitro* bactericidal and antimycotic activity of 1% Decontaminol solution, biocide based on quaternary amines and glutaraldehyde, was studied. Its efficacy for the decontamination of animal rearing areas has also been investigated.

The *in vitro* testing was conducted on 14 bacterial strains and 4 mycetes strains, from FMV Bucharest collection.

The strains, fresh 18-24h culture, were brought into contact with the 1% Decontaminol solution for 10, 30 and 60 minutes. After contact, each solution was reseeded on tomato broth and nutritive agar, tomato broth and VF agar and solid Sabourand mediums.

The *in vitro* antibacterial and antimycotic activity was examined after 7 and 14 days from seeding on nutritive mediums and incubation at 37^{o}C and at 25^{o}C.

The bactericidal and antimycotic activity was 100% after 10 minutes of contact, against *Enterococcus faecalis, Enterococcus faecium, Bacillus anthracis, Listeria monocytogenes, Erysipelotrix insidiosa, Escherichia coli 0 175: H_7, Escherichia coli var Bruxelles, Salmonella enteritidis, Salmonella tyhphimurium*, as well as against *Candida albicans, Malassezia pachidernatis, Aspergillus niger, Penicillinium glaucum.*

After 30 minutes of contact the bactericidal and antimycotic activity was 100% against *Staphylococcus aureus* var *Oxford*, *Staphylococcus intermedius* and *Pseudomonas aeruginosa* too.

The antibacterial and antimycotic activity of 1% Decontaminol solution was tested on 800 square meters of area used for animal rearing and hospitalisation. The samples collected 2 and 24 hours after decontamination were negative, the TGC being below 10 germs/ml. The seeded mediums were free of coliform bacteria or streptococci.

Keywords: biocide, quaternary amines, glutaraldehyde, bactericidal and antimycotic activity

INTRODUCTION

The quaternary amines have been used in the human medicine starting since 1935 as antiseptic as disinfectants and cationic soaps. The first observations on their bactericidal algicide and antifungal activity were done between1950-1955, but no tests have been performed to determine their bactericidal and antimycotic activity. Due to their emulsifying bactericidal and antimycotic properties the quaternary amines have been used worldwide as biocide agents.

The bactericidal and bacteriostatic activity was studied on Gram-positive and Gram-negative germs, which showed that the quaternary amines have a very good bactericidal activity against the Gram-positive germs.

The quaternary amines have been used as disinfecting substances due to the low toxicity of the working solutions on the tegument and due to their lack of noxiousness against the environment, with no danger to the life and health of the operators. Due to their bactericidal, algicide and antimycotic activity, the quaternary amines are used currently for the disinfection of the surgery instruments, protection equipment and surgery rooms. The mechanism of action on the bacteria has yet to be elucidated. The quaternary amines are supposed to act on the enzymes from the cell membrane of the bacteria and mycetes, blocking the mechanisms of trans-membrane transportation. Due to this action, the cells discontinue their metabolism.

Glutaraldehyde is a substance known for its antibacterial and antifungal activity, but poorly teratogenic and oncogenous. Glutaraldehyde is a dialdehyde used as disinfectant and chemical sterilizer due to its biocide activity on a wide spectrum of bacteria, fungi and viruses.

Decontaminol is a biocide prepared from alkyl phenol o/p dimethyl ammonium chloride, glutaraldehyde, isopropyl alcohol, nonylphenol ethoxilate and water, the main component being a quaternary amine. The 1% solution is used to disinfect the animal farms, in the food industry units, for the disinfection of the vehicles used for animal transportation, for the prophylactic and required decontamination of the animal farms.

The *in vitro* virucide activity of the quaternary amines has been determined on 4 viruses frequently met in the poultry farms, where they produce great losses (*Newcastle disease virus, avian infectious bursitis virus, avian influenza virus and avian infectious bronchitis virus*) (2, 3)

The virucide activity of Decontaminol was manifest after 10 minutes of contact (3).

The purpose of the paper is to show the *in vitro* bactericidal and antifungal activity of 1% Decontaminol solution, preparation based on alkyl phenol o/p dimethyl ammonium chloride and glutaraldehyde.

MATERIAL AND METHODS

Strains. Fourteen Gram-positive and Gram-negative bacterial strains and four fungi strains have been selected, from FMV-USAMV Bucharest collection, as follows:

Bacteria

15. *Staphylococcus aureus var Oxford*
16. *Staphylococcus intermedius*
17. *Enterococcus faecalis*
18. *Enterococcus faecium*
19. *Bacillus cereus*
20. *Bacillus anthracis*
21. *Listeria monocytogenes*
22. *Erysipelotrix insidiosa*
23. *Clostridium perfringes A*
24. *Escherichia coli 0 175: H_7*
25. *Escherichia coli var Bruxelles*
26. *Salmonella enteritidis var Oxford*
27. *Salmonella tyhphimurium*

28. *Pseudomonas aeruginosa*

Fungi

29. *Candida albicans*
30. *Malassezia pachidernatis*
31. *Aspergillus niger*
32. *Penicillinium glaucum*

Culture medium. The following culture mediums have been used to test *in vitro* and *in vivo* the bactericidal activity:

- Tomato broth and nutritive agar;
- Tomato broth and VF agar;
- Sabouraud agar.

Test water. The *in vitro* fungicide and bactericidal activity was tested on hard water with 300 mg calcium/l, according to standard *SR EN 14675*.

Decontaminol is a biocide containing 15% alkyl phenol o/p dimethyl ammonium chloride, 5% glutaraldehyde, isopropyl alcohol, nonylphenol ethoxilate as emulsifiers and deionised water.

The commercial solution is a clear, colourless, yellowish with a characteristic smell; it is used in a proportion of 1%. The product is recommended for the prophylactic and required disinfection of the areas from animal farms (animal houses), and in the feed industry (slaughterhouses, meat processing units, sanitary facilities, factory laboratories) and for the vehicles transporting animals. At 1% dilution the product is not toxic, doesn't endanger life and work security, or the environment.

Experimental model

a) "*in vitro" testing*. The bactericidal and antifungal activity of the product was evaluated on fresh, 18-24h, cultures of the strains mentioned earlier. The bacterial and fungal suspensions were put into contact with the solution of 1% Decontaminol in a ratio of 10/1 (9 parts Decontaminol and 1 part bacterial or fungal suspension).

After a contact of 10, 30 and 60 minutes, each mixture (Decontaminol and the bacterial or fungal suspension) were seeded with 0.1 ml in specific growth mediums, i.e., in plates with Sabourand agar, and incubated at 37^{o}C and 25^{o}C, respectively, for 15 days. The samples were examined on a daily basis.

b) *Decontamination testing*. This was done applying the 1% Decontaminol solution on the flooring of an animal house and on the entire surface of a hospital-clinic for pets, after a rigorous mechanical cleaning, using 1 of l 1% solution/square meter of area.

The decontamination state was evaluated using the standard method i.e. areas of 100 cm × 100 cm were decontaminated and wiped with sterile cotton swabs 2 and 24 h after decontamination.

The samples were collected from 5 locations of each objective. The effect of the decontamination was determined using the total germ count (TGC) technique and the presence of coliform bacteria or of staphylococcus, using classical methods for each cotton swab.

RESULTS AND DISCUSSION

Table 1 shows the results of the bactericidal and antifungal activity of the alkyl phenol o/p dimethyl ammonium chloride and glutaraldehyde.

Table 1

Evaluation of the bactericidal and antifungal activity of *DECONTAMINOL*

Nr. crt.	Bacterial strain	Gram tinctoriality	Period of contact (minutes)			Final result
			10	30	60	
1	*Staphylococcus aureus var Oxford ATCC*	+	+	-	-	inactivated
2	*Staphylococcus intermedius FMV 204*	+	+	-	-	inactivated
3	*Enterococcus faecalis FMV 12*	-	-	-	-	inactivated
4	*Enterococcus faecium FMV 30*	-	-	-	-	inactivated
5	*Bacillus cereus FMV 1240*	+	-	-	-	inactivated
6	*Bacillus anthracis FMV 1190 R*	+	-	-	-	inactivated
7	*Listeria monocytogenes FMV 51*	+	-	-	-	inactivated
8	*Erysipelotrix insidiosa FMV 412*	+	-	-	-	inactivated
9	Clostridium perfringes A FMV 214	+	-	-	-	inactivated
10	*Escherichia coli 0 175: H_7, FMV 302*	-	-	-	-	inactivated
11	*Escherichia coli var Bruxelles ATCC*	-	-	-	-	inactivated
12	*Salmonella enteritidis var Oxford ATCC*	-	-	-	-	inactivated
13	*Salmonella tyhphimurium FMV 53*	-	-	-	-	inactivated
14	*Pseudomonas aeruginosa FMV 1420*	+	+	-	-	inactivated
Fungal strain						
1	Candida albicans FMV 218		-	-	-	inactivated
2	*Malassezia pachidernatis FMV 154*		-	-	-	inactivated
3	Aspergillus niger FMV 307		-	-	-	inactivated
4	*Penicillinium glaucum* FMV 402		-	-	-	inactivated

The experimental results show that Decontaminol, product prepared from quaternary amines (alkyl phenol o/p dimethyl ammonium chloride and glutaraldehyde) used in 1% dilution displayed an outstanding *in vitro* effect after 10 minutes of contact against most of the Gram-positive and Gram-negative strains that were used (11/14), while after 30 minutes of contact, the bactericidal activity was 100% against all used strains. The 3 strains which were not activated after a contact of 10 minutes are Gram-positive, but they were eventually inactivated after a contact of 30 minutes.

The antifungal activity of the disinfecting solution was 100% after 10 minutes of contact against the 2 yeast and the 2 mycetes strains.

The intense bactericidal and antifungal activity of the 1% Decontaminol solution against the Gram-positive and Gram-negative germs that were tested, as well as against the fungi, is supported in our opinion by the action of the glutaraldehyde, known to be a strong disinfectant.

The sanitation tests conducted in the animal stable and in the veterinary hospital-clinic, 2 and 24 h from decontamination, produced results within the admitted range, the total germ (mesophyllic organisms) count (TGC) being lower than 10 germs/ml. The collected sanitation samples didn't show coliform bacteria or staphylococci.

CONCLUSIONS

The 1% Decontaminol solution prepared from alkyl phenol o/p dimethyl ammonium chloride and glutaraldehyde manifested a strong bactericidal and antifungal activity after a 10 minutes contact against most Gram-negative and Gram-positive germs that were tested.

Three of the tested bacterial strains, belonging to the Gram-positive group, were inactivated after a contact of 30 minutes.

A contact of 2 hours was sufficient to for the disinfection of the areas from animal rearing and hospitalization units after the mechanical cleaning.

The bactericidal an antifungal action of the quaternary amine is enhanced by the addition of a low proportion of glutaraldehyde.

REFERENCES

1. BâlbâieV., N. Pozsgi (1995). Bacteriologie medicală Vol. II. Ed. Medicală 1995, Bucureşti, pag. 7 – 14.
2. Coman T., I. Țogoe, P. Ştiube, C. Chiurciu, Viorica Chiurciu. Virulicide activity of „*Catiorom*" biocide a preparation from quaternary ammines. Analele *Universității Spiru Haret* Seria Medicină Veterinară Vol. 8, Nr. 8, 2007, pag. 19 - 24.
3. Coman T., I. Țogoe, P. Ştiube, C. Chiurciu, Viorica Chiurciu (2008). The virulicid activity of „*Decontaminol*" biocide. Analele *Universității Spiru Haret* Seria Medicină Veterinară Vol. 9, Nr. 9, 2008, pag. 11 - 16.
4. FRAUD S., A.C. HANN, J-Y MAILARD, A.D.. RUSSEL (2003). Efects of o – fhalaldehide, glutaraldehide and chlorhexidine diaacetat on Mycobacterium chelonae and Mycobacterium abceseus strains with modified permeability. J. of antimicrobial Chematherapy 2003, 51 pag 575 – 584.
5. Kanerva L., T. Estlander, R. Jolanski (2001). Occupational Allergic Contact Dermatitis from Alkilammonium Benzoat. European Day of Dermatology (2001), Vol 11/3, pag. 240- 244.
6. Laopaiboon L., K. Phukotphim, P. Laopaiboon. Effects of glutaraldehide biocide (2006)on laboratory – seale ratarting biologicxal contacors and biocide efficacy. Electronic Journal of Biotehnology 2006, Vol 9/4, pag. 245 – 248.
7. SNACH A., J. GEIER, M. UTER, P.J. FROSH. Preservatives Antimicrobials and (1998) Industrial Biocides. British Dermatology 1998, Vol. 38, pag. 467 – 476.
8. * * * N. Alkil Dimethil Benzyl Ammonium Chloride. Monography. w.w.w. chemical land 21 com.

HISTOLOGICAL CHANGES IN THE EXPERIMENTAL INTOXICATION PRODUCED BY BIOCIDE SUBSTANCES PREPARED WITH QUATERNARY AMINES

MODIFICĂRI HISTOLOGICE ÎN INTOXICAȚIA EXPERIMENTALĂ PRODUSĂ DE SUBSTANȚE BIOCIDE PREPARATE CU AMINE QUATERNARE

Coman T.[1], D. Turcu[1], T. Petruț[1]

[1] University „Spiru Haret", Faculty of Veterinary Medicine, Bucharest

ushmv_coman.toma@spiruharet.ro

REZUMAT

Au fost studiate modificările histologice produse în diferite organe după intoxicația experimentală a șoarecilor SPF cu Catiorom și Decontaminol, substanțe biocide preparate din amine quaternare.

Loturi de câte 10 șoricei SPF linia NMRI, în greutate de 19,5-21,5 g au fost inoculați intraperitoneal cu 0,2 ml din concentrațiile de 2%, 1,5%, 1,25%, 1% și 0,5% din produsele biocide. Șoriceii au fost monitorizați zilnic, timp de 10 zile, înregistrându-se evoluția clinică și mortalitatea, în condițiile furajării și cazării corespunzătoare identice.

Șoriceii care au murit au fost necropsiați, iar cei care au supraviețuit au fost sacrificați și s-au recoltat probe pentru examenul histologic creier, ficat rinichi, cord și gonade. Probele au fost fixate în formol neutru salin, incluse în parafină, secționate la 5 microni și colorate prin metode topografice (HE și tricromic Mallory).

Leziunile histologice cele mai semnificative au fost înregistrate la loturile inoculate cu concentrația 2% (9/10), mai puține leziuni la lotul inoculat cu 1,5% (5/10) și foarte puține la lotul inoculat cu 1,25% (1/10).

Loturile inoculate cu 1% și 0,5%, după sacrificare nu au prezentat leziuni. Modificările înregistrate la animalele moarte inoculate cu 2%, 1,5% și 1,25% au constat în leziuni congestive și hemoragice la nivelul encefalului și al gonadelor însoțite de distrofie neuronală, respectiv a celulelor liniei seminale și celulelor Sertoli, precum și leziuni congestive, distrofice și degenerative în ficat și rinichi.

Leziunile congestive și hemoragice din encefal au fost înregistrate la nivelul meningelui și scoarței cerebrale, însoțite de distrofii la nivel neuronal. La nivelul gonadelor se înregistrează congestii și hemoragii interstițiale cu degenerescența celulelor Sertoli, a spermatogoniilor și spermatocitelor de ordinul I în majoritatea tubilor seminiferi. Celulele endocrine din insulele Leydig suferă procese distrofice. În ficat leziunile congestive și hemoragice sunt prezente intralobular însoțite de intumescența tulbure a celulelor hepatice. În rinichi s-au înregistrat leziuni de glomerulonefrită hemoragică, congestie și hemoragii interstițiale. În tubii renali se constată leziuni degenerative ale celulelor renale însoțite de desprinderea de pe membrana bazală și formarea de cilindrii hialini.

Cuvinte cheie: amine quaternare, intoxicație experimentală, șoricei SPF, modificări histologice.

ABSTRACT

The histological modifications produced in different organs after the experimental intoxication of SPF mice with Catiorom and Decontaminol, biocides prepared from quaternary amines, has been studied.

Groups of 10 SPF mice, NMRI line, weighing 19.5 – 21.6 g, have been inoculated intraperitonealy with 0.2 ml of 2%, 1.5%, 1% and 0.5% concentrations of the biocide products. The mice were monitored on a daily basis for 10 days, recording the clinical evolution and mortality, under the conditions of identical feeding and housing.

The dead mice have been necropsied, and those who survived have been slaughtered and samples were collected for the histological examination of the brain, liver, kidneys, heart and gonads. The samples were fixed in neutrally saline formalin, imbedded in paraffin; 5 microns slices have been cut and stained using topographical methods (HE and Mallory's trichromic).

The most significant histological lesions have been observed in the groups inoculated with the concentration of 2% (9/10); less lesions have been noticed in the group inoculated with the concentration of 1.5% (5/10) and very few lesions have been noticed in the group inoculated with the concentration of 1.25% (1/10).

The groups inoculated with the concentrations of 1% and 0.5% showed no lesions after slaughtering. The changes observed in the dead animals inoculated with 2%, 1.5% and 1.25% consisted in congestive and hemorrhagic lesions in the encephalon and gonads, accompanied by neuronal dystrophy, by dystrophy of the seminal line cells, of the Seroli cells; dystrophic and degenerative congestive lesions in the liver and kidneys have also been reported.

The congestive and hemorrhagic lesions in the encephalon have been found in the meninges and cortex, accompanied by neuronal dystrophies. Interstitial congestions and haemorrhages have been observed in the gonads, with degenerating Seroli cells, first order spermatogones and spermatocytes in most seminiferous tubules. The endocrine cells

of the Leydig islets also underwent dystrophic processes. In the liver, the congestive and hemorrhagic lesions were noticed intralobularly, accompanied by the hepatic cells intumescence. Lesions of hemorrhagic glomerulonephritis, interstitial congestion and haemorrhages have been observed in the kidney. Degenerative lesions of the renal cells accompanied by the detachment from the basal membrane and formation of hyaline cylinders have been observed in the renal tubules.

Keywords: quaternary amines, experimental intoxication, SPF mice, histological changes

INTRODUCTION

Catiorom and DecontaminoL, are two biocide preparations made of quaternary amines used for the disinfection and decontamination of the exposed areas in food processing units, animal production units, veterinary laboratories and clinics, animal transportation vehicles (5). Both substances belong to the same group of biocide antiseptics with indications for disinfection in veterinary units (13).

Catiorom contains alkyl-dimethyl-benzyl-ammonium chloride, while Decontaminol contains alkyl phenol o/p dimethyl ammonium chloride and glutaraldehyde. The ammonium chloride alkyl belongs to the group of quaternary amines being active cations of reversed soaps which, due to their superior properties are used for the hard water too. The quaternary amines are not toxic. They are active against bacteria and fungi, particularly against Gram-positive bacteria and Candida fungi because of their antibacterial, antifungal and antialgal properties. They are frequently used in surgery to disinfect the hands, surgery instruments and the operatory field.

The quaternary amines are known to be substances with a strong bactericidal, fungicidal and algicidal activity (7). Recent studies conducted to assess their virulicide activity have shown that a concentration of 0.1% has a strong capacity of inactivation of the Newcastle disease virus, avian infectious bursitis virus, avian influenza virus after a contact of 10 minutes, and against the avian infectious bronchitis virus after 30 minutes of contact (2, 3).

The quaternary amines have been characterized chemically by Jacobson et al. in 1916 and used in the medical practice as disinfectants since 1935 Domag H, cited by 7). As antiseptic substances, the quaternary amines have bactericidal and bacteriostatic activity (8). They are active against most fungi, protozoa and algae. The studies have confirmed that the quaternary amines have a low toxic activity against the skin (9). They have tensioactive properties being frequently used as softening or detergent agents (7).

Glutaraldehyde is known to have bactericidal, tuberculicidal, slow sporicidal, antifungal and antimycobacterial (1), as well as virulicidal (6) activities. In large concentrations glutaraldehyde has toxic, teratogenic and mutagen potential and is suspected to be cancerigenic. In concentrations lower than 1000 ppm it doesn't display toxic, teratogenic, fetotoxic or embryotoxic activity (10). Studies conducted on 344 rats have shown that glutaraldehyde doesn't have oncogenic and teratogenic (11).

The mechanism of action of the quaternary amines is not properly deciphered. The supposition is that the bactericidal activity is determined by the possibility to block the active centres of the bacterial enzymes or by its capacity to decrease the superficial tension, which enables the substances to cross the cell membrane and denaturate the cell proteins (7).

The purpose of the paper was to determine the histological changes produced by the quaternary amines from the commercial preparations Catiorom and Decontaminol, induced by experimental intoxication in mice.

MATERIAL AND METHOD

1. SPF mice, from NMRI line, weighing 19.5 – 21.5 g have been assigned to groups with identical conditions of housing and feeding. Eleven groups of 10 mice each, have been formed. Five groups were inoculated with Catiorom, 5 groups with Decontaminol, and one group was the control group, with no inoculation.

2. The biocides used to inoculate the mice were Catiorom and Decontaminol. Catiorom is a biocide containing 15g/100 ml alkyl-dimethyl-benzyl-ammonium chloride, while Decontaminol contains 15g/100 ml contains alkyl phenol o/p dimethyl ammonium chloride and 5g/100 ml glutaraldehyde.

3. Experimental model. The groups of mice have been inoculated intraperitonealy (ip) with 0.2 ml of 2%, 1.5%, 1% and 0.5% concentrations of Catiorom and Decontaminol, using 10 mice per group. A group was not inoculated and it used as control group. The biocide substances have been diluted in sterile hard water prepared according to standard SREN 14675 (12). The animals have been individualised by groups.

The mice were monitored clinically for 10 days. The dead mice have been necropsied and samples were collected for the histological examination of the brain, liver, kidneys, heart and gonads. The animals which survived have been slaughtered, necropsied and samples were collected for the histopathological examination. The samples were collected in neutrally saline formalin. The groups of mice were housed in special containers.

4. Histological examination. The samples were imbedded in paraffin and 5-6 micron slices have been cut and stained using the usual methods (Mallory's trichromic and HE).

RESULTS AND DISCUSSION

The mice inoculated with the 2% and 1.5% solution have displayed the following histological changes upon necropsy:

1. Brain. The congestive and haemorrhagic lesions observed in the group inoculated with 2% solution were located at the level of the meningeal membranes and of the grey matter. The neurons from the external molecular and granular layer started necrobiosis ensuing the state of hypoxia (Fig. 1)

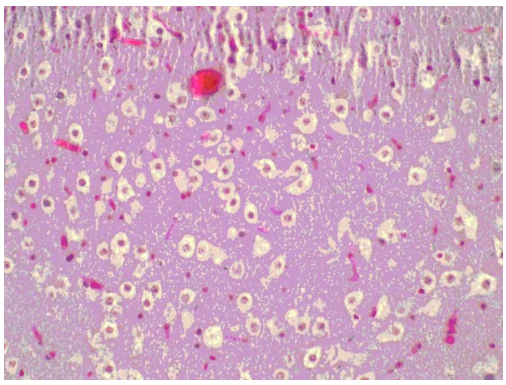


Fig. 1 – Vacuolar dystrophy in the brain; Mallory's trichromic staining technique; Ob. 40x

The Wirchow – Robin spaces around the encephalon vessels are ectasied because of the cerebral oedema which formed. Frequent haemorrhages were observed in the nervous matter (Fig. 2)

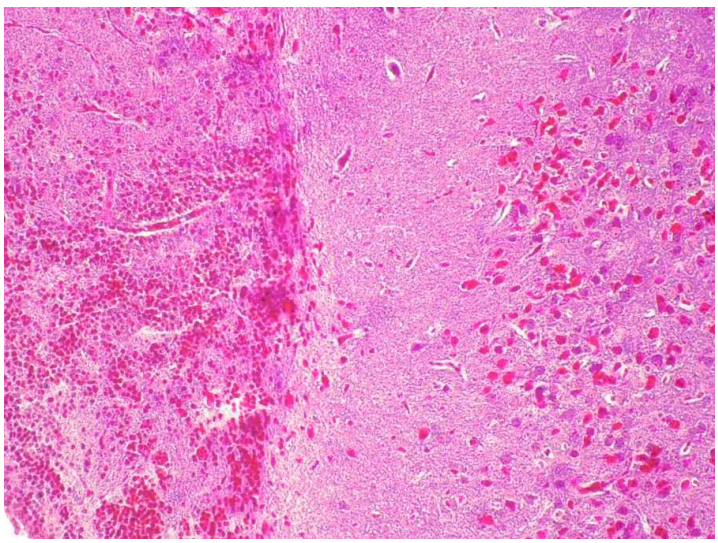


Fig. 2 – Vacuolar dystrophy in the brain; Mallory's trichromic staining technique; Ob. 20x

Areas of degenerescence appeared in the white matter (Fig. 3).

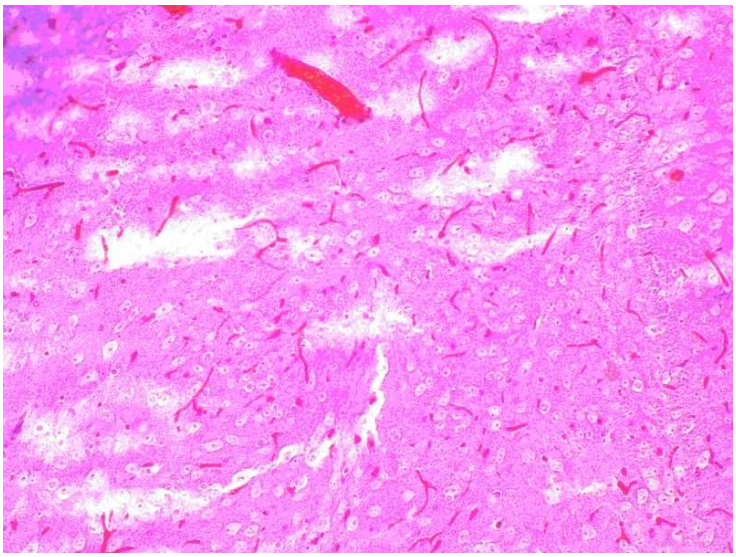


Fig. 3 – Cerebral degenerescence; HE staining technique; Ob. 20x

The choroid plexus is congested and the cerebrospinal fluid is haemorrhagic.

2. Liver. The histological changes observed in the hepatic lobe were congestive and haemorrhagic. The blood accumulated in the liver caused the vascular sinuses to block (Fig. 4).

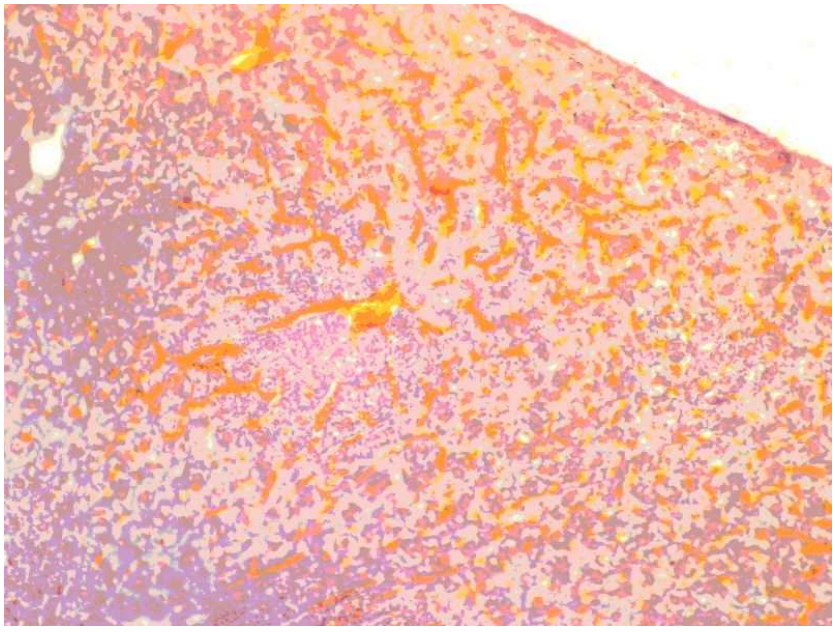


Fig. 4 – Hepatic congestion; Mallory's trichromic staining technique; Ob. 20x

The hepatocytes from Remack chords undergo a dystrophic process of intumescence ensuing the state of hypoxia (Fig. 5)

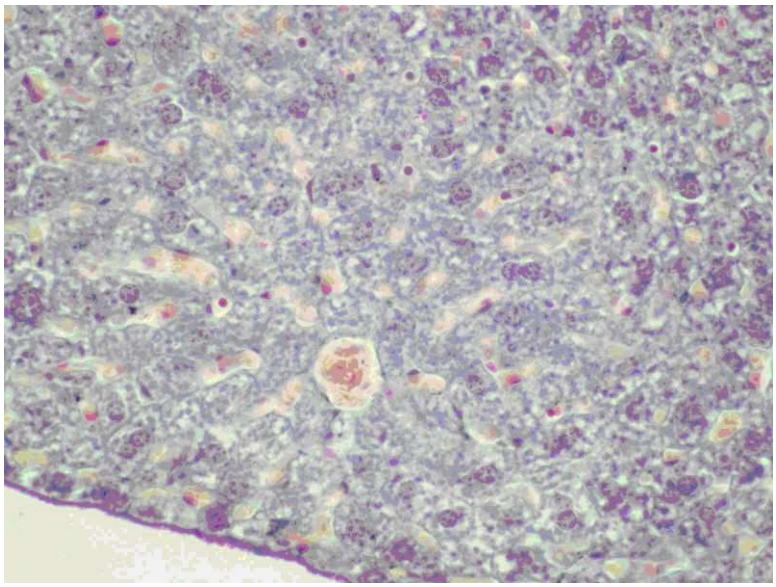


Fig. 5 – Granular hydric dystrophy; Mallory's trichromic staining technique; Ob. 40x

The most serious lesions have been observed in the group inoculated with the 2% concentration of both biocide substances; changes of vacuolar toxic hepatitis have been observed (Fig. 6)

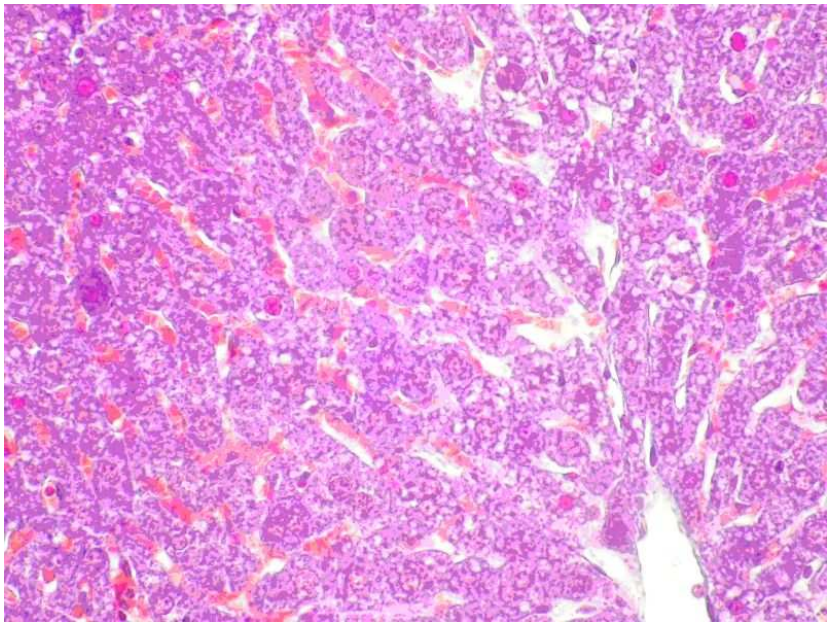


Fig. 6 – Toxic hepatitis; HE staining technique; Ob. 40x

The control groups and the slaughtered animals from the groups inoculated with 1% and 0.5% didn't display liver lesions. Some hepatic cells started the necrobiosis.

3. Heart. The histological changes observed in the hepatic lobe were congestive and haemorrhagic, of ectasied lymph capillaries. The major changes observed in the heart were congestions and haemorrhages, haemorrhagic myocaditis. Due to the state of hypoxia caused by the coronary vessels congestion, the myocardiocytes became dystrophic (Fig. 7)

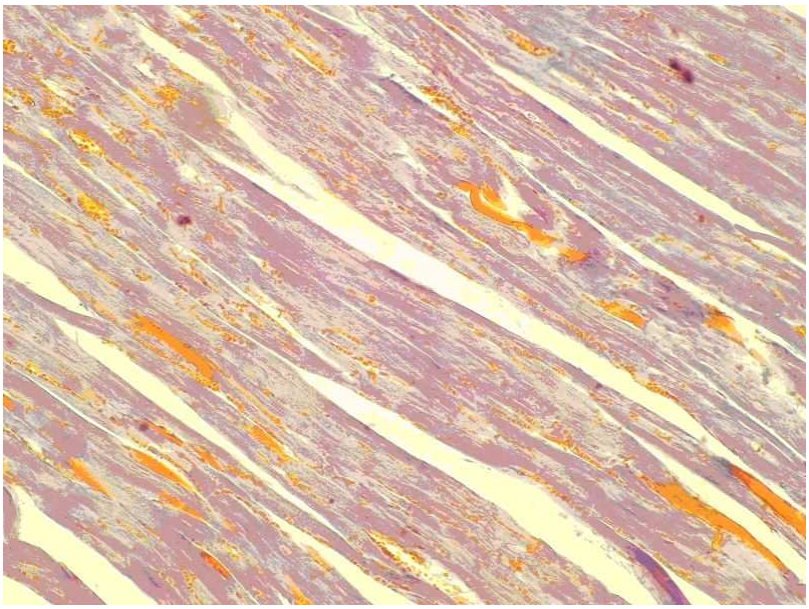


Fig. 7 – Myocardial congestions and haemorrhages; Mallory's trichromic staining technique; Ob. 20x

4. Kidneys. They were strongly affected by the action of the quaternary amines in 2% concentration. The high mortality observed in this group for both biocide substances was determined by haemorrhagic nephrosis and glomerulitis. A severe interstitial congestion and haemorrhage associated with the haemorrhagic glomerulo-nephritis has been observed in these animals (Fig. 8).

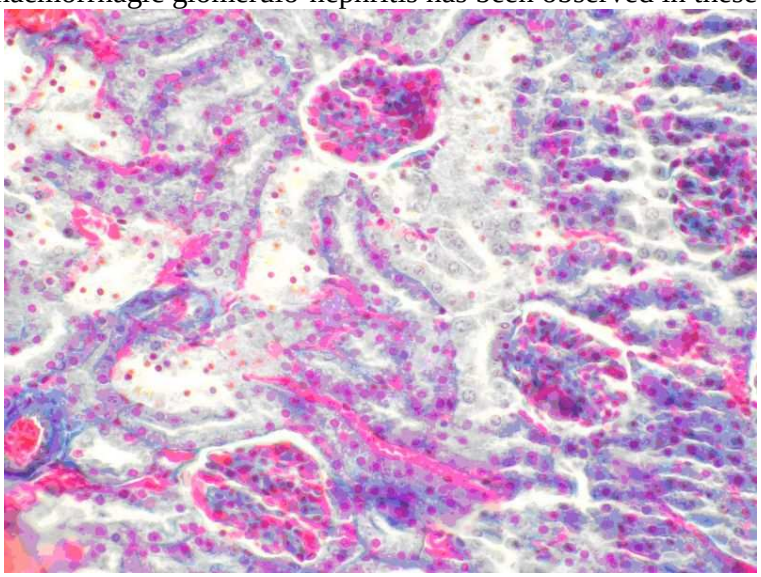


Fig. 8 – Haemorrhagic nephrosis and glomerulitis; Mallory's trichromic staining technique; Ob. 40x

The glomerular vascular networks undergo a process of hyalinosis which makes the vascular walls break and the renal glomerulus to get blocked by blood. The nephrocyes from the distal contorted tubules displayed dystrophic processes which cause necrobiosis and their necrosis accompanied by detachment from the basal membrane, forming the hyaline cylinders which had blocked the reabsorption processes. The collecting ducts also are blocked by the cuboidal cells undergoing different stages of necrobiosis and necrosis (Fig. 9).

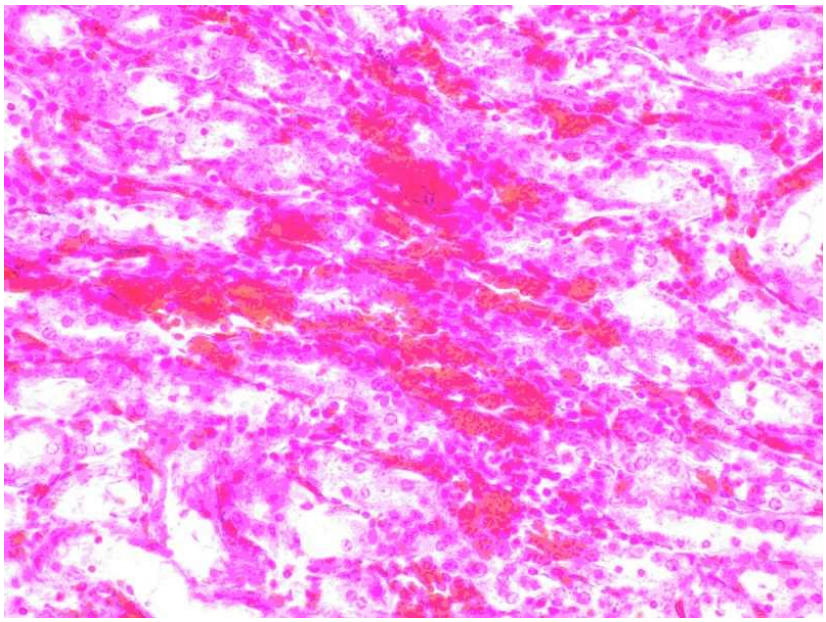


Fig. 9 – Interstitial haemorrhage and nephrosis in to contorted tubules; HE staining technique; Ob. 40x

5. Gonads. The histological changes observed in the testes of the animals inoculated with the 2% and 1.5% concentrations are due to the peritubular interstitial haemorrhages and congestions caused by the toxic effect of the biocide substances. The lesions were observed in the testicular stroma and they consisted in severe haemorrhages of the capillary vessels due to the endothelial walls hyalinosis, which affected both the seminiferous tubes and the Leydig islets.

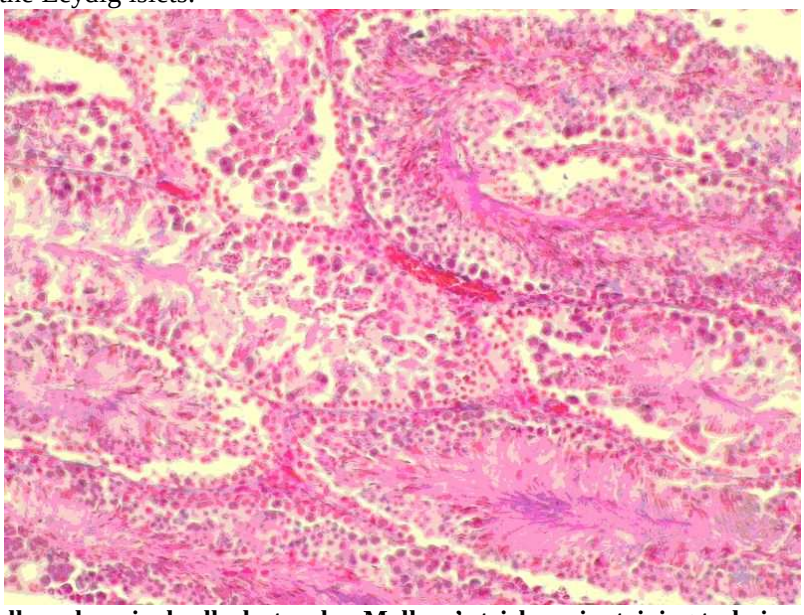


Fig. 10 – Sertoli cells and seminal cells dystrophy; Mallory's trichromic staining technique; Ob. 20x

Inside the seminiferous tubes, a strong dystrophy and necrobiosis of the seminal cells existing in the first compartment of the hemato-cellular barrier was noticed; the first order spermatogones and spermatocytes, as well as the Sertoli cells were affected (Fig. 10).

The Sertoli cells undergo a process of degenerescence ensuing the state of hypoxia. The interstitial cells of the Leydig islets suffer necrobiotic and dystrophic processes. Like in the intoxication with Salinomycin in poultry, the gonads have been strongly affected, which affected the process of reproduction (4).

6. Lung. Congestion of the intralobular vessels and alveolar oedema have been reported in the lung.

CONCLUSIONS

1. The experimental intoxication in SPF mice with biocide substances prepared from quaternary amines produced mortality and histological changes in the groups inoculated with concentrations of 2% and 1.5%.

2. The groups inoculated with 1% and 0.5% concentrations, as well as the animals which survived after the inoculation with 1.25% didn't display changes in the examined organs.

3. The histological changes observed in the dead and necropsied animals were determined by the acute toxic processes, materialized in congestions and haemorrhages in all studied organs.

4. The experimental intoxication with quaternary amines produced dystrophic and necrobiotic changes and necrosis in the parenchimatous cells both in the liver and in the kidneys, as well as in the grey matter of the cerebral hemispheres.

5. Congestive and haemorrhagic changes have been reported in the testes accompanied by dystrophic and degenerative changes of the Sertoli cells and of the sexual cells (spermatides and spermatocytes), as well as in the interstitial glands (Leydig islets).

REFERENCES

1. Collins F.M., 1986, *Bacteridal activity of alkaline glutaraldehyde solution against a number atypical mycobacterial species*, J. Appl. Bacteriol., Comp., 61 (3), pg. 247 – 251.

2. Coman T., I. Țogoe, P. Ştiube, C. Chiurciu, Viorica Chiurciu, 2007, *Virulicide activity of Catiorom biocide, a preparation from quaternary amines*, Analele Universității Spiru Haret, Seria Med. Vet., 8, pag. 19 – 24.

3. Coman T., I. Țogoe, P. Ştiube, C. Chiurciu, Viorica Chiurciu, 2007, *The virulicid activity of Decontaminol biocide*, Analele Universității Spiru Haret, Seria Med. Vet., 9, pag. 11 – 16.

4. Coman T., V. Teuşdea, O. Sicoe, C. Bejan, 1999, *Modificări histologice în intoxicația cu Salinomicină la găini de reproducție rase grele*, Revista Asociației de Patologie aviară, vol. 10/2, pag. 18 – 23.

5. Crivineanu Maria, 2005, *Farmacologie veterinară*, Ed. Fundis, Bucureşti, pag. 85 – 86.

6. Cunliffe H.R., J.H. Blackwell, J.S. Walker, 1979, *Glutaraldehyde inactivation of exotic animal viruses in swine heart tissue*, Applied and Environmental Microbiology, vol. 37/5, pg. 1044 – 1046.

7. Kanerva L.T., T. Eslander, R. Jolansky, 2001, *Occupational Allergic Contact Dermatitis from Alkilammonium Benzoat*, European Day of Dermatology, vol. 11/3, pg. 240 – 244.

8. Missir Al., Ileana Chiriță, Carmen Limban, 2003, *Chimie farmaceutică, vol. 1, Antiseptice şi dezinfectante*, Ed. Tehnoplast Comp., București, pag. 330 – 334.

9. Oniga O., Doina Ghiran, Brândușa Titerciuc, 1999, *Chimie farmaceutică, Antiseptice, dezinfectante şi chimioterapice generale*, Ed. Accent, Cluj - Napoca, pag. 218 – 232.

10. * - www. mindfully/Health/Cidex-Glutaraldehyde withdrawn 22 ian 2002. htm

11. ** - www. tedpella.com/msas_ html/1841 mds. htm

12. *** - Standard SREN 14675 – Antiseptice şi dezinfectante chimice. Testarea cantitativă a suspensiei pentru evaluarea activității virulicide a antisepticelor şi dezinfectantelor chimice utilizate în domeniul veterinar. Metode şi cerințe.

13. **** - H.G. 956/2005.

THE HISTOLOGICAL STRUCTURE OF 50 DAY OLD SWINE EMBRYO
STRUCTURA HISTOLOGICĂ LA EMBRIONUL DE PORC ÎN VÂRSTĂ DE 50 ZILE

Petruț T., T. Coman
University Spiru Haret, Faculty of Veterinary Medicine,
email: ushmv_petrut.tanase@spiruharet.ro

REZUMAT

Studiul s-a efectuat în vederea evidențierii structurii histologice a unor organelor din cavitatea toracică şi abdominală la embrionii de porc în vârstă de 50 zile.

Secțiunile transversale seriate prin cavitatea toracică şi abdominală la embrionul de porc în vârstă de 50 zile au fost colorate prin metodele HE, tricromică Mallory, impregnația argentică Gömöri şi Giemsa la rece.

Organele din cavitatea toracică şi abdominală prezintă celule funcționale, dar fără a avea organizarea structurală postpartum.

Pulmonul prezintă bronhii lobare, fiind în curs de organizare tunicile conjunctivo-cartilaginoase şi mugurii bronşici. Mezenchimul pulmonar care începe diferențierea corionului bronşic înconjoară mugurii epiteliali bronşici. În masa mezenchimului pulmonar se diferențiază numeroase rețele vasculare. Nucleii cartilaginoşi hialini din tunica musculo-fibro-cartilaginoasă sunt absenți. Lobulii pulmonari nu sunt organizați sau delimitați de țesutul conjunctiv.

Esofagul prezintă epiteliul mucoasei în curs de diferențiere, iar absența muscularei mucoasei nu permite delimitarea corionului de submucoasă. Glandele esofagiene lipsesc.

Ficatul prezintă celulele hepatice dispuse sub forma cordoanelor Remack ce converg către vena centrolobulară, nefiind prezentă o delimitare netă a spațiilor Kiernan şi a țesutului conjunctiv perilobular. În capilarele sinusoidalele hepatice se găsesc aglomerări de eritroblaste, eritrocite şi alte elemente figurate.

Stomacul ca şi intestinul subțire este format numai din mucoasă, musculoasă şi seroasă, nefiind diferențiată submucoasa. Ambele organe sunt lipsite de elemente glandulare.

Rinichiul prezintă elementele constitutive ale nefronului diferențiate, nefrocite funcționale cu polul apical diferențiat fiind prezenți microvilii, dar fără a prezenta elementele structurale ale aparatului juxtaglomerular.

Cuvinte cheie*: dezvoltare embrionară, suine, pulmon, ficat, esofag, stomac, intestin, rinichi.*

ABSTRACT

The study is meant to highlight the histological structure of certain organs in the thoracic and abdominal cavity of 50 day old swine embryo.

The serial transversal sections through thoracic and abdominal cavity of 50 day old swine embryo coloured with HE methods, Mallory trichromical, silver impregnation Gomori and cold Giemsa.

The organs of thoracic and abdominal cavity present functional cells, but with no postpartum structural organisation.

The lung has lobar bronchi and the conjunctive cartilaginous tunics and the first bronchial cells are about to develop. The pulmonary mesenchyma which begins the bronchial chorion differentiation, surrounds the bronchial epithelial and the first bronchial cells

In the whole mass of pulmonary mesenchyma are seen multiple vascular nets. The cartilaginous hialinic nucleus from the muscular-fibrous-cartilaginous tunic are missing. The pulmonary lobules are not organised or delimited by conjunctive tissue.

The esophagus presents the mucosa epithelium in course of differentiation and the absence of mucosa muscular doesn't allow the chorion definition from the submucosa. The esophagus glands are also missing. The liver has hepatical cells as Remack strings which converge towards the centrolobular vein, but have no clear delimitation of the Kiernan spaces and the perilobular conjunctive tissue. Into the hepatical sinusoids can be found eritroblasts, eritrocites and other figurative elements

The stomach as well as the small intestine is composed of mucosa, muscular and serous, without submucosa differentiation. Both organs are missing glandular elements.

The constitutive elements of the nephrons are differentiated. The functional nephrocites which have the apical pole differentiated present microviles but no structural elements of the juxtaglomerular apparatus.

Key words: *embryo development, swine, lung, liver, esophagus, stomach, intestine, kidney.*

INTRODUCTION

The research on the ontogenetical development of swine embryo often concentrates on the embrionary period up to 45 days old (2,6), an extremely important period in terms of creating new reproduction biotechnologies (transfer of embrios).

The studies effectuated on 45 days old swine embryo have proved the presence of hepatic cell-strings along with sinusoidal capilars charged with embrionary sanguineous figurative elements - hemocitoblasts and erythroblasts included, proving that at this age, the liver performs hematopoietic function (4,5).

The fetal development of swine embryo is not presently an usual subject of research in the specific literature, a limited number of articles exist on this topic, while most studies effectuated are in fact electronomicroscopic researches on the microstructure of the organs in course of differentiation (1,3).

Thus, studies using electron microscopy on swine embryo aged 26-27 days showed that the male gonads present differentiated components (the seminiferous tubules with the support cells and the spermatogoniae), while the interstitial tissue and testicular Leydig cells are not yet present at the this age (3).

Also, at 45 days-old, the stomach microstructure has an epithelium in course of differentiation with PAS positive granules on its surface and in the structure of gastric epithelium cells (1).

MATERIAL AND METHODS

The purpose of this research is the ontogenetic development of the lung, liver, esophagus, stomach, intestin and kidney in swine embryo 50-days old from the fecundation.

The embryos were picked up from the uteri of the females sacrified by necessity and were classified by length, with special focus on the 7-cm length embryos. This length corresponds to the age of 50 days of intrauterin development, at the limit of embrionary and fetal development.

The histological pieces collected were selected by dissection and fixed in saline neutral formol, being processed later for paraffin inclusion. The paraffin blocks were cut to 6 microns and coloured by the HE methods, Mallory trichromical, silver impregnation Gömöri and Giemsa cold.

RESULTS AND DISCUSSION

The 50 - days old lung shows a slight differentiation of the bronchial tree with the histological structures that differs in function of the level of pulmonary organization. It is formed of pulmonary lobules with epithelial condensation of the bronchial buds that ramify dichotomically. Around the bronchial buds the mesenchyma condenses and induces the differentiation of the bronchial epithelium (fig. 1).

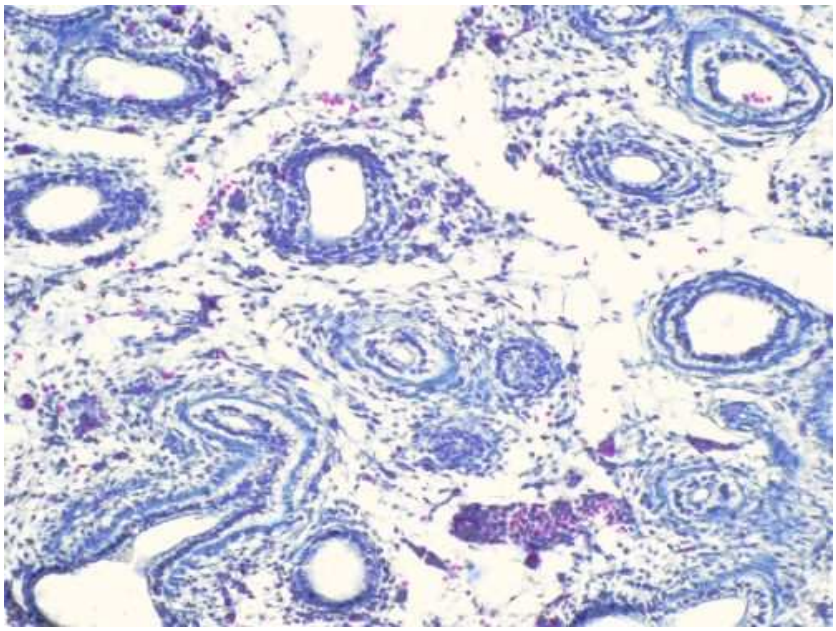


Fig. 1 – Swine embryo - 50 days; section by lung; Col. Mallory trichromical; Ob. 20x

The pulmonary lobulies are bounded by the perilobular conjuctive tissue presenting laxe nets of mesenchymal conjuctive tissue.The functional and trophic blood vessels are present in the lobular mesenchymal peribronchial tissue .(fig. 2)

The bronchial epithelium during this period of embryonic development appears as a simple prismatic epithelium, being present in the bronchiolar muscle. At this age the apical ciles have not appeared yet.

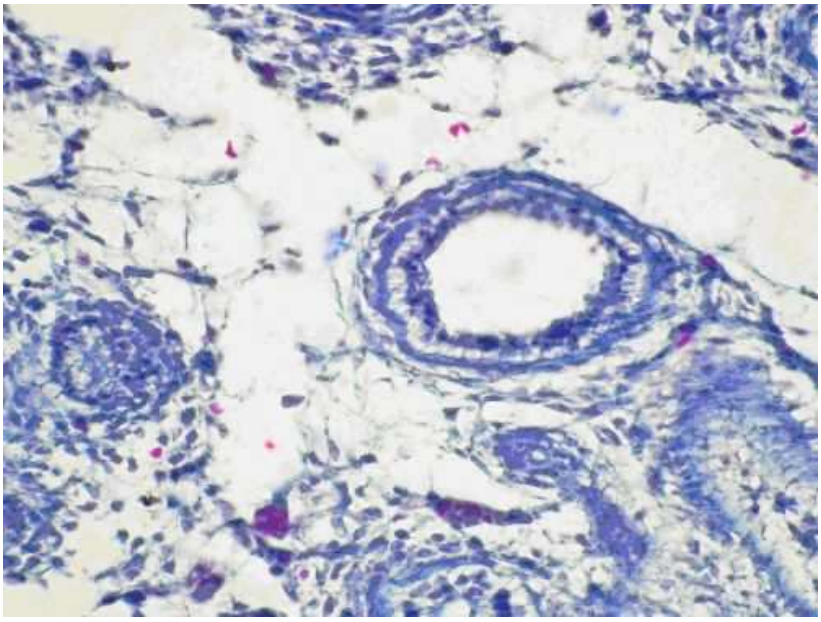


Fig. 2 – Swine embryo - 50 days; section by lung; Col. trichromical Mallory; Ob. 40x

The basal membrane on which lies the epithelium is obvious, and in bronchial chorion there are collagen fibers and fibroblasts, mastocites and lymphocytes. The fibro-muscular tunic is now differentiating, the smooth muscular fibers forming the Reissessen muscle. Tunic fibromuscular tissue continues with perilobular mesenchymal tissue (fig. 2).

The liver – presents centrolobular veins that converge towards the hepatocite strings, the conjunctive walls that delimitate the hepatic lobules are non-differentiated, the liver lobulation, along with the interlobular Kiernan spaces, being less marked. (fig. 3).

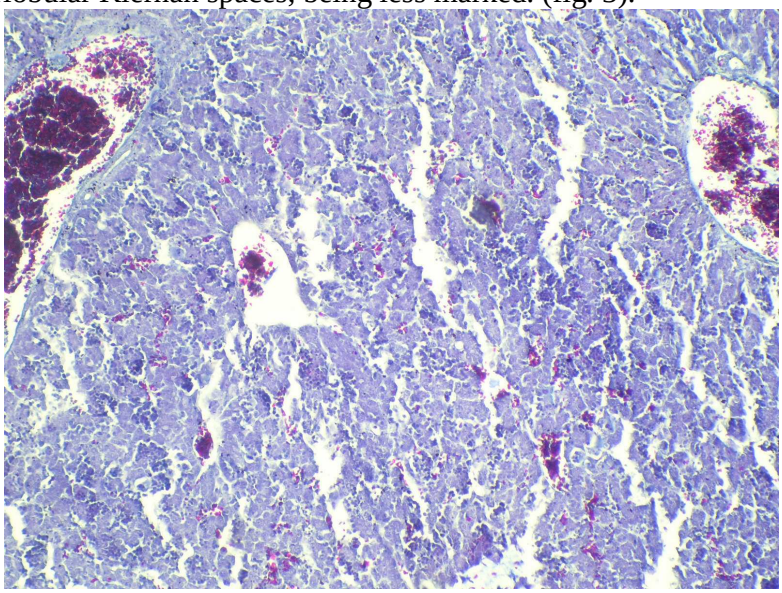


Fig. 3 – Swine embryo - 50 days; section through the liver; Col. trichromical Mallory; Ob. 10x

The sinusoidal capillaries are filled with eritroblasts as during this period the liver has also a hematopoietical function (fig. 4).

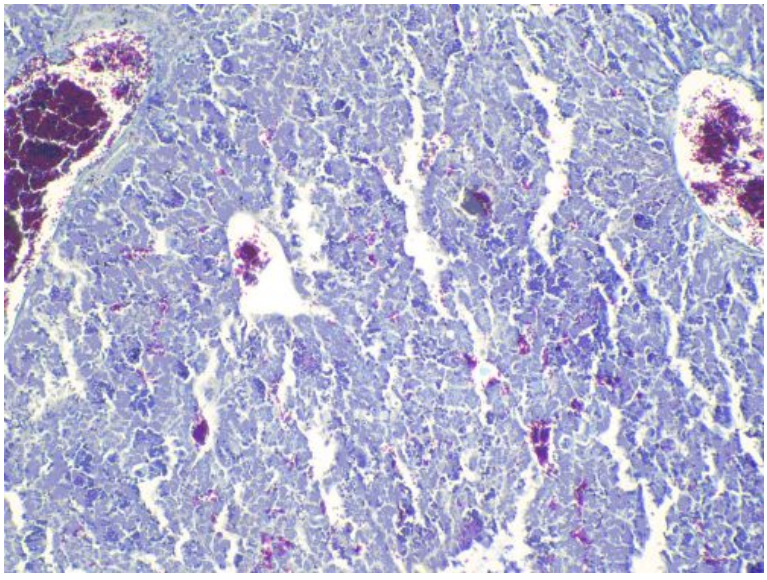


Fig. 4 – Swine embryo - 50 days; section through the liver; Col. cold Giemsa; Ob. 10x

The reticulin fibers are present in the Glisson capsule and also in the wall of the sinusoidal capillaries (fig. 5).

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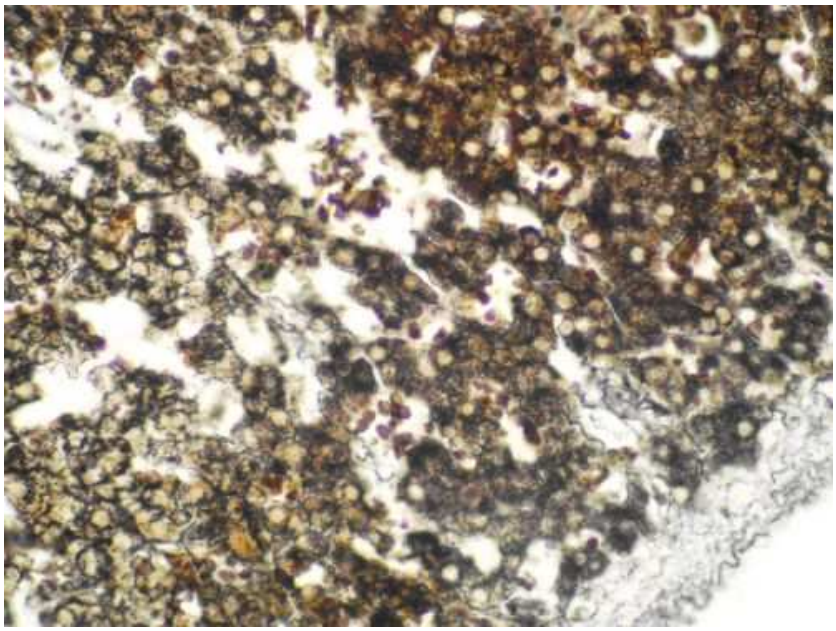


Fig. 5 – Swine embryo - 50 days; section through the liver;
The silver impregnation Gömöri; Ob. 40x

The esophagus is in course of stuctural organization. The esophagus has 3 tunics (mucosa, musculous and adventicea), the submucosal being undifferentiated. The mucosa muscular is not present. The esophagian mucosa is in course of differentiation: the stratified epithelium and the chorion are present (fig. 6), without the mucosa muscular which would delimitate the mucosa from the submucosa.

The epithelium on the basal layer seems to be formed of several cell layers with an intense mitotic activity which generates the cells of the stratum spinosum, while the pavimentous layer is missing. The epithelium is delimited from the chorion by an obvious basal membrane.In the chorion one can notice fibroblasts and fibrocites spread among the mesenchymal cells.

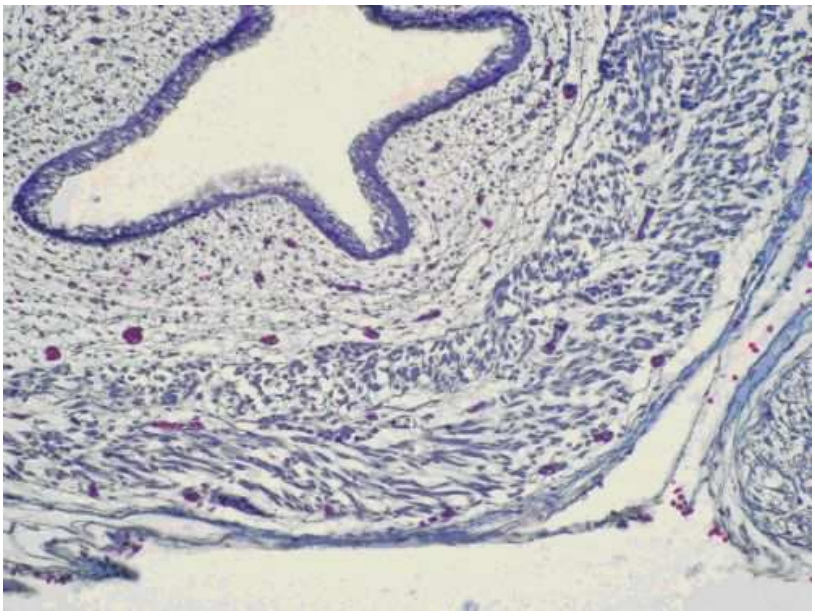


Fig. 6 – Swine embryo - 50 days; section through esophagus; Col. HE; Ob. 20x

The stomach is formed only of mucosa, muscular and serous, the absence of the mucosa muscular does not permit the delimitation of the chorion from the mucosa. The gastric mucosa presents folds that will later transform intoconivent valvules. The gastric folds are covered by a simple prismatic epithelium presenting accumulation of mucine at the apical pole.(fig. 7). The gastric chorion has no glands.At the subepithelial level it is formed by a conjunctive mesenchymal condensed area. Its deeper level is formed by a conjunctive lax area in which the fibroblasts and the fibrocites are predominant, along with the limphatic and sanguineous capillaries.

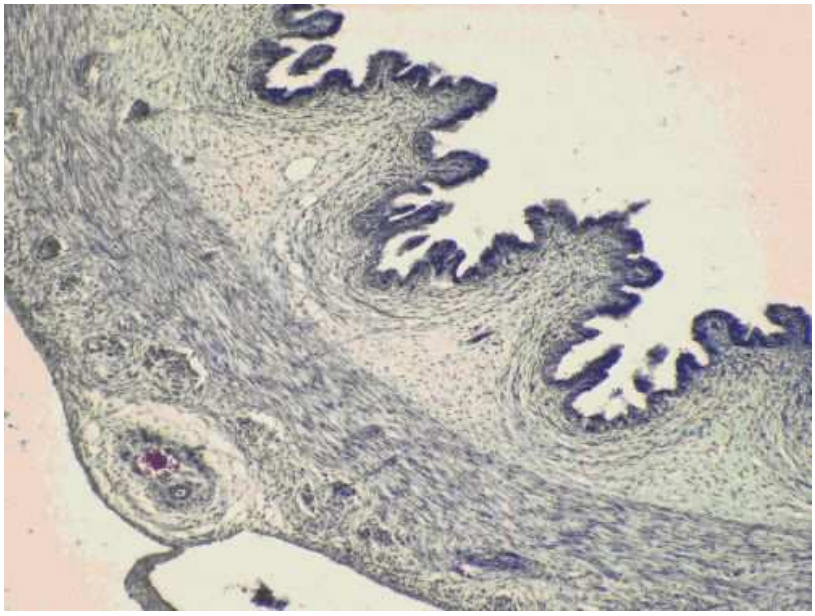


Fig. 7 – Swine embryo - 50 days; section by the stomach;

Col. trichromical Mallory; Ob. 10x

The small intestine presents well developped vilosities and the enterocites have a prismatic shape with the spherical nucleus disposed on the central part of the superior third.The apical pole is not differentiated.The mucosa muscular is in course of differentiation. The tunics of the thin intestine appear differentiated especially at the level of the submucosa , the muscular wall being in course of organization.The chorion of the intestinal mucosa is weakly developped and has no Lieberkühnn glands.The submucosa has thje form o a fine layer of collagen fibers (fig. 8).

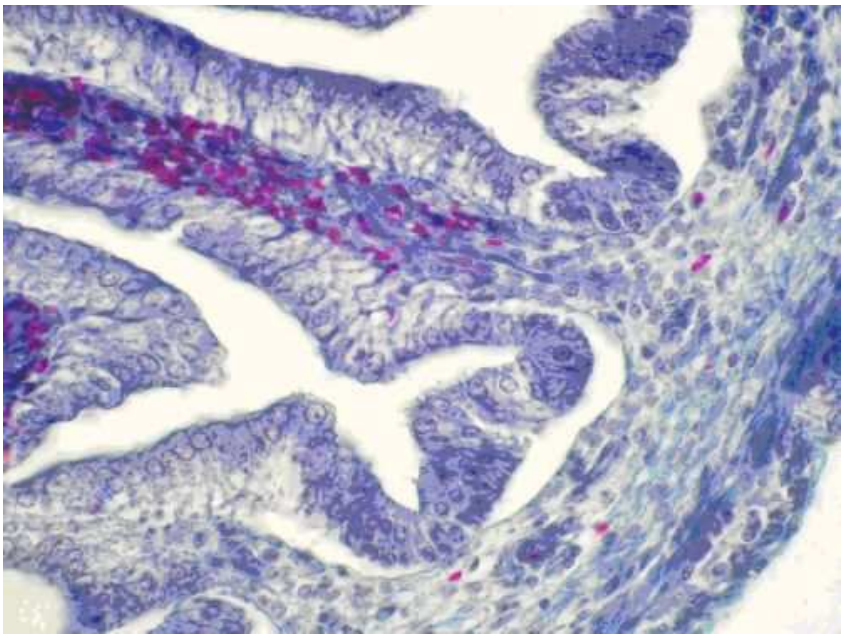


Fig. 8 – Swine embryo - 50 days; section by the intestine;

Col. trichromical Mallory; Ob. 40x

In the vilosity axis, the blood vessels and the lymphatic vessels can be easily noticed and are accompanied by mesenchymal conjunctive tissue populated withmesenchymal cells, fibroblasts, fibrocites and lymphocites.

The kidney-has the morphological structures of the nephron differentiated. One can notice the Malpighi corpuscules- formed of fenestrated capillaries of the vascular ball, inglobated in a renal mesangium- and the Bowman capsule with the podocytes disposed on the basal membrane,the filtration space being present between the two laminae of the Bowman capsule (fig. 9).

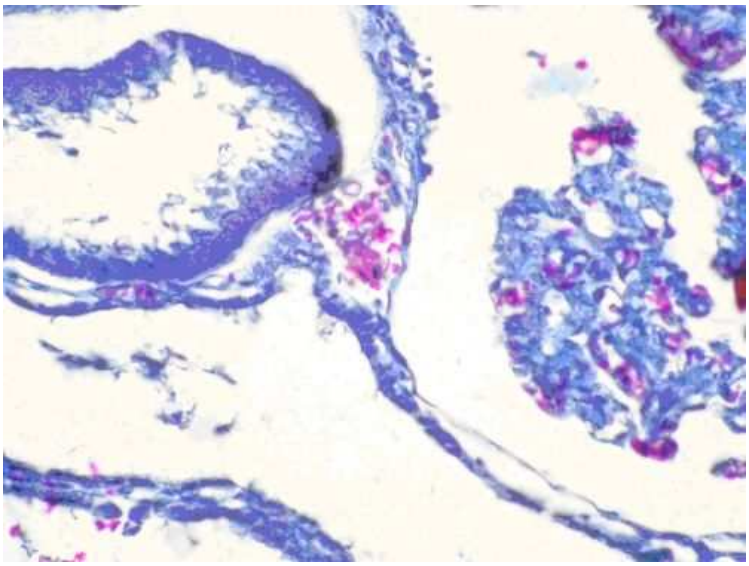


Fig. 9 – - Swine embryo - 50 days; section through kidney;

Col. trichromical Mallory; Ob. 40x

The uriniferous tubes have the nephrocytes disposed on the basal membrane like a simple cubic epithelium. The nephrocytes are present and functional in the contort tubules and at the apical pole

the slightly differentiated microvilli are disposed like a brush border. (fig.. 9) The structural elements of the juxtaglomerular apparatus are not present.

At the level of interstitial conjunctive tissue situated between the uriniferous tubules and at the level of the vascular glomerus ,the reticulin fibers can be noticed .(fig. 10).

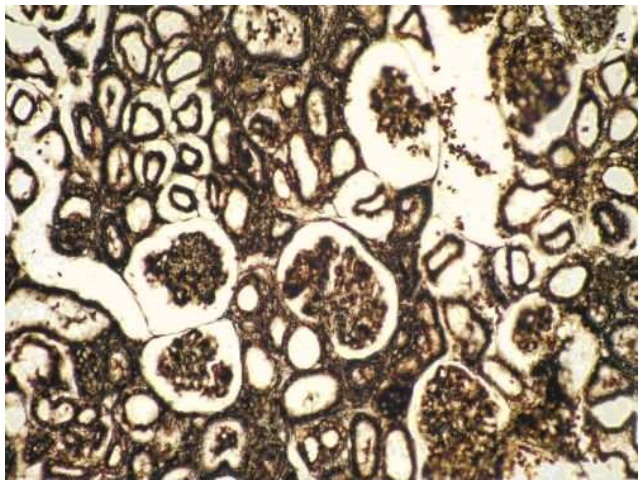


Fig. 10 – Swine embryo - 50 days; section by the kidneys;
The silver impregnation Gömöri; Ob. 40x

CONCLUSIONS

1. The lung presents lobar bronchi; the conjunctive cartilaginous tunics and the bronchial buds are in course of differentiation. The pulmonary mesenchyma begins the differentiation of the bronchial chorion that surrounds the bronchial epithelial buds. The pulmonary lobules are not organized ordelimitated by the conjunctive tissue.

2. The esophagus presents the epithelium of the mucous in course of differentiation and the absence of the mucosa muscular does not permit the delimitation of the chorion from the mucosa.

3. The liver presents the hepatocytes disposed as Remack strings that converge towards the centrolobular vein. The Kiernan spaces and the perilobular conjunctive tissue are not clearly delimitated. In the hepatic sinusoidal capillaries there arte agglomerations of erythroblasts, erythrocytes and other figurative elements.

4. The stomach and the thin **intestine** are formed only of mucous, muscular and serous;the submucous is not differentiated. The mucosa muscular is in course of differentiation. The two organs have no glandular elements.

5. The kidney the constitutive elements of the nephron are differentiated, the nephrocytes are functional with the apical pole differentiated. The microvilli are present but without the structural elements of the juxtaglomerular apparatus.

REFERENCES

1. Georgieva R.K., K. Gerov, 1975, *The morphological and functional differentiation of the alimentary canal of the pig during ontogeny. I. Development and differentiation of the fundic portion of the stomach,* Anat. Anz, 137(1-2):12-15.

2. Hill M., 2003, *6 mm pig embryos*, UNSW Embryology, Australia, http://anatomy med. unsw.edu.an./www. Pig/pig sty.htm.

3. Pelliniemi L. J., 1990, *Ultrastructure of the early ovary and testis in pig embryo*, American Journal of Anatomy, vol. 144, pg. 89-111.

4. Petruț T., T. Coman, M. Cucoaneş, N. Velicu, 2006, *Dezvoltarea ontogenetică intrauterină a rinichiului, pulmonului, intestinului şi ficatului la embrionul suin de 45 de zile,*

Analele Universității Spiru Haret, Seria Medicină Veterinară, Anul VI, nr. 6-7, Ed. Fundației România de Mâine, pag. 15-21.

5. Petruț T., T. Coman, 2007, *The Histological Structure in Swine Embryo Aged 60 Days*, Analele Universității Spiru Haret, Seria Medicină Veterinară, Anul VIII, nr. 8, Ed. Fundației România de Mâine, pag. 25-38.

6. Schoenwolf C.G., 1973, *6 mm pig embryos*, Laboratory studies of vertebrate and invertebrate embryos, Prentince Hall, Englewood Cliffs, New Jersey, 7632, pg. 99-124.

LEUKOCYTES REACTIONS IN RABBITS AS A RESULT OF *HELLEBORUS PURPURASCENS* ROOT IMPLANT
REACȚIA LEUCOCITARĂ LA IEPURI CA REZULTAT AL IMPLANTULUI DE RĂDĂCINĂ DE *HELLEBORUS PURPURASCENS*

VELICU N., D. CONDUR, N. BERCARU, T. PETRUȚ
Faculty of Veterinary Medicine, Spiru Haret University
Email: ushmv_velicu.nicolae@spiruharet.ro

REZUMAT

Introducerea transcutanată a rădăcinii de spânz la animale, mai ales la bovine, porcine, ovine, şi cabaline, denumită în folclor „spânzit" sau „tras cu rădăcină de spânz" este un procedeu terapeutic etnoiatric vechi, cunoscut şi aplicat în toate regiunile din ţara noastră.

Pentru verificarea leucocitozei produsă de spânz, disciplina de Propedeutică veterinară de la Facultatea de Medicină Veterinară Spiru Haret a întreprins o serie de cercetări la iepuri. La această specie scopul cercetărilor a fost compararea leucocitozei produsă de spânz cu leucocitoza indusă de preparate de tip Cantastim şi Polidin.

S-au folosit 4 loturi de iepuri după cum urmează:

Lotul 1 – 10 iepuri în vârstă de 3 luni, injectaţi timp de 3 zile cu Polidin s.c. 0,5 ml/animal/zi;

Lotul 2 – 10 iepuri în vârstă de 3 luni, injectaţi timp de 3 zile cu Cantastim s.c. 0,5 ml/animal/zi;

Lotul 3 – 10 iepuri în vârstă de 3 luni, la care s-a implantat s.c. rădăcină de spânz (un fragment de 2-3 mm) care a fost extras după 24 de ore;

Lotul 4 – 10 iepuri în vârstă de 3 luni, a reprezentat lotul martor.

Examenele hematologice s-au efectuat după 3 zile de la ultima administrare şi au constat în:

- numărarea leucocitelor prin metoda electronică la un aparat de tip Coulter-Counter;

- numărarea diferenţiată a diferitelor tipuri de leucocite prin metoda indirectă, după efectuarea formulei leucocitare.

Rezultatele obţinute au evidenţiat că leucocitoza cea mai modestă se obţine după Polidin (leucocitele au crescut numeric cu 15-20%, neutrofilele cu 40-100%), Cantastimul a avut un efect aproape dublu faţă de Polidin, iar rădăcina de spânz un efect triplu. Nu s-a observat o ascensiune febrilă după spânz. Din aceste date experimentale preliminare se constată o leucocitoză şi neutrofilie pregnantă, efectul produs de implantul de spânz fiind mai puternic decât cel produs de medicaţia clasică.

Cuvinte cheie: implant de spânz, leucocitoză, iepuri.

ABSTRACT

The transcutaneous introduction of Heleborus purpurascens root to animals, especially to calf, pigs, muttons and horses is an old, popular, terapeuthic procedure, known and applied in all regions of our country.

In order to check the leukocytosis produced by Heleborus purpurascens, the disciplin of Propedeutics of Spiru Haret Faculty of Veterinay Medicine has done a series of research in rabbit. The purpose of research was comparing the leukocytosis induced by Heleborus purpurascens with the one induced by substances such as Cantastim and Polidin.

There were used 4 lots of rabbits as it follows:

Lot 1-10, 3month old rabbits, injected for 3 days with Polidin s.c. 0,5 ml/animal/day.

Lot 2-10, 3 month old rabbits, injected for 3 days with Cantastim s.c. 0,5ml/animal/day.

Lot 3-10, 3 month old rabbits, implanted with Heleborus purpurascens root(a fragment of 2-3 mm) which was removed after 24h;

Lot 4-10, 3 month old rabbits, witness lot.

The haematological exams were done after 3 days from the last administration and consisted in:

- counting the leukocytes through electronic method with a Coulter-Counter device;
- counting the different types of leukocytes through indirect method, after obtaining the leukocytes formula.

The results showed that the least leukocytosis is induced by Polidin (the leukocytes number grew by 15/20% and the neutrophiles number by 40-100%). The cantastim had almost doubled the effect of Polidin, while the Heleborus purpurascens tripled it. No feverish reaction was noticed after Heleborus purpurascens intake. From these preliminary experimental data one can notice a steady leukocytosis and neutrophily, the effect of Heleborus purpurascens being stronger than the one induced by classic medication.

Key words: Helleborus purpurascens implant, leukocytosis, rabbits.

INTRODUCTION

The hellebore (Helleborus purpurascens, fam. Ranunculaceae) is a herbaceous plant, spread in the broadleaf forests and in the wet hayfields on the hilly and mountaineous areas.

It has a thick rhizome with many unramified fine roots. During springtime a flowering stem that can grow up to 50 cm, develops out of the rhizome. 2-3 green or reddish odourless flowers develop on the stem; the 2 basal leaves have a long petiole and a palmate-serrated blade.

The drug itself lies in the rhizome(with its roots) and is collected in august-september. The rhizome is up to 10 cm length and 3-8 mm thick and at the tip one can notice the marks of its aereal parts of the past years.

A lot of fine roots branch off from the rhizome, 2-5 mm in diameter, dark brown to black when dry. The drug is odourless with a hot, spicy, galling taste.

It contains 3 types of active principles:

- bufanolide-steroidal glycosides (the most important is the helebrin which generates helebrigenin, glycosis and ramnosis);
- 2 saponosides: heleborin and heleborein
- an unsaturated lactone: protoanemonin.

Due to the bufanolide glycosides, the hellebore has a cardiotonic action, similar to that of digital and strophantin and also vomitive and purgative effects. Helleborus purpurascens also has an irritant, severe purgative and oxytocic effects, due to its saponosides. Apud Neagu et col.(6), heleborin has effects on the nervous system, too.

The active principles of the hellebore (H. purpurascens) are found mostly in the rhizome roots and in the seeds.

MATERIAL AND METHODS

The purpose of the research was the comparative analysis of leukocytosis induced by Helleborus purpurascens and leukocytosis induced by substances like Cantastim and Polidin.

There were used 4 lots of rabbits, as follows:

Lot 1-10, 3 month-old rabbits, injected for 3 days with Polidin s.c. 0.5 ml/animal/day.

Lot 2-10, 3 month-old rabbits, injected for 3 days with Cantastim s.c. 0.5 ml/animal/day.

Lot 3-10, 3 month-old rabbits, implanted with Helleborus purpurascens root (a 2-3 mm fragment) which was removed after 24 hours.

Lot 4-10, 3 month-old rabbits, witness lot.

The haematological exams were done 3 days after the last administration and consisted in:

- counting the leukocytes through electronic method with a Coulter-Counter device;
- counting the different types of leukocytes through indirect method, after obtaining the leukocytes formula.

RESULTS AND DISCUSSION

Leukocytes in rabbit have a morphological structure similar to that of other species. Lymphocytes have a big, spherical nucleus and occasionally azurophile granules(fig. 1,2). Monocytes are the biggest figurate elements with a less condensed cromatine nucleus than the (heterophil) neutrophil (fig.1).

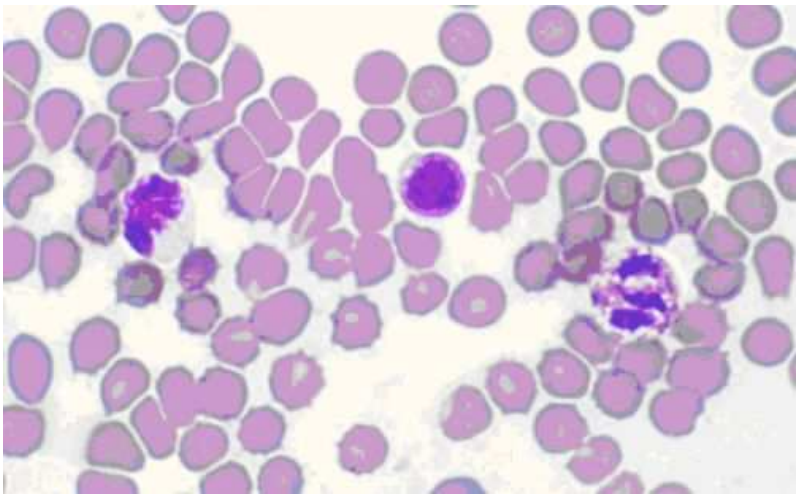


Fig. 1 – Rabbit smear; Hemacolor panoptic stain; 100x Ob.

Heterophils are often characterized by a segmented nucleus, the cytoplasm granules being smaller when compared to eosinophils (fig.1).

Eosinophils often have bilobated nucleus, the intracytoplasmic granules being more numerous and bigger (fig.2).

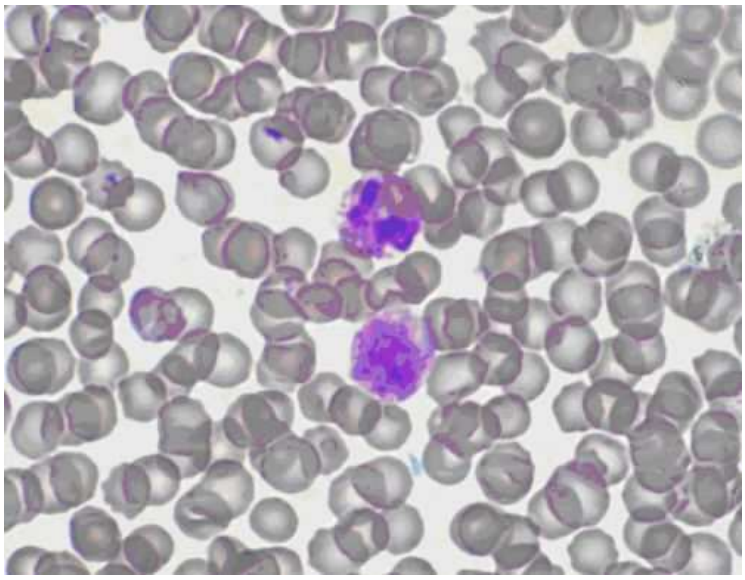


Fig. 2 – Rabbit smear; Hemacolor panoptic stain; 100x Ob.

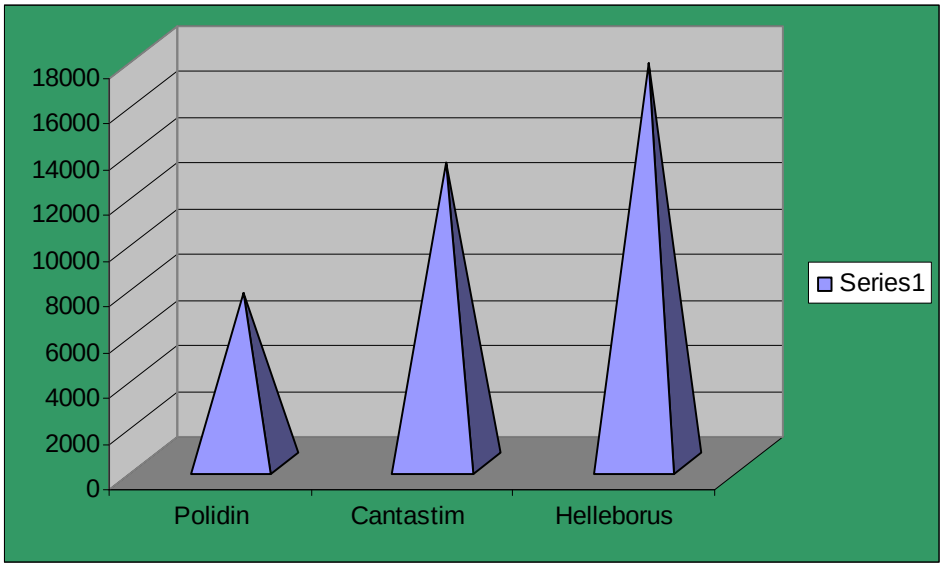
Here are the results by lots, centralised in table 1, with their comparative representation in graphics 1and 2:

Table 1

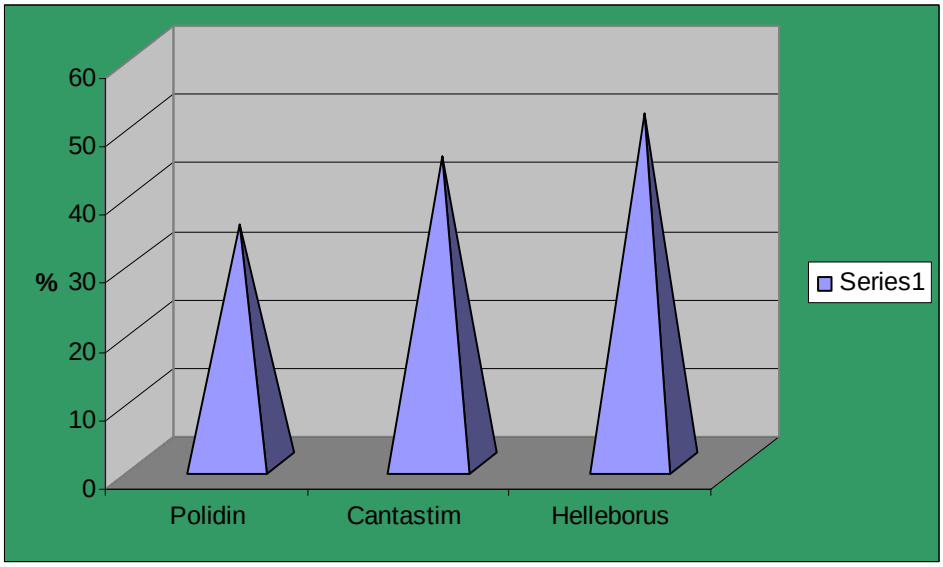
The variation of the leukocyte formula in the witness lot and in the experimental lots:

Lot	Specificație	Leucocite mii/mm^3 sânge	Neutrofile %	Eozinofile %	Bazofile %	Limfocite %	Monocite %

1	înainte de administrare	6.200	29	1.3	13	54.8	1.9
	Polidin	7.440	34.8	1.1	6.2	56	1.9
2	înainte de administrare	9.400	32	1.6	16	48.6	1.8
	Cantastim	13.160	44.8	1.0	3.4	49	1.8
3	înainte de administrare	10.300	33	2.0	12.5	43.2	2.3
	Helleborus purpurascens	17.510	51.1	1.0	1.6	44	2.3
4	martor	4000-14.000	8-50	1.0-3.0	2.3-31	20-90	1.0-4.0



Graphic 1 - Number variation of leukocytes in experimental lots



Graphic 2 - Percentage variation of neutrophils in experimental lots

The following facts can be noticed after the analysis of the results:

- in the lot injected with Polidin, the leukocytosis and the correlated neutrophilia registered a 20% growth;
- in the lot injected with Cantastim, the leukocytosis and the correlated neutrophilia registered a 40% growth;
- in the lot implanted with Helleborus purpurascens root, leukocytosis and the correlated neutrophilia registered a 70% growth.

The increased number of neutrophils was correlated with eosinopenia and basopenia. During the experimental period, monocytemia has not suffered obvious changes. The white elements got back within normal parameters 6-10 days after.

CONCLUSIONS

1. The usage of Polidin, Cantastim and hellebore implant is correlated with severe leukocytosis and neutrophilia.
2. The hellebore implant generates the strongest effect in leukocytosis and neutrophilia (hyperleukocytosis).
3. In veterinary medicine, the hellebore can be used for its hyperleukocytosis effect and also for being cardiotonic, antirheumatic, analgesic, oxytocic, vomitive, etc.).

REFERENCES

1. Bruneton J., 1993, *Pharmacognosie, Phytochimie, Plantes medicinales*, II, Ed. Tec – Doc Londra-Paris- New York, p. 596-597.
2. Campbell T.W., 2004, *Mammalian hematology: Laboratory animals and Miscellaneous species*. In: Thrall MA: *Veterinary Hematology and Clinical Chemistry*, 1 st ed, Lippincott Williams and Wilkins, p. 211-224.
3. Grigorescu E., I.M. Lazăr, Ursula Stănescu, I. Ciulei, 2001, *Index fitoterapeutic*, Ed. Cantes Iaşi, p. 255-256.
4. Mohan Gh., 2001, *Atlasul plantelor medicinale din România*, Ed. Corint, Bucureşti, p. 72.
5. Mozăceni – Vasilca, 2003, *Ghidul plantelor medicinale*, Ed. Polirom, Bucureşti, p. 168-169.
6. Neagu E., C-tin Stătescu, 1985, *Practicum de fitoterapie şi fitotoxicologie în medicina veterinară*, Ed. Ceres, Bucureşti.
7. Nueleanu Veturia Ileana, 2006, *Farmacologie veterinară*, Ed. Risoprint, Cluj Napoca, p. 406-407.
8. *** Farmacopeea Română, Ed. X-a, 1993, p. 872-873.

THE CONTAMINATION WITH MYCOTOXINS OF COMMERCIAL DRY FOOD FOR FOOD USED IN DOGS ALIMENTATION
CONTAMINAREA CU MICOTOXINE A HRANEI COMERCIALE USCATE PENTRU ALIMENTATIA LA CAINI

MANDA Floriana[1], M. SALLAY [1], Violeta-Elena SIMION[1], C. NEGREANU[2], Adriana AMFIM [1]

[1] Veterinary Medicine Faculty, Spiru Haret University, Bucureşti, simionvioleta@zoonutritie.ro
[2] DSV Ilioara Bucureşti

REZUMAT

Micotoxinele sunt metaboliți secundari ai fungilor care afectează sănătatea tuturor speciilor de animale.

Cercetarea a constat în investigarea prin examen micotoxicologic a unor probe de hrană comercială uscată destinată alimentației la câini, în vederea determinării gradului de contaminare cu unele micotoxine.

Au fost recoltate 11 probe de hrană uscată din diverse centre de vănzare a acestor produse şi s-au efectuat 22 de analize, câte două analize pentru fiecare probă.

Analiza micotoxicologică s-a efectuat prin testul imunoenzimatic ELISA pentru determinarea micotoxinelor aflatoxina B_1 şi ochratoxina A.

Din analiza micotoxicologică a rezultat nedetectarea aflatoxinei B_1 în 5 probe (10 analize) reprezentând 45,5% şi detectarea aflatoxinei în 6 probe (12 analize) reprezentând 54,5%; nici una dintre probele analizate nu a depăşit limita maximă admisă. Analiza micotoxicologică pentru ochratoxină a relevat nedetectarea acesteia în 7 probe (14 analize) reprezentând 63,6% şi detectarea în 4 probe (8 analize) reprezentând 36,4%; nici una dintre probele analizate nu a conținut ochratoxină peste limita admisă la această specie.

Din totalul de 22 de analize efectuate, în 6 analize a fost determinată aflatoxina B_1, cu valori sub limita maximă admisă şi în 8 analize a fost determinată ochratoxina A cu valori sub limita admisă la această specie.

Cuvinte cheie: hrană uscată, câine, aflatoxina B_1, ochratoxina A

ABSTRACT

Mycotoxins are fungi secondary metabolits which affect health of all animal species.

The research consisted in the investigation by means of a mycotoxicological exam of some commercial dry food assays meant for dogs alimentation, in order to determine the contamination degree with some mycotoxins.

11 assays of dry food were taken from different centres where these products are sold and 22 analyses were performed, two analyses for each assay.

The mycotoxicological analysis was performed by means of the immunoenzymatic test ELISA for the determination of the mycotoxins: aflatoxin B_1 and ochratoxin A.

The results of the mycotoxicological analysis were the following: aflatoxin B_1 was not detected in 5 assays (10 analyses) representing 45,5% but it was detected in 6 assays (12 analyses) representing 54,5%; none of the analyzed assays exceeded the maximum allowed limit. The mycotoxicological analysis for ochratoxin revealed the following results: it was not detected in 7 assays (14 analyses) representing 63,6% but it was detected in 4 assays (8 analyses) representing 36,4%; none of the analyzed assays contained ochratoxin exceeding the allowed limit for this species.

From the total of 22 analyses that were performed, : aflatoxin B_1 was detected in 6 analyses having values under the maximum allowed limit and ochratoxin A was detected in 8 analyses, having values under the maximum allowed limit for this species.

Key words: dry food, dog, aflatoxin B_1, ochratoxin A

INTRODUCTION

Fungi are microorganisms that grow on a variety of substrata, including in dry food meant for dog alimentation and for other pets (cats, cage birds, rodents). Fungi can develop in the substratum represented by the fodders used as ingredients (especially cereal grains) or in the product itself, during the manufacture process, during shipping or in the storage period.

Mycotoxins are fungi secondary metabolits, extremely agressive for animals health, including for pets health.

The research had as purpose the mycotoxicological analysis regarding the contamination with aflatoxin B1 and ochratoxin A of the commercial dry food for dogs, starting from the premises that the type of mycotoxins and their quantities in fodder are inscrutable from one period to another.

Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) are mycotoxins produced in nature by species of mychetes from the genders *Aspergillus* (*A. flavus* and *A. parasiticus*) and more rarely by some species from the gender *Penicillium* (*P. puberulum*, *P. citrinum*, *P. variable*) and *Rhizopus spp*.

Ochratoxins: A, B, C, D represent a group of compounds in whose chemical structure L-fenilalanine is combined by means of an amidical bond with an isocumarinic derivate (Fuchs, 1988). The production of these ochratoxins is encouraged by musty or hot fodder, the presence of oligoelements in the environment, a temperature of 20 – 28 °C and humidity of 18-19% at wheat, in the case of *P. Viridicatum* or 22% at maize.

MATERIALS AND METHODS

In order to examine two of the hepato-nephrotoxic mycotoxins – aflatoxin and ochratoxin, 22 assays of dry food used for the alimentation of dogs, taken from commercial centers in Bucharest, were studied.

The quantitative identification of the mycotoxins (AFB1 and OTA) from food and fodder was performed using ELISA method, direct competitive immunoenzymatic test.

5g were taken from each analyzed assay which was processed by grinding; these 5 g were extracted with 25 ml of methanol 25%; the obtained essence was filtered using filter paper. Standards, assay essence and the mycotoxin combined with the enzyme were mixed together and then, they were added in the reservoirs coated with antibodies. After wash, the enzymatic substratum was added, the intensity of the blue colour thus obtained was in inverse ratio to the concentration of the mycotoxin from the assay or from the standard. After adding the stopping solution, blue turned into yellow, the intensity of the colour was measured by spectrophotometrical means using a microplates reader having a filter of 450nm.

The optical densities (OD) of the assays were compared to those of the standards, thus determining the concentrations of the assays.

RESULTS AND DISCUSSIONS

After analyzing the 22 assays of dry food for dogs, respectively 11 assays of food analyzed in double assays, aflatoxin B1 (AFB1) and ochratoxin A (OTA) were identified as shown below.

A) Analysis of aflatoxin B1 in the dry food assays.

After the analysis of the dry food assays in order to identify AFB1, the following results were obtained, as shown in table 1 and chart 1.

Table 1

Results obtained at the analysis of mycotoxin AFB1

Number of the assay	Absorbent 1	Absorbent 2	Average of absorbents	Obtained value µg/kg
1.	1.159	1.125	1.142	1.26
2.	1.039	1.038	1.0385	1.57
3.	1.695	1.724	1.7095	0.39
4.	1.743	1.718	1.7305	0.37
5.	1.399	1.359	1.379	0.77
6.	1.576	1.628	1.602	0.48
7.	1.326	1.304	1.315	0.88
8.	1.066	1.028	1.047	1.54
9.	1.685	1.629	1.657	0.43
10.	1.683	1.699	1.691	0.40
11.	0.688	0.653	1.315	0.88

From the 11 assays analyzed for aflatoxin B1, in 5 analyzed assays the results were not detectable (under the detection limit of 0,5 µg/kg of the kit) representing a percentage of 45,5%. At the other 6 assays representing a percentage of 54,5% from the analyzed assays, the results had values over the detection limit between 0,77 – 1,57 µg/kg. If food had been meant for human consumption, it would have had values within tha maximum allowed limits.

In chart 1, the incidence of AFB1 in the analyzed assays is shown.

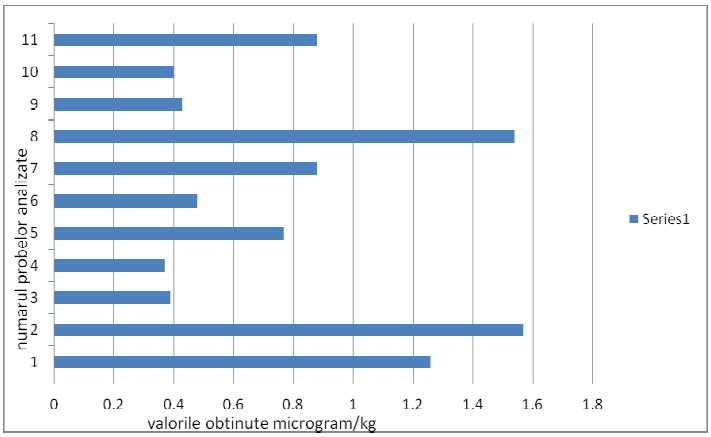


Chart 1. The representation of the contamination degree with AFB1 of the analyzed assays

Aflatoxicosis at dogs was reported for the first time in 1952 in the United States (Newberne, 1973) and it was called ,,hepatitis X'' and then it was reproduced as a disease, in 1955. Liver is the main affected organ in this mycotoxicosis, aggressive both for dogs and for cats. Among pets, cage birds and fish are considered to be the most sensitive (Rumbeiha, 2007).Except for the nephrotoxic effect, AFB1 also has immunosuppressive effects. DL50 for aflatoxin B1 is of 0,5 – 1,0 mg/kg at dogs and respectively of 0,3 – 0,6 mg/kg at cats (Rumbeiha, 2007).

The cases of aflatoxicosis reported at dogs were rare, to a great extent due to the control of the quality of food used in dogs alimentation. Thus, most recently Devegowda and Castaldo (2000) reported a case of aflatoxicosis at dogs (1990, USA), after the consumption during 3-4 months of the food contaminated with 100 – 300 ppb aflatoxin B1.

Cereals are good matrices for fungi growth (Gonzales and col., 1997) but the production of mycotoxins is not always related to the presence of fungi which produced them in that substratum. Toxins can persist much longer after fungi dissapeared from the substratum.

Similar studies regarding the contamination with mycotoxins of food for pets were performed in different countries. The analysis using HPLC method of 20 assays of food for dogs, 20 assays of food for cats and 20 assays of food for cage birds (10 assays of food for canaries and 10 assays of food for parrots) revealed the absence of the mycotoxin AFB1 from the analyzed assays although there was a reduced contamination with fungi, especially from the genders *Aspergillus* (58,3%), *Penicillium* (38,3%) and *Mucor* (38,3%). The analysis of 100 food assays made of cereals and meant for pets revealed the absence of mycotoxins in 84% of the analyzed assays and the presence of AFB1 in quantities under the detection limit in one food assay for cats and in concentration of 370 µg AFB1/kg food in an assay of dry food for birds.

B) The analysis of OTA in the assays of dry food.

The analysis of the ochratoxin A in the assays of dry food revealed its presence in varied quantities as they are shown in table 2 and chart 2.

Table 2

Results obtained at the analysis of mycotoxin OTA

Assay number	Absorbent 1	Absorbent 2	Average of absorbents	Obtained value µg/kg
1.	1.102	1.069	1.0855	0.53728
2.	1.110	1.123	1.1165	0.461845
3.	1.036	0.949	0.9925	0.845888
4.	0.833	0.876	0.8545	1.65883
5.	0.759	0.755	0.757	2.669635
6.	0.575	0.512	0.5435	7.567673
7.	0.917	0.951	0.934	1.125389
8.	1.126	1.123	1.1245	0.444161
9.	1.368	1.316	1.342	0.153657
10.	1.369	1.456	1.4125	0.108925
11.	1.388	1.398	1.393	0.1198

From the 11 assays analyzed for OTA, at 7 analyzed assays the results were undetectable (under the detection limit of the kit of 1 µg/kg) representing a percentage of 63,6%. At the other 4 assays representing a percentage of 36,4% from the analyzed assays, results had values over the detection limit, meaning between 1,12 – 7,56 µg/kg. At assay no. 6, the obtained value exceeded the maximum allowed limit for this mycotoxin in processed cereals (7.5 ppb).

In chart 2, the incidence of OTA in the analyzed assays is shown.

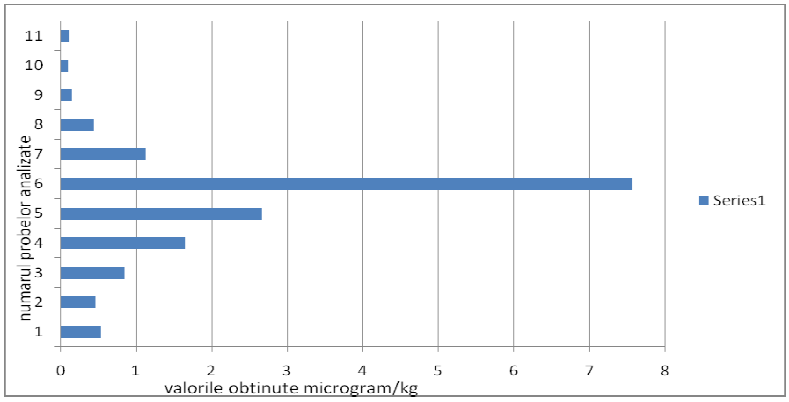


Chart 2. The representation of the contamination degree with OTA of the analyzed assays

Ochratoxicosis is met more rarely at dogs in comparison with aflatoxicosis. The most complete studies regarding the effect of OTA upon dogs were performed in 1977 by Kitchen and col. Although a maximum lethal dose for this mycotoxin was not determined yet, after the ingestion of 0,2 – 3,0 mg OTA/kg food, pathological clinical manifestations were seen and they included muchohemoragic entheritis and bigger limphonodules. As well as at other animal species, kidneys are the main target of OTA in the body.

Other similar studies regarding the incidence of OTA in pets food were performed by numerous researchers. In a study performed in Portugal, the analysis, using the HPLC method, of 20 assays of food for dogs, 20 assays of food for cats and 20 assays of food for cage birds revealed the presence of OTA only in dogs food, in 5 assays. These represented 8,3% from the analyzed assays and they had values between 2,0 – 3,6 μg/kg food. The analysis using HPLC of 30 assays of food for cage birds revealed the presence of OTA in 10% of the analyzed assays, the highest concentration being of 7 μg/kg food (Scudamore and col., 1997).

Parallel studies regarding the contamination level with OTA of dry food for cats and the level of the mycotoxin in the renal tissue did not prove a correlation between these levels. The assays of analyzed food contained OTA in concentration of 0,1 – 0,8 μg/kg food, and in 2 assays there were values of 3,2 and respectively 13,1 μg/kg food; in the renal tissues taken from the cats that consumed this food and showed renal manifestations OTA was determined in concentration of 0,35 – 1,5 μg/kg tissue (Razzazi and col., 2001).

It must be highlighted that, additional to the modification of the pets state of health, the presence of mycotoxins in the food meant for pets consumption leads to major economical problems both for food producers and for pets owners.

In annexes 1 and 2, the charts of the determinations performed for AFB1 and OTA are shown, the determinations were made according to the analysis kit that was used.

CONCLUSIONS

After the tests that were made and after the results that were interpreted within the laboratory, regarding the analysis of the mycotoxins AFB1 and OTA of the 11 assays of dry food for dogs, the following conclusions resulted:

- From the 11 analyzed assays for aflatoxin B1, at 5 analyzed assays, the results were not detectable (under the detection limit of 0,5 μg/kg of the kit) representing a percentage of 54,5% from the analyzed assays, the results had values over the detection limit between 0,77 – 1,57 μg/kg.
- From the 11 analyzed assays for OTA, at 7 analyzed assays, the results were not detectable (under the detection limit of 1 μg/kg of the kit) representing a percentage of 63,6% and at 4 assays representing a percentage of 36,4% from the analyzed assays, the results had values over the detection limit between 1,12 – 7,56 μg/kg.
- Dry food meant for pets consumption does not present high contamination with the involved mycotoxins (AFB1 and OTA) because the obtained results had values corresponding to the communitary legislation in force and to the category of fodder to which they belong (4 ppb for AFB1 and respectively 5 ppb OTA), except for one assay in which OTA had the value of 7,56 ppb.

REFERENCES

1. Gonzalez H.H.L., Martinez E.J., Resnik S.L., 1997 – Fungi associated with sorghum grain from Argentina, Mycopathol. 139, pp.35-41.

2. Razzazi E., Böhm J., Grajewski J., Szczepaniak K., Kübber-Heiss A.J., Iben C.H., 2001 – residues of ochratoxin A in pet foods, canine and feline kidneys, J. Anim. Physiol. Anim. Nutr., 85(7-8), pp. 212-216.
3. Scudamore K.A., Hetmanski M.T., Nawaz S., Naylor J., Rainbird S., 1997 – Determination of mycotoxins in pet foods sold for domestic pets and wild birds using linked-column immunoassay clean-up and HPLC, Food additives and contaminants, Vol., 14, nr.2, pp 1750186.

AN EXPERIMENTAL STUDY OF GASTRIC MUCOSA IN OXIDATIV STRESS
UN STUDIU EXPERIMENTAL AL STRESULUI OXIDATIV LA NIVELUL MUCOASEI GASTRICE

DINU Cristina[1], Cristiana DIACONESCU[2], N. AVRAM[1], D. CUCA[1], C. COMAN[3], V.A. DUMITRU (student ush)
[1]Faculty of Veterinary Medicine Spiru Haret University
[2]USAMV Bucharest
[3] I.N.C.D.M.I. Cantacuzino Bucharest
Email: usamv_dinu.cristina@spiruharet.ro

REZUMAT

Acest studiu dorește să explice participarea speciilor reactive ale oxigenului (ROS) în afecțiunile cronice ale mucoasei gastrie prin administrarea apei de băut cu pH 4 și prin imersarea în apă la temperatura de 20°C de trei ori pe zi timp de 30 minute. După patru săptămâni animalele expuse la cei doi factori stresanți au fost sacrificați și s-a recoltat mucoasa gastrică pentru analiza activității superoxid dismutazei și a peroxidării lipidice. Nivelele malondialdehidei și a 4-hidroxinonenalul folosite ca indicatori ai peroxidării lipidice au crescut de la 5.85±0.04 nmol/g la 12.25±0.95 nmol/g pentru grupul "acid" și de la 5.85±0.04 nmol/g la 14.06±1.20 nmol/g pentru grupul imersat în apă. În grupul de control glutationul total a fost 230.20±20.12 mg/100g și glutationul redus de 170.32±97.66 mg/100g. În grupul "acid" nivelul glutationului total a scăzut la 200.10±19.10 mg/100g și al glutationului redus la 145.56±13.85 mg/100g. În grupul imersat în apă nivelul glutationului total a scăzut la 180.70 ±16.82 mg/100g și al glutationului redus la 130.60±10.64 mg/100g. Superoxid dismutaza în grupul de control a fost 340.30 ± 28.77 U/g. În grupul "acid" a scăzut la 255.18 ± 22.84 U/g și în grupul imersat în apă a scăzut la 215.73 ± 20.60 U/g.
Cuvinte cheie: mucoasa gastrică, superoxid dismutaza, malondialdehida, stres

ABSTRACT

This study aims to explain the participation of reactive oxygen species (ROS) in cronic gastric mucosal damage by drinking water with pH 4 and by undergoing 30 minutes for three times per day of water immersion in temperature 20°C. After four weeks the animals which were exposed at two damaging factors were sacrificed and gastric mucosa was collected for analyzing lipid peroxydation and superoxide dismutase activity. The levels of malondialdehyde and 4-hydroxynonenal used as indicators of lipid peroxidation, increased from 5.85 ± 0.04 nmol/g to 12.25±0.95 nmol/g for "acid" group and from 5.85 ± 0.04 nmol/g to14.06 ± 1.20 nmol/g for water immersion group. In the control group the total glutathione was 230.20±20.12 mg/100g and 170.32±97.66 mg/100g reduced glutathione. In the "acid" group the level of total glutathione decresed to 200.10±19.10 mg/100g and 145.56±13.85 mg/100g reduced glutathione. In water immersion group the level of total glutathione decresed to 180.70 ±16.82 mg/100g and 130.60±10.64 mg/100g reduced glutathione. Superoxide dismutase in control group was 340.30 ± 28.77 U/g of tissue. In "acid" group decreased to 255.18 ± 22.84 U/g and in water immersion group decreased to 215.73 ± 20.60 U/g.
Key words: gastric mucosa, superoxide dismutase, malondialdehyde, stress

INTRODUCTION

A variety of factors produce damage of gastric mucosa, including: systemic events such as psychological and physical stress, or local mucosal application of various irritants nutritional factors. The mucosal barrier is composed by epithelial cells with tight junctions and superimposed layer of mucus. The aim of this barrier is to protect the mucosa against damage of deeper structures by hydrogen ions (H+) and other noxious substances originating from the gastric lumen (9). The endogenous prostaglandins (PGs) play an important role in the maintenance of mucosal integrity, which includes continuous secretion of bicarbonate anions (HCO3-) and a mucus production in the stomach and duodenum (1). The imbalance between gastrotoxic agents and protective mechanisms results in an acute inflammation. This acute inflammation is accompanied by neutrophils infiltration of gastric mucosa. Neutrophils produce superoxide radical anion (O2•-), which belongs to group of reactive oxygen species (ROS). Superoxide radical anion reacts with cellular lipids, leading to the formation of lipid peroxides, that are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). The body has several enzymatic systems, which scavenges ROS and prevents their destructive action. The major antioxidative enzyme is superoxide dismutase (SOD). Three types of superoxide dismutase (SOD) can be distinguished: cytoplasmatic, mitochondrial and extracellular.

SOD catalyzes the dismutation of superoxide radical anion (O2•-) into less noxious hydrogen peroxide (H_2O_2), that is further degraded by catalase or glutathione peroxidase. Catalase is an enzyme which accelerates degradation of H_2O_2 into water and oxygen (7). The second pathway of H_2O_2 metabolism depends on the activity of glutathione peroxidase (GPx) and cooperating glutathione reductase. The reduction of H_2O_2 into water by GPx is accompanied by the conversion of glutathione from reduced form (GSH) into oxidized form (GSSG).

The aim of our present investigations is to demonstrate the participation of ROS in gastric mucosal damage by various irritants.

MATERIALS AND METHODS

Twenty four Wister white rats, weighing 120±20 g from Biobaza Cantacuzino were used. They were housed in plastic cages, 8/cages, in identical conditions of temperature (18°-20°C) and humidity (40-60°C). They were divided into three groups: a control group, which drank only water (pH 6.5), an "acid" group, which drank syrup water with acetic acid (pH 4), and a "water immersion stress" group. In 3rd group of animals underwent 30 minutes for three times per day of water immersion restraint stress in temperature 20°C. After four weeks they were sacrificed and gastric mucosa was collected for analyzing lipid peroxydation and superoxide dismutase activity. The levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were used as indicators of lipid peroxidation. The procedure of MDA and 4-HNE determination was following: 600 mg of gastric mucosa was excised. Then 20ml 0,5 M BHT (butylated hydroxytoluene) was added in order to prevent sample oxidation. This sample was subsequently homogenized in 20 mM Tris for15 sec. in pH=7.4. Then homogenate was centrifuged (3000´g at 4°C for 10 min.). The clear supernatant obtained was stored at -80°C prior to testing. The colorimetric assay for lipid peroxidation (Bioxytech LPO-586, Oxis, Portland, USA) was used to determine of MDA and 4-HNE tissue concentration. This assay is based on the reaction of a chromogenic reagent N-methyl-2-phenylindole with MDA and 4-HNE at 45°C. This reaction yields a stable chromophore with maximal absorbance at 586nm. This absorbance was measured by spectrophotometer S 300, Warsaw, Poland. Results were expressed as nanomol per gram of tissue (nmol/g) (6). To determine activity of superoxide dismutase (SOD), a sample of gastric mucosa was taken, as described above. The colorimetric assay for assessment of SOD activity (Bioxytech, SOD-525, Oxis, Portland, USA) was used. This method is based on the SOD-mediated increase in the rate of autooxidation of tetrahydrobenzofluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm (8). This absorbance was measured by spectrophotometer S 300, Warsaw, Poland. Outcomes were expressed as units per gram of tissue (U/g).

The concentration of total and reduced glutation was found out applying DTNB (5,5′ Dithio-bis-2 nitrobenzoic acid), method forming TNB (5THIO-2 nitrobenzoat) whose absorbtion was measured at λ 412 nm (10). The results were expressed in mg at 100 g gastric tissue.

Results were expressed as means ±SEM and were statistical analysis using „t“Test. Differences with $p<0.05$ were considered as significant.

RESULTS AND DISCUSSIONS

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in tissue are accepted as major products of lipid peroxidation. They are considered indicators of mucosa injuring by ROS. Concentration of MDA and 4-HNE in intact mucosa was at very low level, near to the analytical limit of detection, averaging 5.85 ± 0.04 nmol/g of tissue. After the administration of syrup water with acetic acid (pH 4), the levels of MDA and 4-HNE increased to 12.25±0.95 nmol/g and in case of water restraint stress, the level of lipid peroxides metabolites increased to 14.06 ± 1.20 nmol/g. These outcomes, in all investigated groups, were significantly higher, as compared with the values obtained in the intact mucosa (figure 1).

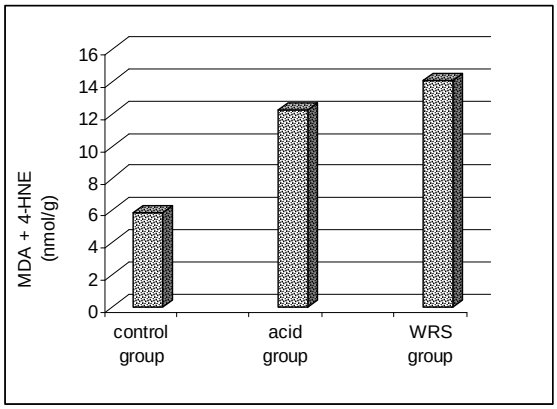


Figure 1. Concentration of MDA and 4-HNE (nmol/g) in the gastric mucosa in rats exposed to application of acetic acid (pH 4) and 30 min. for three times per day of water immersion (temp. 20°C) compared with the control group values. Results are mean ± SEM.

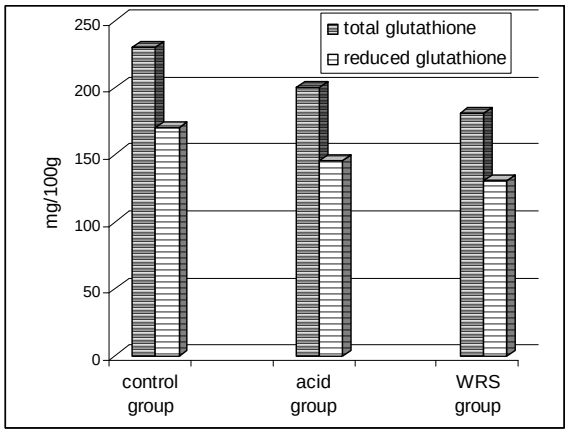


Figure 2. Total and reduced glutathione expressed as mg/100 g of fresh gastric mucosa in rats exposed to application of acetic acid (pH 4) and 30 minutes for three times per day of water immersion (temp. 20°C) compared with the control group values. Results are mean ± SEM.

The total and reduced glutathione were analyzed for three groups. In 1st group the total glutathione was 230.20±20.12 mg/100g and 170.32±97.66 mg/100g reduced glutathione. In 2nd group the level of total glutathione decresed to 200.10±19.10 mg/100g and 145.56±13.85 mg/100g reduced glutathione. In 3rd group- by water immersion stress the level of total glutathione decresed to 180.70 ±16.82 mg/100g and 130.60±10.64 mg/100g reduced glutathione (figure 2).

Enzymatic activity of superoxide dismutase (SOD) is a measure of antioxidative properties of cells. The activity of SOD in intact gastric mucosa reached high level, 340.30 ± 28.77 U/g of tissue. In 2nd group significant decrease resulted to 255.18 ± 22.84 U/g and in 3rd group an insignificant decrease resulted to 215.73 ± 20.60 U/g (figure 3).

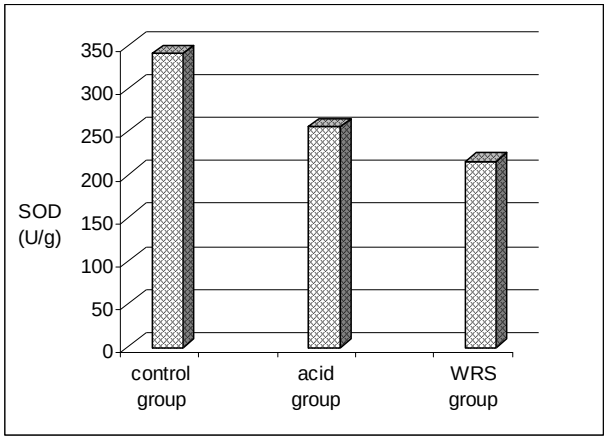


Figure 3. SOD activity (U/g) in the gastric mucosa in rats exposed to application of of acetic acid (pH 4) and 30 minutes for three times per day of water immersion (temp. 23°C) compared with the control group values. Results are mean ± SEM.

These values correlated with those of MDA concentration, namely the decrease of SOD activity induces favourising conditions for cell membrane lipoperoxidase. Consequently, under conditions of moderate oxidative stress, SOD activity increases demonstrating the effort of organisms to balance the oxidative effect, which enhances lipidperoxidative processes. During the process of prolonged or intensive oxidative stress SOD activity increases much owing to the enzyme inactivity.

Previous studies focused on the participation of ROS in pathogenesis of gastric diseases. This disease is more common than we think but they can be difficult to confirm. In digestive system investigations on ROS of pancreas (11), liver and small intestine predominantly concerned (4). Little information is available regarding the formation of ROS into esophagus and gastric mucosa, exposed to various damaging factors.

Erin et al. (5) attempted to explain the mechanism of radical production. He examined pathomechanisms of gastric mucosa damage, resulting from thermal stress. Animals in Erin's model, underwent thermal stress, in temperature 6°C, during 4 h. Erin et al. failed to observe any significant changes in MDA level in stressed stomach. In our investigations we applied different approach, animals underwent 30 minutes for three times per day of water immersion restraint stress (WRS) in temperature 23°C. Under these stress conditions, a significant increase of MDA level after WRS, accompanied by decrease of enzymatic activity of antioxidative enzyme-superoxide dismutase (SOD) were observed.

Previous research on metabolism of ROS in gastric mucosa focused on the effects of Helicobacter pylori infection. Davies at al (3) showed that such infection of human gastric mucosa resulted in an increase of ROS production, measured by chemiluminometry, as compared with healthy mucosa. In our investigations we confirmed that exposured gastric mucosa to oxidative stress, induced by drinking syrup water with acetic acid (pH 4), or water immmersion in temperature 23°C undergoing 30 minutes for three times per day leads to the generation of lipid peroxides, as expressed by an increase of tissue level of MDA accompanied by impairment of antioxidative defense mechanisms, such as decrement in SOD activity. Experiments carried out till now focused on measurement of MDA level, or its derivatives, in rat's liver after ethanol application (2). Alterations in SOD activity in rat cerebellum under an influence of ethanol were also investigated (12).

Our experiments indicate that intensification of ROS production results in lipid peroxidation, expressed by tissue increment of MDA and 4-HNE levels. These phenomena are accompanied by impairment of antioxidative properties of cells, what is supported by our finding of the decrease of SOD activity in gastric mucosa.

CONCLUSIONS

1. MDA concentration increased significantly for the group under water immersion and it is negatively correlated with SOD activity obtained from gastric mucosa.
2. The decrease of SOD activity favourised ROS attack at the level of gastric mucosa.

REFERENCES

1. **Brzozowski T., Konturek P.Ch., Konturek S.J. -** Involvement of cyclooxygenase (COX-2) products in acceleration of ulcer healing by gastrin and hepatocyte growth factor. J Physiol Pharmacol 2000, vol. 51, 751-773.
2. **Chen J., Petersen D.R., Schenker S., Henderson G.I**. - Formation of malondialdehyde adducts in livers of rats exposed to ethanol: role in ethanol-mediated inhibition of cytochrome c oxidase. Alcohol Clin Exp Res 2000, vol. 24, 544-552.
3. **Davies G.R., Banatvala N., Collins C.E.** - Relationship between infective load of Helicobacter pylori and reactive oxygen metabolite production in antral mucosa. Scand J Gastroenterol 1994, vol. 29, 419-424.
4. **Deshmukh D.R., Mirochnitchenko O., Ghole V.S.** - Intestinal ischemia and reperfusion injury in transgenic mice overexpressing copper-zinc superoxide dismutase. Am J Physiol 1997, vol. 273, C1130-1135.
5. **Erin N., Ercan F., Yegen B., Arbak S., Okar I., Oktay S**. - Role of capsaicin-sensitive nerves in gastric and hepatic injury induced by cold-restraint stress. Dig. Dis. Sci. 2000, vol. 45, 1889-1899.
6. **Esterbauer H., et al** – Free Rad. Bio. Med., 1991, 11 (1), 81-128.
7. **Halliwell B**. - How to characterize a biological antioxidant. Free Radical Res. Commun 1990; vol. 9, 1-32.
8. **Archibald F.S**. – Assay of superoxide dismutase appplicable to whole bacterial cells. Methods Enzymol., 1990, 186, 232-237.
9. **Konturek P.Ch**. - Physiological, immunohistochemical and molecular aspects of gastric adaptation to stress, aspirin and to H. pylori-derived gastrotoxins. J Physiol Pharmacol 1997, vol. 48, 3-42.
10. **Harmut J., Jeny Mitchell** – Use of isolated perfused organs in hypoxia and ischemia/reperfusion oxidant stress. Methods Enzymol., 1990, 186, 758-759.
11. **Simovic M.O., Bonham M.J., Abu-Zidan F.M.** - Manganese superoxide dismutase: a marker of ischemia-reperfusion injury in acute pancreatitis. Pancreas 1997, vol. 15, 78-82.
12. **Xia J., Simonyi A., Sun G.Y.** - Chronic ethanol and iron administration on iron content, neuronal nitric oxide synthase and superoxide dismutase in rat cerebellum. Alcohol Clin Exp Res., 1999, vol. 23, 702-707.

CHARACTERIZATION OF THE ROMANIAN SPORT HORSE'S CAPACITY TO ADAPTATION TO TRAINING EFFORT

CARACTERIZAREA CAPACITĂȚII DE ADAPTARE A CALULUI DE SPORT ROMÂNESC LA EFORTUL DE ANTRENAMENT

DINU Cristina[1], Cristiana DIACONESCU[2], N. AVRAM[1], D. CUCĂ[1], Şt.BERECHET[1], Gh. TUDOR[1]
[1]Faculty of Veterinary Medicine, Spiru Haret Univesity
[2]USAMV Bucharest

REZUMAT

Scopul acestui studiu este de a caracteriza evoluția unor parametrii fiziologici la cinci cai sănătoşi din rasa Cal de Sport Românesc în timpul exercițiilor finale dinaintea competiției de sărituri peste obstacole. Frecvențele cardiacă şi respiratorie au atins 110 bătăi pe minut şi respectiv 72 respirații pe minut, imediat după exercițiu şi au revenit la valorile din repaus în aproximativ 30 minute după exercițiu (perioada de recuperare). Numărul de eritrocite a fost 7.4 $\times 10^6$/mm^3 în repaus şi a crescut imediat după exercițiu la 9.8 10^6/mm^3 of blood. Această creştere a fost însoțită de modificarea concentrației hemoglobinei care a crescut de la 11.12 g/dL în repaus la 14.74 g/dL după exercițiu. De asemenea valoarea hematocritului a crescut de la 35% to 46%, revenind la valoarea de 38%, după 30 minutes de la experiment. Numărul total de leucocite a crescut de la 7.15×10^3/mm^3 în repaus la 8.02×10^3/mm^3 imediat după exercițiu. Toate creşterile din timpul efort ale RBC, Hb, Ht, WBC au fost semnificative statistic ($P<0.05$). Examinarea statistică a arătat o creştere de 9% a pCO_2 venos şi o descreştere de 20% a pO_2 venos imediat după exercițiu dar atinge valorile de repaus după 30 minute de la exercițiu. Vlorile SO_2 au fost 88.40% în repaus şi 86.32% după exercițiu dar nu au fost semnificative statistic ($P>0.05$). Concentrația sanguină a glucozei a scăzut semnificativ de la 3.83 mmol/L la 3.34 mmol/L ($P<0.05$). Lactatul plasmatic a crescut de la 1.80 mmol/L în repaus la 5.14 mmol/L imediat după experiment, diferența a fost semnificativă statistic ($P<0.05$).

Cuvinte cheie: cai, antrenament, indici fiziologici

ABSTRACT

This study aims to characterise the evolution of some physiological parameters on five clinically healthy jumping horses of the Romanian Sport Horse breed during the final exercises before competition. Heart and the breathing rates reached 110 beats per minute and, respectively, 72 respirations per minute, immediately after the exercise and returned to the rest values in about 30 minutes after trial (recovery period). The erythrocytes count was 7.4 $\times 10^6$/mm^3 at rest and increased immediately after the exercise to 9.8 10^6/mm^3 of blood. This increase was accompanied by the modification of blood haemoglobin concentration which increased from 11.12 g/dL at rest to 14.74 g/dL after the exercise. The hematocrit value also increased from 35% to 46%, returning to the value of 38%, after 30 minutes to the trial. The total leukocytes count increased from 7.15×10^3/mm^3 at rest to 8.02×10^3/mm^3 immediately after the exercise. All increases during effort of RBC, Hb, Ht, WBC were statistically significant ($P<0.05$). The statistic examination revealed a 9% increase of the venous pCO_2 and a 20% decrease of the venous pO_2 immediately after the exercise but reached the rest values after 30 from the exercise. SO_2 values were 88.40% at rest and 86.32% after the exercise, which weren't statistically significant ($P>0.05$). Blood glucose concentration decreased significantly from 3.83 mmol/L to 3.34 mmol/L ($P<0.05$). Plasma lactate increased from 1.80 mmol/L at rest to 5.14 mmol/L immediately after the experiment, the difference were statistically significant ($P<0.05$).

Keywords: horses, training, physiological indices.

INTRODUCTION

The athletic performance of the horse is a corollary of the functional integration of the major systems of the organism involved in the production and release of energy. The adaptation to a standardized physical effort requires complex physiological modifications in horse's cardiovascular, respiratory, locomotor and endocrine systems, reflected in the values of the physical parameters.

Authors such as Art et al., (1990), Chabchoub et al., (1999), Lekeux et al., (1991), Krumrych (2006), Piccione et al., (2001), Różański et al., (2005), have shown that the exercise parameters (intensity, duration and frequency) induce changes in the haematological and biochemical

parameters, according to the individual reactivity. These changes are used in the evaluation of the capacity to adapt to effort.

This study aims to characterise the evolution of some physiological parameters in the Romanian Sport Horse during the exercises performed to determine its capacity to adapt to training effort.

MATERIAL AND METHODS

The study was carried out on five clinically healthy jump Romanian Sport Horses (RSH): one stallion and four geldings, average age 10±2 years, weight limits 436-560 kg.

RSH nucleus was created in 1965 in Sambata de Jos stud, by crossing several breeds with aptitudes for sports (Arabian and British Thoroughbred, Gidran, Furioso North-Star and Nonius). The nucleus has been transferred to Jegalia stud since 1969. In Jegalia, the breed has been consolidated genetically by repeated crossings and infusions. Now, the breed counts 209 horses in Jegalia and it is currently isolated reproductively.

The experimental horses were provided by Jegalia stud, now belonging to a private horse equitation club. Since January 1st, 2008 the five horses started progressively their practice to obtain the maximal physical condition needed to participate in an annual jump competition. The experiment was realised in June 2008 during a final exercise before competition. The training of horses took place on a sandy ground, during dry and sunny weather (temperature: 22°-26°C, air humidity: 36-48%, atmospheric pressure: 101.2-102.3 kPa). The tested horses underwent the following program: ten minute warm-up walking, fifteen minutes trot and five minutes gallop followed immediately by a show jumping course made from 12 obstacles (height: 100-120 cm) and a course length of 600 m. The horses then relaxed walking for the next 10-15 minutes. Then, they were taken for complete rest in the stable. All effort exercises were conducted during 8:00-10:00 A.M. Forage was withdrawn 14 hours before the exercise to avoid postprandial disorders of glucose and lactate metabolism.

The heart rate (palpation method) and breathing rate (auscultatory method) have been determined at rest, at time zero after exercise, then every five minutes, for 30 minutes after trial (recovery period). The blood was sampled three times: before the exercise, immediately after the trial (time zero) and 30 min after trial which involved horses' relaxation.

The venous blood was extracted in syringes on heparin and assayed for red blood cell (RBC) number, haemoglobin (Hb), hematocrit (Ht), white blood cell (WBC) number, using an automatic haematological analyzer HEMAVET 950FS. Other blood samples have been sampled on lyophilised lithium heparin using a vacutainer system. Plasma was obtained from these samples whizzing at 2,500 rpm for 10 min. Plasma was stored in isothermal bags at 4ºC no longer than two hours before analysis. Blood glucose concentration and blood lactate concentration were determined following the methods described by Manta et al. (1976).

The partial pressures of the carbon dioxide (PCO_2) and oxygen (PO_2), oxygen saturation percentage (SO_2%) of haemoglobin and venous blood pH were determined to evaluate the adaptive response of the horses to exercise. All these determinations were performed using an AVL 995-S analyser.

The data were processed statistically with the Student test.

RESULTS AND DISCUSSION

Fig. 1 shows graphically the evolution of the heart beat rate and respiratory frequency in RSH horses after the standard jumping effort. The graphs show that heart beat rate and respiration rate reached 110 beats per minute and, respectively, 72 respirations per minute immediately after the exercise. Fig. 1 also shows the evolution of these parameters during the recovery period.

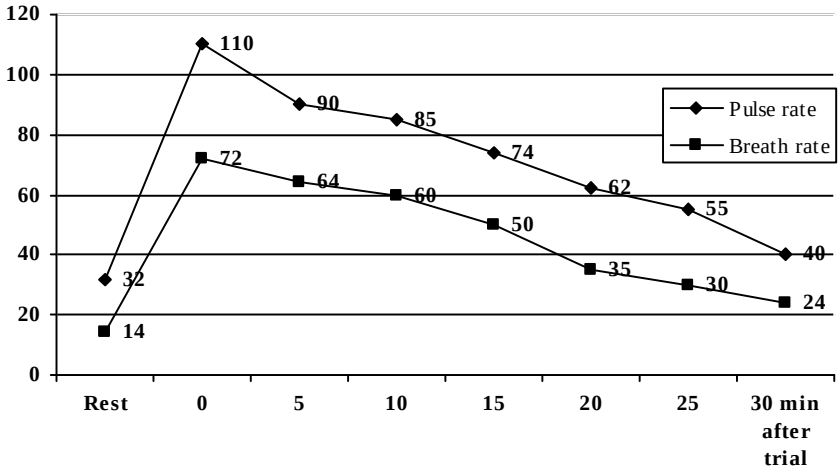


Fig. 1. The recovery curve of the heart beat rate and respiration rate in RSH breed horses after a standard jump exercise with 12 obstacles, 100-120 cm high and 600 m run. The values represent the mean for five horses in experiment

Both heart beat rate and respiration rate reached the rest values in about 30 minutes from the end of the exercise.

Table 1 shows the evolution of the haematological parameters in the five horses trained for jumping exercises.

Table 1

The average values of some haematological parameters in five jumping horses under different experimental conditions ($\overline{X} \pm s_{\overline{x}}$)

Parameters	Experimental conditions		
	At rest	After trial	
		0 minute	30 minute
RBC (10^6/mm^3)	7.40 ± 0.67	9.80 ± 0.88*	8.10 ± 0.76
Hb (g/dL)	11.12 ± 1.10	14,74 ± 1.38*	11.86 ± 0.98
Ht (%)	35 ± 2.48	46 ± 3.63*	38 ± 3.30
WBC (10^3/mm^3)	7.15 ± 0.58	8.02 ±0.92 *	7.47 ± 0.62

*P<0.05

RBC number (7.4×10^6/mm^3 at rest) increased immediately after the exercise to 9.8×10^6/mm^3 of blood. This increase was accompanied by the modification of blood haemoglobin concentration which increased from 11.12 g/dL at rest to 14.74 g/dL at the end of the exercise. The hematocrit also increased from 35% to 46%, returning to the value of 38% in 30 minutes after trial. The total WBC number increased to 8.02×10^3/mm^3 at zero min after trial. All increases during effort of RBC, Hb, Ht, WBC were statistically significant (P<0.05).

Table 2 shows venous blood pH, the partial pressures of CO_2 (PCO_2) and O_2 (PO_2), oxygen saturation percentage (SO_2%), blood lactate and blood glucose concentrations as a function of work intensity in the five RSH.

Table 2

Values of some biochemical's parameters as a function of work intensity in five RSH ($\overline{X} \pm s_{\overline{x}}$)

No	Parameters	Experimental conditions		
		At rest	After trial	
			0 minute	30 minute
1	*p*H	7.46 ± 0.01	7.40 ± 0.01	7.45 ± 0.02
2	PCO_2 (mmHg)	50.50 ± 4.32	55.00 ± 4.84	46.80 ± 3.85

3	PO_2 (mmHg)	42.10 ± 1.21	33.60 ± 3.42	43.20 ± 1.69
4	SO_2 (%)	88.40 ± 2.16	86.32 ± 7.02	86.98 ± 2.42
5	Lactat (mmol/L)	1.80 ± 0.14	5.14 ± 0.48	2.20 ± 0.26
6	Glucose (mmol/L)	3.83± 0.24	3.34 ± 0.23	3.72 ± 0.23

Immediately after the exercise the average *p*H decrease was significant ($P<0.05$) comparatively with the resting period, while 30 minutes after the exercise the *p*H value returned to the resting value. The data revealed 9% increase of the venous PCO_2 and 20% decrease of the venous PO_2 immediately after the exercise. Both PCO_2 and PO_2 values were statistically significant ($P<0.05$) when compared to the rest values. Both PCO_2 and PO_2 reached the rest values 30 min from the exercise. SO_2 values were 88.40% at rest and 86.32% zero minutes after the exercise ($P>0.05$).

Plasma lactate showed an average value of 5.14 mmol/L immediately after the experiment, the difference being significant compared to the resting period ($P<0.05$). Blood glucose decreased significantly from 3.83 mmol/L to 3.34 mmol/L ($P<0.05$).

Heart beat rate and respiration rate are readily monitored parameters. Therefore they are frequently used in evaluating the capacity of adaptation to effort. Heart beat rate decreased suddenly during the first five minutes of the recovery period (Fig. 1). The decrease was constantly and slowly during the next time intervals of recovery. A similar evolution was also reported by Chabchoub *et al*., (1999) and Sloet *et al*., (2006), working on different breeds of sport horses.

The effect of training on the respiratory function was the increase the ventilating flow and the maximal possibilities for oxygen transportation. The hyperventilation noticed during the early minutes of the recovery period (figure 1) allows the payment of the oxygen debt contracted during the first 30 seconds of the intensive exercise. The ventilation changes are positively correlated with the cardiac flow and with the uptake of tissue oxygen according to the intensity of the exercise. After the exercise ceased, the uptake of tissue oxygen diminished gradually but it remained higher than during the rest period to allow the restoration of the phosphocreatine stock, for lactic acid drainage and new synthesis of glucose.

The variations under effort of RBC, Hb, Ht, and WBC were typical for sport horses and were within physiological norms according to Chabchoub et al., (1999), Piccione et al. (2001) and Krumrych (2006).

The increase of RBC under effort shows that RSH were properly trained horses, well adapted to effort. This increase is a consequence of the rapid and large volume mobilization of the spleen erythrocytes. The increase of RBC number was accompanied by the increase of the haemoglobin content, as it was expected.

The significant increase of Ht after trial could be accountable by water translocation from blood plasma outside the vascular system, due to the intensive, long-term training exercises.

In our experiment we found a significant increase of the WBC number. The stress of the exercise induces the increase of blood corticoadrenal hormone (cortisol mainly) and/or the increase of epinephrine, which produced leukocytosis.

The trial is correlated with changes in the acid-base status due to the anaerobic muscle metabolism. The experimental data show a decrease of *p*H immediately after sustained strain because of the respiratory acidosis caused by the higher concentration of HCO_3^- and of the higher PCO_2.

We also observed significant increases of the PCO_2 and decrease of PO_2 during the effort in the RSH.

The phenomenon of higher PCO_2 and lower PO_2 in the venous blood is tightly correlated with the effort capacity. According to the observations of Lekeux et al. (1991) or Art (1993), venous blood oxygen depletion increases with the O_2 capacity capture of the skeletal muscle in exercise. This phenomenon is common in properly trained horses. Increased modifications of PO_2 are in relation with high heart rate and a consecutive blood velocity, too.

Blood lactate concentration showed a maximum value immediately after trial, confirming that the effort involves both anaerobic and aerobic metabolism. With regard to our biochemical studied parameters, we found that they maintained the pattern observed by Art et al. (1990) and Lekeux et al. (1991). Rapidly recovering in athletes (with lactate returning to basal levels 30 min after the trial) shows the good performance of the subjects.

In our experiment, glucose returns to 3.72 mmol/L 30 min after trial. This is in accord to previous studies carried out on jumpers (Różański et al., 2005).

CONCLUSION

The investigated physiological parameters allow to characterise the capacity of effort of the RSH. The evolution of the physiological parameters under strain shows that the Romanian Sport Horse breed has a potential for performance comparable with other breeds, acknowledged in this direction.

REFERENCES

1. Art T., Amory H., D., Desmecht D., , Lekeux P. 1990: Effect of show jumping on heart rate, blood lactate and other biochemical values. *Equine Veterinary Journal*, (suppl. 9), 78-82.
2. Art T., Lekeux P. 1993: Training induced modification in cardiorespiratory and ventilatory measurements in thoroughbred horses. *Equine Veterinary Journal*, 1993, 25, 532-536;
3. Chabchoub A., F. Landolsi, S. Queslati 1999– Comparaison de la frequence cardiaque et des parametres hematologiques et electrocardiographiques chez le cheval pur-sang arabe, *Revue de Médicine Vétérinaire*, 150, 467-472
4. Krumrych, W. (2006) - Variability of clinical and haematological indices in course of training exercise in jumping horses, *Bulletin Veterinary Institute Pulawy*, 50, 391-396.
5. Lekeux P., Art T., Linden A., Desmecht D., Amory H. 1991: Heart rate, hematological and serum biochemical responses o show jumping. *Equine Exercise Physiology* 3, 385-390.
6. Manta, I, Cucuianu M, Benga G, Hodarnau A 1976. *Methods in biochemistry*. Dacia, Bukarest, Romania.
7. Piccione, G., A., Assenza, F., Fazio, E., Giudice, G., Caola (2001) – Different periodicities of some haematological parameters in exercise-loaded athletic horses and sedentary horses, *Journal of Equine Science*, 12, 17-23
8. Różański, P., Nowakowicz-Dębek, B., Saba, L., Ondrašovič, M., Vargová, M., (2005) – Gucoze concentration in the serum of Arabian and Angloarabian horses. *Folia Veterinaria*, 49, 3, 129-132
9. M. Sloet van Oldruitenborgh-Oosterbaan, et al., (2006) - The workload of horses during jumping, *7th International Conference on Equine Exercice Physiology Applied Physiology* : *Training, Methods, Exercice Testing and Selection*, August 26-31, Fontainebleau, France.

STUDIES FOR THE SELECTION OF BULL DAMS CANDIDATES FROM A POPULATION OF FRIESIAN COWS
STUDII PRIVIND SELECȚIA CANDIDATELOR MAME DE TAURI ÎNTR-O POPULAȚIE DE VACI DIN RASA FRIZĂ

PÂRVU Monica, Elena POTECEA, Ioana Cristina ANDRONIE, Elena Luiza BĂDIC

Faculty of Veterinary Medicine *Spiru Haret* University
e-mail: ushmv_parvu.monica@spiruharet.ro

REZUMAT

Studiul a avut ca scop aprecierea fenotipică a vacilor candidate în vederea selecției vacilor mame de tauri. Experimentul s-a efectuat pe 30 vaci din rasa Friză, apreciate genotipic încă de la fătare, pe baza ascendenței și rudelor colaterale. Aprecierea fenotipică s-a efectuat după pe baza datelor individuale privind dezvoltarea corporală, apre-cierea conformației și constituției, examenul ugerului, cantitatea de lapte pe lactație totală și normală, cantitatea de grăsime, cantitatea de proteină, uniformitatea lactației, activitatea de reproducție. Dezvol-tarea corporală a fost apreciată pe baza datelor privind masa corporală și principalele dimensiuni corporale (talia, lungimea trunchiului, adâncimea toracelui, perimetrul toracic, lărgimea crupei la șolduri. Aprecierea conformației și constituției s-a efectuat prin metoda punc-telor. Examenul ugerului s-a efectuat prin inspecție, palpație și proba funcțională. Însușirile productive s-au apreciat pe parcursul primelor trei lactații, prin efectuarea controlului oficial al producției de lapte. Datele au fost prelucrate prin metoda analizei variației. După determinarea mediei și a abaterii standard, 4 vaci au fost selectate ca mame de tauri.

Cuvinte cheie: selecție, vaci mame de tauri, Friză

ABSTRACT

The purpose of the study was to valuate phenotypically the cows that are candidate for selection of bull dams. The experiment was conducted on 30 Friesian cows evaluated genetically from calving based on the ascendance of the collateral kin. The phenotypic evaluation was done using individual data on body development, evaluation of the conformation and constitution, udder examination, milk yield per total and normal lactation, milk fat, milk protein, uniformity of lactation, reproductive activity. Body development was evaluated using the data on body mass and the main body dimensions (height, trunk length, thorax depth, thorax perimeter, width of the croup at hips). The conformation and constitution were evaluated using the scoring method. Udder examination was done by inspection, palpation and the functional test. The productive traits were evaluated throughout the first three lactations using the official test day method. The data were processed by variance analysis. After the mean and the standard deviation have been determined, four cows were selected as bull dams.

Key words: selection, bull mothers, Friesian cow

INTRODUCTION

In the applied practice of cattle improvement one must take into account some particularities of the species, among which the larger interval between generations and the different value of some correlations between the main production traits

The purpose of the study was the phenotypical evaluation of the bull dams candidate cows.

MATERIAL AND METHODS

The biological material studied consisted of 30 Friesian cows reared in a selection farm. The cows were evaluated genetically from calving based on the ascendance of the collateral kin. The animals were reared in an intensive system. The phenotypic evaluation was done using individual data on body development, evaluation of the conformation and constitution, udder examination, milk yield per total and normal lactation, milk fat, milk protein, uniformity of lactation, reproductive activity. Body development was evaluated using the gravimetric method determining the body mass and the method of somatometry, determining the main body dimensions (height, trunk length, thorax depth, thorax perimeter, width of the croup at hips). Udder examination was performed during the

third lactation, six weeks after calving, by inspection, palpation and the functional test. The udder score was determined by somatoscopy. The milking speed was determined by mechanical milking, using the ratio of the milked amount of milk (kg) and the duration of milking (minutes). The functional symmetry was determined using the total amount of milk and the amount of milk obtained from the fore and after quarter. The udder index was determined by calculation using the udder score and the mammary index.

The conformation and constitution were evaluated using the scoring method. The productive traits were evaluated throughout the first three lactations using the official test day method. Milk fat was determined using the butyrometric method. The data were processed by variance analysis.

RESULTS AND DISCUSSION

At the first lactation, the minimal requirements to be selected as bull dam are 4050 kg milk and 148.5 kg fat. In terms of milk amount, seven of the 30 candidates had higher milk yields, as shown in Table 1.

Table 1

Milk yield at the first lactation

No.	Milk, kg	Fat, kg	Protein, kg
1	4057	132.66	125.76
2	4063	133.61	117.00
3	4071	135.93	126.47
4	4095	149.33	126.20
5	4130	150.68	128.03
6	4184	148.83	129.61
7	4200	153.72	128.77

Only four of the seven candidates met the conditions set for milk fat.

At the second lactation, the minimal requirements to be selected as bull dam are 4600 kg milk and 172.5 kg fat. Nine of the 30 candidates had higher milk yields, as shown in Table 2.

Table 2

Milk yield at the second lactation

No.	Milk, kg	Fat, kg	Protein, kg
1	4622	163.32	145.66
2	4763	161.47	157.21
3	4771	168.77	156.74
4	4695	179.87	152.06
5	4730	180.81	158.38
6	4784	184.36	155.16
7	4700	173.28	158.07
8	4615	174.36	148.47
9	4725	168.48	151.36

Only five of the nine candidates met the conditions set for milk fat.

At the third lactation, the minimal requirements to be selected as bull dam are 4950 kg milk and 172.5 kg fat. Seven of the 30 candidates had higher milk yields, as shown in Table 3.

Table 3

Milk yield at the third lactation

No.	Milk, kg	Fat, kg	Protein, kg
1	4982	169.27	165.62
2	5063	171.73	169.17

4	5095	181.54	172.63
5	5030	183.16	171.58
6	4984	185.81	165.52
7	5000	179.80	168.46
8	5015	171.53	158.82

Only four of the seven candidates met the conditions set for milk fat.

Udder evaluation for mechanical milking was done using the following criteria: udder size, udder conformation and fixing, morphological symmetry of the udder quarters, qualitative structure of udder tissue, milking speed and functional symmetry. The first three groups of traits were scored, as shown in Table 4.

Table 4

Udder score

Specification		Udder score		
	3	4	4.5	5
No. of animals (N = 30)	4	22	2	2

Of the 30 candidates, 22 were scored 4 (good), four were scored 4.5 (very good) and two were scored 5 (exceptional). The minimal requirement for bull dams is a scorer of 4.

Table 5 show the data on the functional test.

Table 5

Data on the functional test

Specification					
Milking speed (kg/minute)	1.91	1.98	2.05± 0.07	2.11	2.19
N = 30	1	3	15	7	4
Mammary index	35.98	38.58	41.18±2.60	43.78	46.38
N = 30	1	3	15	7	4
Udder index	92.18	96.18	106.18	109.98	120.18
N = 30	1	3	15	7	4

The average milking speed ranged between 1.91 – 2.19 kg milk/minute. 26 candidates documented the minimal requirement of 2 kg/minute for this trait, as recommended for the bull dams.

The functional symmetry was evaluated by the calculation of the mammary index. One candidate had the average value of 35.98%; three candidates had 38.58%; fifteen had 41.18%; seven had 43.78% and four had 46.38%. The minimal requirement in terms of mammary index is 45% for the bull dams, four of the candidates documenting this requirement, the same which had the adequate milk yield and milk fat.

The udder index was 92.18% at a candidate; 96.18% in three candidates, 106.18% in fifteen candidates; 109.98% in seven animals and 120.18% in four cows. Only these latter animals met the requirement of minimally 110%.

The candidates were assigned to five groups in order to analyse the data on body dimensions. Table 6 shows the minimal values for each group.

Table 6

Main body dimensions observed

	Minimal value by group				
Body dimension	G1	G2	G3	G4	G5
Height, cm	131.41	133.16	134.91	136.66	138.41
N = 30	3	8	9	5	5
Trunk length, cm	146.91	148.58	150.25±2.09	151.92	153.59
N = 30	2	4	14	6	4
Thorax depth, cm	69.98	71.23	72.48±1.56	73.73	74.98
N = 30	1	6	11	7	5
Thorax perimeter, cm	185.47	187.57	189.67±2.62	191.77	193.87
N = 30	3	5	13	5	4
Croup width at hip, cm	51.76	53.08	554.40±1.65	55.72	57.04
N = 30	1	4	16	5	4
Body mass, kg	572.60	593.85	615.10±26.56	636.35	657.60
N = 30	1	7	12	6	4

Group 5 displayed the following maximal values: 140.17 cm for height; 155.27 cm for trunk length; 76.22 cm for thorax depth; 195.95 cm for thorax perimeter; 58.36 cm for croup width at hip and 678.84 kg body mass.

The data from table 6 were used to evaluate the body development of the candidates, four of them outstanding by the values for all traits under investigation. They were the same animals which met the minimal requirements for production (milk yield and milk fat) and for udder aptitudes to mechanical milking (animals 4, 5, 6 and 7).

Animal conformation and constitution were assessed by the evaluation commission, the candidates being divided in four value groups. Table 7 shows the minimal data for each group.

Table 7

Data on conformation and constitution score

Specification	G1	G2	G3	G4
Total	55.0	60.0	65.0±6.6	75.0
FO	11.5	12.9	13.4±1.4	16.2
CPL	8.2	9.2	10.2±1.0	12.2
FU	20.3	21.2	23.0±1.6	26.6
U	15.0	16.7	18.4±2.5	20.0
N = 30	3	11	12	4

where: FO = format; CPL = traits specific to milk yield; FU = fundament; U = udder

Because the minimal requirement for bull dams is of 75 points, of which at least 20 points for the udder, only four of the 30 candidates met these requirements, the maximal values being 80.1 points in all, of which 31.6 points for the udder.

CONCLUSIONS

1. The minimal requirements for the main traits (milk yield and udder aptitudes for mechanical milking) were considered for bull dam selection.
2. Evaluation was done using the secondary criteria (body development, conformation and constitution), considering their positive correlation with the milk yield.
3. Analysing the results for all criteria, four of the thirty cows were selected as bull dams.

REFERENCES

1. Grosu H., Oltenacu P.A, 2005, Programe de ameliorare genetică în zootehnie, Ed Ceres, Bucureşti, pg 644-693
2. Dinescu S., Anne Marie Tontsch, 2005, Creşterea vacilor pentru lapte, Ed Ceres Bucureşti, pg 95-96

CORRELATION BETWEEN THE GENETIC STRUCTURE OF THE EWES AND THE BODY WEIGHT OF LAMBS AT BIRTH
CORELAȚII ÎNTRE STRUCTURA GENETICĂ A OILOR ŞI GREUTATEA MIEILOR LA FĂTARE

PÂRVU Monica[1], **Elena GHIȚĂ** [2], **Mariana REBEDEA** [3] Cristina DINU[1]
[1] Faculty of Veterinary Medicine *Spiru Haret* University
e-mail: ushmv_parvu.monica@spiruharet.ro
[2] ICDNBA Baloteşti;
[3] University Bucureşti

REZUMAT

Materialul biologic luat în studiu a fost reprezentat de 66 oi din rasa Țigaie cu capul negru de Teleorman. Markerii genetici luați în studiu la oile mame au fost hemoglobina și transferina. S-au identificat două genotipuri la locusul hemoglobinei şi opt genotipuri la locusul transferinei. La locusul hemoglobinei, s-a constatat superioritatea animalele heterozigote (AB), la care mieii au avut greutatea la fătare mai mare cu 16,7% decât la animalele homozigote BB. Diferențele au fost semnificative ($p \leq 0,05$). La locusul transferinei, s-a constatat superioritatea productivă a animalelor heterozigote Tf^{M}/Tf^{E}, cu 12,2% mai mult decât genotipul Tf^{C}/Tf^{D} clasat al doilea ($p \leq 0,05$). În urma analizei genotipului agregat, s-a constatat că genotipul $Hb^{A}Hb^{B}/Tf^{M}Tf^{E}$ s-a clasat pe primul loc, mieii înregistrând greutatea la fătare de 6,2 kg ($p \leq 0,05$). Acest genotip s-a clasat pe primul loc și la înțărcare, când greutatea mieilor a fost de 12,7 kg, cu 11,5% mai mult decât genotipul clasat al doilea.
Cuvinte cheie: marker genetic, ovine, hemoglobină, transferina

ABSTRACT

The investigation involved 66 Teleorman Black Head Tsigai sheep. The genetic markers considered by the investigation of the ewes were the haemoglobin and transferrin. Two genotypes were identified at the haemoglobin locus and eight genotypes at the transferrin locus. At the haemoglobin locus, the superiority of the heterozygous ewes (AB) was documented, the lambs having a neonatal body weight 16.7% higher than that of the homozygous ewes (BB). The differences were significant ($p \leq 0.05$). At the transferrin locus, the superiority of the heterozygous ewes Tf^{M}/Tf^{E} was documented, 12.2% more than the Tf^{C}/Tf^{D} genotype, which ranked secondly. The simultaneous analysis of the two studied markers revealed the superiority of the heterozygous ewes Hb^{A} $Hb^{B}/Tf^{M}Tf^{E}$, whose lambs had 6.2 kg at lambing ($p \leq 0.05$). The same genotype ranked first at weaning, when lambs' body weight was 12.7 kg, 11.5% higher than that of the second ranked genotype.
Key words: genetic marker, ewes, haemoglobin, transferrin

INTRODUCTION

The prediction of the breeding value of the farm animals is important for the selection of the most valuable specimens which to yield the next generation and for pair matching. Among the modern methods assessing the productive capacity of the animals and of their breeding value is the use of biochemical markers whose identification allows the determination of the population's genotypes and the correlation with the productive results (3).

MATERIAL AND METHODS

The experiment used 66 Teleorman Black head Tsigai sheep. The sheep were monitored during the pregnancy and calving periods. Each ewe gave birth to two lambs.

The monitored parameters were: duration of the pregnancy, weight lambs at birth and weaning, the amount of milk during the nursing period.

The duration of pregnancy period was determined on the basis of livestock records. The weight of lambs was determined by the gravimetric method. The amount of milk during the nursing period was calculated based on the weight of lambs (at birth and weaning) and using specific coefficients.

The genetic markers under study were the haemoglobin and the transferrin.

The genotype categories were identified from samples of ewe's blood, by vertical electrophoresis using polyacrylamide as migration carrier by technique of Meriaux J.C. (2) adapted by Mariana Rebedea and the biochemistry collective of the Faculty of Biology (1, 3)

The data were processed statistically by variance analysis.

RESULTS AND DISCUSSION

For the haemoglobin marker (Table 1), the frequency of the observed genotypes was 0.3 for the heterozygous genotype HB^AHB^B (P) and 0.7 for the homozygous genotype HB^BHB^B (Q). The frequency of gene A (p) was 0.15 and the frequency of gene B (q) is 0.85, according to the Hardy – Weinberg law.

Table 1

Gene frequency at the haemoglobin locus

Genotype	HB^AHB^A	HB^AHB^B	HB^BHB^B	Total
Genotype frequency	0	0.3	0.7	1
Gene frequency	p (gene A) 0.15; q (gene B) 0.85			

For the transferrin marker, the frequency is shows in table 2.

Table 2

Gene frequency at the transferrin locus

Genotype	Tf^BTf^C	Tf^BTf^E	Tf^BTf^M	Tf^CTf^C	Tf^CTf^D	Tf^CTf^M	Tf^MTf^E	Tf^MTf^M	Total
Genotype frequency	0.18	0,05	0,04	0,14	0,14	0,22	0,14	0,09	1
Gene frequency	p (gene B)= 0.227; q (gene C) = 0.204; r (gene D) = 0.068; s (gene E) = 0.25; t (gene M) = 0.250								

Eight genotypes were identified. The gene frequencies were: 0.227 for gene B, 0.204 for gene C, 0.068 for gene D, 0.251 for gene E and 0.250 for gene M.

Table 3 shows the results concerning the weight of lambs at the birth, in correlation with the aggregate genotype of their mothers.

Table 3

The birth weight of lambs

Ewes aggregate genotype	Lambs birth weight	Ranking
Hb^AHb^B/Tf^MTf^E	6.11 ± 0.63	1
Hb^BHb^B/Tf^CTf^D	5.90 ± 0.51	2
Hb^BHb^B/Tf^MTf^E	5.75 ± 0.47	3
Hb^BHb^B/Tf^BTf^C	5.67 ± 0.37	4
Hb^BHb^B/Tf^CTf^C	5.53 ± 0.56	5
Hb^AHb^B/Tf^CTf^M	5.48 ± 0.41	6
Hb^AHb^B/Tf^MTf^M	5.32 ± 0.22	7
Hb^BHb^B/Tf^BTf^E	5.26 ± 0.31	8
Hb^AHb^B/Tf^BTf^C	5.18 ± 0.23	9
Hb^AHb^B/Tf^BTf^M	5.06 ± 0.30	10

The lambs resulted from ewes with Hb^AHb^B/Tf^MTf^E aggregate genotype had the biggest value at the birth, 6.11 kg, ranking first. On the second place ranked the lambs having 5.90 kg, born from mother with Hb^BHb^B/Tf^CTf^D aggregate genotype. On the last place ranked the lambs born from ewes with Hb^AHb^B/Tf^BTf^M genotype.

Table 4 shows the results concerning the weight of lambs at the weaning, in correlation with the aggregate genotype of their mothers.

Table 4

The weaning weight of lambs

Ewes aggregate genotype	Lambs birth weight	Ranking
$Hb^{A}Hb^{B}/Tf^{M}Tf^{E}$	20.65 ± 1.77	1
$Hb^{B}Hb^{B}/Tf^{M}Tf^{E}$	19.11 ± 1.26	2
$Hb^{B}Hb^{B}/Tf^{C}Tf^{D}$	17.03 ± 1.62	3
$Hb^{B}Hb^{B}/Tf^{B}Tf^{C}$	16.88 ± 1.41	4
$Hb^{A}Hb^{B}/Tf^{C}Tf^{M}$	16.36 ± 1.67	5
$Hb^{B}Hb^{B}/Tf^{C}Tf^{C}$	15.57 ± 1.48	6
$Hb^{A}Hb^{B}/Tf^{M}Tf^{M}$	14.64 ± 1.36	7
$Hb^{B}Hb^{B}/Tf^{B}Tf^{E}$	14.13 ± 1.52	8
$Hb^{A}Hb^{B}/Tf^{B}Tf^{M}$	13.74 ± 1.45	9
$Hb^{A}Hb^{B}/Tf^{B}Tf^{C}$	13.36 ± 1.33	10

The lambs resulting from sheep with $Hb^{A}Hb^{B}/Tf^{M}Tf^{E}$ aggregate genotype had the highest value at weaning, 20.65 kg, ranking first. On the second place ranked the lambs having 19.11 kg, born from mother with $Hb^{B}Hb^{B}/Tf^{M}Tf^{E}$ aggregate genotype. On the last place ranked the lambs born from ewes with $Hb^{A}Hb^{B}/Tf^{B}Tf^{C}$ genotype.

Table 5 shows the data obtained by genotype aggregated from the haemoglobin and transferrin loci, concerning the nursing milk yield.

Table 5

Average performance by aggregate genotype

Genotype	Milk yield	Ranking
$Hb^{A}Hb^{B}/Tf^{M}Tf^{E}$	65.77 ± 5.12	1
$Hb^{B}Hb^{B}/Tf^{M}Tf^{E}$	62.64 ± 5.33	2
$Hb^{B}Hb^{B}/Tf^{C}Tf^{D}$	56.10 ± 4.43	3
$Hb^{B}Hb^{B}/Tf^{B}Tf^{C}$	55.63 ± 4.04	4
$Hb^{A}Hb^{B}/Tf^{C}Tf^{M}$	54.34 ± 5.07	5
$Hb^{B}Hb^{B}/Tf^{C}Tf^{C}$	50.47 ±4.76	6
$Hb^{A}Hb^{B}/Tf^{M}Tf^{M}$	48.02 ± 5.16	7
$Hb^{B}Hb^{B}/Tf^{B}Tf^{E}$	44.65 ± 4.65	8
$Hb^{A}Hb^{B}/Tf^{B}Tf^{M}$	42.13 ± 5.38	9
$Hb^{A}Hb^{B}/Tf^{B}Tf^{C}$	40.52 ± 5.05	10

The analysis of the aggregated genotype showed that genotype $Hb^{A}Hb^{B}/Tf^{M}Tf^{E}$ ranked first, with a nursing milk yield of 65.77 kg, 5% more than genotype $Hb^{B}Hb^{B}/Tf^{M}Tf^{E}$ which ranked second. The differences between groups are not significant ($p \leq 0.05$). On the last place ranked the ewes with the genotype $Hb^{A}Hb^{B}/Tf^{B}Tf^{C}$, having 83.50 kg milk. This category also had the lambs with the lowest weight at weaning.

Table 6 shows the data obtained by genotype aggregated, concerning the total milk yield.

Table 6

Average performance by aggregate genotype

Genotype	Milk yield	Ranking
$Hb^{A}Hb^{B}/Tf^{M}Tf^{E}$	135.61 ± 6.68	1
$Hb^{B}Hb^{B}/Tf^{M}Tf^{E}$	129.15 ± 7.33	2
$Hb^{B}Hb^{B}/Tf^{C}Tf^{D}$	115.65 ± 7.24	3
$Hb^{B}Hb^{B}/Tf^{B}Tf^{C}$	114.62 ± 7.37	4
$Hb^{A}Hb^{B}/Tf^{C}Tf^{M}$	112.05 ± 8.06	5

$Hb^{B}Hb^{B}/Tf^{C}Tf^{C}$	104.08 ±6.87	6
$Hb^{A}Hb^{B}/Tf^{M}Tf^{M}$	99.02 ± 5.72	7
$Hb^{B}Hb^{B}/Tf^{B}Tf^{E}$	92.06 ± 3.16	8
$Hb^{A}Hb^{B}/Tf^{B}Tf^{M}$	86.88 ± 6.23	9
$Hb^{A}Hb^{B}/Tf^{B}Tf^{C}$	83.50 ± 5.9.0	10

The analysis of the aggregated genotype showed that genotype $Hb^{A}Hb^{B}/Tf^{M}Tf^{E}$ ranked first, with a total milk yield of 135.61 kg, 5% more than genotype $Hb^{B}Hb^{B}/Tf^{M}Tf^{E}$ which ranked second. The differences between groups are not significant ($p \leq 0.05$). Taking into consideration the data obtained at the transferrin locus, one may observe that irrespective of the genotype existing at the haemoglobin locus, the $Tf^{M}Tf^{E}$ heterozygous individuals will have better results of the total milk yield.

CONCLUSIONS

1. At the *haemoglobin* locus, two types of migration were observed by electrophoresis, corresponding to two genotypes Hb^{A}/Hb^{B} and Hb^{B}/Hb^{B}. Gene frequency was: 0.15 for gene Hb^{A} and 0.85 for gene Hb^{B}.

2. At the *transferin* locus, eight types electrophoretic movements were noticed, determined by genotypes Tf^{B}/Tf^{C}, Tf^{B}/Tf^{E}, Tf^{B}/Tf^{M}, Tf^{C}/Tf^{C}, Tf^{C}/Tf^{D}, Tf^{C}/Tf^{M}, Tf^{M}/Tf^{E} şi Tf^{M}/Tf^{M}. Gene frequency was: 0,227 for gene Tf^{B}, 0,204 for gene Tf^{C}, 0,068 for gene Tf^{D}, 0,251 for gene Tf^{E} and 0,250 for gene Tf^{M}.

3. At lambing and weaning, the lambs from ewes with the aggregate genotype (haemoglobin/transferrin) $Hb^{A}Hb^{B}/Tf^{M}Tf^{E}$ displayed the highest values.

4. During the nursing period and throughout the entire lactation, the ewes with the genotype HbAHbB/TfMTfE displayed the best milk production, 5% higher than the ewes ranking second.

REFERENCES

1. Costache. Marieta şi Dimischiotu. Anca : Electroforeză. Principii şi concepte metodologice. Ed. Ars Docendi, pg 67 – 73, 2004
2. Rebedea Mariana: Studiul determinismului genetic al unor sisteme biochimice la cabaline - Teză de doctorat, USAMVBucureşti, pg 52 – 58, 1998.
3. Merinaux J. C. – Tehnique de l'électrophorèse, Annales Medecine Veterinaire, Universite Liege, Belgium, vol 34, pg 121 – 127, 2003

STUDIES FOR GROWING HORSES EVALUATION FOR THEIR CLASSIFICATION
STUDII PRIVIND BONITARE TINERETULUI CABALIN ÎN VEDEREA CALIFICĂRII

PÂRVU Monica, Roxana BOGZA (student), Ioana Cristina ANDRONIE, Adriana AMFIM
Faculty of Veterinary Medicine University Spiru Haret
ushmv_parvu.monica@yahoo.com

REZUMAT

Bonitarea tineretului cabalin din rasa Pur sânge englez s-a realizat individual, până la susținerea probelor de calificare, pe baza următoarelor criterii: origine, precum și exteriorul (dimensiuni corporale, conformație și mersuri). Fiecare caracter sau însușire se apreciază printr-un sistem de puncte, pe o scară de la 1 la 10. Aprecierea originii s-a făcut prin metoda punctelor, pe baza analizei pedigreului. Aprecierea după dimensiunile corporale s-a realizat prin biometrie și gravimetrie, stabilindu-se talia, masa corporală, perimetrul toracic și perimetrul fluierului. Aprecierea conformației corporale s-a efectuat pe trei grupe de regiuni corporale: cap, gât și trunchi; membre; constituție, musculatură, tendoane și ligamente. După bonitare, animale au fost clasate astfel: 37% în clasa record; 51% în clasa elită și 12% în clasa I. Tineretul încadrat în clasele record și elită, care au prezentat potențial genetic ridicat și au fost oprite în herghelie pentru a intra în calificare. Restul a fost propus pentru valorificarea prin vânzare.

Cuvinte cheie: Pur sânge englez, bonitare, tineret

ABSTRACT

The evaluation of English Thoroughbred growing horses was done individually, before they were tested for classification, based on the following criteria: origin and exterior appearance (body size, conformation and walk). Each character or trait was evaluated on a scale from 0 to 10. The evaluation of horse origin was done using the method of points, based on pedigree analysis. The evaluation according to body size was done by biometry and gravimetry, determining the height, body mass, thorax perimeter and cannon diameter. Body conformation was evaluated on three groups of body parts: head, neck and trunk; limbs; constitution, muscles, tendons and ligaments. After evaluation, the animals were classified as follows: 37% in record class; 51% in elite class and 12% in first class. The young horses classified in record and elite classes, which displayed a high genetic potential were retained in the stud to be trained for classification. The balance was put out for sale.

Keywords: English Thoroughbred, evaluation, growing horses

INTRODUCTION

Classification is a criterion for growing horses' evaluation according to their phenotypic and genotypic value, playing an important role in the elaboration of the methodology for their improvement. Selection, as means of improvement, aims to better the biological traits of the animals using for reproduction only the specimens with proper dimensions and body conformation, with higher energetic capacity and with increased resistance to the environmental conditions and to diseases. Compared top other species of farm animals, the effect of selection is smaller in horses, because the populations are quite small and homogenous, their heredity is more stable and the variability of their traits is lower. The present study aimed to determine the destination of a population of English thoroughbred horses tested before they performed the qualification trials.

MATERIAL AND METHODS

The study was conducted on 25 English thoroughbred horses (10 males and 15 females) aged 2 – 2.5 years. The horses were evaluated using the following criteria: origin, body dimensions and body conformation.

The evaluation by origin was done by pedigree analysis and the evaluation of the breed was done by the individual examination and by identifying the exterior traits of the English thoroughbred breed.

The exterior traits were evaluated by determining the body dimensions, using biometry, with the zoometer and ruler. The following body measurements were performed: height, thorax perimeter and cannon perimeter.

The body conformation was evaluated under static and moving conditions on a flat and elastic land, of circular shape with a diameter of 25 m. The animals didn't wear harnesses. Examination was performed as follows: animal sides, front and back (width, length and shape), hoofs, skin quality, hair colour and quality. The evaluation of their walk was done by moving the horses with normal walk and trotting on a straight line.

Each character or trait was scored on a scale from 1 to 10.

The data were processed statistically using the variance analysis.

RESULTS AND DISCUSSION

The 25 horses were evaluated individually taking into consideration the genealogical records of the breeder.

Individual scores were given after pedigree analysis, which included the determination of the genetic value of the parents and grandparents and the degree of inbreeding, as shown in Table 1.

Table 1

Scores for origin and breed

Score	10	9	8	7
Males N=10	1	3	5	1
Females N=15	2	3	8	2

Of the 10 tested male horses, 10% were scored 10, 30% were scored 9, 50% were scored 8 and 10% were scored 7.

Of the 15 tested female horses, 13.3% were scored 10, 20% were scored 9, 53.3% were scored 8 and 13.4% were scored 7.

The literature shows that the evaluation by origin aids in horses to evaluate the zootechnical value of the examined animal. The inclusion of the animals in the specific type of breed is directly correlated to their productive aptitudes (1). The scores have the following significance: 9 and 10 show an outstanding expression of the genotype, while 7 and 8 shows a very good expression of the genotype.

Table 2 shows the scores for the average body dimensions.

Table 2

Scores for exterior examination

Specification	No. of animals	Height, cm	Thorax perimeter, cm	Cannon perimeter, cm	Score
Males, N=10	2	158	168	18.5	10
	2	152	162	17.0	9
	5	148	156	16.0	8
	1	144	152	15.5	7
Females, N=15	1	158	170	18.0	10
	2	152	164	16.5	9
	2	150	162	16.0	8
	8	148	158	16.0	7
	2	146	162	16.0	7

The data show that 20% of the male horses were scored 10, 20% were scored 9, 50% were scored 8 and 10% were scored 7.
The evidences show that 6.7% of the female horses were scored 10, 13.3% were scored 9, 13.3% were scored 8 and 66.7% were scored 7. In this latter group, 53.4% of the female horses were scored 7 for all three categories pf measurements and 13.3% had higher scores for the thorax perimeter and for cannon perimeter, but were scored 7 for height. According to the classification instructions, the single score was given considering the lowest score for one of the measured dimensions (2).
Table 3 shows the scores for body conformation and walking.

Table 3

Scores for body conformation and walking

Score	10	9	8	7
Males N=10	1	3	5	1
Females N=15	2	3	8	2

Of the 10 tested male horses, 10% were scored 10, 30% were scored 9, 50% were scored 8 and 10% were scored 7. Of the 15 tested female horses, 13.3% were scored 10, 20% were scored 9, 53.4% were scored 8 and 13.3% were scored 7.

The class of classification was determined using the partial scores for the three criteria of evaluation (origin and type of breed, exterior, conformation and walks), as shown in Table 4.

Table 4

Scores and class of classification

		Origin and type of breed	Exterior	Conformation and walks	Class of classification
Males	1	10	10	10	R
	2	9	10	9	R
	3	9	9	9	R
	4	9	9	9	R
	5	8	8	8	E
	6	8	8	8	E
	7	8	8	8	E
	8	8	8	8	E
	9	8	8	8	E
	10	7	7	7	I
Females	11	10	10	10	R
	12	10	9	10	R
	13	9	9	9	R
	14	9	8	9	R
	15	9	8	8	R
	16	8	7	8	E
	17	8	7	8	E
	18	8	7	7	E
	19	8	7	8	E
	20	8	7	8	E
	21	8	7	8	E
	22	8	7	8	E

	23	8	7	8	E
	24	7	7	7	I
	25	7	7	7	I

Of the 35 tested horses, 9 (4 males and 5 females) were ranked in R (record) class, 13 (5 males and 8 females) were ranked in E (elite) class, and 3 (1 male and 2 females) were ranked in class I. According to the classification instructions, the animals ranked in record and elite classes are to be tested for qualification, while the animals ranked in class I are to be put out for sale.

CONCLUSIONS

1. Young horses classification prior to the qualification tests was done using here criteria: origin and type of breed, exterior, conformation and walks.
2. After evaluation, the animals were classified as follows: 37% in record class; 51% in elite class and 12% in first class.
3. The young horses classified in record and elite classes, which displayed a high genetic potential were retained in the stud to be trained for classification. The balance was put out for sale.

REFERENCES

1. Mărginean Gh. şi col, 2005, Îndrumător de lucrări practice pentru exploatarea cabalinelor, Ed Agrotehnica, pg 112-120
2. Velea C, 1980, Creşterea cabalinelor, Ed Dacia, pg 44-48

WELFARE OF SPORT HORSES DURING TRANSPORT
BUNĂSTAREA CAILOR DE SPORT PE TIMPUL TRANSPORTULUI

ANDRONIE Ioana, Livia TĂNASE (student USH), V.ANDRONIE, Monica PÂRVU

Faculty of Veterinary Medicine Spiru Haret University

email: ioanaandronie@innovet.ro

REZUMAT

Cercetările au urmărit răspunsul unor indicatori de bunăstare a cailor de sport pe timul transportului, prin evaluarea intensității stresului pe durata călătoriei, prin modificarea frecvenței cardiace, al cortizolului, nivelului acidului lactic și creatininei. Caii luați în studiu au fos grupați în două grupe: cai transportați pentru prima dată (A n:12) și cai care au mai fost transportați (B n:10).

Valorile indicatorilor au fost diferite între cele două grupe, chiar dacă durata călătoriei și condițiile în care s-a realizat transportul au fost aproape aceleași. Valoarea frecvenței cardiace și a nivelului cortizolului au fost crescute la caii din grupa A față de cei din grupa B dar și la aceștia din urmă s-au observat creșteri ale valorilor la repetarea îmbarcării și debarcării (100-130 nmol/l). Nivelul acidului lactic înregistrat a fost crescut în cazul primei grupe (2,2 mmol/l), doar până când caii s-au obișnuit cu efortul, după care a scăzut (1,8 mmol/l).

Transportul cailor în anumite condiții ce țin de obișnuirea acestora cu manopera de îmbarcare și debarcare, de durata călătoriei, dar și de temperamentul animalelor, poate fi considerat un factor de stres ce depreciază bunăstarea lor.
Cuvinte cheie: cai, stres, transport, bunăstare

ABSTRACT

Our research monitored the response of some welfare indicators in sport horses during transport by assessing the intensity of stress during travel time, changes in heart rate, cortisol, lactic acid and creatinine levels. The horses included in the study were separated into 2 groups: horses that were transported for the first time (A n: 12) and horses that had been previously transported (B n: 10).

The values of the indicators varied between the two groups regardless of the fact that travel length and transport conditions were almost identical. The heart rate and cortisol levels were elevated in horses from group A as compared to the ones in group B while the latter showed increased levels of these indicators upon repeating the loading and unloading (100-130 nmol/l). The recorded value of lactic acid was higher for the first group (2,2 mmol/l), only until the moment when the horses adapted to the effort, after which it decreased (1,8 mmol/l).

Transport of horses under certain conditions related to their adjusting to manhandling during loading and unloading operations, as well as the animals' temper, may be considered a stress factor with significant depreciating effect on horse welfare.

Key words: horse, stress, transportation, and welfare

INTRODUCTION

The necessity of animal transport with different purposes – commercial, sports related, towards slaughtering or other activities – has imposed the health and welfare assessment of horses during long or short distance transport. Transport related stress effects determine in most cases different physiological and behavioural responses, depending on the horses' ability to adapt. Broom (2000), stated following research that physiological responses are difficult to assess due to the complexity of stress factors the animals are subjected to during transport, such as loading, unloading, the transport vehicle itself, the microclimate on the vehicle, the loading surface, fodder and water deprivation.

An important welfare factor in sport horses during transport is the vehicle, which must be appropriate, according to the animals transported, must be well maintained and the transport equipment must be kept clean and perfectly functional. Loading and unloading manoeuvres are important as well, as they may reduce fear that is relatively easily installed in horses during transport and may generate and account for a high percentage of limb lesions when handled improperly.

Loading/unloading do not represent a problem for most horses, which are more than happy to climb on the platform or walk into the trailer. Easy loading of the horses is a result of caretaker's experience that uses different methods to direct and handle the animals. Some horses are extremely difficult to load particularly because they had been exposed to prior negative travelling experiences, insecurity, which all are conducive of fear and refusal to be loaded.

Oikawa (1995) state that the health condition of transported horses decreases with travel duration, thus increasing the occurrence of respiratory diseases.

This research monitored the assessment of sport horses response to various stress factors during transport on short distances and their impact on the animals' welfare.

MATERIAL AND METHODS

The research was carried out on 22 sport horses (male and female), 3 to 14 years old and weighing on average 510 kg ± 10kg. The horses were transported from their stables to other farming or temporary training facilities.

The horses that participated in the study were grouped in two categories: animals that had been transported for the first time A (n: 12) and animals, which had previously had this experience B (n: 10). We completed our research in spring over the course of eight weeks and the distance covered during travel time varied between 200 and 400km (2-5h depending on quality of road).

Specialized horse vehicles with two or more places transported the animals. The horses' caretakers handled loading and unloading manoeuvres as well as blood sample drawing.

Horse protection norms and standards were observed during transport, in terms of handling manner, allotted surface on the vehicle, vehicle characteristics, fodder and water supplies.

Blood samples were taken by jugular vein puncture on the morning of the journey prior to loading and immediately following unloading, in 1,3 ml Vacutainer tubes with Lithium-Heparin (LH/1,3) that were kept according to working protocol during travel time. This manoeuvre was carried out in the presence of the animals' caretaker, which minimized their stress during blood sampling. Working and analysis of blood tests was performed in the laboratory.

The level of plasmatic cortisol was obtained by ELISA method and level of plasmatic lactate by means of chemical analysis.

The statistical data analysis included the T student test in order to compare the biochemical and haematological parameters of the two horses groups whereas.

Heart rate was measured by a non-invasive method using a cardio-monitor (Polar Electro Oy, Finland). Its electrodes were placed under the girth on each side of the animal and in contact with its skin by means of a gel. The transmitter was horizontally placed on the withers, fixed on the harness together with the recording device. The data recorded throughout the transport was downloaded on a computer and processed by the Polar Equine SW software installed.

RESULTS AND DISCUSSIONS

Horse physiological responses during transport may appear following a large number of stress factors, which impact their welfare.

Rose, (1977) state that during endurance exercises, the increase of heart rate in horses shows metabolic diseases, and proves to be a practical, simple and precise method in assessing the „stressed" horses.

The heart rate monitored during transport on horses that participated in the research shows an obvious increase in those loaded for the first time and unaccustomed with this manoeuvre, compared the other animals, which had previously had this experience (fig 1).

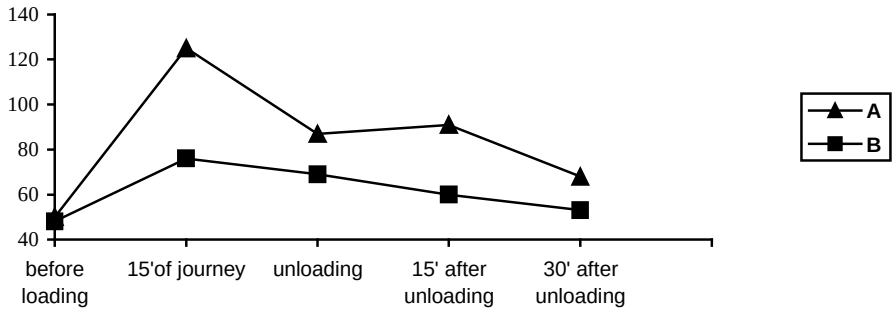


Figure 1. Heart rate variation (bpm) during horse transportation

The increase in heart rate was recorded also in group A horses, as transport was an underlying chronic stress factor. However, the heart rate decreased between loading and unloading time, which shows that animals adapted fairly quickly to the new transporting conditions on the vehicle. The anxiety exhibited by the group B horses was caused by loading onto/unloading from transport vehicles and less by the journey itself.

The heart rate varied with travel time (fig 2), where we noticed the same increase in-group A horses. Hyperpnea and decrease of neurovegetative system control of the heart rate during transport may result in increased heart rate prevalence.

Measurement of heart rate immediately following unloading of the horses from the transport vehicle may be an important indicator of their health condition and physical performance.

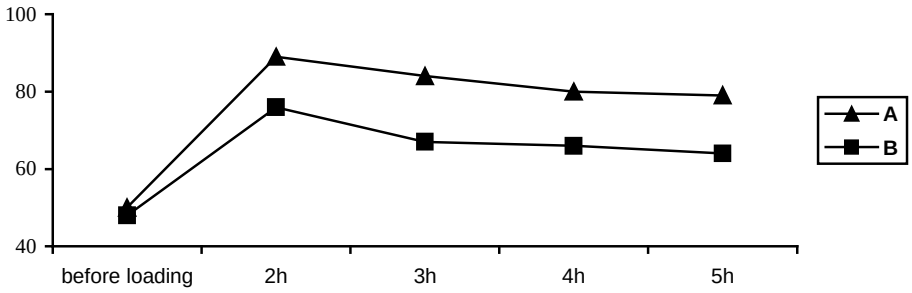


Figure 2. Heart rate variation (bpm) after different journey hours

The cortisol (corticosteroid hormone produced by adrenal cortex) is directly involved in the bodily response to stress factors by an increase of brachial pressure and glycaemia levels. Blood level cortisol ranges with the circadian rhythm, so that the maximum level is recorded in the morning and the minimum level at night (Evans, 1977). Change in cyclic evolution is directly connected to ACTH hormone activity, stress factors, clinical depression, surgical procedures, anxiety, and pain.

The level of plasmatic cortisol measured during transport in the two horse groups showed an increase in concentration (fig 3). Recorded cortisol values were not low in the horses previously

exposed to transport either. The recorded level of plasmatic cortisol did not vary with the travel duration and the horses' effort to adapt to transport conditions were probably the same, as a consequence of the relatively short travel time (2-5h).

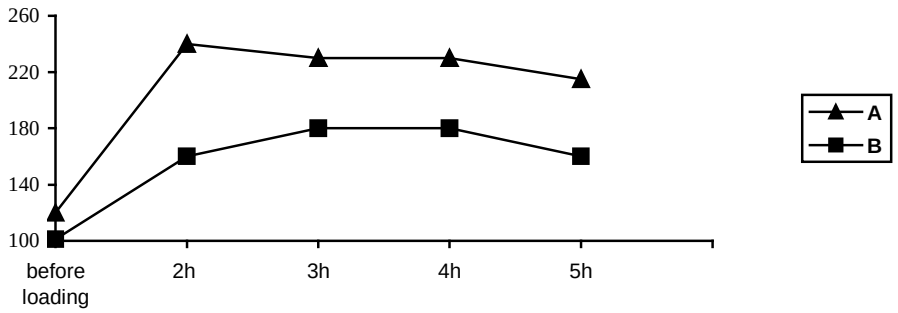


Figure 3. Cortisol level (nmol/l) of horse before and after transport at different hours of the journey

The increased cortisol concentration recorded even after unloading time confirms the fact that this is a stress indicator, as we have showed in other studies. Irvine (1994) state that the cortisol levels in horses removed from their environment increases due to the disruption of the circadian rhythm.

The levels of plasmatic cortisol together with the heart rate may be used as stress indicators in assessing horses' welfare during transport.

Stress, fear, anxiety may elevate lactate concentration at blood levels as glicogenesis is stimulated by catecolamine.

The level of plasmatic lactate in horses participating in the study (fig.4) showed a drop, particularly in those accustomed to transport (1,9mmol/l) as compared to the ones, which had not previously been exposed to it (2,7mmol/l).

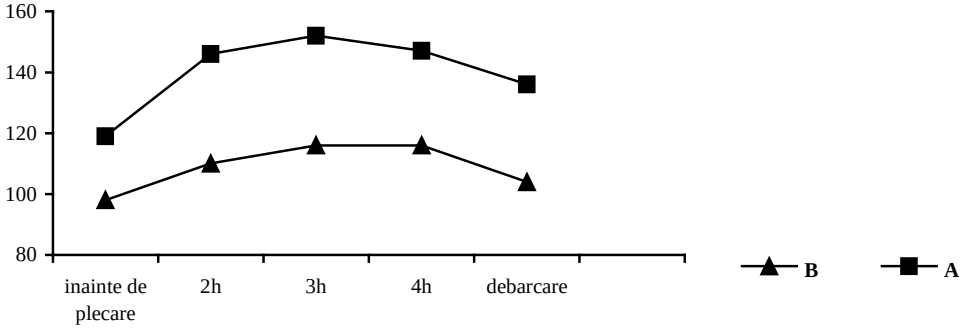


Figure 4. The level of plasmatic lactate (mmol/l) in horses participating in the study

This fact is probably due to the former group being used to the handling manoeuvres, which indicates high adjusting abilities.

CONCLUSIONS

Horse transport and particularly the loading/unloading time determine changes in the physiological indicators of welfare such as heart rate and cortisol.

The heart rate may be considered a physiological marker, an efficient method in assessing the horses' welfare level, as its variation indicates the horses' response to loading/unloading stress.

Under different transport conditions and short travel time, plasmatic cortisol concentrations showed different levels in horses accustomed to transport as compared to the ones travelling for the first time, which were not used to transport related handling. These levels also varied with transport duration.

Lactic acid levels show a drop in horses accustomed to transport thus indicating that a correlation with cortisol levels does not constitute a stress indicator during transportation.

REFERENCES

1. Bachmann, I., P. Bernasconi, R. Herrmann, M.A. Weishaupt, M. Stauffacher, 2003, Behavioural and physiological responses to an acute stressor in crib-biting and control horses. *Applied Animal Behaviour Science*, 82(4): 297-311.
2. Broom, D.M., 2000, Welfare assessment and welfare problem areas during handling and transport. In: Grandin T., editor. *Livestock Handling and Transport.* Wallingford, CABI. pp. 43–61.
3. Collins, M.N., Friend, T.H., Jousan, F.D., Chen, S.C., 1999, Effects of density on displacement, falls, injuries, and orientation during horse transportation. *Applied Animal Behaviour Science*, 67, 169-179.
4. Evans, J. W., C. M. Winget, E. J. Pollak, 1977, Rhythmic cortisol secretion in the equine: Analysis and physiological mechanisms *Biological Rhythm Research*, 8, 111 – 121.
5. Fazio, E., Medica, P., Aronica, V., Loredana Grasso, Adriana Ferlazzo, 2008, Circulating β-endorphin, adrenocorticotrophic hormone and cortisol levels of stallions before and after short road transport: stress effect of different distances. *Acta Vet Scand*. 50 (1): 6.
6. Ferlazzo, A., Fazio, E., Murania, C., Piccione, G., 1993, Physiological responses of stallions to transport stress. *Proceedings of the International Congress on Applied Ethology* Germany, pp. 544–546.
7. Friend T.H., (2000), Dehydration, stress, and water consumption of horses during long-distance commercial transport. *J Anim Sci.*, 78:2568–2580.
8. Irvine, CHG; Alexander, SL., 1994, Factors affecting the circadian rhythm in plasma cortisol concentrations in horses. *Domestic Animal Endocrinology*, 11:227–238.
9. Oikawa, M., Takagi, S., Anzai, R., Yoshikawa, H., Yoshikawa, T., 1995, Pathology of equine respiratory disease occurring in association with transport. *J. Comp. Pathology*, 13:29–43.

10. Pell S.M, McGreevy P.D., (1999), A study of cortisol and beta-endorphin levels in stereotypic and normal Thoroughbreds. *Appl Anim Behav Sci.,* 64:81–90.
11. Stull CL, Rodiek AV., 2004, Physiological responses of horses to 24 hours of transportation using a commercial van during summer conditions. *Journal of Animal Science,* 78:1458–1466.
12. Stull C.L., (1999), Responses of horses to trailer design, duration, and floor area during commercial transportation to slaughter. *J Anim Sci.,* 77:2925–2933.

THE GETTING, THE PURIFICATION AND THE CHARACTERIZATION OF SHEEP'S IMMUNOGLOBULIN G (IgG) AND ALSO OF SHEEP'S ANTI-IMMUNOGLOBULIN G SERUM FOR USING THEM IN DIAGNOSIS TESTS

OBTINEREA, PURIFICAREA ŞI CARACTERIZAREA IMUNOGLOBULINEI G (IGG) DE OAIE ŞI A SERULUI ANTI-IMUNOGLOBULINĂ G DE OAIE ÎN VEDEREA UTILIZĂRII ÎN TESTE DE IMUNODIAGNOSTIC

TURCU D.[1], Mariana OPORANU[1], C. COMAN[2], V. CALIN[1]
[1] Faculty of Veterinary Medicine, Spiru Haret University
[2] I.N.C.D.M.I. Cantacuzino Bucharest

Email: ushmv_turcu.danut@spiruharet.ro

REZUMAT

Purificarea Ig G de oaie s-a realizat prin precipitare cu sulfat de amoniu şi cromatografie pe schimbători de ioni (DEAE celuloză). Verificarea purităţii IgG obţinut s-a efectuat prin imunoelectroforeză (IEF) faţă de un ser de iepure anti-ser total de oaie şi electroforeză în gel de poliacrilamidă verticală în sistem denaturant cu dodecil sulfat de sodiu (PAGE-SDS). S-a obţinut un singur arc de precipitare cu migrare catodică la IEF şi o fracţiune cu masa moleculară de 150 KDa la PAGE-SDS corespunzătoare IgG. Serul anti-IgG s-a obţinut prin hiperimunizarea iepurilor. Cuantificarea nivelului de anticorpi IgG s-a realizat prin testul imunoenzimatic (ELISA) şi a evidenţiat valori ridicate ale densităţilor optice care denotă un titru ridicat de anticorpi. Serul de iepure anti-IgG oaie obţinut se va utiliza ca reagent în teste de imunodiagnostic de mare sensibilitate şi specificitate.
Cuvinte cheie: imunoglobulina G oaie, purificare

ABSTRACT

The sheep's G immunoglobuline purifying was made by ammonium sulfate precipitation and chromatography on ion changers (DEAE cellulose). The purity assay of the obtained IgG was made by ion exchange chromatography and immunoelectrophoresis (IEF) toward a rabbit serum total sheep antiserum and sodium dodecyl sulfate poliacrylamide electrophoresis (PAGE-SDS). A single precipitation arch was obtained with cathodic migration to IEF and a fraction with the molecular mass of 150 KDa at PAGE-SDS suitable to IgG. The anti-IgG serum was obtained by rabbits hyperimmunization. The IgG antibodies level quantification was made by the immunoenzymatic technique (ELISA) and we obtained raised values of the optic densities which showed an high level of antibodies. The rabbit's anti-IgG sheep serum obtained will be used as reagent in the immunodiagnosis tests of high sensibility and specificity.
Key words: sheep's G immunoglobuline, purification

INTRODUCTION

The immunoglobulins (Ig) are glycoprotein with antibodies features obtained through extraction methods from the plasma, interstitial liquids and biological secretions. Most of the fractional and purification methods of Ig are based on the chromatographic techniques – the ion exchange chromatography on and gel filtration (1, 3, 5, 6). The ion exchange chromatography with a cellulose structure has the advantage of devising in pure form the G immunoglobulin from a gamma globulin solution or of devising in two types of IgG parts (Fab and Fc) after their digestion with papain.

Phat and all (6) purified the IgG, IgA and IgM from the sow's milk combining the gel filtration with ion exchange chromatography. When the purity of these Ig was checked using SDS-PAGE and the Ouchterlony immunodiffusion, the S element was distinguished from the IgA molecule and also the J linking piece was present in the IgA and IgM molecules. The authors recommend us to use this purifying method to show all the Igs that can be found in other biological liquids.

Kelly and all isolated the IgG and IgG's subclasses through ammonium sulfate, gel filtration and ion exchange chromatography. The fraction's analysis using SDS-PAGE showed that IgG has 150kDa, five subclasses, two heavy chains of 57kDa and two light chains of 27kDa.

Joshi and all (4) described the IgG purification technique on Sepharose column with protein A after three successive serum precipitations with sodium sulfate; the splited gamma globulin obtained using affinity chromatography for IgG had no signs of contamination with other Ig classes. The immunoenzymatic method was used by many other authors (1, 2, 4) due to the analytical system's sensitiveness and specificity. The specialty literature from the last years (4, 7, 8) certifies the extension of these techniques and their high value, according to the known criteria for the diagnosis methods evaluation.

In this paper the research's results are presented regarding the getting, the purification and the characterization of sheep's IgG and also of sheep's anti-IgG serum for using them in immunodiagnostic tests.

MATERIAL AND METHODS

The sheep's total gamma globulin getting was made using the precipitation technique of the sheep's normal serum with saturated ammonium sulfate solution neutralized until pH=7. Three successive precipitations were done and the final amount of gamma globulin was resumed with a small quantity of water. The gamma globulin obtained was then dialyzed at 4^{o}C with a NaCl solution 0,15 M.

The purification of sheep's IgG was made using ion exchange chromatography (DEAE – cellulose). To obtain the IgG a chromatographic column was used (K 2,5/30 cm) with DEAE-cellulose (with a switch capacity of 0,009+0,1mEq/g) balanced with phosphate buffer 0,075M and pH=6,3. The gamma globulin solution was put into the column, using 3-4 g DEAE-cellulose at 200mg protein. The sample's dilution was made with phosphate buffer 0,0175M, pH=6,3 collecting 2mL/tube. The collected fractions were mixed, concentrated and afterwards tested for purity and specificity using SDS-PAGE) and immunoelectrophoresis (IEF).

The fraction's electrophoresis in poliacrylamid gel was done in unnatural conditions using a SCIE-PLAS TV 100 according with the techniques described by Laemmli. The proteins separation was done in two gels with different concentration and pH values: one for proteins concentration using acrylamid 4% gel in Tris-HCl 0,5M buffer, pH=6,8 and another for theirs separation. After the gels migrated they were colored with Coomassie Brilliant Blue G-250 0,1% solution. The markers molecular mass that we used were: ovalbumin (45kDa), bovine serum albumin (66kDa) and myosin (205kDa).

The immunoelectrophoresis was performed in agarose gel 1,2% which was hurried on a glass mount (7,5cm long/2,5cm wide) prepared in veronal buffer with an ionic force of 0,05. The fractions that had to be studied were put in the gel wells, and then after their electrophoretic migration, in the split created between two gel wells the rabbit's serum sheep's antiserum was put. The mounts were maintained in a wet room, at the labs temperature for 18-24 hours. The precipitation arch was seen using a down-up highlight source. The proteins were colored using Amido Black 100 solution, 0,1 %.

The rabbits immunization (n=3) to obtain the sheep's monospecific anti-IgG serum was done with 4 mg IgG/ml put in complete Freund adjuvant. Three subcutaneous inoculations were done, in several places, on the body's sides. The bleeding was done after seven days, after the last inoculation. The quantification of sheep's antibodies anti-IgG level was made using the immunoenzymatic technique.

The immunoenzymatic technique.

- *The antigen*: to catch and to quantize the sheep's anti-IgG serums we used as antigen sheep's IgG diluted at 10 µg/ml in a NaOH solution 0,1N. In the coated stage we added 100µL IgG in every hole. After the plates were put in the incubator for two hours at 37^{o}C, they were washed up using PBS/Tween in a mindray mv-12a plate cleaner. The sheep's anti-IgG

serums were diluted in a ratio of 1/25, 1/50, 1/200, 1/400, 1/800, 1/1600 and 1/3200 in a PBS/Tween buffer with an addition of 0,5% bovine seric albumin. The plates incubation was done at 37°C, for 60 minutes.

- *The conjugate*: we used a sheep anti-IgG conjugate IgG marked with peroxidase. It was diluted in a ratio of 1/100 in PBS/Tween buffer to which we added bovine seric albumin 1% and then we put 100µl in each well.
- *The substratum*: it contained 0,005 hydrogen peroxide and 0,6mg/mL ABTS in citrate buffer, pH=4. We used 100µl substratum in each well and after one hour the reaction was stopped with 50 µl sodium florure 1,5%.
- *The reading*: the optical densities (DO) were read at 405nm with a multichannel spectrophotometer on ELISA Apollo LB 911 plates (Berthold Technologies).

RESULTS AND DISCUSSIONS

The total gamma globulin fraction on a DEAE-cellulose column showed only one proteic pick. The elution profile obtained after the chromatography is presented in figure 1.

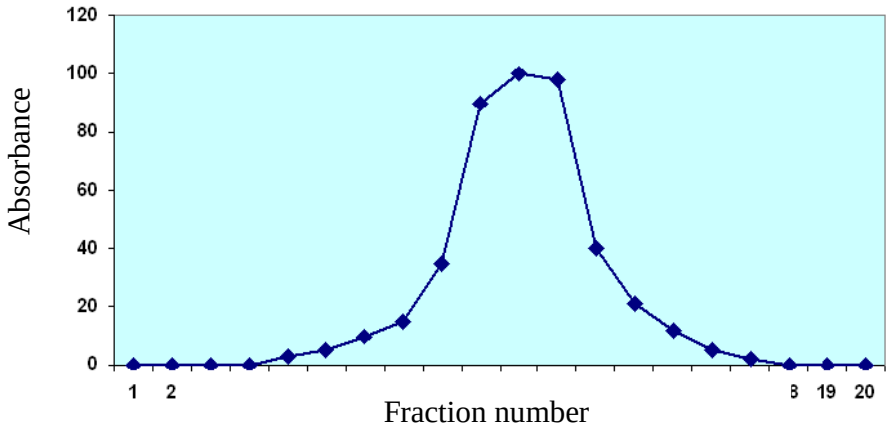


Figure 1. The elution profile of sheep's IgG obtained on a DEAE cellulose column

As the data show, the fractions that are in the pick's maximal area were put together, concentrated and analyzed for their purity using IEF towards the sheep's anti-serum serum. In the diluted part the IgG appeared after collecting 30ml buffer. From the total gamma globulin, the IgG was diluted in the next 20-25 ml buffer. The elution of the others proteins left in the column was done with a NaCl solution 0,25 M.

Using the electrophoretic analyses for the fractions suitable to the pick obtained, we could see a precipitation arch characteristic to IgG with electroforetic migration in the cathodic area of the mount (figure 2).

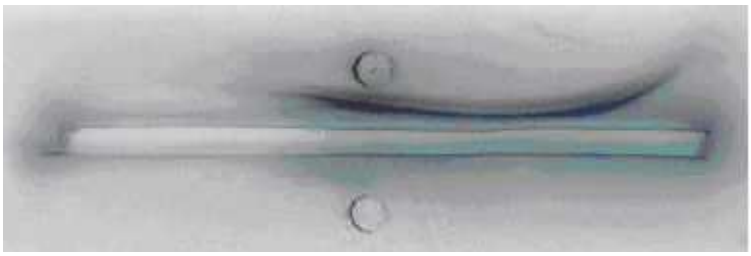


Figure 2. The purity checking of sheep's IgG using immunoeletrophoresis towards the rabbit's serum sheep's antiserum.

It was proven that IEF is a qualitative method very useful in estimating the G immuno globulin purification degree.

Using the SDS-PAGE electroforetic study of the molecular fractions obtained through separation on DEAE cellulose we could see the presence of a single band with the molecular mass of 150kDa (figure 3).

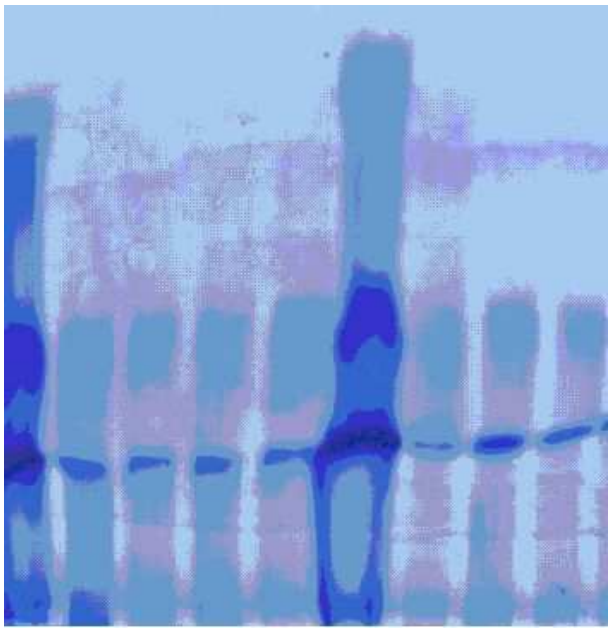


Figure 3. Electrophoretic analysis of sheep's IgG usinf SDS-PAGE

This band is a match to IgG which shows that using the DEAE-cellulose splitting we can do this purification.

The titers of the sheep’s anti-IgG serum (n=3) between 1/25 -1/3200 (values of concentrations and dilutions) are presented in table 1 and figure 4.

Table. 1. The DO values of sheep's anti-IgG serum obtained using the immunoenzymatic technique

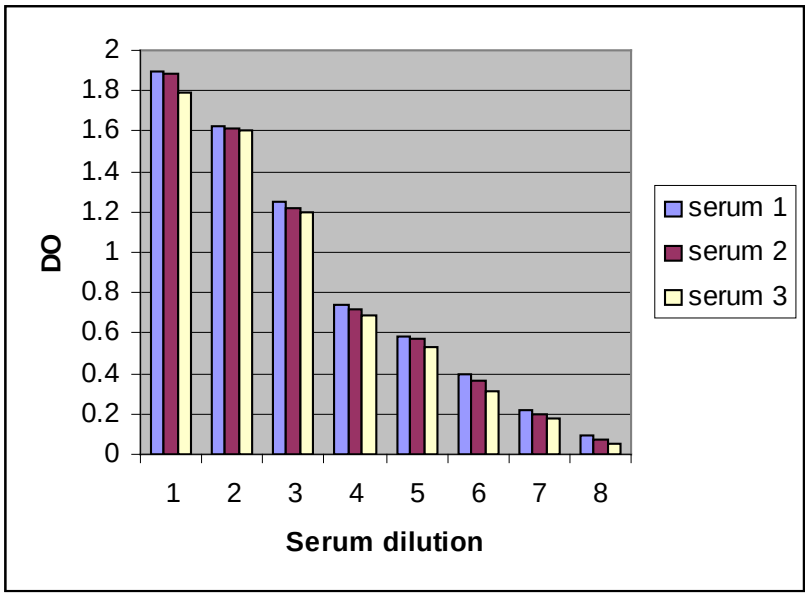


Figure 4. The sheep's anti-IgG serum titration using immunoenzymatic technique

The level's quantification of the sheep's antibodies anti-IgG using ELISA showed high titer of antibodies up to a dilution of 1/800 (DO serum 1=0,395; DO serum 2=0,362 and DO serum 3=0,315). These values show the ELISA test sensitiveness and the possibility to determine the titer of the analyzed anti-sera (1/800 in all 3 sera).

The sheep's anti-IgG serum specificity was studied using the immunoenzymatic technique for the evaluation of antigen-antibody reactions towards other species immuno globulin: ox's IgG, pig's IgG, rabbit's IgG and dog's IgG (10µl/ml).

In figure 5 we showed that DO were negative comparative to the pig's IgG (DO=0,010), rabbit's IgG (do=0,0004) and dog's IgG (DO=0,002) and this fact proves that the sheep's anti-IgG serum have a raised specificity.

No	IgG species	Optic density
1	Sheep's IgG	1.890
2	Ox's IgG	0.200
3	Pig's IgG	0.010
4	Rabbit's IgG	0.004
5	Dog's IgG	0.002

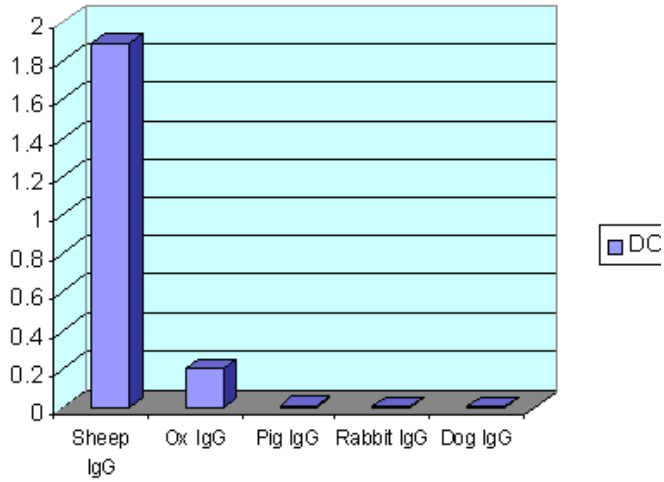


Figure 5. The sheep's anti-IgG serum specificity testing

The sheep's anti-IgG serum presented a weak reaction towards the ox's IgG. The DO=0,2000 value was in the negative reaction area.

The proven qualities of the sheep's serum helped them became valuable reagents used in diagnosis and therapy. Their applicable area can be expanded in the animals pathology, especially in the scientific research to achieve immunodiagnostic techniques of high efficiency (immunoenzimatic, immunofluorescence), structural and ultrastructural immunocitochemistry with marked antibodies, the antibodies research using blotting tests, immunobiosensors, immunoelectronomicroscopy).

CONCLUSIONS

1. The sheep's IgG purification was done using the precipitation reaction with ammonium sulfate and ion exchange chromatography (DEAE –cellulose).
2. The IgG's purity checking was done using the IEF technique towards the rabbit's anti-serum and using also SDS-PAGE.
3. After using IEF we obtained a single precipitation arch with migration in the chatodic area and a fraction with the molecular mass of 150KDa using SDS-PAGE.
4. The sheep's anti-IgG serum were obtained from the rabbit's hyperimmunization
5. The sensitiveness of the sheep's anti-IgG serum tested using ELISA showed high antibodies titers.
6. The sheep's anti-IgG serum testing was done using ELISA test comparative to their species Ig and the results obtained are in the negative area.
7. The sheep's anti-IgG serum prepared will be used in the immunodiagnostic tests of great sensitiveness and specificity.

REFERENCES

1. Fețeanu A., - Anticorpi marcați în biologie şi medicină. Editura ceres, Bucureşti, 1973, 39-68.
2. Hodgkinson A.J., Mc Quoid M.P., Hodgkinson S.C., - Antibody class specific responses in ovine milk measured by ELISA. Proceed New Zeal, Soc-Anim. Prod., 1995, 55, 218-220.
3. Joshi V.B., Sodhi S.S., Saini S.S., - Purification of immunoglobulin G of the Indian buffalo (Bubalus bubalis) on protein A Sepharose. Buff. J., 1997, 13 (1), 87-90.
4. Jeffrey K Actor, - Elsevier's Integrated Immunology and microbiology, Mosby Elsevier, Houston, Texas, 2007, 73-80.
5. Kelly P.J., Carter S.D., Azwai S.M., - Isolation and caracterisation of immunoglobulin G subclases of the African elephant. Comp. Immunol. Microbiol. Inf., 1998, 21 (1), 65-73.
6. Phat T.L., Paraf A., - Purification of three porcine immunoglobulin classes from the same biological source. Ann. Rech. Vet., 1997, 18 (3), 261-267.
7. Vior C., - Biotehnologii medicale. Editura Fundației România de Mâine, Bucureşti, 2000, 250-266.
8. Williams D.L., - Ophthalmic Immunology and Immune Mediated Disease, 2008, 38 (2), 361-387.

HEMATOLOGICAL RESEARCHES, AFTER THE INCREASE THE IMMUN RESPONSE ON PIGS, BY USING SOME IMUNOMODULATORS
CERCETĂRI PRIVIND POTENȚAREA RĂSPUNSULUI IMUN LA SUINE, DUPĂ VACCINARE, PRIN FOLOSIREA UNOR IMUNOMODULATORI

CALIN V., D. TURCU, T. COMAN, T. PETRUT
Faculty of Veterinary Medicine
Spiru Haret University
ushmv_calin.victor@spiruharet.ro

REZUMAT

S-a urmărit reacția hematologică a organismului, ca urmare a potențării cu imunomodulatori nespecifici a răspunsului imun indus de vaccinarea antipestoasă şi antirujetică (imunomodulare specifică) la purcei crescuți în sistem intensiv. Au fost supuşi testării 40 de purcei, sub forma a 4 loturi (A, B, C, D), începând cu vârsta de 52 de zile, testarea fiind realizată într-un complex de creşterea porcului în sistem intensiv.

La lotul A s-a administrat suspensie de corpi bacterieni (Corynebacterium parvum). La lotul B a fost administrat preparatul Levamisol, de uz veterinar, iar la lotul C s-a administrat vitamina E şi seleniu folosindu-se produsul Romselevit. Lotul D a fost folosit ca martor fiind supus doar vaccinării.

Experimentul s-a derulat pe o perioadă de 85 de zile, timp în care s-au efectuat trei prelevări de sânge. Datele au fost prelucrate statistic prin metoda Student-Fischer.

Numărul de leucocite (valori absolute) a prezentat la sfârşitul experimentului o scădere semnificativă la lotul modulat cu Corynebacterium parvum ($p<0,05$), comparativ cu celelalte loturi.

Concentrația de hemoglobină, la lotul A (modulat cu Corynebacterium parvum), a crescut distinct semnificativ ($p< 0,01$), după recoltarea a II a, față de recoltarea I. La recoltarea finală s-a produs o scădere a acestor concentrații, statistic manifestându-se tot distinct semnificativ ($p< 0,01$), față de recoltarea a II a.La loturile B, C, şi D, concentrațiile de hemoglobină au prezentat o creştere importantă din punct de vedere statistic, doar la recoltarea intermediară (înalt semnificativ - $p< 0,001$), după care s-au menținut constante.

La lotul A, modulat cu Corynebacterium parvum, hematocritul a crescut distinct semnificativ ($p< 0,01$), față de recoltarea I. La recoltarea finală s-a produs o scădere a acestor concentrații, statistic manifestându-se semnificativ ($p< 0,05$) față de recoltarea a II a. La loturile B, şi D, hematocritul a crescut din punct de vedere statistic la recoltarea intermediară (semnificativ–$p<0,01$), la recoltarea finală concentrațiile fiind similare celor intermediare. La lotul C s-au produs modificări în sensul că după o creştere distinct semnificativă ($p< 0,01$), la recoltarea intermediară valorile hematocritului au scăzut fără să intereseze din punct de vedere statistic.

Numărul de hematii a prezentat o crescut distinct semnificativ ($p< 0,01$) la lotul A, la recoltarea a II a, față de recoltarea I, la recoltarea finală scăderea statistic fiind semnificativă ($p< 0,05$);

Cuvinte cheie: reacție hematologică, suine, răspuns imun.

ABSTRACT

It was followed the hematological reaction of the organism, as a result of increasing, by using unspecific immunomodulators, of the immune response induced by the Classical Swine Fever and Swine Erysipelas vaccination (specific immunomodulation) on piglets reared in intensive system. We tested 40 piglets, in 4 groups (A,B,C,D), starting with age of 52 days old testing being conducted in an intensive piggery.

In group A it was administrated a bacterial suspension (Corynebacterium parvum). Group B received Levamisol (for veterinary use) and group C received vitamin E and selenium, using Romselevit. Group D was used as a witness group, being submissed only to vaccination.

The experiment was developed in a period of 85 days. During this time, we made 3 blood analysis. The data were statistically processed using Student-Fisher method .

The number of leucocytes (in absolute values) showed at the end of the experiment a significant decrease on group modulated with Corynebacterium parvum ($p<0,05$), comparared with the other 3 groups. The hemoglobin concentration in the same group

A (modulated with Corynebacterium parvum) increased significately distinct ($p<0,01$) after the second blood analysis, comparative with the first blood analysis. At the final analysis these values were decreased, statistically looking as distinctive significative ($p<0,01$), comparative to the second blood analysis.

In groups B, C and D the concentration hemoglobin showed a significately increase from a statistical point of view just at the second blood analysis (high significative $p<0,001$), but after those values remaining constant

in group A, modulated with Corynebacterium parvum, the hematocrit values were significative increased ($p<0,01$) at the second blood analysis, compared to the first blood analysis. At the final blood analysis those concentrations were reduced statistically significant ($p<0,05$) comparative with the second blood analysis.

At the groups B and D the hematocrit was significant increased from statistic point of view at the intermediate blood analysis ($p<0,01$), same values were established at the final blood analysis. At group C some modifications were observed ; after a distinctive significant increasing ($p<0,01$), at the intermediate blood analysis the hematocrit values dropped, without statistically interest.

The red blood cells presented significately distinct increase ($p<0,01$) in group A at the second blood analysis, in opposition with the first one, in which the decrease was statistically significant ($p<0,05$).

Key words: hematological reaction , swine, immune response

INTRODUCTION

The resistance capacity of the animal body can be increased through the use of imunomodulators (3,5), some of which can act specifically inducing different effects (destruction of pathogen germs or the blocking of their activity), in this category falling the vaccines, immune sera, and even antibiotics (4).

The intensity of the immune response can be increase also nonspecifically (1,2) for a certain type of agressor, by the use of a various range of cellular structures, organic or anorganic substances.

The hematomogical reaction of the body after the potentiation with nonspecific substances was observed, over the immune response induced by the the Classical Swine Fever and Swine Erysipelas vaccination (specific immunomodulation), in piglets reared in intensive system.

MATERIALS AND METHODS

32 piglets were submitted to testing, in 4 groups (A,B,C,D), starting with age of 52 days old, testing being conducted in an intensive piggery.

In group A was administered a bacterial suspension (Corynebacterium parvum) in saline solution (2 mg bacterial body dry residue/ml) through the use of *Imunostimulent S.R.E. Corynebacterium parvum*, subcutaneous administration.

Group B received Levamisol (for veterinary use), administered subcutaneous, and lot C received vitamin E and selenium, using the product Romselevit, also administered subcutaneous, according to the experimental scheme (table 1).

Group D was used as a witness group, being submitted only to vaccination.

The vaccination against Classical Swine Fever and Swine Erysipelas was accomplished with a suspension of attenuated Classical Swine Fever virus with the minimal titre of 1000 DICF 50/ml and culture of Erysipelothrix rhusiopathiae (VR_2 strain) with minimal germ concentration of $5x10^7$ UFC/ml, at 60 and 120 days old. The animals had normal feeding and microclimate the entire experiment.

The experiment was conducted on a period of 85 days, during which there were three blood sampling.

Table 1

Experimental models

Stages	Group	Day	Administration C. parvum	Administration Levamisol	Administration Romselevit	Vaccination	Blood draw
Stage 1	A	Day 1	0,2 ml sc/anim	-	-	-	-
		Day 3	0,5 ml sc/anim	-	-	-	-
	B	Day 1	-	0,5 ml sc/anim	-	-	-
		Day 3	-	0,5 ml sc/anim	-	-	-
	C	Day 1	-	-	1,5 ml sc/anim	-	-
		Day 3	-	-	1,5 ml sc/anim	-	-
	D	Day 1	-	-	-	-	-
		Day 3	-	-	-	-	-
Stage 2	A	Day 8	-	-	-	1 ml sc/anim	*
	B	Day 8	-	-	-	1 ml sc/anim	*
	C	Day 8	-	-	-	1 ml sc/anim	*
	D	Day 8	-	-	-	1 ml sc/anim	*
	A	Day 15	0,5 ml sc/anim	-	-	-	-

Stage 3		Day 17	0,5 ml sc/anim	-	-	-	-
	B	Day 15	-	0,5 ml sc/anim	-	-	-
		Day 17	-	0,5 ml sc/anim	-	-	-
	C	Day 15	-	-	1,5 ml sc/anim	-	-
		Day 17	-	-	1,5 ml sc/anim	-	-
	D	Day 15	-	-	-	-	-
		Day 17	-	-	-	-	-
Stage 4	A	Day 61	0,5 ml sc/anim	-	-	-	-
		Day 63	0,5 ml sc/anim	-	-	-	-
	B	Day 61	-	0,7 ml sc/anim	-	-	-
		Day 63	-	0,7 ml sc/anim	-	-	-
	C	Day 61	-	-	2 ml sc/anim	-	-
		Day 63	-	-	2 ml sc/anim	-	-
	D	Day 61	-	-	-	-	-
		Day 63	-	-	-	-	-
Stage 5	A	Day 70	-	-	-	1 ml sc/anim	*
	B	Day 70	-	-	-	1 ml sc/anim	*
	C	Day 70	-	-	-	1 ml sc/anim	*
	D	Day 70	-	-	-	1 ml sc/anim	*
Stage 6	A	Day 84	-	-	-	-	*
	B	Day 84	-	-	-	-	*
	C	Day 84	-	-	-	-	*
	D	Day 84	-	-	-	-	*

* = the group from witch the blood was sampled

Quantified parameters

1. Total white cell count
2. Hemoglobinemia
3. Hematocrit
4. Red blood cell count (absolute values)

The hematological determinations were conducted through the electronical method with a Coulter-Counter CBC-5 analyzer.

RESULTS AND DISCUSSION

White blood cell count.

There were 3 blood sampling for the determination of theese parameters, the first one after 8 days after the experiment started when the piglets were immunized with Swine Erysipelas vaccine, the second sampling after the revaccination, in the 70th day, and the last one at the end of the experiment.

After the statistical processing of the white cell count, there was a statistical significant decrease at the 3rd sampling, in group A, to which was administered bacterial suspension Corynebacterium parvum ($p<0,05$), the count being 16,10±4,94 thou/ mm^3, compared to the witness group that counted 21,01±3,94 thou/mm^3 white blood cells(table 2, graphic 1).

There were no other statistic significant alterations in this constant.

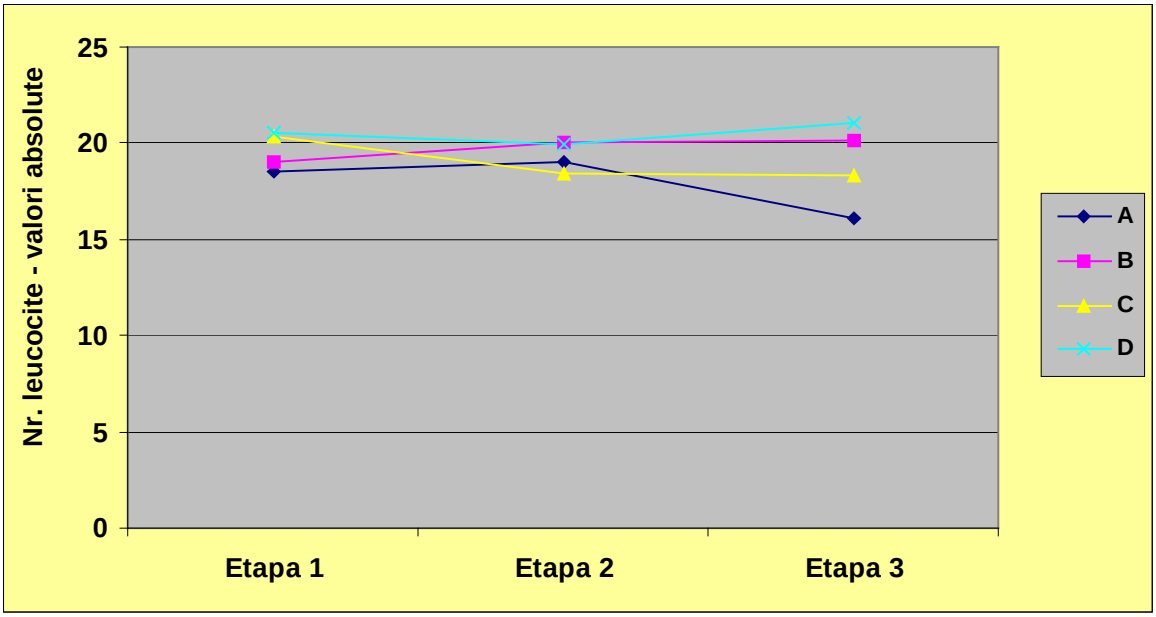
Table 2

White cell count* – absolute values

Group	Stages		
	I	II	III
A	18,53±3,94	18,99±3,66	16,10±4,94*
B	19,03±5,09	20,03±2,84	20,16±4,38

C	20,30±3,93	18,41±4,12	18,27±2,77
D	20,54±10,13	19,91±3,69	21,01±3,94

*** - (average + standard deviation);**
*** = significant difference;**



Graphic 1 – The graphic representation of the white cell count-absolute values

Determination of some hematological parameters

Tabel 3

Hematological parameters. Blood sampling I

Group	Parameter	Number of subjects						
		1	2	3	4	5	6	7
A	Hemoglobin (g /dl)	11,9	10,9	11,3	10,5	9,7	10,2	9,8
	Hematocrit (%)	36,4	34,2	34,0	32,9	29,7	31,9	30,7
	Haematids count (mil/mm^3)	6,52	6,80	5,91	6,49	5,64	6,07	6,17
B	Hemoglobin (g /dl)	11,2	9,2	10,5	11,0	10,5	9,7	10,2
	Hematocrit (%)	34,2	28,2	31,8	33,1	31,9	29,5	31,3
	Haematids count (mil/mm^3)	6,36	5,56	6,78	6,03	6,37	5,97	6,54
C	Hemoglobin (g /dl)	10,7	9,0	10,1	11,7	10,8	9,0	11,3
	Hematocrit (%)	32,1	27,5	30,2	35,0	32,0	27,5	33,2
	Haematids count (mil/mm^3)	6,02	5,93	5,98	6,07	6,22	6,35	6,52
D	Hemoglobin (g /dl)	8,9	9,0	9,6	10,2	9,5	11,6	11,5
	Hematocrit (%)	28,0	28,	29,6	31,6	28,1	35,5	34,4
	Haematids count (mil/mm^3)	5,17	5,11	5,84	6,23	5,26	7,13	6,08

Table 4

Hematological parameters. Blood sampling II

Lot	Parametrul	Number of subjects						
		1	2	3	4	5	6	7

A	Hemoglobin (g /dl)	13,0	13,0	11,8	17,3	12,4	12,6	11,9
	Hematocrit (%)	39,5	38,9	34,2	51,3	37,2	37,7	34,7
	Haematids count (mil/mm^3)	7,4	7,79	6,2	9,47	7,54	7,25	6,66
B	Hemoglobin (g /dl)	11,7	12,7	13,1	12,4	11,7	12,5	10,7
	Hematocrit (%)	34,6	36,8	38,6	36,1	33,8	36,7	30,3
	Haematids count (mil/mm^3)	6,31	6,93	7,56	6,67	6,53	7,03	5,96
C	Hemoglobin (g /dl)	10,9	12,5	12,3	11,9	13,3	13,1	12,8
	Hematocrit (%)	32,0	36,3	35,5	34,5	39,3	38,3	37,3
	Haematids count (mil/mm^3)	6,52	7,05	6,48	6,57	7,48	7,02	7,51
D	Hemoglobin (g /dl)	11,5	10,3	11,9	12,5	12,6	12,5	12,7
	Hematocrit (%)	35,5	30,0	36,5	37,7	37,3	37,1	37,4
	Haematids count (mil/mm^3)	6,92	5,76	7,09	6,96	7,29	3,84	7,24

Table 5
Hematological parameters. Blood sampling III

Lot	Parametrul	Number of subjects						
		1	2	3	4	5	6	7
A	Hemoglobin (g /dl)	12,4	11,4	9,8	9,4	12,2	6,4	-
	Hematocrit (%)	36,5	33,9	30,5	28,4	37,4	19,6	-
	Haematids count (mil/mm^3)	7,52	6,49	5,62	5,2	7,42	3,67	-
B	Hemoglobin (g /dl)	13,2	13,1	12,5	12,2	12,5	11,5	9,9
	Hematocrit (%)	39,4	39,7	37,5	36,3	37,5	34,6	30,3
	Haematids count (mil/mm^3)	7,11	7,16	6,93	6,72	7,15	6,42	5,75
C	Hemoglobin (g /dl)	12,2	13,0	12,9	12,4	12,2	12,5	12,6
	Hematocrit (%)	5,4	38,5	37,8	36,5	35,5	38,0	37,4
	Haematids count (mil/mm^3)	7,15	7,47	7,25	6,52	6,79	7,26	6,83
D	Hemoglobin (g /dl)	10,8	12,1	11,1	13,1	11,3	13,8	13,5
	Hematocrit (%)	31,8	36,2	33,0	38,5	33,3	41,5	38,6
	Haematids count (mil/mm^3)	6,42	6,93	6,22	7,31	6,29	8,17	7,33

The Hemoglobin concentration (table 6, graphic 2) in group A (modulated by *Corynebacterium parvum*), increased significantly distinct ($p<0,01$) in the second blood sampling (13,14±1,89 g /dl), opposite to the first blood sampling (10,61±0,81 g/dl), and in the final blood draw decreasing significantly distinct ($p< 0,01$), from 13,14±1,89 g /dl (2nd sampling) to 10,27±2,26 g /dl at 3rd sampling.

In groups B, C and D, Hemoglobin concentrations were increased with statisctical importance only in the 2nd sampling (significant high $p<0,001$) and after that maintained constant.

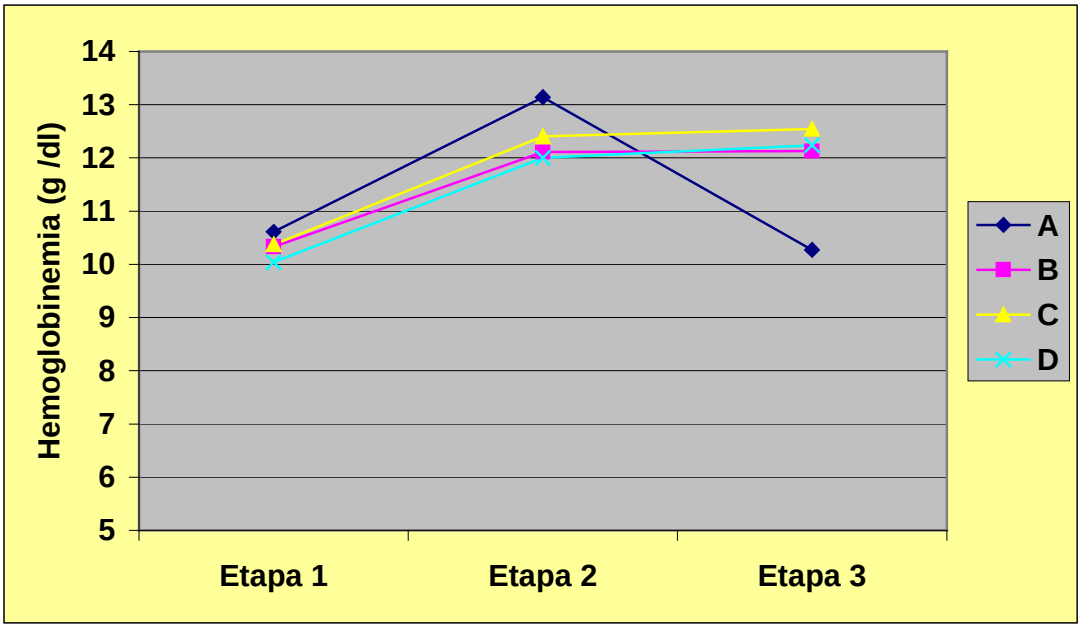
Table 6
Hemoglobinemia (g /dl)*

Group	**Stages**		
	I	II	III
A	10,61±0,81	13,14±1,89**	10,27±2,26**
B	10,33±0,7	12,11±0,81***	12,13±1,14
C	10,37±1,06	12,4±0,81***	12,54±0,32
D	10,04±1,11	12,0±0,87***	12,24±1,23

*** - (average + standard deviation);**
**** = significantly distinct difference; *** = significantly high difference.**

Regarding the hematocrit, the statistic result were :

-in group A, modulated by *Corynebacterium parvum*, the increase was significantly distinct ($p<0,01$) at the second sampling (39,07±5,74%), compared to the first sampling (32,83±2,28%). At the final draw, there was a statistically significant decrease ($p< 0,05$), from 39,07±5,74 % at the second blood draw to 31,05±6,58 % at the 3rd blood sampling.



Graphic 2 – Graphical representation of the hemoglobin concentration

- in groups B and D, the hematocrit increased statistically in the intermediate blood draw (*significantly*–p<0,01), at the final sampling the concentrations were similar to thoseintermediate (table 7, graphic 3);

- in group C, after a significantly distinct increase (P<0,01) in the 2nd sampling (36,17±2,46%), the values dropped without interest from a statistical viewpoint (32,73±12,09 %).

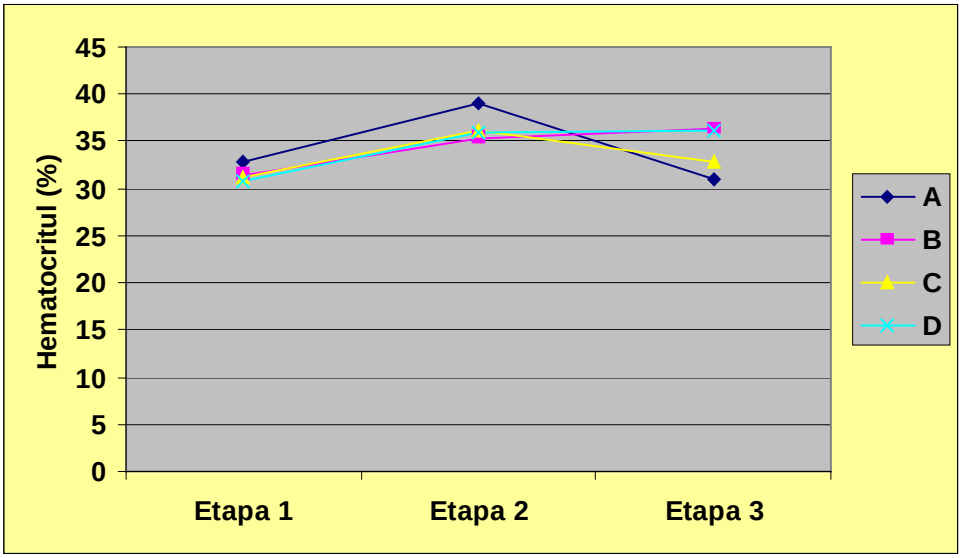
Table 7

Hematocrit (%)

Group	Stages		
	I	II	III
A	32,83±2,28	39,07±5,74**	31,05±6,58*
B	31,43±2,04	35,27±2,69*	36,43±3,26
C	31,07±2,83	36,17±2,46**	32,73±12,09
D	30,74±3,16	35,93±2,71*	36,13±3,59

*** - (average +standard deviation);**

*** = significant difference; ** = significantly distinct difference.**



Graphic 3 – Graphic representation of the hematocrit

The red blood cells count had the following changes :

- in group A there was a significantly distinct increase ($p< 0,01$), at the second sampling (7,47±1,04%), opposite to the first sampling (36,23±0,4%), and in the final one, the increase was statistically significant ($p< 0,05$) (table 8, graphic 4);

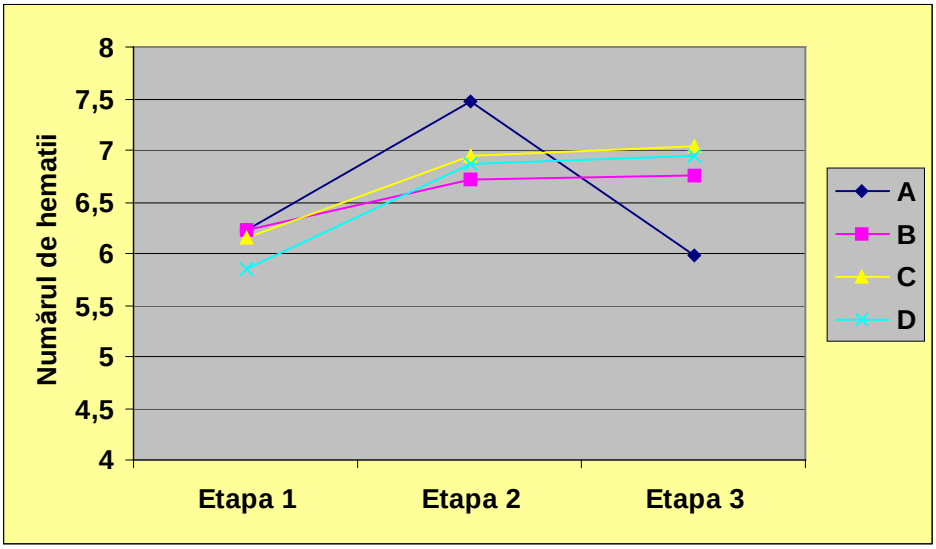
Table 8

Haematids count in absolute values (mil/mm^3)

Group	Stages		
	I	II	III
A	6,23±0,4	7,47±1,04**	5,99±1,47**
B	6,23±0,41	6,71±0,52	6,75±0,52
C	6,16±0,22	6,95±0,44***	7,04±0,33***
D	5,85±0,73	6,87±0,52	6,95±0,71

*** - (average +standard deviation);**
**** = significantly distinct difference; *** = significantly high difference.**

- in group C, the red blood cells count increased significantly high ($p< 0,001$), in the 2nd (6,95±0,44), and in the 3rd blood sampling also(7,04±0,33), compared to the initial one (6,16±0,22);
- in group B the increase was insignificant.



Graphic 4 – Graphic representation of the Haematids count in absolute values (mil/mm^3)

CONCLUSIONS

1. Testing was conducted in an intensive system piggery, the animals beeing subjected to normal feeding and microclimate conditions during the experiment.

2. White blood cells count (absolute values) revealed at the end of the experiment a well-marked decrease in the *Corynebacterium parvum* modulated group compared to the other ones.

3. In the *Corynebacterium parvum* modulated group the hemoglobin concentration, the hematocrit and the red blood cell count had a similar evolution during the experiment, increasing (with statistical value) to the upper physiological limit in the second sampling compared to the first one. In the final sampling , the values decreased to the inferior limit.

4. In the remaining groups, all the tested hematological parameters presented simmilar manifestations during the experiment, beiing observed a statistically important increase only in the intermediate blood sampling , after which they maintained constant.

REFERENCES

1. Beuth, J., Ko H.L., G. Pulverer, (2002), Bacterial peptides as immunomodulators, Old Herborn University Seminar Monograph, 15: 151-157.

2. Kim, J.D., Hyun Y., (2000), Effects of immunostimulators on groth performance and immune response in pig weaned at 21 days of age, Journal of Animal and Feed Sciences, 9(2) 333-346.

3. Lee, D.N., shen T.T., et al, (2000), Effects of chromium supplementation and lipopoly sacharide injection on the immune responses of weanling pigs; Asian-Australian journal of Animal Science, 13(10) 1414-1421.

4. Mikulska-Skupien, E., Szweda, W., Procajlo, Z.; Platt-Samoraj, A.; (2004), Indices of non-specific celular immune response in pigs after intredermal vaccination with deleted Aujeszky's disease vaccine and after experimental infection, Bulletin of the Veterinary Institute in Pulawy 48 (4) 347-354.

5. Xie Ming Quan, Song Chang Xu, (2000), Effects of five immunostimulants on proliferation of peripherial blood mononuclear cells (PBMC) proliferations pigs, Chinese Journal of Vet. Science, 20(5) 485-486.

EFFECTS OF ORGANIC SELENIUM AND VITAMINA E DIET SUPPLEMENTATION ON SOME SERUM ENZYMES ACTIVITIES IN BROILER
EFECTELE SUPLIMENTĂRII DIETEI CU SELENIU ORGANIC ŞI VITAMINA E ASUPRA ACTIVITĂȚII UNOR ENZIME SERICE LA BROILERI

AVRAM N.[1], Eugenia AVRAM [1], Cristina DINU [1], D. CUCĂ[1], Cristina ȚOCA [2]
[1]Faculty of Veterinary Medicine *Spiru Haret* University
e-mail: ushmv_avram.nicolae@spiruharet.ro
[2]IDSA, Bucureşti

REZUMAT

Au fost studiate efectele suplimentării raţiei cu seleniu organic şi vitamina E la un lot de pui broiler asupra unor enzime serice. În acest scop s-au alcătuit două loturi a câte 30 de pui din linia Cobb 500: lotul 1 a cărui raţie a fost suplimentată cu seleniu organic (0,3 ppm Se) şi vitamina E (1500 UI/zi) şi lotul 2 (control), furajat cu raţie deficitară în seleniu şi vitamina E. Au fost prelevate probe de sânge în două etape, la vârsta de 30, respectiv 56 zile (înainte de sacrificarea puilor). Din serul sanguin s-au determinat următoarele enzime: transaminaze (TGO, TGP), gamaglutamyltranspheraza (GGT), fosfataza alcalină (Pal), creatinfosfokinaza (CPK). Analizele s-au efectuat la un analizor biochimic semiautomat. S-au înregistrat diferenţe semnificative între loturi ($p< 0.01$) privind activităţile TGO, Pal (etapa a 2-a) şi CPK, crescute la lotul de control faţă de lotul 1; valorile TGP şi GGT nu au prezentat diferenţe semnificative între loturi. Rezultatele demonstrează efectele benefice ale seleniului şi vitaminei E din raţie ca protectori antioxidanţi hepatici şi ai musculaturii scheletice; la lotul de control datele evidenţiază evoluţia leziunilor distrofice hepatice şi musculare (creşterea activităţii TGO, Pal şi CPK peste limitele maxime de referinţă).

Cuvinte cheie: *seleniu, broiler*

ABSTRACT

Effects of organic selenium and vitamin E supplemented diet on some serum enzymes activities was studied. Two groups of 30 broiler Cobb 500 breed, were formed: lot 1 was fed with organic selenium(0,3ppm) and vitamin E(1500UI/day) supplemented diet; lot 2(control) was fed with selenium and vitamin E deficient diet. Blood samples were drawn in two stages, at 30 days and 56 days of life respectively, before slaughter. Following serum enzymes were determined using a semiautomatic biochemical analyser:transaminases(GOT,GPT),gamaglutamyltranpherase (GGT),alkaline phosphatase(Pal), creatinphosphokinase(CPK). Significant differences($p< 0.01$) among the lots of GOT, Pal (stage 2) and CPK were recorded. GPT and GGT activities not presented significant differences.The dates proved best results in the lot 1 with selenium and vitamin E supplemented diet as liver and muscular antioxidant protector factors; at the control lot results demonstrated the evolution of liver and muscular distrophie(values of GOT, Pal and CPK serum activities were over of the maximum reference values).

Keywords: *selenium, broiler*

INTRODUCTION

Deficiency of selenium and vitamin E in broiler is closely related with their deficiency, especialy of selenium in plants. Deficiency of selenium and vitamin E in hen and chicken has complex actions and is associated with a large variety of pathological desorders (3,5,6,9) Glutathionperoxidase (GSH-Px) and selenium have important part in chicken exudative diathesis prevention (5,6,7,9). The mechanism of GSH-Px and vitamin E action was well established. Diferent target organs of selenium and vitamin E deficiency, as liver, skheletal

muscles, heart, contains especially GSH-selenodependent form(10,12). It is well proved capacity of selenium to intensify vitamin E activity in chicken encefalomalacie (5,8).

In poultry farms are known selenium and vitamin E sources, their biological requirement, their values for production, reproduction and health (2,4,10,11,12,13,14).

The hope of the study is to establish some serum enzimes activites in broiler as a result of organic selenium and vitamin E supplementation diet especially for diagnosis purpose.

MATERIALS AND METHODS

Two groups of 30 broiler Cobb 500 breed were formed:

- lot 1 was fed with organic selenium (0,3 ppm) and vitamin E (1500 UI/day) supplemented diet;
- lot 2 (control) was fed with selenium and vitamin E deficient diet.

Blood samples were drown in two stages, at 30 days and 56 days of life, respectively, before slaughter.

Following serum enzimes were determined using a semiautomatic bichemical analyzer: glutamic oxalacetic transaminase (GOT), glutamic piruvic transaminase(GPT), gamaglutamyltranspherase (GGT), alcaline phosphatase (Pal), creatinphosphokinase (CPK)

RESULTS AND DISCUSSION

The values recorded was centralised in the tables 1 and 2 and graphically presented in the figures 1 and 2. The dates were statistically processed by "t" test, student.

Tabel 1

Values of serum GOT, GPT and GGT in broiler($\bar{x}\pm s$)

Lots	No	TGO(u/l)		TGP (u/l)		GGT (u/l)	
		Stage 1	Stage 2	Stage 1	Stage 2	Stage 1	Stage 2
1	30	64.5 ± 27^{a}	68.6 ± 15.4^{c}	8.72±2.6	7.40±0.9	14.8±2.3	17.5±3.8
2	30	102.1 ± 35^{b}	118.4 ± 26.8^{d}	11.9±2.4	9.40±1.5	12.7±3.0	13.5±2.4
Reference values (Avram ,2004)		70±40		12±8		10±5	

a -› b- $p < 0,001$

c -› d- $p < 0,001$

Tabel 2

Values of serum Pal and CPK in broiler($\bar{x}\pm s$)

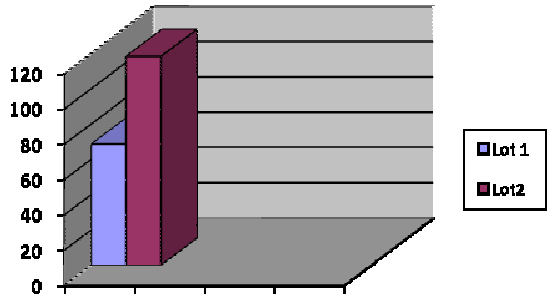
Lots	No	Pal (u/l)		CPK(u/l)	
		Stage 1	Stage 2	Stage 1	Stage 2
1	30	1166±102	806 ± 94^{a}	80 ± 25.4^{c}	78.5 ± 30.0^{e}
2	30	1240±300	1140 ± 120^{b}	398 ± 126^{d}	418 ± 141.70^{f}
Reference values (Avram N ,2004)		820±440	620±440	150±100	

a -› b – $p < 0.001$ e –› f – $p < 0,001$

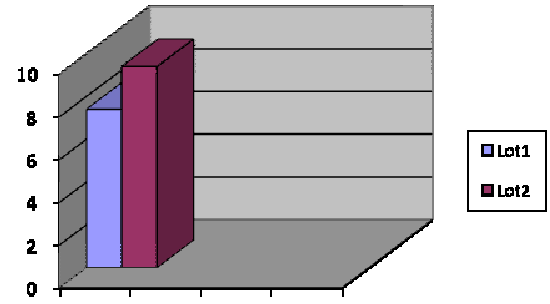
c -› d - $p < 0.001$

Fig1 Mean values of GOT, GPT and GGT in broiler (stage 2)

TGO u/l



TGP u/l



GGT u/l

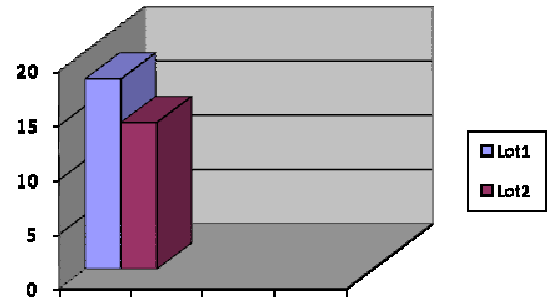
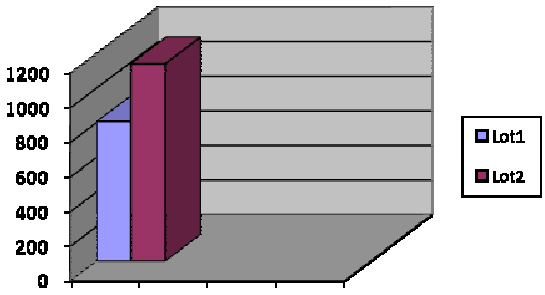
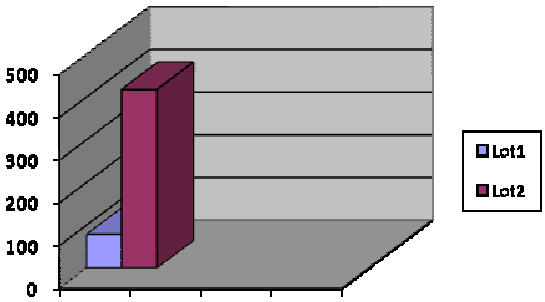


Fig 2 Mean values of Pal and CPK in broiler (stage 2)

Pal u/l



CPK u/l



GOT is a ubiquity enzyme formed especially in the liver, skeletal muscle and myocardium . In nutritional hepatopathies and myopathies due to selenium and vitamin E deficiency GOT activity is increased by tissue affection (distrophie or even necrosis) – (1). In our study increasing of GOT activity in the chicken at the control group was recorded with high values of 102,1±35 U/l (stage 1) and 118,4±26,8 U/l (stage 2) that demonstrated evolution of liver and muscular desorders of nutritional origin. In the group 1 GOT activities were in the reference limits being significantly lower by the group 2 ($p < 0.001$) that indicate benefical effect of organic selenium and vitamin E suplementted.

GPT don't present particular specificity in poultry with nutritional hepatopathies and myopathies due to selenium/vitamin E deficiency point confirmed also in our study. Thus GPT presented close values between the two groups of broiler in the reference limits (table 1). GPT activitie may increase in defferent liver and muscular desorders but her diagnosis value rest limited (1,5).

GGT is present in higher quantites in liver, kidney and pancreas. GGT activitie may increase in several hepatic lesions due to some disfunction of celular liver membrane (1). In our study was established that GGT values exceeded the reference limits to both groups but the defferences between the groups were not significantly ($p< 0.5$).

Pal may present in poultry increased values in some phisiological state by osteoclastes or osteoblastes activity ar well as in different pathological state as:rickets, osteomalacia, osteofibrosis(5). Increasing in Pal activities may be also observed in different liver desorders. Interpretation of Pal values had to be made in corelation with the age of the tested birds. In our study could be established, especially at the group 1 decreasing of Pal activitie in the second stage of analysis($p< 0.001$), table 2. It is possible that some increased values of Pal, in corelation with GPT values to point out the presence and evolution of some subclinical liver desorders but the diagnosis value these enzymes for selenium/vitamin E deficiency is rather reduced.

CPK is enzyme localizeted especially in skeletal muscle and myocardium (5,10). Increasing of CPK activitie represent an usefull and precocius marker for nutritional myodistrophie wich is frequently formed in selenium/vitamin E deficiency. CPK may indicate the integrity of celular membrane especially of skeletal muscle.

In our study the differences between the groups of CPK were significantly in both stages ($p<0,01$). CPK values in the control group was significantly higher by the reference values wich indicate nutritional myodistrophie evolution at the broiler with selenium/vitamin E deficiency diet.

The recorded results demonstrate benefical effect of diet supplemented with selenium and vitamin E as antioxidative protectors of the liver and skeletal muscles; to the group wich was fed a selenium/ vitamin E deficiend diet was recorded distrofic lesions of the liver and skeletal muscles (GOT; Pal, CPK activities were significantly increased over the maximum reference limits). The results were also confirmed by histology exams.

CONCLUSIONS

1. Effects of organic selenium and vitamin E supplementation diet was studied to a group of 30 broiler Cobb 500 breed on some serum enzymes activities comparatively with a control group fed a selenium/vitamin E deficient diet.

2. At the supplemented group significantly lower values ($p<0.001$) of GOT, Pal(stage 2) and CPK was recorded versus the control group; GPT and GGT don't present significantly differences betwen the groups.

3. Recorded results demonstrate benefical effects of organic Se/vitamin E supplemented diet as antioxidatives liver and skeletal muscle protectors; at the nonsuplemented group increasing of GOT, CPK and Pal activities demonstrate evolution of some liver and muscular distrophic lesions.

REFERENCES

1. Avram N, Eugenia Avram, Maria Serdaru, D. Cucă, B. Nicolae, Cristina Dinu (2007). – Teste paraclinice pentru evaluarea deficitului în seleniu la broileri.
   Rev . Română de Medicină Veterinară N:3, pag 141-146
2. Edens F. W., Growdy K. M (2004)- Selenium sources and selenoproteins in practical poultry production.
   Nutritional Biotechnol. in the Feed and Food Industries
   Proc. of Alltech's 20 –th. Annual Symp. 36-55
3. Edens F. W., T. A. Carter, C. R. Parkhurst (2000)- Effect of selenium source and litter type on broiler feathering.
   J. Appl. Poul Res. 9: 407-413.
4. Ferencik M., L. Ebringer (2003) – Modulatorry effects of selenium and zinc on the immune system
   Folia. Microbiol.; 48 (3): 417-426.
5. Ghergariu S., (1980) – Oligominerale şi oligomineraloze
   Ed. Acad RSR., Bucureşti
6. Jones G., V. G Stanley(2000) – Comparative evaluation of the effects of selenium – yeast and vitamin E on ascites syndrome in broilers.
   Ass. Res. Dir . 12th Res. Symp Washington D.C.
7. Naylor A. J., M. Choct, K . Jacques (2000). Effects of selenium source and level on performance, mortality and meat quality in male broilers,
   Southern Poultry Sei,Symp.Atlanta, Georgia, Ian. 16-17.

8. Orăşanu Adriana, Jenica Bucur, N. Alexandru, S. Nicolae (2003)
Observații privind capacitatea seleniului de a potența activitatea vitaminei E în encefalomalacia puilor.
Rev. Rom.de Med. Vet. Vol 13, 3-4, p 173

9. Roch. G., M. Boulianne (2000)- Effects of dietary vitamin E and seleniu on incidence of ascites, growth performance and blood glutathione peroxidase in cold stressed broilers.
Poult. Scy, 79:115

10. Sevastre B., A. I, Baba, L. Pantă (2005) Seleniul organic- mecanisme de acțiune şi efecte.
Rev. Rom. de Med. Vet. Vol 15, N:2, 51-62.

11. Surai P. F. (1999)- Vitamin E in avian Reproduction
Poultry and Avian Biology Rev. , 10, 1, 1-60.

12. Surai P. F. (2004)- Selenium in Nutrition and Health
Nottingham Univ. Pres. Nottingham UK.

13. Todorovic M., M. Mihailovic, S Hristov (1999) – Effects of excesive levels of sodium selenite on daily wright gain, mortality and plasma selenium concentration in chickens
Acta vet. (Belgrade) 49: 313- 320.

14 Țăranu Ionelia, Rodica Criste , Gh. Burlacu (1994). Formarea vitaminelor la puii broiler. Analele IBNA, vol 17, Bucureşti.

MONITORING OF SOME LIVER DISEASES IN DOG BY PARACLINICAL TESTS
MONITORIZAREA UNOR AFECȚIUNI HEPATICE LA CÂINE PRIN TESTE PARACLINICE

MANDA Nicoleta (masternad USH), N. AVRAM
Faculty of Veterinary Medicine *Spiru Haret* University
Email – eugeniaavram@yahoo.com

REZUMAT

Într-un cabinet veterinar au fost monitorizați 10 câini cu diferite afecțiuni hepatice, prin examene clinice şi paraclinice efectuate în mai multe etape până la vindecare, eventual moartea animalelor.

Testele de laborator efectuate la un analizor biochimic Metrolab 1600 DR (biochimie umedă) au constat în: determinarea activității unor enzime serice-transaminaze (TGO, TGP), gamaglutamiltransferaza (GGT), fosfataza alcalină (Pal) şi a altor parametri biochimici serici: proteine totale, albumină, colesterol total, trigliceride, glucoză, bilirubină totală.

Dintre cei 10 câini, la 5 activitatea enzimelor serice modificate a revenit în limite normale după 60-200 de zile de tratament şi dietă; 2 câini au prezentat evoluție favorabilă a bolii fiind în curs de monitorizare, iar 3 câini, care au decedat (doi cu ciroză hepatică asci-togenă şi unul cu tumoră hepatică), au prezentat valorile parametrilor investigați modificate până la ultima etapă de control.

Testarea paraclinică s-a dovedit a fi esențială pentru urmărirea evoluției şi orientarea tratamentului afecțiunilor hepatice la câine.

Cuvinte cheie: *transaminaze, gamaglutamiltransferaza, fosfataza alcalină, câine*

ABSTRACT

In a vet surgery 10 dogs with some liver diseases were evaluated by clinical and paraclinical exams performed in several stages till recovery or death of the animals.Laboratory tests perfomed at a biochemical analyser Metrolab 1600 DR (wet biochemestry) were: some serum enzymes activities determination:transaminases (GOT, GPT) gamaglutamyltransferase (GGT), alcaline phosphatase (Pal) and other serum biochemical parameters as: total protein, albumin, total cholesterol, triglicerides, blood glucose, total bilirubin.

By the 10 dogs examinated ,to 5 altered serum enzymes activities returned to normal limits after 60-200 days of treatment and diet, 2 dogs within monitoring presented favorable evolution of the disease and 3 dogs dead (2 with liver ascites cirrhosis and one with liver neoplasia) presented analised parameters altered til the last stage of control.

Paraclinical investigation proved to be essential for evolution and treatment control of the liver diseases in dogs.

Key words: *transaminases, gamaglutamyltransferase, alcaline phosphatase, dog*

INTRODUCTION

Liver functions may be disturbed by numerous etiological factors: nutritional, toxic viruses, bacteriums, metabolics, neoplastic (2, 4, 5, 6, 9).

Functions are indifinite as variety and complexity and may be classiefed as well as: synthetics, catabolics, detoxified, secretives, excretives.

Due to complex etiopatogenesis, a insiduous evolution, fregvently underclinic, laboratory diagnosis is absolutely necessary in liver diseases (3, 5, 7, 8, 10).Estimating of some serum biochemical tests gives the clinician useful dates for diagnosis, monitoring the evolution of liver disease and use of the treatment.

In a private vet cabinet equiped with clinic laboratory, during 3 years, 120 dogs with different liver diseases were diagnosed. Among these 10 dogs were monitorised by complex paraclinical tests in different stages until recovery or, eventually, death of the animals

MATERIAL AND METHODS

The animals were examined, after anamnesis by: inspection, palpation, percussion, examination, thermometry.

For biochemical exams some 5 ml blood for serum were preserved by radial vena.

Most important liver laboratory tests were grouped like this:

- tests depended on estimate enzimatic activities: glutamic oxalacetic transaminase (GOT), glutamic piruvic transaminase (GPT), gamaglutamyltransferase (GGT), alcaline phosphatase (Pal);
- tests depended on secretion and secretion of the liver: total bilirubin;
- tests depended on estimate of specific biochemical activities of the liver: total protein, albumin, blood glucose, triglicerides, total cholesterol.

Laboratory tests were performed at a biochemical analyser Metrolab 1600 DR (wet biohemestry) – Foto.

Fig.1 Metrolab 1600 DR

RESULTS AND DISCUSSION

The results of paraclinical tests were centralised in tables 1-6. Among the 10 dogs, 3 died (one with liver tumour and 2 with major liver insufficiency and cirrosis), 4 recovered and 3 were in evolution with favorable prognosis. In the tables 1-6 , 2 dogs for each of the 3 mentioned situations were presented in dinamics (monitorised).

Table 1

The results of paraclinical testing at the dog 1 (recovered)

No	Parameter	u/m	Recorded values				Reference values *
			initial	after 34 days	after 69 days	after 126 days	

1.	GOT	u/l	35,9	33,9	34,9	4,5	0-25
2.	GPT	u/l	454,4	161,0	57,6	15,9	3-45
3.	GGT	u/l	62,5	56,9	5,6	6,2	1-9
4.	Pal	u/l	3407	963,2	151,9	51,2	30-120
5.	Bilirubin	mg/dl	0,2	0,4	0,3	0,2	0,1-0,6
6.	Total protein	g/dl	5,04	5,43	5,4	5,6	5,4-7,5
7.	Albumin	g/dl	3,07	3,2	3,4	3,4	2,6-4,1
8.	Glucose	mg/dl	87,3	75,2	92,3	78,9	65-110
9.	Triglicerides	mg/dl	79,5	60,5	120	72	50-200
10.	Cholesterol	mg/dl	161,2	119,6	130	110	100-150

* Avram N, 2004

Table 2

The results of paraclinical testing at the dog 8 (recovered)

No	Parameter	U/M	Recorded values					Reference values*
			Initial	after 5 days	after 12 days	after 60 days	after 90 days	
1.	GOT	u/l	75,7	80	26,9	15,1	17,1	0 - 25
2.	GPT	u/l	50,5	66,3	96,4	58,6	45,5	3 - 45
3.	GGT	u/l	4,6	5,4	3,1	9,6	7,3	1 - 9
4.	Pal	u/l	176,3	153,5	139,4	932,3	240	30 - 120
5.	Bilirubin	mg/dl	0,5	0,5	0,5	0,5	0,5	0,1 – 0,6
6.	Total protein	g/dl	5,4	6,4	7,1	6,5	6,7	5,5 – 7,5
7.	Albumin	g/dl	3,2	3,2	2,9	2,7	3,0	2,6 – 4,1
8.	Glucose	mg/dl	100,7	97,2	80,0	90,0	85	65 - 110
9.	Triglicerides	mg/dl	130,4	122,1	150	102	132	50-200
10.	Cholesterol	mg/dl	138	140	123	142	147	100 - 150

*Avram N. ,2004

Table3

The results of paraclinical testing at the dog 6 (favorable progresis-in evolution)

No.	Parameter	U/M	Recorded values				Reference values*
			Initial	after 93 days	after 127 days	after 157 days	
1.	GOT	u/l	31,7	60,5	60	35,7	0 - 25
2.	GPT	u/l	100,8	134,2	550,0	94,6	3 - 45
3.	GGT	u/l	6,1	8,5	102,5	4,7	1 - 9
4.	Pal	u/l	30	150	389,2	33,5	30 - 120
5.	Bilirubin	mg/dl	0,2	0,2	0,4	0,4	0,1 – 0,6
6.	Total protein	g/dl	8,2	7,4	7,4	7,0	5,5 – 7,5
7.	Albumin	g/dl	3,7	3,0	3,08	3,08	2,6 – 4,1
8.	Glucose	mg/dl	84,9	102	63,0	75	65 - 110
9.	Triglicerides	mg/dl	30,1	100	100	50	50-200
10.	Cholesterol	mg/dl	96,9	57	80,3	212,5	100 - 150

*Avram N. , 2004

Table 4

The results of paraclinical testing at the dog 7 (favorable progresis-in evolution)

No	Parameter	U/M	Recorded values			Reference values*
			Initial	after 10 days	after 35 days	
1.	GOT	u/l	97,9	16,4	20,3	0 – 25
2.	GPT	u/l	362,5	78,1	62,5	3 – 45
3.	GGT	u/l	7,6	26,0	16,5	1 – 9

4.	Pal	u/l	367,7	841	196	30 - 120
5.	Bilirubin	mg/dl	0,4	0,4	0,4	0,1 – 0,6
6.	Total protein	g/dl	6,2	5,2	6,1	5,5 – 7,5
7.	Albumin	g/dl	3,0	3,0	3,0	2,6 – 4,1
8.	Glucose	mg/dl	154,6	72,9	119	65 - 110
9.	Triglicerides	mg/dl	70,4	20,1	78	50-200
10.	Cholesterol	Mg/dl	317,5	288,6	476	100 - 150

*Avram N. , 2004

Table 5

The results of paraclinical testing at the dog 2 (dead)

No	Parameter	U/M	Recorded values		Reference values*
			Initial	after 41 days	
1.	GOT	u/l	140,9	775,5	0 - 25
2.	GPT	u/l	688,8	1733,0	3 - 45
3.	GGT	u/l	188,7	700	1 – 9
4.	Pal	u/l	1578	2421	30 - 120
5.	Bilirubin	mg/dl	0,3	1,6	0,1 – 0,6
6.	Total protein	g/dl	4,8	4,9	5,5 – 7,5
7.	Albumin	g/dl	3,1	2,4	2,6 – 4,1
8.	Glucose	mg/dl	135	304	65 - 110
9.	Triglicerides	mg/dl	900	600	50-200
10.	Colesterol	Mg/dl	782	397	100 - 150

* Avram N., 2004

Table 6

The results of paraclinical testing at the dog 3 (dead)

No	Parameter	U/M	Recorded values			Reference values*
			Initial	after 30 days	after 44 days	
1.	GOT	u/l	81,7	49,2	97	0 – 25
2.	GPT	u/l	83,7	55,7	291	3 – 45
3.	GGT	u/l	16,2	7,9	35,8	1 – 9
4.	Pal	u/l	259,9	104,1	118,8	30 – 120
5.	Bilirubin	mg/dl	0,3	0,3	0,2	0,1 – 0,6
6.	Total protein	g/dl	9,0	7,8	8,8	5,5 – 7,5
7.	Albumin	g/dl	2,7	2,8	2,9	2,6 – 4,1
8.	Glucose	mg/dl	70	80	86	65 – 110
9.	Triglicerides	mg/dl	60	90	50,8	50-200
10.	Cholesterol	mg/dl	32	170	115	100 – 150

*Avram N. , 2004

At the studied dogs, according established diagnosis took action with liver diet and vitamine therapy, perfusion with physiological solution and glucose, prednisolon and sometimes antibioterapie and chimioterapie.

The treatment was permanent applied depending on the results of the paraclinical tests. At dog 1 and 8 the values of the parameters: bilirubin, total protein, albumin, glucose, trigliceride, cholesterol were in the limits of reference values.

GOT, at the begining with signiphicantly high values, returned, in normal limits after 60 days of diet and treatment.

GPT presented high values with maximum of 454,4 u/l at the dog 1 in initial stages and returned to normal after 126 days including 90 days of diet and treatment respectively.

GGT activitie registered with high values at the dog 1 until 34 days of treatment and returned to normal; at the dog 8 GGT activitie were nonmodified.Pal activitie presented initial values very high at the dog 1 (3407,6 u/l) and returned progressively to normal limit after 126 days. Both animals were recovered and the values of modified parameters remited.

The dog 6 and 7, in evolution with favorable prognosis, presented generally the same"picture" of values for: bilirubin, total protein, albumin, glucose, triglicerides; a slight increasing of cholesterol recorded at the dog 6 at 157 days.

Significantly changes recorded in the values of tested enzymes. GOT and GPT presented high values in all stages of monitoring at the dog 6; at the dog 7 GOT values remited after 10 days and GPT presented further high values.

GGT recorded temporary increased values with maximum (102,5 u/l0 at the dog 6 in third stage of control (127 days).

Pal had high values in all the stages at the dog 7 with maximum 841 u/l.

At the dogs 2 and 3, with nonfavorable evolution most of analysed parameters presented modified values, especially the dog 2 (table 5) except albumin and at the dog 3 except glucose and triglicerides. General disturbance of the body functions can be observed and these conducted to the dead of the dogs.

The dates recorded confirm value of the biochemical tests, specially serum enzymes determination, for the diagnosis and prognosis of liver diseases; they also are usefful for treatment orientation.

The changes in celular permeability, liver celular distrophy, necrosis and inflamation can determined releasing of GOT and GPT from hepatocyte and consegvently increasing of their level in blood serum.

Among GOT and GPT the last one is an accuracy biomarker for liver diseases; GOT is also presented in heart muscles, skeletal muscle and kidney too (2, 5, 7).Level of GPT in hepatocytes is also more high than GOT.

Studies demonstrated that GOT can returned to normal after 2-3 weeks before these of GPT in diseases evolution (5, 7); these were confirmed also in one research. Pal increases in liver celular necrosis and their values ussualy returned to normal after 2-3 weeks (5, 7).

High values of Pal are noted in colestatic troubles, biliary obstruction and liver neoplasic.Was also noted increasing of Pal activitie in liver inflamation and systemic infection (5, 7). Pal increasing can precede increasing of serum bilirubin in dogs.

The liver is an important important producer of serum GGT; GGT increases in intraliver or extraliver cholestatic and also in kidney or pancreatic trouble as well as after glucocorticoid therapy.

The rest of analysed parameters had a secundary diagnosis value and represent the association of proteic, glucidic and lipidic metabolic desorders at the liver disturbed function.

CONCLUSIONS

1. In a private vet cabinet equiped with clinic laboratory, during 3 years, 120 dogs with different liver diseases were diagnosed. Among these 10 dogs were monitorised.
2. Ten serum biochemical parameters were determined at each dog using a biochemical analiser Metrolab 1600 DR.
3. Among 10 dogs, 3 died (one with liver tumour and 2 with major liver insufficiency and cirrosis), 4 recovered and 3 are in evolution with favorable prognesis.
4. The main parameters for liver diseases diagnosis are: transaminanases, especially glutamic piruvic transaminase, alcaline phosphatase and gamaglutamil transaminase.
5. GOT and GPT presented high values in all stages of monitoring. At recovered dogs enzymes activities returned to normal after 60-126 days.

REFERENCES

1 Amstrong P.J. (1994) – Hepatic lipidosis Vet. Prev: 1: 10-11.

2.Avram N. (2002) - Compendiu de fiziopatologie specială , Editura Fundației Romania de Mâine , Bucureşti,

3. Avram N.(2004) – Explorări paraclinice . Lucrări aplicative . Editura Fundației România de Mâine , Bucureşti .
4. Bârză H., Câlmău F.(1999) – Patologie medicală veterinară. Editura Fundației România de Mâine , Bucureşti .
5. Center S.A. (1995) - Pathophysiology, laboratory diagnosis and diseases of the liver. In : Ettinger, S.J., Feldman , E.C.,ds. Textbook of Veterinary Internal Medicine, Philadelphia, W.B. Saunders ,pp 1261-1312.
6. Codreanu M. (2002) **–** Bolile ficatului – Aparuta in Tratatul de Medicina Veterinara ,vol.2 Editura Tehnica , Bucuresti .
7. Duncan J.R., Prosse K.W., Mohafei E.A.(1994) – Veterinary Laboratory Medicine **in** Clinical Pathology 3 rd Ed. Ames., Iowa State University Press., pg 9-112.
8. Kirk I., Robert W. (1998)- Curent veterinary therapy, small animal practice, Philadelphia-London, W. V. Sanders Company.
9. Mihai D., Andronie V .(2000) - Medicina animalelor, Vol. 1, Editura Geea, Bucuresti .
10. Rothuizen J. (1985) – Hiperbilirubinemia în boala hepatobiliară la câine, L. von Jean Rothuizen, Utrecht.

MONITORING OF CULTURAL DEVELOPMENT AND IDENTIFICATION *YERSINIA ENTEROCOLITICA* STRAINS THROUGH MASS SPECTROMETRY

MONITORIZAREA DEZVOLTĂRII CULTURILOR DE *YERSINIA ENTEROCOLITICA* ŞI IDENTIFICAREA TULPINILOR PRIN SPECTROMETRIE DE MASĂ

NECŞULESCU M.[1], V.ORDEANU[1], C. LUPESCU[2,], Lucia IONESCU[1], Diana POPESCU[1], Simona BICHERU[1] , C. CUMPĂNĂŞOIU[3], Gabriela DUMITRESCU[1]

[1]*Army Center for Military Medical Research, Bucharest*
[2]*NSVFSA, Bucharest (email* - lupescu@ansv.ro)
[3]*Hygiene and Veterinary Public Health Directorate, Valcea*

REZUMAT

Spectrometria de masă utilizată pentru determinarea masei moleculare a proteinelor analizează moleculele prin ionizare şi ulterior observarea comportamentului acestora în câmp electric sau magnetic**.** Echipamentul Microflex LT 20 este un spectrometru de masă utilizat pentru identificarea şi clasificarea rapidă şi precisă a agenților biologici după prelevarea şi pregătirea specifică a probelor. Elementul de bază este un aparat MALDI TOF având domeniul de masă cuprins între 2000-20000 daltoni şi este utilizat pentru identificarea şi caracterizarea automată a proteinelor 16S din ribozomi. Domeniile de aplicație ale tehnologiei Maldi Tof cuprind, printre altele, controlul microbiologic al alimentelor şi apei, controlul calității microorganis-melor din colecții şi analiza relațiilor taxonomice precum şi diagnosticul microbiologic în domeniul medical uman şi veterinar.

Cercetările au urmărit evidențierea modificărilor apărute în ex-presia proteinelor ribozomale la trei tulpini de Yersinia enterocolitica, ca urmare a variației compoziției mediilor de cultură, temperaturilor de incubare, vârsta culturilor şi influența acestora asupra rezultatului identificării prin spectrometrie de masă. Compararea spectrelor de masă ale tulpinilor luate în studiu, cu spectrele tulpinilor de referință din baza de date, a relevat un set de proteine constant indiferent de condițiile de cultivare, care poate reprezenta semnătura proteică de referință pentru specia Yersinia enterocolitica.

Cuvinte cheie: Maldi Tof, spectrometrie de masa, identificare, *Yersinia enterocolitica*

ABSTRACT

Mass spectrometry is used in order to determined molecular weight protein, analyze molecules through ionization and observe their behavior in electric or magnetic field. The Micro flex LT 20 is a mass spectrometer used for identification and classification of biological agents. The base elements is MALDI TOF mass range of 2000-20000 Daltons and is used for automatic identification and characterization of proteins from 16S ribosome's. Fields of application Maldives Tof technology include the microbiological control of food and water, quality control of micro-collection and analysis of taxonomic relationships and microbiological diagnosis in human and veterinary health.

The study indicates that the changes arising ribosome's in protein expression in three strains of Yersinia enterocolitica as a result of the change of culture media composition, temperature of incubation, cultures and age influence. Comparison of mass spectra of the strains made with spectra strains of reference revealed a set of proteins regardless of the constantly growing, which may represent the protein of reference for the species Yersinia enterocolitica.

Keywords: Maldi Tof, spectrometry, identification, *Yersinia enterocolitica*

INTRODUCTION

The MALDI-TOF (Matrix Assisted Laser Desorption Ionization - Time of Flight) technology used to identify microorganisms, based on the "protein signatures" provides an excellent alternative to traditional methods of laboratory analysis, applicable in various areas such as the microbiological control of food and water, quality control of micro-collection and microbiological diagnosis in human and veterinary health. A key requirement is to create a database of spectra for microorganism identification. The speed and the low cost for sample preparation and analysis itself make this method to be recommended for routine use and high reliability. A new field of application is the analysis of taxonomic relationships. Profile analysis using MALDI-TOF, leads to the achievements of like family tree, in contrast with the classical methods of analysis, such as morphological and biochemical reaction or sequencing of 16S ribosomal DNA. Because of the presence of many ribosomal proteins and their high stability, the protein model enables direct visualization of translated DNA sequence. Therefore the method that is based on determining the molecular weight can be considered as having the value of a difficult analysis of multifocal sequencing. One of the most encountered applications of Microflex LT 20 equipment with MALDI-TOF technology is to the identification of bacteria, spores, viruses and toxins in various samples. This was pointed at NBC International Symposium, Tampere 2006.

The research has started from the question: *Can the microorganism cultivation, on different culture media, culture age and cultivation conditions, induce changes in 16S ribosomal protein expressions and in this sense, may it influence the result of identification by mass spectrometry?* For the experimental model there have been selected three strains of *Yesinia enterocolitica* (YE VL0108, VL0408 and YE ATTC 27729) which were cultivated on minimal and selective culture media (solid and liquid) at different temperatures (26 and 37°C). The cultures obtained were analyzed with the Microflex LT 20 MALDI TOF equipment in order to achieve the characteristic "protein signatures".

MATERIAL AND METHODS

The study was performed between 2008-2009. There were used the classical bacteriological techniques for cultivation of Yesinia enterocolitica strains on solid and liquid, minimal and selective medium. The cultures obtained were analyzed with the Microflex LT 20, MALDI-TOF technology.

Materials

• Yersinia enterocolitica strains (YE VL0108, YE VL0408) isolated from swine in the microbiology laboratory of DSV Vâlcea and standard YE 909574 strain. Warning! Yersinia enterocolitica is a biological agent classified as Biosafety Level 2 (BSL 2) and can be handled only in laboratories with special equipement.

• Standard Culture Media - Columbia Agar with 5% blood (Biomerieux), nutrient agar and simple broth produce by NIRDMI Cantacuzino, Bucharest.

• Reagents - Trifluoracetic acid (TFA), Acetonitrile (AN), HCCA matrix solution (α-cyano-4-hydroxy-cinnamic acid), Phosphate Buffer Saline (PBS), sterile watter.

• Equipement: System Microflex LT 20 (Bruker Daltonics), Class II Microbiological Safety Cabinets with laminar flow, incubators with adjustable temperature, centrifuges for Eppendorf tubes, wet autoclaves for sterilization, vortex tubes, adjustable automatic Eppendorf pipetts.

• Supplies: inoculating sterile loops, Eppendorf tubes, sterile tips for pipettes, specific disinfectants and disposable containers for medical waste.

Methods

Yersinia enterocolitica cultures studied (YE VL0108, VL0408 YE) were cultivated by streak plate method with sterile inoculating loop on Columbia agar with 5% blood and nutrient agar in order to obtain isolated colonies. Cultures in liquid medium were obtained by inoculation of simple broth. The incubation of the media was made at 26 and 37°C temperature, for 72 hours. From each Petri plate there were scraped two colonies of Yersinia enterocolitica at 24, 48, 72 hours and 7 days interval, each of them considering as double sample for ribosomal protein extraction.

Two samples of 5 ml from the broth cultures, were distributed in Corning tubes and centrifuge 5 minutes at 5000 rpm for sedimentation of bacteria. The supernatant was removed and the bacterial pellet was washed twice with PBS, to remove the protein remains in the culture. After washing, the culture was scraped with sterile inoculating loop from the bacterial pellet.This sample was considered as double sample for ribosomal protein extraction.

The extraction of 16S ribosomal protein was done accordance with "Microorganism Profiling " extraction TFA 80% procedure, as follows:

Yersinia enterocolitica culture scraps and put into Eppendorf tube. Add 50 µl TFA 80%. The suspension homogenize to complete denaturation (suspension remains cloudy). Wait 10-30 minutes. Add the bidistilated watter (3 vol) and 200 µl of AN. Next it is centrifuge at 2500-3000 rpm for about 2 minutes and the supernatant is transfer in a new Eppendorf tube. After this 1 µl of supernatant was pipeting on a steel target plate and dry in biosafety cabinets with laminar flow. After drying, each well was covered with 2 µl matrix solution and air dried in biosafety cabinets with laminar flow, for subsequently examination at mass spectrometer Microflex LT 20.

The method is used for extraction of ribosomal proteins from bacteria in vegetative form and for the spores, too. To prevent any oxidation reactions, it is very important to work quickly, especially after drying extracts when HCCA matrix solution must immediately added. Warning! - trifluoracetic acid is corrosive and can cause severe burns to the skin!

Two samples of each strain were displayed on the Micro Scoud plate. In the end there resulting a 24 well test, for each experiment and 96 samples in total. In order to eliminate errors, results from examined field, there were made 2 "shots" (75 laser shots) for each well. The mass domain used for identification was established between 800 and 12.000 Daltons. The parameters set in the Flex Control identification program (m/z - molecular weight, SN - Signal Noise, Quality Fac - Quality Factor, Res - Resolution, Intens - Intensity, Area - Area) were introduced according to the data for the bacteria section. Mass spectra were processed and identified with the Bio Expert Profiler program and were compared between them using Flex Analysis soft.

For each strain of Yersinia enterocolitica there have been efectuated:

• a graphic representation of mass spectrum, in which the characteristic peak of polypeptides fragments analyzed, can be viewed as well as molecular weight quantified in Daltons;

• a table with detection parameters and molecular weights obtained for each strain of Yersinia enterocolitica tested;

• a comparative graphical representation of the spectra of analysed strains.

RESULTS AND DISCUSSION

Spectra of the bacterial strains studied were identified as belonging to the species Yersinia enterocolitica, in that way confirming the results obtained by classical bacteriological techniques. Analysis of the spectra of the 16S polypeptides fragments from ribosome of the Yersinia enterocolitica strains has revealed a range of mass between 800 and 12,000 Daltons, with characteristic peaks in the domain 1500 - 10,000 Daltons. Average of characteristic peaks for this species was included in the following interval: 2100-2700 Daltons; 3100-3650 Daltons; 4350-4850 Daltons; 6000-6500 Daltons, 7100-7750 Daltons, 8300-8900 Daltons; 9100-9650 Daltons, 10,500-11000 Daltons. The average score obtained for the 3 strains of Yersinia enterocolitica studied was 130, with a minimum of 110 and maximum of 150. This score is representative and attests a correct identification. By comparing Yersinia enterocolitica strains studied with the strain from the equipment database we can conclude that our strain belongs to the same species of bacteria. Comparing the isolated strains (YE VL0108, VL0408 YE and ATTC 27729) between themselves, we can observe that they are very close in terms of ribosomal protein structure demonstrating a high degree of similarity.

Different composition of growth media (Columbia or nutrient agar) does not have very important effect on the model of distribution of the peak. Between 3000 - 10000 Da is observed the apparition of new peak that no interfere with characteristic peak of the "protein signature" of Yersinia enterocolitica species. The presence of the culture medium in adherent colonies (scraped together with the colony of bacteria) also does not translate into a signal to demonstrate its influence on the outcome of the testing.

The cultivation of the three strains of Yersinia enterocolitica studied at different temperature (26 and 37 ° C) and also at 24, 48, 72 and 7 days intervals, revealed no significant signal changes at testing the dynamic cultures, although at a very careful analysis, there are some features of the obtained spectra. These features do not affect the protein signature of the species and will be subject to the another research.

The stage of cells development (growth) influences to a small extent the test performance. Cells in the lag phase of growth present a very similar pattern, like cells in stationary phase or phase which succeeds the cell death. Moreover, because sample preparation and their analyses done in standardized conditions, the values of spectra obtained for the same samples are comparable.

Next, there are presented the mass spectra and drop characteristic values (Da) of Yersinia enterocolitica strains studied.

For a better processing of spectral data, spectra obtained were analyzed, taken by 3 (for each variant of medium and temperature) through comparing overlap and individual way that allowed more accurate assessment of the degree of correlation between strains of Yersinia enterocolitica studied. It is extremely important that the characteristic drop to be reproducible, result quantified by the frequency of apparition of the same peak at successive measurements.

In the tables with molecular weights of the protein fragments and the acquisition parameters for each strain of Yersinia enterocolitica analysed, are presented the molecular weights that making up the protein signature of the strain, the minimum and maximum domains (until 2000 Da and over 10,000 Da) being excluded.

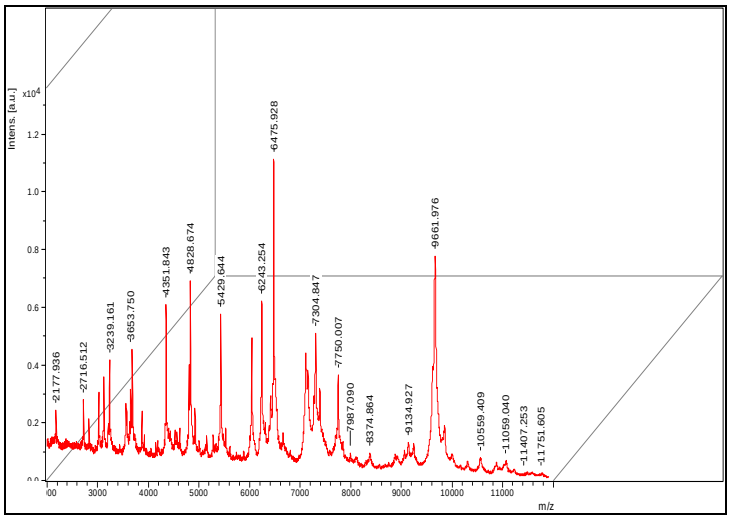


Figure no. 1. The graphic representations of ATTCC 27729 Yersinia enterocolitica spectrum (mass spectra)

Table 1.

The molecular weight and the aquisition parameters for ATTCC 27729 Yersinia enterocolitica strain

m/z	SN	Quality Fac.	Res.	Intens.	Area
2716.573	20.3	1216	29	1542	7531
2828.672	18.5	1165	19	1411	8902
3026.08	22.2	4881	48	1688	12842
3099.855	20.5	8417	67	1557	14412
3210.567	27.5	3981	53	2091	13453
3554.768	96.2	62644	101	7322	76205
3647.67	42.2	7598	48	3210	26438
4351.743	57.3	111783	229	4365	56022
4530.905	34.7	49178	74	2643	57943
4829.696	119.4	116725	103	9085	140185
5281.218	34.4	8569	13	2617	67836
5428.71	56.7	21830	53	4315	65409
6045.975	46.7	20146	45	3556	69772
6232.642	58.2	75776	58	4427	135111
7110.64	161.7	1699504	50	12310	1267602
7293.326	111.5	176679	62	8489	307728

7763.523	30.5	29052	9	2319	185203
8102.652	23.7	38068	14	1802	157540
8887.996	36.1	158909	24	2747	323787
9148.457	50.2	80411	17	3822	322047

Legend:

- m/z - Mass
- SN - Signal Noise
- Quality Fac - Quality Factor
- Res - Resolution
- Intens - Intensity
- Area - Area

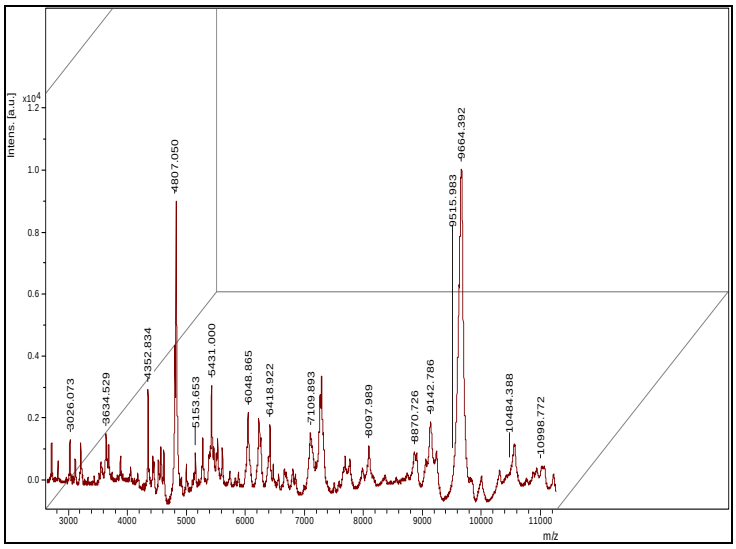


Figure no. 2. The graphic representations of YE VL0108 Yersinia enterocolitica spectrum (mass spectra)

Table 2.

The molecular weight and the aquisition parameters for YE VL0108 Yersinia enterocolitica

m/z	SN	Quality Fac.	Res.	Intens.	Area
2717.1	17.4	37476	442	1195.44	7676
3026.073	19	47705	516	1306.19	8738
3634.529	21.8	17301	416	1494.84	7951
4352.834	42.7	122541	485	2934.87	28966
4807.05	63.4	178822	359	4356.34	57000
5153.653	13	11666	567	893.23	5298
5431	43.3	50655	310	2975.81	32850
6048.865	31.2	30897	250	2144.07	26717
6234.381	26.8	38591	118	1841.28	43505
6418.922	25.9	26442	363	1782.7	19496
7109.893	20.9	33244	97	1436.82	44376
8097.989	15.9	11898	216	1094.18	16551
8870.726	12.5	12522	168	861.53	18866
9142.786	27.4	48317	153	1884.25	57601
9515.983	1.5	446	5360	102.35	158
9664.392	145.2	419215	113	9978.99	470474
10484.39	3.6	557	365	250.54	1597
10998.77	2.7	141	672	186.67	461
11220.02	1.8	391	1817	126.05	403

Legend:

- m/z - Mass
- SN - Signal Noise
- Quality Fac - Quality Factor
- Res - Resolution
- Intens - Intensity
- Area - Area

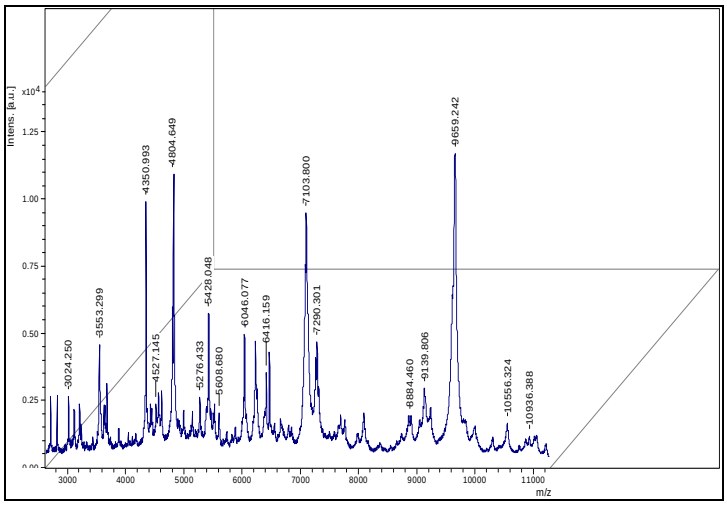


Figure no. 3. The graphic representations of YE VL0408 Yersinia enterocolitica spectrum (mass spectra)

Table 3.

The molecular weight and the aquisition parameters for YE VL0408 Yersinia enterocolitica

m/z	SN	Quality Fac.	Res.	Intens.	Area
2715.251	33.7	127190	386	2636	22367
3024.250	34	54815	290	2661	20397
3553.299	58.5	23559	136	4579	30871
4350.993	126.5	661882	486	9908	116197
4527.145	30.5	15767	73	2385	30406
4804.649	76.4	137743	261	5985	74541
5276.433	33.3	17177	107	2607	31370
5428.048	73.4	345690	299	5747	108147
5608.680	26.1	9074	87	2045	22431
6046.077	63.4	148660	261	4968	80080
6227.912	60.2	61639	118	4711	79338
6416.159	45.1	72996	126	3530	72655
7103.800	121.3	80696	103	9500	164314
8884.46	22.5	67148	33	1765	143109
9139.806	34.9	33917	39	2735	119519
9659.242	149.3	343341	116	11691	445056
10556.32	20.1	19034	85	1573	50043
10936.39	14.4	6002	47	1126	32651
11222.5	10.6	9740	95	828	26249

Legend:

- m/z - Mass
- SN - Signal Noise
- Quality Fac - Quality Factor
- Res - Resolution
- Intens - Intensity
- Area - Area

As seen observed in the figures no. 1, 2, 3 and in the tables 1, 2, 3, the characteristic peaks of Yersinia enterocolitica species (2100-2700 Da; 3000-3500 Da, 4350-4850 Da, 6000-6500 Da; 7100-7750 Da, 8300 - 8900 Da, 9100-9650 Da, 10500-11000 Da) is found in all compared spectra, which confirms the association of isolated strains (YE VL0108, VL0408 YE) with the standard strain of Yersinia enterocolitica (ATTC 27729) and with strains from the database of the device (fig.no.4).

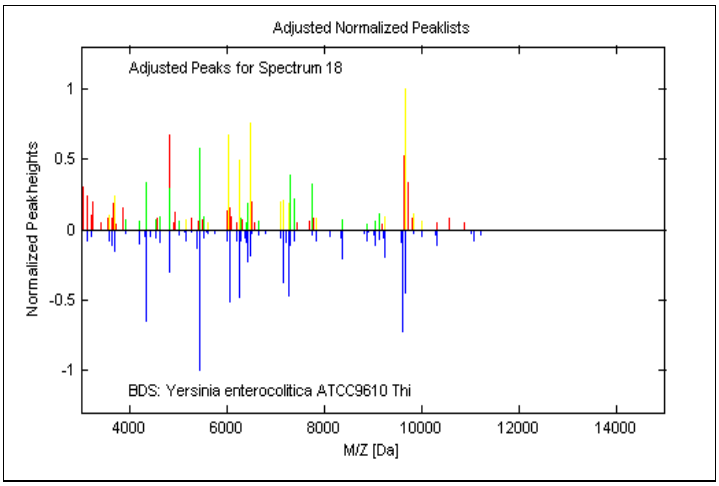


Figure no. 4 The comparation between YE VL0108 and ATCC9 610Thi Yersinia enterocolitica mass spectrum

The analysis of the mass values, there was obtained separately for each strain, shows three peaks that are present in all strains. This peaks belong to 4828-5200 Da, 6227-6474 Da and 9659-9673 Da domains, which can be considered the mass characterisitc values for the Yersinia enterocolitica species. The complete results are presented in the following table:

Table. 4
The characteristic mass values for the Yersinia enterocolitica species

Nr.crt	Yersinia enterocolitica	Valori de masă (Da)		
		Pic 1	Pic 2	Pic 3
1	ATTC 27729	4829	6232	9662
2	YE VL0108	4807	6418	9664
3	YE VL0408	4804	6416	9659

The software of the device, allows the individually comparation of the obtained spectra by grouping of them (maximum 4 spectra) or by the overlap (fig. no 5). The last method allows the easy observation of the images of the spectra of strains studied.

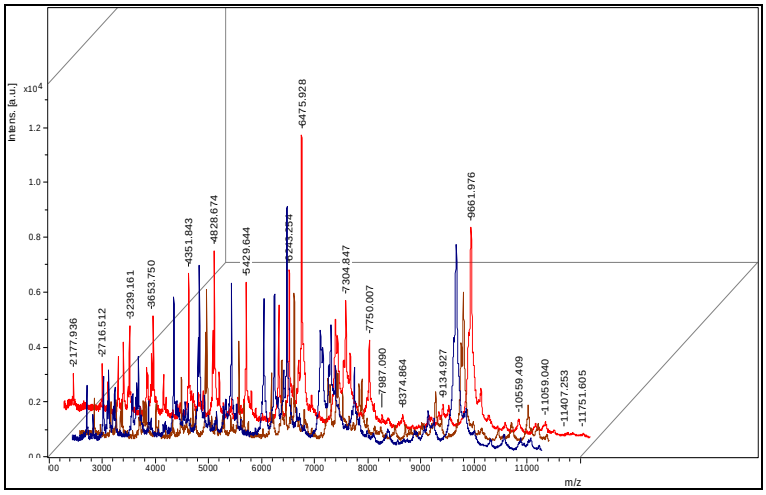


Figure no. 5. The graphic representations of ATTC 27729, YE VL0108, YE VL0408 Yersinia enterocolitica spectrum (mass spectra)

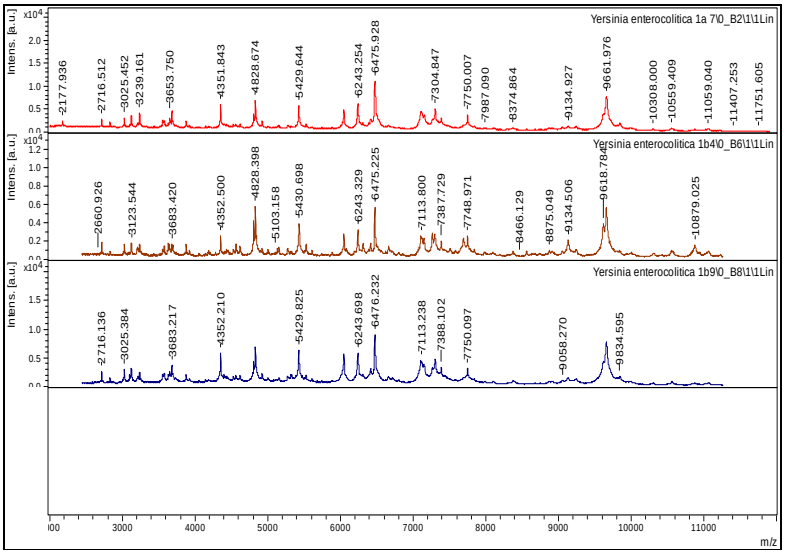


Figure no. 6. The graphic representations of the compared mass spectrum for ATTC 27729, YE VL0108, and YE VL0408 Yersinia enterocolitica strains

The raw spectra of analyzed Yersinia enterocolitica strains were compared between them and the results show a significant correlation of peaks, that represents the polypeptides fragments (molecular weights) obtained by analyzing with the Microflex LT 20 Maldi Tof technology (fig. no. 5, 6). The characteristic peaks that represent protein signature of the species (4828-5200 Da, 6227-6474 Da and 9659-9673 Da) can be

observed in the following comparative charts, because the synthetic presentation of the results give us the possibility of facility analysis of the obtained data.

CONCLUSIONS

From the analysis of the obtained results we are drawing the following conclusions:

- The strains of Yersinia enterocolitica (ATTC 27729, YE VL0108, VL0408 YE) studied and identified by classical bacteriological methods, were confirmed by mass spectrometry, using Maldi Tof (Matrix Assisted Laser Desorbtion ionization - Time of Flight) technology.
- The average of characteristic peaks of species was included in the following mass domains: 2100-2700 Da, 3100-3650 Da, 4350-4850 Da, 6000-6500 Da, 7100-7750, 8300-8900 Da, 9100-9650 Da; Yes 1050-11000.
- The medium score obtained for all three strains of Yersinia enterocolitica was 130, with a minimum of 110 and a maximum of 150. This is a representatives score that reveals a good identification.
- The comparation between them of mass spectra of the Yersinia enterocolitica strains studied, and also with the spectra of reference strains from the database of the device revealed a high degree of association, represented by a significant correlation of polypeptides fragments, the overlapping of spectra beign highly relevant.
- The relative importance of the individual peaks is reproducibility of spectra (the frequency of apparition), this property being used to construct the reference protein signature.
- The strains of Yersinia enterocolitica tested for confirmation with the Microflex LT 20 system, were validated as a percentage of 100%.
- The Microflex LT 20 system, allows the rapid and accurate identification of biological agents and it is an important instrument of the confirmation /validation methods for microbiological diagnosis.

REFERENCES

1. **Arnold, R., J. Karty, A. Ellington, J. Reilly**, Monitoring the growth of a bacteria culture by MALDI-MS of whole cells, Anal. Chem., 1999, 71:1990– 1996
2. **Ellwood, D. C., Tempest D. W.,** Effects of environment on bacterial wall content and composition, Adv. Microb. Physiol., 1972, 7:83–117
3. **Fenselau, C., Demirev P. A.,** Characterization of intact microorganisms by MALDI mass spectrometry, Mass Spectrom Rev., Appl. Environ.microbiol., 2001, 20:157–171
4. **Hawley, R. J., Eitzen E. M. Jr.,** Biological weapons - a primer for microbiologists., Annu. Rev. Microbiol, 2001, 55:235–253
5. **Holt, J. G., Krieg, N. R.. Sneath P. H. A, Staley J. T., Williams S. T.** (ed.). 1994, Bergey's manual of determinative bacteriology, 9th ed. Williams & Wilkins, Baltimore, Md.
6. **Jarman, K. H., Cebula S. T., Saenz A. J.,. Petersen C. E, Valentine N. B., Kingsley M. T., Wahl K. L.,** An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 2000, 72:1217–1223
7. **Kievit O., Arjan van Wuijckhuijse, Kientz C.**, Fiedable biodetectionszstem using MALDI-TOF MS, Symposionum on Chemical, Biological, Nuclear and Radiological Threats, 2006, 112-114, Tampere, Finland
8. **Lay, J. O., Jr.,** MALDI-TOF mass spectrometry of bacteria. Mass Spectrom. Rev., 2001, 20:172–194
9. **Popa M. I.**, Diagnosticul de laborator în microbiologie, 2004, Editura Medica, 211-214, Bucureşti
10. **Wahl, K. L.,. Wunschel S. C, Jarman K. H., Valentine N. B., Petersen C. E., Kingsley M. T, Zartolas K. A., Saenz A. J.**, Analysis of microbial mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem., 2002, 74:6191–6199

ABILITY OF ADJUVANTS TO ENHANCE THE SYSTEMIC ANTIBODY RESPONSES AGAINST H1N1 AND H3N2 SWINE INFLUENZA VIRUSES

BOH Chang Lin, DVM, MS, PhD; R. Gene WHITE, DVM, MS; Karen K. BROWN, PhD
Leann SIEDLIK; Colette HRABIK; Jack MCGONIGLE, BS
MVP Laboratories, Inc., Omaha, Nebraska

REZUMAT

Studiul a urmărit alegerea unui adjuvant care să crească în mod semnificativ nivelul anticorpilor serici după aplicarea vaccinurilor mono sau bivalente pentru prevenirea infecției cu swine influenza viruses subtipurile H1N1 şi H3N2. Datele obținute prin tehnica de inhibare a hemaglutinării au arătat că cel mai corespunzător adjuvant s-a dovedit a fi Emulsigen -D, care este o emulsie de ulei în apă conținând dimetyldioctadecylammonium bromide (DDA).

Cuvinte cheie:

ABSTRACT

The study followed the election of an adjuvant to increase the level of serum antibodies after the application of the mono or bivalent vaccines to prevent infection by the virus of swine influenza subtypes H1N1 and H3N2. Data obtained by the technique of hemagglutination inhibition showed that the best adjuvant proved is Emulsigen®-D, witch is an emulsion of oil in water containing dimetyldioctadecylammonium bromide (DDA).

Keywords: swine influenza virus, adjuvants, Emulsigen®-D, inhibarea hemaglutinării

INTRODUCTION

Swine influenza (SI) is an acute infectious disease in pigs caused by swine influenza virus (SIV). During the past decade, SI has become a widespread and endemic disease in pig populations worldwide. The currently available method for the control of SI in a pig herd is the vaccination of young pigs with an inactivated whole virus vaccine containing an adjuvant. A monovalent vaccine containing SIV subtype H1N1 and a bivalent vaccine containing subtypes H1N1 and H3N2 are available commercially. These vaccines are well accepted. It is also well accepted that a hemagglutination inhibition (HI) titer of >1:40 is protective (3). However, it has been reported that inactivated SIV vaccines do not consistently provide complete protection to virus challenges in vaccinated pigs (6). One possible way to improve protection provided by SIV vaccines is to add more potent adjuvants that stimulate higher immune responses.

During the past few years, novel adjuvants, such as virosomes (4), muramyl peptides (3), MF59 (2), and ISCOMS (7) have been tested with influenza vaccines in both animal and human models with variable efficacy. In response to market requirements, a variety of promising new adjuvants have been developed. These include EMULSIGEN® -D, EMULSIGEN®/Rehydragel-LV, EMULSIGEN®-BCL, and POLYGEN™ (MVP Laboratories, Inc.).

This study was conducted in order to evaluate some of these adjuvants with SIV antigens, comparing the capability of each adjuvant to enhance immune responses in young pigs vaccinated with currently available inactivated SIV antigens. A commercial vaccine containing inactivated freeze-dried H1N1 and H3N2 swine influenza antigens was used to supply the antigen mass. Five test vaccines were prepared by adding the recommended amount of either the manufacturer's adjuvant (ADJUVANT A) or one of the four new adjuvants to the inactivated freeze-dried bivalent antigens. With test vaccines containing a constant antigen, any variability in immune response would be related to the effect of the adjuvant. Immune responses stimulated in pigs by each adjuvant were evaluated using HI and ELISA anti-body specific for H1N1 and H3N2.

MATERIALS AND METHODS

Adjuvants

The adjuvants used in this study were EMULSIGEN®-D, EMULSIGEN®/Rehydragel-LV, EMULSIGEN®-BCL and POLYGEN™ (MVP Laboratories, Inc.), plus the adjuvant that was supplied by the manufacturer of the freeze-dried SIV antigens used in this study. EMULSIGEN®-D is an oil-in-water emulsion containing dimethyldioctadecylammonium bromide (DDA) for added immune stimulation. EMULSIGEN®/Rehydragel-LV contains a controlled particle size emulsion plus aluminum hydroxide gel. EMULSIGEN®-BCL is a novel oil-in-water emulsion containing an immune-stimulant proprietary to the company supplying the adjuvant. POLYGEN™ is a low molecular weight, non-particulate copolymer adjuvant that has been demonstrated to stimulate excellent gamma interferon responses in cattle (1).

Preparation of experimental vaccines

A commercial vaccine that contained inactivated freeze-dried H1N1 and H3N2 SI viruses with the manufacturer's adjuvant (ADJUVANT A) was purchased from a veterinary distributor. Just prior to vaccination, each experimental vaccine was prepared by adding and mixing the calculated amount of ADJUVANT A or one of the other four adjuvants to rehydrate the freeze-dried vaccine according to the manufacturer's recommendations.

Vaccination protocol

Prior to the study, approximately 20 sows were bled and their sera were evaluated for HI titer. Six pigs from each of the 6 sows having the lowest HI titers for SIV were selected and identified as to litter by ear tags. Pigs were transported to the research facilities at the University of Nebraska for testing. When the pigs were 21 days of age, they were assigned randomly to 6 vaccine groups with one pig from each litter being assigned to each vaccine group. Pigs of group 1 through 5 were given vaccines containing EMULSIGEN®-D, EMULSIGEN®/Rehydragel-LV, EMULSIGEN®-BCL, POLYGEN™, and ADJUVANT A, respectively. Pigs in group 6 were given PBS only. The latter group of pigs served as the negative control group. On Day 0, all of the vaccinated pigs were injected intramuscularly with a 2.0 ml dose. On Day 21, all pigs were given a second dose of vaccine. All pigs were bled on Days 0, 21, and 42. The sera were processed and frozen.

HI assay

The HI assays were performed at the Veterinary Diagnostic Laboratory at Iowa State University (Swine Influenza Virus H1N1 HI Pfizer Test, and Swine Influenza Virus H3N2 HI). All sera were coded so that the evaluations were blinded.

ELISA for detection of antibodies to H1N1 and H3N2 swine influenza viruses

ELISA antibody responses were evaluated using the IDEXX HerdChek Swine Influenza Antibody Test Kit-H1N1 and Test Kit-H3N2. The test procedures and interpretation of results were performed according to the manufacturer's instructions. The sera from vacci-nated pigs were diluted 1:40 with Sample Diluent and the positive and negative control sera were tested undi-luted. One hundred microliters of the prepared serum samples were added to each well of ELISA plates precoated with SIV antigen specific for either H1N1 or H3N2. Positive and negative control sera were added to appropriate wells and were used to determine the S/P ratio for calculation of seroconversion. All samples were run in duplicate. Plates were incubated at room temperature for 30 minutes after which the liquid from each well was aspirated off and discarded. Each well was washed with 350 ul of Wash Solution 3 to 5 times. After removing the Wash Solution, 100 ul of Anti-Porcine: HRPO Conjugate was dispensed into each well. Plates were incubated again for 30 minutes at room temperature. Washing as described above was repeated and then 100 ul of TMS Substrate Solution was dispensed into each well. Plates were incubated for 15 minutes at room temperature after which 100 ul of Stop Solution was dispensed into each well to stop color reaction. The absorbance at 650 nm was measured and recorded. Seroconversion to SIV positive was determined by calculating the sample/positive (S/P) ratio for each sample. If the S/P

ratio was less than 0.4, the sample was classified as negative for SI antibody. If the S/P ratio was greater than or equal to 0.4, the sample was classified as positive for SI antibody.

Statistical methods

A one-tailed Student's T Test was used for analysis of significance between groups.

RESULTS AND DISCUSSION

Hemagglutination inhibition (HI) responses

All pig sera from Days 0, 21 and 42 were tested for the presence of HI antibody titers against either H1N1 or H3N2 SI viruses. On Day 0, all 36 pigs were seronegative to both subtypes with HI titers <1:10. On Days 21 and 42, the HI titers of Group 6 pigs (negative control group) remained seronegative at <1:10 except for one pig that developed a titer to H1N1of 1:20 by Day 42. Pigs vaccinated with experimental vaccines formulated with either EMULSIGEN®-D or EMULSIGEN®-BCL gave enhanced HI responses when compared to all other adjuvants in this study (Tables 1, 2 and Figures 1, 2). The H1N1 Geometric Mean Titer (GMT) of the EMULSIGEN®-D group was 1437 as compared with GMTs of 640, 320, 127 and 320 for groups receiving EMULSIGEN®-BCL, EMULSIGEN®/Rehydragel-LV, POLYGEN™ and ADJUVANT A, respectively. The H3N2 GMT of the EMULSIGEN®-D group was 1613 as compared with GMTs of 453, 180, 57 and 226 for groups receiving EMULSIGEN®-BCL, EMULSIGEN®/Rehydragel-LV, POLYGEN™ and ADJUVANT A, respectively.

HI seroconversion

Tables 3 and 4 show that EMULSIGEN®-D and EMULSIGEN®-BCL produced the best results with H1N1 seroconversions of 50% and 33% respectively by Day 21, whereas none of the pigs in the other groups seroconverted by Day 21. EMULSIGEN®-D and EMULSIGEN®-BCL were again the only adju-vants that seroconverted pigs to H3N2 by Day 21 (83% for EMULSIGEN®-D and 33% for EMULSIGEN®-BCL). All pigs in all vaccinate groups seroconverted by Day 42.

ELISA responses

All pigs were seronegative to both H1N1 and H3N2 on Day 0, confirming the HI results. ELISA antibody could not be detected in any of the Group 6 pigs throughout the study. ELISA antibody titers as indicated by optical densities showed the same general pat-tern as the HI titers (Figures 1, 3, and figures 2, 4). For both H1N1 and H3N2, the highest ELISA ODs were produced by the vaccine containing EMULSIGEN®-D, with the next highest results being produced by EMULSIGEN®-BCL. On Day 42, H1N1 geometric mean OD values were 0.314, 0.121, 0.241, 0.090, 0.156 and 0.069 for EMULSIGEN®-D, EMULSIGEN®/Rehydragen-LV, EMULSIGEN®-BCL, POLYGEN™, ADJUVANT A and the Negative Control Group, respectively. All pigs remained seronegative on Day 21 to H1N1. H3N2 geometric mean OD values were higher. Day 42 values were 0.669, 0.606, 0.661, 0.548, 0.651 and 0.073, respectively for the groups listed above. Day 21 values were 0.254, 0.121, 0.202, 0.077, 0.131 and 0.060, respectively for the above listed groups. This indicates that pigs were responding to H3N2 by Day 21, especially pigs in the EMULSIGEN®-D and EMULSIGEN®-BCL groups.

ELISA seroconversion

Tables 3 and 4 illustrate the results of this testing. The H1N1 and H3N2 Positive Control optical density values were 0.488 and 0.490, respectively. These values were used for calculation of the S/P ratio. All pigs remained seronegative to H1N1 on Day 21. By Day 42 (three weeks after the second vaccination), 100% of the EMULSIGEN®-D and EMULSIGEN®-BCL pigs seroconverted to H1N1, whereas 33% of the EMULSIGEN®/Rehydragel-LV, 0% of the POLYGEN™ and 50% of the

ADJUVANT A group seroconverted. Pigs vaccinated with H3N2 antigen began to seroconvert by Day 21. The seroconversion percentage by Day 21was 83% for the EMULSIGEN®-D group, 17% for the EMULSIGEN®/Rehydragel-LV group, 33% for the EMULSIGEN®-BCL group, 0% for the POLYGEN™ group and 17% for the ADJUVANT A group. All of the vaccinated pigs in all five vaccine groups seroconverted to H3N2 by Day 42.

Discussion

Vaccination of pigs against swine influenza is generally carried out by administering two intra-muscular injections of an inactivated whole virus vaccine containing an adjuvant. The purpose of the present study was to determine the comparative adjuvant effects of five different adjuvants, when added to a constant antigenic mass of bivalent, freeze-dried H1N1 and H3N2 SIV antigens from a commercial source. Adjuvants evaluated in this study were EMULSIGEN®-D, EMULSIGEN®-BCL, EMULSIGEN®/Rehydragel-LV, POLYGEN™ and the adjuvant supplied by the manufacturer of the vaccine (ADJUVANT A). All of the adjuvants except POLYGEN™ contained an oil and water base. There-fore, this study evaluated the enhancement of the im-mune responses when immunostimulants were added to the oil and water base. The study also provided a comparison of oil and water-based adjuvants with a co-polymer base adjuvant (POLYGEN") for use with SIV vaccines.

Each of the adjuvants was used as a diluent for a bottle of the freeze-dried combination antigens after which they were used to vaccinate groups of young pigs (6 pigs per group). An additional group of pigs was injected with PBS and served as a negative control group. Serum samples from all of the pigs were evaluated for specific H1N1 and H3N2 HI titers and ELISA anti-body at Days 0, 21 and 42.

All vaccine/adjuvant groups stimulated protective HI titers to both H1N1 and H3N2 in all pigs by Day 42 post vaccination. Additionally, all adjuvants stimulated a significant increase in HI and ELISA antibody responses when compared with the negative control group at the $p \leq 0.05$ level. EMULSIGEN®-D was shown to be the most effective adjuvant for use with SIV antigens. The SIV vaccine containing EMULSIGEN®-D produced significantly higher HI titers against both H1N1 and H3N2 on Days 21 and 42 than the positive control group containing ADJUVANT A ($p \leq 0.05$ for all values). Further, the ELISA antibody stimulated by EMULSIGEN®-D was significantly higher than that stimulated by ADJUVANT A for both H1N1 and H3N2 on Day 21 and for H1N1 on Day 42 ($p \leq 0.05$ for all values). Finally the vaccine containing EMULSIGEN® -D produced a higher seroconversion rate to both H1N1 and H3N2 than the vaccine containing ADJUVANT A. EMULSIGEN® -BCL was the second best adjuvant for use with SIV antigens. It produced Day 42 antibody responses that were significantly higher than those produced by ADJUVANT A using the H1N1 ELISA ($p \leq 0.05$) and en-hancement of all other antibody responses when com-pared to ADJUVANT A. Also, vaccine containing EMULSIGEN® -BCL produced the second best rate of seroconversion by both HI and ELISA.

Table 1: Systemic antibody responses against swine influenza virus, subtype H1N1, in young pigs vaccinated with one of the five experimental SIV vaccines.

Vaccine adjuvant	Day 0		Day 21		Day 42	
and pig ID	ELISA*	HI	ELISA	HI	ELISA	HI
1. EMULSIGEN® -D						
#1	0.053 (–)	<10	0.059 (–)	20	0.560 (+)	1280
#14	0.055 (–)	<10	0.057 (–)	20	0.192 (+)	2560
#27	0.053 (–)	<10	0.084 (–)	80	0.330 (+)	320
#40	0.053 (–)	<10	0.073 (–)	40	0.381 (+)	1280
#11	0.052 (–)	<10	0.057 (–)	10	0.244 (+)	1280
#24	0.052 (–)	<10	0.058 (–)	40	0.289 (+)	5120
GMT	0.053	<10	0.064	28	0.314	1437
2. EMULSIGEN® /Rehydragel-LV						
#2	0.052 (–)	<10	0.055 (–)	20	0.184 (+)	320
#15	0.055 (–)	<10	0.059 (–)	10	0.096 (–)	320
#28	0.052 (–)	<10	0.053 (–)	10	0.161 (–)	160
#41	0.055 (–)	<10	0.058 (–)	20	0.048 (–)	320
#12	0.051 (–)	<10	0.066 (–)	10	0.125 (–)	320
#31	0.049 (–)	<10	0.058 (–)	20	0.276 (+)	640
GMT	0.052	<10	0.059	14	0.121	320
3. EMULSIGEN® -BCL						
#3	0.050 (–)	<10	0.052 (–)	20	0.353 (+)	1280
#16	0.056 (–)	<10	0.055 (–)	10	0.350 (+)	2560
#29	0.049 (–)	<10	0.065 (–)	40	0.195 (+)	160
#42	0.048 (–)	<10	0.073 (–)	20	0.238 (+)	320
#19	0.056 (–)	<10	0.058 (–)	20	0.170 (+)	320
#32	0.050 (–)	<10	0.071 (–)	80	0.201 (+)	1280
GMT	0.051	<10	0.062	25	0.241	640
4. POLYGEN™						
#4	0.050 (–)	<10	0.053 (–)	10	0.098 (–)	160
#17	0.050 (–)	<10	0.054 (–)	<10	0.072 (–)	160
#30	0.051 (–)	<10	0.056 (–)	<10	0.082 (–)	80
#7	0.053 (–)	<10	0.054 (–)	<10	0.086 (–)	80
#20	0.050 (–)	<10	0.056 (–)	<10	0.082 (–)	80
#33	0.051 (–)	<10	0.053 (–)	20	0.126 (–)	320
GMT	0.051	<10	0.054	0.5	0.090	127
5. ADJUVANT A (from commercial vaccine)						
#5	0.049 (–)	<10	0.054 (–)	20	0.094 (–)	160
#18	0.057 (–)	<10	0.052 (–)	10	0.089 (–)	160
#37	0.049 (–)	<10	0.050 (–)	10	0.275 (+)	1280
#8	0.045 (–)	<10	0.059 (–)	10	0.171 (+)	320
#21	0.049 (–)	<10	0.054 (–)	10	0.218 (+)	320
#34	0.047 (–)	<10	0.053 (–)	10	0.168 (–)	320
GMT	0.049	<10	0.054	11	0.156	320
6. PBS (negative control group)						
#13	0.052 (–)	<10	0.056 (–)	<10	0.065 (–)	<10
#26	0.054 (–)	<10	0.060 (–)	<10	0.062 (–)	<10
#39	0.052 (–)	<10	0.054 (–)	<10	0.063 (–)	<10
#10	0.047 (–)	<10	0.053 (–)	<10	0.084 (–)	20
#23	0.046 (–)	<10	0.051 (–)	<10	0.057 (–)	<10
#36	0.045 (–)	<10	0.056 (–)	<10	0.092 (–)	<10
GMT	0.049	<10	0.055	<10	0.069	<10

* According to the ELISA Kit instructions, the presence or absence of antibody to SIV subtype is determined by calculating the S/P ratio for each sample. If the S/P ratio is greater than or equal to 0.4, then the sample is positive for SIV antibody. In this study, the Positive Control mean is 0.488 for H1N1and 0.490 for H3N2, and the Negative Control is 0.059 for H1N1 and 0.059 for H3N2.

Table 2: Systemic antibody responses against swine influenza virus, subtype H3N2, in young pigs vaccinated with one of the five experimental SIV vaccines.

Vaccine adjuvant	Day 0		Day 21		Day 42	
and pig ID	ELISA*	HI	ELISA	HI	ELISA	HI
1. EMULSIGEN® -D						
#1	0.053 (--)	<10	0.318 (+)	40	0.832 (+)	5120
#14	0.055 (--)	<10	0.278 (+)	40	0.588 (+)	2560
#27	0.052 (--)	<10	0.394 (+)	40	0.631 (+)	640
#40	0.053 (--)	<10	0.263 (+)	40	0.685 (+)	2560
#11	0.056 (--)	<10	0.113 (--)	<10	0.633 (+)	640
#24	0.059 (--)	<10	0.259 (+)	40	0.667 (+)	1280
GMT	0.055	<10	0.254	22	0.669	1613
2. EMULSIGEN® /Rehydragel-LV						
#2	0.057 (--)	<10	0.157 (--)	10	0.731 (+)	320
#15	0.057 (--)	<10	0.140 (--)	20	0.564 (+)	80
#28	0.056 (--)	<10	0.099 (--)	20	0.601 (+)	160
#41	0.073 (--)	<10	0.054 (--)	<10	0.476 (+)	160
#12	0.055 (--)	<10	0.131 (--)	10	0.644 (+)	160
#31	0.055 (--)	<10	0.199 (+)	20	0.653 (+)	320
GMT	0.058	<10	0.121	10	0.606	180
3. EMULSIGEN® -BCL						
#3	0.057 (--)	<10	0.129 (--)	10	0.778 (+)	640
#16	0.059 (--)	<10	0.117 (--)	10	0.727 (+)	640
#29	0.053 (--)	<10	0.480 (+)	160	0.628 (+)	320
#42	0.051 (--)	<10	0.138 (--)	20	0.544 (+)	320
#19	0.060 (--)	<10	0.157 (--)	10	0.635 (+)	320
#32	0.056 (--)	<10	0.432 (+)	160	0.678 (+)	640
GMT	0.056	<10	0.202	62	0.661	453
4. POLYGEN™						
#4	0.055 (--)	<10	0.071 (--)	<10	0.516 (+)	80
#17	0.053 (--)	<10	0.066 (--)	<10	0.571 (+)	40
#30	0.055 (--)	<10	0.084 (--)	<10	0.614 (+)	40
#7	0.057 (--)	<10	0.101 (--)	<10	0.506 (+)	40
#20	0.057 (--)	<10	0.067 (--)	<10	0.463 (+)	40
#33	0.053 (--)	<10	0.078 (--)	<10	0.640 (+)	160
GMT	0.055	<10	0.077	<10	0.548	57
5. ADJUVANT A (from commercial vaccine)						
#5	0.060 (--)	<10	0.213 (+)	20	0.694 (+)	160
#18	0.065 (--)	<10	0.107 (--)	10	0.566 (+)	80
#37	0.055 (--)	<10	0.099 (--)	<10	0.696 (+)	640
#8	0.053 (--)	<10	0.115 (--)	20	0.730 (+)	160
#21	0.052 (--)	<10	0.146 (--)	10	0.674 (+)	320
#34	0.051 (--)	<10	0.132 (--)	20	0.566 (+)	320
GMT	0.056	<10	0.131	10	0.651	226
6. PBS (negative control group)						
#13	0.073 (--)	<10	0.065 (--)	<10	0.061 (--)	<10
#26	0.058 (--)	<10	0.059 (--)	<10	0.060 (--)	<10
#39	0.059 (--)	<10	0.056 (--)	<10	0.058 (--)	<10
#10	0.058 (--)	<10	0.061 (--)	<10	0.160 (--)	20
#23	0.054 (--)	<10	0.060 (--)	<10	0.063 (--)	<10
#36	0.054 (--)	<10	0.062 (--)	<10	0.070 (--)	<10
GMT	0.059	<10	0.060	<10	0.073	<10

Figure 1: Adjuvant stimulation of HI antibody to SIV H1N1 in swine receiving bivalent SIV vaccines containing a constant antigen mass.

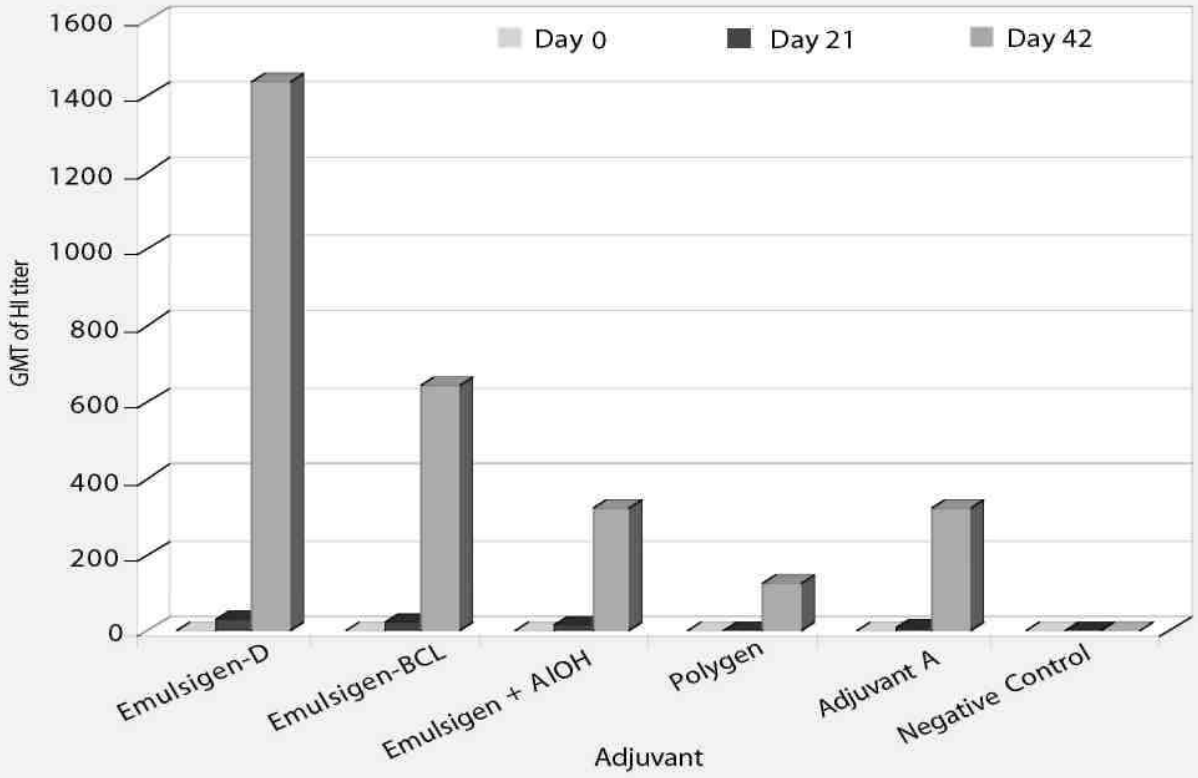


Figure 2: Adjuvant stimulation of HI antibody response to SIV H3N2 in swine receiving vaccines containing a constant antigen mass.

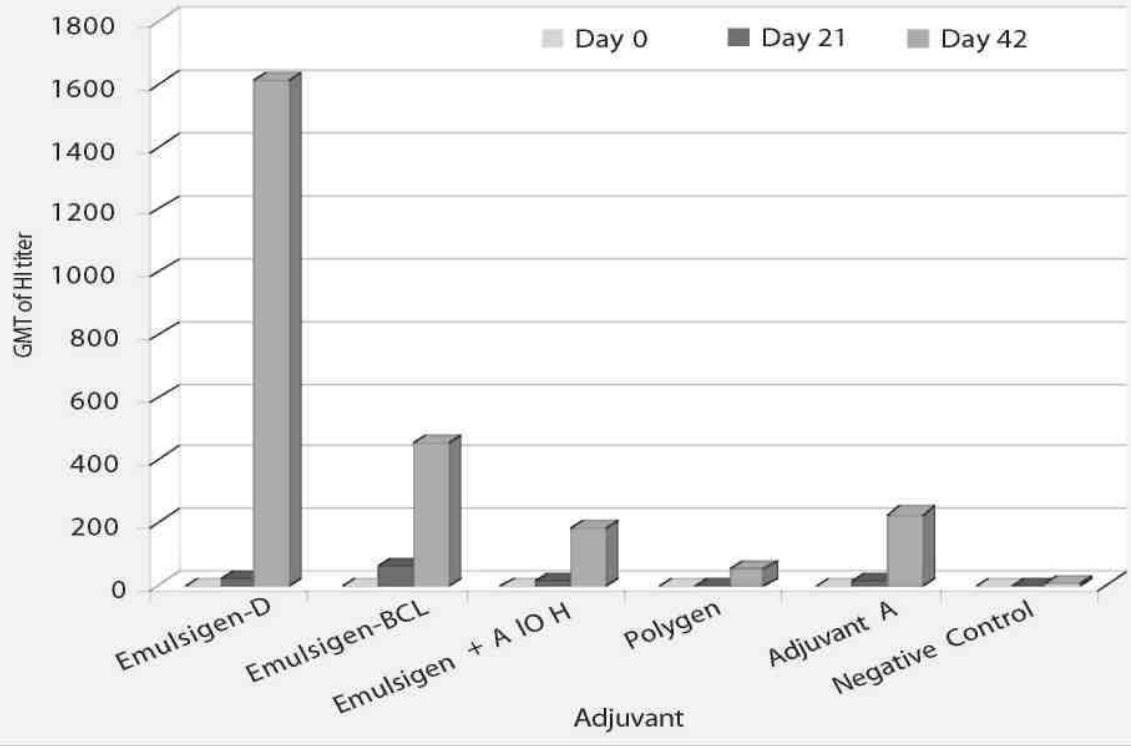


Figure 3: Adjuvant stimulation of ELISA antibody response to SIV H1N1 in swine receiving vaccines containing a constant antigen mass.

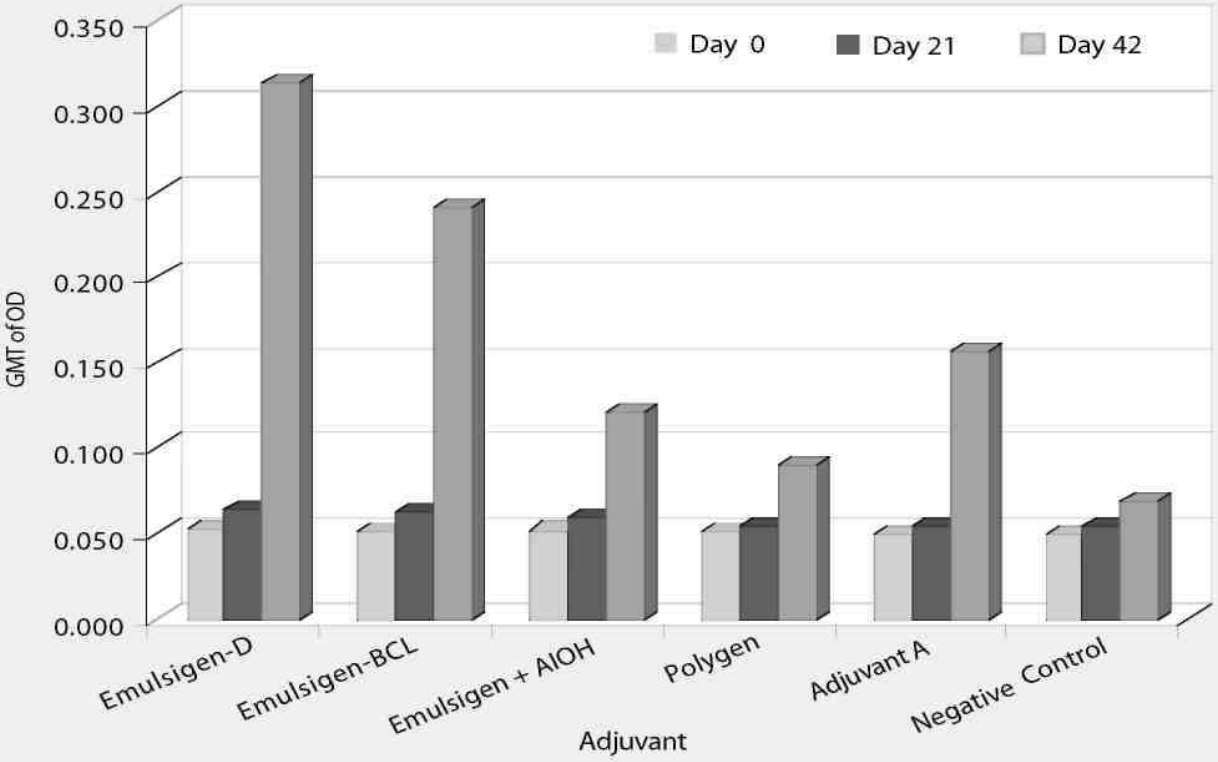


Figure 4: Adjuvant stimulation of ELISA antibody response to SIV H3N2 in swine receiving vaccines containing a constant antigen mass.

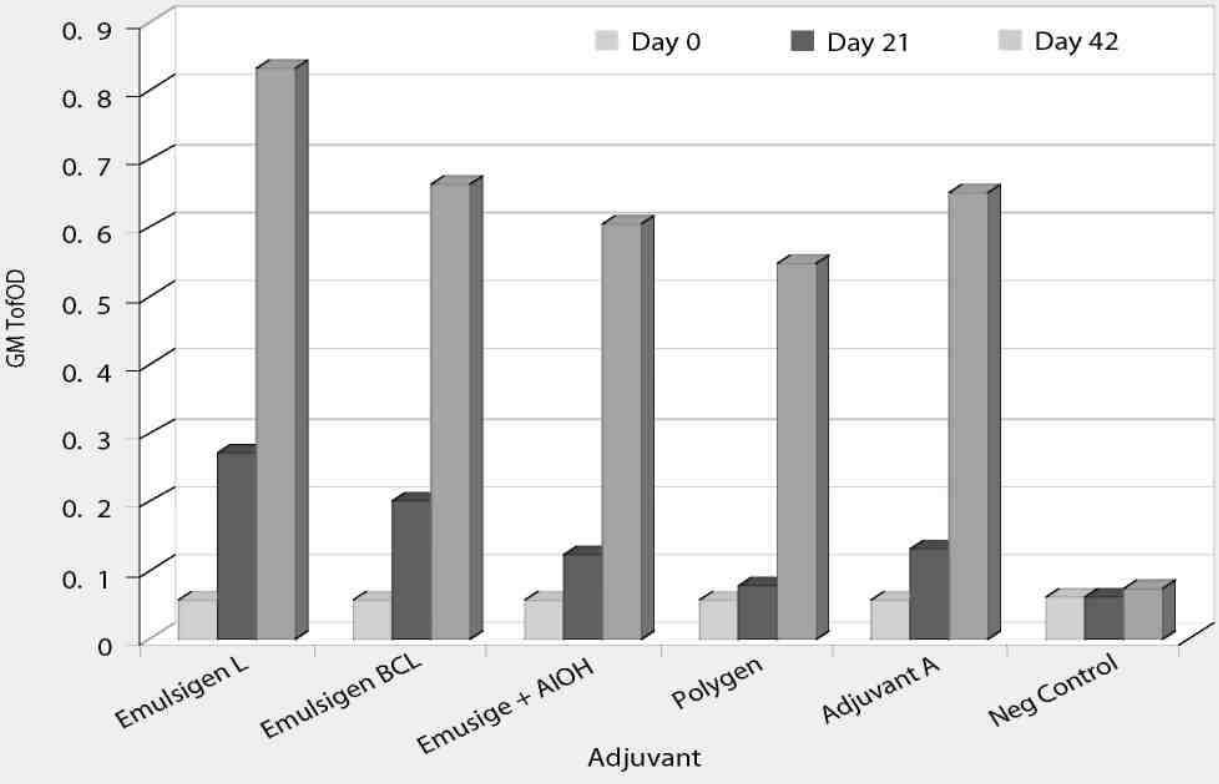


Table 3: Comparison of ELISA and HI seroconversion to SIV H1N1 antigen in pigs receiving vaccines containing various adjuvants.

Vaccine adjuvant	Percentage of swine converting to positive H1N1					
	Day 0		Day 21		Day 42	
	ELISA	HI	ELISA	HI	ELISA	HI
EMULSIGEN® -D	0	0	0	50	100	100
EMULSIGEN® /Rehydragel-LV	0	0	0	0	33	100
EMULSIGEN® -BCL	0	0	0	33	100	100
POLYGEN™	0	0	0	0	0	100
ADJUVANT-A	0	0	0	0	50	100
Negative control	0	0	0	0	0	0

HI seroconversion is considered to be a titer of >1:40

Table 4: Comparison of ELISA and HI seroconversion to SIV H3N2 antigen in pigs receiving vaccines containing various adjuvants.

Vaccine adjuvant	Percentage of swine converting to positive H3N2					
	Day 0		Day 21		Day 42	
	ELISA	HI	ELISA	HI	ELISA	HI
EMULSIGEN® -D	0	0	83	83	100	100
EMULSIGEN® /Rehydragel-LV	0	0	017	0	100	100
EMULSIGEN® -BCL	0	0	33	33	100	100
POLYGEN™	0	0	0	0	100	100
ADJUVANT-A	0	0	0	0	100	100
Negative control	0	0	0	0	0	0

HI seroconversion is considered to be a titer of >1:40

REFERENCES

1.Andrianarivo, A. G., Choromanski, L., McDonough, S.P, Packham, AE., and Conrad, PA. 1999. Immunogenicity of a killed whole Neospora caninum tachyzoite preparation formulated with different adjuvants. International Journal for Parasitology 29:1613–1625.

2.Higgins, D.A., Carlson, J. R., and Van Nest, G. 1996. MF59 adjuvant enhances the immunogenicity of influenza vaccine in both young and old mice. Vaccine 14:478–484.

3.Janke, B. H. 2000. Diagnosis of swine influenza. Swine Health and Production. 8:79–83.

4.Kaji, M., Kaji R., Ohkuma, K., Honda, T., Oka, T, Sakoh, M., Nakamura, S., Kurachi, K., and Sentoku, M. 1992. Phase 1 clinical tests of influenza MDP-virosome vaccine (KD5382). Vaccine 10:663–667.

5.Keitel, W, Couch, R., Bond, N., Adair, S., Van Nest, G., and Dekker, C. 1993. Pilot evaluation of influenza virus vaccine (IVV) combined with adjuvant. Vaccine 11:909–914.

6.Larsen, D.L., Karasin, A., Zuckermann, F, and Olsen, C.W. 2000. Systemic and mucosal immune responses to H1N1 influenza virus infec-tion in pigs. Veterinary Microbiology. 74:117–131.

7.Sundquist, B., Lovgren, K., and Morein, B. 1988. Influenza virus ISCOMs: antibody response in animals. Vaccine 6:49–53.

ANTIBODY RESPONSE OF YOUNG PIGS TO AUTOGENOUS *HAEMOPHILUS PARASUIS* VACCINE

Boh Chang Lin, DVM, MS, PhD; R Gene White, DVM, MS; Karen K Brown, PhD
Leann Siedlik; Colette Hrabik; Jack McGonigle, BS
MVP Laboratories, Inc., Omaha, Nebraska

REZUMAT

Cercetările au fost făcute în scopul evaluării antigenicității a 5 vaccinuri autogene folosite în prevenirea infecției cu *Haemophilus parasuis* la tineretul porcin. Răspunsul imun în anticorpi serici la animale vaccinate a fost verificat prin ELISA. Datele obținute au arătat că după folosirea unui vaccin standardizat, prin ELISA, la 42 zile de la administrarea vaccinului se poate evalua eficacitatea unui vaccin autogen folosind antigen omolog.

Cuvinte cheie: *Haemophilus parasuis,* vaccin autogen, ELIZA, antigen omolog

ABSTRACT

Research was conducted to assess the antigenity of 5 autogenous vaccines used to prevent infection by *Haemophilus parasuis* in young pigs. The immune response in serum antibodies in vaccinated animals was checked by ELISA. The data obtained showed that using a vaccine standardized by ELISA, at 42 days of vaccine administration can evaluate the effectiveness of a vaccine with the homologous antigen autogenous

Keywords: *Haemophilus parasuis,* autogenous vaccine, ELISA, homologous antigen

INTRODUCTION

Infection of immune-naive pigs by *Haemophilus parasu-is* has become one of the most signifcant swine diseases in the past few years. Good, homologous protection against some strains of *H parasuis* in pigs vaccinated with an autogenous vaccine has been reported (1,2) and the early vaccination of young pigs with an autogenous *H* p*arasuis* vaccine has been adopted in many high health status pig farms. However, due to a lack of an appropriate serological test, an evaluation of the antigenicity of the autogenous vaccine and the antibody response in vaccinated pigs is seldom done on most pig farms. The published data about the serologic profile of pigs vaccinated against *H parasuis* is very limited. In 1991, Miniats et al had indicated that their attempts to detect the presence of specific antibodies against *H* p*arasuis* strains in the sera of the vaccinated or exposed pigs by the passive hemagglutination test or by ELISA were unsuccessful.

The antigens employed in Miniats' ELISA serology were either supernatants from boiled bacteria or dialyzed hot phenol water extracts of the *H parasuis* (3) Although Tadjine et al recently described a protocol to screen for mouse monoclonal antibodies against *H parasuis* by ELISA using whole cell suspension, boiled cell suspension, and sonicated cell suspension as the coated antigens (5) a standardized ELISA that can be used to evaluate the antibody response in vaccinated pigs still needs to be developed.

The aim of this study was to use an indirect enzyme-linked immunosorbent assay (ELISA) developed at MVP Laboratories (Omaha, NE) to evaluate the antigenicity of four different *H parasuis* field strains used in autogenous vaccines through the determination of specific antibody titer in individual vaccinated pigs. This protocol may also be used to monitor the time of infection with *H parasuis* in commercial pig farms and help swine veterinarians determine the optimal time of vaccination.

MATERIALS AND METHODS

Test vaccines: Five autogenous vaccines, each containing one of the five *H parasuis* isolates (isolate #6204803, serovar 7; #6204536, serovar 13; #6205360, serovar 2; #6204553, serovar 4; and #6200077, sero-nontypeable) were prepared at MVP Laboratories. Each *H parasuis* culture was inactivated with formalin and adjuvanted with 12% Emulsigen (MVP Laboratories) and 4% Rehydragel.

A standardized vaccination study: Twenty five 18-day-old pigs, testing seronegative for *H parasuis*, were purchased from a high health status pig farm with no history of *H parasuis* infection. These pigs were weaned at 18 days of age and moved to the University of Nebraska Veterinary Research facility in Lincoln, Nebraska. The pigs were individually identifed with a unique ear number. The pigs were allowed to acclimate to the new surroundings for 14 days and then were randomly assigned to one of the fve groups. The test vaccines and group assignments were: 1) no vaccine for the un-treated controls; 2) bacterin prepared from *H parasuis* serovar 7, isolate 6204803; 3) bacterin prepared from *H parasuis* serovar 13, isolate 6204536; 4) bacterin pre-pared from *H parasuis* serovar 2, isolate 6205360; and 5) bacterin prepared from *H parasuis* serovar 4, isolate 6204553. On day 0, a blood sample was collected from all pigs and each pig from groups 2, 3, 4, and 5 was vaccinated with a 2.0 ml dose of one of the four autogenous vaccines, subcutaneously. They were vaccinated again at day 21. On day 42, day 63, and day 84, all pigs were bled again. The serum was separated by centrifugation and stored at -20˚C before use.

A feld vaccination study: Ten 14-day old pigs were selected from a commercial pig farm that had outbreaks of *H parasuis* infection. An autogenous vaccine was prepared using a sero-nontypeable strain of *H parasuis* (#6200077) isolated from the same farm. Each pig was individually identified with a unique ear tag number. On day 0, a blood sample was collected from all of the ten pigs and all of the pigs were vaccinated with a 2 ml dose of the autogenous *H parasuis* vaccine subcutaneously. They were vaccinated again on day 14. On day 14 and day 35, all of the ten pigs were bled again. The serum was stored at -20˚C before use.

Enzyme-linked immunosorbent assay (ELISA): Anti-bodies directed against seven commonly seen serotypes of *H parasuis* (serovar 2, 4, 5, 7, 12, 13 and 14) in the US (4) were detected by use of an indirect ELISA. Anti-bodies against the vaccine strain were also detected using the plate coated with the soluble proteins obtained from the vaccine strain. Briefy, each of the seven standard strains as well as the five field isolates of *H parasuis* were grown on Frey Chocolate agar plates and harvested into sterile PBS (pH 7.2). The washed bacterial cells were treated with CelLytic reagent (Sigma Chemical Co.) and the soluble protein antigens obtained from each isolate or standard strains were adjusted to 1 mg/ml using sterile PBS. A negative control antigen was made in the same way. Each well of a 96-well plate (Immulon 2, Dynatech) was either coated with 2 ug of the mixed soluble antigens obtained from *H parasuis* isolates as positive antigens (PA) or 2 ug of the negative control antigen (NA). Some plates were coated with the soluble proteins obtained from a single field isolate as the homologous antigens. Both the test sera and control sera were first diluted to 1:200 using a dilution buffer (PBS Tween buffer with 0.5% BSA) and two fold serial dilutions were made from there. A rabbit antiserum against all seven commonly seen serovars (serovar 2, 4, 5, 7, 12, 13, 14) of *H parasuis* was used as the positive control and a pig serum from an unexposed pig was used as a negative control. Each well of the plate was blocked with 50 ul of a blocking agent (PBS buffer with 1%BSA) for 30 minutes. After washing with PBS-Tween buffer 3 times, each diluted test serum sample and control serum sample was dispended into two wells of the PA and two wells of NA in an amount of 50 µl per well. The plate was incubated at 37˚C for 90 minutes. After washing with PBS-Tween buffer, 50 µl of alkaline phosphatase labeled goat anti-pig IgG (KPL Laboratories), diluted at 1:100, was added to each well of the plate containing pig serum. Fifty microliters of alkaline phosphatase labeled goat anti-rabbit IgG (KPL Laboratories), diluted at 1:200, was added to each well of the plate containing rabbit serum. The plate was incubated at 37˚C for 90 minutes. After washing, 100 ul of a chromagen containing phosphatase substrate (Sigma Chemical) was added to each well of the plate and incubated at room temperature for thirty minutes. The optical density (OD) was measured at 405 nm. As the OD reading of the positive control serum (1:200) reached around 1.50, the color reaction was stopped by adding 50 ul of a 5N sodium hydroxide

to each well and the plate was read at 405 nm. The average OD of the NA for each test sample was subtracted from the average OD of PA of the same test sample and was used for evaluation of its antibody titer. All of the serum samples showing larger than 50% of the OD value of the positive control were considered sero-positive (S/P > 0.5). The antibody titer was reported as the re-ciprocal of the last positive dilution. For the assay to be valid, the adjusted OD readings of the positive control serum must be around 1.50 and the negative control must be around 0.20 at 1:200 dilution. Any serum sample that had a titer equal to or less than 400 was considered to be sero-negative against *H* p*arasuis* in this assay system.

Demonstration of an acceptable antigenicity of the vaccine strains: In order to evaluate the antigenicity of the *H parasuis* strain used in an autogenous vaccine, an arbitrary criterion was set as follows: If on day 42 at least 80% of the pigs from a vaccinated group could demonstrate at least a four fold increase in antibody titer over the pre-vaccinated serum samples, the antigenicity of that *H parasuis* strain used to test in that group would be acceptable. The negative control pigs should remain sero-negative throughout the whole test period.

Statistical method: Two-tailed Student's t test was con-ducted to determine the statistical significance of the serological data obtained from a standardized vaccination study and a field vaccination study. *P* values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Serologic evaluation for a standardized vaccination study: A summary of the titer of each of the 25 pigs is shown in Table 1 and Figure 1. In order to calculate the increase in antibody titer from day 0 to day 42 (conversion factor), pigs showing titers of < 200 were considered to be 200. If a pig was found to have a titer < 200 on day 0 and 800 on day 42, it would have a conversion factor of 4. It was found that all pigs were sero-negative against *H parasuis* on day 0. Each of the pigs in the non-vaccinated group (group 1) remained sero-negative throughout the study. At least 4 out of 5 pigs (80%) in each vaccinated groups sero-converted by day 42, exhibiting at least a four-fold increase in titer over day 0. On day 35, a pig from group 2 developed an umbilical hernia and was sold on day 42. Either on day 63 (six weeks after second vaccination) or day 84 (nine weeks after second vaccination), 13 out of 19 vaccinated pigs (68.4%) still showed at least a fourfold increase in titers over day 0. This serologic study indicated that the antigenicity of the four autogenous vaccine strains of *H. parasuis* was acceptable.

Serologic evaluation for a field vaccination study: The EL1SA titers for anti- *H parasuis* antibodies are shown in Table 3. Three out of ten 2-week old pigs were found to be sero-positive and had high antibody titer against *Hparasuis* before vaccination. Two weeks after the first vaccination, EL1SA titers for aii ten pigs remained unchanged on day 14. Three weeks after two vaccinations, eighty percent of the ten vaccinated pigs reached a high ELISA titer of 6400.

In a standardized vaccination study, the geometric mean of anti - H. *parasuis* antibody titers of 20 vaccinated pigs showed a significant *(P* < 0.01) increase from day 0 to day 42, day 63, and day 84 (Table 1). The level of specific antibodies against *H. parasuis* increased significantly *(P* < 0.01) on day 42 in vaccinated pigs as compared with non-vaccinated control pigs and this tendency persisted for a minimum of 9 weeks after two vaccinations. Although the antibody titers of the vaccinated pigs obtained from the ELISA using homologous antigens were significantly *(P* < 0.05**)** larger than the titer of the same serum obtained from the ELISA using heterologous antigens (Table 2), antibody titers obtained from plates coated with either homologous or heterologous antigens all indicated that 80% of the vaccinated pigs sero-converted by day 42. Due to a high complexity in the antigenic structures of *H parasuis* field strains, ELISA plates coated with homologous antigens significantly increase the sensitivity of the as-say system. However, ELISA plates coated with soluble antigens extracted from 7 commonly seen *H parasuis* serovars (heterologous ELISA) have been tested in this study and demonstrate the ability to show the same trend of antibody increase in vaccinated pigs as the homologous ELISA.

Table 1: Serum antibody titers and conversion factors for each pig in a standardized vaccination study using ELISA plates coated with homologous antigens.

Group	Pig ID	Day 0 titer	Day 42 titer	CF	Day 63 titer	CF	Day 84 titer	CF
1	1	< 200	< 200	1	< 200	1	< 200	1
1	2	< 200	< 200	1	< 200	1	< 200	1
1	3	< 200	< 200	1	< 200	1	< 200	1
1	4	< 200	< 200	1	< 200	1	< 200	1
1	5	< 200	< 200	1	< 200	1	< 200	1
2	6	< 200	6400	32	6400	32	800	4
2	7	< 200	6400	32	6400	32	6400	32
2	8	< 200	200	1	< 200	1	< 200	1
2	9	< 200	6400	32	400	2	400	2
2	10	< 200	6400	32	200	1	200	1
3	11	< 200	3200	16	400	2	400	2
3	12	< 200	6400	32	800	4	800	4
3	13	< 200	6400	32	ND		ND	
3	14	< 200	400	2	800	4	6400	32
3	15	< 200	6400	32	6400	32	6400	32
4	16	< 200	6400	32	6400	32	6400	32
4	17	< 200	6400	32	6400	32	6400	32
4	18	< 200	6400	32	3200	16	3200	16
4	19	< 200	200	1	< 200	1	400	2
4	20	< 200	6400	32	6400	32	6400	32
5	21	< 200	6400	32	6400	32	6400	32
5	22	< 200	6400	32	6400	32	6400	32
5	23	< 200	6400	32	6400	32	6400	32
5	24	< 200	200	1	400	2	400	2
5	25	< 200	6400	32	6400	32	6400	32
Geometric mean		< 200	1838		1165		1199	

CF = Conversion factor
ND = Not done

Figure 1: Geometric mean antibody titers of pigs vaccinated with autogenous *H parasuis* vaccines in a standardized vaccination study.

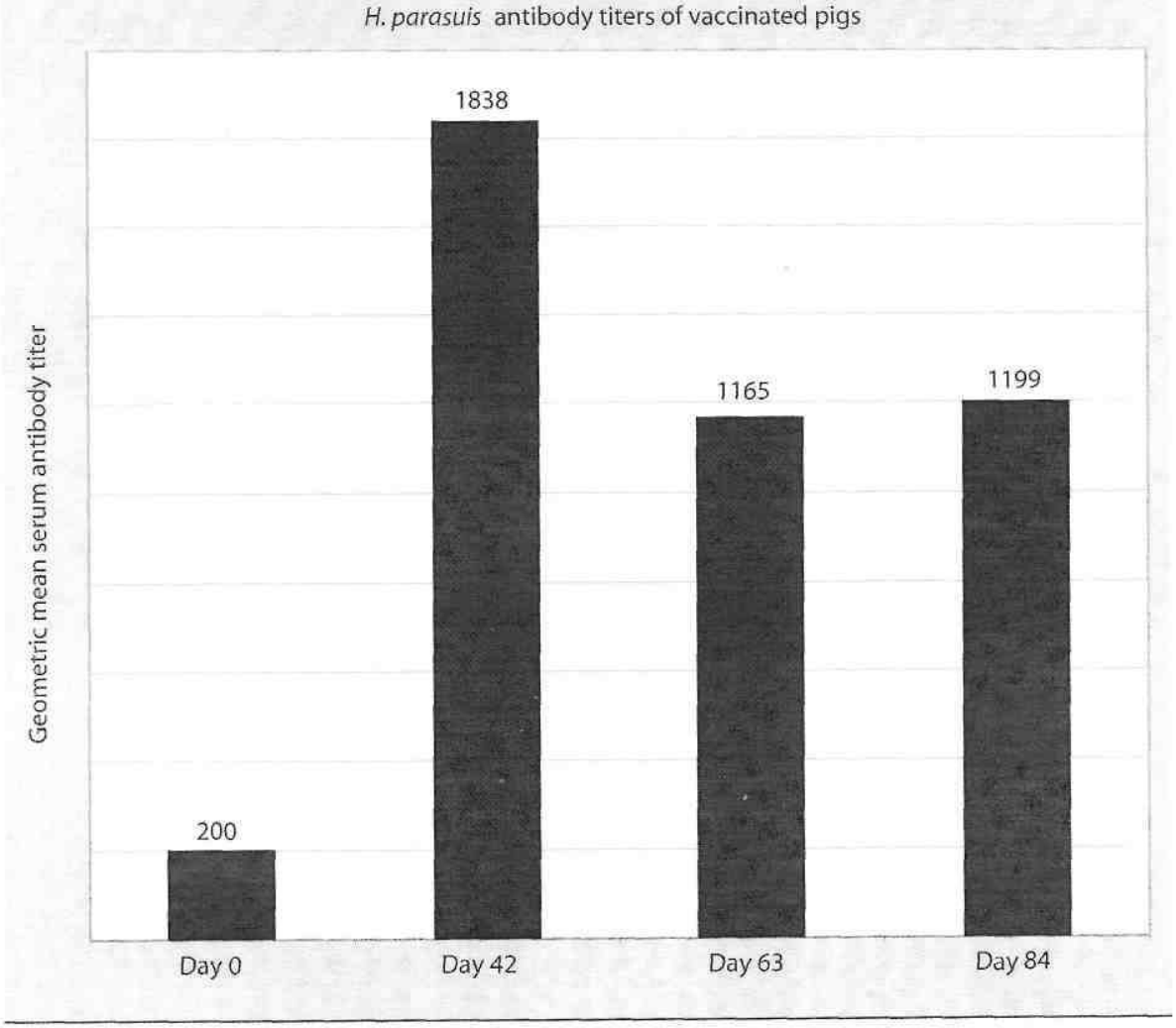


Table 2: Determination of serum antibody titers using ELISA plates coated with either homologous or heterologous antigens in a standardized vaccination study.

Pig ID	Homologous antigen		Heterologous antigen	
	Titer		Titer	
	Day 0	Day 42	Day 0	Day 42
6	<200	6400	<200	1600
7	<200	6400	<200	3200
8	<200	200	<200	400
9	<200	6400	<200	800
10	<200	6400	<200	800
21	<200	6400	<200	6400
22	<200	6400	<200	6400
23	<200	6400	<200	800
24	<200	200	<200	200
25	<200	6400	<200	6400
Geometric mean	<200	3200	<200	1493

Table 3: Antibody titers and conversion factors for young pigs vaccinated with an autogenous *H parasuis* vaccine at a commercial pig farm.

Pig ID	Day 0 (2 weeks old)	Day 14 (4 weeks old)		Day 35 (7 weeks old)	
	Titer	Titer	CF	Titer	CF
1	200	200	1	6400	32
2	6400	6400	1	6400	1
3	6400	6400	1	6400	1
4	6400	6400	1	6400	1
5	200	200	1	6400	32
6	200	200	1	6400	32
7	200	200	1	200	1
8	200	200	1	6400	32
9	200	200	1	6400	32
10	200	200	1	200	1
Geometric mean	566	566		3200	

In a field vaccination study, the geometric mean of anti-*H parasuis* titer increased significantly ($p < 0.05$) on day 35 in pigs after 2 vaccinations as compared with the same pigs before vaccination. In this study, three out of ten test pigs were sero-positive against *H parasuis* before vaccination on day 0 when they were 2 weeks old. These 3 pigs might carry maternal antibodies from vaccinated sows or infected sows. Even after one or two vaccinations, all three pigs still showed the same titer of 6400 on day 14 and day 35. It appears that serum antibody titers in these 3 pigs were not interfered by maternal antibodies and their titers did not drop down during the five weeks period after first vaccination. Among the 7 pigs that showed sero-negative before vaccination, a sero-conversion could be seen in 5 pigs on day 35, while none of the 7 pigs became sero-positive on day 14. One vaccination may not be enough to induce sero-conversion in young pigs.

In the standardized vaccination study, four vaccinated pigs did not show any sero-conversion on day 42 (3 weeks after second vaccination). Among them, only one pig became sero-positive on day 63 and day 84. The other 3 pigs did not show any sero-conversion from day 42 through day 84. Two out of the ten pigs in a field vaccination study also did not show sero-conversion from day 14 through day 35. This may imply that perhaps 20% of the young pigs will not respond to the vaccination with *H parasuis* vaccines. Further study is needed to search for the causes of the inability to have antibody response to *H parasuis* vaccines in some percentage of younger pigs.

In this study it has been demonstrated that ELISA using soluble proteins obtained from homologous *H parasuis* strains as coated antigens can be used for the investigation of antibody titers in young pigs after two vaccinations. This serologic study can also be used for the evaluation of the antigenicity of bacterial isolates used in *H parasuis* vaccines.

REFERENCES

1.Kielstein, P, and Rassbach, A. 1991. Serologische Typisierung und Nachweis immunogener Kreuzreaktionen von *Haemophilus parasuis* (Glassersche Krankheit). Mh Vet-Med 46:586–589.

2.Miniats, O.P, Smart, N.L., and Ewert, E. 1991. Vaccination of gnotobiotic primary specifc pathogen-free pigs against *Haemophilus parasuis.* Can J Vet Res 55:33–36.

3.Miniats, O.P, Smart, N.L., and Rosendal, S. 1991. Cross protection among *Haemophilus parasuis* strains in immunized gnotobiotic pigs. Can J Vet Res 55:37–41.

4.Rapp-Gabrielson, VJ., and Gabrielson, D.A. 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. Am J Vet Res 53:659–664.

5.Tadjine, M., Mittal, K.R, Bourdon, S. and Gottschalk, M. 2004. Production and characterization of murine monoclonal antibodies against *Haemophilus parasuis* and study of their protective role in mice. Microbiology 150:3935–3945.

INTRASPECIES DIFFERENTIATION OF *MYCOPLASMA HYOPNEUMONIAE* FIELD STRAINS ISOLATED IN THE UNITED STATES

BOH Chang Lin, DVM, MS, PhD MVP Laboratories, Inc., Ralston Nebraska

REZUMAT

În perioada 1998 şi 1999, 51 tulpini de *Mycoplasma hyopneumoniae* izolate de la efectivele de porci situate în principalele state producătoare de carne de porc din Statele Unite au fost utilizate pentru un studiu privind diferențiere intraspecii. Rezultatele analizelor privind profilul proteinelor totale totalul de proteine profil, glicoproteinei şi diferențele de dimensiune existente prin amplificarea PCR produse de p97 a genei adezină în regiunea repetitivă R1 indică faptul că există o variație intraspecii în cadrul populației naturale de *M. hyopneumoniae* izolată în Statele Unite. Amplificare PCR a regiunii R1 de la şapte tulpini de vaccin comercial produce, de asemenea, diferite dimensiuni ale ampliconilor ADN.

Amplificarea PCR a regiunii repetitive R1 a p97 gena adezină din ADN-ul cromozomal al tulpinilor de *M. hyopneumoniae* va da o dimensiune particulară a fiecărui amplicon ADN de la fiecare tulpină, care poate fi folosit ca unul din criteriile de diferențiere intraspecii. În cazul în care ampliconii ADN au dimensiuni similare la diferitele forme izolate, studiu suplimentar al profilului de proteine totale şi glicoproteină pot ajuta la diferențierea formelor izolate.

Cuvinte cheie: porc, *Mycoplasma hyopneumoniae,* ADN

ABSTRACT

During 1998 and 1999, 51 field strains of *Mycoplasma hyopneumoniae* isolated from pig herds located in the major pork producing states in the United States were used for an intraspecies differentiation study. Results from the analysis of total protein profile, glycoprotein profile, and size differences in the amplified PCR product of p97 adhesin gene Rl repeat region indicate that there exists an intraspecies variation among the natural population of *M. hyopneumoniae* isolated in the United States. PCR amplification of the Rl region gene from seven commercial vaccine strains also produces different sizes of DNA amplicons.

PCR amplification of the Rl repeat region of p97 adhesin gene from the chromosomal DNA of *M. hyopneumoniae* field strains will give a particular size of DNA amplicon from each strain, which may be used as one of the criteria for making intraspecies differentiation. In the case of having similar size DNA amplicons among different isolates, a further study of the total protein and glycoprotein profiles may help differentiate the isolates.

Wordkey: pig, *Mycoplasma hyopneumoniae*, DNA

INTRODUCTION

Despite the introduction of commercial *Mycoplasma hyopneumoniae* vaccines into the market many years ago, mycoplasmal pneumonia in swine is still a significant threat to the worldwide pig industry. *M. hyopneumoniae* alone can cause an important chronic respiratory disease called "swine enzootic pneumonia" (SEP). In cases of mixed infection with other respiratory pathogens, *M. hyopneumoniae* produces more severe pneumonia than that after a single infection with either pathogen alone (1). For this reason, vaccination against *M. hyopneumoniae* is becoming more common in the United States for the control and prevention of swine respiratory diseases.

A previous report indicated that some commercial *M. hyopneumoniae* vaccines can provide protection for pigs against experimental *M. hyopneumoniae* challenge, but

cannot completely eliminate pneumonia or significantly reduce the colonization of the microorganism (13). The reasons are not known. However, a lack of full understanding of the virulence mechanism that is used by *M. hyopneumoniae* to cause disease in pigs could be a major factor. With the advances in molecular biology during the past few years, it has been found that the genome of *M. hyopneumoniae* encodes several immunogenic proteins, including a cytosolic protein (p36), membrane proteins (p46, p65, p74), and an adhesin (p97). Although the biological functions of these proteins in the pathogenesis of SEP have not *yet* been determined, some recent studies indicate that a p36 has lactate dehydrogenase activity (2,4), a p74 membrane protein can elicit neutralizing antibodies (1), and an antigenic variation of adhesin may be a pathogenic mechanism utilized by *M. hyopneumoniae* to evade the porcine immune system (15).

The aim of this study is to make an inquiry into the genetic diversity of the adhesin gene of the field strains of *M. hyopneumoniae* isolated in the United States. The protein profiles of some of riiese field strains will also be examined using electrophoresis, silver stain, and an Immun-Blot® kit (BioRad Laboratories, Hercules, CA).

MATERIALS AND METHODS

Bacterial strains

The reference strains used in this study were the ATCC 25934 strain of *M. hyopneumoniae,* ATCC 27717 strain of *M. hyorhinis,* ATCC 27716 strain of *M.flocculare,* and ATCC 27720 strain of *M. hyosynoviae* obtained from the American Type Culture Collection (Rockville, MD). Some 51 field strains of *M. hyopneumoniae* used in this study are listed in Table 1. These were isolated from porcine lung tissues sent to the Diagnostic Laboratory at MVP Laboratories, Inc., from hog farms in the United States during 1998 and 1999- *M. hyopneumoniae* was isolated and grown in modified Friis medium as described earlier (3). In addition, seven *M. hyopneumoniae*'vaccines that could be purchased over the counter in the United States were also used for comparison.

DNA extraction

All field strains were identified by polymerase chain reaction (PCR) assays as described (8). Briefly, mycoplasma species is grown in modified Friis media for an appropriate rime to ensure purity and quantity of test microorganism before beginning the PCR assay. One milliliter of the culture broth is centrifuged at 14,000 x g for three minutes and the pellet is resuspended in 250 microliters of sterile PBS (pH 7.2) in a screw capped tube. The tube is incubated in boiling water for 10 minutes and then stored at —20° C for ten minutes. The tube is then centrifuged at 14,000 x g for three minutes and the supernatant is removed and used as the DNA template.

PCR assay

The oligonucleotide primers that were used to amplify the species-specific DNA sequences for porcine mycoplasmas are listed in Table 2. The PCR reaction mix for one test sample contains 5 µl of 10X PCR buffer, 2 µl of dNTP (each deoxynucleoside triphosphate at a concentration of 2.5mM), 0.25 µl each of forward and reverse primers (each at a concentration of 40 µM), 4 µl of 25 mM magnesium chloride, 0.25 µl Taq DNA polymerase at a concentration of 5U/µl, 33.25 µl of deionized water and 5 µl of DNA template. The PCR conditions are as follows: denaturation of the DNA at 94° C for five minutes, followed by 40 cycles (94° C for 30 seconds, 55° C for 30 seconds, and 72° C for 60 seconds), and a final 5 minutes at 72° C. Aliquots of 18 p.1 of the amplified products are analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and recorded using UV transillumination and Polaroid® film.

The oligonucleotide primers that were used to amplify the Rl repeat region of p97 adhesin gene are listed in Table 3. The PCR is performed in a 50 µl reaction mixture that contains 10 µl of DNA template, 5 µl of 10X PCR buffer, 2 µl of dNTP (2.5 mM each), 0.625 µl each of forward and reverse primers (each at a concentration of 40 µM), 4 µl of 25 mM magnesium chloride, 0.4 µl of Taq DNA polymerase at a concentration of 5 U/ µl, and 27.35 µl of deionized water. The PCR conditions are as follows: One cycle at 94° C for five minutes, followed by 35 cycles (94° C for 60 seconds, 55° C for 90 seconds, and 72° C for 60 seconds), and a final 5 minutes at 72° C. Amplified products are analyzed as described above.

SDS-PAGE and detection of total protein and glycoprotein

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is performed by the method of Laemmli (7) with a 10% separating gel and a 4% stacking gel. The separated proteins are visualized by staining the gels with a silver stain kit (BioRad, Hercules, CA). An Immun-Blot® kit (BioRad, Hercules, CA) is used to identify the glycosylated proteins from the separated mycoplasma proteins. In brief, mycoplasma proteins from each field strain are separated by SDS-PAGE and electrophoretic transfer of proteins from gel to nitrocellulose membranesis performed as described by Towbin et al (14). The detection of glycosylated proteins is based on the periodate oxidation of carbohydrate groups followed by biotinylation and a final detection of biotinylated glycoproteins with a streptavidin-alkaline phosphatase and NBT/BCIP detection system as described by the manufacturer. A scanner (HP ScanJet 6300C) and the UN-SCAN-IT gel software (Silk Scientific, Inc., Orem, Utah) are used to analyze the gel images.

RESULTS AND DISCUSSION

The 51 field strains of *M. hyopneumoniae* that were isolated from porcine lung tissues and identified by species-specific PCR assays are summarized in Table 1. Most of the field strains were isolated from lung tissues that showed typical SEP lesions, but about 12% of the culture-positive lungs did not have SEP lesions. About 55% of the field strains were isolated from lung tissues that had secondary infections with other respiratory pathogens, such as *Streptococcus suis, Pasteurella multocida, Actinobacillus pleuropneumoniae, Arcanobacteriumpyogenes, Haemophilus parasuis, Bordetella bronchiseptica, Salmonella cholerae-suis* and *Mycoplasma hyorhinis.*

Table 4 shows the results of the PCR amplification of Rl region of p97 adhesin gene from the chromosomal DNA of 51 field strains and an ATCC type strain of *M. hyopneumoniae.* The size of the Rl region varies from 0 bp to 308 bp. Most of them are between 230 bp and 280 bp. Table 5 shows the size variation of the p97 adhesin gene Rl region among seven commercial vaccine strains. The size of the Rl region varies from 0 bp to 337 bp; most are above 300 bp.

Table 6 show the total protein profiles of seven *M. hyopneumoniae* field strains. Table 7 show the glycoprotein profiles of five field strains.

Vaccination of pigs against *M. hyopneumoniae* with commercial vaccines does not always prevent colonization or protect pigs sufficiently against respiratory disease. The reason is not fully known. However, some recent studies have indicated that a 97 kD membrane protein could be one of the most important antigens. Other membrane proteins and p97, as well as a few accessory factors in a coordinated fashion (5, 6,15), could mediate the adherence of *M. hyopneumoniae* to the cilium membrane of pig trachea epithelial cells. It was further reported that the cilium binding site of *M. hyopneumoniae* is located in the AAKPV(E) repeat sequence of the p97 membrane protein, which is referred to as Rl repeat region (5). It was reported that the convalescent-phase swine sera also recognize the Rl repeat structure (9).

PCR amplification of the Rl region gene from 51 field strains and seven vaccine strains produce different sizes of DNA amplicons. The relationship between the size of Rl repeat regions and the cilium adherence activities was not fully known. But, a recent report also indicated that three repeat units (15 amino acids) are needed for generating a proper p97 epitope (9). Therefore, it can be expected that any change in the Rl region gene may prevent the binding of antibodies that are specific for the original epitope. Mycoplasma species may make use of this size variation strategy to evade the humoral immune response of the infected hogs (11). Further study is needed to establish this relationship.

It has been reported that the specific functions of glycoproteins in prokaryotes include maintenance of cell shape, protein stability, protection against proteolysis, and adherence to the target structure of the host cells (10). Figures 4 and 5 also indicate that there exist some differences in the total protein and glycoprotein profiles among field strains of *M. hyopneumoniae.* The degree of glycosylation in a few particular proteins in each strain is also different.

It was also found that some immunogenic proteins, such as p97, p74, and p46, are well glycosylated in MVP857 and MVP843 strains, and not well glycosylated in MVP754 and MVP878 strains.

Collectively, the findings in this study provide evidence of a genetic variation in natural populations of *M. hyopneumoniae* in the U.S. The variation of surface proteins among *M. hyopneumoniae* field strains might indicate an increase of antigenic diversity in this species, and such diversity in immunogenic surface proteins among field strains might play a role in the inconsistent efficacy of vaccination in pigs.

Table 1

M.hyopneumoniae field strains isolated at MVP Laboratories in 1998 and 1999

M. hyopneumoniae strain	State of Origin	Typical SEP Lesion	Other Bacterium Isolated
MVP701	IL	YES	YES
MVP745	NC	YES	YES
MVP754	NC	YES	YES
MVP757	IL	YES	NO
MVP761	NC	YES	YES
MVP809	NC	YES	YES
MVP813	IA	YES	NO
MVP817	ND	YES	YES
MVP818	IL	YES	NO
MVP822	OH	YES	NO
MVP843	OH	YES	YES
MVP849	KS	YES	YES
MVP857	NE	NO	YES
MVP859	NC	YES	YES
MVP863	ND	YES	YES
MVP868	IL	YES	YES
MVP878	IN	YES	YES
MVP904	NC	YES	NO
MVP906	IL	YES	NO
MVP908	IL	YES	NO
MVP910	IL	NO	NO
MVP915	OH	NO	NO
MVP916	MO	YES	NO
MVP919	IL	YES	NO
MVP927	NC	NO	YES
MVP930	OH	YES	YES
MVP937	NC	YES	NO

MVP940	NC	YES	YES
MVP945	NC	NO	YES
MVP946	IL	YES	NO
MVP947	NC	YES	NO
MVP948	IL	YES	NO
MVP949	ND	YES	NO
MVP951	NC	YES	NO
MVP953	IL	YES	NO
MVP954	Ml	NO	YES
MVP960	IL	YES	YES
MVP966	OH	YES	YES
MVP971	CA	YES	YES
MVP973	IL	YES	NO
MVP983	NE	YES	YES
MVP996	IL	YES	NO
MVP1014	MN	YES	YES
MVP1015	NE	YES	YES
MVP 1026	IL	YES	YES
MVP 1029	NC	YES	YES
MVP 1051	IL	YES	NO
MVP 1065	SC	YES	YES
MVP 1074	OH	YES	NO
MVP 1094	IL	YES	NO
MVP 1470	KS	YES	YES

Table 2.

Oligonucleotide primers used for porcine mycoplasma PCR assays

Organism	Primer
Mycoplasma hyopneumoniae	MHPNF 5-GAG CCT TCA AGC TTC ACC AAG-3' L 234-256 MHPNR 5'-TGT GTT AGT GAC TTT TGC CAC C-3' L 889-867
Mycoplasma hyorhinis	MHRHF 5'-GAA CGG GAT GTA GCA ATA CATTC-3'L74-117 MHRHR 5'-AGC GGA CTG AAG TTG AGC TTC AG-3' L678-646
Mycoplasma flocculare	MFLF 5'-ATT AGG TAG GGA ATG ATC TAA TC-3'L 490-512 MFLR 5'-GCT GCG CTA GTG ACT TCT G-3' L 891-872
Mycoplasma hyosynoviae	MHSYF 5'-CAGTTG AGG AAA TGC AAC TGAAC-3'L491-513 MHSYR 5'-CGT CAG TGA TTG GCC ACC G-3' L 887-86

Table 3

Oligonucleotide primers used for amplifying R1 repeat region of p97adhesin gene

TH120 5'-AAG GTA AAA GAG AAG AAG TAG-3' TH121 5'-TTG TAA GTG AAA AGC CAG TAT-3'

Table 4

Analysis of the size of the p97 adhesin gene Rl region of *Mycoplasma hyopneumoniae* field strains isolated in the U.S.

M. hyopneumoniae strain	Size of R1 region Gene	*M. hyopneumoniae* Strain	Size of R1 gene
MVP701	264 bp	MVP937	264 bp
MVP745	264 bp	MVP940	301 bp
MVP754	264 bp	MVP945	264 bp
MVP757	264 bp	MVP946	0 bp
MVP761	285 bp	MVP947	280 bp
MVP809	255 bp	MVP948	280 bp
MVP813	264 bp	MVP949	280 bp
MVP817	280 bp	MVP951	275 bp
MVP818	264 bp	MVP953	280 bp
MVP822	280 bp	MVP954	230 bp
MVP843	269 bp	MVP960	264 bp
MVP849	250 bp	MVP966	280 bp
MVP857	275 bp	MVP971	275 bp
MVP859	275 bp	MVP973	264 bp
MVP863	280 bp	MVP983	275 bp
MVP868	264 bp	MVP996	301 bp
MVP878	250 bp	MVP1014	264 bp
MVP904	308 bp	MVP1015	275 bp
MVP906	264 bp	MVP 1026 .	285 bp
MVP908	281 bp	MVP 1029	275 bp
MVP910	264 bp	MVP1051	264 bp
MVP915	269 bp	MVP1065	275 bp
MVP916	264 bp	MVP1074	275 bp
MVP919	264 bp	MVP 1094	264 bp
MVP927	255 bp	MVP 1470	0 bp
MVP930	280 bp	ATCC25934	254 bp

Table 5

Analysis of the size of the p97 adhesin gene R1 region of seven *Mycoplasma hyopneumoniae* strains isolated from seven commercial vaccines in the U.S.

Commercial Vaccine	Size of R1 Region Gene
A	329 bp
B	306 bp
C	317 bp
D	321 bp
E	337 bp
F	0 bp
G	325 bp

Table 6

Total protein profiles of seven *M. hyopneumoniae* field strains

MVP 849	MVP 754	MVP 904	MVP 843	MVP 910	MVP 857	MVP 996
140 kD*		140 kD	140 kD	140 kD	140 kD	
-------		130 kD	130 kD	-------	130 kD	
-------		-------	-------	-------	-------	
98 kD		100 kD	99 kD	99 kD	99 kD	
90 kD		-------	90 kD	93 kD	92 kD	
74 kD		76 kD	78 kD	74 kD	77 kD	
65 kD		65 kD	65 kD	65 kD	65 kD	
-------		-------	61 kD	-------	-------	
-------		-------	-------	-------	-------	
-------		48 kD	-------	-------	-------	
-------		-------	46 kD	46 kD	46 kD	
-------		-------	-------	-------	-------	
-------		-------	36 kD	36 kD	36 kD	
-------		-------	30 kD	30 kD	30 kD	
-------		27 kD	27 kD	27 kD	27 kD	

*Each number stands for the size of a protein in kilo daltons

Table 7

The glycoprotein profile of five *M. hyopneumoniae* field strains shown in Fig. 5

MVP 843	MVP 904	MVP 857	MVP 754	MVP 878
140 kD*	140 kD	140 kD		
130 kD		130 kD		
99 kD	100 kD	99 kD	99 kD	97 kD
90 kD		92 kD	93 kD	90 kD
78 kD	76 kD	77 kD		
46 kD	48 kD	46 kD	45 kD	45 kD

*Each numberstandsforthesizeofa glycoprotein in kilo daltons.

CONCLUSION

PCR amplification of the Rl region of p97 adhesin gene from the chromosomal DNA of *Mycoplasma hyopneumoniae* field strains will give a particular size of DNA amplicon for each strain, which may be used as one of the criteria for making intraspecies differentiation.

In cases of similar size DNA amplicons among different isolates, a further study of the total protein profile and glycoprotein profile may differentiate the isolates.

REFERENCES

1. Brooks, E. and D. Faulds. 1989. The Mycoplasma hyopneumoniae 74.5 kD Antigen Elicits Neutralizing Antibodies and Shares Sequence Similarity with Heat-Shock Proteins. Vaccines 89: 265-269.
2. Frey, J., A. Haldimann, M. Kobisch, and J. Nicolet. 1994. Immune Response against the L-lactate Dehydrogenase of *Mycoplasma hyopneumoniae* in Enzootic Pneumonia of Swine. Microb. Pathog. 17: 313-322.

3. Friis, N.F., 1975. Some Recommendations Concerning Primary Isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculate.* Nord. Vet. Med. 27: 337-339.
4. HaJdimann, A., J. Nicolet, and Frey, J. 1993. DNA Sequence Determination and Biochemical Analysis of the Immunogenic Protein p36, the Lactate Dehydrogenase (LDH) of *Mycoplasma hyopneumoniae.* J. Gen. Microbiol. 139: 317-323.
5. Hsu, T. and EC. Minion. 1998. Identification of the Cilium Binding Epitope of the *Mycoplasma hyopneumoniae* p97 Adhesin. Infect, tmmun. 66: 4762-4766.
6. Krause, D.C. 1996. *Mycoplasma hyopneumoniae* Cytoadherence: Unraveling the Tie That Binds. Mol. Microbiol. 20: 247-253.
7. Laemmli, U.K., 1970. Cleavage of Structural Proteins During die Assembly of die Head of Bacteriophage T4. Nature (ILondon) 227: 680-681
8. Lauerman, L.H., 1998. Mycoplasma PCR Assays. R 41-48. In: Lloyd H. Lauderman (ed.), Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases. Life Technologies and American Association of Veterinary Laboratory Diagnosticians.
9. Minion, EC, C. Adams, and T. Hsu. 2000. Rl Region of p97 Mediates Adherence of *Mycoplasma hyopneumoniae* to Swine Cilia. Infect. Immun. 68: 3056-3'060.
10. Moens, S. and J. Vanderllevden. 1997. Glycoproteins in Prokarvotes. Arch. Microbiol. 168: l69-175.
11. Neyrolles, O., I. Chambaud, S. Ferris, M. Provost, T. Sasak., L. Montagnier, and A. Blanchard. 1999. Phase Variations of the *Mycoplasma penetrans* Major Surface Lipoprotein Increases Antigenic Diversity. Infect. Immun. 67: 1569-1578.
12. Ross, R.F. Mycoplasmal Diseases, p 537-551. In A.D. Leman, B. Straw, W. Mengeling, S. D'Allaire, and D. Taylor (ed.) Diseases of Swine, 7 ed. 1992. Iowa State University Press, Ames.
13. Thacker, E.L., B. J. Thacker, T.B. Boettcher,, and H. Jayappa. 1998. Comparison of Antibody Production, Lymphocyte Stimulation, and Production Induced by Four Commercial *Mycoplasma hyopneumoniae* Bacterins. Swine Health and Production. 6: 107-112.
14. Towbin, H., T. Staehelin, and]. Gordon. 1979. Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. Proc. Nad. Acad. Sci USA *76:* 4350 - 4354.
15. Zhang, Q., T. Young, and R.F. Ross. 1995. Identification and Characterization of a *Mycoplasma hyopneumoniae* Adhesin. Infect. Immun. 63: 1013-1019.

DIAGNOSIS AND TREATMENT OF CHRONIC FATIGUE SYNDROME (C.F.S.) AT DOGS

ANDRONIE V., Ioana ANDRONIE

Faculty of Veterinary Medicine University „Spiru Haret”

viorelandronie@innovet.ro

REZUMAT

Diagnosticul si tratamentul sindromului de oboseala canin(SOC). A fost diagnosticate trei cazuri de sindrom canin de oboseala cronica (SOC) dupa criteriile curente din medicina umana. Simptomele de durere si oboseala au fost asociate cu piodermita, prezenta organismelor tip micrococci din sange si recuperarea a 2 tulpini de Staphylococcus xilosus rezistente la vancomicin dintr-o pustula si din apa de baut. A fost administrat intravenos Tiacetarsamide sodium in doza mica (0.1 mg/Kg/zi) timp de 3 zile tuturor cainilor. Parametrii clinici si hematologici din zilele 4, 7 si 10 de dupa terapie au confirmat remisia completa a sindromului care fusese prezent timp de 2 ani si a fost tratat in aceasta perioada cu diversi agenti chimioteapeutici. Studiul va lua in discutie posibilul rol al stafilococilor coagulazo negativi in etiologia SOC si actiunea antimicrobiana a arsenicelor.

Cuvinte cheie: Sindromul de Oboseala Cronica, caine, zoonoza, tiacetarsamida

ABSTRACT

A cluster of canine Chronic Fatigue Syndrome (CFS) was diagnosed according to current criteria accepted in human medicine. The fatigue and pain symptoms were associated with pyoderma, presence of micrococci-like organisms in the blood and the recovery of two vancomycin-resistant Staphylococcus xilosus strains, from a pustule and from drinking water. Thiacetarsamide sodium, was administered intravenously at low dosage (0.1 mg/Kg/day) for three days in all dogs. Clinical and hematological parameters at days 4, 7 and 10 after therapy confirmed complete remission from the syndrome, which had lasted for more than 2 years and had been treated previously with severalchemotherapeutics agents. The possible role of coagulase-negative staphylococci in the aetiology of CFS and the antimicrobial action of arsenicals are discussed.
Key-words: Chronic Fatigue Syndrome, thiacetarsamide, dog, zoonosis.

INTRODUCTION

Chronic Fatigue Syndrome (CFS), as originally defined by the American Centers for Disease Control and as recently redefined (Fukuda et al., 1994) is a human illness in which patients experience severe, debilitating fatigue for more than 6 months. Chronic Fatigue Syndrome (NIAID,1996) is defined by the presence of the following criteria:

• unexplained, persistent or relapsing chronic fatigue;

• the concurrent occurence of 4 or more of the following symptoms, all of which must have persisted or recurred during 6 or more consecutive months of illness and must not have predated the fatigue:

a) impairement in memory or concentration,

b) sore throat,

c) tender cervical or axillary lymph nodes,

d) muscle pain or multijoint pain without swelling or redness,
e) headaches,
f) unrefreshing sleep, and
g) postexertional malaise lasting more than 24 hours.

Most CFS cases are sporadic but, occasionally, close contacts, including family members, become ill with CFS at about the same time (Bell, 1994). Also, cluster of CFS-like illnesses have been reported during the past 60 years in various families and communities (NIAID, 1996).

Initial epidemiologic studies failed to identify a viral aetiology (Johnson, 1996) and recent advances seem to indicate a bacterial ethiology (Butt et al., 1998; Dunstan et al., 1999). During the past decade, substantial evidence has been generated to support the existance of a CFS-like illness among animals (Ricketts et al., 1992; Glass, 1998). Although Chronic Fatigue Syndrome has never been clinically described in dogs, prevalence surveys indicate that a remarkable number (97%) of patients with CFS had animal contact, particularly with dogs and cats, and that 75% of these pets appeared sick, with signs and symptoms which mimicked CFS in humans, strongly suggesting a zoonotic transmission (Glass,1998). A recent report describe CFS in horses: as with the disease in humans, Equine Fatigue Syndrome is associated with long-term exhaustion , difficult treatment and immune dysfunction (Ricketts et al., 1992).

Increased cariage of coagulase-negative Staphylococci (McGregor et al, 1998) has been found in humans with chronic pain/fatigue symptoms. The Staphylococci recovered from 89% of these patients produced membrane-damaging toxins, delta- and /or “horse”- haemolysins, whereas
control subjects did not (Butt et al, 1998).

Recently, four horses diagnosed with CFS and resistant to standard therapies, were all found to carry unusual micrococci-like organisms in the blood (Tarello, 2000). Their CFS-resembling
lethargy had a complete remission after a treatment with thiacetarsamide sodium (0.1mg/Kg/day
IV) for 2-3 days. The clinical response was associated with the disappearance of micrococci from
follow-up blood samples.

Little is known about the syndrome in animals and therefore, in this study, dogs fulfilling the current human criteria for CFS and resistant to extensive prior therapies were checked for
similar blood abnormalities and therapeutical responses. The first aim of this paper is to report the
clinical history, symptoms, microbiological findings and method of treatment in a cluster of dogs
diagnosed with CFS. Information about environmental risk factors are given as partial explanation
and importance of the presence of micrococci-like organisms in the blood is discussed.

MATERIALS AND METHODS

The study comprised three related dogs with similar symptoms dominated by unexplained persistent fatigue and 5 other symptoms (muscle and multi-joint pain, sore throat, somnolence, postexertional malaise and lymphadenopathy) lasting more then 6 months. All dogs had relapsed after previous standard therapies that included several antibiotics (doxycycline, enrofloxacin, ceftazidime, amoxacillin + clavulanic acid), imidocarb, antihelmintics, vitamins and glucocorticoids. These animals were visited in their kennel to perform clinical and environmental examination and to collect blood

samples for haematologic and serologic analysis. Drinking water for bacteriological examination was collected too.

During additional visits, further clinical examinations were performed and blood samples were collected from the dogs at day 4, 7 and 10 after therapy.

Complete blood counts (CBC) were performed on samples collected at the first visit (day 0) and at days 4, 7 and 10 after therapy. Two fresh blood smears, stained with May-Grunwald-Giemsa
and Wright techniques, were prepared each time . A Knott test for microfilariae was also performed at each visit.

Serum collected at day 0 was tested for circulating antigens of Dirofilaria immitis

Serum values of total protein (TP), albumin and globulin, serum CK and LDH were evaluated at days 0, 4, 7 and 10 after therapy. These values are summarized in table I and II.

Two swabs were taken, from drinking water and from an interdigital pustule of dog,immediately after the lesion was opened with a sterile needle.

The culture established from the drinking water were subcultured onto three different agar plates for 24 h at 37oC. Representative colonies were transplanted onto two plates and submitted for identification and antibiotic sensitivity testing. Ten microliters of the culture established from the pustule were subcultured onto two separate agar plates for identification + antibiotic sensitivity testing. All samples were subject to Gram stain and Catalase test.

Thiacetarsamide sodium was administered intravenously at 0.1 mg/Kg/day for 3 days. No other medication was given.

RESULTS AND DISCUSSIONS

The 3 dogs comprised in the study were 2 adult female and 1 juvenile male less than 5 months old, all living together in a cement-floored kennel subdivided into 2 runs, separated by chain-link fencing and covered with a tin roof. The adult females had been vaccinated and dewormed every year. The kennel has been built inside the area of a mushroom cultivation farm.

All dogs had access to water coming from a well, situated near an artificial lake where the waters from the mushroom cultivation were discharged.

A sterile swab taken from the kennel’s water and immediately incubated into brain-heart infusion for 24 h at 37oC generated bacterial growth which was subcultured (10 microliters) onto three specific agar grounds for further 24 h. at 37oC. Gram positive and catalase positive cocci were identified in all three plates. Representative colonies from the plate revealed them to be (99.9%) Staphylococcus xilosus . This strain produced acid from mannitol, so it was considered pathogen. The bacteria showed a partial sensitivity to gentamycin and were not sensitive to spiramycin, kanamycin, amoxacillin, chloramphenicol, doxycycline, sulpha-trimethoprim and vancomycin.

The first dog investigated was a 6-year old female, with chronic undefined illness lasting more than 2 years and resistant to extensive prior therapy. The symptoms were : episodic weakness, muscular pain, sore throat, exercise intolerance, periodic fever, weight loss, pyoderma, shedding and dull haircoat and lymphadenopathy. The dog had poor tolerance to moderate exertion and was reluctant to run for more than 1 minute.

Physical exam revealed tender and enlarged lymph nodes and poor body condition. Serology for Dirofilaria immitis and the Knott test for microfilariae was negative.

The CBC was unremarkable. Serum creatine kinase (CK= 188.8 IU/L) and lactate dehydrogenase (LDH= 477.1 IU/L) were elevated.

Material taken with a sterile swab from an interdigital pustule and immediately incubated in Brain-Heart infusion at 37oC for 24 h produced bacterial growth. These bacteria were then recognized as gram-positive and catalase-positive cocci after subculture , identified as of Staphylococcus xilosus (99.8%). This strain was also mannitol-ermenting. This strain was partially resistant to gentamycin, chloramphenicol, doxycyllin, sulpha-trimethoprim, amoxacillin and resistan to spyramicin, kanamycin and vancomycin .

Examination of fresh blood smears stained with May-Grunwals-Giemsa were negative for babesiosis and ehrlichiosis, but small, micrococci-like organisms, 0.3-0.5mm in diameter, were found attached to the external surface of red blood cells (RBCs) in quantity varying from 10 to 15 %. Thiacetarsamide sodium (0.1 ml/Kg/day) was given intravenously for three days.

In a few days the weakness decreased and interdigital pyoderma began to heal, the appetite improved leading to total weight gain and better locomotion.

A physical examination made at day 4 showed that general health status was improved. Creatinekinase activity was still high (CK=176.8 IU/L) and 2 fresh blood smears revealed a decreasing percentage of micrococci upon RBCs (2-5%).

The clinical response appeared satisfactory at day 7, when muscular pain and resistance to
physical activity had ceased and pyoderma pustules had completely disappeared. Biochemistry
examinations still revealed elevated activity of creatine kinase (CK= 291.8 IU/L). A reduced
percentage of RBCs (1-5%) was carrying micrococci and the globulin fraction was rising (3.61
gr/dl).

At day 10, blood smears were micrococci-negative, creatine kinase (CK = 33.4 IU/L), lactate-dehidrogenase (LDH= 156.3 IU/L) activities and hematocrit (PCV= 41.2%) were now within the reference ranges. Following complete clinical remission, no exercise resistance could be observed, lymph node size were decreasing and the haircoat was bright.

The second gog was a 7-year old, the sister of first dog, with a similar 2-year history of
chronic illness , characterized by muscular pain, lethargy, sore throat, severe interdigital
pyoderma, lameness, weight loss, lymphadenopathy, abundant scurf and shedding hairs and post-
exertional malaise lasting more than 24 hours.

Serological and Knott's testing proved negative for heartworm disease. There was a normocytic normochromic anaemia (PCV= 31%, MCV = 64.9 fl., MCH = 22.04 pg) and all other laboratory examinations gave results within normal limits, with the exception of increased CK (380 IU/L) and LDH (449 IU/L) activities at rest.Fresh blood smears showed 10% of RBCs with micrococci on their surface.

Treatment was performed as usual with thiacetarsamide sodium in low dosage (0.1/ml/Kg) for three days.At day 4 the PCV improved (35%), the CK reduced (75.4 IU/L) and decreased number of RBCs with micrococci were seen (5%). The dog was less reluctant to perform exercise and did not
showed muscular pain on palpation.At day 7, pyoderma and lameness had completely disappeared and smaller number of RBCs (2%) appeared parasitized by micrococci.

At day 10, the PCV was improved (36.5%) and the CK (41.0 IU/L) and LDH (211.5 IU/L)
activities were normal. A very low number of micrococci (0.5%) could be detected in fresh blood

smears . Physical examination revealed a complete recovery from weakness and exercise
intolerance, improved condition of the haircoat and moderate diminution of the peripheral lymph
nodes dimensions.

The last dog was a 3 month-old, affected since birth by lethargy, poor appetite and severe
pododermatitis at all feet. The dog always had difficulty rising and was unable to climb stairs or to
move faster than a clumsy walk. The puppy the smallest of the litter and was hand reared for the
first month.

Two similarly affected littermates had to be put asleep previously. Despite a normal hematocrit, high muscle enzyme activity (CK= 182.8 IU/L) and presence of micrococci on 5-8% of RBCs were findings similar to those of the mother and her sister. Arsenical treatment was done as usual for three days. In the following week a second course was required in order to achieve a complete remission from the pododermatitis. Fresh blood smears resulted negative for micrococci and the puppy was able to play vigorously with the other littermates.In the absence of a specific test, a diagnosis of Chronic Fatigue Syndrome (CFS) in human medicine is currently done by exclusion of other known fatigue-related diseases and by compliance with a clinical definition (Fukuda et al., 1994; NIAID, 1998; Dunstan et al.,1999).

In this report, the dogs referred as having CFS apparently matched the official definition of CFS in humans (NIAID, 1996) and the clinical picture of CFS in horses (Ricketts et al., 1992; Tarello, 2000).

During the previous 2 years, these dogs had progressively deteriorated despite treatment with several antimicrobials. The primary features of fatigue and pain, were accompanied by chronic
skin lesions and pyoderma, as occurs in 10-35% of human patients (Rebora & Drago, 1994). The canine cases here described shared haematological (anemia) and biochemical (high muscular enzymes at rest) abnormalities together with the unexpected presence of micrococci in blood smears.

These micrococci were similar to those previously observed in horses diagnosed with CFS (Tarello, 2000). No other typical blood canine parasite was detected . Micrococci in the blood were not found in smears made after thiacetarsamide sodium therapy, suggesting an underlying chronic bacterial infection. The isolation of a vancomycin-resistant mannitol-fermenting Staphilococcus xilosus strain from a pustule in association with CFS-related symptoms was similar to the association between coagulase-negative Staphylococcus spp. and chronic fatigue/chronic pain disorders in humans (Butt et al., 1998; Dunstan et al., 1999), and between pyoderma and CFS in horses with micrococci in the blood (Tarello, 2000).

In this study, an environmental aspect of interest is the living place of the subjects: a farm where edible mushrooms were artificially cultivated on beds of manure coming from intensive breedings of cows, pigs and poultry. The drinking water for the dogs was collected from a well near to the artificial lake in wich the cultivation waters were discharged every day. Manure used for such
cultivation may be a source of vancomycin-resistant enterococci (VRE).

The identification of a vancomycin-resistant Staphylococcus xilosus strain from the drinking water suggested acquisition from an environmental source and also a relationship with the clinical picture, because the CFS-related symptoms and pyoderma recovered following therapy and a
change in the water supply. An increased incidenc of pediatric CFS cases has been described in clusters of people drinking unpasteurized goat milk (Bell et al.,1991).

In my experience , the presence of such microrganisms is the only remarkable difference between fresh blood smears taken from healthy and chronically fatigued animals. This finding seem to confirm the report of a newly-identified human blood bacterium (HBB) which is claimed to be present in high number in the blood of persons who have CFS or Multiple Sclerosis (Lindner L. & McPhee K., 1999). These authors have no evidence that the bacterium can be completely eliminated using the standard FDA approved antibiotics and they affirm that under certain circumstances antibiotics can actually stimulate bacterial growth and make the patient worse. In the present study, the lack of responsiveness to previous standard therapeutic agents, suggested an antimicrobial-resistance of the underlying agents.

The therapeutic efficacy of arsenic on pyoderma is acknowledged in both human (Stone & Willis, 1968) and veterinary (Hutyra et al.,1949) medicine. Furthermore, The Merck Index (1976) lists several arsenical veterinary preparations as tonics, useful against general debility and various dermatological diseases. However, no relationship has ever been established with underlying chronic bacterial infections or with the presence of micrococci in the blood. Recently, a 50% of successful rehabilitation has been described in human patients with chronic fatigue syndrome treated with a staphylococcus toxoid vaccine (Anderson et al., 1998).

In this report, high serum CK (>100 IU/L) and LDH (>300) at rest, were detected at first examination but not following therapy, when the symptoms had disappeared. The laboratory results and the symptoms observed (weakness, muscular pain, lameness) seem to indicate a possible systemic myopathy. Interestingly, myopathy with atrophy of both types of the fibre and severe mitochondrial abnormalities have been described in goats artificially raised in conditions of dietetical arsenic deficiency (Schmidt et al.,. 1984) .

The size and shape of mitochondria reveal abnormalities in up to 75% of CFS patients (Behan et al.,1991) and in dogs with episodic weakness associated with myopathy and high muscle enzyme activities (Breitschwerdt et al., 1992). High CK values have been already observed in a subset of CFS patients , particularly in those with seizure, ataxia and sudden onset of symptoms (Preedy et al., 1993). Human sufferers have shown also high GOT values (Komaroff, 1988) in 25% and relevant LDH activity in 0.3% (Bates et al., 1995) of cases.

At present time it is difficult to undestand the peculiar mechanism of action of thiacetarsamide sodium, an organic trivalent arsenical drug, in these CFS canine cases. Arsenic, which is known to selectively bind to thiol or -SS- groups of proteins (Cobo & Castineira,1997) in particular concentrations with iron in solution have showed to inhibit bacterial growth in mixed culture of thermophilic bacteria, with As(III) reported to inhibit bacteria to a greater degree than AS(V) (Breed et al., 1996). Arsenical have been used as medicines for thousands of years, in particular against bacterial infections, syphilis, tubercolosis and scrofulosis (Kasten, 1996; The Merck Index, 1976) and have shown to have beneficial actions when fed in very small amount to
laboratory animals (Anke, 1986). Experiments with different species have provided circumstancial
evidence that arsenic is essential and that it may have a role in methionine metabolism (Uthus,
1994).

CONCLUSIONS

In summary, a multi-drug resistant cluster of canine chronic fatigue syndrome showed complete clinical and hematological remission 7 days after treatment with thiacetarsamide sodium, an

organic trivalent arsenical given intravenously in low dosages (0.1/ml/Kg/day) for 3 days.

Severe skin lesions were associated with CFS-related symptoms, presence of micrococci-like organisms in the blood, high muscle enzymes and the recovery of two vancomycin-resistant strains of Staphylococcus xilosus from drinking water and a lesion. Although serologic tests for CFS do not exist, it seems worthy to suggest that the presence of micrococci in the blood could be used as a diagnostic for this syndrome, because they apparently are the main hematological difference observed between healthy and chronically fatigued animals. The striking degree of activity of an arsenical drug in this cluster and in previous animal cases, seem to indicate a novel antimicrobial approach to CFS-like conditions in veterinary medicine.

REFERENCES

1.Anderson M., Bagby J.R., Dyrehag L. & Gottfries C. (1998). Effects of staphylococcus toxoid
vaccine on pain and fatigue in patients with fibromyalgia/chronic fatigue syndrome. European
Journal of Pain, 2, 133-42.

2.Anke M. (1986). Arsenic. In Trace Elements in Human and Animal Nutrition. Mertz W. (ed.)
Academic Press, pp.347-372. Orlando, FL.

3.Bates D.W., Buchwald D., Lee J., Kith P., Doolittle T., Rutherford C., Churchill W.H., Schur
P.H., Wener M.& Wybenga D. (1995). Clinical laboratory test findings in patients with chronic
fatigue syndrome. Archives of Internal Medicine, 155, 97-103.

4.Behan W.M.H., More I.A.R. & Behan P.O. (1991). Mitochondrial abnormalities in the post-viral
fatigue syndrome. Acta Neuropathologica , 83, 61-5.

5.Bell K.M., Cookfair D., Bell D.S., Reese P., & Cooper L. (1991). Risk factors associated with
chronic fatigue syndrome in a cluster of pediatric cases. Clinical Infectious Diseases, 13,(Suppl.
1), 32-8.

6.Bell D.S.(1994). The Doctorís Guide to Chronic Fatigue Syndrome. Addison-Wesley Publishing
Co., U.S.A

7.Cobo J.M. and Castineira M. (1997). Oxidative stress, mitochondrial respiration, and glycemic
control: clues from chronic supplementation with Cr3+ or As3+ to male Wistar rats. Nutrition,
13, 965-70.

8.Glass T. (1998). The human/animal interaction of Chronic Fatigue and Immune Dysfunction
Syndrome: a look at 127 patients and their 462 animals. Medical Professional with CFIDS
(MPWC) News , 3, 2.

9.Hutyra F., Marek J. & Manninger R. (1949). Patologia speciale e terapia degli animali domestici.
Pp. 645-646. Vallardi, Milano.

10.Levine P.H. (1998). What we know about chronic fatigue syndrome and its relevance to the

practicing physician. American Journal of Medicine, 105, 100-103.

RESEARCHES REGARDING THE HONEY-BEE *APIS MELLIFERA CARPATHICA* HEMOLYMPH ELECTROPHORESIS
CERCETĂRI PRIVIND ELECTROFOREZA HEMOLIMFEI LA SPECIA DE ALBINĂ MELIFERĂ *APIS MELLIFERA CARPATHICA*

CONDUR D., T. PETRUȚ, N. VELICU
Facultatea de Medicină Veterinară
Universitatea *Spiru Haret*
e-mail: ushmv_condur.dan@spiruharet.ro

REZUMAT

Hemolimfa reprezintă echivalentul sângelui la artropode şi marea majoritate a moluştelor, care au un sistem circulator deschis, fără o delimitare precisă între sânge şi lichidul interstiţial. Hemolimfa umple tot interiorul (hemocelul) corpului insectei şi înconjoară toate celulele. De asemenea, prezintă un rol important în apărarea imunitară a insectelor. De aceea, studiul nostru s-a bazat pe caracterizarea pro-teinelor din hemolimfa albinelor, putând forma baza pentru ulterioare investigaţii clinice legate de starea de sănătate sau de boală a familiilor de albine, cu răsfrângere asupra cantităţii şi calităţii mierii şi produselor apicole.

Recoltarea hemolimfei s-a efectuat de la albine provenite din familii sănătoase din punct de vedere clinic, în lunile iulie-august.

Experimentul s-a efectuat pe 6 familii de albine. S-au prelevat 10 probe individuale pentru fiecare familie de albine, folosindu-se probe de câte 3,5 µl de la fiecare albină lucrătoare. Din hemolimfa recoltată s-au efectuat determinări de fracţiuni proteice hemolimfatice cu ajutorul electroforezei orizontale, pe gel de agaroză, folosindu-se aparatul de electroforeză EP Line 1.1. Iniţial s-a folosit ca martor de migrare serul uman standardizat. Toate proteinele, la un pH al soluţiei tampon de migrare de 8,6, au migrat spre anod, demonstrând o încărcătură electronegativă.

Rezultatele au demonstrat o viteză de migrare mai mică a pro-teinelor hemolimfatice, care s-au încadrat între α- şi γ-globulinele standardului uman ca viteză de migrare. De asemenea, s-a observat o foarte mare constanţă a electroforegramelor individuale la albinele din aceeaşi familie.

Cuvinte cheie: *Apis mellifera, hemolimfă, electroforeză*

ABSTRACT

Hemolymph is the blood analogue used by all arthropods and most mollusks that have an open circulatory system, with no distinction between blood and interstitial fluid. The hemolymph fills all of the interior (the hemocoel) of the body and surrounds all cells. It also it plays a major role in immunologic defense of insects.

For this purpose, our research have been based on honey-bee hemolymph proteic characterization, in order to represent the base of further clinical investigation related to health or diseased status of honey-bee families, reflected on quantity and quality of honey and bee products.

Hemolymph ingathering was made from clinical healthy families, in July and August months. The experiment was done on six healthy honey-bee families. 10 individual probes were gathered from each family, 3,5 µl from each worker bee. From the gathered hemolymph were made proteic fractions detections using agarose electrophoresis, using the EP Line 1.1. electrophoretic device.

For the beggining, human standardised serum was used as a control for further migration timing. All proteic fractions, to a buffer pH of 8,6, migrated throw the anod, proving a electronegative charge. The results revealed a smaller hemolymph migration speed then the human control, between α- and γ-globulin fractions. It also can be mentioned a very good consistency of individual electroforegrams in the same family.

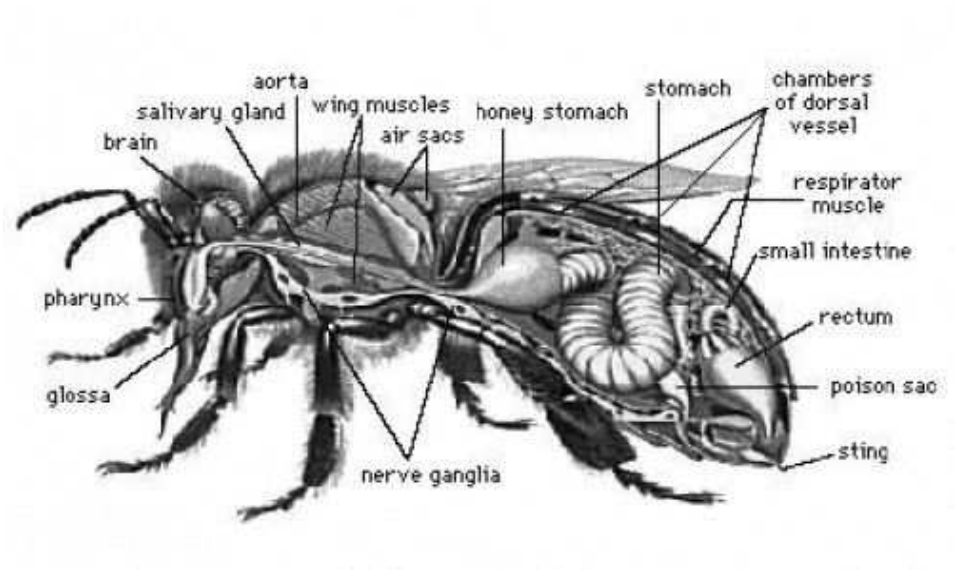
Keywords: Apis mellifera, hemolymph, electrophoresis

INTRODUCTION

The honeybee, *Apis mellifera*, is an invaluable helper for the agriculture. Its utility consists in honey production and for its role in pollination. Excepting some electronical microscopy studies, honeybees are largely unexplored at the molecular level.

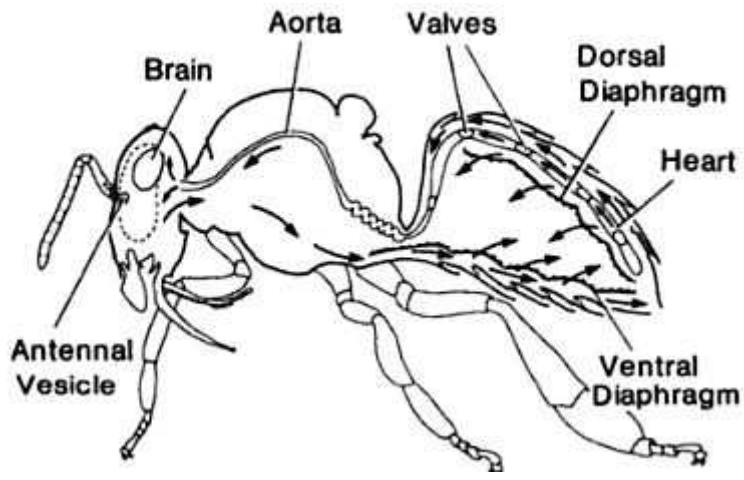
Like other social insects, honeybees can be divided into several categories: the queen (fertile female), workers (sterile females) and drones (males). Each category has different metabolic activity and each differs in its sensitivity to pathogens.

Anatomical structure lacks in fine structure knoledges, seventies being the last with massive insects structural researches (picture 1).



Picture no 1: Internal anatomy of the worker honey-bee (1)

Hemolymph is the blood analogue used by all arthropods and most mollusks that have an open circulatory system, with no distinction between blood and interstitial fluid. The hemolymph fills all of the interior (the hemocoel) of the body and surrounds all cells (picture 2). It also it plays a major role in immunologic defense of insects.



Picture no 2: Honey-bee circulatory system (1)

The aim of our study was to do the first steps in order to correlate the hemolymph proteic composition with some physiological and pathological issues (4). This is a part of an extensive research, involving biochemical, histological, and immunological studies, regarding to future use the hemolymph in the same way as human blood. This could be useful for health/disease status estimations of the complex beehive organism.

Our research have been based on based on firstly gross characterisation of worker honey-bee hemolymph proteic characterization (2,3), in order to represent the

base of further clinical investigation related to health or diseased status of honey-bee families, reflected on quantity and quality of honey and bee products.

MATERIALS AND METHODS

The apiary used for this study is destined to produce bio-chemical clean honey. Hemolymph ingathering was made from clinically healthy families, in July and August months. The experiment was done on six healthy honey-bee families.

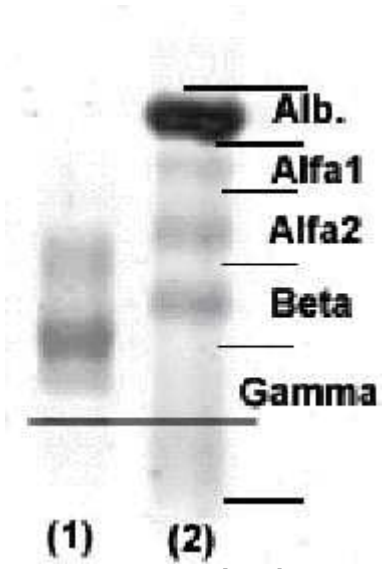
The bees were anesthesiated in cool air for 5 minuites, the torax was fixed in a device that only let the abdomen outside. After the beginning of insect releasing, a special curved glass needle was put between 2nd and 3rd abdominal rings, in the dorsal vessel, and, under bee abdominal movings, let to fill with hemolymph. 10 individual probes were gathered from each family, 3,5 µl from each worker bee.

Total protein was concluded by using 10 probes from each beehive. The method used was a refractometric method (a low resolution method – 0,2 g/dl), and the protein levels were between 4,6 g/dl and 5,4 g/dl.

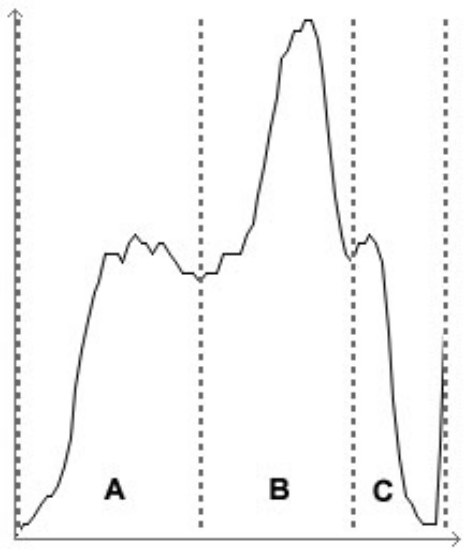
From the gathered hemolymph proteic fractions detections were made. The EP Line 1.1. electrophoretic device orizontal agarose electrophoresis was used. The parameters used were: tension 100V, time 30 minutes, and the intensity remained fixed to 37mA. For the beggining, human standardised serum was used as a control for further migration timing.

RESULTS AND DISCUSSIONS

All hemolymphatic proteic fractions, to a buffer pH of 8,6, migrated throw the anode, proving an electronegative charge. The results revealed a smaller hemolymph migration speed then the human control, between α- and γ-globulin fractions (picture 3). It also can be mentioned a very good consistency of individual electroforegrams in the same family.



Picture no 3: Hemolymph protein migration (1), set beside human standard serum (2)



Picture no 4: Worker honey-bee electrophoregram (A, B, and C fractions)

Three main fractions were established (noted by us with „A”, „B”, and „C”), theese fractions being constantly observed in all electrophoregrams (picture 4).

There was also observed a good omogenity between individuals in the same family. Standard deviation in protein A values had been established between 1,522

(family 5) and 2,600 (family 2). In B protein, standard deviation layed between 1,351 (family 5) and 3,222 (family 2). C protein had a standard deviation between 0,596 (family 5) and 1,710 (family 4) (Table 1, chart 1):

Table no 1

The average and standard deviations of the families (10 bees/family) involved in the experiment

	A		B		C	
	Average	St. deviation	Average	St. deviation	Average	St. deviation
Family 1	40,32	2,241	47,52	2,623	12,16	0,859
Family 2	41,43	2,600	45,69	3,222	12,88	0,959
Family 3	40,84	1,877	45,56	2,076	13,60	1,100
Family 4	41,72	2,452	46,14	3,194	12,15	1,710
Family 5	40,64	1,522	46,67	1,351	12,69	0,596
Family 6	41,57	2,360	45,54	1,986	12,89	1,008

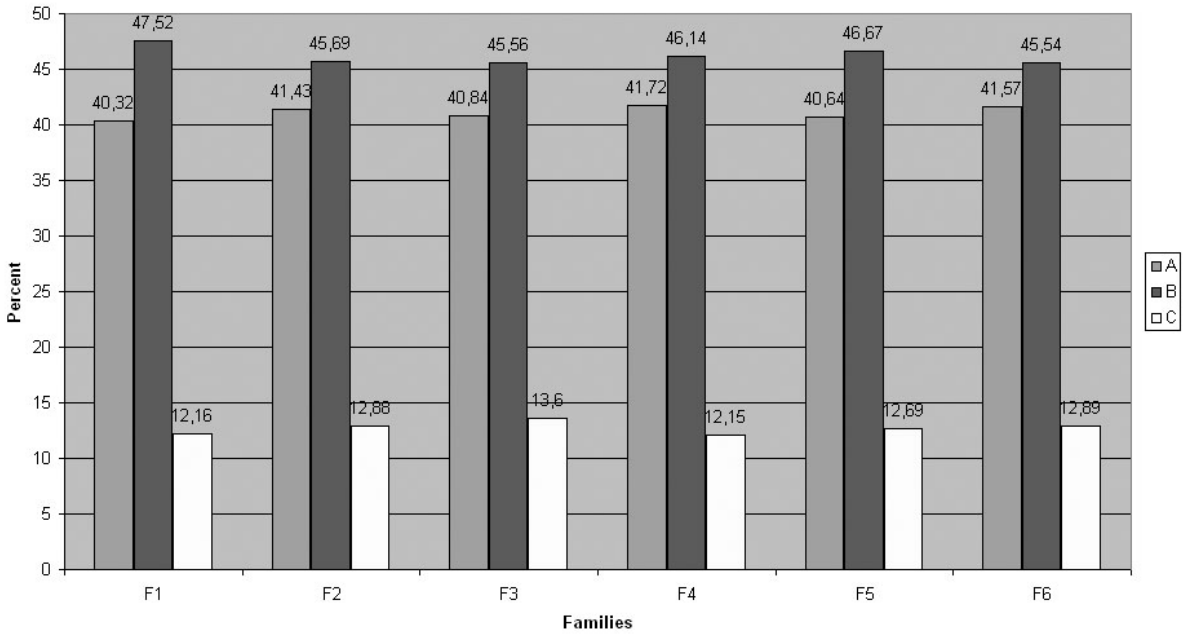


Chart no 1

The overall distribution of the 3 electrophoretic protein compounds of hemolymph was: compound A - around 47%, compound B – around 40%, compound C – around 13% (chart 2). The function of each compound is a target for our next researches.

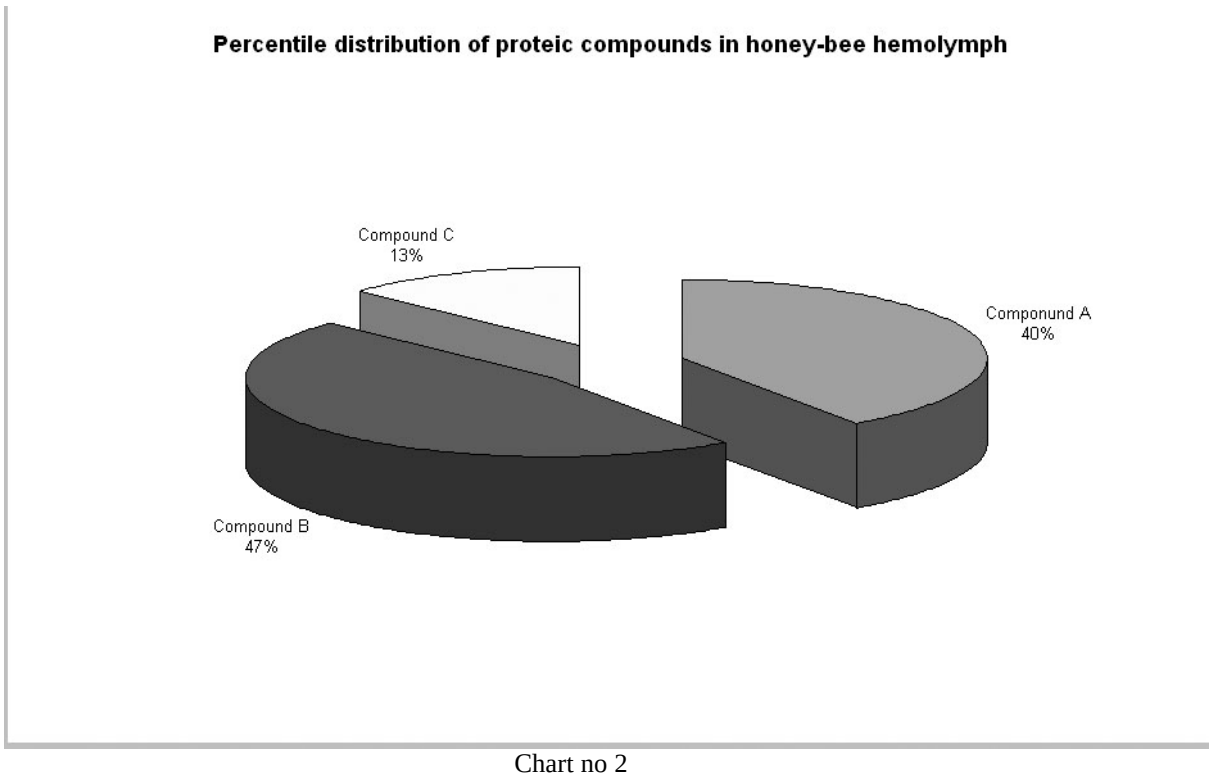


Chart no 2

CONCLUSIONS

1. All hemolymphatic proteins migrated to anode (+) pole, proving a negative charge to pH 8,6.

2. Migration speed of hemolymph protein in agarose gel is smaller then human seric proteins.

3. Hemolymph proteins speeds were distributed between α- and γ-globulins of human standard.

4. There is a good consistency of individual electrophoregrams in honey-bees in the same family, and also between related healthy families.

5. The best represented part of hemolymph proteins (about one half of them) were the ones with a medium migrating speed, noted by us with „B", followed by „A" proteins, and the fiewest were the „C" proteins, that migrated the shortest lenght.

6. For the future, we will try to follow two main directions:

6.1. An increase of electrophoretic precision and the number of the separated fractions.

6.2. Electrophoregrafic testing of genetic different families and from families with clear ethiological diagnostic of different kind of diseases.

REFERENCES

1. Dade, H. A., 1977, *Anatomy and Dissection of the Honeybee*. London: International *Bee* Research Association.

2. Kubicz, A., H. Galuska, 1971, *Polyacrylamide gel electrophoresis of proteins and the acid phosphatase Isoenzymes from haemolymph of honey bees, Apis mellifera.* L. Zool. Po., 21:51-57.

3. Weber, K., M. Osborn, 1969, *The reability of molecular weight determination by sulfate polyacrylamide gel electrophoresis.* J. Biol. Chem., 244: 4406-12.

4. Zakaria, M. E., 2007, *Factors Affecting On The Food Metabolism In Some Honey Bee Races*. Journal of Applied Sciences Research, 3(4): 311-316.

INCIDENCE AND THERAPY ASPECTS IN ACARIOSES IN DOGS AND CATS

INCIDENȚA ŞI ASPECTE TERAPEUTICE ÎN ACARIOZE LA CÂINI ŞI PISICI

COMAN Sofia, B. BĂCESCU
Faculty of Veterinary Medicine *Spiru Haret* University
ushmv_coman.sofia@spiruharet.ro

REZUMAT

Cercetările s-au efectuat pe un număr de 115 câini cu leziuni cutanate şi 46 pisici cu otite externe. Investigațiile de laborator s-au efectuat prin examen clinic, examen microscopic din raclatul cutanat şi a cerumenului prelevat din conductul auditiv extern. Combaterea acariozelor diagnosticate s-a efectuat cu produse acaricide sub formă de soluții, emulsii sau unguente aplicate zilnic sau la un interval de 2-3 zile timp de 2-3 săptămâni.

În formele clinice generalizate s-a instituit tratamentul pe cale generală, cu antibiotice imunostimulente, hepatoprotectoare, vitamino-terapie, însoțite de alimentație corespunzătoare.

Examenul microscopic efectuat la 115 câini cu leziuni cutanate a evidențiate dermatite cu etiologie parazitară la 58 câini (50,4%), din care la 29,3% s-a diagnosticat râie sarcoptică şi la 70,6% dermodicoză. Incidența râiei sarcoptice a fost de 41,1% la cățeii de 1-6 luni, 11,7% la cățeii de 7-11 luni şi de 17,6% la câinii de 1-3 ani şi de 4-6 ani. La câinii din rasa comună incidența râiei sarcoptice a fost de 35,2%.

Incidența demodicozei uscate forma localizată la câini a fost de 43,9%, a demodicozei uscate forma generalizată de 24,3%, în forma umedă localizată de 4,8% şi a formei umede generalizată de 26,8%. Incidența demodicozei la tineretul în vârstă de 3 luni-2ani a fost de 78,04%, la câinii de 3-5 ani de 17,03% şi la exemplarele de 6-8 ani de 4,8%. La câinii din rasa comună s-a înregistrat o incidență de 29,2% a demodicozei.

Incidența râiei otodectice la pisică a fost de 17,3%, din care 37,5% la pisici din rasa Persană şi de 25% la rasele Birmaneză şi comună europeană.

Combaterea râiei sarcoptice s-a realizat cu Dectomax în 5 administrări s.c. la 10 zile asociată cu terapia locală cu Amitraz 1% în 4-5 aplicații şi s-a obținut o eficacitate de 100%.

În demidicoza uscată s-a utilizat Dectomax în 3 administrări s.c. la 10 zile şi 4-5 aplicații locale cu Amitraz 1% cu o eficacitate de 100%.

În demodicoza umedă forma generalizată combaterea s-a efec-tuat timp de 45-60 zile prin terapie generală cu Dectomax, Imaverol, produse hepatoprotectoare, vitamine şi antibioterapie, asociate cu aplicații locale cu Amitraz 1% şi s-a înregistrat o eficacitate de 98%.

Combaterea râiei auriculare la pisici s-a efectuat cu produse Oticure, Mitex şi Otoguard în instilații locale zilnice sau la 2-3 zile şi s-a obținut vindecări de 100%.

Cuvinte cheie: *râia sarcoptică, otodectoza, câini, pisici, demodicoză*

ABSTRACT

The investigations were conducted on 115 dogs with skin wounds and 46 cats with external otitis. The investigations were conducted through clinical examination, microscopic examination of the curetted material obtained from the skin and of the cerumen collected from the outer auditive duct. The diagnosed acarioses were treated with miticide solutions, emulsions and ointments applied daily or at 2-3 days intervals, until healing. In the generalised clinical forms, a general therapy was started with antibiotics, according to the antibiogram, immunostimulants, liver protectors, vitamin-mineral therapy and a proper feeding.

The microscopic examination conducted on 115 dogs with skin wounds showed dermatitis with parasitic etiology in 58 dogs (50.4%), of which 29.3% with sarcoptic mange and 70.6% with demodicosis. Sarcoptic mange incidence was 41.1% in 1-6 months old puppies; the common breed dogs displayed a 35.2% incidence of sarcoptic mange.

The localised dry demodicosis was observed in 43.9% of the dogs, the generalised dry demodicosis was observed in 24.3% of the dogs and the generalised wet

demodicosis was observed in 26.8% of the dogs. Demodicosis was observed in 78.04% of the dogs aged 3 months to 2 years.
Otodectic mange was noticed in 17.3% of the examined cats, of which 37.5% in Persian cats.
Sarcoptic mange was treated with Dectomax, 5 SC administrations at 10 days, associated with a local therapy with Taktic 1%, 4-5 applications at 2-3 days. The efficiency was 100%.

The dry demodicosis was treated with Dectomax, 3 SC administrations at 10 days, and 4-5 local applications with Amitraz (Taktic)1%. The efficiency was 100%.

The generalised wet demodicosis was treated for 60-90 days by parenteral administration of Dectomax, immunostimulants (Levamisol), liver protecting agents (Aspatofort), vitamin-mineral therapy (vitamin A, biotin, Se, Zn) and antibiotics associated with local applications with Taktic 1%. The efficiency was 98%.

The auricular mange in cats was treated with Oticure, Mitex and Otoguard using local instillations, daily or at 2-3 days interval. The efficiency was 100%.
Keywords: sarcoptic mange, otodectes, digs, cats, demodicosis

INTRODUCTION

The increased incidence of parasitic dermatitis in dogs and cats produced by acari was noticed by many authors (1, 3, 4, 7, 8). In some geographical areas the sarcoptic mange is the most frequent clinical form of manifestation of dermatitis in dogs (2, 4, 8). The zoonotic character of the sarcoptic mange was noticed in 30-50% of the children coming into contact with infested dogs (4, 5) and it manifested as pruritus papules on the forearms, ankle, thighs or abdomen.

In the dogs of improved breeds, 74.2% of the sarcoptic mange cases were noticed in long-hair individuals, mainly in the puppies below one year of age (2). Rataj et al. (2004) (10) reported a 34.6% incidence of the sarcoptic mange in 101 examined cats.

The otodectic mange was noticed more frequently in the cats than in dogs, the prevalence being 25% (1).

Demodex canis is considered to be a skin symbiont in the healthy dogs, because this species has been identified in 60% of the microscopic preparations from curetted material obtained from the skin of lesion-free dogs (3, 5). The symbiotic balance is broken when factors occur which decrease the resistance of the organism, frequently the state of immunodepression (7).

Demodex canis has an immunospuressive effect, and by its irritative mechanical action it causes a hypersecretion of sebum which is favourable to the multiplication of the acari; it also has a pyogenic effect which contributes to the purulent aspect of the secretions from the wet form of the demodicosis (5).

The onset of demodicosis is favoured by the congenital or acquired hypoactivity of the T lymphocytes as proven by the experiments conducted on dogs used to reproduce tumours, which receive antilymphocyte serums which produce the inset of acariosis (3, 5).

Nayak et al. (9) reported a 60% incidence of demodicosis in puppies below the age of one year, 23% in the dogs aged 1-2 years and 17% in the dogs over 2 years.

MATERIAL AND METHODS

The investigations conducted during February 2007 – April 2008 at the clinic of the Faculty of Veterinary Medicine of the Spiru Haret University, Bucharest, on a total of 115 dogs of different breeds and ages with skin lesions and on 46 cats with external

otitis. The animals have been examined clinically to determine the body regions that are affected and the aspect of the lesions.

The acari species has been identified by the microscopic examination of the preparations made from curetted material obtained from the skin and of the cerumen collected from the outer auditive duct. The microscopic preparations were cleared with sodium hydroxide 10%.

The incidence of acariosis in dogs and cats was classified according to the evolutive clinical forms by age categories and breeds.

The following therapeutic designs were used to control the acariosis diagnosed in the dogs and cats:

- the dogs with sarcoptic mange were treated with Dectomax, 4-5 SC administrations at 10 days, associated with a local therapy with Taktic 1%, 4-5 applications at 2-3 days;
- the dry demodicosis was treated with Dectomax, 3 SC administrations at 10 days, and 4-5 local applications with Amitraz (Taktic)1%;
- the generalised wet demodicosis was treated for 60-90 days by parenteral administration of Dectomax, immunostimulants (Levamisol), liver protecting agents (Aspatofort), vitamin-mineral therapy (vitamin A, biotin, Se, Zn) and antibiotics associated with local applications with Taktic 1%;
- the auricular mange in cats was treated by cleaning the outer auditive duct of the cerumen, followed by daily local instillations with one of the specific products, Oticure, Mitex or Otoguard

Results and discussion

Table 1 and graph 1 show the incidence of the parasitic dermatitis in the group of 115 dogs: 58 dogs (50.4%) have been diagnosed with the parasitic aetiology.

Table 1

Incidence of the parasitic dermatitis in dogs

Number of examined dogs	parasitic dermatitis	
	No.	%
115	58	50.4

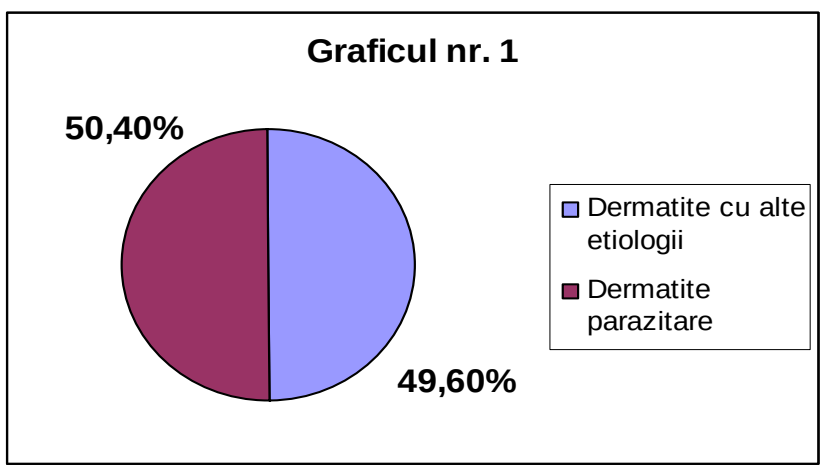


Table 2 and graph 2 show the incidence of acariosis observed in 58 dogs: 29.4% were diagnosed with sarcoptic mange and 70.6% with demodicosis.

Table 2

Incidence of the parasitic dermatitis in positive dogs

Number of infested dogs	Sarcoptic mange		Demodicosis	
	No.	%	No.	%
58	17	29.3	41	70.6

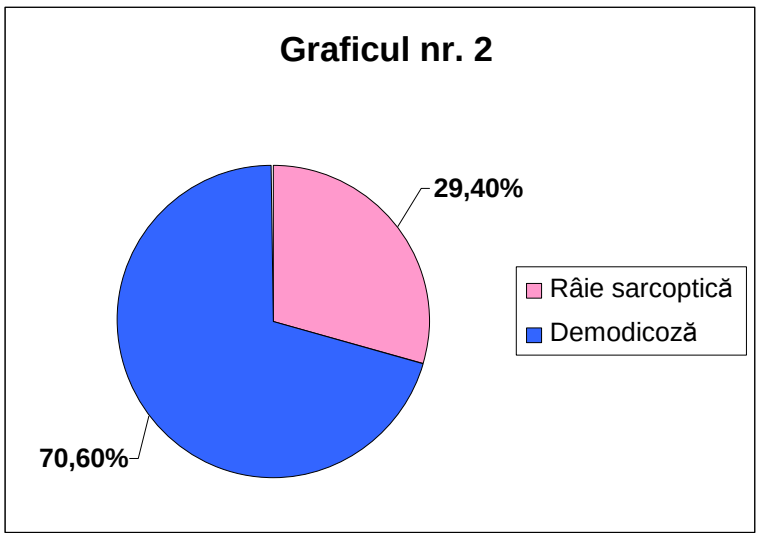
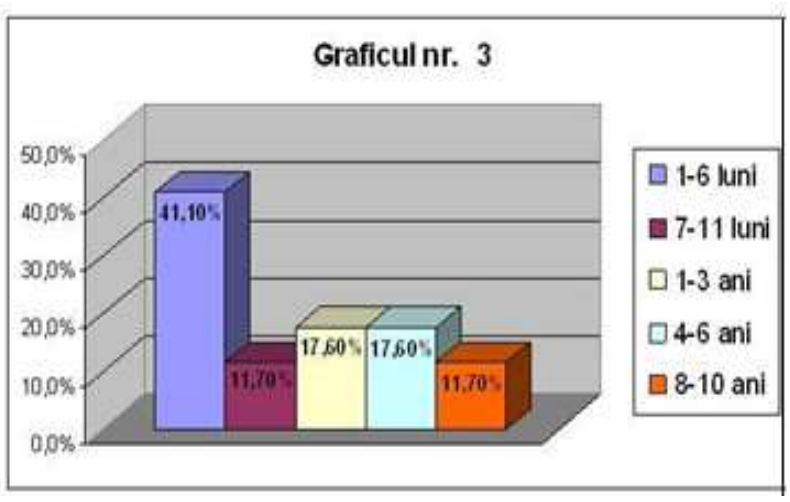


Table 3 and graph 3 show the incidence of the sarcoptic mange by age category. The highest prevalence was noticed in puppies aged 1-6 months (41.1), 11.7% in 7-11 months old puppies and in dogs aged 8-10 years, and 17.6% in 1-6 years old dogs

Table 3

Incidence of sarcoptic mange by age category

Positive dogs	1-6 months		7-11 months		1-3 years		4-6 years		8-10 years	
	No.	%	No.	%	No.	%	No.	%	No.	%
17	7	41.1	2	11.7	3	17.6	3	17.6	2	11.7



The common breed dogs displayed the highest rate of sarcoptic mange incidence, 35.2% (Table 4).

Table 4

Incidence of sarcoptic mange by breed

Positive dogs	Common breed	Pekinese	Teckel	Bichon	German shepherd	Cocker	Show Show	Caniche

	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
17	6	35.2	3	17.6	2	11.7	2	11.7	1	5.8	1	5.8	1	5.8	1	5.8

Table 5 shows the prevalence of the evolutive clinical forms of the demodicosis. The localised dry demodicosis was observed in 43.9% of the infested dogs, the generalised dry form in 24.3% of the dogs; the localised wet demodicosis was observed in 4.8% of the infested dogs, while the generalised wet form in 26.8% of the dogs.

Table 5

Incidence of the demodicosis by evolutive clinical forms

Dogs with demodicosis	**Dry form**				**Wet form**			
	localised		**generalised**		**localised**		**generalised**	
	No.	%	No.	%	No.	%	No.	%
41	18	43.9	10	24.3	2	4.8	11	26.8

Demodicosis is an acariosis specific to the young animals, as shown by the results of the investigations: 78.04% in puppies aged 3 months to 2 years (Tables 6, 7 and graphs 4 and 5)

Table 6

Incidence of the demodicosis by age category

Positive dogs	3-11 months		1-2 years		3-5 years		6-8 years	
	No.	%	No.	%	No.	%	No.	%
41	17	41.4	15	36.5	7	17.03	2	4.8

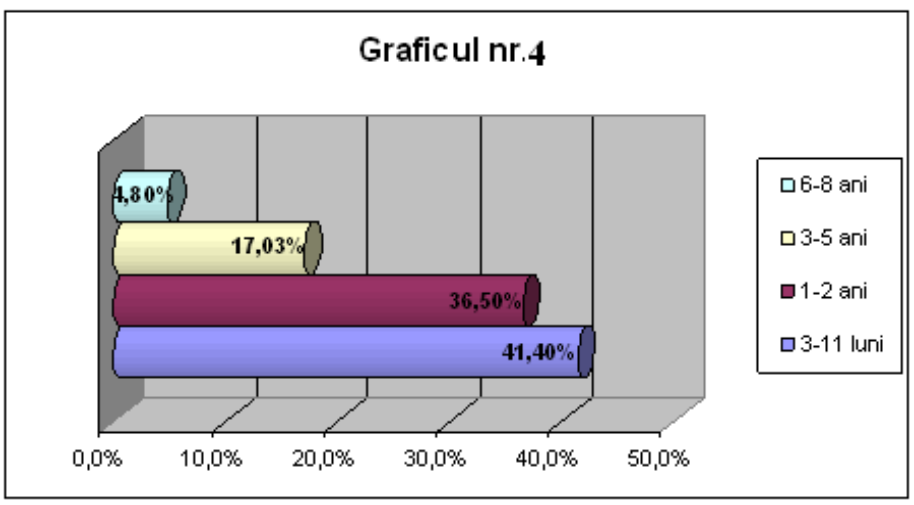
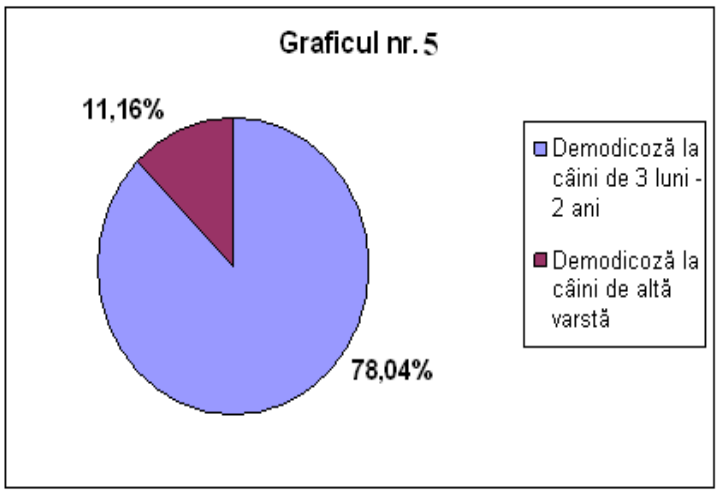


Table 7

Incidence of demodicosis in dogs aged 3 months – 2 years

Positive dogs	**3 months – 2 years**	
	Nr.	**%**
41	32	78.04



The incidence of demodicosis by age category was as follows: the highest prevalence was noticed in the puppies aged 3 – 11 months (41.4%), followed by 36.5% in the dogs 1-2 years old, 17.3% in the dogs aged 3-5 years and 4.8% in the dogs aged 6-8 years (Table 5).

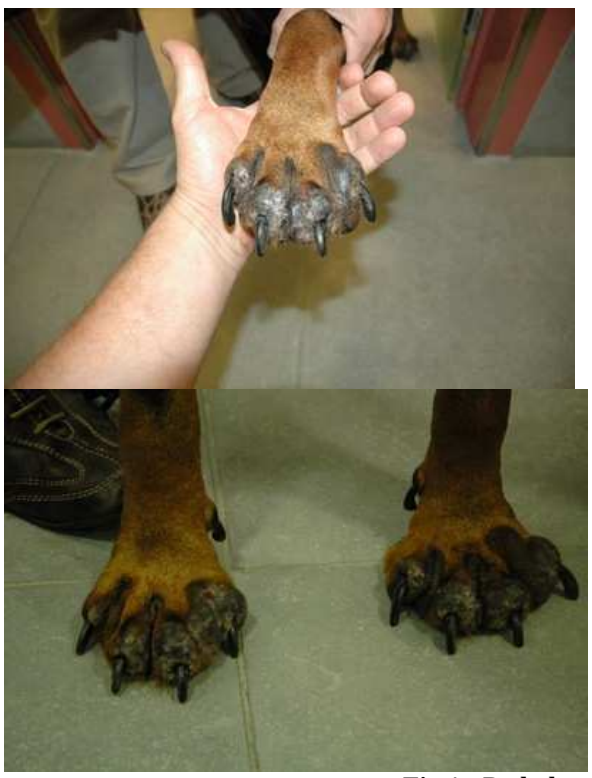


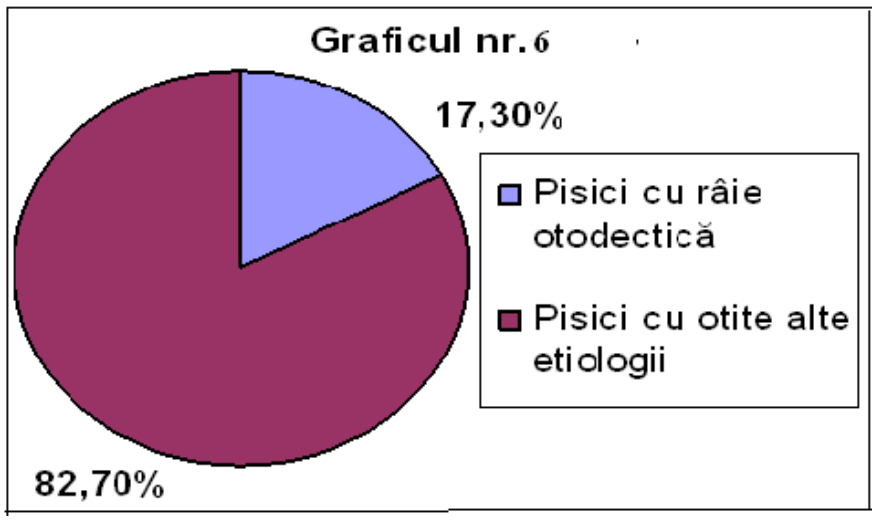
Fig.1 - Pododermatitis in demodicosis

Table 8 and graph 6 show the incidence of otodectic mange in cats. The microscopic examination of the crusts collected from 46 cats with external otitis diagnosed the presence of the acari Otodectes cynotis in 8 cats, which represents an incidence of 17.3%.

Table 8

Incidence of otodectic mange in cats

Examined cats	Cats with otodectic mange	pERCENTAGE
46	**8**	**17.3 %**



The general treatment applied for the localised dry demodicosis and for the localised wet demodicosis, and the local applications for 21-25 days according to the mentioned therapy healed the animals with 100% efficiency.
The generalised wet demodicosis was treated for 60-90 days by a general therapy with Dectomax, liver protecting agents, immunostimulants, long-term supplementation of vitamins and minerals, associated with antibiotics; the treatment had an efficiency of 98%. Two percent of the cases relapsed with points where the lesions persisted on the ventral side of the tail, an area hardly accessible to the local therapy.
The cases of external otitis with parasitic aetiology in cats were healed 100% after the local applications with the specific products.

CONCLUSIONS

1. Dermatitis with parasitic aetiology were reported in 50.4% of the dogs with skin lesions that were examined.
2. Sarcoptic mange was diagnosed in 29.4% and demodicosis was diagnosed in 70.6% of the dogs with ectoparasitoses;
3. 41.1% of the sarcoptic mange cases were reported in puppies aged 1-6 months;
4. 78.04% of the demodicosis cases were reported in dogs aged 3 months – 2 years;
5. 17.3% of the cats with external otitis were diagnosed with otodectic mange
6. The treatment applied to the localised forms of demodicosis were 100% efficient;
7. The general and local treatment applied to the generalised wet demodicosis was 98% efficient;
8. The specific products used to control the parasitic otitis were 100% efficient.

REFERENCES

1. Bourdeau P.(1990) – Dermatologie feline – aspects d'actualité, Rec.Med.Vet.166, 6/7, 665 – 697.
2. Castro R. et all.(2005) – Retrospective study of canine and feline scabies presented in Sao – Paulo in the period 1984 – 2002. Brasilian Jour.Vet.Res. and Animal Science 42, (2),135 – 142.
3. Coman Sofia, B.Băcescu, N.Bercaru,T.Petruț (2008)– Boli parazitare şi micoze la animale de companie , Ed. FRM, ISBN 978-973-725-982-0.

4. Cosoroabă I. (2000) – Parazitologie veterinară – acarioze entomoze, Ed.Mirton, Timişoara, ISBN 973-585-271-3, 645 pg.
5. Euzeby J. (1999) – Les parasites agents de dermatoses humaines d'zoonosique et leur role pathogene, Flammarion Paris.
6. Franco M.B.,Hamann W.(2004) – Doramectin in the treatment of dogs with sarcoptic mange and gastrointestinal nematodes , Archives of Vet.Science 9, (1), 23 – 29.
7. Gothe R. (1989) – Die Demodikose des Hundes eine Faktorenkrakheit, Berl.München, Tierärztl.,102, 293 – 297.
8. Meleney W.P. (1985) – Mange mite and other Parasitic Mite, In Gaafar Parasites, Pests and Predators Elsevier, Amsterdam.
9. Nayak D.C.et all.(1997) – Prevalence of canine demodicosis in Orissa (India)Vet.Parasitol. 73, (3/4), 347 – 352.
10. Rataj A.V., (2004) – Ectoparasites: Otodectes cynotis, in the ear of cats, Slovenian,Vet.,Rec. 41, (2), 89 – 92.

ANTIHELMINTIC ACTIVITY OF ROMBENDAZOL F 10% IN POULTRY AND PIGLETS
ACTIVITATEA ANTIHELMINTICĂ A ROMBENDA-ZOLULUI F 10% LA PĂSĂRI ŞI PURCEI

DIDĂ I.[1], Sofia COMAN [2], C.CHIURCIU [3], Viorica CHIURCIU [3]

[1] USAMV, Bucureşti

[2] Faculty of Veterinary Medicine
Spiru Haret University
ushmv_coman.sofia@spiruharet.ro

[3] Romvac S.A.

REZUMAT

Rombendazolul F este un produs antiparazitar pe bază de Flubendazol preparat de Romvac Co. SA.

Produsul a fost testat asupra eficacității pe efective de păsări, găini adulte, pui de găini, curci, gâște, diagnosticate cu singamoză, heterakioză, ascaridioză, capilarioză şi pe efective de purcei în vârstă de 6 – 10 săptămâni diagnosticați cu ascaridioză.

Tratamentul a fost efectuat prin administrarea produsului în furaje în doză de 10 mg/kg greutate vie la păsări şi 5 mg/kg greutate vie la porc, timp de 5 zile. Examenele helmintologice au fost efectuate înaintea tratamentului, la 5 zile după tratament şi apoi din 7 în 7 zile timp de 27 zile.

Rombendazolul 10% a avut o intens eficacitate de 100% față de Heterakis galline şi Syngamus traheea la controlul după 5 zile şi de 100% față de Ascaridia galli şi Capillaria spp. după controlul de la 12 zile de la tratament. La puii de curcă şi la gâște după primul control, au persistat infestațiile cu Amidostomum anseris şi Syngamus traheea. La porci intenseficacitatea a fost de 100% la primul control după tratament față de infestația cu Ascaris suum şi de 100% după 12 zile de la tratament față de Strongiloydes ransoni. Intens eficacitatea față de Dicroanotaenia collaris şi Railletina spp. a fost de 100% după 5 zile de tratament la rațe. Toleranța produsului a fost foarte bună la toate speciile.

Cuvinte cheie: *Rombendazol, nematodoze la păsări şi porc, intenseficacitate*

ABSTRACT

Rombendazol F is an antihelminthic product manufactured by Romvac Co. SA. It appears in two forms, powder or pills containing Flubendazole (Methyl N-[6-(4-fluorobenzoyl)-1H-benzimidazol-2-yl] carbamate).

The product has been tested on poultry, adult hens, chicken, turkey hens, geese, diagnosed with singamosis, heterakiosis, ascaridiosis, capillariasis, and infestations with cestode (tapeworms), as well as on 6-10 weeks piglets diagnosed with ascaridiosis and strongyloidiasis.

The treatment was done by including the antiparasitic product into the feeds in amounts of 10 mg/kg live weight for the poultry, and 5 mg/kg live weight for the piglets, for 2 consecutive days. The helminthological examinations were conducted before the treatment, 5 days post- treatment and then every 7 days for 27 days, using the flotation Willis and McMaster techniques.

Rombendazol 10% was 100% efficient against heterakiosis, singamosis and capillariasis at the check up after 5 days and 100% efficient against ascaridiosis at the check up after 12 days post-treatment. In the young turkey and geese, after the first checking, the infestation with *Amidostomum anseris* and *Syngamus traheea* persisted.

In the piglets, efficiency was 100% at the first checking post treatment against the infection with *Ascaris suum* and 100% 12 days post treatment against *Strongiloydes ransoni*. The efficiency against *Dicroanotaenia collaris* and *Railletina* spp. was 100% after 5 fays of treatment in ducks.

The product was properly tolerated by all species.

Keywords: Rombendazol F10%, efficiency poultry, pigs, helminthiasis

INTRODUCTION

Rombendazol F is an antihelminthic product manufactured by Romvac Co. SA. It appears in two forms, powder or pills containing Flubendazole (Methyl N-[6-(4-fluorobenzoyl)-1H-benzimidazol-2-yl] carbamate).

Rombendazol is an antihelminthic product from the class of benzimidazole products with a wide range of action against species of nematodes and cestodes observed in dogs, cats and poultry (5). It acts by inhibiting the enzymes that coordinate the contraction of the intracytoplasmatic microtubule networks and in forming the division spindle in the prophase of the mitotic division (8).

The product has a low toxicity, the toxic dose in pigs being 20 times higher than the therapeutic dose; it has no teratogenic, embryotoxic, cancerigenic or mitogenic activity (7, 8).

The absorption, distribution, metabolism, excretion and residues of Rombendazol have been studied by radioactive marking of the product with ^{14}C on groups of rats, dogs, pigs and poultry (1, 2, 3, 6). The metabolites are excreted through urine and faeces. The highest concentration of residues was noticed in the pig liver, while in the poultry they are stored in the egg yolk. (4).

The residues are eliminated from the organism over a longer period, the withdrawal time for the residues being 28 days (9).

The purpose of the paper was to determine the efficacy of Rombendazol F powder, in the dose recommended by the manufacturer, to control the parasitic infestations with nematodes and cestodes in growing pigs and in different poultry species.

MATERIAL AND METHODS

The investigation was conducted on a total of 1,334 poultry specimens reared in household system, assigned to 7 groups according to the species and age: 97 hen chicks, 104 adult hens, 102 turkey chicks, 51 adult turkeys, 62 goose chicks, 406 ducks, 512 pheasants and on 104 growing pigs reared in a semi-intensive system.

The laboratory investigations conducted with the Willis flotation helminths egg count technique determined the level of helminthic infestation before, and after the treatment with Rombendazol F 10% powder.

The treatment of helminths – nematodes and cestodes infestations in poultry was done by including the antiparasitic product into the feeds in amounts of 10 mg/kg live weight for 2 consecutive days, while a dose of 5 mg/kg live weight was used for the piglets, also for 2 consecutive days.

The post treatment egg count examination was performed at 5, 12, 19 and 26 days, when the efficacy of the antihelminthic product was assessed.

RESULTS AND DISCUSSION

The coproscopic examination conducted on the groups of poultry before the treatment showed infestations with *Ascaridia* sp., *Heterakis* sp. and *Capillaria* sp. in hens and turkey hens, with *Ascaridia, Syngamus* and *Capillaria* in the hen and turkey chicks; with *Amidostomum anseris* in the goose chicks; with cestodes species in ducks (*Dicroanotaenia collaris*) and with *Ascaridia* and *Syngamus* in pheasants (Table 1).

Table 1 shows the efficacy of Rombendazol F 10% powder in the helminths infestation in poultry. Five days after treatment a poor infestation with *Ascaridia galli* was observed in the group of adult hens and a poor infestation with *Amidostomum anseris* was noticed in the group of goose chocks. In the other groups of poultry, the coproscopic examinations yielded negative results in all the post-therapy checking.

The efficacy of Rombendazol F 10% powder was 100%, 12 days post-therapy against all species of nematodes and cestodes diagnosed in poultry.

Table 1

Efficacy of Rombendazol F 10% in poultry

Species and category	Number of treated specimens	Parasitic infestations	Level of infestation				
			Before treatment	After treatment (days)			
				5	12	19	26
Adult hens	**104**	***Ascaridia***	++	+	-	-	-
		Heterakis	++	-	-	-	-
		Capillaria	+	-	-	-	-
Hen chicks	**97**	***Ascaridia***	++	-	-	-	-
		Syngamus	+	-	-	-	-
		Capillaria	++	-	-	-	-
Adult turkey hens	**51**	***Heterakis***	++	-	-	-	-
		Capillaria	+	-	-	-	-
		Ascaridia	++	-	-	-	-
Turkey chicks	**102**	***Capillaria***	+	-	-	-	-
		Ascaridia	+	-	-	-	-
		Syngamus	+	-	-	-	-
Goose chicks	**62**	***Amidostomum***	++	+	-	-	-
Ducks	**406**	***Dicroanotaenia***	++	-	-	-	-
Pheasants	**512**	***Ascaridia***	+	-	-	-	-
		Syngamus	+	-	-	-	-
TOTAL	**1,334**						

Legend: strong infestation ++; poor infestation +

Table 2 shows the efficacy of Rombendazol F 10% powder in the growing pigs infested with *Ascaris suum* and *Strogyloides ransomi*. The coproscopic examination conducted before the treatment showed an infestation with ascarids and with strongyloides. Five days after treatment the coproscopic examination was negative for the infestation with *Ascaris suum* and poor for the infestation with Strogyloides.

Twelve days post-therapy the efficacy of the product was 100% against both nematodes species.

No side effects were noticed during the therapy, the tolerance was very good both in the poultry and in the pig groups.

Table 2

Efficacy of Rombendazol F 10% in pigs

Species and category	Number of treated specimens	Parasitic infestations	Level of infestation				
			Before treatment	After treatment (days)			
				5	12	19	26

Growing pigs	**104**	***Ascaris***	++	-	-	-	-
		Strongyloides	++	+	-	-	-

Legend: strong infestation ++; poor infestation +

CONCLUSIONS

The efficacy of Rombendazol F 10% powder was 100% five days after treatment in the hen chicks and in the adult hens infested with *Heterakis gallinae*, *Syngamus trachea* and *Capillaria sp.* and twelve days for the infestation with *Ascaridia galli*.

The efficacy was 100% in adult turkey hens and in turkey chicks, five days after treatment, against the infestations with *Ascaridia, Heterakis, Syngamus* and *Capillaria*.

The efficacy was 100% in goose chicks, 12 twelve days after treatment against the infestation with *Amidostomum anseris*.

The efficacy was 100% in ducks, five days after treatment, against the infestations with cestodes.

The efficacy was 100% in pheasants, five days after treatment, against the infestations with *Ascaridia* and *Syngamus*.

In the growing pigs the efficacy was 100% five days after treatment for the infestation with *Ascaris suum* and twelve days after treatment for the infestation with *Strongyloides ransomi*.

The tolerance of the product during therapy was very good in all investigated species.

REFERENCES

1. Lee I –J. (1981) – Mebabolism of Flubendazol in swine, Excretion pattern of (2 – C 14) Flubendazol and its metabolites and deplation Kinetics of drug residues in edible tissues. Unpublished raport V 7902. Submitted to FAO by Janssen Pharmaceutical, Beerce, Belgium.

2. Meulder Mans W., Hurkmans R.,Swiysen E.,Heykants J.(1997) – A comparative study on the excretion and metabolism of flubendazole and mebendazole in the rat. Unpublished raport V 2940. Submitted to FAO by Janssen Pharmaceutical, Beerce, Belgium.

3. Meulder Mans W., Lee I-Y , Hendricks J. et all (1982) – Metabolism of flubendazole in swine ; Excretion patern of (2 C 14) flubendazole and its metabolites and depletion kinetics of drug residues in edible tissues. Unpublished raport V 4485. Submitted to FAO by Janssen Pharmaceutical,Beerce, Belgium.

4. Michiels M., Hendricks R.et all (1983) – Flubendazole residues in eggs and tissues of laying hens. Unpublished raport V 4925. Submitted to FAO by Janssen Pharmaceutical, Beerce, Belgium.

5. Moreno I.,Mottier L.et all (2004) – Integrated pharmacological assessment of flubendazole potential for use in sheep : disposition kinetics, live metabolism and parasitic diffusion ability. J.Vet.Pharmacology and therapeutics 27, 299 – 308.

6. Tornoe N.,Christensen S.(1991) – Flubendazole residues in hens after treatment with flubendazole medicated food. Unpublished raport . Submitted to FAO by Janssen Pharmaceutical, Beerce, Belgium.

7. Van Lee M. Put, Meas L., Meulder Mans W., Haykants J. (1991) – Pharmacokinetics profile of flumendazole: critical reveiw of available data. Unpublished review from Department of Drug Metabolism and Pharmacokinetics and Department of Parasitology, Janssen Pharmaceutics.Submitted to Who by Janssen Research Products, Janssen Pharmaceutical B – 2340, Beerce, Bergium.

8. (2006) – Flubendazole Monografie – Institut für Veterinär Pharmakologie und Toxicologie. Winterthurerstrasse 260, 8057 Zürich, Schweiz.

9. ANSVSA order 82/2005 for the approval of the Sanitary veterinary norm concerning the monitoring and control norms of some substances and their residues, published in M.O., 173 Nr. 832 bis part I/14.09.2005.