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SUMMARY

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DYNAMICS OF BIRD POPULATIONS ON THE SACALIN ISLAND - DANUBE DELTA

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Abstract

Sacalin Island is an area of major importance because it is situated in the path of one of the most important bird's migration routes in the world, covering three continents: Asia, Europe and Africa, the route with a great abundance and biodiversity of avifauna including sedentary species, passage species, and summer and winter guest species. In order to demonstrate the necessity of preserving this area and to establish the biodiversity of ornithological fauna, this article presents the results obtained from the data collection in a period of 6 years and analyzed in terms of the status, abundance and biodiversity of birds, highlighting the need to protect this habitats. Our result showed that the birds collected form Sacalin Island belonging to a wide variety of birds including 17 orders, 46 families and 201 species; 53% of the total of 382 bird species identified in our country. The frequency of bird populations in relation with biogeographic regions during winter and vernal season showed that the dominant species are the species typical for Palearctic regions. By analysis of trophic level, our results showed that there is a definite dominance of insectivore species (72%), followed by carnivore (9%), omnivore (5%) insectivore – larvivore (4%), and larvivore (2%) species. Summarizing all the results it is clearly that Sacalin Island should maintained the strictly protected area status; here are present 85% of strictly protected bird's species.

Key words*: avifauna, biodiversity, Danube Delta, Sacalin Island.*

INTRODUCTION

Conservation and protection of wild birds and their habitats setting up networks of protected areas that include also Sacalin Island (Munteanu, 1998; Hansell, 2005). This area remains of major importance because it is situated in the path of one of the most important migration routes in the world, covering three continents: Asia, Europe and Africa, the route with a great abundance and biodiversity of avifauna including sedentary species, passage, and summer and winter guests. (Kiss, 1973, 1976, 2006, Alerstam, 2001). Sacalin Island is situated to the south of Sf. Gheorghe delta (Stanescu, 1973). The impact of human regarding the use of the same territories or consumption the same resources disturbed the bird habitats (Radu, 1957; Rudescu, 1955). In order to demonstrate the necessity of preserving this area and to establish the biodiversity of ornithological fauna, this article presents the results obtained from the data collection in a period of 6 years and analyzed in terms of the status, abundance and biodiversity of birds,

highlighting the need to protect habitats in the studied area.

MATERIALS AND METHODS

Ornithological data used in the present study were collected from 2007 to 2013 by observations, surveys, and measurements done in the Sacalin Island, Danube Delta (Figure 1). Data were obtained with the support of the Romanian Ornithological Centrala and Nos Oiseaux Institute, Switzerland. Were captured over 4,000 birds every year using ornithological nets (L=6m, H=2.5m, mesh=19x19mm) (Gibbons et. al., 1996; Sutherland, 2004). The observations were made using binoculars (Swarovski EL42) and telescope (Swarovski ATX) and photographs were captured using Nikon D300 camera with Nikkor 70-200mm f / 2.8 lens. For data collection methods were used transects in line, point counts method and bird's ringing. (Sutherland, 2006; Emlen and Dejong, 1992; Buckland et al., 2001; Newton, 2010). Birds were separate by stages and sex, and identified based on morphological characters using the specific identification keys (Svensson et al., 2010; Voous, 1986).

Figure 1. Map of Sacalin Island areas where birds were collected

RESULTS AND DISCUSSIONS

Our result showed that the birds collected form Sacalin Island belonging to 17 orders, 46 families and 201 species. This data come in support to other data who sustain that this number of species represents 53% of the total of 382 bird species identified in our country, highlighting the importance of this area (Munteanu et al., 1998; Gogu–Bogdan and Marinov, 1997) (Figure 2).

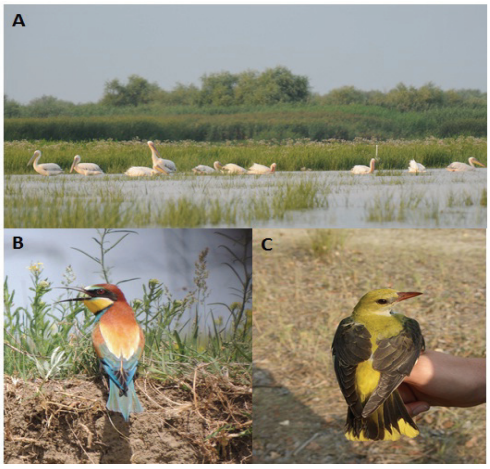

Figure 2. (A) Pelican (*Pelecanus onocrotalus*) colony. (B) European bee-eater *Merops apiaster*. (C) *Oriolus oriolus*

The frequency of bird populations in relation with biogeographic regions during winter season shows that the dominant species in terms of numbers are the species typical for northern regions, which are Palearctic regions (49%), with subregions: European (18%), Holarctic (12%), Arctic (7%), European - turchestanian (6%) Cosmopolitan (4%), and Nearctic (2%) (Figure 3).

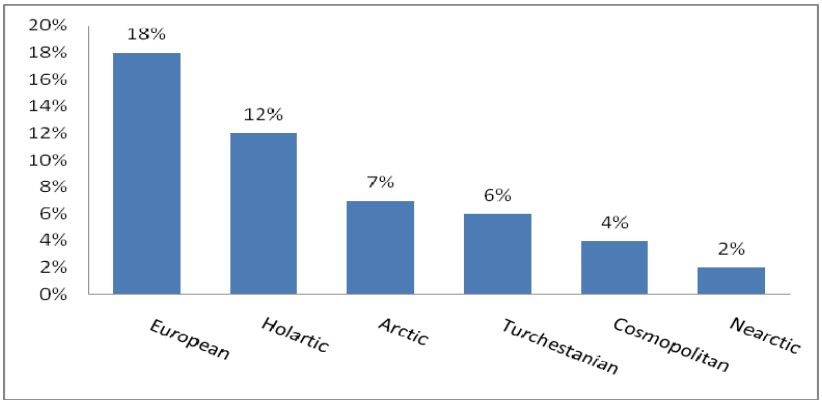

Figure 3. Incidences of bird populations in relation with biogeographic regions during winter season

Unlike winter season, during vernal season the most dominant species are from the Palearctic regions with subregions: European (16%), European - turchestanian (13%), Holarctic (8%), Cosmopolitan (3%), Mediterranean-turchestanian (3%), Mediterranean (2%), Indo-African (1%), and China (1%), which are species wintering in the southern hemisphere (Figure 4).

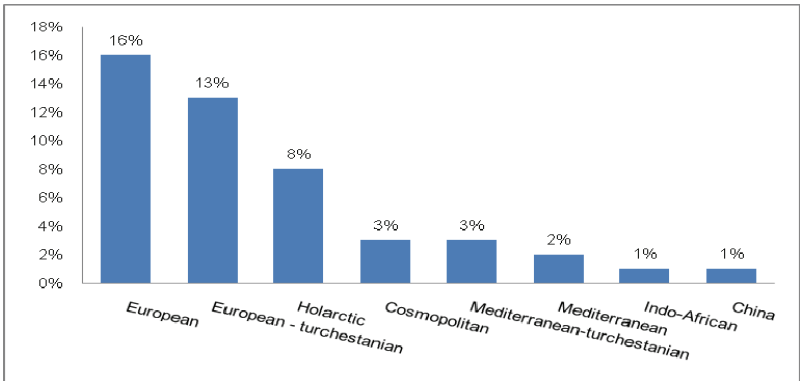

Figure 4. Incidences of bird populations in relation with biogeographic regions during vernal season

By analysis of trophic level, our results showed that there is a definite dominance of insectivore species (72%), followed by carnivore (9%), omnivore (5%) insectivore – larvivore (4%), and larvivore species (Figure 5). These data shows that Sacalin Island contains ideally biotopes for birds where they can rest and feed being characterized by the abundance and quality of food.

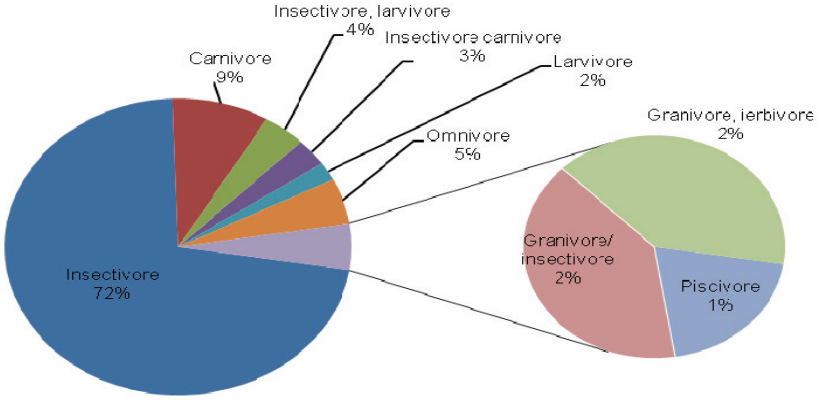

Figure 5. Trophyic levels distribution of avifauna from Sacalin Island

The great value of Sacalin Island is given essentially by the number of birds that are in passage, spring and autumn (43% of the species observed), representing a very important feeding and resting place. Also, the study area is important for wintering species (12% of the species observed), Sacalin Island is a body of brackish water, and at the same time is in direct contact with seawater from Black Sea (Ciochia, 1984; Catuneanu, 1954). Therefore freezing brackish water and seawater is much higher in absolute terms than the freezing point of freshwater and allowed many birds such as *Anseriformes* species to find a source of food and rest for a long periods of time (Ionac and Ciulache, 2004; Newton, 2007). Summarizing all the results it is now clearly that Sacalin Island should maintaine the strictly protected area status, since here are presented 85% of strictly protected birds species. The unic chareacteristics of Sacalin Island are particularly important to maintaining biodiversity and genetic variety of the bird's species (Paspaleva et al., 1985; Speek et al., 2003).

CONCLUSIONS

Our data highlight the importance of Sacalin Island for birds biodiversity. The unic characteristic of this area create for birds a shalter and a place for food during the migration seasons. Also, Sacalin Island represents the habitat for a variety of marine species with stictly protected status.

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INVESTIGATION THE ECOLOGY AND DISTRIBUTION OF PASSERIFORMES POPULATION FROM SACALIN ISLAND - DANUBE DELTA

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Abstract

Danube Delta is the second largest river delta in Europe, after Volga Delta, and the best preserved on the continent. Sacalin Island is a strictly protected area of the Danube Delta, declared biosphere reserve since 1938. The impact of human regarding the use of the same territories or interest in eating the same food resources disturbed bird's habitats. The aim of this study was to investigate the distribution and ecology of passerine birds (Passeriformes) on the Sacalin Island in relation with anthropic changes. Data were obtained through the establishment of a permanent ringing station in Sacalin Island, with the support of the Romanian Ornithological Central and Nos Oiseaux Institute in Switzerland. As a working methodology, observations and bird ringing were made throughout the years 2007-2013, and distribution and ecology were analyzed. A total number of 6619 Passeriformes were collected, belonging of 13 familia. Among Passeriformes collected from Sacalin Island, Sylvidae familiae was the most divers with 14 species, followed by Muscicapidae with 8 species, Fingilidae (5 species), Turdidae (4 species), Paridae (3 species), Paradoxornithidae and Motacillidae (2 species) and Emberizidae, Oriolidae, Corvidae, Laniidae, Troglodytidae, and Hirundinidae with 1 species respectively. The predominant number of birds was found in Muscicapidae familiae (36.51%), followed by Sylviidae (29.65%), Turdidae (13.05%), Paridae (8.68%), Laniidae (6.78%), Fringiliidae (2.07%), Troglodytidae (0.98%), Motacilidae (0.60%), Paradoxomithidae (0.51%), Oriolidae (0.50%), Emberizidae (0.40%), Corvidae (0.18%) and Hirundinidae (0.05%). In conclusion, our data suggest that the Sacalin Island is an area with a wide diversity of bird's fauna, uncovering the ecology and distribution of birds could greatly improve the knowledge of bird's dynamics and behavior.

Key words*: Biosphere reserve, birds, Danube Delta, Passeriformes.*

INTRODUCTION

The greater part of Danube Delta lies in Romania (Tulcea county), whiles its northern part, on the left bank of the Chilia arm, is situated in Ukraine (Odessa Oblast). The approximate surface area is 4,152 km², and of that, 3,446 km² are in Romania. With the lagoons of Razim-Sinoe (1,015 km² with 865 km² water surface), located south of the main delta, the total area of the Danube Delta reaches 5,165 km². The Razelm - Sinoe

lagoon complex is geologically and ecologically related to the delta proper and their combined territory is part of the World Heritage Sites (Giosan et al., 2012). Over 300 species of bird have been recorded, of which over 176 species breed, the most important being cormorant, pygmy cormorant, white pelican and Dalmatian pelican (Covaci et al., 2006; Ion et al., 2002). There are numerous multi-species heron colonies and raptor species including white-tailed eagle. The marsh tern colonies are especially notable. The delta holds huge numbers of geese in the winter, white-fronted geese, red-breasted geese (a globally threatened species with almost all the world wintering population present), teal, mallard and pochard (Kalosca et al., 2007; Cramp et al., 1992). Ecological changes in the Danube Delta including the creation of a network of canals through the delta to improving access and water circulation, and the reduction of the wetland area by the construction of agricultural polders and fishponds which reduced biodiversity, altered natural flow and sedimentation patterns, and diminished the ability of the delta to retain nutrients (Aurigi et al., 2000; Sarbu, 2005). Therefore the aim of this study was to investigate the distribution and ecology of passerine birds (*Passeriformes*) on the Sacalin Island in relation with anthropic changes, in order to improve the measures for protecting the bird's fauna.

MATERIALS AND METHODS

Data were obtained through the establishment of a permanent ringing station in Sacalin Island (Figure 1), with the support of the Romanian Ornithological Central and Nos Oiseaux Institute, Switzerland. As a working methodology, observations and ringing were made throughout the years 2007-2013, the station having fixed opening hours. The nets were opened and checked within 30 minutes in good weather conditions and temperature and within 15 minutes unfavorable climatic conditions, from sunrise to sunset. Under extreme conditions, the nets were closed (temperatures above 26°C or high winds). Data processing was performed using Microsoft Office Excel and ANOVA software.

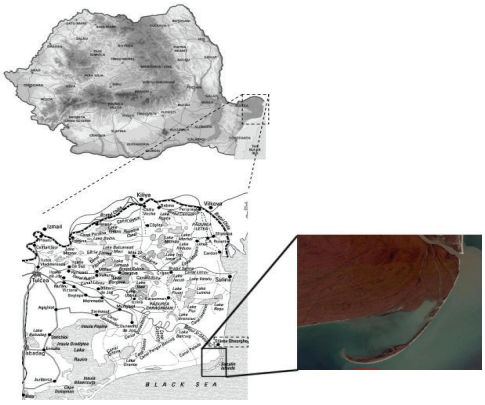

Figure 1. Map of Sacalin Island areas where birds were collected

RESULTS AND DISCUSSIONS

During 2007-2013, a total number of 6619 *Passeriformes* were collected, belonging of 13 familia (Tabel 1). Among *Passeriformes* collected from Sacalin Island, Sylvidae familiae was the most divers with 14 species, followed by Muscicapidae with 8 species, and Fingilidae (5 species), Turdidae (4 species), Paridae (3 species), Paradoxornithidae and Motacillidae (2 species) and Emberizidae, Oriolidae, Corvidae, Laniidae, Troglodytidae, and Hirundinidae with 1 species respectively (Figure 2; Tabel 1).

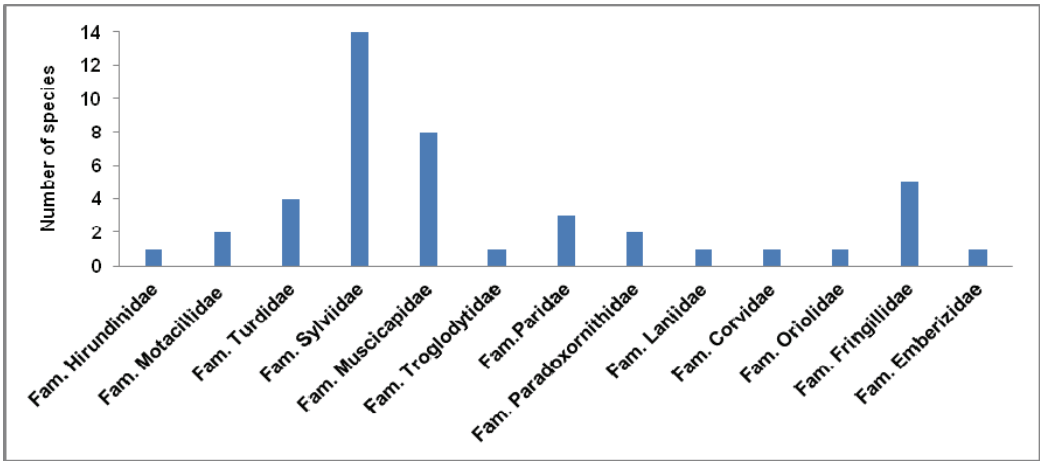

Figure 2. Number of species of different families of Passeriformes identified in Sacalin Island

Tabel 1. Details on Passeriformes species collected from Sacalin Island, stratified by phenological type, frequency, and biogeography type.

No. of species	Species	Number of specimens	Phenological type	Status	Frequency	Biogeography type
Fam. **Hirundinidae**						
1.	*Delichonurbica*	3	S.G., UnH.	strict protected species	* *	Palearctic
Fam. **Motacillidae**						
2.	*Anthustrivialis*	15	S.G., P. UnH.	strict protected species	* *	European-Turkestan
3.	*Motacilla alba*	25	S.G., P., H.	strict protected species	* * * *	Palearctic
Fam. **Turdidae**						
4.	*Turdusmerula*	439	S.G., P. UnH.	protected species	* * * *	Palearctic
5.	*Turduspilaris*	8	W.G., P. UnH.	protected species	* *	Siberian
6.	*Turdusiliacus*	21	W.G., P. UnH.	protected species	* **	Siberian
7.	*Turdusphilomelos*	396	W.G., P. UnH.	protected species	* * *	European
Fam. **Sylviidae**						
8.	*Locustellaluscinioides*	16	S.G., P. H.	strict protected species	* *	European-Turkestan
9.	*Locustellafluviatillis*	9	S.G., P. H.	strict protected species	* * *	Palearctic
10.	*Acrocephalusscirpaceus*	14	S.G., P. H..	strict protected species	* * *	European-Turkestan
11.	*Acrocephalusschoenobaenus*	12	S.G., P. H.	strict protected species	* * *	European-Turkestan

12.	*Acrocephaluspalustris*	59	S.G., P. H.	strict protected species	* *	European
13.	*Acrocephalusarundinaceus*	23	S.G., P. H.	strict protected species	* * * *	European-Turkestan
14.	*Hippolaispallida*	46	S.G., P. UnH.	strict protected species	* *	Mediteranean
15.	*Sylvia communis*	87	P. UnH.	strict protected species	* * *	European-Turkestan
16.	*Sylvia curruca*	207	P. UnH.	strict protected species	* * *	European-Turkestan
17.	*Sylvia borin*	237	P. UnH.	strict protected species	* * *	European
18.	*Sylvia nisoria*	39	P. UnH.	strict protected species	* *	European-Turkestan
19.	*Sylvia atricapilla*	485	P. UnH.	strict protected species	* * *	European
20.	*Phylloscopussibilatrix*	44	S.G., P. UnH.	strict protected species	* *	European
21.	*Phylloscopustrochilus*	685	S.G., P. UnH.	strict protected species	* **	Palearctic
Fam. **Muscicapidae**						
22.	*Muscicapastriata*	250	S.G., P. UnH.	strict protected species	* * *	European-Turkestan
23.	*Ficedulahypoleuca*	21	S.G., P. UnH.	strict protected species	* *	European
24.	*Ficedulaparva*	347	P. UnH.	strict protected species	* * *	Palearctic
25.	*Ficedulaalbicollis*	37	P. UnH.	strict protected species	*	European
26.	*Phoenicurusphoenicurus*	188	S.G., P. UnH.	strict protected species	* *	European
27.	*Luscinialuscinia*	306	S.G., H.	strict protected species	* * *	Palearctic
28.	*Phoenicurusochruros*	60	S.G., P. UnH.	strict protected species	* *	Paleo-Xeromontan
29.	*Erithacusrubecula*	1208	P. UnH.	strict protected species	* * * *	European
Fam. **Troglodytidae**						
30.	*Troglodytes troglodytes*	65	P. UnH.	strict protected species	*	Transpalearctic
Fam. **Paridae**						
31.	*Aegithaloscaudatus*	68	S.G., H.	strict protected species	*	Palearctic
32.	*Parus major*	205	S.G., P. H.	strict protected species	* * *	Palearctic
33.	*Cyanistescaeruleus*	303	S.G., P. H.	strict protected species	* * *	European
Fam. **Paradoxornithidae**						
34.	*Panurusbiarmicus*	32	S.G., P. H.	strict protected species	* *	Palearctic
35.	*Remizpendulinus*	2	S.G., P. H.	strict protected species	*	Palearctic
Fam**. Laniidae**						
36.	*Laniuscollurio*	449	S.G., P. H.	strict protected species	* * *	Palearctic
Fam. **Corvidae**						
37.	*Pica pica*	12	S. H.	-	* * *	European-Turkestan
Fam. **Oriolidae**						
38.	*Oriolusoriolus*	33	S.G., H.	strict protected species	* * *	Old world
Fam. **Fringillidae**						
39.	*Fringillacoelebs*	64	P.,W.G. UnH.	protected species	* * * *	European
40.	*Cardueliscarduelis*	2	S.G., P., W.G., UnH.	strict protected species	* *	European-Turkestan
41.	*Carduelisspinus*	28	S.G., P., W.G., UnH.	strict protected species	* * * *	Palearctic
42.	*Coccothraustescoccothraustes*	3	P., UnH.	strict protected species	*	Palearctic
43.	*Passer montanus*	40	S., UnH.	-	* * *	Palearctic
Fam. **Emberizidae**						
44.	*Emberizaschoeniclus*	27	S.G., P. H.	strict protected species	* * *	Palearctic

S.G. - summer guest; W.G. - winter guest; P.- passage; UnH. - unhatched species; H. - hatched species; S. – sedentary; * accidentally; ** rare; *** frequently; **** very frequently

From all *Passeriformes* collected the biggest number was found in Muscicapidae familiae (n = 2417/6619, 36.51%), followed by Sylviidae (n = 1963/6619, 29.65%), Turdidae (n= 864/6619, 13.05%), Paridae (n =575/6619, 8.68%), Laniidae (n = 449/6619, 6.78%), Fringiliidae (n =137/6619, 2.07%), Troglodytidae (n = 65/6619, 0.98%), Motacilidae (n = 40/6619, 0.60%), Paradoxomithidae (n = 34/6619, 0.51%), Oriolidae (n = 33/6619, 0.50%), Emberizidae (n =27/6619, 0.40%), Corvidae (n = 12/6619, 0.18%) and Hirundinida (n = 3/6619, 0.05%) (Figure 3).

Dominance index showed that eudominant species were *Erithacus rubecula* (18.25%) and *Phylloscopus trochilus* (10.34%), followed by dominant species such as *Sylvia atricapilla* (7.32%), *Lanius collurio* (6.78%), *Turdus merula* (6.63%), *Turdus philomelos* (5.98%), *Ficedul aparva* (5.24%). Seven species were classified as subdominat species (2-5%), 2 species were recedente (1-2%) and 28 subrecedente (0-1%).

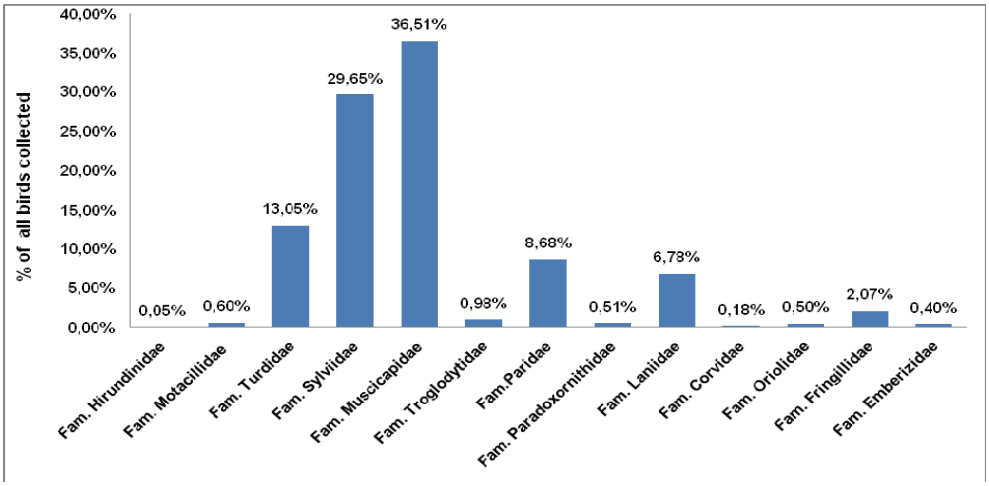

Figure 3. Families' distribution within Passeriformes order collected from Sacalin Island

In the last 10 years natural disasters have become increasingly common in Danube Delta, and the years 2005 - 2006 was recorded the highest values of pollution in Danube river over the last 100 years. Pollution, damming, industrializations, agriculture, livestock, and urban settlements disrupt the fragile ecology of the Danube Delta. Over-exploitation of birds, frogs, and introduction of exotic species constitute other significant threats. Recent human conflicts in the Danube basin have also had negative impacts on this ecoregion. All this will have a significant impact on unique biodiversity of the Danube Delta (Sinclaire et al., 2006). To prevent the destruction of these valuable ecoregions, which represent the habitat for over 300 species of birds, we should improve the

nesting and wintering condition for birds and protect them from illegal hunting.

Order Passeriformes is well represented in Danube Delta; a total number of 44 species were identified. Many of them were summer or winter guest and are protected species (Trevor et al., 2010).

CONCLUSIONS

In conclusion, our data suggest that the Sacalin Island is an area with a wide diversity of Passeriformes, uncovering the ecology and distribution of this group of birds could greatly improve the knowledge of bird's dynamics and help to develop better protection measures for bird's conservation.

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MARKET SURVEY CONCERNING ORGANIZATIONAL CONDITIONS OF VETERINARY PHARMACIES IN BUCHAREST

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Abstract

Currently, in Romania, retailing medicinal and other veterinary products is an important part of veterinarians' activity. The study was conducted from March to September 2013 in 20 veterinary pharmacies in Bucharest. The analyzed issues were related to: location, size, connection to mandatory utilities, drugs regime, arrangement of medicinal products on the shelf, conditions of microclimate, holding the required documents, filing records, employed personnel. Of the 20 veterinary pharmacies analyzed, 15 were located at the basement of residential buildings and had separate access than the one of tenants, while the remaining 5 pharmacies were located in separate buildings. All veterinary pharmacies were connected to sewer, water and electricity, and 13 pharmacies were secured with anti-theft systems. None of the analyzed veterinary pharmacies had laboratory, which means that none of these pharmacies prepared medicines. Veterinary pharmacy's warehouse was equipped with temperature and humidity insurance systems in 12 pharmacies. In 8 pharmacies, microclimate parameters were recorded in special registers. Veterinarians were employed in all studied pharmacies, while in 7 units the personnel includes also veterinary technicians. Lockers for the storage of substances included in Separanda and Venena lists were present in 16 pharmacies. The study of 20 veterinary pharmaceutical units in Bucharest showed that they largely respect the organizational conditions specified by law; however, there is a relatively small number of units fully complying with legislative requirements.

Key words: *veterinary pharmacy, veterinary pharmaceutical units, organizational conditions, market survey*

INTRODUCTION

Nowadays, the veterinarian should be a good practitioner and, in addition, a good businessman, a good organizer and leader in order to develop a successful business which can survive in an increasingly competitive environment. In this context, veterinary pharmacists have a particularly challenging task, the decisions that they need to take in organizing the work being the key point of development, survival or bankruptcy of their pharmaceutical unit.

According to ANSVSA Order nr. 41/2012, veterinary pharmacy is the sanitary-veterinary unit which holds and sells veterinary medicinal products, cosmetic and hygiene products for animals, instruments, herbal products, feed, medicated feed, complementary feed and other products for animals, in compliance with legal provisions in force.

The aim of this study was the analysis of compliance with the organizational conditions of veterinary pharmacies in Bucharest, according to regulations specified by national legislation in force (ANSVSA. Order nr. 41/2012, ANSVSA Order nr. 64/2012, ANSVSA Order nr. 11/2013, ANSVSA Order nr. 4/2004).

MATERIALS AND METHODS

The study was carried out between March and September 2013 in 20 veterinary pharmacies in Bucharest. We tried to get accurate data by talking to each veterinary pharmacist and by analyzing factual conditions of the pharmacies.

We evaluated veterinary pharmacies in terms of organizational conditions and noted in tables if they fulfill or not legal requirements in relation to certain criteria: location, number of rooms, size, connection to mandatory utilities, drugs regimen, conditions of microclimate, required documents, employed personnel, etc. (Table 1).

Table 1. Organizational conditions of the veterinary pharmacies analyzed in the study

No.	Specification		Yes	No
1	Veterinary pharmacy has sanitary-veterinary authorization			
2	The firm respects all the instructions provided by the College of Veterinarians			
3	The rooms of veterinary pharmacy are functionally linked together, without being spread inside the building			
4	The access in the veterinary pharmacy is by using a different entrance from the tenants of the building - in the case of pharmacies located on the ground floor of residential buildings			
5	Veterinary pharmacy	is secured with anti-theft systems		
		is connected to a source of water		
		is connected to sewerage		
		is connected to electricity		
6	Oficina is equipped with appropriate furniture			
7	Veterinary pharmacy has laboratory			
8	In the veterinary pharmacy are made divisions of veterinary medicinal products			
9	The veterinary pharmacy prepares magistral and officinal recipes			
10	Veterinary pharmacy warehouse is equipped with temperature and humidity insurance systems			
11	In veterinary pharmacy warehouse, microclimate parameters are recorded in special registers			
12	Veterinary pharmacy warehouse is equipped with shelves, cabinets and pallets made of easily washable materials, fire- and corrosion-resistant			
13	In the warehouse, veterinary products are placed on shelves by groups of substances			
14	In the warehouse, the groups of products are labeled with name, batch, validity and marketing / manufacturing authorization number			
15	Veterinary pharmacy warehouse is equipped with fridges with thermometers metrological checked and with temperature recording sheets			
16	Veterinary pharmacy opening hours is displayed in a visible place			
17	In the veterinary pharmacy operates personnel consisting of:	staff with specialty higher education studies - veterinarians		
		staff with higher education studies - other than veterinarians		
		staff with secondary studies – veterinary technicians		
18	Veterinary pharmacy has a useful area sufficient and appropriate for the specific activity			
19	Veterinary medicinal products sold in the veterinary pharmacy have marketing authorization			
20	In the veterinary pharmacy there are metal cabinets with locking systems, for the storage of toxic substances, narcotics, psychotropic substances and their precursors and for substances and pharmaceutical preparations listed in Separanda and Venena tables			
21	There is a numbered, stamped and sealed register for toxic substances, narcotics, psychotropic and other therapeutic groups that are issued based on a prescription which is retained in the veterinary pharmacy			
22	Veterinary medicinal products in the pharmacy are accompanied by official documents stating:	product name		
		series and batch of manufacturing		
		validity period		
		manufacturer		
		quality certificate		
23	Veterinary pharmacy keeps track of prescriptions for magistral and officinal preparations			
24	In the veterinary pharmacy exists:	a register recording magistral and officinal preparations		
		a register recording veterinary medicinal products used for making magistral and officinal formulas		
		a register recording prescriptions not retained in the pharmacy		
		documents evidencing the release of toxic substances, narcotic drugs and psychotropic on prescription only		
		list of suppliers		
25	Medical prescriptions which are retained in veterinary pharmacy are properly archived and kept throughout the period provided by law			
26	Veterinary medicinal products are placed on shelves by action groups, allowing separation of batches and stock rotation			
27	Veterinary pharmacy's technical library contains, on paper or electronically:	Romanian Pharmacopoeia, current edition, if magistral and officinal formulas are prepared		
		Nomenclature of veterinary medicinal products in force		
		Sanitary/veterinary legislation in force and, in particular, that relating to veterinary medicinal products		

RESULTS AND DISCUSSIONS

All analyzed veterinary pharmacy had sanitary-veterinary authorization (20 out of 20, 100%).

Of all the pharmacies surveyed, a total of 11 (55%) had on the company firm all the elements specified by the College of Veterinarians, while the firm of the remaining 9 pharmacies (45%) contained only some elements.

The rooms of veterinary pharmacies were functionally linked together, not being spread inside the building in case of 14 pharmacies (70%).

Of the 20 veterinary pharmacies analyzed, 15 (75%) were located at the basement of residential buildings and had separate access than the one of tenants, while the remaining 5 pharmacies (25%) were located in separate buildings.

All veterinary pharmacies studied (100%) were connected to sewerage, water and electricity, while 13 pharmacies (65%) were secured with anti-theft systems.

Oficina was equipped with appropriate furniture in case of 18 veterinary pharmacies (90%).

None of the analyzed veterinary pharmacies had laboratory (0%), which means that none of these pharmacies prepared magistral and officinal medicines. As a consequence, analyzed veterinary pharmacies didn't keep track of prescriptions for magistral and officinal preparations and didn't have registers recording magistral and officinal preparations or veterinary medicinal products used for making magistral and officinal formulas.

None of the studied veterinary pharmacies (0%) made divisions of veterinary medicinal products.

Veterinary pharmacy warehouse was equipped with temperature and humidity insurance systems in case 12 pharmacies (60%). In 8 pharmacies (40%), these microclimate parameters were recorded in special registers.

Veterinary pharmacy warehouse was equipped with shelves, cabinets and pallets made of easily washable materials, fire- and corrosion-resistant in case of 13 pharmacies (65%).

In the warehouse, veterinary products were placed on shelves, by groups of substances in 14 pharmacies (70%). Also, veterinary products in the warehouse were labeled with name, batch, validity and marketing / manufacturing authorization number in care of all pharmacies (100%).

Fridges and thermometers metrological checked were found in the warehouses of 15 pharmacies (75%).

19 veterinary pharmacies (95%) displayed opening hours in a visible place.

Veterinarians were employed in all studied pharmacies (100%), while in 7 units (35%) the personnel included also veterinary technicians. Staff with higher education studies - other than veterinarians was not identified in the analyzed veterinary pharmacies.

Veterinary pharmacies had a useful area sufficient and appropriate for the specific activity in 15 cases (75%); for the remaining 5 pharmacies (25%), useful space was considered too small.

Veterinary medicinal products sold in all studied veterinary pharmacies (100%) had marketing authorization.

Metal cabinets with locking systems, for the storage of toxic substances, narcotics, psychotropic substances and their precursors and for substances and pharmaceutical preparations listed in *Separanda* and *Venena* tables were present in 16 pharmacies (80%).

In all the 20 analyzed pharmacies (100%), veterinary medicinal products were accompanied by official documents stating product name, series and batch of manufacturing, validity period, manufacturer and quality certificate.

All veterinary pharmacies had the register recording prescriptions which are not retained in the pharmacy, documents evidencing the release of toxic substances, narcotic drugs and psychotropic on prescription only and list of suppliers.

Medical prescriptions which are retained in veterinary pharmacy were properly archived and kept throughout the period provided by law in case of 17 pharmacies (85%).

Veterinary medicinal products were placed on shelves by action groups, allowing separation of batches and stock rotation in 14 pharmacies (70%).

Veterinary pharmacy's technical library contained, on paper or electronically, the following documents: Romanian

Pharmacopoeia, current edition – 7 pharmacies (35%), nomenclature of veterinary medicinal products in force – 17 pharmacies (85%) and sanitary/veterinary legislation in force and, in particular, that relating to veterinary medicinal products – 18 pharmacies (90%).

CONCLUSIONS

The study of 20 veterinary pharmaceutical units in Bucharest showed that they largely respect the organizational conditions specified by law; however, there is a relatively small number of units fully complying with legislative requirements.

None of the analyzed veterinary pharmacies prepared magistral and officinal medicines, aspect sustained by the absence of the special room for this purpose, namely the laboratory.

All the studied pharmaceutical units have in the staff list employees with specialty higher education studies, namely veterinarians.

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CLINICAL SCIENCES

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NOVEL ASPECTS OF THE CANINE MAST CELL TUMORS

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Abstract

The authors present the morphological and clinical casuistry of canine mastocytoma diagnosed and treated in the last few years at the Clinics of the Faculty of Veterinary Medicine in Bucharest.

Keywords: *diagnosis, mastocytoma, therapy.*

INTRODUCTION

The scholarly literature (Baba, 2002; Hottendorf, 1968 1969; Magnol, 1990; Patnaik, 1982, 1984; Standard, 1978; Toulemonde-Bassede, 1986) sets the frequency of canine mastocytoma at 4% of the total oncological manifestations in dogs. The localization of mastocytoma may be cutaneous or visceral. This type of tumour usually metastasizes to the lymph nodes, liver and kidney. Mast cell leukaemia has also been described.

The analysed morphological and clinical forms were as follows:

- with mature cells;
- with intermediately differentiated cells;
- with anaplastic cells.

Regardless of the cellular form of the mastocytoma, the frequency of dividing cells is considerably high.

MATERIALS AND METHODS

Within the oncological casuistry at the Medical Clinic of the Faculty of Veterinary Medicine in Bucharest, during 2008 – 2014, out of 931 cancer cases, the type of cancer identified as mastocytoma was diagnosed in a number 17 cases, with a clear dominance of the cutaneous form.

The mastocytoma diagnosis was given on the basis of the morphological and clinical aspect, complemented by fine needle aspiration for cytomorphological examination. The staining employed was May-Grünwald-Giemsa.
A special mention is due to the identification of a cutaneous mastocytoma form that has metastasized in the mammary gland parenchyma.

RESULTS AND DISCUSSIONS

In our casuistry, canine mastocytoma registered a frequency of 1.8%, which,

compared to the data provided by the scholarly literature, representing half of the announced and published frequency. The 17 cases diagnosed with mastocytoma had the following topographical distribution:

- Cutaneous form – 9 cases, representing 55% of the total number of mastocytoma diagnosed cases;
- Mucosal form – 1 case, representing around 5.5% of the total number of mastocytoma diagnosed cases;
- Subcutaneous form – 3 cases, representing around 15% of the total number of mastocytoma diagnosed cases;
- Lymph node metastasis (without the identification of the primary tumour) – 1 case, representing around 5.5% of the total number of mastocytoma diagnosed cases;
- Mast cell leukaemia - 1 case, representing around 5.5% of the total number of mastocytoma diagnosed cases;
- Metastasis in somatic cavities – 2 cases, representing around 11% of the total number of mastocytoma diagnosed cases.

The average age at which the disease develops, according to our statistics, ranges between 8 and 14 years. Out of our total casuistry, this range was attributed to 70% of the cases.

Concerning the matter gender predisposition, the dominating prevalence is registered in males.

The breeds most commonly affected are boxer, german shepperd and mixed breed dogs, registering three cases for each of the previously mentioned.

Investigations have shown that the prevalent cytomorphological form is that of malignant mastocytoma with mature cells, having a low mitotic index. Nevertheless, even this type of neoplasm had shown a high level of aggressiveness. The anaplastic forms with blast mast cells (and precursor cells) are rarer; these register a high mitotic index and consequently a higher level of aggressiveness.

As a special note in canine mastocytoma, we have identified two morphological and clinical forms:

- cutaneous mastocytoma metastasis in the mammary gland parenchyma
  and
- mast cell leukaemia.

CONCLUSIONS

Mastocytoma is a tumoral form of the connective tissue which registers a moderate frequency in canine specie (1.8% of the total malignant tumours diagnosed at the Clinics of the Faculty of Veterinary Medicine in Bucharest).

The most frequent morphological and clinical forms form of mastocytoma is the cutaneous form, the mucosal type being extremely rare;

Mast cell leukaemia is an extremely rare form of malignant haemopathy;

The cutaneous mastocytoma metastasis in the mammary gland parenchyma was a surprise diagnosis as it has not been described in the scholarly literature.

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STAPHYLOCOCCI RESISTANT PHENOTYPES OF THE INTERMEDIUS GROUP ISOLATED FROM DOGS

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Abstract
Dogs have frequently infections with coagulase-positive and coagulase-negative staphylococci with different locations. A total of 49 samples consisting of pathological skin, auricular and genital secretions were bacteriologically examined using standard methodology.
The isolates were tested by disk diffusion Kirby-Bauer method, on which were used biodiscs with 19 antibiotics from different groups.
The staphylococci strains isolated from dogs included in Intermedius Group were susceptible to the antibiotics rarely or not used in the therapy of diseases in this species.
The isolates were methicillin-resistant strains, thus emphasizing the movement of these strains in the canine population, confirming the zoonotic risk of these strains.
After this study several resistant phenotypes of staphylococci strains included in Intermedius Group were identified, whose frequency was variable.

Key words: *antibiotic resistance, Grup Intermedius, methicillin-resistant.*

INTRODUCTION

Dogs have frequently infections with coagulase-positive and coagulase-negative staphylococci with different locations. Most common are the Intermedius Group staphylococci, including *S. intermedius* and *S. pseudintermedius*. They are coagulase-negative staphylococci and are part of the resident flora of the dogs. (Schissler, 2009)
Staphylococci in this group have also been found to pathogens for other animal species, including humans, and methicillin-resistant strains are considered as zoonotic risk strains, regardless of the species to which they belong. (Frank and Müller, 2012, Degi et al., 2012)
The research followed the frequency of resistant phenotypes of staphylococcal strains to antibiotics, included in this group, isolated from dogs with various diseases.

MATERIALS AND METHODS

A total of 49 samples consisting of pathological skin, auricular and genital secretions were taken from dogs with various diseases. The primary inseminations were made on agar with 5% defibrinated sheep blood and the isolated strains were initially sorted based on cultural, morphological and tinctorial characters.
Isolated and purified staphylococcal strains were biochemically tested on Chapmann medium, on Difco agar with maltose, pH indicator and, finally, through the API Staph system.
The examined pathogenic factors were represented by hemolysins and clumping factor. (Degi et al., 2012)
The isolates were tested by disk diffusion Kirby-Bauer method, on which were used biodiscs with 18 antibiotics from different groups (table 1) and the results were interpreted according to the CLSI Standard. (Bemis et al., 2009)

RESULTS AND DISCUSSIONS

The bacteriological examination performed by the described methodology allowed the isolation of 45 strains included in Intermedius Group based on the main phenotypic tested characters.
The strains of staphylococci unexposed to the pressure of antibiotics are susceptible to these substances, while the strains isolated from dogs with various conditions, under the

pressure of antibiotics, due to therapy, may exhibit the occurrence of multiple resistance.

The results obtained by testing the susceptibility to antibiotics of staphylococcal strains isolated from dogs are shown in Table 1.

Resistant phenotypes and the frequency of methicillin-resistant and cefoxitin-resistant strains were pursued at a total of 44 staphylococcal strains, using 19 antibiotics (Table 1).

Table 1. Methicillin-resistant phenotypes isolated from dogs

No.	Antibiotic	Antibiogram results						Total strains
		Susceptible		Intermediary		Resistant		
		No.	%	No.	%	No.	%	
1	Methicillin	35	79,5	2	4,5	7	15,9	44
2	Ampicillin / Sulbactan	44	100	-	-	-	-	44
3	Amoxicillin / Clavulanic acid	8	18,18	30	68,18	6	13,63	44
4	Ceftioxone	43	97,72	-	-	1	2,72	44
5	Cefoxitin	36	81,81	8	18,18	-	-	44
6	Cefaclor	36	81,81	8	18,18	-	-	44
7	Cefuroxime	-	-	36	81,81	8	18,18	44
8	Erythromycin	5	11.3	6	13,6	33	75	44
9	Lincomycin	6	13,6	6	13,6	32	72,72	44
10	Rifampicin	8	18,18	36	81,81	-	-	44
11	Gentamicin	7	15,9	37	84	-	-	44
12	Kanamycin	-	-	36	81,81	8	18,18	44
13	Tetracycline	2	4,5	9	20,4	33	75	44
14	Doxycycline	-	-	36	81,81	8	18,18	44
15	Ciprofloxacin	43	97,7	-	-	1	2,27	44
16	Vancomycin	8	18,18	36	81,81	-	-	44
17	Polymyxin B	-	-	8	18,18	36	81,81	44
18	Novobiocin	5	11,3	39	88,6	-	-	44
19	Pristinamycin	36	81,81	8	18,18	-	-	44

Analyzing the results from the table it can be seen that the rate of resistant phenotypes was variable depending on the groups of antibiotics.

Against the β–lactam group used (methicillin, ceftioxone, cefoxitin, cefaclor, ampicillin with sulbactan and amoxicillin with clavulanic acid) antibiotic susceptibility was highest to ampicillin, followed by cefuroxime, cefaclor, cefoxitin, methicillin and minimum to ceftioxone.

Intermediary antibiotic susceptibility was to ceftioxone and amoxicillin with clavulanic acid, and antibiotic resistance was shown to cefuroxime, amoxicillin with clavulanic acid and methicillin and absent to cefoxitin, cefaclor and ampicillin with sulbactan.

The various behaviour of the strains, tested to this group of antibiotics, is influenced, in dogs, by use of antibiotics mentioned in therapy, some of which are being used only for humans.

CHROBAK and col. notify similar results, observing that the staphylococci from Intermedius Group were resistant, in high proportions, to amoxicillin with clavulanic acid, ciprofloxacin, clindamycin and gentamicin.

The occurrence of antibiotic resistance in β-lactam group is based on genetic determinants located in plasmids and chromosomes, which governs the synthesis of β-lactamases with large spectrum, that provides, thus, the staphylococci resistance.

Analyzing the results it can be observed that at the tested strains, methicillin resistance was of 15.9% and to cefoxitin resistant strains were not found.

The resistance to methicillin is governed by *mec* gene and transmitted by R plasmid, also having a common pattern to other β-lactams, therefore, oxacillin and cefoxitin can also be used for testing the presence of methicillin resistance in staphylococci, regardless of species or coagulase activity. (Frank and Müller, 2012)

For this reason, methicillin-resistant strains of staphylococci are considered strains with high zoonotic risk, that have a complex circuit, which is human-animal-human.

Against aminoglycosides used (gentamicin and kanamycin) antibiotic resistance was between 15.9% and 18.18%. Only these two antibiotics were used for testing, because in dogs aminoglycosides are rarely used, sometimes being used even products for humans, which may explain the high proportion of strains with intermediary behaviour.

The antibiotic resistance to this group of antibiotics is determined by a gene located either plasmidic or chromosomal, responsible for the synthesis of aminoglycoside acetyl transferase, enzyme that induce the resistance,

which explains the behaviour of isolated strains.
The resistance to macrolides (erythromycin) was of 75%, also indicating an inducible resistance to macrolides with 14 atoms that can be used in dogs, other animals and humans.
The resistance to tetracyclines was of 75% to tetracycline and 18.18% to doxycycline, procedure that can be explained by the fact that the doxycycline is less used in dogs.
Resistance to tetracycline group is governed by the *tet* gene present in the plasmid and the chromosomal genetic material and is common to all antibiotics of this group.
The resistance to fluoroquinolones, at the staphylococci isolated from dogs, it is rarely indicated, as this group of antibiotics gives various side effects to this species. Resistance was tested only to ciprofloxacin, which was in a proportion of 2.27%.
The resistance to lincomycin was of 72.72%, to polymyxin was of 81.81% and to vancomycin was absent. This behaviour is difficult to explain because these antibiotics are rarely used in dogs.
In case of novobiocin, rifampicin and pristinamycin, considered to be the elective antibiotics for staphylococci, there was no antibiotic resistance to the isolated and tested strains. This suggests that, in veterinary therapy, these three antibiotics are not used, but represents a kit for staphylococci in human pathology.
In case of staphylococci, the increase of resistance to various antibiotics is a consequence of the abuse in treatment of diseases in dogs, or of dog contamination with strains of human origin (from hospital).
As a result of testing the staphylococcal strains isolated from dogs to 19 antibiotics, there were identified methicillin-resistant strains and more resistant phenotypes to the groups of antibiotics used in the form of biodiscs.

CONCLUSIONS

The staphylococci strains isolated from dogs included in Intermedius Group were susceptible to the antibiotics rarely or not used in the therapy of diseases in this species.
The isolates were methicillin-resistant strains, thus emphasizing the movement of these strains in the canine population, confirming the zoonotic risk of these strains.
After this study several resistant phenotypes of staphylococci strains included in Intermedius Group were identified, whose frequency was variable.

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PHENOTYPIC CHARACTERIZATION OF STAPHYLOCOCCI ISOLATED FROM SMALL RUMINANTS

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Abstract
Staphylococcal infections are common in small ruminants and are represented by localized infections of the skin, hooves and mammary gland.
The pathological samples for bacteriological examination were taken from a total of 37 sheeps and goats with different lesions and the primary inseminations were made on agar with 5% sheep defibrinated blood. Biochemical properties were revealed by API Staph system. The isolates were tested against novobiocin and methicillin using the Kirby-Bauer method with biodiscs.
37 strains of staphylococci were isolated and included in *S. aureus* ssp. *aureus* species (32 strains) and in *S. xylosus* species (5 strains).
16 methicillin-resistant strains were identified that belong to those two staphylococci species, confirming thus the epidemiological circuit of these strains also in small ruminants.

Key words: *S. aureus* subsp. *aureus, S. xylosus, small ruminants.*

INTRODUCTION

Staphylococcal infections are common in small ruminants and are represented by localized infections of the skin, hooves and mammary gland. These infections are caused by *S. aureus.* subsp. *aureus* and less often by other staphylococci species. (Cătana, 2001; Velescu, Tănase and Irina, 2010)
These infections have a variable clinical evolution and the staphylococci strains that produce them have a complex epidemiological circuit, some of which are pathogenic for humans, too. (Mørk, 2012; Velescu, Tănase and Irina, 2010)
In the last years, there have also been isolated, from small ruminants, methicillin-resistant strains of staphylococci, which proves their intra and interspecific movement and that the occurence of methicillin resistance represents a remarkable zoonotic risk. (Mørk et al., 2012; Leitner et al., 2011)
The research was made in order to identify and characterize phenotypically the strains of staphylococci isolated from small ruminants.

MATERIALS AND METHODS

The pathological samples for bacteriological examination were taken from a total of 37 sheeps and goats with different lesions and the primary inseminations were made on agar with 5% sheep defibrinated blood.
The isolated strains were sorted based on cultural, morphological and tinctorial characters.
The clumping factor was revealed by Prolex Staph Latex fast kit, mannitol fermentation was tested on Chapmann medium and the biochemical properties were revealed by API Staph system. (Codiţă, 2008)
After the final biochemical identification, the isolates were tested against novobiocin and methicillin using the Kirby-Bauer method with biodiscs. (Codiţă, 2008)

RESULTS AND DISCUSSIONS

After the primary insemination from the pathological samples, 37 strains were isolated of which 32 strains formed yellow colonies and produced β-haemolysis while 5 strains

formed white unhaemolytic colonies. All strains were Gram positive and on the bacterioscopic examination, the bacterial cells were grouped in clusters and, rarely, in pairs.
These preliminary tests showed that bacterial strains with cultural, morphological and tinctorial features typical for staphylococci were isolated from samples of pathological material taken from sheep and goats with different lesions.
The isolated strains fermented the mannitol and produced clumping factor, and with API Staph system were standardized and included in the following species: *S. aureus* subsp. *aureus* and *S. xylosus*. All the strains standardized and included in these species were susceptible to novobiocin, and a total of 21 strains were methicillin-resistant, 16 of *S. aureus* subsp.. *aureus* and 5 of *S. xylosus*.
The methodology used for phenotypic characterization of staphylococci isolated from small ruminants is fast and can be taken in routine diagnosis. Staphylococcal strains, phenotypically classified in those two species, were isolated from sheeps and goats, most of them belonging to *S. aureus* subsp. *aureus* species.
The strains included in *S. xylosus* species are ubiquitous and less pathogenic in small ruminants. (Velescu, Tănase and Irina, 2010)
The strains of *S. aureus* subsp. *aureus* were isolated from mammary and hooves infections, but also from the skin without problems.
Identification of methicillin-resistant strains demonstrates the porting of *mec* gene, but also a complex epidemiological circuit of these strains, which was demonstrated by several researchers.

CONCLUSIONS

37 strains of staphylococci were isolated by the used methodology, which based on the phenotypic characters were included in *S. aureus* subsp. *aureus* species (32 strains) and in *S. xylosus* species (5 strains).
The isolated strains were susceptible to novobiocin, a constant character that differentiates the *Staphylococcus* genus from *Micrococcus* and *Streptococcus* genus.
16 methicillin-resistant strains were identified that belong to those two staphylococci species, confirming thus the epidemiological circuit of these strains also in small ruminants.

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ENDOSCOPICAL MONITORING OF THE MARE'S REPRODUCTIVE TRACT FOR ESTRUS AND OVULATION DETECTION

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Abstract

Detecting the right moment of estrus in witch ovulation occurs it is extremely important for mare's biotechnology reproductive management and especially for mare breeding soundness evaluation. Endoscopic examination of mare's reproductive tractus makes possible the visualization of the vagina, the external cervical ostium, the lumen of the uterine body and the horns up to the uterotubal junctions, in order to obtain a large amount of information related with mares reproductive status. The endoscopic reproductive examination was performed at the Faculty of Veterinary Medicine Bucharest, for 15 mares from different races, examined for a research study concerning the best ways to monitor the ovulation. The examination was performed with a Olympus GIF K2, a flexible endoscope.

Keywords**:** *mare, reproduction, endosccopy, estrus, ovulation.*

INTRODUCTION

The main objective of this paper is to present the changes occurring in the genital tract during the course of estrus and ovulation, in terms of direct observations obtained using endoscopic examination of mares. Various endoscopic aspects from cervical level and uterine cavity where compared with the reproductive phase of the mares from this study in order to establish a possible protocol for detection of the best moment for insemination, respectively of ovulation and for mare breeding soundness evaluation.

MATERIALS AND METHODS

The endoscopic reproductive examination was performed at the Faculty of Veterinary Medicine Bucharest, for 15 mares from different races. The examination was performed with a Olympus GIF K2, flexible endoscope, 130 cm length, 13 mm diameter and a 150 W light source. After contention and local preparation, air was infused in the uterine cavity for a good distention of the uterine walls. Every mare was examened during one estrus cycle.

RESULTS AND DISCUSSIONS

A number of 15 Romanian Sport Horse mares and mix breed were examined. The cervix of oestrus mares appared by endoscopy relaxed, soft with cervical ostium opened, associated with the time preceding ovulation during estrus (Figure 1.) In the luteal phase by endoscopy the appearance was of a closed cervical ostium associated with post ovulation period, subsequent to the formation of the corpus luteum and the secretion of progesterone (Figure 2.)

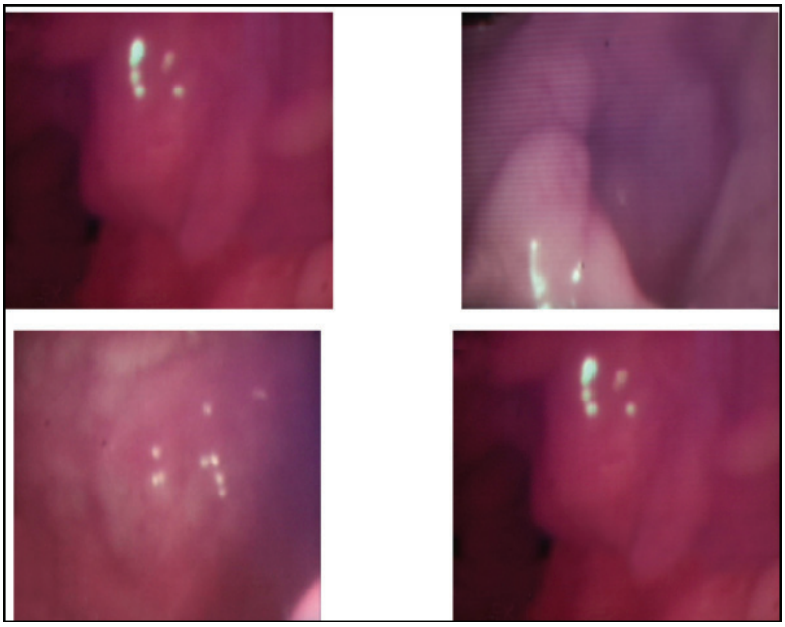

Figure 1. Endoscopic view-relaxed cervix, mare during estrus

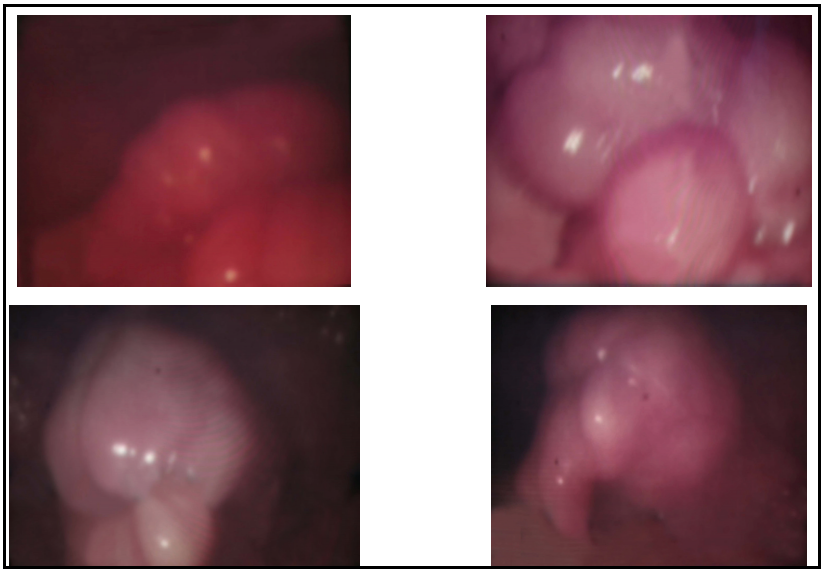

Figure 2. Endoscopic view-ferm cervix, mare during diestrus

Uterine folds of the mares in oestrus phase showed a endoscopic edematous appearance, associated with the time preceding ovulation (Figure 3.) and an erased appearance associated with the luteal phase (Figure 4.).

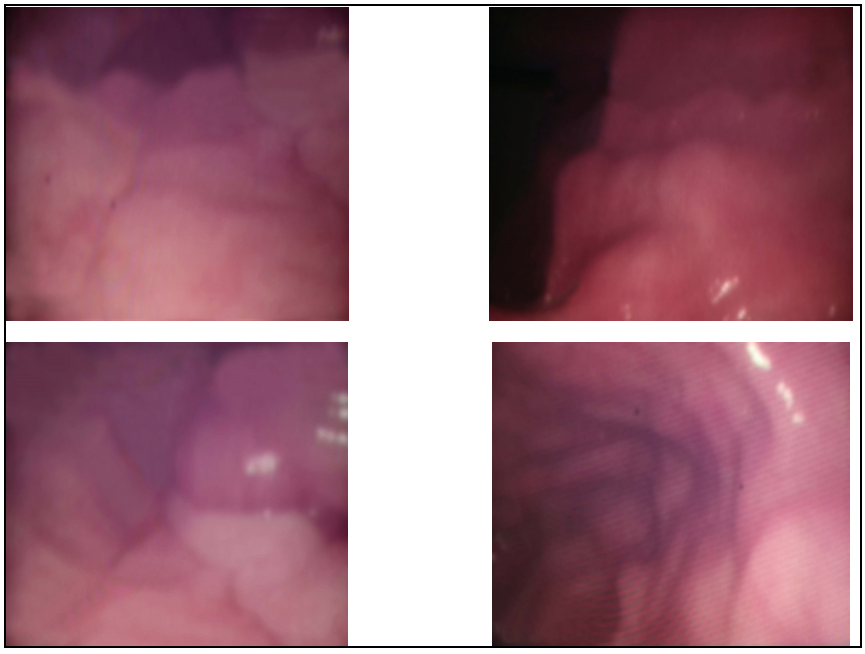

Figure 3. Uterine view-edema of the uterine falds, mare during estrus

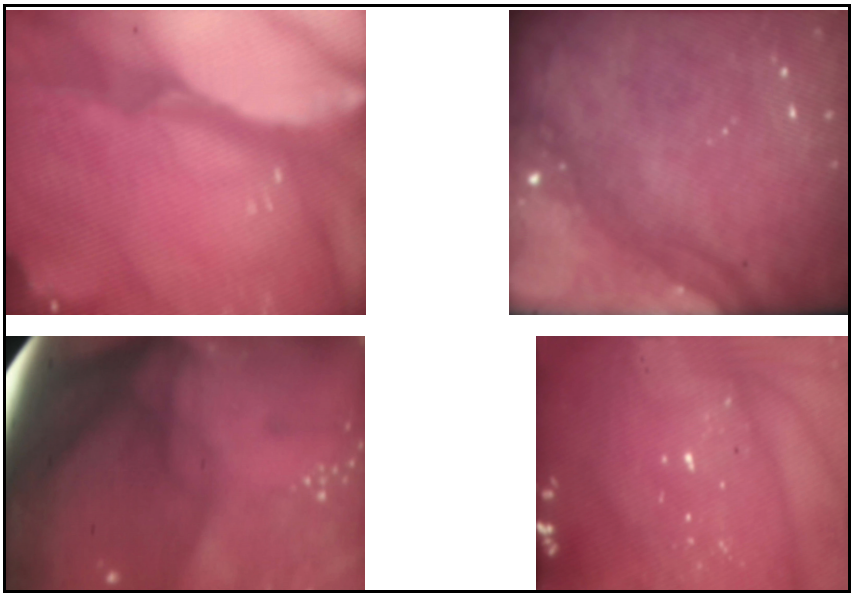

Figure 4. Uterine endoscopic view-erased uterine falds, mare during diestrus

We established a direct correlation between endoscopic appearance of the cervix and endometrial folds and different times of the estrous cycle so that it can be considered that endoscopy, as a screening method is particularly useful in the management of equine reproductive biotechnologies. At the uterine bifurcation level and the oviductal papilla site we could not establish a clear correlation of the endoscopical aspects obtained and the status of the mares examined.

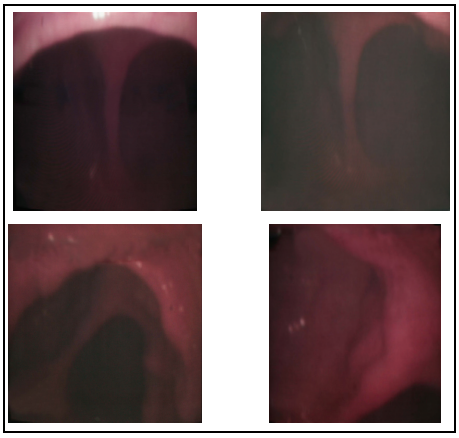

Figure 5. Endoscopic view of the uterine bifurcation

CONCLUSIONS

Detecting the right moment of mare's estrus in witch ovulation occurs it is extremely important

A direct correlation between endoscopic appearance of the cervix and endometrial folds and different times of the estrous cycle was established.

It can be considered that endoscopy can be a screening method useful in the management of equine reproductive biotechnologies and this observations can have a applicability to other species in order to maintain biodiversity.

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GENERAL CONSIDERATIONS OF BLOOD BANKING AND TRANSFUSION IN DOG: A REVIEW

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Abstract

This review is a practical aspect of transfusion therapy for dogs and of the blood banking process and transfusion standards. Many of these aspects are based of the current veterinary and human standards.

The first documented transfusion occurred in 1665, and was made by withdrawing blood from one dog and replaced it with blood from another dog. Since then veterinary transfusion medicine has made remarkable progress, following close to our human contra parts.

Whole blood refers to blood that has not been separated. Blood products are composed from blood components and these are prepared either by centrifugation or by apheresis. The use of blood components allows several patients to benefit from one donation and reduces the risk of transfusion reactions to unnecessary components.

Both whole blood and blood components may be used shortly after the collection or after storage, Blood banking allowing the user access to both blood and blood components immediately. This procedures may be feasible to obtain and process blood on demand. However for emergency clinics with a large requiring caseload of transfusion therapy, blood banking is essential.

Key words: *transfusion, blood, blood banking, donor, blood group.*

INTRODUCTION

Transfusion is defined as an intravenous therapy with blood products or whole blood. Blood banking allows immediate access to whole blood and blood components in any cases (Abrams-Ogg, 2000; Gibson, 2007)

The blood banking procedures that must be followed refer to the blood donors, the dog blood groups, the blood typing, cross-matching anticoagulant (preservative solution), the blood donation procedure, collection systems, the blood product preparation and storage, transfusion of dog whole blood and blood components. (Abrams-Ogg, 2000; Gibson, 2007).

Blood donors

The source of blood donors may be obtained from clinic owned donors (depending on the anticipated needs), from a donor program (client owned pets that donate for benefits), animal controlled facilities (animal shelters or pounds that may allow stray dogs to donate), terminal donors (animal that are being euthanized for behavior problems or medical disorders that do not affect the quality of the donated blood). (Gibson, 2007; Abrams-Ogg, 2000; Wardrop, et al., 2005; Slichter, et al., 1986)

An ideal blood donor should be clinically normal, large breed with normal weight (25 – 28kg), friendly and has easily accessible veins

and a universal blood type, has to be current on the vaccination status, free of parasites and infectious disease (depending on the geographical location) and do not suffer of other disorders (immune mediated, cancer, systemic disorders, organ failure) and did not receive any drug therapy or had previous transfusions. (Gibson, 2007; Palmer, et al., 2014; Abrams-Ogg, 2000; Palmer, et al., 2014; Chervier, et al., 2012; Horgan, et al., 2009; Hackner, 2015)

Blood groups

The definition of blood groups are made by the inherited antigens of the RBC surface. They are of crucial importance in the transfusion medicine because of the risk of hemolytic reactions that occur when there is an antibody directed against a blood group antigen, depending on the complement activation by IgM and IgG. (Abrams-Ogg, 2000; Slichter, et al., 1986; Lynel, et al., 2009).

The blood groups are classified in the DEA (Dog Erythrocyte Antigen) and there are sex antigens (DEA 1.1, 1.2, 3, 4, 5, 7) defined by the current standardization antisera and a new antigen Dal, but 20 or more specificities have been described. DEA`s have not been extensively characterized for composition and structure. (Corato, et al., 1997; Abrams-Ogg, 2000; Gibson, 2007; Blais, et al., 2007).

Blood typing

The compatibility of donor recipient should be considered when selected a donor. To prevent DEA incompatibility is to blood type the donors using antisera. The antisera is produced by allow-immunizing a dog negative for a given DEA. Blood typing is not performed at a regular basis in veterinary medicine because of the prices and is performed on a small number of donors. The universal blood donor is negative to DEA 1.1, 1.2, 3, 5 and 7 and the minimal requirement to prevent a moderate or a severe DEA hemolytic reaction needs a minimum for the donor to be negative for DEA 1.1. (Lynel, et al., 2009; Abrams-Ogg, 2000; Gibson, 2007).

Cross-matching

The cross-matching procedure test for anti-RBC antibodies through hemolysis and agglutination. Cross-matching is an adjunct to blood typing and is not a substitute, but probably is the only incompatibility test available. (Lynel, et al., 2009; Abrams-Ogg, 2000).

There are numerous cross-matching procedures, but two of these methods are adapted into the general practice, the rapid slide method and the tube method. Both of this methods function on the agglutination and hemolysis process. (Beth, 2013; Abrams-Ogg, 2000; Gibson, 2007).

Blood donation system

The estimated blood volume that can be donated safely it is between 15% and 20%, the maximum donation it is about 16-18 ml/kg. Determine for a standard donation in the dog is 450ml referred as a canine unit of blood. Dogs can donate every month as long as they receive a good nutrition and iron supplementation in the diet. In case of the client own dogs the usual donation period it is every two months and does not require a nutritional supplementation. (Gibson, 2007) (McMichael, 2015) (Abrams-Ogg, 2000).

In the recent studies, guidelines have not been established of how frequently a dog could donate plasma or pellets if the RBC are returned to the donor. The standard suggestion is that we should use the whole blood donation guidelines. (McMichael, 2015) (Abrams-Ogg, 2000).

Collection system

The collection system refers to the standard human blood collection packs and they are the most suitable collection packs, using a closed system that has no potential for environmental contact with the blood as it flows from the

vein to the container. The same collection packs are also available for preparation of blood components, these bags having satellite bags for the extraction of the erythroconcentrate. In an emergency, if the regular systems are not available they can be collected using a 60ml syringes collected to an infusion kit. (Gibson, 2007) (Abrams-Ogg, 2000).

The blood collection procedure is made with a venipuncture of the jugular vein, cephalic vein (large breed dogs) or the femoral artery also can be used, but technically the femoral artery puncture is the most difficult and it has an increased chance of hematoma formation and scarring of the vessels. (Abrams-Ogg, 2000).

The blood collection procedure has a minimum requirement of four people: a phlebotomist, two restrainers and another person to handle the collecting bag. (Abrams-Ogg, 2000).

After care of the donor, refers to the observation of the dog for 15 to 30 minutes for weakness, pale mucous membranes, weak pulse or other signs of hypotension. In case of hypotension we can replace the volume with saline or a similar crystalloid solution after the donation. It is most necessary to apply moderate pressure over the vein puncture sight for about five minutes and to apply a neck bandage. (Gibson, 2007) (Abrams-Ogg, 2000).

Blood product preparation and storage

Whole blood packed RBC, fresh frozen plasma and frozen plasma are the most important blood products. Collected blood can be given for transfusion as fresh whole blood, stored or transferred into various components to be used fresh or refrigerated. (Abrams-Ogg, 2000).

The latest veterinary and human blood banking practices requires that only the blood collected in the closed system should be used for storage or blood product preparation and this should be standardized to minimize the risk of microbial growth. (Abrams-Ogg, 2000) (Gibson, 2007).

The open collection system should be only used for immediate transfusions or emergency transfusion procedures. The requirements of blood product preparation after the collection of the blood refers that the pack should be held at room temperature while awaiting the separation into the components. (Mollison, 2000); (Abrams-Ogg, 2000); (Gibson, 2007).

Every blood product should be labeled with the type of product, the donor, the blood type if it is known and the collection and expiration dates. (Gibson, 2007); (Abrams-Ogg, 2000).

RBC products are represented by: whole blood and packed RBC.

Whole blood is the most common used blood product and should be kept under refrigeration at 1 to 4 degrees Celsius and should be kept on a dedicated refrigerator. (Abrams-Ogg, 2000).

The principal indication of whole blood, fresh or stored is for acute hemorrhage and for replacement of RBC plasma in case if volume deficit appears. The whole blood volume that should be transferred it is estimated on ongoing future losses. The usual dosage that should be administrated is between 10 and 22 ml/kg and the volume should not be modified or exceed unless the outgoing losses are severe, in this case we should consider using massive transfusion. (Jutkowitz, 2015) (Gibson, 2007).

Packed RBC. There are two principals to obtain packed RBC from fresh whole blood: centrifugation or sedimentation.

Centrifugation procedure requires a 5000G`s for 5 minutes at 4 degrees Celsius. On slower centrifuges 2000G`s for 10 minutes and the plasma should be removed with a plasma extractor and kept on a designed refrigerator for a maximum of 30 days.

The sedimentation process for whole blood it`s obtained by vertically suspension of the blood pack in a refrigerator for a minimum of 12 hours, procedure that lasts from 3 days to 2 weeks. To speed up the process we should

consider the addition of a synthetic colloid that would maximize the RBC and plasma separation. (Gibson, 2007).
The RBC transfusion is indicated in cases of anemia without hypovolemia or deficits in other blood components, but has the benefits of not overloading the circulatory system. (Abrams-Ogg, 2000).
Plasma products are obtained the separation of RBC from the whole blood product. This could be processed into various products. (Abrams-Ogg, 2000).
Fresh plasma and fresh frozen plasma. The fresh frozen plasma is the plasma that has been separated and placed at -18 degrees Celsius in maximum 8 hours from the collection. Keep in mind the time restrictions, this product should be normally prepared by centrifugation. (Hackner, 2015); (Abrams-Ogg, 2000).
The transfusion of fresh frozen plasma has an interval between 10 to 30 ml/kg and it should be supplemented if a hemorrhage or a coagulation deficit persists.
Frozen plasma. Liquid plasma should be stored under 1 to 6 degrees Celsius refrigeration and the maximum storage time is approximately 6 weeks. (Abrams-Ogg, 2000) (Gibson, 2007).

Transfusion of whole blood and blood products

The whole blood and blood components that had been refrigerated should be warmed before administration. The requirement is to warm the products gradually to room temperature during the administration, but to proceed with extreme caution because the excessive warming may decrease the RBC viability and increase the risk microbial growth. (Abrams-Ogg, 2000).
The whole blood should be mixed by a gentile inversion before the transfusion. The canine packed RBC`s that had been stored in CPDA1 has a PCV of approximately 70-80% and a very high viscosity that makes the product difficult to transfuse, even allows the formation of RBC clumps. To reduce this problem we should add 100ml of NaCl at 37 degrees Celsius, to facilitate the transfusion process. (Abrams-Ogg, 2000) (Gibson, 2007).
Frozen plasma should be reheated in an incubator or a water bath at 37-38 degrees Celsius for approximately 30 minutes per unit of canine plasma. Frozen plasma products may be more rapidly defrosted even in a microwave oven (Hurst et al. 1987); (Abrams-Ogg, 2000).

Transfusion rates

The initial transfusion rate of whole blood and its components should be 0.25ml/kg/hour for the first 30 minutes and then should be transfused at a rate of 5 to 10 ml/kg/hour. For the cardiac patient it should not exceed 0.3 to 3 ml/kg/hour. (Gibson, 2007).
The maximum rate of transfusion should not exceed 22 ml/kg/hour used in emergency situations except the situations where massive transfusion is required, at a maximum of 90 ml/kg/24hours. (Jutkowitz, 2015); (Gibson, 2007).
It is recommended that during the higher transfusion rates that the patient should be connected to a monitor and it should be monitored during the transfusion. In the presence of the increased risk of volume overload the transfusion rates must be slowed down. To minimize the bacteria proliferation in the RBC and plasma products the veterinary and human transfusion standards recommend that every transfusion should be completed in a maximum of 4 hours. (Abrams-Ogg, 2000); (Jutkowitz, 2015); (Abrams-Ogg, 2000).

Delivery

The delivery of whole blood and its components in most veterinary clinics is by gravity and it is recommended that we use infusion pumps for an accurate dosage. Alternatively the blood product may be gently

drawn of the bag in 60ml syringe and then slowly administrated. (Abrams-Ogg, 2000).

Record keeping of each transfusion should be kept for each transfusion and also it would have to contain general information from the blood product recipient, the date of the transfusion and transfusion reaction if they appear. A highly visible notation should be added to the medical record to show that the patient received the transfusion. (Abrams-Ogg, 2000); (Gibson, 2007).

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EFFECTS OF CIS-PLATINUM ON THE BIOCHEMICAL HOMEOSTASIS IN RATS NOTE I. INVESTIGATIONS ON THE HEPATIC DNA AND ON SERUM PROTEINS

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Abstract

Cytostatic chemotherapy induces changes in the homeostasis of the hepatic DNA and of serum proteins. Among the various drugs used in the chemotherapy the alkilating agents, antimetabolites, steroid hormones and antibiotics are better known. In the last decades there were discovered other new compounds such as platinum derivates which representia distinct class of compounds, having specific antitumoral action. Among platinum derivatives an essential effect has cis-platinum. Research on the cytostatic activity of cis-platinum implies the knowledge of the pharmacokinetic and biochemical effects. Experiments "in vivo" performed on Wistar strain rats pursued the action of the intraperitoneally injected cis-platinum on the hepatic deoxyribonucleic acid (DNA) biosynthesis and on serum proteins. The statistically processed analytical data revealed non-significant decreases of the hepatic DNA and of serum proteins concentrations. Electrophoretical fractions showed hypoalbuminemia and hyperglobulinemia. In case of globulin subfractions the decrease of α_1 - and α_2 - and increase of β - and γ - globulins was observed.

Key words: *cis-platinum effects - hepatic DNA and serum proteins in rats*

INTRODUCTION

In the acceptation of WHO, oncotherapy is aimed at the annihilation, elimination or neutralization of all cancer cells.

The main modalities of the antitumoral therapy - in actual use - are: surgical intervention; radiotherapy; chemotherapy and immunotherapy.

Presently, in the antitumoral chemotherapy there are used various types of drugs, such as: alkylating agents, e.g.: cyclophosphamide, chlorambucil, nitrosourea a.o.; antimetabolites, e.g. : 5-flurouracil, 6-mercaptopurine, methotrexate a.o.; steroid hormones**,** e.g.: estrogens and androgens; antibiotics, e.g. actinomycin D, bleomycin a.o.; alkaloids, e.g.: vincristine, vinblastine a.o. (Gârban et

al., 1997; Neidle and Waring, 2000; Manolescu, 2003).

The class of platinum coordination complexes was discovered during the investigations of the electric field effects on bacterial growth. Cis-platinum has the chemical denomination cis-diamminedichloroplatinum (abbreviated as cDDP) is the most important inorganic coordination compound (Rosenberg et al., 1969; Haiduc and Silvestru, 1989; Lippert, 1999; Gârban et al., 2014).

Secondary effects of cis-platinum include nausea, vomisment, nephrotoxicity, ototoxicity, neuropathia and mielosupression. In some cases appear arithmias, ischemic stroke, glucose intolerance and pancreatitis (Manolescu, 1997; Kelland and Farrell, 2000). Nephrotoxicity can be ameliorated by hydration, renal injury at the level of glomerules and tubules is cummulative, reason why serum creatinine is not longer a conclusive parameter for the glomelular filtration rate.

Nowadays a domain of major interest in comparative medicine and in biochemistry is represented by the study of metallomics related to xenobiotics of food interest, e.g. metals in foods and of pharmaceutic interest, e.g. metal based drugs like cis-platinum (Haiduc et al., 2008, Gârban, 2011).

MATERIALS AND METHODS

Experimental model. In vivo experiments were performed on laboratory animals - Wistar strain male rats, included in three groups : one control - C and two experimental (E_i) groups - noted E_1 and E_2. Each group comprised 10 animals with an average weight of 200±10 g. Animals of group C were injected intraperitoneally (i.p.) with physiological saline and those from groups E_i with cis-platinum in physiological saline, as follows : E_1 - a dose of 5 mg/kg b.w. and E_2 - a dose of 10 mg/kg b.w. After 72 hours the animals were anesthetized and killed. Blood samples were taken by the puncture of vena cava caudalis and a liver fragment for DNA analysis were excized.

Requirements for the protection of animals used in scientific or other experiments were respected according to Council Directive 86/609/EEC and National Governmental Ordinance No.37/30.01.2002.

Biochemical investigations. The hepatic DNA concentration was determined by the Ogur Rosen method modified by Spirin (1958) and adapted by us for UV spectroscopical methods (Gârban et al., 1986). From blood samples the total serum proteins by the biuret method and the electrophoretic protein fractions by paper electrophoresis (veronal-medinal buffer, bromo-phenol blue staining) were determined (Franke et al., 1977; Kaplan and Pesce, 2010).

Statistical evaluation. All the obtained experimental data were statistically processed, mean values (X) and standard deviations (SD) were calculated. One way ANOVA (Analysis of Variance) was also used.

RESULTS AND DISCUSSIONS

Investigations on the DNA interaction with cis-platinum revealed the possibility of complexes formation named "adducts". These adducts of DNA-cDDP type perturb the secondary structure of the macromolecule (Kelland and Farrell, 2000; Gârban, 2004).

In the present experiment we found the decrease of DNA concentration in the liver tissue of rats after the cis-platinum administration – see Table 1. The observed decrease was more obvious in group E_2 - animals to which a higher concentration of cis-platinum was administered.

Table 1 . Hepatic DNA concentration after cis-platinum administration in Wistar rats

Groups	No. of animals	Admin.dose (mg/kg b.w.)	Duration (hours)	DNA (μg/mg tissue) X ± SD	ΔX $X_C - X_{E_i}$
C	10	-	72	2.74 + 0.19	-
E_1	10	5.0	72	2.65 + 0.20	- 0.09
E_2	10	10.0	72	2.61 + 0.17	- 0.13

The decrease of hepatic DNA concentration might be explained by the interaction of cis-platinum with the cellular DNA. Bindings occur to the nucleobases of DNA, i.e. guanine (G), cytosine (C), adenine (A) and thymine (T). As a consequence of this interaction structural modifications will occur in DNA which are followed by the disturbance of the replication process and consequently of other biological functions, too.

Aquation of cDDP in two steps leads to the formations of its pharmacologically active form which will bind to the nucleobases of DNA. These steps are given below :

$$cis\text{-}Pt(NH_3)_2Cl_2 \xrightarrow[-\,Cl^-]{+H_2O} \left[cis\text{-}Pt(NH_3)_2(OH)Cl^-\right]^+ \xrightarrow{-\,Cl^-}$$

$$\longrightarrow \left[cis\text{-}Pt(NH_3)_2(OH)_2\right]^{+2} \xrightarrow[-2H_2O]{+\,2H} \left[cis\text{-}Pt(NH_3)\right]^{+2} \longrightarrow$$

$$\longrightarrow cis\text{-}Pt(NH_3)\left[nucleobase\right]_2$$

It is to mention that $[\ cis\text{-}Pt(NH_3)_2\ ]^{+2}$ binds to the N and O atoms of the nucleobases, preferentially to N_7(G), N_3(C), O_2(C), O_6(G), N_3(A), N_7(A), O_4(T) and O_2(T), i.e. in the decreasing order of the binding energy level. These bindings will destabilize the secondary structure of the DNA and thus the replication-transcription-translation processes, involved in the protein synthesis, will be disturbed, too. One can consider that effect of cis-platinum on the protein synthesis is due to the changes in the chemical structure-biological activity relationship at the level of DNA by resulting adducts (Gârban, 2011).

The results obtained in our experiment revealed homeostatic changes in serum proteins and electrophoretic fractions and are given in Table 2. One can observe that with the increasing dose of the administered cis-platinum the concentration of serum proteins decreased. It is to notice the existence of an inverse proportionality: the increase of the cis-platinum dose induces the decrease of serum proteins concentration.

Table 2. Concentration of serum proteins and electrophoretic fractions

Specification		C (n = 10) X ± SD	E_1 (n =10)		E_2 (n = 10)	
			X ± SD	ΔX	X ± SD	ΔX
Serum proteins (g%)		5.87± 0.23	5.39 ± 0.35	- 0.48	5.15 ± 0.42	- 0.72
Electrophoretic fractions (%)	Albumin	54.30± 1.70	53.60 ± 2.06	- 0.70	52.60 ± 1.71**	- 1.70
	Globulins - total	45.70± 1.70	46.40 ± 2.06	+ 0.70	47.40 ± 1.71**	+ 1.70
	α_1-globulins	12.90± 1.28	12.70 ± 1.63	- 0.20	12.70 ± 1.05	- 0.20
	α_2-globulins	10.70± 0.82	10.50 ± 0.84	- 0.20	10.80 ± 0.91	+ 0.10
	β-globulins	14.80± 1.54	15.10 ± 1.52	+ 0.30	15.20 ± 1.22*	+ 0.40
	γ-globulins	7.30± 1.15	8.10± 1.44*	+ 0.80	8.70 ± 1.05*	+ 1.40

Note: n - number of animals; * $P < 0.01$; ** $P < 0.05$

In case of electrophoretic fractions a decrease of albumin and increase of total globulins in the experimental groups (E_1 and E_2) as compared with the control group (C) was found. As to globulin subfractions α_1 - and α_2 - globulins decreased while β - and γ - globulins increased in E_1 group. In case of animals from E_2 group - experiment with a higher cis-platinum dose, the globulin subfractions showed a decrease of α_1 - globulin and increase of α_2 -, β- and γ - globulins. Regarding the concentration of serum proteins and of the albumin fraction Saleh et al. (2014) found also a decrease after the administration of cis-platinum in rats. The occurred biochemical homeostasis changes in hepatic DNA, serum proteins and electrophoretic fractions are the consequences of the cis-platinum interaction with DNA and tissue proteins, more exactly with certain target functional groups, e.g. amino, hydroxyl a.o. (Lippert, 1999; Keeland and Farrell, 2000). At molecular level these interactions concern with the pharmacon-receptor interaction and explain the mechanism of cytostatic action of cis-platinum.

CONCLUSIONS

The in vivo interaction of cis-platinum with DNA evidenced a statistically non-significant decrease of DNA concentration in case of both doses: 5 mg/kg b.w., 10 mg/kg b.w., respectively. These slight decreases could be due to the low administered dose of cis-platinum. The effect is explained by adducts formation which binds a part of DNA resulting cis-platinum-DNA type adducts.

In both experimental groups (E_1 and E_2) the concentration of serum proteins decreased. The electrophoretic fractions revealed decrease in case of albumin concentration and increase of globulins concentration. Globulin subfractions in case of E_1 group showed a decrease of α_1 - and α_2- globulins, and an increase of β- and γ-globulins. In case of group E_2 only α_1-globulins decreased while α_2- , β- and γ-globulins increased. Higher increases were found in case of γ-globulins (immunoglobulins) which confirm the effect of cis-platinum.

Note. This paper was elaborated within the activity of the *Comparative Medicine Forum – Romania*. The next note will approach the homeostasis of the non-protein nitrogen metabolites.

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STUDY ON ANTICANCER ACTIVITY OF DEUTERIUM-DEPLETED WATER (DDW) IN EXPERIMENTAL ONCOLOGY

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Abstract

The authors demonstrated the effect of deuterium depleted water (DDW) with a concentration of 60 ppm to be a very good inhibitor of the neoplastic cell proliferation in outbred Wistar rats inoculated with two strains of the highest tumor aggressiveness (Walker 256 and T8 Guérin).
For this experiment were organized groups, in equal parts by sex of outbread Wistar rats, with an average weight of 100g, which were established both control groups and experimental groups.
Through the experiment, animals were monitored in anatomoclinical terms. Laboratory tests were executed periodically, especially cytomorphological. Dead animals were subjected to a careful pathological examination.
It is well known that high malignant Walker 256 and T_8 Guérin tumour strains develop a solid, ulcerated subcutaneous cancer, after an incubation of 5-6 days. The reproductibility of this type of cancer is of 95%. Therefore the death of the rats occurs within a short period of 40-60 days. The cumulative effect of DDW 60 ppm on the rats grafted with Walker 256 and T_8 Guérin strains was about 28-30 %. This percentage comprises both the animals in which the effect of primary reject of the tumour graft was noticed and the healing effect after an important development of the tumour.

Key words: cancer, DDW 60 ppm, experimental, oncotherapy.

INTRODUCTION

Regarding the application of deuterium-depleted water (DDW) effects in biology, oncology and therapy, the first theoretical and laboratory data are found in the papers provided by Somlyai G. et al, Bild W. et al and Gyöngyi Z (1998-2002) followed by well-known research data in other scientific papers. (1, 2, 3, 4, 5, 6). Many attempts to cure Wistar outbred rats, inoculated with high malignity 256 Walker and T_8 Guérin strain tumour cells, by means of various therapies, within the Cancer Biology Department from the Oncological Institute Bucharest (IOB), were unsuccessful; therefore, this experiment was carried out in order to provide a new therapy.

Therefore, as all these therapies were ineffective, DDW was taken into account, with different deuterium concentrations, in relation to the biological components of normal and/or pathological life.
Consequently, DDW with a deuterium concentration of 60 ppm (DDW 60 ppm) was chosen in order to carry out the experiment, due to the previous results showing its possible anti-cancer protection, superior to all other deuterium concentrations.

MATERIALS AND METHOD

500 Wistar outbred homogenous rats from IOB own lot, all with an average weight of 100 g, males and females in equal parts, were used. These were divided in two groups, initially as follows: control group (250 rats), which drank exclusively tap water (TW) with a deuterium concentration of 150 ppm (TW 150 ppm); and experimental group (250 rats), drinking exclusively deuterium-depleted water (DDW) with a deuterium concentration of 60 ppm (DDW 60 ppm). Differences between the initial number of 500 rats in the experiment and the experiment onset values of 320 rats are due to the following facts:

1. some rats had to be taken from the control group, in order to carry out other experiments, therefore only 100 rats were left for this experiment;

2. from the initial experimental group consisting of 250 rats, only 220 out of 250 rats remained alive at the experiment onset – the rest representing mortality due to an enterotoxaemia.

DDW used in the experiment was provided by the National Research and Development Institute for Cryogenics and Isotopic Technologies - ICSI Rm. Valcea.

The daily water intake was approximately 15 ml/day. After 60 days, considered "preparatory" for the experiment, the animals were divided in four study groups, as follows:
Groups one and two were subjected to latero-dorsal subcutaneous grafting with 1 X 10^7 ascites cells in 0,5 ml of normal saline solution of 256 Walker (ascitogenous) (tumour cells with a viability over 98%). Viability determination for the tumour cells to be grafted was evaluated by counting the dead cells (trypan blue stained) under microscope, and calculating the ratio corresponding to 1000 cells displayed on the slide.

Group one (experimental) – consisting of 150 rats which drank DDW 60 ppm, both prior to (over 60 days), and during the entire experiment.

Group two (control) – consisting of 50 rats which drank exclusively TW 150 ppm, during the entire experiment.

Groups three and four were subjected to latero-dorsal subcutaneous grafting, with a saline suspension of ascites tumour cells from T_8 Guérin strain with 1 X 10^7 tumour cells with 98% viability percentage. Viability determination for the tumour cells to be grafted was evaluated by the same method (trypan blue stained).

Group three (experimental) – consisting of 79 rats which drank DDW 60 ppm exclusively.

Group four (control) – consisting of 50 rats which drank exclusively TW 150 ppm.

The food ingested by the rats was consistent with IOB bio-basis standards, during the entire experiment.

The surveillance protocol of the four groups included:

Graft status was assessed daily by inspection and palpation in all animals, beginning with day four post-grafting, in order to identify and note the onset day of malignant neoformation; furthermore, this day represented the subcutaneous grafting day. Consequently, after identification of the tumour, the tumour growth was evaluated objectively by measuring the two perpendicular axes (major and minor). Also, the tumour volume was calculated daily using the formula:

$$V = 0,4(constant)\ X\ (a\ X\ b^2),$$

where – V represents the volume in mm^3 ; a, and b represent the values of the two axes, namely major (a) and minor (b).

The animals were weighed daily, monitoring their weight evolution, and clinical assessments on the grafted animals were carried out in order to note any change in their clinical status. Mortality was recorded daily, as well.

Cytomorphological examinations were performed periodically in order to establish the leukocyte formula, and to identify the "*blast cells"*, as well as dendritic and NK cells, followed by cytological evaluations from haematopoietic bone marrow and lymph nodes.
The following assessments of the tumour growth were performed periodically, usually on a twenty-days basis, according to NCI criteria (National Cancer Institute – Bethesda, USA - 1990), by calculating:

- tumour growth index (TGI %), provided that values of 50% and above represented a significant inhibition of tumour growth;

- mean survival time (MST) of the grafted animals;
- T/C ratio (%) (T = treated, C=control X 100); the values had to be > 125;
- it was very important to assess the "*survival time* (ST) *increasing*", which had to be over 25%, evaluated in days, since the tumour graft onset, and until the death of the last animal from each group or until the maximum day of the experiment (700 days in our case);
- establishing the number of long term surviving animals, versus number of treated animals.

Note that standard operating procedures (SOP) were fully respected in order to validate the good manufacturing practices (GMP) currently used in pharmacological studies. Consequently, for quantitative estimation of the anti-cancer effect of the product, two variables required by GMP were taken into account:

- - the independent prediction variable, therefore minimum 1 X 10^6 tumour cells must be present in each tumour grafting, under the same standard terms;
- - the dependent prediction variable – concerning the following criteria:
- -latent period (in days) – representing the number of days from grafting until the smallest tumour growth onset;
- -tumour incidence, estimating the number of animals with/without tumours, tumour rejections;
- -mean survival time (MST), representing the days since grafting, and until the death of the last animal in the each experimental group.
- -monitoring the physiological status of the animals:
- -weekly weighing of the rats;
- -monitoring of water and food consumption of the animals in every group;
- -performing complete haematological exams; these consisted of Coulter Counter cell counting, whole blood and leukocyte concentrated (LCT) blood smears, in order to establish the leukocyte formula;
- -post-mortem examinations of the animals (necropsy), including lymphadenograms and medulograms, in order to notice the local development of the tumour, with or without metastasis of lymph nodes; ratio of virgin lymphocytes (antigenic unexposed) and those antigenic exposed; furthermore, the ratio between total immunologic competent cells and mast cells was evaluated.

RESULTS AND DISCUSSIONS

The results of this study are relevant as all the rats in the "*control groups"* - which consumed TW 150 ppm - grafted with both T_8 Guérin, and Walker 256 strains tumour cells, died within a post-grafting period of 40-60 days. In conclusion, their death was due to some malignant ulcerous and invasive tumour development, including regional lymph nodes metastasis. On the contrary, the groups grafted with the same tumour cells, but which consumed exclusively DDW 60 ppm for "*a long time*", recorded exquisite results as shown in tables 1 and 2.
Therefore, our experiment managed to emphasize some specific actions of DDW 60 ppm in comparison to TW 150 ppm, as follows:

1. The onset of inoculated graft and its anatomoclinical visualization increased from 5-6 days in the control group to 10-12 days in the groups of animals that consumed DDW 60 ppm for 60 days pre-grafting.

2. A DDW 60 ppm consumption exclusively for 700 days was shown to have a beneficial effect on the animals, consequently leading to an *"immunologic boom".*

3. Furthermore, a broad antineoplastic activity, both by initiation and completion of "*graft early reject*", and an increase in neoplastic development time, for both tumour strains, was demonstrated by the experiment. Thus, both grafted tumour strains were lysed

either early or late leading to a less intended survival time of approximately 28% from the total grafted rats, over the entire experiment period of 700 days. In conclusion this result is compared with the 100% morbidity of the control groups within a short post-grafting period of 40-60 days.

4. The experiment demonstrated that these actions are based on an intense activity of immune modulation of "*the cellular immune system*" (CIS) by clonal proliferation of lymphocytes B, of NK cells, and of the dendritic-mast system. This activity was identified both in haematopoietic bone marrow, lymph nodes, and peripheral blood.
Our results are broadly similar to data from specialized literature.

Our experiments brought some novelties concerning:

- Demonstration of no toxic effect or life discomfort for the animals in the experimental groups, under the exclusive administration of DDW 60 ppm, for a long time, to unusual digits such as 700 days.
- Exclusive administration on the long term of DDW 60 ppm allowed the identification of primary graft reject of the tumour graft in about 20% of inoculated rats, on the one hand, and on the other hand, achieved a significant therapeutic effect in about 10% of inoculated animals providing that the inoculated and developed tumour was lysed in time, thus leading to a very good healing of the tumour site.
- The extension of the time in which the smallest post-grafting tumour evolved, from 5-6 days for control group, to 10-12 days for experimental groups (thus proving the graft attachment)
- In the control group, after 5-6 days following the tumour onset, there could be noticed the tumour development and subsequent death of all the inoculated rats occurred within 40-60 days.

Table 1. Summary of the experiments general data

Inoculated rat line	No. of days with DDW 60 ppm before grafting	No. of post-grafting days after the tumour growth onset	No. of animals			Remarks
			total	males	females	
Walker 256	60	11	150	75	75	Experimental group
Walker 256	-	6	50	45	45	Control group
T8 Guerin	60	11	79	40	39	Experimental group
T8 Guerin	-	6	50	25	25	Control group

Table 2. Summary of the final results regarding the experimental anticancer value of DDW 60 ppm in Wistar outbred rats

Grafted tumour cell line	No. of animals experimental group/ control group	Dead animals control group during 40-60 days	T_8 Guerin grafted surviving animals over 500 days	Walker 256 grafted surviving animals over 500 days	Remarks
Walker 256	150/50	50	-	28,5%	Morphoclinical assessments were conducted on an 100 days basis over the 700 experimental days
T_8 Guerin	79/50	50	28%	-	

CONCLUSIONS

The most important effect of DDW 60 ppm consumption for 60 days before tumour grafting was to induce the graft primary reject in about 20% of the inoculated animals.
Consequently, the intake of DDW 60 ppm for 60 days in advance of the experiment, and afterwards for 700 days, showed an inhibiting

action of the cancer development in the subcutaneous inoculated Wistar outbred rats with Walker 256 and T_8 Guérin strains at the rate of approximately 8 – 10 %.
The cumulative effect of DDW 60 ppm on the rats grafted with Walker 256 and T_8 Guérin strains was about 28-30 %. This percentage comprises both the animals in which the effect of primary reject of the tumour graft was noticed and the healing effect after an important development of the tumour.
DDW 60 ppm showed a special activity of "*immune modulation"* of the cellular immune system (CIS), by clonal stimulation of mast-dendritic system, of NK cells, and lymphocytes B;
Therefore, note that DDW 60 ppm produced no toxic or inhibiting effect to the animals even in the long run consumption (700 days) exclusively.

Fig.1 rat with tumor in flank area after a tumoral inoculation

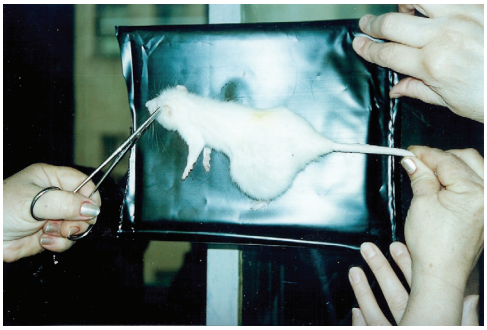


Fig.2 tumor ulcera after 40 days

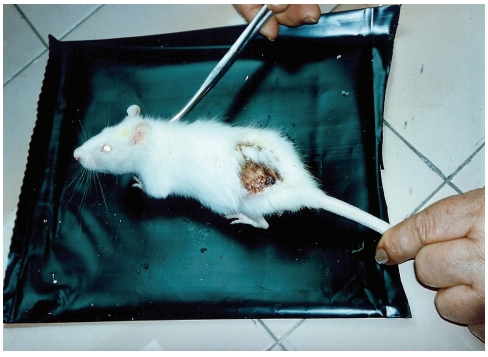
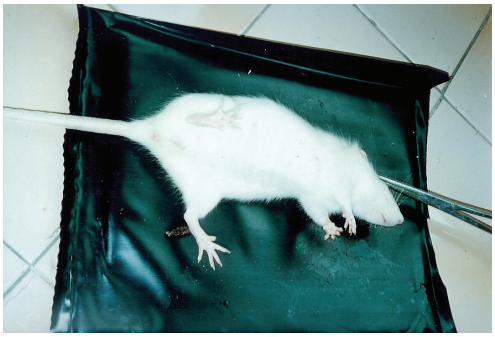


Fig.3 day 70-reepithelization of the ulceral area



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ASSESSMENT OF MESENCHYMAL STEM CELLS EFFECTS ON DENDRITIC CELLS MATURATION

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Abstract
Introduction. Mesenchymal stem cells (MSCs) are adult stem cells (Pittenger et al., 1999, Zhang et al., 2009) able of self-renewal, with low immunogenicity and immunoregulatory property (Jorgensen et al, 2003, English et al., 2008, Zhang et al., 2009). Dendritic cells (DCs) present in the bone marrow play a crucial role in the instruction of adaptive immunity (Nauta et al., 2006, Zhi-Gang et al., 2012) DC have the unique capacity to stimulate naive and memory T cells (Banchereau et al., 2000, Nauta et al., 2006, Wang et al., 2013). The aim of the present study was to assess the effect of MSCs on DC maturation.
Materials and Methods. MSCs were collected from femurs of male Wistar rats. Cells suspension were cultured in DMEM/F12 supplimented with 10% fetal calf serum (FCS), 5% horse serum and 1% antibiotic–antimycotic (Gibco). DCs were prepared from rat bone marrow after red cells lysis and cultured in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 1% antibiotic–antimycotic (Gibco), 10 ng/mL GM-CSF (Sigma), and 5 ng/mL IL-4 (Sigma). MSCs and DCs were cultured for 7d at 37°C. DCs ($5x10^3$) were grown in two different conditions: co-culturing with MSCs and 25 ng/ml TNF-α (I) or without MSCs and 25 ng/ml TNF-α (Sigma) (II) for 48d. Cell phenotype were characterized by flow cytometry (FACSCanto II) using CD11b, CD44, CD86 (Becton Dickinson) antibodies.
Results and Conclusion. After co-culture with MSCs, DC showed a decrease in CD86 expression compared with culture supplemented only with TNF-α which showed an increase in expression of this marker.
Acknowledgements-This work was supported by Forerunner Federation

Key Words: *dendritic cells, differentiation, mesenchymal stem cells*

INTRODUCTION

Mesenchymal stem cells (MSCs) are adult stem cells (Pittenger et al., 1999, Zhang et al., 2009) able of self-renewal (Soleymaninejadian et al., 2012), with low immunogenicity and immunoregulatory property (Jorgensen et al, 2003, English et al., 2008, Zhang et al., 2009). MSCs can replicate for a long time while maintaining their multilineage differentiation potential (Eslaminejad et al., 2008, De Miguel., et al., 2012).
Dendritic cells (DCs) the most potent of the antigen presenting cells (Toubai et al., 2014) present in the bone marrow play a crucial role in the instruction of adaptive immunity (Nauta et al., 2006, Zhi-Gang et al., 2012). DCs can be divided into distinct subsets based on differential phenotype and function (Bjorck, 2001, Toubai et al., 2014). DCs have the unique capacity to stimulate naive and memory T cells (Banchereau et al., 2000, Nauta et al., 2006, Wang et al., 2013) and play a major role in development of cell-mediated immunotherapy (Pion et al., 2010). Recent studies indicate that MSCs can inhibit cell proliferation of T cells, B-cells, natural killer cells (NK) and dendritic cells (DC), De Miguel., et al., 2012 and can stop some of the immune cell functions: including the maturation and activation of DCs (De Miguel., et al., 2012)
The aim of the present study was to assess the effect of MSCs on DC maturation.

MATERIALS AND METHODS

MSCs were collected from femurs of male Wistar rats. Cells suspension were cultured in 25-cm^2 tissue culture flasks at a concentration of 10^6 cells/mL in DMEM/F12 supplimented with 10% fetal calf serum (FCS), 5% horse serum and 1% antibiotic–antimycotic (Gibco). After 72h incubation at 37°C in a 5% CO_2 humidified atmosphere (60%), nonadherent cells were removed and the adherent fraction were cultured in fresh medium basal medium. The MSCs were used only after 5 passages in order to eliminate monocytes.
DCs were prepared from rat bone marrow. After red cells lysis mononuclear cells were separated by Hypaque 1077 density gradient centrifugation (Sigma). Then cells were washed, counted, and

plated at 1× 10^5 onto T25 tissue culture plates in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 1% antibiotic–antimycotic (Gibco), 10 ng/mL GM-CSF (Sigma), and 5 ng/mL IL-4 (Sigma). Culture medium were replaced, and the nonadherent cells were after 48h hours of initial culture and every 4 days thereafter.
MSCs and DCs were cultured for 7d at 37°C. DCs ($5x10^5$) were grown in two different conditions: co-culturing with MSCs and 25 ng/ml TNF-α (I) or without MSCs and 25 ng/ml TNF-α (Sigma) (II) for 48d. Cell phenotype were characterized by flow cytometry (FACSCanto II) using CD11b, CD44, CD86 (Becton Dickinson) antibodies. Quantified data are presented as the mean ±SD. Significance were accorded at $p< 0.05$.

RESULTS AND DISCUSSIONS

Microscopic examination of the bone marrow derivated cells after 72 h revealed cellular heterogeneity. A high percentage of cells were elongated or oval/round shape with smooth borders and a part of the cells were in suspension. After a few days were observed an intense proliferation of bipolar fibroblastoid cells with a significant reduction of the cells with round morphology (fig.1).

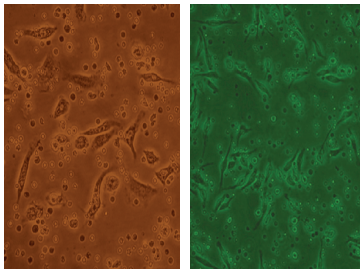

Fig. 1 – Mesenchymal- like cells derived from rat bone marrow

The MSCs were expanded for 5 passages in normal culture medium and were analyzed for the expression of cell surface molecules by flow cytometry (fig 2).

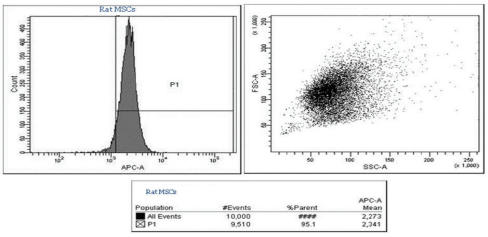

Fig. 2 -Immunophenotypic profile of rat MSCs (CD44 positive cells)

Freshly isolated mononuclear cells were cultured overnight at 37 °C and 5% CO_2 in standard media. Microscopic analysis revealed cells with irregularly shaped eccentric nuclei and abundant cytoplasmic extensions (fig.3).

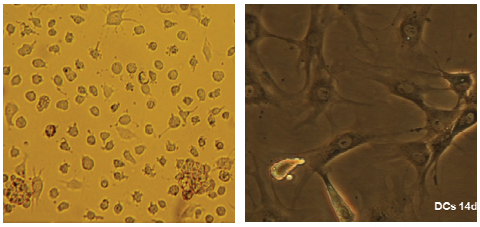

Fig. 3 - Morphological characteristic of cells derived from rat bone marrow

After co-culture of DCs cells with MSCs, were observed a flattening and widening of dendrites with a reduction in their length and a slight rounding of the cells. Also, DCs cells showed a decrease in CD86 expression compared with culture supplemented only with TNF-α which showed an increase in expression of this marker (fig.4).

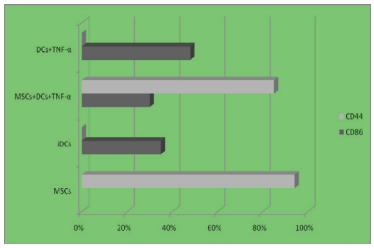

Fig. 4 - Immunophenotypic changes according to different cultural conditions

Mesenchymal stem cells are plastic adherent multipotent cells characterized by their expression of surface markers, capable of differentiating into various lineages, (Pittenger et al., 1999, Wang et al., 2006, Bydlowski et al., 2009, Szade et al., 2011, de Vasconcellos Machado et al., 2013, Majumdar et al., 2013, Wen-hua et al., 2013). MSCs have the ability to potently suppress immunological activity. This capacity is possible by activating on various cells of the immune system (Wen-hua et al., 2013). MSCs are capable of interfering in the differentiation, maturation and function of dendritic cells (de Vasconcellos Machado et al., 2013). Many studies show that the inhibitory effect of MSCs is dose-dependent; a high concentration of stem cells is more pronounced inhibitory effects (Krampera et al., 2003, Le Blanc et al., 2003, de Vasconcellos Machado et al., 2013). The inhibitory effect of

MSCs may also affect the expression of DCs surface markers and cytokine secretion profile and obviously the development of DCs function (English et al., 2008, Wen-hua et al., 2013). After co-culture with MSCs, DCs showed a decrease in CD86 expression compared with culture supplemented only with TNF-α which showed an increase in expression of this marker. These aspects are very important and need further studies to reveal all the involved mechanisms. This study demonstrates that MSCs interfered with the DCs maturation

CONCLUSION

Mesenchymal stem cells are characterized by stable undifferentiated phenotype under normal culture conditions; these cells have the capacity to prevent upregulation of DCs maturation markers. DCs cultured with MSC showed significantly reduced of CD86 ($p < 0.05$), but after maturation by TNF- resulted in increased surface expression of these markers. Our study demonstrates that MSC have an influence in DCs maturation through cell-to-cell contact, and secretion of specific cytokines.

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TRANSGENIC PIGS FOR BIOMEDICAL APPLICATIONS

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Abstract
The purpose of this article is to review the most modern techniques used in obtaining transgenic pigs as important models in the study and understand of mechanisms involved in the pathogenesis of various human diseases. Genetically engineered animals have a significant role in biomedical research (Wheeler 2007, Robl et al., 2007, Vajta et al., 2012). Genetically modified swine are recognized as outstanding models for the study of various human diseases. These animals have high reproductive capacity and adequate precocity, short farrowing interval, parturition of multiple offspring (an average of 10–12 piglets per litter) (Wolf et al., 2000, Walters et al., 2012). Besides all these aspects the completed sequencing of the swine genome (Schook et al., 2005), made possible precise representations of human diseases based on propinquity of genes between the two species (Whyte, Prather 2011). Pigs are recognized as excellent models in a variety of areas, including nutrition, toxicology, dermatology, diabetes, cancer, atherosclerosis, cardiovascular disease, cutaneous pharmacology, ophthalmology, degenerative disease etc. (Lunney et al., 1999, Ishii et al., 2006, Herkenne et al., 2006, Dyson et al., 2006, Du et al., 2007, Matsunari, Nagashima 2009, Aigner et al., 2010, Noel et al., 2012, Prather et al., 2013). Due to the emergence of modern genetic engineering techniques, the genome of these animals can be modified to serve as a potential xenograft donor (Sachs, Galli 2009).

Key words: biomedical, pig, transgenic,xenograft

INTRODUCTION

Transgenic (TG) pigs generated using assisted reproductive techniques are favorable animal models often used in various fields of biomedical research (Douglas 1972, Furth et al., 1994, Phelps et al., 2003, Furth et al., 1994, Aigner et al., 2010, Hemann et al., 2012, Watanabe et al., 2012, Yeom et al., 2012, Giraldo et al., 2012, Jin Y.X et al., 2014).

The human and and pig have a large number of likeness in anatomy, physiology and pathophysiology (Larsen et al., 2004), thus genetically modified swine are recognized as excellent models for the study of various human diseases (Jin Y.X et al., 2014) and the development of new strategies for disease prevention and treatment.

This is due to the fact that these animals have high reproductive capacity and precocity, short farrowing interval, parturition of multiple offspring (10–12) combined with the availability of techniques for oocyte manipulation and artificial insemination (Wolf et al., 2000, Sachs et al., 2009, Walters et al., 2012).

The goal of transgenic technology refers to the insertion of foreign genes into livestock and its stable integration into the germ line (Wheeler et al., 2003) followed by the expression in tissues of the resulting individual.

Using such methods have already received numerous genetically modified strains of animals useful for xenotransplantation (Sachs et al., 2009).

Pronuclear microinjection

The first method to produce transgenic pigs, pronuclear microinjection, was originally initiate in mice (Brinster et al., 1981, Whyte, Prather 2011) and then extended in other species. This technique is based on the microinjection of DNA into the pronuclei of zygotes collected from a superovulated female, and then transferred to recipient animals by embryo transfer (Hammer et al., 1985, Whyte & Prather 2011).

This technique permits the addition of large transgene at a random location (Prather et al., 2013), but the major drawback is that only about 1% of injected eggs produce transgenic pigs (Niemann, 2004, Prather et al., 2008, Whyte & Prather 2011).

Sperm mediated gene transfer (SMGT)

SMGT is a technique to produce multitransgenic pigs with high efficiency based on the intrinsic ability of epididymal sperm cells to bind, internalize and integrate exogenous nucleic acid during fertilization (Lavitrano et al., 2005).

SMGT in the pig was carried out by collection of sperm, incubation of sperm with exogenous DNA, and artificial insemination of gilts with modified semen. A very important aspect refers to the careful selection of semen donor animals (Aigner et al., 2010). However, this method has limitations which refers limitations to the inability to prescreen embryos for transgene integration prior to embryo transfer (Whyte & Prather 2011)

Viral mediated transgenesis

Viral-mediated transgenesis are extremely efficient, with 80–100% of the animals being born transgenic after oocyte or embryo infection (Whitelaw et al., 2004), or somatic cell culture infection. The method requires the use of viruses to transduce cells with various transgenes. The viral strains that are often used are retroviruses belong to the family *Retroviridae.*

These viruses insert a DNA copy of their genetic material, produced from RNA as a template, into the host cell DNA following infection (Wheeler &Walters 2001).

The main advantage of retroviral-mediated gene transfer into animals is the technical ease of presenting a virus to embryos at various developmental stages (Wheeler &Walters 2001), but the major disadvantage of this method refers to the risks associated with multiple integration including oncogene activation, insertional mutagenesis and silencing of lentiviral sequences (Hofmann et al., 2006) and high frequency of mosaicism in obtained animals.

Somatic Cell Nuclear Transfer (SCNT)

Somatic cloning is emerging as a new biotechnology, has been established using blastomeres from early stage embryos as donor cells (Niemann & Lucas-Hahn 2012). SCNT works better in pigs than in other large animals (Lagutina et al., 2007). Embryonic stem cells (ESCs) and embryonic germ cells (Shim et al., 1997, Piedrahita et al., 1998) are important sources of cells for the production of transgenic animals. This transfer method involves injection of embryonic cells into expanded blastocysts to produce chimeric embryos composed of two or more distinct cell lines (Robertson et al., 1986).

Transgenesis by SCNT involves the following steps: (1) genetic modification and selection of donor cells in culture; (2) recovery and enucleation of in vivo or in vitro matured oocytes (metaphase II); (3) nuclear transfer by electrofusion and activation; (4) in vitro culture of the reconstructed embryos; and (5) embryo transfer to synchronized recipients (Aigner et al., 2010).

ESCs has many advantages for transgenesis (Robertson 1991); these advantages include: ESCs can be isolated from a single cell, the capacity of self-renewal without senescence, transformed ES cells can be screened and selected using reporter genes, ESCs cells can be transformed *in vitro* with foreign DNA, these cells can be expressed in tissues and organs.

The ability of the isolated ESCs cells to participate in embryogenesis of porcine chimeras was tested in different stage of embryos development (morula, blastocyst and expanded blastocyst stage embryos injected with embryonic stem cells). No differences were observed between embryonic stages. The major limitations of this method are represented by the lack of embryonic stem cell (ESCs) technologies.

Adult cells can be used for transfer but they have a limited lifespan, thus restricting the time the cells can be cultured *in vitro* for genetic manipulation (Sachs et al., 2009).

The production of transgenic organisms represented a major technical advance in many research areas. The numbers of animals obtained by these methods have grown exponentially in recent years, also have been developed standardized protocols globally accessible to more researchers (Whyte & Prather 2011).

The development of genetically modified pigs in various medical purposes has a significant impact for the scientific community and improving the development of treatments and new therapies for human diseases. Currently, genetically modified pig models are used for the analysis of gene function in a variety of human diseases, the development of new therapeutic strategies, and the production of biopharmaceuticals (Walters et al., 2012).

Complete pig genome sequencing and the modern techniques, now offer very useful tools for veterinary and human medicine. The research results conducted on these animals can be extrapolated in various medical fields.

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SPERMOGRAMS ANALYSIS AS A MANDATORY PART OF THE CANINE SEMEN CRYOPRESERVATION PROTOCOLS

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Abstract

Quality of fresh semen is a crucial element for the succes of assisted reproduction techniques in all species. 57 ejaculates were collected manually from 25 dogs. Motility and concentration (CASA SpermVision®, Minitübe, Germany, morphology and acrosome status (Spermac® stain) were determined. Semen analysis showed a strong relationship between male's weight and size (testes dimensions) and the volume of the sperm rich fraction, but did not identify a correlation between concentration and male's size. Motility parameters, morphology and acrosome status for the ejaculates included in our study were within the accepted standard values. Age did not significantly influence the volume or the morphology of canine semen.

Key words*:spermogram, canine, cryopreservation*

INTRODUCTION

Quality of fresh semen in the male dog is one of the crucial elements for the succes of assisted reproduction techniques. Semen analysis (spermogram) has to steps: macroscopical (volume, colour, smell and pH of semen) and microscopical exam (concentration, motility, viability and morphology of sermatozoa).

Semen parameters vary betwen individuals and even for the same male, being influenced by numerous endogenous (endocrin, genetic factors etc.) and exogenous factors (food, stress, the reproduction regimen etc.) (de Souza et al., 2007; Goericke-Pesch and Failing, 2013; Rijsselaere et al., 2004).

To confirm infertility in a male, semen parameters should be reevaluated 2-3 times at 1-2 weeks time intervals. On the other hand, a correct estimation of *in vitro* potential of a male dog is not always possible, a good spermogram not being an absolute equivalent of individual fertility (Payan-Carreira et al., 2011).

The goal of the paper was to establish and verify the quality of canine semen based on spermograms in order to cryopreserve the collected semen.

MATERIALS AND METHODS

Research was carried out in the Clinic of the Faculty of Veterinary Medicine of Bucharest on 57 ejaculates collected from 25 dogs. The age of the male dogs varried between 1,5 and 9,5 years, with a mean of 4,8 years.

Before the collection of semen, the males were examined clinically (general physical exam and genital examination) and serologically (Brucella canis testing). The time interval between two collections for a dog was of minimum 3 days. A male was not collected multiple times in the same day.

Semen was collected manually, in the absence of a bitch in estrus, according to the method described by Kutzler (Kutzler, 2005). 3 of the 25 dogs did not respond initially to the digital manipulation. In order to stimulate them, we used swabs collected from bitches in estrus and stored in a freezer.

The sperm rich fraction was kept at 37^{o}C all the time in order to avoid the termic shock. Each of the three fractions of the ejaculate were evaluated macroscopically and microscopically. The macroscopic exam determined the volume and the colour of the fraction. The microsocopic exam of the sperm rich fraction established the concentration and

motility of semen (computer assisted sperm analyzer, SpermVision®, (Minitübe, Germany) (Schafer-Somi and Aurich, 2007).
The following motility parameters were assessed with CASA:
1) Curvilinear velocity (VCL, µm/s), the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time.
2) Linear velocity (VSL, µm/s), the straight trajectory of the spermatozoa per unit of time.
3) Mean velocity (VAP, µm/s), the mean trajectory of the spermatozoa per unit of time.
4) Mean coefficient (STR, %), which indicates the linearity of the mean trajectory and is defined as (VSL/VAP) x 100.
5) Linear coefficient (LIN, %), the ratio of the straight displacement in the sum of elementary displacements during the time of the measurement and it is defined as (VSL/VCL) x 100.
6) Wobble coefficient (WOB, %), which indicates the oscillation of the curvilinear trajectory upon the mean trajectory and is defined as (VAP/VCL) x 100.
7) Frequency of head displacement = beat cross frequency (BCF, Hz), the number of lateral oscillatory movements of the sperm head around the mean trajectory.
8) Amplitude of lateral head displacement (ALH, µm), which is the mean width of sperm head oscillation.
9) Distance curved line (DCL, µm), the actual distance that the sperm cell moved during the analysis period.
10) Distance straight line (DSL, µm), the distance from the point in which the cell was first found in the analysis to the location of the cell at the last frame of the analysis in a straight line.
11) Distance average path (DAP, µm), the measured distance using a smoothed line as a reference.
12) Average orientation change (AOC, degrees), the average number of degrees that the head of the sperm moved from left to right during the analysis.

Morphology and acrosome status were evaluated using the Spermac® stain kit (Stain Enterprises, Onderstepoort, South Africa) (Goericke-Pesch and Failing, 2013). A drop of semen was placed on a glass slide and a thin smear was prepared and air-dried for 5 min on a warm plate at 37°C. The slide was then fixed for 5 min and washed with distilled water 5-6 times. Excess water was removed with a piece of absorbent paper and the slide was placed into stain solution A for 1 min. This procedure was repeated for solutions B and C. Finally, the slide was air dried. 200 spermatozoa were evaluated for abnormal acrosome, head, mid-piece and tail forms under a light microscope at x1000 magnification. Under the microscope, the acrosome is dark green, the nucleus is stained red, the equatorial region is pale green and the midpiece and tail are green. Morphological abnormalities were classified as primary and secondary (Johnston et al., 2001).
Statistical analysis was done using the IBM SPSS – ver. 19 for Windows (IBM, New York, SUA) – paired student T test and one way ANOVA. In some cases, in order to establish the degree of correlation between data groups, Pearson coefficient (R) was calculated. On the dot-plot graphics, the linear trendline (r^2) is represented in order to estimate the evolution of the two data sets.
Results are presented as mean values ± standard deviation. The statistical signifance level was $P < 0,05$.

RESULTS AND DISCUSSIONS

Semen was collected from 25 male dogs of different breeds (fig. 1), resulting a total of 57 ejaculates.

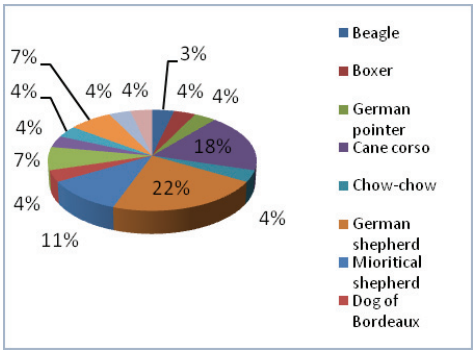

Figure 1. Breed distribution of the dogs used for the collection of semen.

The colour of the ejaculate was characteristic for each fraction: the first fraction was

clear,while the sperm rich fraction was milky-white, opaque to watery-white. The milky-white aspect of the ejaculate was correlated to a higher semen concentration. The third fraction of the ejaculate (prostatic fraction) was clear, transparent for 53 of the 57 ejaculates collected. 4 ejaculates presented a red colour for the prostatic fraction. The microscopical exam showed the presence of a high number of red blood cells, phenomenon correlated with the presence of benign prostatic hyperplasia.
The volume of the 3 fractions of the ejaculate was measured immediately after collection.

Table 1. Mean volume of the ejaculates collected from the 25 dogs included in this study.

Nr.	Breed	Age (years)	Sperm rich fraction (ml)	Prostatic fraction (ml)
	Beagle	4	0.8	5.2
	Boxer	3	2.0	7.8
	German pointer	3.5	1.3	7.5
	Cane corso	4	4.2	8.2
	Cane corso	3.5	3.8	7.9
	Cane corso	3	4.9	9.3
	Chow-chow	5	1.4	4.5
	German shepherd	3	2.5	5.5
	German shepherd	9	1.9	6.4
	German shepherd	8	2.2	7.1
	German shepherd	6	2.0	5.8
	German shepherd	5.3	2.7	8.9
	German shepherd	2.5	3	8.6
	Romanian shepherd	6	4	9.8
	Romanian shepherd	4	4.2	8.7
	Romanian shepherd	5.5	5	8.5
	Dog of Bordeaux	4	2.1	4.2
	Grand dane	9.5	3.9	10.3
	Grand dane	2.8	4.1	9.9
	Golden retriever	1.5	2.6	4.5
	Greyhound	3.5	1.7	3.0
	Labrador	5.5	1.7	4.7
	Labrador	6.8	2	6.7
	Samoyede	7	1.9	5.4
	Terra Nova	4.6	4.3	3.4
Mean		4.82	2.81	6.87
Standard deviation		2.05	1.22	2.15

Analyzing the relationship between the sperm rich fraction of the ejaculate and the male's breed, we found a strong correlation between the two (r^2 = 0,9021), more precisely a direct proportionality between the volume of the sperm rich fraction and the breed's weight (fig. 2). Even since 1983, Olar et al. had signaled the existance of a relationship between the size of the testes and the volume of the sperm rich fraction (Olar et al., 1983). Even though the volume of the ejaculate is not an indicator of the quality of canine semen, it represents a preliminary step in calculating the total number of spermatozoa in the sample.

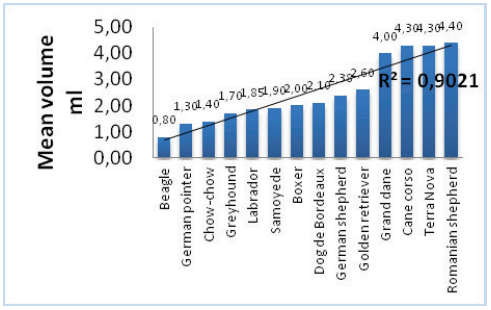

Figure 2. Correlation between breed and the volume of the sperm rich fraction.

The age did not significantly influence the volume of the sperm rich fraction, the liniar correlation between the two being almost 0 (r2 = 0,009) (fig. 3).

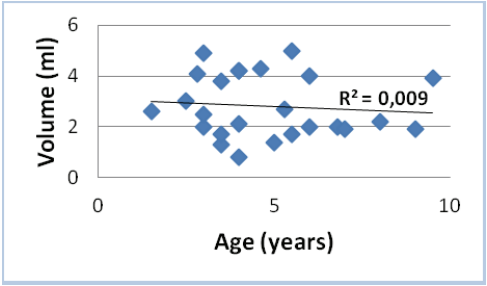

Fig. 3. Correlation between the age of the male and the volume of the sperm rich fraction.

Semen concentration and the total number of spermatozoa were determined automatically with the SpermVision® CASA. Semen concentration was determined for the sperm rich fraction and the total number of spermatozoa was established by multipling

the volume of the sperm rich fraction with its volume. The concentration of the ejaculate is very important for the assisted reproduction technoques, representing the base for the calculus of insemination doses. Fresh semen concentration varied between 202,2 x 10^6 and 1750,3 x 10^6 spermatozoa/ml. We did not identify a correlation between semen concentration and the age or weight of the male dogs (fig. 4).

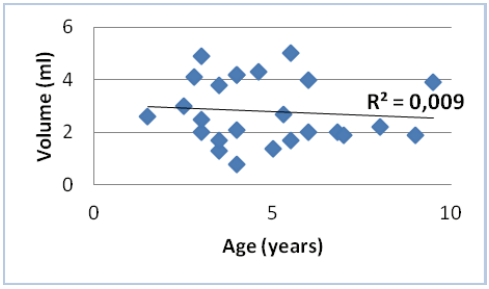

Figure 4. Correlation between the age of the male and the volume of the sperm rich fraction.

Motility of semen is a crucial element for fertility and represents one of the essential elements for establishing the quality of an ejaculate. Mean value for motility in our study was 90,16% (minimum 75,15% and maximum 98,87%) and for progressive motility was 85,14% (minimum 70,38% and maximum 96,22%). This values of motility are according to the standard of the American Society of Theriogenology.

SpermVision® allowed the determination of a complex set of values for canine semen motility, the most important being the velocity parameters (VCL, VSL, VAP). The sperm that deviate with less than 10% from the liniar trajectory are considered to have a straight motility. Excepting the liniar trajectory, the rest of movements are considered non-progressive motility. Since some motility parameters were associated with *in vitro* fertility (VAP, VSL, BCF), we analyzed this values for the fresh semen collected (Silva et al., 2006).

There is a strong correlation between progressive motility, VSL and VAP, this being a good indicator for fertility. For some of the males, VAP has net bigger values than progressive motility going over 160 µm/sec. Although this may look like a good factor for fertility, it can also be a negative factor, a high velocity leading to a rapid depletion of the energetic resources of the sperm cells.

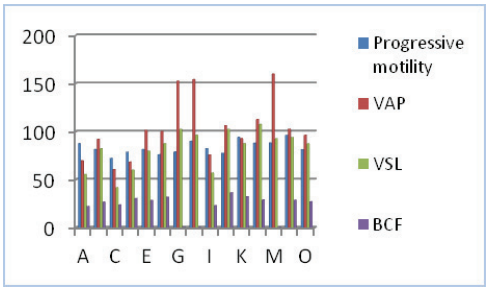

Figure 5. Relationship between progressive motility and certain velocity parameters.

Semen morphology and acrosome status were evaluated using the Spermac stain and the percentages of normal morphology were in the limits of the standard for all the ejaculates. The main types of abnormal forms were detached head, coiled tails, proximal and distal droplet.

Acrosome's integrity is a must for *in vivo* fertility. The percentage of semen with acrosome reaction was 2,37%.

For canine semen it is considered that a percentage lower than 60% of normal semen will negatively influence fertility (Oettle, 1993).

For human semen it has been showen that age has a negative effect on semen morphology, this decline being obvious after 42 years of age (Pasqualotto et al., 2005). In dogs this relationship was not identified, data confirmed also by our study were the correlation between the age of the dogs and semen morphology is almost null (r^2 = 0,0073) (fig. 6).

Other studies showed a positive correlation between normal morphology, progressive and total motility and acrosome intact for canine semen (Agarwal et al., 2003; Ellington et al., 1993; Veznik et al., 2003). For our study, the correlations between semen morphology and motility (fig. 7) and between motility and acrosome status (fig. 8) were weak (r^2 = 0,265, respectivly r^2 = 0,1486), data being in partial agreement with the above mentioned studies.

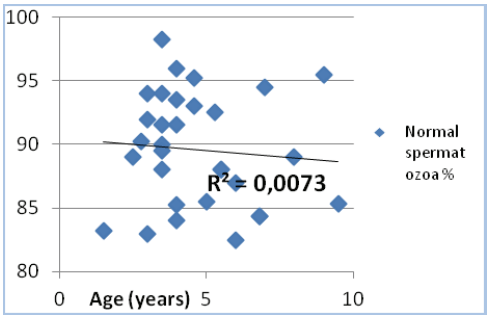

Figure 6. Correlation between the age of the dogs and the percentage of sperm cells with normal morphology.

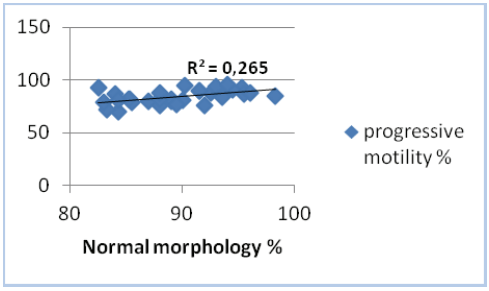

Figure 7. Correlation between intact acrosomes and total motility of sperm.

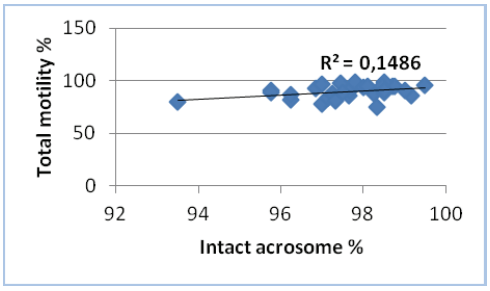

Figure 8. Correlation between intact acrosomes and total motility of sperm.

CONCLUSIONS

Canine semen collection can be easily achieved by manual collection, without needing any special equipment.

Semen analysis showed a strong relationship between male's weight and size (testes dimensions) and the volume of the sperm rich fraction, but did not identify a correlation between concentration and male's size.

Opposed to human medicine, age did not significantly influence the volume or the morphology of canine semen.

Motility parameters, morphology and acrosome status for the ejaculates included in our study were within the accepted standard values.

ACKNOWLEDGEMENTS

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OPTIMIZATION OFREPRODUCTIVE BIOTECHNOLOGIES IN BITCHES BY IMPROVING THE PROTOCOLS REGARDING ESTABLISHMENT OF THE OVULATION TIMING

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Abstract

Determining the optimal time of mating in bitches has become a routine in veterinary practice. Endogenous LH has an important role in ovulation induction and the beginnings of fertile period in bitches but its dosing is difficult and veterinary clinics don't apply it widely.The indirect methods of establishing fertile period are: examination of vaginal smear, determination of progesteron blood levels, vaginoscopy, or the simple examination of clinical changes of external genital segment and characteristics of vulvar discharge and receptivity. The purpose of this study is to determine the connection between estrus clinical signs and the examination methods most frequently used to assess the exact time of ovulation (vaginal smear, vaginoscopy, serum progesterone dosing).

***Key words**: Ovulation timing, Exfoliative citology, Vaginoscopy, P4 assay*

INTRODUCTION

Establishing the exact ovulation moment as accurately as possible is the most important factor when an artificial insemination is made, especially when using frozen/thawed semen since sperm cells survive short periods of time in female genital tract after insemination.

Knowing exactly the physiology of sexual cycle and its variations in bitches is essential. Practically, the fertile period in bitches in which oocytes are fully mature and ready to be fertilised it lasts only 2-3 days during oestrus (Phemister, Holst & Spano, 1973; Verstegen, Silva, & Onclin, 2001).

The variable length of proestrus and estrus in bitches can cause errors regarding ovulation onset if the ovulation moment and beginning of fertile period is based only on appearance of vulvar discharge and its modifications and on behavior changes of the bitch.

Use of vaginal cytology, hormone dosage or vaginal endoscopy are very important methods that allow us to estimate as accurately as possible the appearance moment of preovulatory LH peak and beginning of fertile period.

MATERIALS AND METHODS

There were evaluated 46 females from different breeds presented in the Clinic of Obstetrics and Ginecology - Faculty of Veterinary Medicine from Bucharest in order to determine the optimal time of mating. All females were evaluated in order to determine the best mating time by using vaginal smear, vaginoscopy and serum progesterone assays and receptivity.
Female receptivity was determined by history the owners without any further investigation.
Vaginal smears were made for all females starting the third day since vaginal discharge occurred. The frequency of making smears was once every 2-3 days during the monitoring period. Citovaginal smears were made using sterile cotton-tipped swabs to take samples on the surface of vaginal epithelium. Samples obtained were applied on a glass microscope slide and after drying they were May Grunwald Giemsa stained (MGG). Microscopic examination of dry and stained smears was made using 20X and 40X objective lens to evaluate the percentage of anucleated keratinized superficial cells.
Vaginoscopy was performed using a rigid endoscope the moment samples were taken for citovaginal smears. The endoscopic examination's purpose is to identify the changes of vaginal mucosa appearance and its folds produced under the influence of ovarian steroids during follicular phase until ovulation.
The dosage of serum progesterone concentration was made in Synevovet laboratory by chemiluminescence. Blood samples were sent and processed in the same day, the results being expressed in ng/ml. Testing of serum progesterone concentration was performed when anucleate superficial epithelial cells appeared in vaginal smear in a rate over 70%.

RESULTS AND DISCUSSION

By history, female receptivity was observed since the sixth day after the thrush infection until the 14th day.
In proestrus, citovaginal smear was dominated from the beginning by the presence of parabasal cells of small sizes and round shape and intermediate cells of various sizes. At the beginning of proestrus the smear shown small nucleated intermediate cells, round or oval shaped, with slightly irregular contour. In middle and late proestrus, citovaginal smear appearance tends to modify. The number of parabasal cells is significantly reduced and intermediate big cells with squamous aspect and irregular contour are present almost exclusively. Some superficial large cells with irregular contour and pyknotic nuclei appear too. During entire estrus period a large number of erythrocytes were present probably as a result of progressive estrogenic stimulation that leads to erythrodiapedesis phenomenon on endometrium and vaginal mucosa in a smaller rate (Johnston, Root Kustritz, & Olson, 2001). Increased number of neutrophils is present in early and middle proestrus probably due to the presence of an increasingly number of desquamated cellular debris and bacteria. In late proestrus neutrophils disappear from citovaginal smear probably due to an intense mitotic activity of estrogens over vaginal mucosa that leads to its thickening so that neutrophils can't cross anymore the big number of cell layers, remaining

trapped at lower levels of thickened vaginal epithelium (Concannon, 2010).

In estrus, for all females the general aspect of smear is dominated almost exclusively by the presence of big superficial cells with irregular contour, anucleated and keratinized. Neutrophils are absent during estrus.

In some cases erythrocytes can be seen in the citovaginal smear at the beginning of estrus, probably because of estrogen increased concentrations. But their number decreases and they even disappear until the end of estrus (Concannon, 2010).

Vaginoscopy was performed for all females the moment samples for citovaginal smear were taken allowing an accurate assessment of changes in vaginal epithelium induced by estrogen/progesterone ratio. In early proestrus the increased level of plasma estrogen cause vaginal mucosa edema that first leads to primary vaginal folds formation that are parallel arranged to vaginal lumen. In middle and late proestrus, the increasingly higher level of plasmatic estrogen cause the accentuation of vaginal mucosal edema leading to appearance of secondary vaginal folds that are arranged in transverse manner over the primary vaginal folds (Rehm, Stanislaus, & Williams, 2007; Jeffcoate & Lindsay, 1989). In estrus, due to early luteinizing of ovarian follicles, vaginal edema starts to reduce also leading to reduction of primary and secondary vaginal folds edema, offering vaginal mucosa a wrinkled appearance (Lindsay & Concannon, 1986). The change is progressive starting from the end of late proestrus and beginning of estrus. Maximum intensity of these specific changes can be observed between 3 and 4 days after ovulation.

During late proestrus due to preovulatory luteinizing, serum progesterone concentration started increasing progressively maintaining itself to a level between 1-3 ng/ml for a period that varies between 3 up to 14 days.

In the moment of ovulation, medium serum progesterone level was 4.61 ng/ml.

Table 1. Results obtained after applying monitoring methods of sexual cycle in the bitch

Case no.	Ovulation day after:			
	Citovaginal smear	Vaginoscopy	P4 dosage(ng/ml)	Receptivity
1	9	11	3,9	6
2	7	10	3,6	12
3	11	12	4,2	9
4	10	13	3,8	7
5	8	11	4,1	11
6	10	12	4,4	12
7	11	12	3,7	8
8	7	10	4,8	7
Case no.	Ovulation day after:			
	Citovaginal smear	Vaginoscopy	P4 dosage(ng/ml)	Receptivity
9	14	18	5,9	8
10	12	13	5,1	9
11	9	10	4,7	13
12	7	9	3,9	7
13	9	12	4,9	9

14	11	13	5,8	12
15	16	17	6,1	14
16	10	11	4,1	7
17	7	8	3,6	11
18	8	11	4,2	6
19	6	9	3,9	6
20	8	9	4,1	10
21	11	14	5,8	9
22	9	11	5,3	9
23	7	10	4,7	8
24	10	11	4,4	13
25	8	9	3,7	6
26	7	8	4,6	9
27	9	11	5,5	12
28	10	12	4,9	8
29	13	15	6,4	10
30	8	11	4,9	7
31	10	12	5,1	8
32	8	10	4,5	10
33	11	13	5,7	8
34	6	8	4,0	6
35	10	13	4,8	11
36	11	12	5,1	14
37	8	10	4,4	6
38	9	11	4,2	11
39	8	10	3,8	10
40	10	14	5,6	8
41	8	10	4,6	6
42	9	10	3,9	7
43	11	13	4,1	12
44	10	12	5,5	14
45	9	10	3,7	9
46	8	10	4,1	12

Based on sexual receptivity, ovulation was estimated at 9,28±2,43 days after onset of vaginal discharge.

Examining the citovaginal smear, ovulation and onset of fertile period in bitches was estimated between 9,3 ± 2,02 days.

According to vaginoscopy, ovulation time was estimated on an average of 11,33 ± 2,11 days. Progesterone dosage revealed a medium level of 4,61 ± 0,75 ng/ml in the moment of ovulation.

Statistical analysis was made using the IBM SPSS – ver. 19 for Windows (IBM, New York, USA - paired student T test and one way ANOVA. In some cases, in order to establish the degree of correlation between data groups, Pearson coefficient (R) was calculated.

Results are presented as mean values ± standard deviation. The statistical significance level was $P < 0,05$.

Table 2. Value of the Pearson correlation coefficient between the 3 evaluation methods for the ovulation time in the bitch

	Receptivity-Vaginoscopy	Receptivity - P4 dosage	Receptivity-Vaginal cytology
Value of Pearson coefficient	0,24	0,27	0,35

-1 negative correlation; 0 uncorrelated; 1 positive correlation

Obtained results indicate a poor degree of correlation between the 3 methods of investigation and monitoring of sexual cycle in the bitch and clinical receptivity. Current implementation of these 3 metods allows a better assessment of the beginning of fertile period.

CONCLUSION

46 bitches were subjected to monitoring their sexual cycle by making citovaginal smear, vaginoscopy and serum progesterone dosage.

Based on the examination of citovaginal smear in all 46 bitches, ovulation took place on the average of 9,3 ± 2,02 days. Vaginoscopy evaluation established that ovulation takes place on an average of 11,33 ± 2,11 days. Progesterone dosing revealed a medium value of 4,61 ± 0,75 ng/ml in the moment of ovulation, with limits between 3,6-6,4 ng/ml. Based on sexual receptivity, ovulation was estimated at 9,28±2,43 days.

The obtained results indicate that there is no examination method that taken separately can accurately establish the ovulation timing. It is indicated to obtain an overview from the concomitant use and correlation of the results of several examination methods in order to determine the time of ovulation, providing more accurate results.

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CRYOPRESERVATION OF RED BLOOD CELLS: A REVIEW

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Abstract

In the past years the concern related to transfusional medicine in veterinary practice has thoroughly increased due to the development of emergency medicine and also to veterinarians' practical abilities. Cryopreservation of blood or its products defines the procedures that allow blood to keep its viability in a sterile state for an indefinite period of time without damaging the biological properties.

***Key words**: blood, cryopreservation*

CRYOBIOLOGY OF ERYTHROCYTES

There are numerous cryopreservation methods of blood products known and applied, especially for erythrocytes. Erythrocytes cryopreservation so that all metabolic and biochemical reactions stop, along with the storage at temperatures under $0^{o}C$ for a long period of time, were intensively researched in 1950-1960, maybe due to the fact that at that time keeping erythrocytes and blood products refrigerated was not possible more than 21 days.

The success of red blood cells preservation for a longer period of time depends on knowing and understanding the physiology of erythrocytes and the biochemical and biomechanical changes that occur during freezing-thawing process.

In 1866, french naturalist Felix Archimede Pouchet was the first that described the fact that after thawing, erythrocytes are being destroyed. For a long period of time it was believed that this was happening because of the mechanical effect produced by ice crystals formed during the freezing process (Pouchet, 1866).

In 1950, Lovelock proved that erythrocytes are destroyed after thawing not mainly because of mechanical effect of ice crystals, but rather because of increased concentration of ions in the extracellular space. This leads to osmotic pressure changes of the extracellular space followed by dehydration of cells and drastic pH changes. This mechanism causes irreversible lesions of cellular membrane structure before the ice crystals are formed (Huggins et al., 1969; Lecak et al., 2004; Lovelock, 1954; Sputtek, 1996).

Based on these findings, in 1972 Mazur et al. issued the hypothesis that cell survival during freezing process will be maximum if both ice crystals formation in intracellular space and cells dehydration as a result of osmotic shock will be minimized by using an optimal cooling rate (Mazur et al., 1972).

Erythrocytes are cells with highly permeable membrane for water, so the optimal cooling rate is around 3500C/min. The use of cryopreservatives will induce a significant increase of environmental osmolarity allowing lowering the optimal cooling rate. According to their effect, there are two categories of cryopreservatives: intracellular and extracellular.

Intracellular cryoprotectant substances have a relatively simple chemical structure and do not exhibit cytotoxic effects at low concentrations, most used today being glycerol, dimethylsulfoxide (DMSO) or certain types of glycol.

The main mechanism of action consists in reducing the formation of ice crystals by modifying their shape and size. During the cooling phase, they penetrate the intracellular space where they create a hyperosmotic

environment that will determine water removal from intracellular space (Kim et al., 2004).
Therefore, cell progressive dehydration occurs and osmotic stress is reduced by maintaining a balance between the intracellular and the extracellular environment (Pert et al., 1963; Valeri et al., 1966).
Extracellular cryoprotectant substances have a high molecular weight that does not allow them to cross the cellular membrane, the most used today being dextran, modified gelatin, hydroxyethylamidon (HES) or albumin. The mechanism of action for these sustances is relatively simple and consists in their ability to stabilize the cellular membrane by interacting with its polar components (McGann, 1978; Sputtek, 2007).
Electrolytes in the frozen extracellular environment will be concentrated in these areas. As a consequence of modifying the osmotic ballance in extracellular environment, the water will be slowly eliminated from the intracellular space. Balancing the osmotic difference makes the structural and functional integrity of cellular membrane to be kept (Horn et al., 1997; Sputtek et al., 1995).

CRYOPRESERVATION OF ERYTHROCYTES

The important findings regarding cryoprotectant effect of various groups of substances made possible the development of some efficient preservation techniques by freezing using almost exclusively glycerol. These techniques can be divided into three main groups: cyto agglomeration technique, high glycerol concentration – low rate of freezing and low glycerol concentration – high rate of freezing (Brecher, 2003; Krijnen et al., 1968; Meryman et al., 1972; Rowe, 1973; Rowe et al., 1968).
However the impossibility to add and remove cryoprotective substances in conditions of absolute sterility in a closed system, has made the erythrocites cryopreservation to be practically applied only at the end of 1950 when Tullis et al. described the first continuous flow centrifuge (Tullis et al., 1966). At the beginning of 1960, Huggins has implemented a set of principles regarding continuous flow centrifugation and developed a cyto agglomeration technique that allows cryopreservatives to be removed by precipitation of erythrocites in a hypo-osmotic solution with low pH and re-suspension of erythrocytes in an isotonic environment (Huggins, 1963).
At the begining of 1970, Meryman and Hornblower simplified and improved the cyto aglomeration technique. Therefore the freezing of erythrocytes units it is made in whole blood collection bags after separation of blood components. Glycerol used as cryopreservative is added in very high concentration (40%) and the freezing is performed slowly at -80^{o}C (around 1^{o}C/min) in mechanical freezers. This way erythrocytes units can be stored up to 21 years (Meryman, 1972; Valeri, 1972). Thawing it is made by immersion of frozen erythrocyes units in 37^{o}C warm water baths for 10 minutes. Removal of glycerol after thawing is made through repeated washings with saline solutions of different concentrations (Valeri et al., 1970).
An altenative technique of freezing red blood cells was described by Pert et al. in 1963 and by Rowe et al. in 1968. The technique is based on the addition of glycerol in low concentrations of 15-20% and applying a rapid cooling rate for freezing (about 100^{o}C/min) by immersion in liquid nitrogen at -196^{o}C and storage of units frozen this way in nitrogen vapors at -165^{o}C. Thawing is made in warm water bath at 42-45^{o}C for 3 minutes and the removal of cryopreserative is made by washing erythrocytes with saline solutions of different concentrations (Pert et al., 1963; Rowe at al., 1968).
A different approach regarding the cryopreservation of erythrocytes refers to the use of extracellular cryoprotectant substances such as hydroxyethylamidon (HES), dextran or polyvinylpyrrolidone (PVP). The major benefits of using them consists in biocompatibility properties in increased concentrations, making possible that erythrocytes units to be transfused immediately after thawing without being necessary any additional processing (Robson , 1970; Sputtek et al., 1993). The idea of using extracellular macromolecules in order to protect erythrocytes of freezing effects was

first implemented by Reinfert (1963). Sputtek et al. were the ones that made thorough studies about the use of HES for the cryopreservation of erythrocytes reporting an acceptable survival rate of erythrocites after transfusion without any post thawing processing (Sputtek et al., 1993).

Despite the fact that erythrocytes can be frozen and preserved almost indefinitely no matter the technique and the cryoprservatives used, setting an optimal administrative storage time was needed. So, in 1987, for human blood products, the optimal storage period for frozen erythrocytes has been set and accepted to be 10 years according to "Guide to the preparation, use and quality assurance of blood components" (Recommendation No. R (95) 15 Council of Europe). In 2010, european legislation extended the storage period of frozen erythrocytes up to 30 years, depending on storage method (Directive 2044/33/EC) (Lecak et al., 2004).

From a practical point of view, the most important clinical parameter is the usability of erythrocytes after thawing and removing the cryoprotectant substance. Until recently, after thawing and cryoprotectant removal, erythrocytes could be used for very short periods, only 24 hours. This is because of the removal of blood plasma that used to offer erythrocytes buffering capacity and protection against lysis. The washing process to remove the cryoprotectant substance deprives red blood cells of some of its intracellular metabolites. Therefore their metabolic needs are different from whole blood (Moore et al., a, b, 1987).

The discovery and use of additive solutions ment to meet specific metabolic needs of frozen-thawed erythrocytes may increase applicability of erythrocytes after thawing up to 3 weeks (Hess et al., 2001, Valeri et al., 2001; Valeri et al., 1970).

In conclusion, preservation of erythrocytes by freezing is a feasible method in transfusional medicine due to implementation of modern blood processing techniques in closed system and also due to significant contribution regarding the maximum usability of red blood cells units after thawing by using modern resuspension solutions.

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HARVESTED EMBRYOS ASSESSMENT FOLLOWING POLIOVULATION USING FSH IN COWS WITH A VIEW TO THE BIODIVERSITY OF THE SPECIES

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Abstract

The paper aimed to present the possibility of obtaining a larger number of calves from valuable cows, increasing their contribution to genetic progress, completing the amelioration programs by artificial insemination, using poliovulation and embryo transfer. We used in this study 15 donor cows from the Montbeliarde breed belonging to three different farms from southern Romania. The current paper presents poliovulation results with a view to the number and quality of embryos harvested following the FSH poliovulation in decreasing doses. In the 16-th day of the protocol, the embryos were harvested and morphologically assessed before transferring or freezing them. After harvesting the embryos through the lavage of the uterine horns, they were transferred in special plates and they were identified employing a Nikon magnifier loupe. They were also evaluated employing an inverted Nikon Eclipse TS 100 microscope. Following the morphological assessment of the 116 harvested formations, they were classified as follows: 2 compact morulas (1.72%), 94 early blastocysts (81.03%), 13 blastocysts (11.21%) and 7 unfertilized ovules (6.03%). By employing this protocol, there were no instances of cystisation of ovarian follicles. This fact proves that the FSH administered in decreasing doses leads to a very uniform ovulation and, implicitly, to a smaller number of late ovulated follicles as compared to other poliovulation protocols.

Key words: *cow, embryos, poliovulation, protocol, FSH*

INTRODUCTION

Embryo transfer provides the possibility of obtaining a larger number of calves from valuable cows, increasing their contribution to genetic progress, completing the amelioration programs by artificial insemination (Betteridge, 1986, 2003; Evans, 2005).

Embryo transfer is very effective in the selection programs, in countries with advanced animal husbandry, approximately 95% of tested bulls from milk breeds come from embryos transfer. This percentage reflects the impact of biotechnology in the selection programs (Hasler, 2003).

The importance of this study lies in the possibility of determining a poliovulation in cows subjected to embryo transfer in order to improve its results (Bîrţoiu et all, 2006, 2007).

It also seeks, through this paper, standardization of protocols to be applied in the Laboratory of Biotechnologies of the Clinic of Obstetrics and Gynaecology of the Faculty of Veterinary Medicine Bucharest and externalization of the services to individuals and businesses, farms and breeders alike (Viţălaru, et all, 2008, Viţălaru and Bârţoiu, 2008).

MATERIALS AND METHODS

15 donor cows belonging to the Montbeliarde breed, from three different farms in southern Romania, were included in a poliovulation survey.

The current paper presents the poliovulation results with a view to the number and quality of harvested embryos following a FSH poliovulation in decreasing doses.

Day 0 of the poliovulation protocol in the donor cow was actually the 9th day of its sexual cycle. On day 0 of the protocol, the Periodestrol was introduced. On day 4 of the protocol, the cow received FSH (Folltropin) 4

ml (140 U.I.) i.m., in the morning at 08:00, and 4 ml (140 U.I.), in the evening at 20:00. On days 5, 6 şi 7 of the poliovulation protocol, the treatment was repeated in decreasing doses. On day 5 of the protocol, the cow received FSH (Folltropin) 3 ml (105 U.I.) i.m., in the morning at 08:00, and 3 ml (105 U.I.), in the evening at 20:00. On day 6 of the protocol, the cow received FSH (Folltropin) 2 ml (70 U.I.) i.m., in the morning at 08:00, and 2 ml (70 U.I.), in the evening at 20:00. On day 7 of the protocol, the cow received FSH (Folltropin) 1 ml (35 U.I.) i.m., in the morning at 08:00, and 1 ml (35 U.I.), in the evening at 20:00. On day 7 of the protocol, $PgF2_{\alpha}$ was added, 500 mcg at every 12 hours, usually at 08:00 and 20:00, and the pridoestrol was retrieved.

The oestrus appeared within an average of 48 hours from the last $PgF2_{\alpha}$ dose. The female donor was artificially inseminated when the oestrus was detected, and two other additional times 12 hours apart.

2,5 ml Buserelin acetate were administered per cow before performing the first artificial insemination, immediately after detecting the oestrus. The employed product was Receptal, from Intervet.

On day 16 of the protocol, the embryos were harvested and they were morphologically assessed before transferring or freezing them.

After harvesting the embryos through the lavage of the uterine horns, they were transferred in special plates and identified with a Nikon magnifier loupe and later on evaluated using an inverted Nikon Eclipse TS 100 microscope. The harvested embryos were rinsed after the identification process in order to be morphologically assessed. The embryonic formations were examined using a Nikon magnifier loupe within the farm and, when possible, they were frozen and brought to the Biotechnology and Reproduction Laboratory within the Faculty of Veterinary Medicine, where they were examined using an inverted Nikon Eclipse TS 100 microscope.

RESULTS AND DISCUSSIONS

Using the decreasing FSH protocol, we have harvested several formations inserted in Table 1.

Table 1. Evolution of Dairy Cows during the period 1990-2010 (thousand heads)

Total	EMBRYOS						Unfertilized oocytes	
	Compact morulas		Ealry Blastocysts		Blastocysts			
	No.	%	No.	%	No.	%	No.	%
116	2	1.72	94	81.03	13	11.21	7	6.03

Following the morphological assessment of the 116 harvested formations, they were classified as follows: 2 compact morulas (1.72%), 94 early blastocysts (81.03%), 13 blastocysts (11.21%) and 7 unfertilized ovules (6.03%).

By employing this poliovulation protocol, various results will be obtained. The 15 donors undergoing this protocol produced 158 corpora lutea. The distribution of these corpora lutea on the two ovaries was unequal (85 on the left ovary, so 53.8%, and 73 on the right ovary, so 46.2%).

116 formations were harvested in total from both ovaries. Only 7 oocytes were identified as being unfertilized (6.03%), representing a smaller percentage than the one offered by the literature in the field (Viţălaru, 2008).

With regard to the embryonic formations, the majority was represented by early blastocysts, namely 94 (81.03%), as compared to the 13 blastocysts (11.21%) and the 2 morulas (1.72%).

One needs to underline the fact that by using this protocol there was no follicle cystisation present.

This fact demonstrates that the FSH administered in decreasing doses leads to a very uniform ovulation and, implicitly, to a smaller number of late ovulated follicles as compared to other existing poliovulation protocols.

When closely examined with an inverted Nikon Eclipse TS 100 microscope, the embryonic formations had various aspects as presented in Figures 1-6.

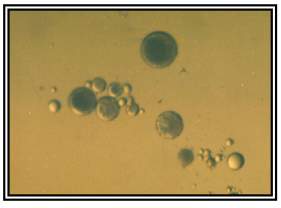

Figure 1. Embryos examined in Nikon Stereo Magnifier Loupe (compacting morulas and blastocysts) (orig.)

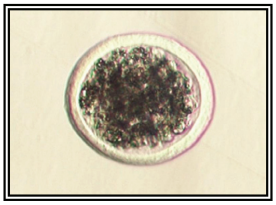

Figure 2. Compacting morula examined with Nikon Inverted Microscope (magnification 40x) (orig.)

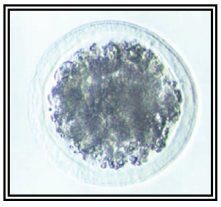

Figure 3. Compacting morula examined with Nikon Inverted Microscope (magnification 40x) (orig.)

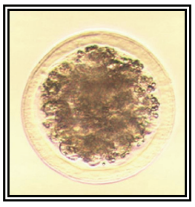

Figure 4. Compacting morula examined with Nikon Inverted Microscope (magnification 40x) (orig.)

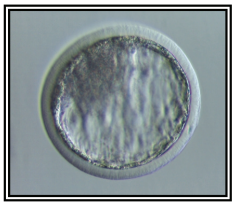

Figure 5. Blastocyst examined with Nikon Inverted Microscope (magnification 40x) (orig.)

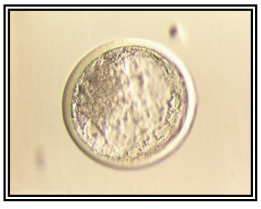

Figure 6. Blastocyst examined with Nikon Inverted Microscope (magnification 40x) (orig.)

CONCLUSIONS

A batch of 15 cows was submitted to an induced poliovulation by using a FSH protocol in decreasing doses (Folltropin + Pridoestrol + $PGF2_{\alpha}$ + Gn-RH) for all animals in the survey.
Following the FSH doses, embryos in all stages were harvested, from compact morulas, to blastocysts, and to a small number of unfertilized oocytes resulting from a late ovulation.
The 15 donors submitted to this protocol produced 158 corpora lutea which were unequally distributed on the two ovaries (85 on the left ovary, so 53.8%, and 73 on the right ovary, so 46.2%).
The majority of the harvested formations was represented by early balstocysts (81.03%).
This protocol did not result in any follicle cystisation.
The number of harvested embryos did not correspond to the number of corpora lutea diagnosed on the ovaries (the number is smaller). It is possible that a number of embryos still remained in the uterus, thus making necessary the administration of $PGF2_{\alpha}$ to all donors after harvesting in order to avoid any unwanted gestation.

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CONFIRMATORY METHOD OF RESORCYLIC ACID LACTONES IN URINE AND TISSUE BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Abstract

This paper presents a liquid-chromatography negative ion electrospray tandem mass-spectrometry (LC-MS/MS) method to determine resorcylic acid lactones (RALs) in urine, muscle, fish, liver and kidney samples. The method comprises an extraction step followed by the clean-up of the samples in two steps using SPE cartridges: C18 Chromabond and Strata Amino from Phenomenex. The RALs were separated on a Pursuit C18 column. in isocratic mode with acetonitril/ 0.1% formic acid aqueous solution. The time for analysis was 12 minutes. A triple quadrupole mass spectrometric from Varian equipped with a ESI source in the multiple reaction monitoring was used for detection. The method was developed according to EU legislation and the parameters assessed were: selectivity, specificity, linearity, recovery, repeatability, within-laboratory reproducibility, decision limit (CCα), and detection capability (CCβ), ruggedness, control chart, uncertainty of measurement. The validation was performed at 1µg/kg for each compound. CCα and CCβ values were 0.16-0.79 µg/kg and the range of mean recoveries were 72.2-119.7% depending of the compound. The relative standard deviation (RSD) of the measurements was below 20%.The method is used to analyze samples originating from the Romanian Residue Control Monitoring Program or private samples. The main purpose of this method is the detection, quantification and confirmation of 6 RAL's in urine and tissue.

Key words: *acid lactones, LC-MS/MS spectrometry.*

INTRODUCTION

The group of resorcylic acid lactones comprises α-zearalanol- zeranol (α ZAL) and its metabolite β-zearalanol- taleranol (βZAL), zearalanone (ZAN). zearalenone (ZON) α/β zearalenol (α/β ZOL), presented in Figure 1.

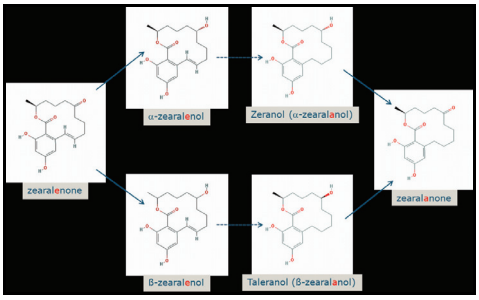


Figure 1 - Zearalenone and its metabolites

Substances with anabolic action are used to increase feed conversion, growth rate or muscle tissue. The EU banned the use of hormones as growth promoter or for fattening. The implantation of Zeranol in calves causes an improvement in mean live weight gain. The use of zeranol is allowed as a growth promoter in livestock in USA and Canada, but it is forbidden in Europe because of its non-steroidal estrogenic properties. RAL's were discovered when it noticed the pigs fed with moldy corn had a higher growth rate. Then, some fungal species were isolated in lab, included Ralgro, the commercial name for Zearanol. This is usually obtained from Zearalenone which is common produced by several species of Fusarium fungi grown in cereals like corn, wheat as Fusarium culmorum and Fusarium graminaerum. The both compounds, ZON and α ZAL give identical metabolites and they are often found in urine from bovine, pigs, ovine and horses. Zearalenone was reported at quantifiable levels in 15% of 20000 samples in grain in a recent EFSA (European Food Safety Authority) opinion. The chronic total dietary exposures to zearalenone ranges 2.4-29 ng/kg body weight/day.Metabolism produces α-

Zearalenol, a metabolite with greater affinity for estrogen receptors than the parent compound, and β- Zearalenol, a metabolite with lower affinity. A liquid chromatography negative ion electrospray tandem mass spectrometry was developed according to EU legislation in order to monitor the presence of RAL's and to know the cause of any illegal treatment with zeranol or natural environment contamination due to moldy feed.

MATERIALS AND METHOD

Reagents and samples

Zearalenol (αZOL), β Zearalenol (βZOL) and Zearalanone (ZAN) were purchased from Sigma-Aldrich, α Zearalanol (αZAL), β Zearalanol (βZAL) and Zearalenone (ZON) were purchased from NIM Australia. Zearalenone d6 (ZONd6) from TRC Canada and α/ β Zearalanol d4 (α/ β ZAL d4) from EURL (European Union Reference Laboratory) Rikilt, the Netherlands were used as internal standards. Stock solutions were prepared by dissolving 10 mg of standards into 10.0 mL of ethanol to obtain 1mg/mL concentration. For zearalenone d6, 1mg was dissolved into 1 ml etanol and it was obtained 1mg/mL concentration. For α/ β Zearalanol d4, 0.1 mg were dissolved into 1 ml etanol. The concentration obtained was 100µg/ml. These solutions were stored at −20 ºC. Intermediate solutions of 10 µg/ml concentration were prepared. For working standards, 250 µL of the intermediate solutions were diluted with ethanol to 25mL into volumetric flasks for a final concentration of 0.1µg/mL for each component. Working standard solutions were stored at 4 ºC. Methyl *tert*-butyl ether (TBME), ethanol, potassium dihydrogen phosphate and beta-glucuronidase from Helix Pomatia were obtained from Sigma-Aldrich, acetonitrile (CAN) and acetone from Merck, methanol and di-sodium hydrogen phosphate from Scharlau and formic acid from Lach:ner. For sample cleaning procedure there were used SPE cartridges: Chromabond C18ec 45 µm 500mg/6ml from Macherey-Nagel and Strata NH2 55 µm 1000mg/6ml from Phenomenex. The samples were collected started with 2011 up to present and were stored at −20 ºC until they were analyzed.

Samples preparation and clean-up Internal standards were added at urine samples and they were hydrolyzed by adding 1ml phosphate buffer 0.1M to 5.0 ml urine. The ph was adjusted at 7.0±0.3 using drops of NaOH 1M or HCl 1M, before adding 0.05 ml Helix Pomatia -β glucuronidase. The mixture was omogenizated by vortex mixing and then incubation at 37 ºC/2h. The samples had been cooled down at room temperature before performing the extraction with 10 ml TBME, vortex- mixing and centrifugation for 10 min/4000RPM. The upper layer was transferred in a glass tube and evaporated at dryness at 60 ºC under a gentle stream of nitrogen. For tissue, was weighted 5.0g well-homogenized sample and the internal standards were added. For extraction, 10 ml CAN were added in the tube, vortex- mixing and centrifugation for 10 min/4000RPM. The upper layer was transferred in a glass tube and evaporated at dryness at 60 ºC under a gentle stream of nitrogen. The residue from urine or tissue was redissolved in 5 ml methanol/water 50/50 v/v by carefully vortex mixing and added 2 ml of water to make the polarity of the solution suitable for the next step (SPE). The SPE column C18 was well-conditioned by passing 5 ml of methanol followed by rinsing with 5 ml water. The sample was passed through the column and washed with 5 ml 40/60 v/v-% methanol/water. The column was dried before the next step by applying a slight vacuum. The RAL's were eluted with 5 ml 80/20 v/v-% methanol/water. The eluate was collected in a test tube by applying a slight vacuum and evaporated at 50 ºC under a gentle stream of nitrogen. The dry residue was redissolved in 5 ml of 80/20 v/v-% acetone/methanol and passed through the SPE amino column which was conditioned before with 5 ml 80/20 v/v-% acetone/methanol. The sample was passed through the column and followed by the collection of the eluate. It was evaporated at 50 ºC under a gentle stream of nitrogen and the residue was redissolved in 100µl, 20% acetonitrile aqueous solution and injecting of 25 µl into LC-MS/MS. The

temperature for column thermostat was: 40 ºC, and the temperature at injection was 5 ºC.

Instrument conditions

The RAL's were separated using a column Varian Pursuit C18 (150mmx3mm, 3µm) equipped with a guard column Metaguard Pursuit 3u C18 4.6mm in isocratic mode 50%(v/v) B-CAN with 0.350 ml/min flow on the column. The mobile phase A was 0.1% formic acid aqueous solution. The analysis time was 12 min. LC–MS system was a Varian Triple Quad equipped with a detector 320MS, a Prostar 410 binary pump LC. Data analyses were performed using MS workstation version 6.9.3. The ion source was in negative ESI mode. Nitrogen gas was used for drying and argon for collision. The MS detector settings were as follows: housing temperature: 50ºC, shield voltage: - 500V, needle voltage: - 4000V, drying gas: 320 ºC, 18psi, nebulising gas : 40 psi

RESULTS AND DISCUSSIONS

According to CRL Guidance Paper 7december/2007 which is the Community Reference Laboratories (CRL's) view on state of the art analytical methods for national residue control plans established in accordance with Council Directive 96/23/EC, for A4 Resorcylic acid lactones and derivates **(**this document is to serve as technical guidance for analytical methods in residue control) the recommended concentration that are presented in Table 1.

Tabel 1 - Recommended concentrations for Zeranol and Zearalanone

Substances	Marker residue-metabolite	Matrix	Recommended concentration
Zeranol	Taleranol	Urine	2 ppb
		Liver	2 ppb
		Muscle	1 ppb
Zearalanone		Urine	2 ppb
		Liver	2 ppb

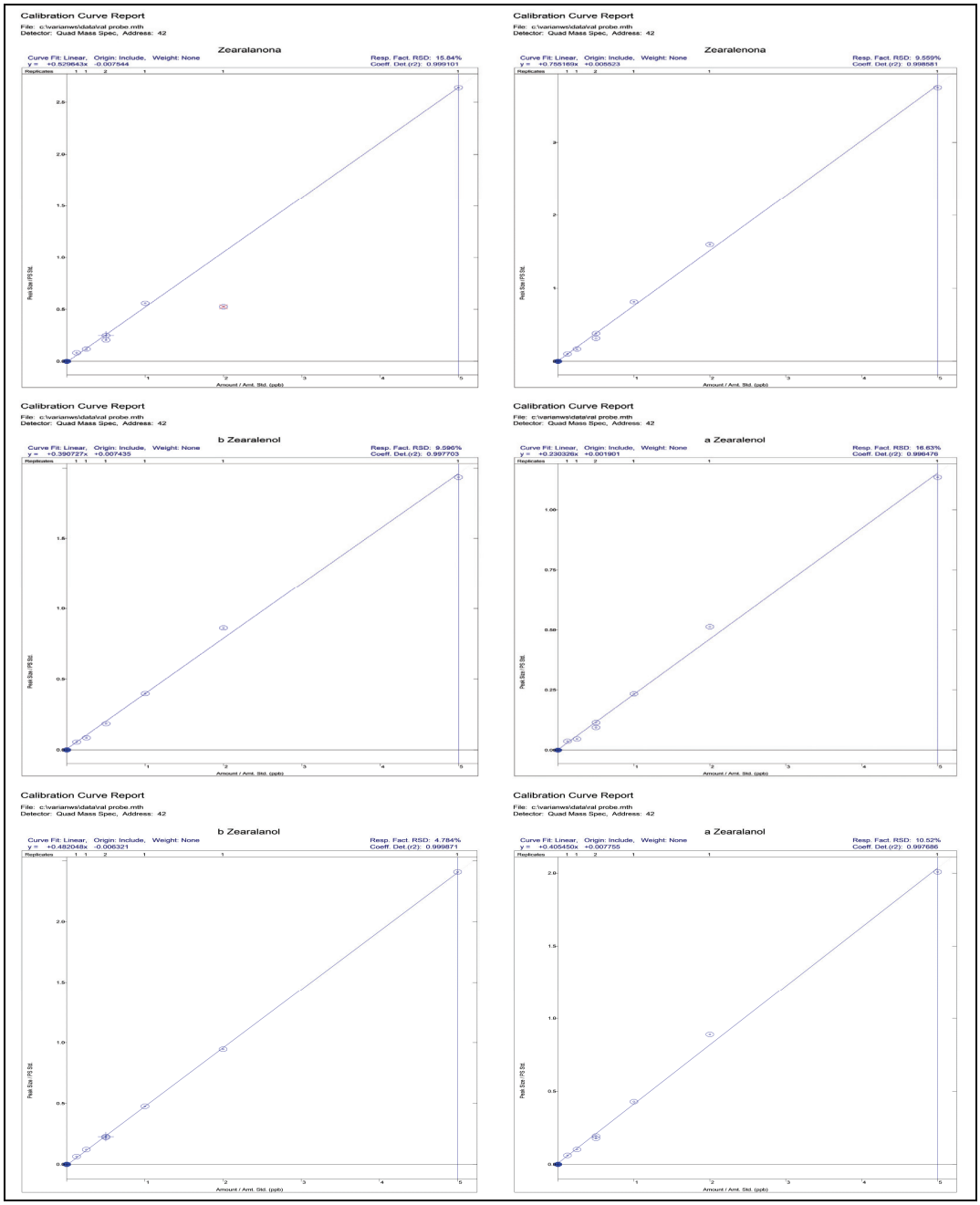
(In case both zeranol and zearalenone are present, the presence of zeranol is considered as the result of mycotoxin contamination). Therefore RAL's are banned substances beside to other anabolic compounds according to 96/22/EC, and the level of validation was chosen at 1ppb for every compound, each matrix. The seven points, including zero, were prepared for quantification of the blank and the spiked samples between 1.25 ng and 50 ng.The mixture of the three internal standards was added at the beginning of the sample preparation at 2ng/ml. The analytical method was validated according to the 2002/657 EC and the parameters assessed were: selectivity, specificity, linearity, recovery, repetability, within-laboratory reproducibility, decision limit (CCα), and detection capability (CCβ), rugedness, control chart, uncertainty of measurement. To demonstrate if the method is suitable, the validation was performed according to 657/2002/EC. It were analyzed many samples from different species to control the interferences and to check the compounds and the internal standards and their behavior in different matrix. The separation of RAL's could be observed without interference in Figure 3. Calibration was based on a standard solution curve. The calibration curves were formed with standard solutions of different concentrations. A seven-point calibration (including zero) was performed at levels 0.25-10ppb. Correlation coefficients were better than 0.98, as can be observed in Figure 2.

Four identification points were obtained using the MRM (Multiple Reaction Monitoring) mode with one precursor ion and two product ions, in negative mode. First, the precursor ions were optimized in Scan mode. The mass spectra of the molecules were recorded and after choosing the optimized fragment there were determined the precursor ions. The collision energies of the ion transitions were optimized and presented in Table 2.

24 blank samples were spiked as follows: 6 at 0.5ppb, 6 at 1ppb, 6 at 1.5ppb, 6 at 2ppb. The data obtained were used to demonstrate the

recovery and the repeatability. Within-laboratory reproductibility was also evaluated by repeating the recovery test and employing the same method on two different days. Within-laboratory reproductibility was calculated for each level. According to the residue legislation, the recovery must be 50–120% for a concentration of 1ppb or lower and the RSD% lower than 20%. These conditions were observed for all RAL's.

Figure 2 - The separation of RAL's



Tabel 2 - The optimized collision energies of the ion transitions

Analyte	CAS	Retention time (min)	MRM I (quantification)	Collision energy (V)	MRM II (confirmation)	Collision energy (V)	Dwell time(S) MRM1/MRM2
ZON	17924-92-4	9.530	317>131	28.5	317>175	23	0.300/0.300
a ZOL	36455-72-8	6.067	319.1>275	18.5	319.1>301	19	0.400 /0.400
b ZOL	71030-11-0	5.025	319.1>275	18.5	319.1>301	19	0.400 /0.400
ZAN	5975-78-0	8.841	319.1>275	18.5	319.1>301	19	0.400 /0.400
a ZAL	26538-44-3	5.805	321.1>277	20.5	321.1>303.0	19	0.300/0.400
b ZAL	42422-68-4	4.878	321.1>277	20.5	321.1>303.0	19	0.300/0.400
ZON d6(SI)	1185236-04-7	9.126	323>131	28			0.100
a ZAL d4(SI)		5.760	325>281	19.5			0.200
b ZAL d4(SI)		4.878	325>281	19.5			0.200

To calculate the decision limit (CCα) and detection capability (CCβ), 8 blank samples were analyzed at: 2x0.5ppb, 2x1ppb, 2x1.5ppb, 2x2ppb. The calculation was done according to ISO 11843 using an EXCEL support. According to the 2002/657 EC, the decision limit of a substance should be lower than MRPL (maximum residue permitted limit) and the detection capability must be lower to MRPL. As shown in Table 3, the results of both the decision limit and the detection capability met the conditions of the 2002/657EC. The sources of uncertainty quantified were: within - laboratory reproductibility, relative standard deviation obtained from calibration curve.

Tabel 3- Asessed performance parameters

No.	Compound/matrix	ccα ug/kg	Ccß ug/kg	r ug/kg	R ug/kg	RSD_r %	RSD_R %	U %	Rec %
1.	Zearalenone/urine	0.29	0.36	0.228	0.287	7.95	10.39	20.83	83.8-114.3
2.	b Zearalenol/urine	0.28	0.35	0.176	0.217	7.11	8.47	17.02	81.5-109.4
3.	a Zearalenol/urine	0.33	0. 40	0.096	0.207	3.24	7.02	16.68	91.5-116.6
4.	Zearalanone/urine	0.24	0.30	0.161	0.385	5.20	13.74	29.74	75.5-119.7
5.	b Zearalanol/urine	0. 16	0. 19	0.181	0.190	5.82	6..32	15.73	95.9-119.7
6.	a Zearalanol/urine	0.21	0.26	0.099	0.190	3.40	6.74	19.98	88.7-111.3
7.	Zearalenone/liver	0.30	0.37	0.239	0.340	9.45	12.24	24.51	72.2-116.8
8.	b Zearalenol/liver	0.37	0. 46	0.147	0.348	6.03	12.90	25.85	80.8-116.6
9.	a Zearalenol/ liver	0.27	0.33	0.079	0.368	3.40	13.86	29.15	81-116.8
10.	Zearalanone/ liver	0. 49	0. 61	0.083	0.471	3.88	18.52	38.75	73.1-115
11.	b Zearalanol/ liver	0. 25	0.31	0.242	0.266	8.84	9.46	21.10	81.7-119.7
12.	a Zearalanol/ liver	0. 24	0. 30	0.306	0.333	11.09	11.55	27.42	80.6-116
13.	Zearalenone/fish	0. 54	0. 67	0.142	0.233	5.47	8.78	17.62	83.4-110.4
14.	b Zearalenol/ fish	0. 64	0. 79	0.169	0.204	7.28	8.43	16.94	76.5-97.6
15.	a Zearalenol/fish	0. 61	0. 76	0.154	0.179	6.13	6..96	16.57	85.9-105.9
16.	Zearalanone/ fish	0. 35	0. 43	0.181	0.208	7.07	8.06	19.73	83.1-110.5
17.	b Zearalanol/ fish	0. 38	0. 48	0.105	0.184	4.20	7.10	17.00	83.9-105.5
18.	a Zearalanol/ fish	0. 33	0. 40	0.122	0.157	4.64	5.78	18.73	86.8-110,6
19.	Zearalenona/kidney	0. 22	0. 27	0.239	0.340	9.45	12.24	24.51	72.2-116.8
20.	b Zearalenol/ kidney	0. 52	0. 65	0.147	0.348	6.03	12.90	25.85	80.8-116.6
21.	a Zearalenol/ kidney	0. 27	0. 33	0.079	0.368	3.40	13.86	29.15	81-116.8
22.	Zeralanone/ kidney	0. 51	0. 63	0.083	0.471	3.88	18.52	38.75	73.1-115
23.	b Zearalanol/ kidney	0. 42	0. 52	0.242	0.266	8.84	9.46	21.10	81.7-119.7
24.	a Zearalanol/ kidney	0. 34	0. 52	0.306	0.333	11.09	11.55	27.42	80.6-116

There were analyzed 5 liver blank samples spiked with the RALs at 1ppb to check if the metod is suitable and robust toward some minor changes. One sample was worked folowing the all steps of the procedure, for 2 samples it changed the methanol percent with

+/-10% at the washing phase at C18 column, and for 2 samples it changed the acetone percent with +/-10% at the washing phase at amino column. It noticed that the deviation standard obtained was lower than the reproductibility standard deviation. In conclusion the method is enough robust to the chosen modifications.

Detection/Identification

The presence of RALs in a sample is confirmed if there are fulfilled the next criteria:

- the ratio of the retention time of the analyte to that of the internal standard, the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ±2.5% for LC;
- the both transitions MRM I and MRM II must be presented;
- for screening (quantification) it uses MRM I.

For confirmation measurements is performed on both MRM I and MRM II.

Both methods can be combined. If a screening result looks non-compliant, MRM II is used for confirmation. The ratio MRM I/II should fulfill 2002/657/EC, described in Table 4.

Table 4 – MRM I/II ratio according to 2002/657/EC

Relative intensity(%)	LC-MS
>50 %	± 20%
from >20 % to 50 %	± 25%
from >10 % to 20 %	± 30%
≤10 %	± 50%

When the two ion traces appeared with the same defined ion ratios and expected retention times, the compound is positively identified. Confirmation of the substance was conducted using a minimum of four identification points. According to 657/2002/EC one precursor ion earned 1 point and each of the both 2 product ions earned 1.5 points which satisfy the condition of the substance identification.

Tabel 5 - The ion ratios in the standard solution and in the samples

COMPOUND	Ion ratios of standard solutions	Criteria 657/2002/EC	Maximum permitted tolerances	Ion ratios of spiked urine samples
ZON	97	± 20 %	77.6-116.4	91.2-103.8
α ZOL	26.8	± 25 %	20.10-33.5	23.3-32.7
β ZOL	27.3	± 25 %	20.48-34.13	23.4-32.7
ZAN	27.4	± 25 %	20.55-34.25	24.4-29.2
α ZAL	26.7	± 25 %	20.03-33.38	23.2-31.8
β ZAL	27.4	± 25 %	20.55-34.25	24.1-32.9

The ion ratios in the standard solution and in the samples during the validation were applied. As shown in Table 5, the ion ratios of each spiked sample fell within the maximum permitted tolerances for positive identifications. MRM chromatograms are shown in Figure 3. There are 2 transitions for compounds and one transition for internal standard.

The internal control of the results

- The suitability of the LC-MS-MS system is checked by autotune conform the manual of the instrument.
- Injection of a standard at 1.25ng. S/N must be >6 for all the compounds. In that case the system is considered as suitable for analysis of all samples
- Injection of a standard at 5ng
- a blank sample spiked at 1ng/ml; the results were introduced in the control chart.

The injection of the extracts into the anlytical instrument were made in the following order: standard blank, standards for calibration curve including a standard at 5ng, reagent blank with internal standard, compliant control sample with internal standard, samples to be quantified including a sample in duplicat, compliant control sample with internal standard, non-compliant control sample spiked at 1ng/ml, standard at 5ng.

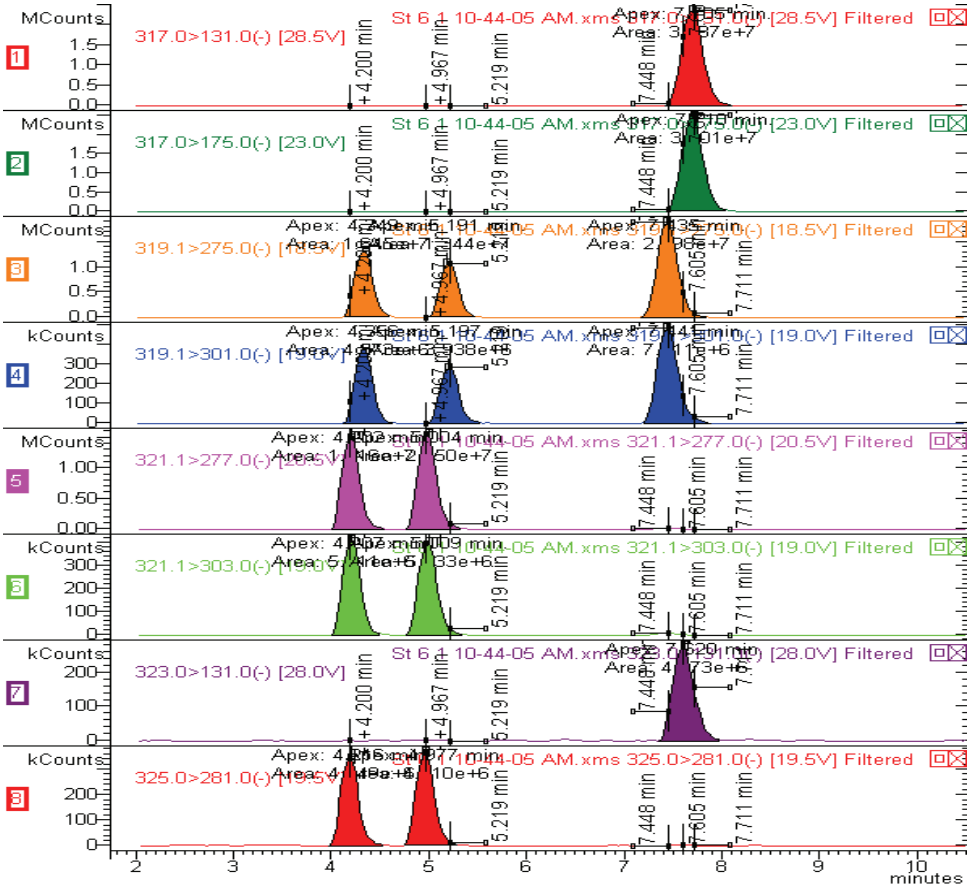


Figure 3 – MRM chromatograms for each compound

Calculation

Areas of the selected ion of the standard and of the internal standard were calculated by the software.

The ratio is a response variable.

Quantitative results are obtained by constructing linear curve fitting using least squares linear regression calculation of the response variable versus the concentration. Unknown concentrations are calculated by interpolation.

According to 2002/657 EC quantification is valid only if:

- In the blank control sample all the internal standards are present (S/N ratio >3 for internal standards).
- In the spiked control sample all components are present (S/N ratio >3 for internal standards and for the non-deuterated compounds).
- In samples all the internal standards are present; for the positive samples, the S/N ratio>3 for the identified analytes

Interpretation

Considering the results interpretation it has been made different approaches by authorities and can be reminded two different opinions made by EFSA and SENASA (National Service for Food Safety and Quality of the Argentine Republic).

To distinguish illegal use of Zeranol from the consumption of *Fusarium spp.* toxin contaminated 8000 samples were analysed by different countries within Europe and there were made different interpretations.

In EFSA's opinion, samples were classified as follows:

- False-positive: Fusarium spp. toxins present, zeranol (or taleranol) absent.

- Equivocal: Fusarium spp. toxins present, zeranol (or taleranol) present.
- True-positive: Fusarium spp. toxins absent, zeranol (or taleranol) present.

In Argentina's case, samples were classified as follows,:

- False-positive: Fusarium spp. toxins present, zeranol (or taleranol) absent.
- Equivocal: Fusarium spp. toxins present, zeranol (or taleranol) present.
  - Ratio (a/ß-zearalanol)/(a/ß-zearalenol +zearalenone)>10 positive
  - Ratio (a/ß-zearalanol)/(a/ß-zearalenol +zearalenone)>1-10 farm
  - Ratio (a/ß-zearalanol)/(a/ß-zearalenol +zearalenone) <1 negative.
- True-positive: Fusarium spp. toxins absent, zeranol (or taleranol) present.

The both criteria give the same results, but at the moment, there is no legal basis for these tools, just screening. It is recommended to do the investigation in farms, also.

Application of the method

About 700 samples, originating from the romanian Residue Control Monitoring Program of 2012 and 2013 or private samples were subjected to the analysis of RAL's using the developed method.

4. CONCLUSIONS

Urine samples were analyzed and RAL's were detected in twenty-three urine samples from horse, pig and bovine.

Zeralenone and its metabolites were detected and confirmated according to the both criteria presented upper, the zeranol abuse was not confirmated and the presence of RAL's in urine sample had a natural environment contamination cause. Also liver samples were investigated and neither zeranol nor taleranol were presented.

The method was developed for the determination of RAL's in urine and tissue. The method was validated successfully, based on the 2002/657 EC. Selectivity, specificity, linearity, recovery, repeatability, within-laboratory reproductibility, decision limit (CCα), and detection capability (CCβ), ruggedness, control chart, uncertainty of measurement were the requirements evaluated. The values obtained during the validation were also in agreement with limits set by the EU.

The method was successfully applied to the proficiency test and to the intercomparison test and it has been accredited by the Romanian National Accreditation Body.

This method was also applied to screening and confirmation of resorcylic acid lactones in urine and tissue.

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COMPARATIVE STUDY BETWEEN THE ANTIBIORESISTANCE OF CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI

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Abstract

The spread of antibiotic resistances and the appearance of multiple-antibiotic-resistant pathogenic bacteria has been recognized by the WHO as a serious problem that complicates medical treatment of bacterial infections. In last years, when campylobacteriosis became the most frequently disease the antibioresistance of this microorganism represents a serious problem. Campylobacter's antibioresistance was carried out through the determination of minimal inhibitory concentration using Sensititre system. This analysis were performed in panels dedicated for these microorganisms. There were tested 132 Campylobacter jejuni and Campylobacter coli strains previously isolated from chicken meat. The species of Campylobacter was perform according ISO 10272/2006.Of the 132 analyzed Campylobacter strains, 39 strains were susceptible to all antimicrobial substances tested, and 93 strains showed resistance to at least one antimicrobial agent; 4 strains were resistant to 6 antimicrobial substances. C. coli showed a higher degree of resistance than C. jejuni to all antimicrobial substances that were tested.

Key words*:* antibioresistance, antimicrobials, *Campylobacter*.

INTRODUCTION

Campylobacter is one of the most common causes of diarrheal illness in Europe and the United States. Campylobacteriosis is generally associated with sporadic diarrhea linked with the consumption of improperly handled or cooked food. Animals such as swine, cattle, and poultry are potential reservoirs for the bacteria. Although *C. jejuni* is predominant in broiler chickens and cattle, it is infrequent in pigs, in which *C. coli* predominate (Nielsen et. al, 1997). Transfer of *Campylobacter* from animals to humans has been demonstrated (Blaser, 1997; Nadeau et. al, 2002). Some authors have suggested that the use of antimicrobial agents in animal production plays a key role in the dissemination of antimicrobial resistances genes from animals to the human population (Swartz, 2002). Most cases of campylobacteriosis occur as isolated, sporadic events, not as part of recognized outbreaks. Nevertheless the number of reported confirmed cases of human campylobacteriosis in the EU in 2011 was 220.209, which was an increase of 2.2 % compared to 2010 (EFSA and ECDC, 2013). The interest in campylobacteriosis in Romania started very recently, and its laboratory diagnostics, followed by recording, began in 2006. Whether in 2006 and 2007 in our country did not recognize any case, in 2011 there were recorded 149 disease induced by Campylobacter. (EFSA and ECDC, 2013) Having in consideration the high number campylobacteriosis reported in EU, the low number of isolates recorded in Romania suggests that only a small number of infections are diagnosed and recorded. In addition to expanding of campylobacteriosis, another worried subject is the antibioresistance of this microorganism. The spread of antibiotic resistances and the appearance of multiple-antibiotic-resistant pathogenic bacteria has been recognized by the WHO as a serious problem that complicates medical treatment of bacterial infections. Transmission of antimicrobial resistance from food animals to humans can occur via the food chain. It is difficult to determine the precise extend of the

risk posed to human health (Harada et. al, 2006). The aim of this study was to evaluate the incidence and the distribution of antimicrobial resistance in *Campylobacter* isolates from chicken meat samples. We present the results on antimicrobial susceptibility measured by MIC assay of *Campylobacter jejuni* and *Campylobacter coli* isolated in Romania. Also, the aim of this study was to realize a comparison between the antibioresistance of *Campylobacter jejuni* and *Campylobacter coli.*

MATERIALS AND METHOD

In our study we tested 72 *Campylobacter jejuni* and 60 *Campylobacter coli* strains isolated from from retail. All strains were stored until use at -80°C and after that, they were cultivated on Columbia agar with 5% horse blood in microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). The species identification was performed using the biochemical tests accordingly ISO 10272/2006. (ISO 10272-1, 2006) Of each strain were obtained a bacterial suspension, from which it was got an amount so that the final inoculum to be 5 x 10^5 cfu/ml.
The minimum inhibitory concentrations (MICs) of tetracycline, ciprofloxacin, nalidixic acid, erythromycin, chloramphenicol, gentamicin, and streptomycin were carried out by the microdilution method acoordingly ISO 20776-1 and CLSI (CLSI M31-A3, 2008). The MIC represent the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in microdilution wells as detected by the unaided eye. (Piddock et. al, 2003) Growth appears as turbidity or as a deposit of cells at the bottom of a well (CLSI M45-A; M100-S16, 2006). *C. jejuni* ATCC 33560 was used as a control. The range of antimicrobials concentrations used for determining MICs were different depending of antibiotic substances (Andrews, 2001).
The epidemiological cut-off values are established by EUCAST (the European Committee on Antimicrobial Susceptibility Testing); in case of *Campylobacter*, the cut-off points are different for *Campylobacter jejuni* and *Campylobacter coli*. These values and the range tested for each antimicrobial agent are presented in table 1 (CLSI M45-A; M100-S16, 2006). For quantitative MIC data, an isolate is defined as 'resistant' for a selected antimicrobial when its MIC value is above the epidemiological cut-off value as indicated in table 1.

Table 1
Antibacterial substances that were tested, their concentration range and cut-off values of Campylobacter jejuni and Campylobacter coli (microdilution method)

Antimicrobial substance	Abbreviation	Range of antibiotics' concentrations tested (μg/ml)	The cut-off values (μg/ml) R >	
			Campylobacter jejuni	*Campylobacter coli*
Tetracycline	TET	0,06 - 64	2	2
Erythromycin	ERY	0,03 - 64	4	16
Gentamicin	GEN	0,12 – 32	1	2
Ciprofloxacin	CIP	0,015 – 64	1	1
Nalidixic acid	NAL	4 - 64	16	32
Chloramphenicol	CHL	2 - 32	16	16
Streptomycin	STR	1 - 16	2	4

RESULTS AND DISCUSSIONS

Of the 132 analyzed *Campylobacter* strains, 39 strains were susceptible to all antimicrobial substances tested, and 93 strains showed resistance to at least one antimicrobial agent; 4 strains were resistant to 6 antimicrobial substances. The results of *Campylobacter jejuni* and *Campylobacter coli* antibioresistance and the percent of antibioresistance depending of antibiotic concentration are presented in table 2 and 3.
The most common the strains were resistant to ciprofloxacin and nalidixic acid. Generally the resistant strains to ciprofloxacin were, also, resistant to nalidixic acid. This aspect is explicable through the appearance of the mutations at *gyr*A and *par*C gene, which determine the resistence of strains both the quinolone of first generation (nalidixic acid) and the fluoroquinolone (ciprofloxacin) (Minihan, 2004; Yan, 2006). Ciprofloxacin is the second choice drug for treatment of campylobacteriosis in humans although resistance rapidly evolves (EFSA, 2011). Also, o quite high resistance was recorded to tetracycline both for C. jejuni (30,6%) and C.

coli (48,0%). Regarding to erythromycin, gentamicin and streptomycin the level of antibioresistance was low. Erythromycin or another suitable macrolide is the first choice drug for the treatment of campylobacteriosis in humans (EFSA, 2011).

Table 2

Antimicrobial resistance in *Campylobacter jejuni* (n = 72) from broiler skin

Distribution (%) of MIC values (µg/ml)

Antimicrobial	Resistance (%)	The procentual (%) distribution of minimal inhibitory concentration (µg/ml)										
		0.064	0.125	0.25	0.5	1	2	4	8	16	32	64
TET	30,6			14,5	0,0	25,8	29,0	14,5	8,0	4,8	3,2	
ERY	6,4				9,6	11,3	50,0	22,5	3,2	3,2	0,0	
STR	3,2					50,0	46,7	0,0	3,2	0,0		
GEN	4,8		0,0	53,2	41,9	0,0	3,2	1,6	0,0	0,0		
CIP	58,0	0,0	12,9	14,5	0,0	14,5	30,6	24,1	3,2			
NAL	56,4						17,7	0,0	19,3	6,4	35,5	20,9
CHL	11,2						25,8	27,4	35,4	0,0	6,4	4,8

The upright bolded lines represent the cut-off values (the value above which the strain is considered resistant) and white fields represent the range of tested antimicrobials.

Table 3

Antimicrobial resistance in *Campylobacter coli* (n = 60) from broiler skin

Distribution (%) of MIC values (µg/ml)

Antimicrobial	Resistance (%)	The procentual (%) distribution of minimal inhibitory concentration (µg/ml)										
		0.064	0.125	0.25	0.5	1	2	4	8	16	32	64
TET	48,0			6,0	0,0	28,0	18,0	4,0	32,0	12,0		
ERY	12,0				20,0	10,0	10,0	12,0	24,0	12,0	12,0	
STR	4,0					20,0	30,0	46,0	2,0	2,0		
GEN	6,0		12,0	16,0	18,0	44,0	4,0	4,0	2,0	0,0		
CIP	68,0	4,0	0,0	0,0	10,0	18,0	24,0	34,0	10,0			
NAL	66,0						2,0	8,0	6,0	12,0	6,0	66,0
CHL	16,0						24,0	16,0	24,0	20,0	16,0	

The most worrying aspect is represented by the multidrug resistance of some strains. Thus, from all strains tested 4 (3%) were resistant at 6 antimicrobial substances and 6 were resistant at 5 antimicrobial substances (4,5%). About 70 % of tested strains were resistant at less than 3 antimicrobial substances. A resume of these is presented in table no. 4.

Table 4

Multiple resistance to antibacterial substances of the tested Campylobacter strains

Resistant to:	*C. jejuni*	*C. coli*	Total *Campylobacter*	%
3 antimicrobial substances	7	9	16	12,1
4 antimicrobial substances	5	6	11	8,3
5 antimicrobial substances	2	4	6	4,5
6 antimicrobial substances	1	3	4	3,0

Regarding to the differences between the *C. jejuni* and *C. coli* antibioresistance it is distinguished that *C. coli* strains are more resistant to antimicrobials compared to *C. jejuni* strains. Thus, *C. coli* strains were more likely to be erythromycin-resistant compared to *C. jejuni* (12 % compared with 6,4 %). This aspect is a potential result of the treatment with erythromycin applied to pigs, knowing that *C. coli* is a specie frequently meet to these. (Harada et. al, 2006; Lin, 2009) *C. coli* were also more likely to be tetracycline, streptomycin and ciprofloxacin-resistant compared to *C. jejuni* (48 % compared with 30.6 % for tetracycline, 4 % compared 2 % for streptomycin and 68 % compared with 58 % for ciprofloxacin). The lowest levels of resistance were found to gentamicin (*C. jejuni* 6 % and *C. coli* 4.8 %), streptomycin (*C. jejuni* 2 %) and erythromycin (*C. jejuni* 6,4 %).

CONCLUSIONS

Of the 132 analyzed *Campylobacter* strains, 39 strains were susceptible to all antimicrobial substances tested, and 93 strains showed resistance to at least one antimicrobial agent; 4 strains were resistant to 6 antimicrobial substances.

C. coli showed a higher degree of resistance than *C. jejuni* to all antimicrobial substances that were tested and especially to tetracycline, nalidixic acid, ciprofloxacin, erythromycin and chloramfenicol.

The highest degree of antibioresistance of *C. jejuni* and *C. coli* strains was recorded to

ciprofloxacin and nalidixic acid (58 %, respectively 68 %), while the resistance against streptomycin and gentamicin was low (2 %, respectively 4,8 %). These findings suggest the use of aminoglycosides and the avoidance of quinolones in the treatment of pathological conditions caused by bacteria of the genus *Campylobacter* in human patients.

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EN ISO 20776-1. Clinical laboratory testing and in vitro diagnostic test system – susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices – part 1: Reference method for testing in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases.

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CAMPYLOBACTER'S PREVALENCE IN ROMANIA – A COMPARISON WITH THE PREVALENCE IN EUROPE

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Abstract

Microbial food safety is an increasing public health concern worldwide. Campylobacter *is a bacterium that can cause an illness called campylobacteriosis in humans. With over 200000 human cases annually, this disease is the most frequently reported food-borne illness in the European Union (EU). Campylobacter bacteria are a major cause of foodborne diarrheal illness in humans and are the most common bacteria that cause gastroenteritis worldwide. Campylobacteriosis are largely perceived to be food-borne, with poultry meat as a major source.*
The interest in determinations of Campylobacter in Romania started very recently, the first monitoring recording in 2007. Since then, every year there are tested about 450 samples represented either carcass skin from slaughterhouse or retail raw chicken meat. All samples have been performed accordingly ISO 10272/2006.
The highest incidence of Campylobacter (63%) was observed in 2008, whereas in next years it was remarked a decrease. This decrease appeared especially consequently of implementation of hazard analysis of critical control point (HACCP). In the same period the Campylobacter's prevalence in Europe was different, depending on country. The most frequently strains isolated were Campylobacter jejuni and Campylobacter coli, these being the main strains involved in human campylobacteriosis pathology.

Key words: *Campylobacter, foodborne, prevalence.*

INTRODUCTION

In 2011, *Campylobacter* continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the EU since 2005. The number of reported confirmed cases of human campylobacteriosis in the EU in 2011 was 220,209, which was an increase of 2.2 % compared to 2010. The EU trend in confirmed cases of ampylobacteriosis showed a statistically significant (p <0.001) increase in the last five years (2008–2012) The EU notification rate of confirmed cases of campylobacteriosis showed a slightly fluctuating, but stable, trend in the last five years (EFSA, 2013).
Campylobacter is considered to be the most common bacterial cause of human gastroenteritis in the world (Newell and Wagenaar, 2000). An acute infection can have serious long-term consequences, including the peripheral neuropathies, Guillan-Barre syndrome and Miller-Fisher syndrome (Pope et al., 2004; Poropatich et al., 2010). In many countries, the organism is isolated 3-4 times more frequently from patients with alimentary tract infections than other bacterial enteropathogens (such as *Salmonella* or *Escherichia coli*). Campylobacteriosis is largely perceived to be food-borne, with poultry meat as a major source (Jore et al., 2010). However, it is often difficult to trace sources of exposure to *Campylobacter* because of the sporadic nature of the infection and the important role of cross-contamination. Thermophilic *Campylobacter* species are widespread in nature. The primary reservoirs are the alimentary tract of birds and mammals including food producing animals (poultry, cattle, pigs and sheep). However the most source in Campylobacter transmission remains the chicken meat (Waldenstrom et al., 2002). Taking in considerations this aspect, since 2008 have been conducting in Europe different monitoring programs to estimate the *Campylobacter*'s prevalence. The aim of this study was to evaluate and compare the

Campylobacter's prevalence recorded in Romania with the *Campylobacter*'s prevalence from other Member States.

MATERIALS AND METHODS

First monitoring of Campylobacter in all States Member was conducted in 2008 accordingly Decision EU 516/2007. During the EU baseline study, the neck and breast skin were also examined for presence of and numbers of *Campylobacter* (Decision EU 516/2007). Since 2008, in Romania there are annually tested about 450 samples represented by carcass skin from slaughterhouse or retail raw chicken meat in order to establish the *Campylobacter*'s prevalence in our country. Initially the monitoring programs were conducted in slaughterhouse and starting from 2010 have been analyzing the chicken meat at retail level.
Campylobacter detection and isolation methods were based on the ISO 10272 procedure. From each sample were weighed 25 g and placed in stomacher bag. Samples were enriched at 1:9 ratio (w:v) in Bolton broth supplemented with cefoperazone (20 mg per l), vancomycin (20 mg per l), trimethoprim lactate (20 mg per l), amphotericin B (10 mg per l) and 5% sterile lysed defibrinated horse blood. The bags with samples and Bolton broth were incubated for 48 h at 41,5°C under microaerobic conditions (10% CO_2, 5% O_2 and 85% N_2) (ISO 10272-1/2006).
Enriched samples (broth) were plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) and Preston agar for isolation and identification of *Campylobacter* spp. All agar plates were incubated at 41,5°C under microaerobic conditions for 48 h. Suspected *Campylobacter* colonies were streaked onto Columbia blood agar (sheep blood 5%) and incubated in the same conditions in order to allow the development of well-isolated colonies. After that, the suspected colonies were observed under phase contrast microscopy for their spiraling motility and characteristic morphology (ISO 10272-1/2006). For confirmation of *Campylobacter* presence were observed also the incapability of suspected colonies to grow at 25°C under microaerobic conditions. Detection of oxidase was another test performed in order to confirm the presence of *Campylobacter*.
Identification of *Campylobacter* species was carried out by biochemical tests as detection of catalase, detection of hippurate hydrolysis and indoxyl acetate hydrolysis (ISO 10272-1/2006). There were used commercial kits to perform these tests.

RESULTS AND DISCUSSIONS

Table 1 shows the prevalence of *Campylobacter* spp, *Campylobacter jejuni* and *Campylobacter coli* in the samples analyzed in period 2008-2012 in Romania.

Table 1
The annual incidence of *Campylobacter* spp., *Campylobacter jejuni* and *Campylobacter coli*

Year	No. samples	% positive *Campylobacter* spp.	*C. jejuni* (%)	*C. coli* (%)
2008	400	63	63,5	36,5
2009	225	37,7	43,5	56,5
2010	225	30,2	52,9	47,1
2011	490	23,4	44,3	53,0
2012	490	31,0	43,4	52,6

The highest incidence of *Campylobacter* (63%) in Romania was observed in 2008, whereas in next years it was remarked a decrease. This decrease appeared especially consequently of implementation of hazard analysis of critical control point (HACCP). Since 2009, the annual *Campylobacter*'s incidence has been varied, with an average about 30%. Initially, due to the high incidence recorded in 2008, it could not provide a correlation between the Campylobacter's presence and climate. In next years, it was observed a high incidence of Campylobacter in period may-september.
At the Community level the prevalence of *Campylobacter*-contaminated broiler carcasses was 75.8% (EFSA, 2011). The MS-specific prevalence varied markedly. MS prevalence ranged from a minimum of 4.9% to a maximum of 100.0%. Consequently, the incidence of *Campylobacter* in Europe was recorded an decrease; in next years the Campylobacter's incidence did not recorded high variations being situated between 26-32% (EFSA/ECDC, 2013). However, the values of

Campylobacter-positive samples in Europe represent a mean of values reported by Member States, between these being high differences. Generally the proportions of *Campylobacter*-positive broiler meat samples (single or batch), at any sampling level, varied widely among Member States, with the prevalence ranging from 3.2 % to 84.6 %. While in the states from Northern Europe (Denmark, Sweden, Finland, Norway) it was recorded a low Campylobacter's prevalence, in other states who has a mild climate, the prevalence was high (Spain) (EFSA, 2011). A possible explanation of this reduction of Campylobacter's incidence in the years after 2008, is the implementation of a lot of programme developed to reduce the Campylobacter's contamination from farm to market.

Both in Romania and Europe, the most frequently strains isolated were *Campylobacter jejuni* and *Campylobacter coli*, these being the main strains involved in human campylobacteriosis pathology. Although in Europe the most met species was *C. jejuni*, in Romania *C. coli* was most frequently isolated.

In figure 1 is presented the dynamic of *Campylobacter* prevalence for period between 2008-2012.

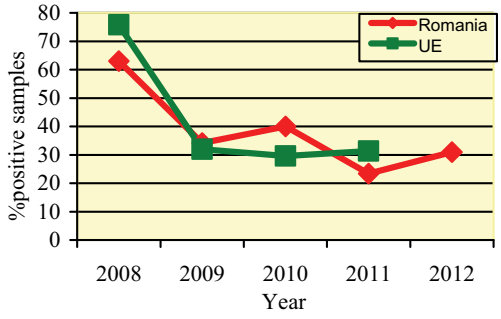

Figure1 Diagram of Campylobacter's prevalence in Romania and European Union

The *Campylobacter*'s importance was unappreciated for many years, but, since *Campylobacter* is the main bacterial agent involved in occurring of gastroenteritis there are lots of monitoring program over world. The monitoring programs are useful both for the annual incidence establishing and for the estimation of the relative contribution of different sources to the burden of human illness (Muller, 2012) On the other hand, it is very useful to know the dynamic of *Campylobacter*'s prevalence consequently applying the control measures in order to reduce the incidence of this microorganism both in farms, slaughterhouses and processing units.

CONCLUSIONS

Campylobacter's incidence is likely to be different in Member States located in the northern of Europe compared to other countries where the climate in milder.

The highest incidence of *Campylobacter* in Romania (63%) was observed in 2008, whereas in next years it was remarked a decrease of this, with a variation of the average about 30%. During the warm months, the Campylobacter's incidence is higher. The *Campylobacter*'s incidence in States Membre in 2008 was 75,8 and in next years was situated between 26-32%.

In order to reduce the *Campylobacter'*s incidence is very important to apply the control measures and the following the effects of these.

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IMPORTANCE OF FARM ANIMAL BIODIVERSITY IN HUMANKIND SECURITY

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Abstract:
Humans are live beings. In order to live they need food. Disposing of creative mind Homo sapiens became the top consumer in the trophic chain on the Earth and multiplied faster than their food. Then people have started to cultivate plants and to breed animals to ensure their food. Their struggle for life developed inside human species and conducted to wars which became more and more destructive. The last two World Wars were tremendously hard. So the United Nations' Organization disposing of a Security Council has been made up to secure people against new World Wars. But recognizing that lack of food stays at the wars' origins ONU included a special Food and Agriculture Organization (FAO), dedicated to secure people against famine anywhere on the Earth. On the above basis, the present paper trays to explain scientifically, the importance of farm animal biodiversity for the food security of the world. How FAO acted to sustain farm animal biodiversity is exposed, as well. Some controversial questions and misunderstanding concerning relations between environment protection especially referring to the Earth's global heating and farm animal biodiversity are answered, too. At the end opinions and hopes related to the contributions of the future Conference on the Sustainable Development that will have place in June of the next year and the food security of the World are emphasized. The final conclusions are: natural animal biodiversity secures the biological balance on the Earth; farm animal artificial biodiversity helps human food security and the social sustainability.

Key words: biodiversity, animal, farm, humankind

Introduction

In science like in jurisdiction words must have precise meanings. Then let's clarify the content of 3 terms we have to use in this report which is intended to be a scientifically one. These three terms are: live beings, biodiversity and security.
Live beings are existing things able for metabolism with the surroundings that need food to live and reproduce as genotypes, under their genetic information control.
Biodiversity means the multitude of genetic information species (species = kind of) having support biological populations formatted in nature as biological species through natural selection and evolution. In farming biodiversity is given by artificial populations (breeds and lines) created trough artificial selection.
Security is the feeling of tranquility what animals able of cognitive perception exercise when there is no danger in their habitat.
Then, on this basis, let us try to understand the importance of farm animal biodiversity for the human live beings' security.

MATERIALS AND METHODS

Biological nature of the humankind
Humans are superior animal live beings. In order to exist, to live and to reproduce, they need housing and food.
As superior animals their genetic information can't command synthesis of 7 essential amino acids needed for the proteins of the cells of their bodies. Their organisms have to receive these amino

acids with the food of animal origin (meat, milk, eggs) they consume. Few plants are able to synthesize essential amino acids. They are synthesized mostly by inferior animal species. (Stan Simona et alii, 2011)

A peculiar trait of human physiology is the development of their brain functions ensuring relations of organisms with the environment over cognitive one, up to the superior stage of creativity. Their cognitive information became creative an humans became able to art things, which not exist in nature. That allows humankind to become de dominant biological species on the Earth and the top consumer in the trophic chain of the planet. Par consequence number of people increased and the natural food resources became scarce.

In time humans learned to breed animals, and to cultivar plants, creating breeds and lines of domestic animals or sorts of cultivated plants as artificial biological populations. The created artificial biodiversity allowed people to satisfy their needs for food better than the nature did. Agriculture developed more and more.

Nevertheless people multiplication was faster than the increase of the food resources. Some human communities migrated to other places already populated with humans. The struggle for life started to be directed against other human communities.

Struggle for life of human communities, what means wars, started with the struggle for food. Humankind security got in danger (Stan Simona et alii, 2009).

Progress of knowledge concerning needed nutrients for human organisms and of medicine resulted in a longer life hope of people.

People continue to multiply. In the former century a real demographic explosion had place (Figure 1). (Atlas Classique. Pierre Gourou Classiques Hachette)

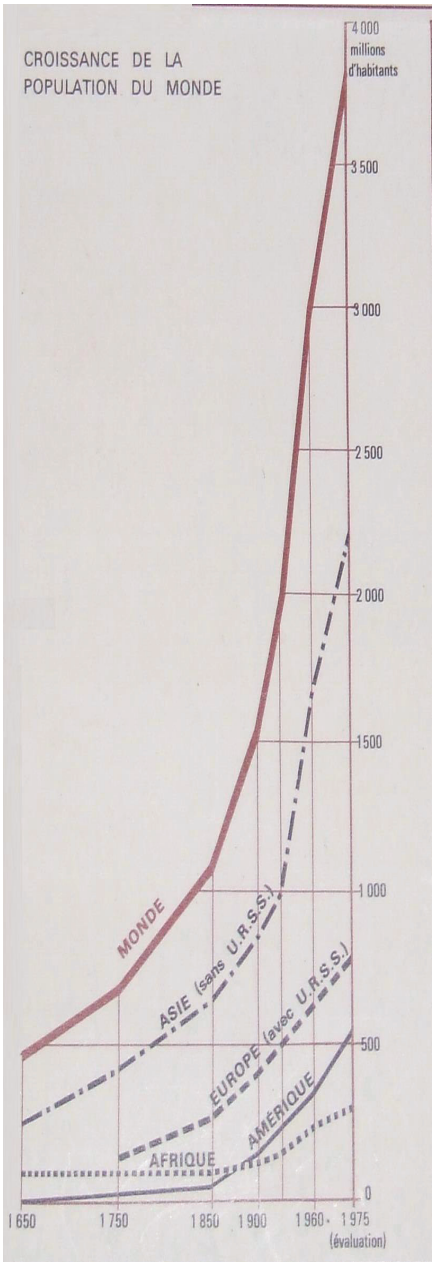

Figure 1. Development of world population

Wars, what in old times were local fights, involved more and more people until they became "world wars". Par consequence of the demographic explosion of the humankind in the former century some main events related to the humans' security should be noticed. Firstly two world wars, at less than twenty years interval, have broken out. Secondly intensive agriculture and the industrial farming in animal production were developed. New wanted types of farm animals have been created.

Farm animal biodiversity

From nutritional point of view human species is omnivorous. Humans eat both vegetable and animal food. From food human organism has firstly to receive energy to maintain its basal functions and to complete its biological production depots.

It also needs amino acids for the plastic proteins that ensure the growth of its body and of its progeny and the replacement of the used cells.

The third requirement refers to the chemical macro elements for skeleton formatting and to the micro elements for enzyme synthesis conditioning a normal metabolism.

There is also the need for vitamins, a kind of enzymes that animal organisms have to take in the food because they are not able to synthesize. Vegetable foods ensure energy, macro and micro elements and hydro soluble vitamins. Foods of animal origin ensure energy, amino acids, both kind of elements and lipoid soluble vitamins. Diversity of food helps a good nutrition. It is result of biodiversity of genetic species cultivated by people to producing food (Paraschivescu M.Th. et alii, 2009).

Concerning farm animal biodiversity we must have in view, at first, the natural biodiversity given by the domesticated biological species. These species have been genetically created by natural selection that means a more fertility of the better accommodated live forms to the habitat. The natural biodiversity is mostly a qualitative one. The organic substances synthesized by different genetic species differ until their molecules' formulas. Fat substances differ concerning their content in oleic acids' molecules. Sweet substances differ with their monosaccharide content. Animal proteins are as different as they are engaged in the Major Histocompatibility Complex (MHC), which is one of the main mechanisms isolating the reproduction of biological species. Maternal organisms have to recognize the proteins of embryo before accepting the pregnancy. This kind of diversity is good from the human nutritional point of view.

The number of farm animal species given by the number of genetic information species of farm animals' ancestors. This number is rather small. There are some species like cattle, sheep, goats, swine, or horses, or poultry, met everywhere on the Earth. These are called eurobiontic because they have a large area where live. Other species, as buffalo, yak, camel, llamas or reindeer are stenobiontice occupying narrow living space.

A higher biodiversity means a larger living area and a chance for more feed resources for the farm animals. (Paraschivescu M.Th. et alii, 2009)

In principle where it is possible to produce food is better to produce food not feed for animals. But there are locations where the vegetable production can't be used as food. Biodiversity of farm animals helps to use such vegetal organic substances as feed stuffs. Aquatic animal species are very important from this point of view, as well.

On the other hand there is an artificial farm animal biodiversity. Disposing of domesticated animals, humans bred, what means have permitted to reproduce, the ones able to produce over the needs of their origin biologic species. (Paraschivescu M.Th. et allii, 2011).

Such groups of animals, formatted by artificial selection, were kept in closed reproduction by artificial means and became artificial biological populations,

which are called breeds. In time biological production of farm animal breeds increased as effect of artificial selection.

Young animals grow faster and higher daily gain of the body mass was registered. Cows and goats were given more milk. Hens laid more eggs.

In the 19th century England developed meat production in part of cattle and in sheep formatting beef cattle breeds and mutton ewe's breeds.

They were not milked and so they required less labor force. That time England needed labor force to develop industry. Breeding of beef cattle has been transferred in the English colonies. In the North America English colonies took place a specialization of cattle breeds as beef cattle selected to produce good and cheep meat and dairy cattle selected to produce much milk. Appling Artificial Insemination, progeny testing the sire bulls and using MOET biotechnologies that was a success (Paraschivescu M.Th. et alii, 2008).

There are now specialized dairy cows yielding over 30 000 kg of milk per year. The idea of specialized artificial populations extended to other genetic species. In poultry were selected lines to reproduce by crossing them hybrids of laying hens and lines to obtain broiler chicken hybrids.

In order to produce good but cheaper commercial pork paternal breeds or lines with much muscle and fertile maternal breeds or lines to multiply the paternal traits have been selected. In ewes there are breeds for lean mutton, breeds for wool, breeds for pellets and breeds for milk. This kind of artificial biodiversity is continuing in farm animals.

By specializing the artificial farm animal populations breeders decreased evidently the quantity of dry matter feed consumed per production unit. One kg of live weight of pork is obtained consuming less than 3kg of dry matter including the feed consumed by the parents of piglets. A kg of broiler chicken is obtained with less than 1, 5 kg of grains. Similar effects have been registered concerning egg or milk production.

The explanation of this fact is found in the action of genetic information. Factors determining the quantity of deposited biological production (meat and fat) and of excreted biological production (progeny and milk) are genetically contradictory. Artificial selection for only one of the two kinds of biological production gives way to increase the selected trait and reduce the quantity of nutrients used for the opposite production. (Paraschivescu M.Th. et alii, 2009). That reduces the needed feed per unit of biological production of the desired type. The future trend pertains to the specialized artificial populations of farm animals. Less feed consumed by the farm animals means more food produced for human consumption. That helps the food security of humankind and the social peace.

We can conclude that natural biodiversity of farm animals, the genetic species of ancestor species of farm animals, is helping to ensure more feed stuffs for animals while artificial biodiversity of the bred farm animals (the breeds and lines) or the resulted crosses and hybrids, reduce the quantity of feed consumed for food production.

RESULTS AND CONCLUSIONS

The human need for security

The need for humankind security appeared in the former century after the First World War. Then idea of a Nations' League was promoted by some prestigious politicians, including the Romanian Nicolae Titulescu. The convention was broken by Nazis German Party and after not more than 20 years the Second World War burst out.

This time the number of deaths and the material damages were tremendous and all people understood that there is a need for a security organization.
Thus at 1945 October 24 the United Nations' Organization (UNO) has been founded. Inside UNO a Security Council successfully acted and continues to act preventing wars (Stan Simona et alii, 2009).
Recognizing that at the origin of wars stayed the famines of human communities caused by lack of food, the Food and Agriculture Organization (FAO) has been created inside ONU, from its foundation.
The target of FAO was and remained to fight against famine anywhere on the Earth as a necessity for humankind security.
The food security concept was clearly promoted in 1963 when in Rome FAO has claimed for "the right of anyone to eat as much as its needs are".
That right of individuals is a duty of each state for its inhabitants. Food security is attaint when all people have direct access to their food.
That isn't an easy task. In 1925 human population counted 1500 million of souls. In 1960 Earth's population got up to 3000 millions. In 1975 it went to 4500 million and now is over 7000 million. This demographic explosion explains the proclamation.
Afterwards cultivated surface extended in the detriment of natural ecosystems. Woods have been especially affected. Some biological species became vulnerable up to the risk of extinction, a sign of danger for the food security of the future (Paraschivescu M.Th. et alii, 2009).

FAO is a strong and active organization involved in many actions connected with the food security. It received the task of surveillance of biodiversity, as well (Figure 2)

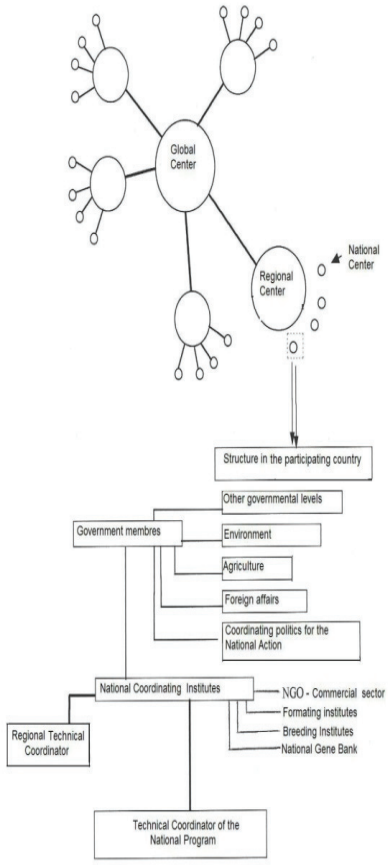

Figure 2. FAO Program structure for Farm animal resources management

The question of animal biodiversity was extended from the wild animals to the farm animals, too. The schedule of the FAO's Management program of Farm Animal Resources exposed above determined the idea of the sustainable development of the world economy what means with human production activities not to show damage to the natural environment.
The sustainable world economy requires to maximally satisfying the needs of the actual human generation without affecting future generations' interests. Environment protection is a basic condition for the sustainable development.

This statement generated the Ecologist social movement dedicated to natural environment protection as civic or political organizations (Paraschivescu M. et alii ,2009).

Unfortunately Ecologist Organizations are not always acting in the favor of the sustainable development of the human society. They limit their action to protecting nature but ignoring human existence, what is dangerous for the world security.

For instance ecologist papers have been published considering that highly producing dairy cattle are causing global heating of the Earth because they are emitting methane (CH_4) as digestion gas out of the concentrated feeds they consume (Jonson E.D.et alii, 1991).

Scientifically experiments with ruminants didn't confirm such hypothesis. More than that other ecologist papers claimed that a greater livestock of cattle will increase the carbon dioxide (CO_2) emission and the green house effect of atmosphere will become more intensive. That is wrong. On the Earth the quantity of Carbon atoms is constant (Paraschivescu M. et alii, 2009). Part of Carbon atoms are blocked underground as diamonds, mineral compounds, coal, oil or natural gases, part of them are stored on the Earth's surface in the organic lifeless substances, and part of them are deposited in the bodies of live beings.

Only the Carbon atoms present in the atmospheric gases CO_2, CH_4, CO and CFC molecules are acting as global heating agents of the planet.

If live beings are less than more Carbon atoms will be free to format gas molecules in the atmosphere.

The true is that no live being, except humans, could cause misbalances on the Earth (Paraschivescu M.Th. et alii, 2011).

Sustainable development and world security

UNO as the world security organization has got to the stage of acting against a very large scale of insecurity sources as: politics of states, terrorism, fishery, food production, energy sources, environment protection and so on. All these targets have conducted together to the sustainable development concept.

The UNO Conference on Sustainable Development that has take place between 4 and 6 June last year, in Rio de Janeiro had two items submitted to discussion:"The Green Economy in the sustainable development and poverty exclusion context" as the first subject and "The Institutional Frame of the Sustainable Development" as the second one.

Green Economy suggests the fact that green plants production is inexhaustible since it is formatted from mineral substances (CO_2, N_3 and H_2O) using sun light energy in the presence of chlorophyll green pigment.

So must be all regenerating inexhaustible production. Example given the energy obtained from hydro-electric power stations is a "green energy", because it is inexhaustible. Aeolian energy, wave's energy, and sun energy deserve to be included in the "green economy" (Paraschivescu M.Th. et alii, 2011).

But green plants are perishable. Desertification caused by global heating of the Earth will decrease the vegetal organic production of natural flora and cultivars. That will cause less feed for farm animals, too. The danger of famine for people will increase.

Reducing the quantity of burned fossil fuel is the only one solution to diminish transferring blocked underground Carbon atoms to atmosphere. Substitution of fossil fuel with Biofuel stops the mentioned transfer of Carbon atoms.

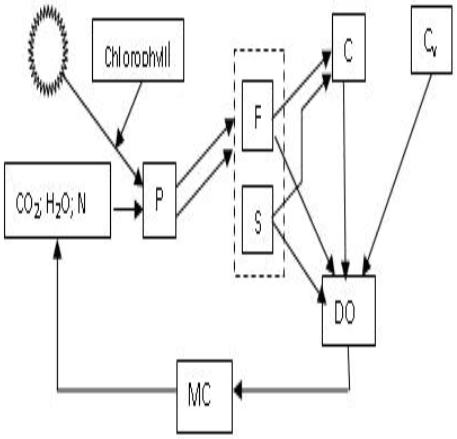

Fig. 3. Primary trophic net (V. Tufescu - 1981)

Nevertheless with Biofuel the chain of gas mineral compounds of Carbon trough organic compounds and again to mineral gas is a short one: atmospheric CO_2 – vegetal organic substance – CO_2, while the trophic chain is much longer (see Fig.3):CO_2 – first producers of vegetal organic substances – first consumers (phytophagous and saprophagous) – second consumers – top consumers - died organic matter – micro consumers – CO_2.
On the other hand producing Biofuel means reducing food or fodder cultivar areas and, unfortunately, the target of famine exclusion in the world wasn't completed yet.
Not long ago FAO officially declared existing of famine in Somalia. In order to have a good balance between the human population and food resources some actions like birth control and family planning have to be taken, at world level. Let us hope other solutions for the future of human society was be suggested by the UNO Conference on Sustainable Development in June 2012.
More difficult will be the second item of the conference. Specific institution has to be proposed and accepted by the participant states.
Or, there are great differences from state to state concerning resources, scientifically development, and inner politics and so on, or there are great differences from state to state concerning resources, scientifically development, and inner politics and so on. At the same time many involved phenomenon are global and have no boundaries.

CONCLUSION

Food security is one of the main conditions of humankind sustainability. Famine has to be excluded everywhere on the Earth.
FAO has proclaimed “the right of anyone to eat as much as its needs are” what is a difficult target.
This target requires more animal production. Natural biodiversity of farm animals given by genetic species involved favor to have more fodder production while artificial biodiversity resulting in specialization of breeds allow a more efficient using of feed.
Birth control and family planning could help in balancing human population to the food resources. Increased livestock of farm animals can not contribute to the global heating of the Earth.
On contrary animal bodies are depositing Carbon atoms reducing their presence in the atmospheric gazes (CO_2 and CH_4) having green house effect.
Maine attention has to be paid to reducing fossil fuel consumption because this way large amount of carbon atoms are shifted from the underground depots to atmosphere increasing its green house effect.
In order to sustain humankind security UNO Conference on Sustainable Development has proposed some “Institutional Frame of the Sustainable Development” to be founded.
This time consents on solutions are very important but concluding will be difficult. Let’s hope for the best.

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THE ASSESSMENT BY AVOIDANCE TEST OF THE HUMAN-ANIMAL RELATIONSHIP IN DAIRY CALVES

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Abstract

The positive effect of a good human-animal relationship was demonstrated on the production, health, behavior and mental state of farm animals, especially when positive interactions take place in the early stages of life. The on-farm management system in dairy farms can potentially have influence on the relation between people and calves, given the different schedule of the daily procedures. The aim of this study was to conduct a comparative assessment of the human-animal relationship in dairy calves in farms with tie-stalls and loose housing based on the results of avoidance testing. A number of 146 dairy calves (in three different age categories) were assessed in five farms during the cold season. A standardized technique of human avoidance test was used, awarding scores depending on the individual avoidance distance of the observer by the calves. The results were statistically processed with the SPSS software. The youngest calves (up to two months old) had the highest scores within the standardized avoidance test, meaning less avoidance toward the observer. When the tie-stall farms were compared with the loose farms, no statistically significant difference ($P>0.05$) was found regarding the human-related behavior of the calves. The assessment of the calves' behavioral response toward humans using the avoidance testing showed that in this study the human-animal relationship was not influenced by the housing system, most probably because the management of the calves was similar in all the five farms.

Key words: *dairy calves, human-animal relation, human avoidance test.*

INTRODUCTION

The human-animal relation was defined as the degree of closeness or distance between the animal and human (Estep and Hetts, 1992), meaning the reciprocal perception of the animal and human that develops and expresses by their mutual behavior (Waiblinger et al., 2006). According to Estep and Hetts (1992) the human-animal relationship is a dynamic process in which the previous interactions between the animal and humans ensure a basis for a stable relationship and which have than a feedback effect on the nature and perception of future interactions. In principle, a relationship of this type needs either individual mutual recognition or the generalization by the animal of the experiences with a specific human person also on other people (Waiblinger at al., 2006). As regards young animals, these cumulate experience and the interactions with humans represent a novelty for them. For this reason, the quality of human-animal relationship is important in these for the formation of some perceptions, for the development of certain attitudes towards a person but also generally towards humans, as a response to the quality of interactions with people. In calves, it was proven that early positive interaction with humans reduces their fear, human related reactivity and stress levels during handling (Boissy and Bouissou, 1988; Boivin et al., 1998). It seems that gentle human handling has long-lasting positive effects (stress reduction) especially when it takes place in early life stages, after the calves are born (Probst et al., 2013). It seems that simpe habituation with the human presence have a beneficial effect on the behavioral response of the calves towards people, as the majority of the fear responses are trigerred in those calves that had minimal contact with people, comparing with those handled more frequently, irrespective if the interactions with humans were negative or positive (Petherick et al., 2009a,b). The lack of human contact in the early period of the calves' lives is associated especially with a defensive behavior (Le Neindre et al., 1996).

Given the fact that the every-day management can be different depending on the housing and

management system of the farm (loose and tie-stall systems), the aim of this study was to conduct a comparative assessment of the human-animal relationship in dairy calves in farms with tie-stalls and loose housing based on the results of avoidance testing.

MATERIALS AND METHODS

This study was accomplished between January 2012 and May 2013. During this period five farms were visited (Bistrita-Nasaud and Cluj counties), evaluating a total number of 146 calves of different ages. For selecting the farms and deciding the moment of the visits it was taken into account the accessibility of the location, numbers of the animals, and agreement of the farmer to take part in the study and the possibility for the observer to spend time near the calves without disturbing the usual daily activities of the farms.

Farm 1 had loose housing system, but with permanent housing (without outdoor access of the cows). In the moment of the visit, there were 30 Holstein Friesian calves (5 calves between 0 and 2 months, 13 between 3 and 5 months and 12 between 6 and 7 months), were kept in three collective stalls, according to their ages, in the same barn with the cows.

In farm 2, also with loose housing, 42 calves were assessed (16 calves with ages between 0 and 2 months, 14 between 3 and 5 months and 12 between 6 and 7 months). Part of the calves was Holstein Friesian and others were mixes between Holstein and beef cattle.

The other 3 farms (3, 4 and 5) had tie stalls for the cows and the calves were kept in collective stalls. There were 5 calves in farm 3 kept all together; 36 calves in farm 4 (13 calves up to 2 months of age, 17 between 3 and 5 months and 6 calves older than 6 months) and 36 calves in farm 5 (a collective stall with calves up to 2 months of age, two stalls with a total number of 17 calves between 3 and 5 months of age and a stall for the calves older than 6 months of age). In all of the farms, the calves were separated from their mothers immediately after birth and all the male calves were sold when they reach the age of 2 months. They were fed with milk up to the age of 2 months and then with hay and cereal meals. The only farm where the calves were released to suck from their mothers was the farm 3. The hay and cereal meal was presented to calves about one week before weaning. None of the farms allow access to water for the unweaned calves, but only after weaning.

In order to assess the human-animal relationship in calves the avoidance test was used, respecting the methodology and technique proposed by Leruste et al. (2012). The assessor entered in the collective stall, waited for one minute, for the calves to get used with his presence, then chose one calf at an approximate distance of 1.5m, having the head oriented in the direction of the assessor. The behavioral response of the calf was scored according to four possible categories: (1) the assessor is able to establish visual contact with the calf; (2) the assessor is able to make a step towards the calf, with the arm flexed in 45 degrees ahead from the bodyline, and the calf stays still at least one second; (3) the assessor is able to make the second step towards the calf which stays still at least another second; (4) the assessor is able to touch the head/nose of the calf without startling it. The test ended when the calf moved in the opposite direction from the assessor. At the end of the test scores were recorded from 0 to 4 (0 = impossible to establish visual contact with the calf; 1 = visual contact; 2 = approaching with one step; 3 = approaching with two steps; 4 = touching the calf without startling it).

The recorded scores were processed by calculating the descriptive statistical indicators and by comparing the results obtained for the calves in the two different housing systems. For the statistical processing of the data the SPSS statistical software was used.

The differences were considered significant if $P<0.05$.

RESULTS AND DISCUSSIONS

The descriptive statistical parameters for the scores obtained in the calves' avoidance test are presented in table 1.

Table 1. The descriptive statistical indicators for the scores of human avoidance test and the significance of difference between the two housing systems

Age category	Farm with loose housing			Farms with tie stalls			P value
	Mean ± sd	Median	Range	Mean ± sd	Median	Range	
0-2 months	3.28 ± 1.21	4.00	0.00-4.00	3.00 ± 1.24	3.00	0.00-4.00	>0.05
3-5 months	2.33 ± 1.51	3.00	0.00-4.00	2.31 ± 1.53	2.00	0.00-4.00	>0.05
6-7 months	2.46 ± 1.58	3.00	0.00-4.00	2.50 ± 1.50	3.00	0.00-4.00	>0.05

sd = standard deviation

If $P < 0.05$, the difference between the two systems is significant

As shown in table 1, no significant differences ($P > 0.05$) were found between the results of the human avoidance test in the calves from the two housing systems. In the study of Leruste et al. (2012), for calves of approximately 15 weeks old the authors obtained a mean score of 1.7 ± 0.1, with intervals from 1.0 to 2.8. In both housing systems assessed in the present study, higher scores were obtained for any of the age categories. These results could indicate an adequate interrelation between the farm workers and the calves. However, interpreting the results of human-animal relationship assessment needs always precaution, because many environmental and individual factors can influence these. As Boissy et al. (2007) highlights that the behavior of the calves towards humans can be shaped by curiosity, exploratory behavior. In the case of the youngest calves (between 0 and 2 months of age) this aspect could have a role, explaining why their mean scores were the highest within this study (Table 1). In the same time, the older calves could have been afraid of humans if they had negative experiences in the past in relation with people. In usual conditions (as it was in the assessed farms), the calves' contact with humans is short, only during feeding and barn cleaning, not enough for building a positive human-animal relationship.

Table 2 presents the proportion of the calves that could be touched by the assessor in the human avoidance test. In a study investigating the effect of early manipulation of calves by humans Schütz et al. (2012) asessed the calves at the age of 4 weeks by the human avoidance test. In the group in which the calves were manipulated positively in their first days of life, 45% accepted to be touched by the assessor, comparing with only 20% from the group in wich the calves were handled negatively immediately after their birth.

Table 2. The percentage of the calves that could be touched in the human avoidance test

Age category	Percentage of calves that could be touched	
	Loose housing	Tie stall housing
0-2 months	57.14	44.82
3-5 months	44.44	34.48
6-7 months	37.5	31.25

In this study within the age category of 0-2 months almost half of the calves from tie stall barns and even more in loose housing could be touched (Table 2) by the assessor. These values can be considered high and could indicate positive early experiences of the calves in relation to humans. Yet, it should be mentioned that in the study of Schütz et al. (2012) the testing of the calves took place in an unfamiliar environment (in a fenced arena), not in their familiar surrounding, as it was made in the present study. The familiarity of the testing environment could influence the results of behavioral tests in animals. For example, it was demonstrated that the social circumstances (presence or absence of the conspecifics, possibility or impossibility of visual contact with consepecifics) affects the behavioral response of cattle towards humans in test conditions (Grignard et al., 2000). Otherwise it is difficult to compare the data obtained in this study with the results of other researchers due to the fact that the studies in this area are not very extended.

The absence of the significant diferences within the human-animal relationship testing

results in the calves in the two different housing systems is most probable due to the fact that even if the housing system was different in the farms, the calves were kept similarly, irrespective if the cows were tethered or not.

CONCLUSIONS

The assessment of the behavioral response of the dairy calves towards people by the human avoidance test showed that the human-animal relationship is not influenced by the housing system used in the investigated farms, most probable because the fact that the calves were kept similarly in all farms.

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THE PREVALENCE OF LAMENESS IN THE ASSESSMENT OF TRANSYLVANIAN DAIRY HERDS BY LOCOMOTION SCORE AND ACCORDING TO THE FARMERS' ESTIMATES

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Abstract

Lameness in dairy cows represents one of the most serious animal welfare problems. Monitoring on-farm lameness prevalence is important for dairy producers and veterinarians in their efforts to reduce lameness. The aim of this study was to compare the prevalence of lame dairy cows assessed by locomotion score and estimated by farmers. For the on-farm lameness assessment a five point locomotion score was used. A number of 751 dairy cows were assessed in the cold season in 10 Transylvanian dairy farms. The results were statistically processed using the SPSS software, version 17. Out of 751 assessed cows 33.49% presented normal locomotion; 40.38% presented slight lameness; 18.03% were moderately lame; 5.17% were lame and 2.93% presented severe lameness. The prevalence of lameness established using the locomotion score varied from 4.76% to 68% (median 23.38%), and that estimated by the farmers ranged between 0 and 15% (median 7.50%). There were statistically significant differences ($P<0.05$) between the prevalence of lameness assessed using the locomotion score and that estimated by the farmers. It follows from the findings of this study that the dairy farmers are not aware about the locomotion problems of their cows.

Key words: *dairy cows, lameness prevalence, locomotion score.*

INTRODUCTION

Lameness represents one of the most severe and frequent problems encountered in the commercial dairy farms worldwide, with a strong negative impact on the welfare and production of the animals (Whay et al., 2003). Lame cows are also prone to other health problems (Walker et al., 2008), decrease in milk production (Warnick et al., 2001), impairment of the body condition (Ozsvari et al., 2007) and finally the dramatic shortening of their productive lives (Booth et al., 2004).

Despite the fact that lameness is a serious welfare and economic problem, some studies (Wells et al., 1993; Whay et al., 2003; Espejo et al., 2006; Rutherford et al., 2009; Leach et al., 2010; Sarova et al., 2011; Richert et al., 2013) have shown that farmers tend to underestimate the prevalence of lameness, contributing to the increase of the lame cows' percentage in their farms. Improved detection of lameness, so the farmer would estimate better the actual lameness prevalence, could play a significant role in persuading farmers on the importance of lameness on their farms. Additionally, the identification of the slightly lame cows may reduce considerably the economic losses represented by the longer duration of the treatments and the higher costs in the cases of severe lameness.

Although the Romanian farms face the same problems, as those in other countries, the researches regarding lameness in dairy cows are extremely limited in Romania. The insufficiency of the studies and the lack of information, knowledge and concernment of the farmers regarding the importance of this problem determine significant economic losses.

The aim of this study was to compare the prevalence of lame dairy cows assessed by locomotion score and estimated by farmers in Transylvanian farms.

MATERIALS AND METHODS

This study was accomplished in 10 dairy farms (5 farms with loose housing and 5 farms with tie stalls) from Transylvania (Cluj, Bistriţa-Nasaud, Sibiu and Satu-Mare counties), between December 2012 and February 2013.

The farms were selected with the help of the

veterinarians in the area, based on the following criteria: the housing system (loose and tie stall barns); the numbers of the animals in the farms (at least 20 dairy cows); easy access to the farm during the winter; the agreement of the farmers to participate to the study. The mean characteristics of the farms are presented in Table 1.

Table 1. The main characteristics of the investigated farms

Barn	No. dairy cows	Breed	Mean milk production (l/head/day)	Resting surface	Bedding	Manure evacuation	Access to paddock/pasture
1	75	Holstein Friesian + Red Holstein	15	Cubicles	-	Mechanical	Paddock
2	70	Holstein	13	Cubicles	Straw	Mechanical	-
3	70	Holstein	15	Cubicles	Straw	Mechanical	-
4	96	Romanian Spotted Cow	10	Cubicles	Straw	Mechanical	-
5	104	Holstein Friesian + Red Holstein	12	Cubicles	Straw	Mechanical	-
6	21	Romanian Spotted Cow	15	Medium long stall	Sawdust	Manual	Pasture
7	42	Romanian Spotted Cow	11	Medium long stall	Straw	Manual	Pasture
8	100	Romanian Spotted Cow + Holstein	16	Short stall	Straw	Mechanical	-
9	13	Romanian Spotted Cow + Holstein	18	Short stall	Straw	Mechanical	-
10	60	Romanian Spotted Cow + Holstein Friesian	15	Medium long stall	Straw	Mechanical	-

Farms 1-5: loose housing system
Farms 6-10: tie stall system

The farms with loose housing, having between 70 and 104 milking cows, with a mean number (SD) of 83 (15.9), had closed barns (in 2 farms) and half-opened barns (in 3 farms). In all of these farms the cows were mechanically fed and watered. All of the farms with tie stalls had closed barns and the numbers of milked cows varied in these farms between 21 and 113, with a mean number (SD) of 67 (38.71) animals. The feeding and watering was made manually (in 2 farms) and mechanically (in 3 farms). The cows were milked (manually or mechanically) twice per day in all the farms included in the study.

The lameness of different degrees in the cows was assessed using the locomotion score (LS) elaborated by Sprecher et al. (1997). This system is based on the evaluation of the cows' dorsal line, and of the position of their feet, giving scores from 1 to 5. All the milked cows were assessed in the investigated farms, 336 in the farms with tie stalls and 415 cows the farms with loose housing. Each animal was observed standing and in movement, at a slow pace on a hard flat surface, where it was possible. For the locomotion assessment of the cows kept in tie stalls, these were loosened and were taken out of the barns. The cows from the farms with loose housing were assessed after the morning milking (as they exited the milking parlor). Were considered lame cows those that presented obvious lameness and obtained scores from 3 to 5 (Amory et al., 2006). It was calculated the percentage of the cows with different locomotion scores and that of the lame cows per barn and per overall number of assessed cow. A range of data was provided by the farmer (mean milk production, access of the animals in paddock and/or pasture, the estimated prevalence of lameness, etc).

The habituation of the assessor with the locomotion score was realized in a preliminary study, in a farm with 36 dairy cows kept in loose housing system. The locomotion scoring was carried out until a correlation coefficient of 0.80 was obtained among the determinations (intra-observer agreement).

The data obtained was statistically processed, using the SPSS statistical software, version 17. The descriptive statistical indicators (mean, standard error of the mean, median, minimum and maximum) were calculated for the determined parameters (different locomotion scores, lameness prevalence). The obtained values were compared with the Mann-Whitney test or the t test, depending on the data distribution. The differences were considered significant if $P<0.05$.

RESULTS AND DISCUSSIONS

The results of the cows' locomotion assessment in the 10 investigated farms are presented in table 2. The percentage of the cows with different locomotion scores varied in the investigated farms due to the different housing and management conditions of the animals (Cook and Nordlund, 2009).

Table 2. The percentage of the cows with different locomotion scores in 10 Transylvanian farms

Farm	Locomotion score				
	1	2	3	4	5
1	13,33	18,67	24.00	28.00	16.00
2	25.96	53.85	16.35	1.92	1.92
3	22.86	30.00	41.43	4.28	1.43
4	28.57	35.72	30.00	5.71	0.00
5	31.25	55.21	11.46	1.04	1.04
6	40.48	47.62	7.14	2.38	2.38
7	25.00	53.00	15.00	4.00	3.00
8	37.17	38.05	16.82	4.42	3.54
9	15.00	71.67	13.33	0.00	0.00
10	95.24	0.00	4.76	0.00	0.00
Mean	33.49	40.38	18.03	5.17	2.93
SEM	7.37	6.52	3.50	2.61	1.51
Median	27.26	42.84	15.67	3.19	1.67
Minimum	13.33	0.00	4.76	0.00	0.00
Maximum	95.24	71.67	41.43	28.00	16.00

Farms 1-5: loose housing system
Farms 6-10: tie stall system
SEM = standard error of the mean

Out of the 751 assessed cows 252 presented normal locomotion (LS=1); 303 were slightly lame (LS=2); 135 showed moderate lameness (LS=3); 39 were lame (LS=4), and 22 presented severe lameness (LS=5), respectively.

In order to evaluate the locomotion of the cows in this study the system suggested by Sprecher et al. (1997) was used because its objective and clear descriptions that differentiate each score. The proportion of the cows with normal gait (LS=1) was slightly higher than that obtained by Espejo et al. (2006) in the evaluation of the cows from 50 farms from Minnesota and lower than the value reported by Cook (2003) following the investigation of the cows in 30 dairy farms in Wisconsin (54.9%). Less than a half of the cows assessed in this study presented abnormal locomotion (SL=2), but were not clinically lame. Similar results were recorded by Espejo et al. (2006). The percentage of the cows with a locomotion score of three (LS=3) was much less than recorded in Chile by Tadich et al. (2010). The percentage of the cows with score 4 (LS=4) was similar to that obtained by Yalylaket al. (2010) in Turkey and slightly higher than that recorded by Cook (2003) in the USA. The proportion of the cows with the locomotion score of 5 was higher than that reported by Tadich et al. (2010). It is considered that the presence of at least two cows with severe lameness in a farm represents a good indicator of the lameness

problems (von Keyserlingk et al., 2012). In this study at least two severely lame cows were found in half of the evaluated farms.

By classifying all of the cows that obtained a locomotion score higher than two in the „lame" category (Amory et al., 2006) it was established the lameness prevalence at farm level. In the studied farms, the lameness prevalence varied from 4.76% to 68% (Figure 1). Reported to the overall number of the assessed cows, the lameness prevalence was 26.10%.

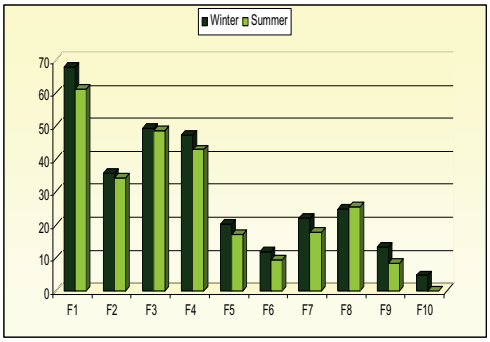

Figure 1 Lameness prevalence in the investigated farms

The prevalence of lameness was significantly higher (P>0.05) in the farms with loose housing system (44.07%) than in those with tie stalls (15.35%). Some studies reported a lower rate of lameness in the tethered cows than in those kept loose (Cook, 2003; Sogstad et al., 2005). This finding suggests that the loose system expose the cows to adverse environmental conditions that present importance in lameness epidemiology (Cook and Nordlund, 2009).

The studies accomplished in different countries of the world report various prevalence of lameness in dairy cows. It is possible that these high variations, both national and regional, in the estimation of lameness prevalence to be caused by the use of different assessment systems (Amory et al., 2006). Recent reports establish o scale of lameness prevalence in dairy cows from 20% (Espejo et al., 2006) to 54.8% (von Keyserlingk et al., 2012) for the cows kept in loose systems and from 1% to 21% for the systems in which the cows are tethered at least periodically (Sogstad et al., 2005; Zurbrigg et al., 2005). The new investigations accomplished in Romania indicate a prevalence of lameness of 15.12% in dairy cows kept in tie stalls with access to free movement in paddocks and/or pasture and of 22.21% in those with access to move (Popescu et al., 2013b). In the Transylvanian dairy farms with loose housing a lameness prevalence of 31.04% was reported for the winter (Popescu et al., 2013a). The results of the present study indicate a slightly higher prevalence of lameness in the cows kept in loose housing systems.

Some authors (Raven, 1985) consider that the differences in the prevalence and severity of lameness in cows would be related more to hereditary factors and farm practices than to housing conditions. Other authors (Phillips and Schofield, 1994) claim the effect of flooring quality in the emergence of foot problems of the cows and the relatively hard surface of the resting bed. Generally, the increase in the lameness prevalence is associated with the hard, concrete floors, slippery traffic alleys (Cook and Nordlund, 2009), dirty and uncomfortable barns (Chapinal et al., 2013), improper body hygiene of the cows (Cook, 2002) and permanenet stabulation, without outside access (Cook, 2003; Zurbrigg et al., 2005; Haskell et al., 2006).

One of the key factors reducing lameness in dairy farms is its detection. The frequent assessment of lameness in dairy cows using animal-based measurements (locomotion score) presents several advantages, such as: implementation of some preventive measures at herd level, individual assessment and of the herd's welfare, but also the detection and early treatment of the lame cows. All these will contribute to increase the welfare degree of the dairy cows by reducing the incidence of lameness and of the pain and discomfort associated with it.

The lameness prevalence according to the farmers' estimates in each investigated farm is presented in figure 2.

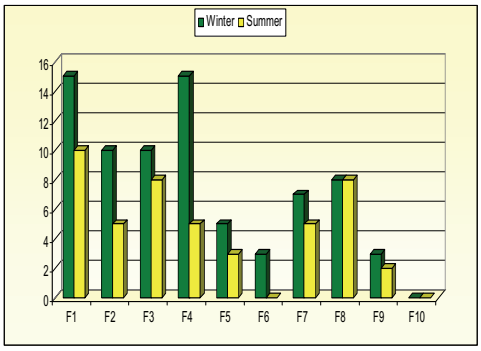

Figure 2 The lameness prevalence estimated by the farmers in 10 dairy farms

The prevalence of lameness estimated by the farmers was significantly higher ($P<0.05$) in the farms with loose housing system than in those with tie stalls.

Table 3 presents the descriptive statistical indicators for the lameness prevalence determined by the locomotion score and that estimated by the farmers. Significant differences ($P<0.05$) can be observed among the medians of the determined values.

Table 3. Descriptive statistical indicators for the lameness prevalence determined by the locomotion score and that estimated by the farmers in 10 dairy farms

Parameter	Locomotion score	Farmers' estimate
Mean	29.71	7.60
SEM	6.30	1.59
Median	23.38*	7.50
Minimum	4.76	0.00
Maximum	68	15.00

*$P<0.05$ – the difference between the lameness prevalence determined by the locomotion score and that estimated by the farmers is significant

In this study the lameness prevalence estimated by the farmer was 3 and up to 9.5 times lower (with a mean value of 3.1 times) comparing with that determined using the locomotion score. Different researchers reported similar results. Wells et al. (1993) found a lameness prevalence of 13.7% in summer and 16.7% in spring, these values being of 2.5 times higher than the prevalence estimated by the farmers. In a subsequent study Espejo et al. (2006) reported prevalence 3.1 times higher than that estimated by the farmers, in 50 dairy cows kept in loose system in Minnesota, using the scoring system of Sprecher et al. (1997). In the Great Britain Whay et al. (2003), using a locomotion scoring system with 4 points, reported a lameness prevalence of 22.1%, almost 4 times higher than that estimated by the farmers (5.7%). More recently, Sarova et al. (2011), using a 3 point scale locomotion scoring tools (0 – does not presents lameness, 1 – moderate lameness, 2 – severe lameness), reported that, in the Czech dairy farms, the mean prevalence of lameness was of 31%, comparing to 6% estimated by the farmers. These studies demonstrate that in the dairy farms from the United States and Europe the workers of the farms and the farmers perceive a much lower prevalence of lameness in the cows than it is in reality (determined using different locomotion scoring systems).

The farmers and farm employees seem to be unable to recognize about 30% of the lame cows (Whay et al., 2003; Espejo et al., 2006). In addition to the difficulty to identify the lame cows, the subtle changes of their behavior and also changes in their gait are not identified until the lesions of their hooves are advanced (O'Callaghan et al., 2003).

The results of the accomplished research show that the proportion of the lame cows identified using the locomotion score, but unidentified by the farmers and farm personnel varies between 60 and 80%. The main impact of the farmers' inability to recognize lameness is on the delay of treatment initiation. Leach et al. (2012) reported that a two-week delay in the beginning of treatment reduced the healing rate from 75% to 60% and shortened the time interval between the treatment and relapse from 4.5 weeks to 3.5 weeks in the cows that become lame again after the treatments. The same authors showed that the identification of the lame cows based on the locomotion score and not on the recognition by the farmers reduced the relapse rate from 58.3% to 36.9%.

CONCLUSIONS

In 70% of the investigated farms (100% with loose system and 40% with tie stall system),

the prevalence of lameness was unacceptable (≥ 15%).

The lameness prevalence was significantly higher in the loose dairy farms than in those with tie stalls.

Both in the farms with loose housing and in those with tie stalls the degree of lameness varied from mild to severe.

In all of the farms included in the study the farmers underestimated the prevalence of lameness.

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STUDY ON THE MICROSCOPE METHOD AND THE VIEW OF THE ANALYTICAL TECHNIQUES FOR IDENTIFICATION AND ESTIMATION OF THE PROCESSED ANIMAL PROTEINS IN ANIMAL FEED

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Abstract

Hypothesis universally accepted as the most likely route of infection with ESB appeared due to the consume of the animal by-products which are not intended for human consumption that contained prion protein derivative - infected led to ban the feeding of farmed animals with processed animal protein (PAP) that focused primarily feed for ruminants and later expanded to all feed for all farm animals. Entry into force of the ban on the use of the processed animal proteins (PAP) in feed for farmed animals and especially in ruminants is considered an important measure of prophylaxis to prevent BSE so the identification and the microscopic estimation of the constituents of animal origin became the official method and mandatory in all Member States.

The microscopic analysis depends upon the identification of the hsitological characters macro-and microscopic structural of the processed animal tissue added in feed mixtures.To identify the microscopic animal constituents, some technical conditions are essential: optical microscope, stereo microscope, high-density solvent (chloroform or tetrachloroethane) clarifying agents (phenol-glycerol, paraffin), microscope with digital visual images support as decision support .

The method allows the identification of bone fragments, muscle tissue, hair, feathers, shell fragments and plant and mineral components.

Nowadays four different approaches are applied to control the compliance on the prohibition of feeding with PAP: microscopic analysis, immunological analysis, infrared spectroscopy and microscopy (NIR), polymerization chain reaction (PCR). In this stage, the microscopic method is the only method validated and able to identify the nature of the animal in feed components with detection limit of <0.1%, but it cannot accurately detect the species of origin.

Key words: *feed, microscopic identification, polymerization chain reaction, processed animal proteins*, *transmissible spongiform encephalopathy.*

INTRODUCTION

So far it has been shown that animal by-products not intended for human consumption, resulting in a row processing, more or less correct, cadavers, products and by-products from animals, constituting a potential source of risk to public health and the animal. This way the probable infection with prionic protein derivative-infected, has led to a ban on the feeding of farm animals with processed animal proteins (PAP) which focused primarily on feeding stuffs for ruminants, and was subsequently extended to all feed for all farm animals. Safety use of proteins derived from animals in feed at European level constitutes an important decision factor in the prevention of transmissible spongiform encephalopathy's (TSEs). Properties of infectious agents of TSEs have proven to be very unusual. Infectious agents of TSEs, manifested in humans or in animals are actually made from a single type of protein called PrP^{Sc}, are devoid of nucleic acids.

Use of protein meat-and-bone type as an ingredient in animal feed is regulated according the European legislation (Commission Decision 97/534/EC) since 1997, and effective heat treatment to which they are subjected has been set to, 3 bar, 133^{o}C, 20 minutes (Regulation (EC) no 1069/2009). European regulations prohibit feeding of animals of farm intended for human consumption, intraspecie with proteins derived. Considering the fact that the methods of analysis for protein supplements have carried out a series of inconveniences in selectivity (in particular for the identification of protein derived from ruminants) the ban was extended for proteins derived from all

species. Extending the ban, he called for better identification methods and more accurate for effective sterilization control as well as for specific detection of meat-and-bone type in compound feeding stuffs.

At present there are applied or are under development in the following ways: microscopic analysis for the identification of particles derived from animal by-products (blood, hair, tissue, muscular, and others), the polymerization chain reaction (PCR) for the detection of species specific DNA, immunological analysis (ELISA) for the detection of specific proteins, spectroscopic methods in the near infrared (NIR) for the detection of protein flour in compound feeding stuffs.

All methods have the advantages and disadvantages of specific technique used.

Taking into account the specific proteins, nucleic acids and are still present in processed flour, have been tested and molecular biology techniques. In this context two main techniques are used which are focused on the detection of proteins with specific antibodies by ELISA (Ansfield et al, 2000) and taxonomical identification of nucleic acids based on genetic methods of amplification by PCR (Chikuni et al.,1990). Near-infrared spectroscopy (NIR) is used for a long time (Baeten et al., 2001) for identification of certain major components (water, fat, protein, etc.) of the food or feed substrates.

The scientific community's interest for NIR spectroscopy is based on the eventual possibility of accidental contamination of feeding stuffs with consistent meal, bearing in mind that this technique is one of the most used for the routine exams. By conducting comparative tests (8-STRATFEED European Project, 2000) with different substrates and different concentrations of fodder of animal protein, it was noted that the processing temperature and duration thereof constitutes a major impediment to the successful application of enzyme immunoassay methods. In cases where the processing temperature is more than 140^{o} C, ELISA techniques performances but even PCR greatly decrease with increasing percentage of false-negative results .Unlike these, NIR spectroscopy is not influenced by temperature, but the limit of detection is 10 times greater than what makes use for routine analysis is not possible.

In 1998, the Committee of Experts on Methods of Analysis (CEMA) of the Directorate-General for Agriculture, put into question the comparative performance of microscopic analysis, the immunoassay analysis (ELISA), polymerase chain reaction(PCR) and infrared spectroscopic methods (NIR). As a result of this analysis and the results of tests carried out at the time, in 2001, he was the European STRATFEED project, whose purpose was to assess the performance of the different methods described above, in a series of tests of interlaboratory comparison with the participation of several European laboratories. Our Institute, by laboratory of microscopy, participated in phase II of the project STRATFEED, in 2003-2004, which included tests of microscopic method validation (DC 126\/2003 EC).

MATERIALS AND METHODS

In this paper are presented analysis methods as development in the Institute of Hygiene and Public Veterinary Health, microscopic identification method and a proper system of visual comparison and interpretation of digital images taken under a microscope and stereomicroscope and polymerization chain reaction method.

Since 2004 has been continually developed as analytical method microscopic method, which is the method of reference at European level for the identification of animal proteins in feeding stuffs and validates alternative method based on the polymerase chain reaction (PCR) for the detection of protein derived from ruminants with the prospect of enlargement and to identify the species of origin derived from non-ruminants.

Microscopic analysis now routinely applied to our laboratory, is based on the identification of structural macro-and microscopic animal tissue derived from consistent flour added to compound feeding stuffs. In principle, the microscopic identification is based on microscopic characteristics of animal tissues. Microscopic identification of animal

derivatives is based on knowledge of basic histology. Histological structure of components that have undergone heat treatment as is the case PAP is different from the normal structures. The differences can be accentuated, such as soft structures (muscle, epithelial tissue, connective tissue, etc.) or less accentuated, some unaltered, such as hard structures (bone tissue, teeth, scales, feathers, etc.). Drying and grinding are changed not only the initial histological structure but also macrostructure, so generally available for identification only small fragments. Because these fragments are then included in complex matrices (cereals, legumes, seeds ground and their derivatives, minerals, vitamins, feed additives etc.) the extraction and separation of these fragments becomes all too important and time consuming to be identified. Bone fragments present in the mixture should be differentiated based on typical lacunae between the bones from fish and terrestrial animals (mammals and birds).

Separation of constituents is facilitated by sieving and concentrated sediment technique, which consists in suspending the sample in a solvent with high density (chloroform, tetrachloroethane) which makes the bones, minerals and other high-density fragments to settle and plant tissues and other low-density constituents, to be found in the floating. Alizarin red staining for identification is very important because only colored bone and cartilage making them easy to recognize separately. Observation of lacunae by clarifying preparation is a densely agent (paraffin, glycerol) that cannot penetrate the capillary so that they remain filled with air and appear black on a light background of the bone fragment.

Alternative, our laboratory has implemented and is in the process of validating DNA detection from ruminants through Real-Time PCR in products intended for animal feeding, aiming a genetic sequence of repetitive nuclear level 85/86 pairs of bases, test developed and validated by the reference Laboratory of the European Union for animal proteins (EURL-AP) Belgium. Target region of plasmid DNA is amplified by specific primers, which, with each cycle of amplification is evidenced in real time with the help of a marked fluorescent probes. Fluorescent signal passes a threshold (threshold) value after a certain number of PCR cycles. The value of this threshold cycle (Ct-cycle threshold) is likened to a predetermined figure after calibration of the Real-time PCR platform, which includes the equipment and reagents for PCR to determine if the result is positive or negative.

RESULTS AND DISCUSSIONS

Constituents of animal nature are identifying by macroscopic examination on stereomicroscope and by microscopic examination on compound microscope. Community methods, as determined by the European law (Regulation EU No 53/2013) indicate the scope of the technical guide for the separation and examination of the constituents of nature of these principles. The more difficult for our laboratory was establishment of a protocol and a database for Visual comparison. In this sense have been selected several methods and tests to confirm through chemical reactions of components identified. Thus was constituted a supplementary material for analysts, supplemented with a Visual support with digital images from the microscope, as decision support.

The tissues that are important to the microscopic identification are bones, teeth, muscle tissue, cartilage tissue, cornified epithelial tissue, feathers, feathers, egg shells fragments, hair and hard tissues of the hoof, guiding elements analysis and identification. The main elements for microscopic diagnosis are bone fragments.

In stereomicroscope mammal and bird bone fragments appear as irregular particles, opaque, yellowish white, pearl and fragments of fish bones appear translucent, with slightly

glazed surface, often lamellar shape, sharp (Figure 1).

On compound microscope, in embedding media (paraffin, glycerine) filled with air lacunae remain and are visible by contrast. Characteristic mammals form longitudinal lacunae size of ~ 5/30 μm, and their arrangement in the form of linear (Figure 2).

Figure1.Bone fragments on stereomicroscope

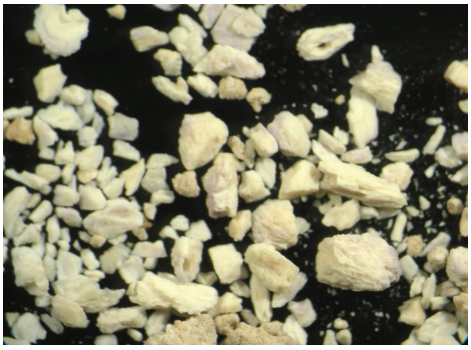
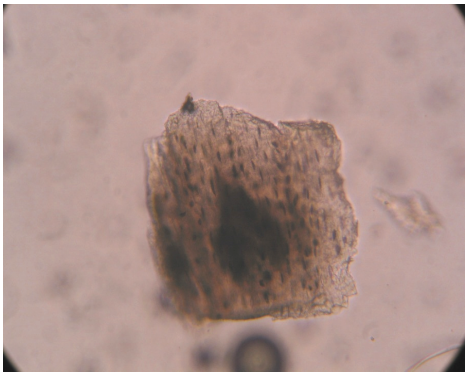
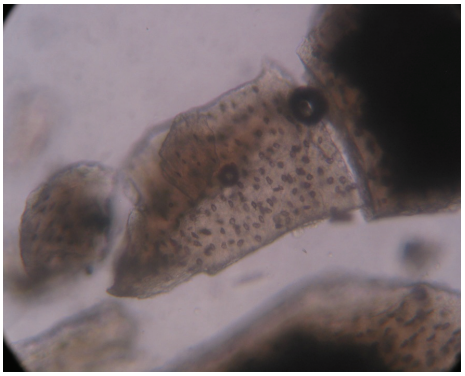


Figure 2.Mammal bone aspect on microscope



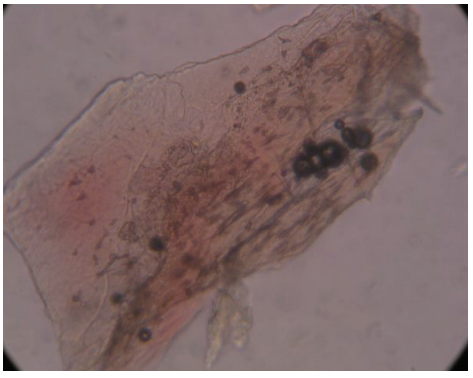
In birds histological bone structure is identical to mammals, the difference is globular lacunae with dimensions of ~ 15/25 μm and lacunae arrangement is in the form of cluster radial aspect and less linear as the mammalian (Figure 3). Other diagnostic elements present bird constituents are represented by presence of egg shell fragments, presence of feather (barbs and barbules) that shows great importance for orientation of identification.

Figure 3.Bird bone aspect on microscope



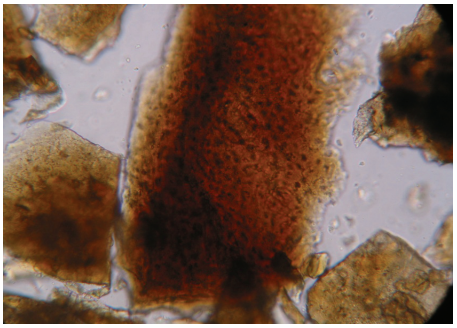
For fish are important microscopic identification of characteristic components of bones and scales. One can distinguish two categories of fish bones: bones and bone lacunae containing osteocytes without lacunae (Figure 4).

Figure 4.Fish bone aspect on microscope



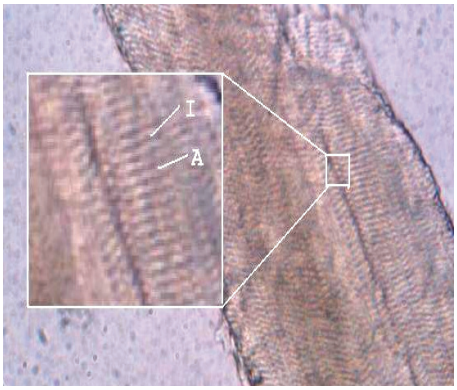
In fishmeal can be found various tissues from clams, shrimp, crabs, snails originating from marine fish harvested with or emanating from the stomach of the fish. Calcified exoskeleton of crabs and shrimps shows generally symmetrical structure and similar arrangement of cells in plant tissues (Figure 5).

Figure 5 – Exoskeleton of crabs



Microscopic examination of muscle tissue, both freshly prepared (in polarized light) show myofibrils that appear with a periodic structure, made of alternating zones or clear discs (I) called isotropic, monorefringents, dark disc (A) called anisotropic, birefringents (Figure 6).

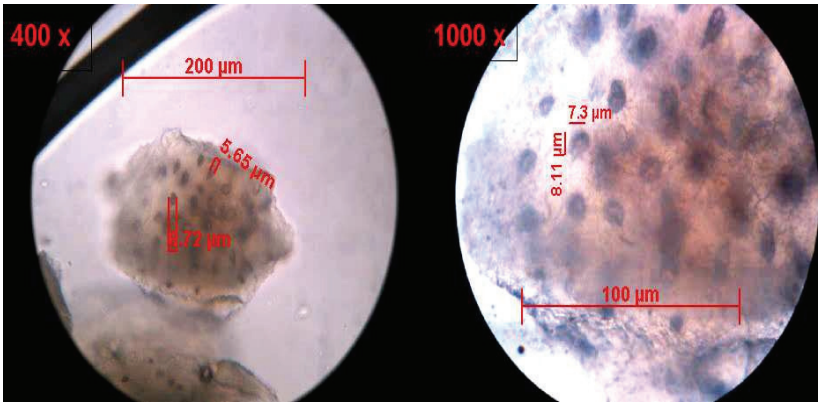
Figure 6. Muscle tissue



To support differentiation of constituents identified, was built an image database as decision support, using program acquisition AxioVision LE and editing of existing laboratory photomicrographs. For this purpose were conducted and evaluated over 1,500 photomicrographs acquired with camera fitted to the microscope, and images were selected by features and images very frequently encountered in routine examinations and were then labelled and compiled in a manner accessible for comparison during work.

If fish bones before the differentiation of the terrestrial species can be made with relative ease, differentiating between species of mammal bone or bones of mammals of the poultry involves a more complex examination. In this respect it is necessary to assess gaps form (with magnification from 400 to 1000 x) and second coordinate dimensions. In general, the size of the gaps are between 15-25/8-30 µm. The appreciation of these dimensions is possible through the treatment procedures based on digital images. For this purpose was established a procedure of evaluation and description of structures in digital image (Figure 8).

Figure 8. Morphometric measurements



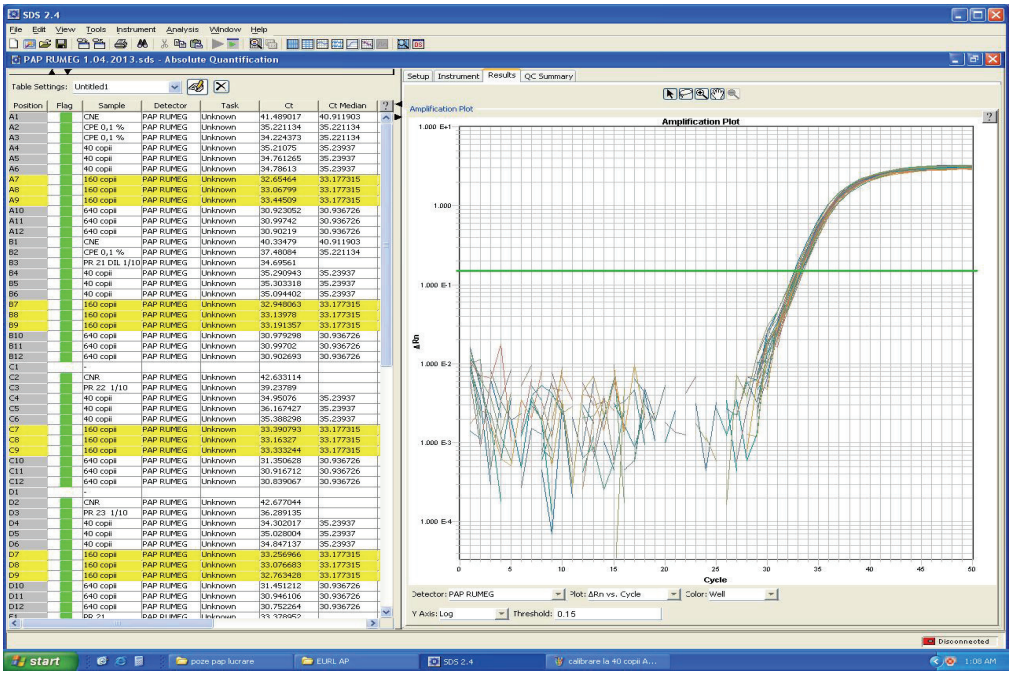
The sensitivity of the method depending on the type of constituents of animal nature can be detected very small amounts (<0.1%) of constituents in feed mixtures as assessed by microscopic identification detection capability demonstrated in the validation of the method. The size of the fragments identified on compound microscope is about 30 µm. Details included are identifiable dimensions between 2.5-5 µm.

Internal validation and accreditation methods were used samples of different mixtures to which have been added to the known applied bone flour, meat or fish. Bone fragments to differentiate between species of mammals, the Visual comparison are required. For this purpose were formed 25 packages of selected images to meet specific characters with a probability of 95%. Such characters shall be represented in the form of gaps, their layout and size of the two plans.

Laboratory records-for samples analysed are kept digital micrographs recorded in a folder with the number of the sample that is applied by editing (AxioVision LE 4.1) sample number and corresponding objective and micrometer scale that worked.

in Detection of DNA derived from ruminants through Real-Time PCR in products intended for animal feeding is a technique of 5 '-nuclease and it consists of selective amplification of DNA sequences through a succession of amplification cycles, each cycle having in turn three steps: denaturation of DNA chains, the delimitation of the amplicon with the help of very short sequences of approx. 20-25 of the complementary DNA database called sense and antisense primer that takes place at a temperature which is calculated according to the structure to develop the extension, polymerization. In accordance with the procedure of EURL-AP, working platforms (equipment and reagents) used to perform this method, must be calibrated, this means the testing of 16 times a set of 3 calibrants this tests obtaining the cut-off method in your own laboratory (Figure 9).

Figure 9. Calibration to 160 copy of DNA rumegator (real-time PCR Applied System)



The samples tested on a calibrated (e.g. with the cut-off value of 38.15) will have a qualitative result of type "present" = samples with Real-time amplification cycle PCR below cut-off or "absent" = samples with Real-time amplification cycle PCR over cut-off value.

CONCLUSIONS

Currently compliance on the prohibition of feeding with PAP, are based on four different approaches: microscopic analysis, immunoassays, spectroscopy and infrared microscopy (NIR), the polymerization chain reaction (PCR), all methods have advantages and disadvantages of technique used and performance parameter.

Testing laboratory has developed a laboratory microscopy applied to control feed with original contributions to the evaluation of digital photomicrographs, future work is focused on microscopic identification by using an expert system to correct recorded images.

In Romania, microscopic method is the only method accredited to ISO/CEN 17025 by RENAR (Accreditation Association Romania) and able to identify and the nature of animal feed components with a detection limit of <0.1%, but cannot accurately detect the species of origin.

Feed ban on the use of processed animal proteins interspecies constitutes a new challenge for analytical methodologies and identification methods will require better and more precise control.

Testing laboratory which formed the basis of this study is accredited for microscopic method and validates alternative method based on polymerase chain reaction (PCR) for detection of ruminant constituents with the prospect of enlargement and to identify species from non-ruminant origin.

Method for detection of DNA derived from ruminants through Real-Time PCR in products intended for animal feeding must be calibrated in the laboratory's own type of equipment and reagents used, in order to obtain the cut off and the result is qualitatively as “present" or “absent".

Our task for the future is focused on validation and accreditation methods for DNA detection through Real-Time PCR products intended for feeding farm animals, so that Romania can fulfill the requirements of the ban on the use of future animal proteins derived from ruminants but also and prohibition of feeding an intraspecific (ban of feeding an animal species with proteins derived from the bodies or parts of bodies, of the same species).

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PROJECT HRDSOP 155/1.2./S/139950 "IMPROVING QUALITY OF NATIONAL HIGHER EDUCATION SYSTEM IN ACCORDANCE WITH CHANGING KNOWLEDGE-BASED SOCIETY AND LABOR MARKET DYNAMICS"

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Abstract

Paper presents the progress of the project IMPROVING QUALITY OF NATIONAL HIGHER EDUCATION SYSTEM IN ACCORDANCE WITH CHANGING KNOWLEDGE-BASED SOCIETY AND LABOR MARKET DYNAMICS, co-financed by European Social Fund through the Human Resources Development Sectoral Operational Programme 2007-2013, implemented by the Ministry of Education, University of Agronomical Sciences and Veterinary Medicine Bucharest, University Politehnica Bucharest, University of Agricultural Sciences and Veterinary Medicine Cluj Napoca, University Politehnica Timisoara.
The project aims to reorganize and improve the national higher educational system by developing an implementing an integrated approach - National Higher Education Consortium and quality management by increasing access to higher education for a number of 1000 students and by delivering training programs to 500 key actors involved in university processes and activities. Relevant information about the project can be found at the address: www.proiect-impact.ro .

Key words*: quality management, National Higher Education Consortium, teachers lifelong learning, high education access*

INTRODUCTION

The lifelong learning education of human resources is the condition for performing a competitive labor market. A highly skilled workforce is essential to a knowledge-based competitive and sustainable economy.(National Development Programme 2007 – 2013, Lisabona Strategy, National Reference Strategic Framework 2007 – 2013, Study regarding the implementation of Bologna higher education process 2009). In the broader European plan for transforming the EU into a smart, sustainable and inclusive growth, which is based on the Europe 2020 Strategy, increasing employment and labor market participation rate is a major concern nationwide, necessitating the creation of a favorable labor markets for new jobs, improving adaptability of enterprises and workers, boosting employment and improving the quality and competitiveness of human resources entering the labor market and develop a permanent adaptation.
The specific objectives are:

- Strengthening innovation and reform in higher education system, by developing and implementing the National Higher Education Consortium between universities / public institutions / research centers / socio-economic environment in order to adapt the higher education system to the needs of the labor market and knowledge based society.
- Improving university management by developing a number of 10 tools / procedures / methodologies / mechanisms of quality management and by delivering training programs for 500 higher education key actors in order to increase the capacity of higher education institutions to provide higher qualifications according to labor market changes. (Standards and Coordinates for Quality Insurance in the European Higher Education Area; Standards of Quality Assurance in Higher Education, Helsinki, 2005; Integrated Strategy for

Human Resource Development from the perspective of lifelong learning 2009-2020- Ministry of Labor, 2011)

- ❖ Increasing access to higher education for a number of 1,000 students in order to promote and correlate higher education to labor market.

MATERIALS AND METHODS

The project offers a systemic and strategic approach in the design and implementation of reforms in higher education. The project aims to improve managerial training of teachers in order to gain a strategic management, to improve relations with the socio-economic and facilitating access to higher education for vulnerable groups.

The project aims improving professional skills of key actors involved in the higher education system in order to improve university management and student services delivery by focusing on teaching-learning process. In order to achieve the improvement of university management, a team of very experienced experts developed a number of 12 courses dedicated to university teachers:

- Risk Management
- Human Resources Management
- Financial Management
- Time Management
- Strategic and Educational Management
- Quality Management
- Benchmarking
- Equal treatment and access in higher education and sustainable growth of higher education
- The impact of the usage of information technology in higher education, including e-learning
- European and national legislation and policies in higher education
- Develop study programs and innovative methods of teaching / learning in order to increase the relevance of qualifications in higher education

The added value of the project comes from the commissioning of the knowledge triangle by linking higher education, research and socio-economic environment. It aims at producing excellence, developing partnerships between socio-economic and higher education by reforming the national system of higher education in accordance with labor market needs.

RESULTS AND DISCUSSIONS TNR 12

- ➢ 500 university teachers beneficiaries of several training courses – " Risk Management", "Human Resources Management", "Financial Management", "Time Management", "Strategic and Educational Management", "Quality Management", "Benchmarking", "Equal treatment and access in higher education and sustainable growth of higher education", "The impact of the usage of information technology in higher education, including e-learning", "European and national legislation and policies in higher education", "Develop study programs and innovative methods of teaching / learning in order to increase the relevance of qualifications in higher education".
- ➢ 21 universities members of the National Higher Education Consortium
- ➢ 1 comparative analysis between Higher Education Quality Management in Romania and Higher Education Quality Management in Europe
- ➢ 10 tools / procedures / methodologies / mechanisms of quality management developed
- ➢ 1000 students participants in " Career Days" and "Open days"
- ➢ 4 peer learning meeting debating relevant subjects for national higher education system
- ➢ 1 seminary "Encouragement and improvement of vulnerable groups to high education"
- ➢ 4 National Training Centers dedicated to key actors in higher education

CONCLUSIONS TNR 12

The National Higher Education Consortium will improve cooperation and interaction between universities, socio-economic and Ministry of Education in order to reform and lead to a competitive higher education for the

alignment to European standards and the needs of knowledge society.
The creation of elite of 500 key actors in higher education will lead to the modernization and to the restructuring of the national education system.
The development of the innovative mechanisms of "Career Days" an "Open Days" will increase access for all undergraduates to higher education.
The improvement of university quality management will reform the higher education system and it will promote higher standards in line with labor market needs.

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PROJECT HRDSOP 160/2.1./S/139928 "NOW A STRUDENT! TOMORROW A PROFESSIONAL! – THE IMPROVEMENT OF LABOUR MARKET INSERTION OF STUDENTS ENROLLED IN HIGHER TECHNICAL/ ECONOMIC/ VETERINARY EDUCATION"

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Abstract

Paper presents the progress of the project NOW A STRUDENT! TOMORROW A PROFESSIONAL! – THE IMPROVEMENT OF LABOUR MARKET INSERTION OF STUDENTS ENROLLED IN HIGHER TECHNICAL/ ECONOMIC/ VETERINARY EDUCATION, co-financed by European Social Fund through the Human Resources Development Sectoral Operational Programme 2007-2013, implemented by University Politehnica of Bucharest, University of Agronomical Sciences and Veterinary Medicine Bucharest, University ' Lucian Blaga" Sibiu, University 1 December Alba Iulia, Romanian American University, Craiova University, Ovidius University Constanta.
The project aims to facilitate and improve labor market insertion process and working skills development of a number of 10,000 students enrolled in higher technical / economic / veterinary medical education programs by developing and providing integrated guidance, professional counseling and development partnerships with 500 potential employers.. Relevant information about the project can be found at the address: www.proiectimpuls.ro .

***Key words**: : labour market studies, XXI century student profile, guidance and career counseling; practical stages, labour market insertion portal*

INTRODUCTION

The initial higher education of human resources is the precondition for performing a competitive labor market. A highly skilled workforce is essential to a knowledge-based competitive and sustainable economy.(National Development Programme 2007 – 2013, Europe 2020, National Reference Strategic Framework 2007 – 2013,National Sustainable Development Strategy 2013-2020-2030). The main objectives of higher technical / economic / veterinary education institutions are to provision of career guidance and adequate practical training, to enable graduates acquire "day one" skills upon completion of their studies, to enable professional skills according to the actual labor market requirements and to the demands of sustainable development.
The specific objectives are:

- Facilitate the transition from school to working life by providing vocational guidance and counseling services for a total of 10,000 students enrolled in higher technical / economic / veterinary medical education
- Develop and strengthen partnerships in order to increase learning outcomes acquired at work by involving a total of 500 institutions / companies / research institutes / companies in order to improve the transition process from school to working life
- Development of working skills through internships for 2,000 students enrolled in higher technical / economic / veterinary medical education, in order to increase skill levels and a more rapid labor market insertion

Monitoring employability of graduates in order to correlate higher education competencies with labor market needs

MATERIALS AND METHODS

The project aims to correlate initial vocational training system to labor market needs, helping to facilitate the transition from school to working life by creating opportunities on a flexible labor market, for the participation of 10,000 students enrolled in higher technical / economic / veterinarian education system, and having a long-term positive effect for students by creating an integrated program of guidance and counseling, creating practical training, and a system of communication and relationship between students and potential employers.

Through its activities, the project aims to achieve added value elements such as:

- a pressing need to cover the training of future professionals in terms of increasing awareness of personal qualities, the structure of professional interests and correlation with educational training and professional placement in the labor market;
- teamwork and communication skills, familiarity with the rigors and demands of a professional working environment, forming an overview of the international organizations and Romanian companies;
- reform of practical training based on performance and correlation with labor market needs;
- Possibility of direct relationships with employers through workshops and internships;
- achieve a true picture student - academic – labor market, through monitoring study on graduates labor market insertion;
- Innovative professional computing portal that allows students to get familiar with new methods of personal promotion in front of a potential employer.

RESULTS AND DISCUSSIONS

- 10000 students enrolled in higher technical / economic / veterinary medical education beneficiaries of counseling and career guidance services
- 500 partnerships with social – economical environment for best practices exchange and for student internships
- 2000 students beneficiaries of internships and practical stages
- 1 study - “21st Century student profile - prospective, opportunities, development”
- 1 study – “The analysis of the educational system components in the technical/economic/veterinary fields”.
- 1 study – “The analysis of labor market dynamics in the technical/economic/veterinary fields”
- 1 study – “Vulnerable groups access to higher education and labor market integration”.

CONCLUSIONS

Counseling and career guidance services in the transition from school to working life is crucial to the extent that increases the chance of becoming employable graduates by empowering their skills in areas falling outside the academic and practice related to communication, decision-making techniques for solving problems and conflict management, stress management and professional development planning.
The development and implementation, among employers, of a system of practical training specific to job vacancies in order to increase employability and to acquire ‘day one” skills after graduation.
The 4 studies will reflect a better image of student – academic system – labor market requirements.

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vention 2.1. "Transition from school to work".

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PROJECT HRDSOP 155/1.2./G/136748 "VETERINARY EMERGENCY MEDICINE – INNOVATION AND NEW SKILLS IN HIGHER VETERINARY EDUCATION SYSTEM"

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Abstract
Paper presents the progress of the project VETERINARY EMERGENCY MEDICINE – INNOVATION AND NEW SKILLS IN HIGHER VETERINARY EDUCATION SYSTEM, co-financed by European Social Fund through the Human Resources Development Sectoral Operational Programme 2007-2013, implemented by the University of Agronomical Sciences and Veterinary Medicine Bucharest, University of Agricultural Sciences and Veterinary Medicine Cluj Napoca.
The project aims to improve curriculum in higher veterinary education for a number of 400 students, in accordance with CNCIS, by introducing new disciplines and by developing a Veterinary Sectorial Network in order to achieve a better correlation between labor market and higher education system.
Relevant information about the project can be found at the address: www.muv.fmvb.ro .

Key words*: veterinary higher education, emergency system, Veterinary Sectorial Network, labor market study in veterinary field*

INTRODUCTION

The lifelong learning education of human resources is the condition for performing a competitive labor market. A highly skilled workforce is essential to a knowledge-based competitive and sustainable economy.(National Development Programme 2007 – 2013, Europe 2020, National Reference Strategic Framework 2007 – 2013, Integrated Strategy for Human Resource Development from the perspective of lifelong learning 2009-2020- Ministry of Labor, 2011). The objectives of veterinary medical education institutions are the provision of adequate veterinary training, ethics and science-based, which allows graduates to practice veterinary profession in all recognized areas of veterinary medicine.
The specific objectives are:

- The extension of learning opportunities for 400 students in higher veterinary education by developing and implementing the new discipline of emergency medicine according to labor market.
- The development and strengthening of the Veterinary Sectorial Network by involving all veterinary universities and a number of 10 companies/public institutions/professional associations/ student association.

MATERIALS AND METHODS

In Romania, this project represents an absolute novelty, considering the integrated approach concept on two components: a theoretical one, represented by academic achievement and teaching curricula for all emergencies and a practical one represented by the sectorial network between universities/public institutions/ companies / professional associations/ students association.
In the context of world motion, in which the risks are everywhere, emergency medicine plays an important role for saving life. As a

natural extension, emergency medicine begins to gain ground in veterinary medicine also. This specialization has emerged from the desire to create uniform and standardized protocols actions to save animals. Emergency Medicine is a complex specialization, involving that knowledge in all areas of veterinary medicine is used in a proper, specific and standardized manner. Emergency Medicine involves the simultaneous intervention of a team and a complex and specific equipment.
The new curriculum will cover major areas of action in emergency medicine, namely: the developing of a Guide of conduct in major emergencies, Respiratory Emergencies, Neurological Emergencies, Gastrointestinal Emergencies, Urinal Emergencies, Endocrine and Metabolic Emergencies, Accident Emergencies.
The added value of the project comes from the integration of new skills and knowledge in the compulsory curriculum, form the increasing involvement of socio-economic environment in updating higher education veterinary system and from the increasing of qualification relevance according to labor market requests.

RESULTS AND DISCUSSIONS

The Veterinary Sectorial Network was created. It will will strength the professional skills of personnel involved in the development of university curricula, the development of modern curricula and the alignment of veterinary medical education system to European level.
The new Emergency Medicine curriculum was implemented. It will increase graduates skills and integration on labor market due to the employers demand for veterinary specialists in emergency medicine and due to the incretion of emergency cases in the activity carried out in hospitals/cabinets/farms.
The introduction of Emergency Medicine in the compulsory curriculum represents a real improvement for the higher veterinary education system and more opportunities for graduates to perform better practice.
A number of 400 students already studied and were trained in the field of this new discipline.
Also, a study " The need and requirements of labor market in veterinary medicine field" was initiated.

CONCLUSIONS

New discipline – veterinary emergency medicine – was introduced in the compulsory curriculum.
A number of 400 students were trained and examined in the field of veterinary emergency medicine.
A functional Veterinary Sectorial Network was implemented.

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RETRACTION of the paper *Spiroxys contortus parasitism associated with dog bite trauma in a captive red-eared slider (Trachemys scripta elegans) – a case report*

Due to the pertinent observations of Dr. Beate Pfau, from German Chelonia Group, and Dr. Albert Martinez-Silvestre and his colleagues from Catalonian Reptile and Amphibian Rehabilitation Center, the paper *Spiroxys contortus parasitism associated with dog bite trauma in a captive red-eared slider (Trachemys scripta elegans) – a case report,* by Andrei Constantin Stoian, Gabriel Predoi, Raluca Ioana Rizac, Mariana Ionita, Daniela Elena Braslasu, Emilia Ciobotaru, which had been publishes in: Scientific Works. Series C. Veterinary Medicine, Vol. LX (1), ISSN 2065-1295, 99-106, was re-evaluated and retracted with the consent of all the authors.

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Executive Editor

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