COMPARATIVE STUDY BY MALDI TOF MASS SPECTROMETRY OF SOME SPECIES OF THE GENUS *BACILLUS*, FAMILY *BACILLACEAE*

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Summary

In recent years, mass spectrometry MALDI TOF (Matrix Assisted Laser Desorbtion Ionization - Time of Flight / desorption ionization matrix laser and calculation of mass by measuring the time of flight) was used for the rapid identification of microorganisms in clinical microbiology laboratories. The germs of the genus *Bacillus* are ubiquitous bacteria, because the spores they confer increased resistance to environmental factors.

The cultures of *Bacillus* spp studied (*Bacillus anthracis, Bacillus subtilis, Bacillus megaterium*) were seeded via loop prick on the simple agar and blood agar, in Petri plates in order to obtain isolated colonies. Bacterial colonies obtained were processed for 16S ribosomal proteins extraction, by the "*Microorganisms Profiling*" extraction trifluoroacetic acid (TFA 80%) procedure and analyzed with Microflex® LT 20 equipment.

Spectra analysis of the genus *Bacillus* biological agents representing the polypeptide fragments of the 16S ribosome revealed a mass range between 800 and 12000 Daltons. The characteristics average peaks has been included between: 1100-1300 Daltons; 3550-3650 Daltons; 4600-4950 Daltons; 5400-5900 Daltons; 6300-7300; 9300-10300 Daltons; 11500-12000 Daltons; 10500 to 11500 Daltons.

Mass spectra obtained were identified as belonging to the tested bacterial species and further confirmed by classical bacteriological methods. Analyzing the results of studied strains, obtined by MALDI TOF technology of studied strains, we can conclude that they are comparable in terms of structure and composition of 16S ribosomal subunit; thus demonstrating a high degree of phylogenetic relatedness.

Keyword: *Bacillus spp.,* diagnosis, MALDI TOF

Genus *Bacillus* includes rod shaped Gram-pozitive aerobice or facultative anaerobic bacteria, wich are forming intracelular spores. This bacterias can normaly be fund in soil, plants and accidentaly in food products making them improper for consumption. (3) Evan if, most of these bacteria are inoffensive

saprophytes can be fund species like *Bacillus subtilis and Bacillus cereus* which are often associated with infections caused by food products. The most known exponent of the genus is Bacillus *anthracis,* anthrax causal agent. *Bacillus subtilis* is best scientifically defined of genus *Bacillus,* beaing used like model microorganism in many genetics reserch sudyes. Precise identification of bacteria is an important step in medical therapy and food industry.(3) Classic microorganism identification methods like growing and identification based on biochemical characteristics, differential coloring or immunoassay technique (ELISA) are time consuming, costly and requires intensive labor. Among modern microorganism identification technique very important is also mass spectrometry, a simple and specific method, well known like a validation method for other less precise techniques. This method is able to offer molecular information on bacteria, subject of this investigation. While in the past was used in bacterial analyze mass spectrometry pyrolysis, the current methods are based on the desorption ionization laser array and mass calculation by measuring the time of flight (MALDI-TOF).(2, 6) This technology won Nobel Prize in 2002, and being easy to use and in the same time giving quick results on microorganism identification and taxonomy. (1,4,7,8,10)

Purpose of this study was to generate a MALDI-TOF working protocol for measuring protein spectrum of bacteria belonging to *Bacillus (Bacillus anthracis* IC 29, *Bacillus subtilis* IC 13390 , *Bacillus megaterium* IC 11550) genus and protein spectrum comparison against a reference spectra data base (13) to identify the degree of sequence similarity of ARNr 16S gene. (5,13)

Material and methods

Materials

Main materials used for study: Trifluoroacetic acid (TFA), acetonitrile (ACN), saturated alpha cyano 4 hydroxycinnamic acid (HCCA α-), absolute ethanol, distilled water; (12)

Growing substrate preparation

14 g of nutritious agar (Fisher Scientific Ltd. Loughborough, UK) was dissolved in vial containing 500 ml of water, followed by strong homogenization. The vial is then autoclaved at 121° C for 15 minutes and then poured into Petri dishes. This was utilized for obtaining bacterial cultures; *Bacillus anthracis* IC 29, *Bacillus subtilis* IC 13390, *Bacillus megaterium* IC 11550. (12)

Bacterial growing

Studied *Bacillus spp*. cultures (*Bacillus anthracis* IC 29, *Bacillus subtilis* IC 13390, *Bacillus megaterium* IC 11550) was seeded by ansa scarification (plate exhaustion technique) on simple agar and blood agar in Petri dishes in order to obtain isolated colonies. Incubation was made at 37°C for 24 hours. Microorganism growing conditions in spectrum obtaining puposes are very important in the MALDI TOF for obtaining precise reproducibility and equal terms of

comparison between strains and database. For having correct final results have to be used fresh culture or slow gown bacteria, cultured for couple of days. (12,15,16)

Protein 16S extraction from ribosomes

From each Petri dish was collected 3 colonies, each colony becoming finaly a double sample. For 16S ribosomal protein extraction was used "Microorganisme Profiling" procedure, trifluoroacetic acid (TFA) 80% extraction. This method is used for bacteria culture in vegetative state, but is suitable also for sporulated biological material. From culture dish take approximately 10 mg of biomass using an Eppendorf tube on which is added 50 µl TFA 80. The mix is then pipetted to became homogeneous and is stored at room temperature for 10 to 30 min. Then 150 µl H2O şi 200 µl acetonitril (AN) is added. The sample is centrifuged at full speed for 2 min. then the supernatant is transferred in an Eppendorf tube. A volume of 1-2µl supernatant is putted on target steel plate (inside the well), is dried in laminar flow hood. After drying every well is covered with 2 µl matrix solution and dried in hood.

Matrix Solution

The matrix solution used for spectrum obtaining is having the same composition nomater extraction technique. The Matrix, is a α-HCCA la 50% acetonitril (AN) 2,5% tri-fluoracetic (TFA) acid saturated solution. The composition of 50% AN/ 2.5% TFA is called basic organic solvent (OS). Matrix once prepared can be stored in the refrigerator up to 1 week.

Data analysis

The dish was inserted in mass spectrometer Microflex LT 20. Mass range in which the identification was executed was set between 860 and 14000 Dalton, most of the strains having peaks between 1500 and 10000 Dalton. Parameters set in identification program Flex Control – m/z – molecular mass, SN – noise (Signal Noise), Quality Fac – Quality Factor, Res – Resolution, Intense – Intensity, Area – The area of the determined zone, have been introduced according with working data for bacteria section. For each sample have been executed between 3 and 5 laser shut resulting approximately 20 specters for each strain.

The specter has been analyzed and compared with the reference specter from the database of the device or with other examined strain belonging to the same bacteria species from the device library, selecting the most "clean" (without noise) specter, with a score at least ≥ 80. Resulted specters has been processed using BioProfiler Expert program and Flex Analysis program, the mass specters of studied bacteria strains was compared between them to see the similarities and differences.

Results and discussions

Bacillus genus biological agents mass analysis, represented by polypeptide 16S fragments from ribosomes have revealed a mass range between

860 and 14000 Dalton, with characteristic peaks in a range between 1500 and 14000 Dalton. Genus characteristic peaks average was comprising between the fallowing mass domain: 1100 -1300 Dalton; 3550 - 3650 Dalton; 4600 - 4950 Dalton; 5400 - 5900 Dalton; 6300 – 7300 Dalton; 9300 - 10300 Dalton; 11500-12000 Dalton; 10500-11500 Dalton.

Average score, taken in to consideration for introducing spectrum in database was ≥ 75, a representative score which certify a correct identification. Comparing test results using MALDI TOF for studied strains, we can conclude that most are similar from ribosomal protein 16S structure point of view, these facts are showing a certain degree of kinship.

Different composition of growing environment (blood agar or nutrient agar) have no significant effect over the distribution model of the peak, however standing out certain differences of specter especially in range between 3000 and 10000 Dalton, difference which can be assigned to various components of the tested growing environments and their implications in the bacterial metabolism.

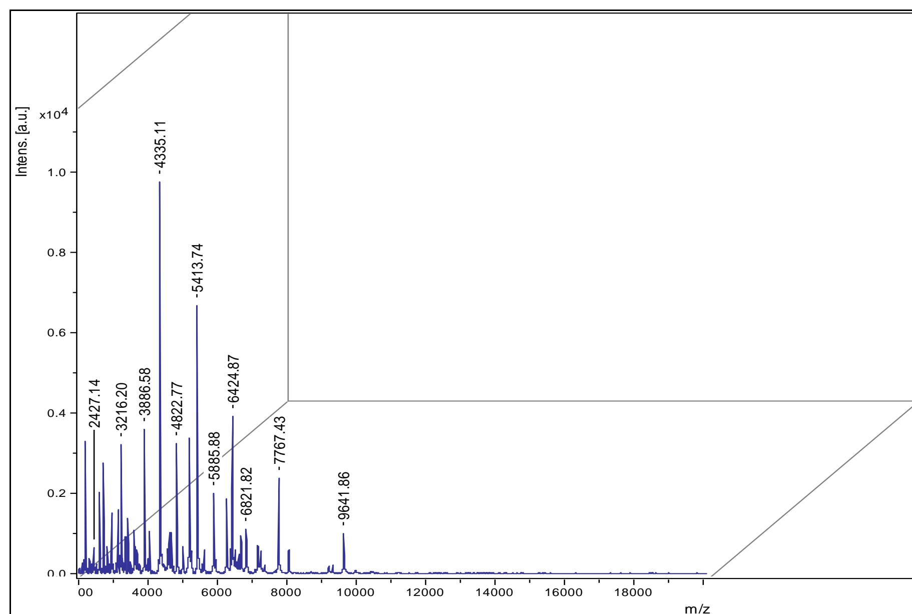

Fig.1. Graphyc representation of *Bacillus anthracis* IC 29 spectrum

Table 1

Molecular mass and aquisition parameters of *Bacillus anthracis* IC 29 strain

m/z	SN	Quality Fac.	Res.	Intens.	Area
9640.17	12.7	5040	1468	183.53	1439
9323.48	37.7	15037	1357	545.76	4132
9208.70	11.2	4141	1204	162.65	1320
8052.70	101.3	47338	1207	1464.76	10892
7766.46	255.1	125506	1159	3690.45	28031
7162.89	61.5	35751	945	888.98	7971
6820.70	164.7	78950	966	2382.98	19217
6609.04	57.4	25532	1090	830.01	5732
6260.82	243.7	148220	866	3525.16	28958
5884.90	288.9	199304	860	4178.98	34107
5613.03	69.7	24690	987	1008.89	6106
5170.11	384.2	216857	854	5558.34	38632
4820.26	68.4	18605	875	988.99	5394
4663.22	156.0	71215	853	2256.78	14425
4477.93	71.3	10668	602	1031.27	4666
4333.64	1322.2	880895	650	19127.95	143211
4027.73	132.3	64285	753	1913.59	11861
3884.41	288.9	137336	760	4180.05	24195
3669.92	77.1	31044	574	1115.42	7346
3582.65	95.0	37870	559	1374.05	8993

Legend: m/z – molecular mass (Mass); SN – noise (Signal Noise); Quality Fac – Quality Factor (Quality Factor); Res – Resolution; Intens - Intensity; Area – Area

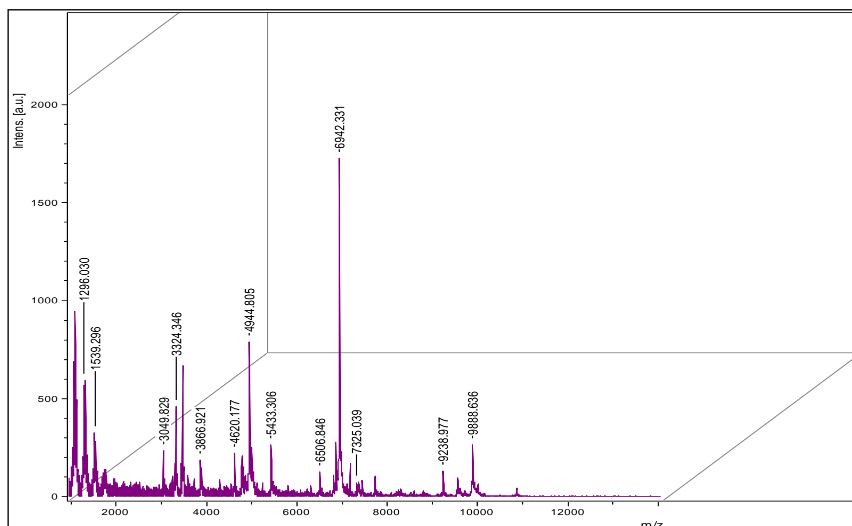

Fig. 2. Graphyc representation *Bacillus subtilis* IC 13390 spectrum analisys

Table 2

Molecular mass and aquisition parameters of *Bacillus subtilis* IC 13390

m/z	SN	Quality Fac.	Res.	Intens.	Area
9888.636	15.9	5714	925	265.90	2778
9549.115	2.4	40	1472	40.39	65
9257.070	1.5	109	3977	24.29	51
9238.977	7.4	1108	917	124.29	798
9207.352	1.3	58	1869	22.31	48
7325.039	4.1	265	1396	69.28	199
6942.331	103.0	65940	925	1725.18	15461
6506.846	6.0	898	2824	100.12	259
5433.306	15.8	1413	880	264.28	872
4944.805	47.2	18914	752	791.24	5409
4620.177	13.2	2866	687	221.12	1135
3866.921	11.1	345	591	185.58	377
3324.346	27.6	5567	499	462.39	2183
3049.829	14.0	401	497	235.20	410
1539.296	16.9	154	171	283.82	278
1296.030	33.9	4803	163	568.31	2011
1051.962	16.9	903	348	282.82	390

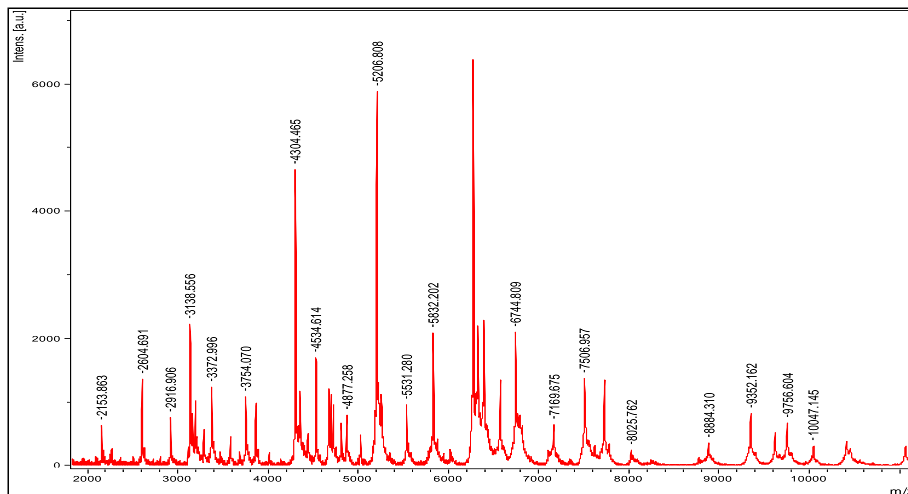

Fig. 3. Graphyc representation *Bacillus megaterium* IC 11550 spectrum analisys

Table 3

Molecular mass and aquisition parameters of *Bacillus megaterium* IC 11550 strain

m/z	SN	Quality Fac.	Res.	Intens.	Area
2153.863	18.8	8318	426	607.76	2212
2604.691	41.8	53818	509	1352.82	8094
2916.906	23.5	24322	571	759.51	4287
3138.556	68.8	75714	561	2225.99	13655
3372.996	37.9	18575	387	1226.91	7030
3754.070	33.4	10629	328	1081.05	6077
4304.465	143.7	210025	613	4649.14	37843
4534.614	52.5	50366	677	1698.74	12040
4877.258	24.2	9505	837	783.02	3693
5206.808	182.0	260127	773	5888.13	48644
5531.280	29.4	34472	1003	952.61	6787
5832.202	64.3	100097	766	2080.01	20048
6744.809	64.8	58429	628	2097.20	21644
7506.957	42.4	20891	324	1371.71	16119
7730.281	41.7	63160	781	1350.63	16449
8025.762	7.5	1962	851	241.78	1422
8884.310	10.8	3617	564	349.50	3167
9352.162	25.0	10161	625	809.51	8004
9756.604	20.6	33339	692	665.02	10925
10047.145	9.3	1827	622	299.30	2271

Analasing mass spectrum and also values with measurement parameters of molecular mass of polypeptide characteristic fragments can observe that the values of characteristic peaks for *Bacillus anthracis* IC29 strain are situated in the range between 2500 and 9600 Dalton, for *Bacillus subtilis* 13390 between 1050 and 9900 Dalton and for *Bacillus megaterium* 11550 strain in a range between 2150 and 10047 Dalton. For common molecular mass identification for species and for strain will present the values for mass and aquisition parameters for the analised strain belonging to *Bacillus* spp genus. Confronting the spectrum of the three strains studied, we have highlited proteic signatures for species and strain, making a complete picture regarding the degree of similarity and differentiation between them. Has been identiffied common peaks for three strains from *Bacillus* spp genus between 6200 and 6600 Dalton, with a common value of mass weight at 6200 Dalton, value wich can be considered like characteristic for genus *Bacillus* spp.

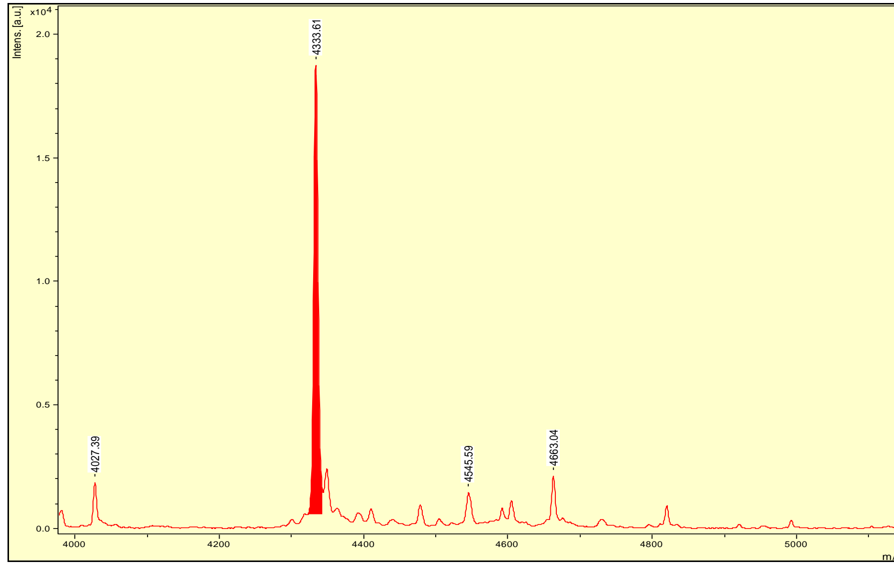

Fig. 4. Graphys representation of spectrum analisys *Bacillus anthracis* IC 29 “peak” characteristic strain (4333 Da)

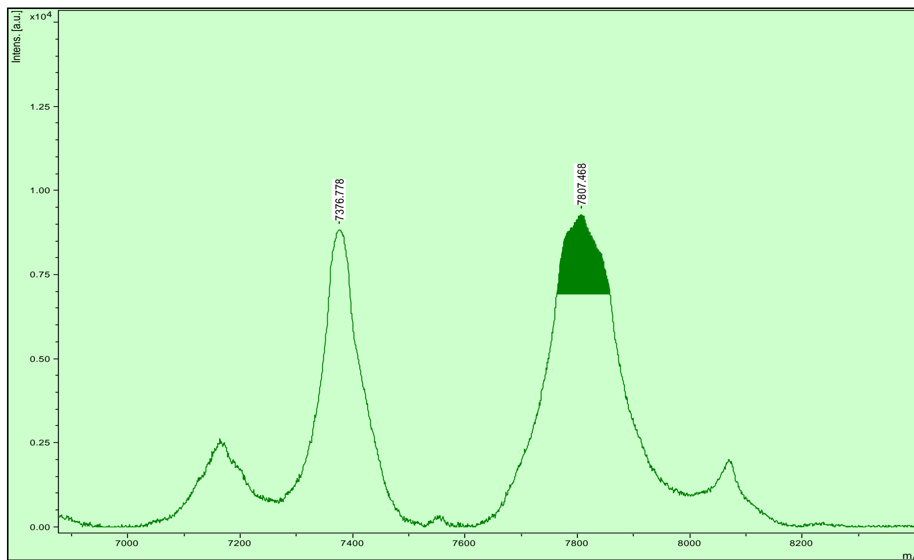

Fig. 5. Graphys representation of spectrum analisys *Bacillus subtilis* IC 13390 “peak” characteristic strain (7807 Da)

In fig. 4 and 5 are shown in hatch representation the species characteristics which represents „proteic species signature” with specific molecular weights for each analised agent: 48333 Dalton for *Bacillus anthracis* IC 29, 7807 Dalton for *Bacillus subtilis* IC 13390.

Conclusions

Bacteria strains for *Bacillus* spp. genus originated from the collection INCDMI “Cantacuzino”, identified through classic bacteriological methods have been taken in to study for confirmation by mass spectrometry, MALDI TOF technology (Matrix Assisted Laser Desorption Ionization – Time of Flight).

16S ribosomal protein extraction has been made using the ‘’Microorganism Profiling” procedure, extraction trifluoroacetic acid (TFA) 80%. Characteristic peak average of the genus was in the fallowing mass range: 1000 – 12000 Dalton. Characteristic peak average of the species was in the fallowing mass range: 1100 - 1300 Dalton; 3550 - 3650 Dalton; 4600 - 4950 Dalton; 5400 - 5900 Dalton; 6300 - 7300; 9300 - 10300 Dalton; 11500-12000 Dalton; 10500-11500 Dalton.

Resulting spectrum have been inserted in device database, based on an average score with a value over 75, a representative score which gives the certitude of a correct identification. The results obtained through MALDI TOF demonstrate that the strains taken in to study are similar from the point of view of 16S ribosomal protein structure, this fact demonstrating a certain degree of kinship.

Mass spectrum intercomparison of *Bacillus* spp strains taken into study and also the comparison with the reference strain from the database have revealed a high degree of association, represented trough a significant correlation of measured polypeptide fragments, graphic superposition of brut spectrum being extremely relevant.

Bacillus spp strain tested for confirmation using Microflex LT 20 system, have been validated 100%.

MALDI-TOF MS is a rapid identification technique, reliable and objective for pathogen microorganism, being used in scientific purposes and also in diagnosis.

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MICROBIOLOGICAL MONITORING OF LACTOSE USED IN THE PREPARATION OF BIOLOGICAL AND DRUG PRODUCTS

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Summary

Lactose is a disaccharide derived from galactose and glucose used in the production of biologicals and medicinal products.

Microbiological tests done to monitor lactose are counting mesophilic bacteria and fungi that may grow under aerobic conditions (the determination of TAMC of casein soya bean digest agar, the determination of TYMC of Sabouraud-dextrose agar) and determine the presence of *E. coli* (subculture on a plate of MacConkey agar and confirmation by biochemical tests).

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality.

Key words: Lactose, *E. coli,* biochemical tests, TAMC, TYMC

Lactose is a disaccharide derived from the condensation of galactose and glucose, which form a β-1→4 glycosidic linkage. Lactose exists in two isomeric forms, known as alpha and beta (designated a-lactose and b-lactose).

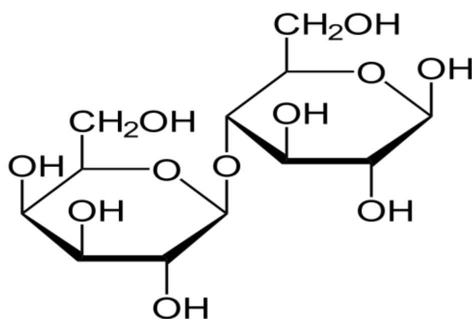


Fig. 1. Chemical formula of lactose

Pharmaceutical-grade lactose is highly pure lactose specifically produced to meet the standards of identity and purity set down in the lactose monographs of the various pharmacopoeias, including the European Pharmacopoeia.

Lactose is widely used as a filler or diluent in tablets and capsules, and to a more limited extent in lyophilized products.

The general properties of lactose that contribute to its popularity as an excipient are its: cost effectiveness, availability, bland taste, low hygroscopicity, compatibility with active ingredients and other excipients, excellent physical and chemical stability and water solubility.

Various lactose grades are commercially available that have different physical properties, such as particle size distribution and flow characteristics. The most common form of lactose used in pharmaceutical formulation is crystalline a-lactose monohydrate (4).

The tests are designed primarily to determine whether lactose used in the preparation of veterinary biological products complies with an established specification for microbiological quality (Eu. Ph. 2.6.13).

Materials and methods

Microbial enumeration tests. The methods will allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions. The tests are designed primarily to determine whether lactose complies with an established specification for microbiological quality.

Dissolve 10 g lactose in casein soya bean digest broth. Further dilutions, where necessary, are prepared with the same diluent. If necessary, adjust to pH 6-8.

Prepare for each medium at least 2 Petri dishes for each level of dilution. Incubate the plates of casein soya bean digest agar at 30-35 °C for 3-5 days and the plates of Sabouraud-dextrose agar at 20-25 °C for 5-7 days.

Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts and calculate the number of CFU per gram of product.

The total aerobic microbial count (TAMC) is considered to be equal to the number of CFU found using casein soya bean digest agar; if colonies of fungi are detected on this medium, they are counted as part of the TAMC. The total combined yeasts/mould count (TYMC) is considered to be equal to the number of CFU found using Sabouraud-dextrose agar; if colonies of bacteria are detected on this medium, they are counted as part of the TYMC.

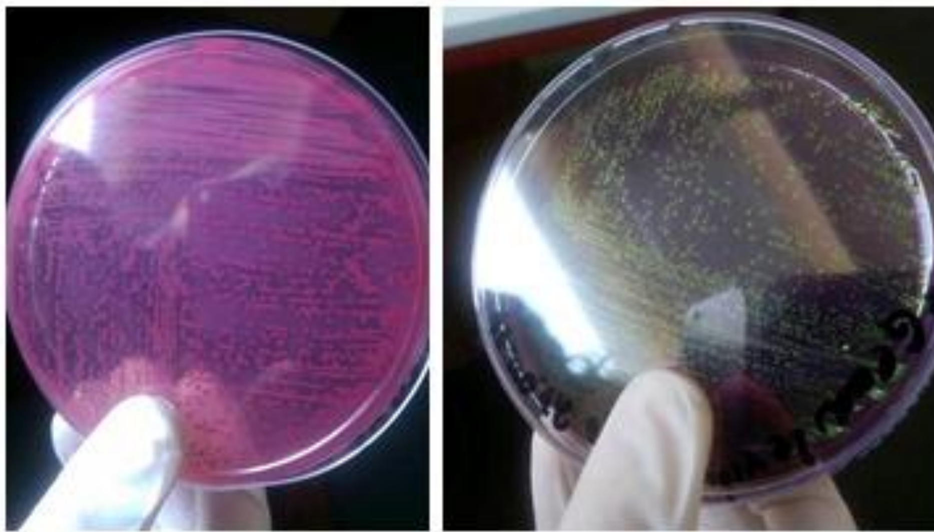
- TAMC: acceptance criterion 10^2 CFU/g (Eu. Ph. 2.6.12).

Test for *Escherichia coli.* The tests described will allow determination of the absence or limited occurrence of specified micro-organism *Escherichia coli* that may be detected under the conditions described. The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality.

The culture medium MacConkey Broth and MacConkey Agar must be sterile and checked with a strain of *Escherichia coli.*

Nutrition medium control of MacConkey Agar was made by the means of bacterial strain *Escherichia coli* G205. From the tube culture it was done the dispersion of the bacterial strain on a plate with MacConkey Agar medium, and on a plate with Geam Levine medium.

The MacConkey Agar medium was incubated at 35 ºC for 18 – 24 hours and the Geam Levine medium at 35 ºC for 24 – 48 hours. The color of the *Escherichia coli* colony on MacConkey Agar medium is pink to red and on Geam Levine medium is dark green with metallic sheen characteristic.



a) b)

Fig. 2. Nutritional value medium control for detection *E. coli*
a) MacConkey Agar: the color of the *Escherichia coli* colony medium is pink to red.
b) Geam Levine medium: the color of the *Escherichia coli* colony medium is dark green with metallic sheen characteristic

The control medium MacConkey Broth is distributed in sterile test tubes fitted with Durham tubes. They are inoculated with the strain *Escherichia coli* G205 and are incubated at 35 - 37°C for 18 – 24 hours. After that, proceed to analyze the development of the germs, the acidification of the medium and the gas production.

Fig. 3. Nutritional value control for MacConkey Broth

Glucose Fermentation: The tube is red originally from the phenol red acid/base indicator. If the *Escherichia coli* ferments glucose, acids are produced and the tube turns yellow. An inverted tube traps gas and shows that gas is produced as well as acids.

Determination *E. coli.* For this is necessary to do few steps.

Sample preparation and pre-incubation. Prepare a sample using a 1 in 10 dilution of the product to be examined, 10 g lactose are introduced into 100 ml casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

Selection and subculture. Shake the container, transfer 1 mL of casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h. Subculture on a plate of MacConkey agar and on Geam Levine medium at 30-35 °C for 18-72 h.

Interpretation. Growth of colonies indicates the possible presence of *E. coli.* This is confirmed by identification tests.

Biochemical test. Biochemical tests are performed from colonies developed on the surface of the specific medium MacConkey agar on Triple Sugar Iron Agar (T.S.I), Mobility Indole Urea (M.I.U), and Simmon's Citrate Agar (Himedia manual microbiology).

Confirmation of *E. coli* on biochemical tests:

T.S.I - medium color changes from brick-red to yellow-orange, presence of air bubbles and gas emission.

M.I.U – the confirmation of *E. coli* is proved by the color changing of medium in red when the Erlich Kovacs reagent is added. This reaction is due to the presence of indole.

Simmon's Citrate Agar - inhibits the growth of *Escherichia coli* colonies so the medium don't change and don't modify the color.

The product lactose complies if in the subculture made on the plate of MacConkey agar no colonies are present. The acceptability limit specified in the European Ph. is:

- Absence of *Escherichia coli* (Eu. Ph. 2.6.13.), (4).

Results and discussions

For microbiological monitoring of lactose, five batches were analyzed to determine the possible microbial contamination and were tested for absence of *E. coli.*

The obtained results are presented in Table 1.

Table 1

Microbiological monitoring of lactose

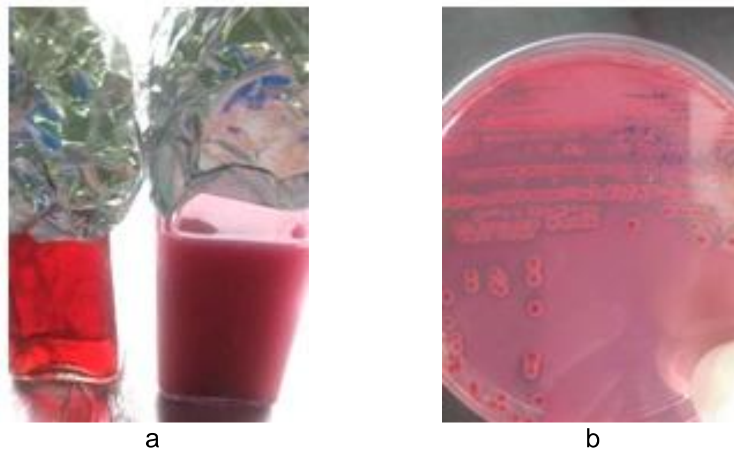
Batch	Test Manual Ref.	RESULTS				USED CODE S -satisfactory U - unsatisfactory I -inconclusive
		TAMC		*Escherichia coli*		
		Acceptance	Obtained	Acceptance	Obtained	
S. 1	Eur. Pharm.	≤10^2 CFU/g	<10^2 CFU/g	A	D	**U**
S. 2	Eur. Pharm.	≤10^2 CFU/g	<10^2 CFU/g	A	A	**S**
S. 3	Eur. Pharm.	≤10^2 CFU/g	<10^2 CFU/g	A	A	**S**
S. 4	Eur. Pharm.	≤10^2 CFU/g	<10^2 CFU/g	A	A	**S**
S. 5	Eur. Pharm.	≤10^2 CFU/g	<10^2 CFU/g	A	A	**S**

Legend: A – absent: D – Detected

Sample 1 didn't comply with the requirements of the European Pharmacopoeia (*E. coli* detected; fig. 4, 5, 6, 7).

Fig. 4. Casein soya bean digest broth, $Sample_1$.

TSB medium with lactose sample contaminated with *E. Coli* shows turbidity.



a b

Fig. 5. The contamination with *E. coli*. on MacConkey broth (a) and MacConkey agar (b), $Sample_1$.

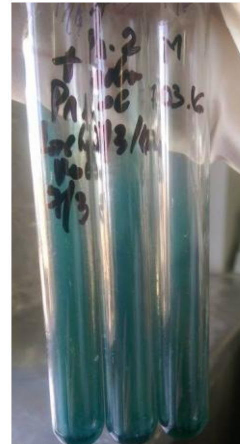
The color of the *Escherichia coli* colony on MacConkey agar is red.
MacConkey broth medium with lactose sample contaminated with *E. coli* shows turbidity.

A

Results are obtained with the inoculation of TSI Agar slants
E. coli shows an acid reaction (yellow) and gas formation in the butt of the test tube and an acid reaction (yellow) on the slant surface.

B

E. coli is positive - indole test
it can be detected by adding Kovac's reagent, which turns red in the presence of indole.



C

E. coli were unable to grow in the Simmons citrate medium
Simmons citrate medium inoculated with a citrate-negative organism will appear identical to the uninoculated Simmons citrate medium: color green.

Fig. 6. Biochemical tests of $Sample_1$: A) Biochemical test on TSI
B) Biochemical test on MIU with Kovach’s reagent C) Biochemical test on Simmons citrate agar

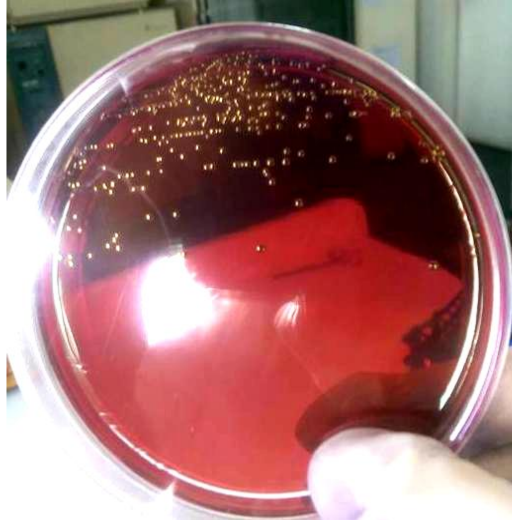
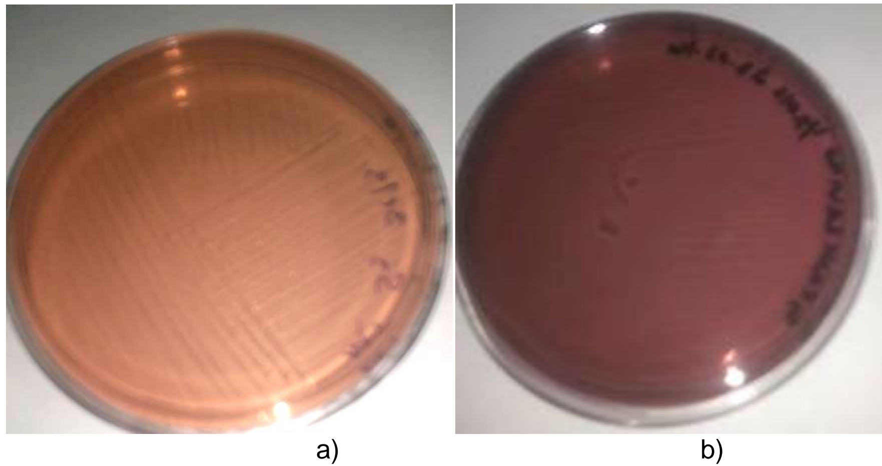


Fig. 7. The confirmation test of the presence of *E. coli* on Geam Levine in the case of $Sample_1$.

The color of the *Escherichia coli* colony on Geam Levine medium is dark green with metallic sheen characteristic.

Absence of *E. coli from* $Sample_2$:



a) b)

Fig. 8. Tests for absence of *E. coli* ($Sample_2$)

a) MacConkey agar b) Geam Levine

In the subculture made on the plate of MacConkey agar and Geam Levine medium no colonies are present.

Conclusions

From five tested batches of lactose the samples 2, 3, 4 corresponded to the requirements: TAMC/ *E. coli* with European Pharmacopeia Edition 8, Chapter 2.6.12/2.6.13.

The first sample (batch) of lactose was not in accordance with European Pharmacopeia Edition 8, Chapter 2.6.13, because a contamination with *E. coli* was detected.

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IDENTIFICATION OF SALMONELLAS BY COMPARING CLASSICAL METHOD WITH THREE RAPID METHODS

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Summary

Thanks to an innovative technology involving bacteriophage recombinant proteins, the test VIDAS® UP *Salmonella* (SPT) (Biomerieux), allows the specific detection of *Salmonella* in food, environmental and material from primary production samples.

The present study was done comparing the classical method SR EN ISO 6579: 2003 with the polymerase chain reaction (PCR), immunoenzymatic enzyme (miniVidas) and impendance (µ-Trac), through sampling of faeces from pigs and sanitation tests on the surface of carcasses.

Analyzing the results obtained by the classical method and rapid methods (PCR miniVidas and µ-Trac) a number of of 43.13% positive samples resulted by PCR, followed by miniVidas method, which obtained 41.17% positive samples, compared to the bacteriological and inductance method (µ-Trac), where where obtained only 35.29% positive samples.

Key words: Identification, method, samples, *Salmonella*

The microbiological methods used as standard for identifying the presence of salmonellas in food, are known and commonly used today, although these methods require 4-5 days to get the results, which themselves being presumptives, have a drawback, that they need time (might take up to 7 days), depending on the biochemical and serological confirmation (6).

Even if they don't require expensive infrastructure, and supplies are relatively cheap, these methods have a number of shortcomings: prolonged time since having the results; heavy working technique, which requires compliance with standard procedures; many culture media and reagents; possibility of affecting the sensitivity of the method by treating the animal with antibiotics during it's lifetime, by the improper way of collecting the samples, as well as by having a low number of viable microorganisms in the samples (6).

These techniques is important for fast identification of the *Salmonella* spp. carrier from pigs, especially when there are free of *Salmonella* spp. groups, or when it is desired to control the efficiency of the cleaning and disinfection procedures in a short time.

The question of whether these methods can be used in pig farms and feces samples, dust, and so on, which are naturally contaminated with an unknown amount of *Salmonella* spp. containing a large amount of bacterial DNA.

Based on the subject was considered appropriate to conduct investigations of molecular biology and the conditions of our country, in order to make new contributions to the knowledge of *Salmonella* present in Romania.

Materials and methods

The study was conducted on a total of 51 samples (21 samples of faeces and 30 sanitation samples collected from the surface of carcasses).

Samples were examined by the classical method SR EN ISO 6579: 2003.

In parallel, to identify *Salmonella* were used: μ-Trac method that allows detection of *Salmonella* spp. in 48 hours Rapid Polymerase Chain Reaction (PCR).

A rapid technique for the detection of *Salmonella* spp receptor using the principle Enzyme Liked Fluorescent Assay (ELFA) it was the method that uses the SPT Vidas® analyzer, which is an immunoenzymatic method.

At the end of the test, the results are automatically analyzed by the machine, which calculates a test value for each sample. This value is then compared with internal references (thresholds) and each result is then interpreted (positive, negative).

Results and discussions

Of the 51 samples, examined by the four methods, there were obtained a total of 22 (43.13%) samples positive by molecular method, 21 (41.17%) samples positive by the miniVidas method and a number of 18 (35.29%) by bacteriological method and μ-Trac.

Comparing the results between the four methods, they found significant differences ($p > 0.005$).

These results could be attributed to microorganisms (*Salmonella* spp.) which were active because it has been revealed the presence of DNA but have not been able to develop on the culture medium (4).

Table 1 and Fig. 1 shows the results obtained from the comparison of the four methods, PCR molecular method, miniVidas, bacteriological and μ-Trac, of the 51 samples analyzed.

Salmonella isolation from faeces by bacteriological method may be inhibit due to the relatively low microoorganisme of *Salmonella* in faeces (3).

Benetti et al. (1), discovered that the method miniVidas® compared with the bacteriological method is able to identify a small number of viable microorganisms present in the sample (1).

Lepper et al. (5), after a study in which they compared the method miniVidas® *Salmonella* with the bacteriological method from many types of products have found a match for 1266 samples from the 1440 samples analyzed (5). A similar study was also conducted by Blackburn et al. (2) on a total of 141

naturally contaminated samples, in which the authors found an agreement between the two methods, 92.6%.

Table 1

Comparison of rapid methods PCR, miniVidas, µ-Trac with ISO

Probe	NR	
	n(+)	%
PCR	22	43.13
miniVidas	21	41.17
ISO	18	35.29
µ-Trac	18	35.29

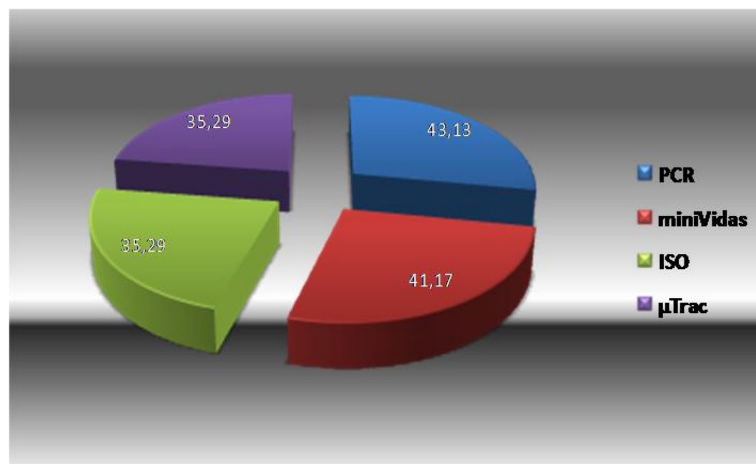

Fig. 1. Comparison of rapid methods PCR, miniVidas, µ-Trac with ISO

So far, the bacteriological method is considered as the "golden standard" for the isolation and identification of *Salmonella* in various products.

However, this method requires a longer time to obtain results, and a large amount of work, so is not recommended to examine an increasing number of samples.

Conclusions

By molecular methods (PCR) was obtained 43.13% positive samples, followed by miniVidas method with 41.17%, while with the bacteriological method and the µ-Trac was obtained 35.29% positive samples.

Following the results obtained, the molecular method (PCR) can be used for a more rapid identification of *Salmonella* spp microorganisms, from different

samples taken from pigs, and the method miniVidas, can be used as a screening method, providing benefits in marketing safe products for the manufacturer.

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MOLECULAR BIOLOGY RESEARCH REGARDING LYOPHILIZED *FRANCISELLA TULARENSIS* REVITALIZED AFTER 40 YEARS

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Summary

Francisella tularensis is a highly contagious Gram-negative bacteria that causes tularemia or "rabbit fever" and it is contagious to humans. There are four known subspecies of *Francisella tularensis,* two of them are the most studied: *A Type strain* that is the more virulent (found in North America) and *B Type* (subspecies *holarctica*, also referred to as *palearctica* strain, found in Europe) that is the less virulent. The two other non-virulent subspecies are: *mediasiatica*, found in central Asia and *novicida*, of which is not know very much.

Our researches have been based on the revitalization of lyophilized strains of *Francisella tularensis* in order to obtain the positive controls required for the in hause real time PCR kit for CCHFv and TBEv and *Francisella tularensis* and *Borrelia burgdorferi s.l.* agents transmitted by ticks.

Lyophilized strains were rehydrated in nutrient broth, cultured in *Francisella tularensis* specific medium (CHAB-PACCV) and passed on nutrient medium. Microbiological diagnosis (including optical microscopy) was confirmed by immunoassay (Tularemia biothreat Alert kit, Tetracore) and molecular tests: Real Time PCR with TaqMan *Francisella tularensis* detection kit, Applied Biosystems for two genes (*fop*A and *tul*4) and TicKitqPCR (in house kit) for one target insertion sequence-like element (IS*Ftu*2).

The methods have confirmed the presence of *Francisella tularensis* strain in revitalized samples after 40 years of storage.

Key words: *Francisella tularensis*, identification, Real Time PCR, lyophilized, revitalized

Francisella tularensis (*F. tularensis*) is a highly contagious Gram-negative bacteria that causes tularemia or "rabbit fever" and it is contagious to humans. (1)

Among domestic animals, sheep are the most common host, but clinical infection has also been reported in cats, dogs, pigs, and horses. Tularemia is seen

more often in cats than dogs, and in young versus adult animals. A pet with a mild infection may show no symptoms or may suffer briefly from lack of appetite, lethargy, and a low-grade fever.

More serious infections can cause dehydration, high fever, swollen lymph nodes, eye infections, ulcers in or around the mouth, draining abscesses, jaundice, and an enlarged liver or spleen.(*)

Humans can become infected through several routes, including: tick and deer fly bites, skin contact with infected animals, ingestion of contaminated water, inhalation of contaminated aerosols or agricultural dusts, laboratory exposure.

Symptoms vary depending on the route of infection. Although tularemia can be life-threatening, most infections can be treated successfully with antibiotics.

Steps to prevent tularemia include: use of insect repellent, wearing gloves when handling sick or dead animals, avoiding mowing over dead animals. (**)

F. tularensis is classified as a Tier 1 Select Agent by the U.S. government due to its low infectious dose, ease of spread by aerosol, and high virulence.

F. tularensis is considered a monomorphic extracellular pathogen, molecular genotyping studies have shown that there are numerous differences between subspecies of *F. tularensis*.

There are four known subspecies of *F. tularensis,* two of them are the most studied: *A Type strain* that is the more virulent (found in North America) and *B Type* (subspecies *holarctica*, also referred to as *palearctica* strain, found in Europe) that is the less virulent. The two other non-virulent subspecies are: *mediasiatica*, found in central Asia and *novicida*, about this subspecies very little is known.

More recent studies showed that in within sub - type/species from genetical point of view there are significant differences particularly in the case of repetitive sequences (SNP-Single Nucleotide Polymorphism) of genome which affect the content of AT/GC of certain target genes involved in virulence. (3)

In addition, *F.tularensis* coexist on cellular and/or tissular lever in the bodies of vector (ticks) with other bacterial as Wolbachia persica, which has led to finding genetic targets very specific to pathogenic subspecies of *Francisella.* (2)

Materials and methods

For present experiments were used lyophilized samples in which *F. tularensis* was supposed to be still present and viable, lyophilized which are over 40 years old. Lyophilizates were rehydrated with liquid nutrient agar, 24 hours at 37^0C. Cultivation of samples after rehydration was done on *F. tularensis* specific medium: cysteine heart agar with 9% chocolatized sheep blood, containing polymyxin B, amphotericin B, cyclohexamide, cefepime and vancomycin.(4)

The current methods for detection of *F. tularensis* are: microbiological diagnosis (including optical microscopy), immunoassay and molecular tests included molecular biology techniques based on Real Time PCR (usually in multiplex approach for specific gene targets as Is*Ftu2*, *tul4* and *fopA*).

Immunoassay detection was made with Tularemia *BioThreat Alert*™ Test Strip who is a hand-held biological agent detection and identification device for *F. tularensis,* the causative agent for tularemia. The Tularemia *BioThreat Alert*™ Test Strip is a rapid qualitative test who utilizes a combination of monoclonal and polyclonal antibodies to selectively detect the presence of biological threat agents in aqueous samples.

DNA extraction from bacterial culture was performed with PureLink® Genomic DNA Mini Kit, Invitrogen which enables rapid and efficient purification of genomic DNA. Real Time PCR tests was made with TaqMan® *Francisella tularensis* detection kit, Applied Biosystems for two genes (*fop*A and *tul*4) and TicKitqPCR for one target insertion sequence-like element (IS*Ftu*2).

Results and discussions

Our 12 lyophilized were analyzed using classical methods - bacterial culture on specific media and modern methods - rapid immunoassay and Real Time PCR.

After rehydration the samples were grown on specific medium, 48-72 h on 37°C (Fig. 1).

Fig. 1. *Francisella tularensis* colonies on CHAB-PACCV after 72 hours

Rapid immunoassay method revealed possible presence of *F.tularensis* which was confirmed by Real Time PCR.

The test is positive if two colored lines appear. One colored line will appear in the sample window and one in the control window (Fig.2).

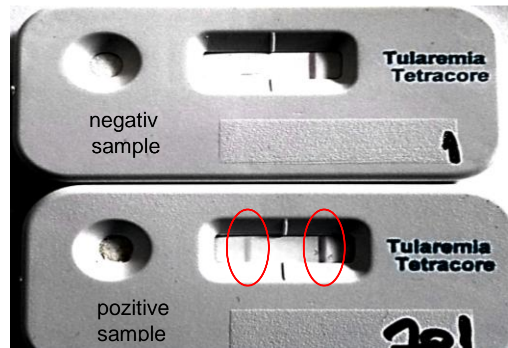

Fig. 2. Immunoassay test using Tularemia Biothreat Alert™ kit, Tetracore

DNA extraction from bacterial culture was performed with PureLink® Genomic DNA Mini Kit, Invitrogen. The total amount of DNA was quantified with NanoDrop One^{C}, Thermo Scientific.

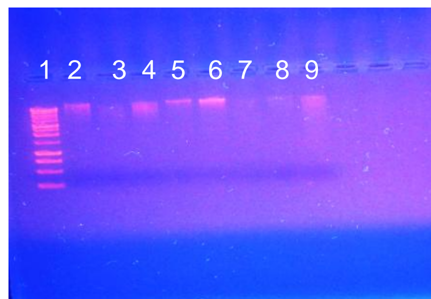

Fig. 3. DNA extraction using PureLink® Genomic DNA kit (1% agarose), Lane 1 -1kb DNA ladder; Lane 2-9 - genomic DNA

Gene amplification was performed using a Cepheid® SmartCycler Real Time PCR machine using a commercial kit with TaqMan® *Francisella tularensis* detection kit, Applied Biosystems for two genes (*fop*A and *tul*4) and TicKitqPCR for one target insertion sequence-like element (IS*Ftu*2). As a positive control for genomic DNA we used Amplirun® *Francisella tularensis* DNA control (LVS strain, 1400copies/µl, CT = 17.05), Vircell (Fig.4).

The reaction outcome was visualized in real time using the software supplied with amplification system by monitoring the fluorescence after each amplification cycle and CT values were registred (Table 1).

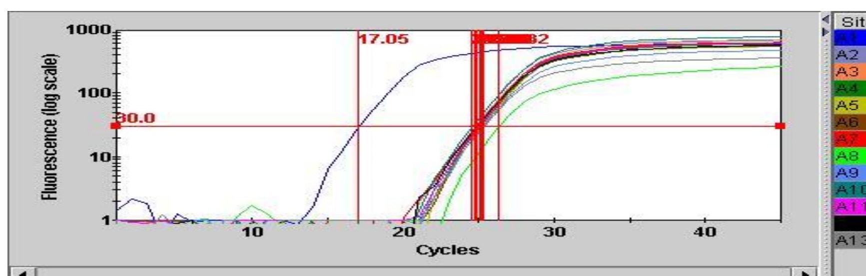

Fig. 4. Detection of IS*Ftu2* gene from *F. tularensis* by Real Time PCR; FAM channel; Cepheid® SmartCycler Real-Time PCR

Table 1

CT value for analyzed samples

Target	1	2	3	4	5	6	7	8	9	10	11	12	positive control
CT value *tul4*	35.37	31.98	0	0	0	43.73	0	0	-	-	-	-	27.61
CT value *fopA*	0	0	31.61	31.89	0	0	0	0	-	-	-	-	27.61
CT value IS*Ftu2*	24.54	24.76	25.08	25.16	25.09	24.80	26.32	25.24	24.53	25.04	24.89	25.34	17.05

Molecular methods had confirmed the presence of specific genes *tul4* (three positive samples), *fopA* (two positive samples) and IS*Ftu2* (in all samples) in analysed vials.

We wondered why IS*Ftu2* has so small values of all isolates. The possible answer is that IS*Ftu2* is an insertion sequence-like element that is in a number of 16 copies per genom of *F. tularensis* (5) and thus is about 16x more sensitive than *fopA* and *tul4* who are one copy per genome.

Conclusions

Our research has shown, through microbiological methods, immunoassay and Real Time PCR, that the *Francisella tularensis* strain (classified as level 4 biological agents) can be revitalized after a period of 40 years from liophylized form.

Acknowledgments

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SENSORIAL AND QUALITY PARAMETERS ASSESSMENT IN SOME TRADITIONAL MEAT PRODUCTS FROM ROMANIA

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Summary

Nowadays, traditional meat products are a well-consumed foodstuff in Romania, so the assessment of the sensorial and quality parameters is very important for food safety.

The traditional meat products samples (peasant file - 15, peasant neck -12 and peasant chest – 10) were collected from a large meat processing plant in Romania.

For all samples, sensorial assessment (shape and dimensions, general aspect, aspect on section, smell and taste) was performed. Using the Corona 45 Visnir analyzer, water, fat, total protein and NaCl content were determinate.

The sensorial assessment revealed normal parameters. The results ranged from 57.9 to 70.2% for water content, 5.1 to 17.4% for fat content, 15.5% to 19.8% for total protein content and 2.4 to 3.2 % for salt content.

All results were within the limits established by legislation, respectively maximum 5% for NaCl and minimum 15% for total protein (10). For water and fat content, there are not legislative limits, but the results were according to the product specification.

From this limited study, it can be considered that there is no risk for human health linked to the consumption of traditional meat products, but a continuous assessment of quality parameters should be performed, in order to ensure good products for consumers and the traditional meat products quality remains an exciting subject which has a stake in the near future.

Key words: traditional meat products, sensorial assessment, quality parameters

Between 2005 and 2013, 4402 traditional products were registered in the certification of traditional products register in concordance with Order 690/2004 (6). Out of the 4402 certified and nationally registered traditional products the highest number is owned by meat products – 1541 followed by dairy products - 1535 and bakery products -750. Also, 285 drinks, 193 products from fruits and vegetables and 11 traditional fish products were recorded (4).

In 2013, for certification of traditional products, Order 724/2013 (7) was implemented and since then 516 traditional were registered, most of them meat products- 211.

In the first decade of 2016, 14 traditional products were recorded, out of which 6 meat products (3).

Since 2007, Romania, as an EU State Member, is obliged to full application of European legislation on veterinary certification and official controls on products of animal origin, including traditional ones (1).

National legislation is in accordance with European legislation and is focused on the recording mode of traditional products and requirements for establishments where these products are produced and stored (5,7,9).

Taking into consideration the fact that traditional meat products are a well-consumed foodstuff in Romania, the aim of this study was to perform a short characterization of some quality parameters in Romanian traditional meat products related to food safety.

Materials and methods

The traditional meat products samples (peasant file - 15, peasant neck -12 and peasant chest – 10) were collected from a large meat processing plant in Romania.

For all samples, sensorial assessment (shape and dimensions, general aspect, aspect on section, smell and taste) was performed. Using the Corona 45 Visnir analyzer, water, fat, total protein and NaCl content were determinate.

Results and discussions

The sensorial assessment was done according to the technical specification of each product (2).

For this analyze were monitored the following parameters:

- shape and dimensions: corresponding to the anatomical region for peasant file and peasant neck and rectangular pieces with 220/220 mm dimensions for peasant chest;
- general aspect: clean surface, red-brownish color specific for smoked products;
- aspect on section: light pink muscular mass for peasant file and peasant neck and light pink muscular mass alternatively with fat for peasant chest;
- smell and taste: pleasant, specific for each type, smoked, without foreign taste and smell (moldy, sour, stale).

For all types of traditional meat samples, the sensorial assessment revealed normal parameters according to the type of product.

For all samples, quality parameters were analysed: water, fat, total protein and NaCl content (Table 1).

Table 1

Results of quality parameters assessment

Product	Water %	Fat %	Total protein %	NaCl %
peasant file 1	70.1	5.1	19	2.4
peasant file 2	72.3	5.9	18.6	3.2
peasant file 3	71.2	7.7	17.7	2.7
peasant file 4	67.2	8.8	18.8	2.7
peasant file 5	69.2	8.6	17.7	2.5
peasant file 6	69.1	10.4	16.8	2.4
peasant file 7	70	6.5	18.5	2.7
peasant file 8	70.2	6.2	18.7	2.7
peasant file 9	68.5	6.3	19.8	2.9
peasant file 10	67.8	8.9	19.5	2.6
peasant file 11	69.1	7.8	18.9	2.5
peasant file 12	68.4	8.1	19.4	2.4
peasant file 13	70.2	8.3	19.2	2.8
peasant file 14	69.3	8.5	19.3	2.9
peasant file 15	68.9	9.2	19.1	3
peasant neck 1	64.3	15.6	16.6	2.8
peasant neck 2	69.5	6.6	19	2.8
peasant neck 3	68.8	9	18.2	2.9
peasant neck 4	68.1	11.8	16.6	2.6
peasant neck 5	65.4	13.8	16.9	2.7
peasant neck 6	69.7	7.2	18.4	2.7
peasant neck 7	67	9	18.6	2.8
peasant neck 8	65.6	8.7	16.1	3.1
peasant neck 9	66.8	9.3	16.5	3.2
peasant neck 10	67.8	10.2	17.8	3
peasant neck 11	69.2	9.5	18.1	2.9
peasant neck 12	68.6	8.9	18.2	2.8
peasant chest 1	65.3	10.7	17.7	2.7
peasant chest 2	62.7	13.6	18.1	2.5
peasant chest 3	61.9	17.4	15.6	2.7
peasant chest 4	57.9	15.6	18.7	2.9
peasant chest 5	58.6	14.8	19.1	3
peasant chest 6	59.4	13.7	18.6	3.1
peasant chest 7	61.2	15	18.9	3.2
peasant chest 8	60.3	12.6	19.2	2.8
peasant chest 9	61.8	13.4	19.4	2.7
peasant chest 10	62.1	11.6	18.8	2.6

The results ranged from 57.9 to 70.2% for water content, with an average of 64.05 % (Figure 1).

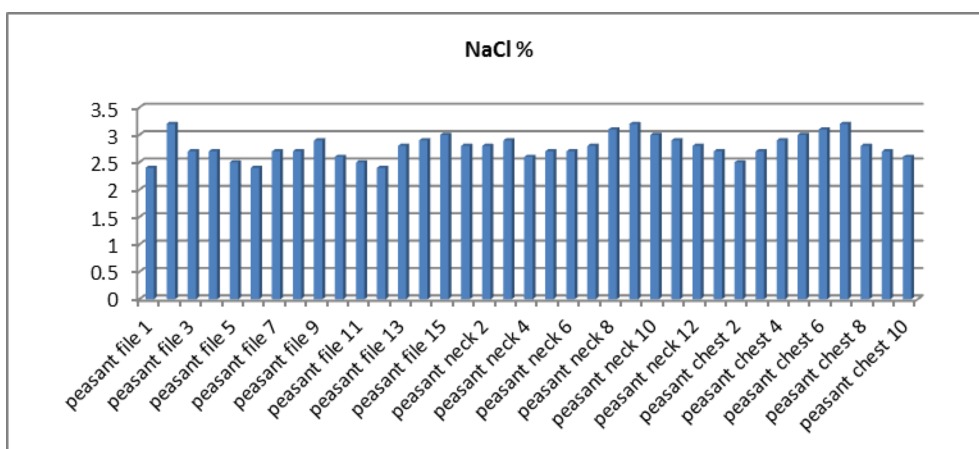

Fig. 1. Results for water content (%)

For fat content, the results ranged from 5.1 to 17.4%, with an average of 11.2 % (Figure 2).

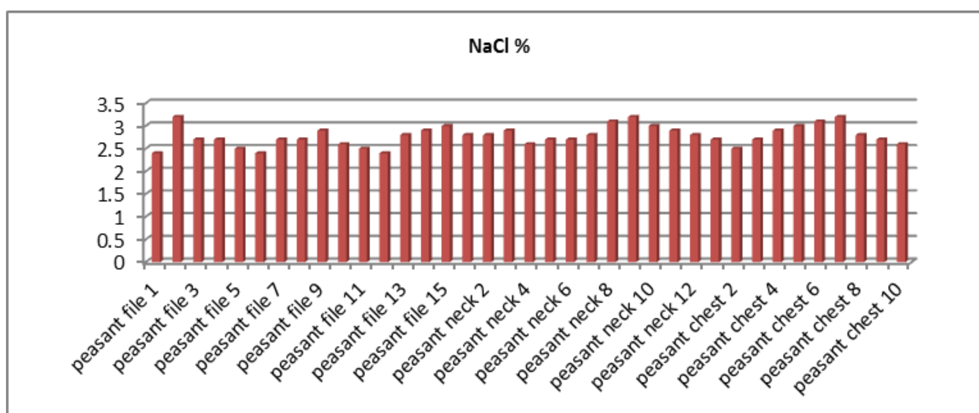

Fig. 2. Results for fat content (%)

The results ranged from 15.5 to 19.8% for total protein content, with an average of 17.6 % (Figure 3).

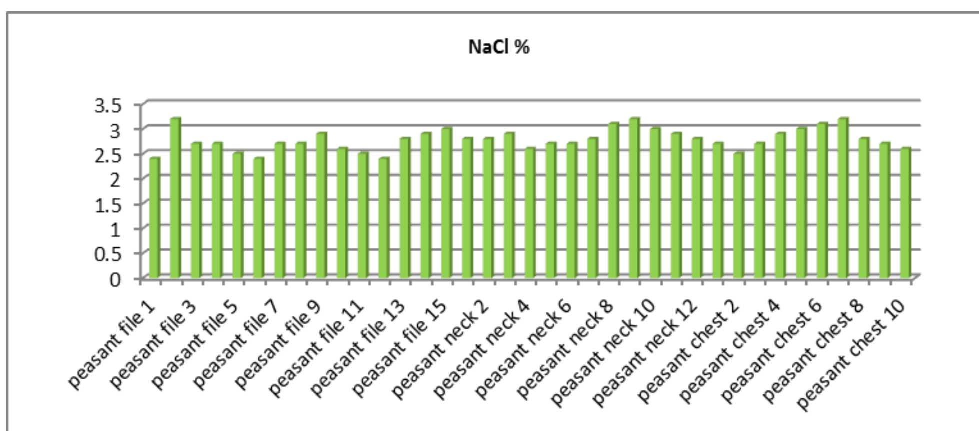

Fig. 3. Results for total protein (%)

For NaCl content, the results ranged from 2.4 to 3,2%, with an average of 2.8 % (Figure 4).

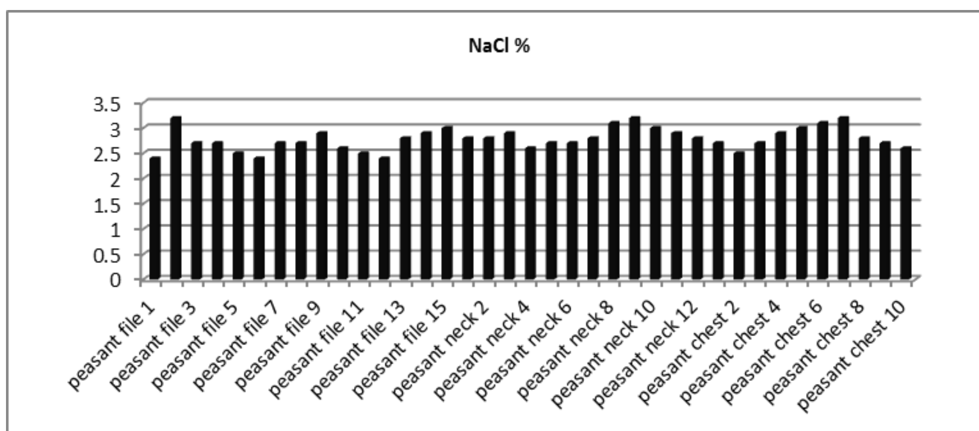

Fig. 4. Results for NaCl (%)

All quality results were within the limits established by legislation, respectively maximum 5% for NaCl and minimum 15% for total protein (8,10). For water and fat content, there are not legislative limits, but the results were according to the product technical specification.

Conclusions

According with the official data concerning traditional products, it can be seen that the meat traditional products own the first place and that is an increasing demand for these kinds of products.

From this very limited study, it can be considered that there is no risk for human health linked to the consumption of traditional meat products.

However, a continuous assessment of quality parameters should be performed, in order to ensure good products for consumers.

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6. Ordinul 690/2004 pentru aprobarea Normei privind condiţiile şi criteriile pentru atestarea produselor tradiţionale.
7. Ordinul nr. 724/2013 privind atestarea produselor tradiţionale.
8. Ordinul nr. 560/2006 pentru aprobarea normelor cu privire la comercializarea produselor din carne.
9. Regulamentul Parlamentului European şi al Consiliului nr.852/2004/CE privind igiena produselor alimentare, precum şi de stabilire a procedurii de acordare a derogărilor şi de înregistrare sanitară veterinară şi pentru siguranţa alimentelor a unităţilor în care se realizează produse alimentare ce prezintă caracteristici tradiţionale.
10. Regulamentul Parlamentului European şi al Consiliului nr. 1333/2008 al privind aditivii alimentari.

RESEARCHES REGARDING AIR CONTAMINATION WITH MESOPHILIC BACTERIA IN POULTRY HOUSE REARED ON ENRICHED CAGES

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Summary

The results of a study done on air load aerobic bacteria from a house for hens reared on enriched cages over one breeding cycle are presented in this paper.

The mean value of total number of mesophilic bacteria from hens house presented large oscillation from , from 3.61 x 10^4 CFU / m^3 of air in the first month, to 1.94 x 10^5 CFU / m^3 air in the sixth month, when it was recorded the maximum value, just to slightly decrease after, but still remaining at values of around 10^5

For this type of rearing hens, the total number of aerobic mesophilic bacteria ranged, for a long period of time, under the recommended values as some researchers referenced.

Key words: hens house, air, aerobic mesophilic bacteria, dynamics, enriched cages

Shelters airborne microflora is very diverse, systemic and quantitative. A small amount of it comes from the atmospheric air (approximately 1.000 germs/m^3 air). The majority of the germs derive from feces, bedding, feed and pathologic secretion and excretion of birds. Bacteria air load is directly proportional with the airborne dust. Total number of germs increases with the time of keeping the effective into the shelter, considered from the last disinfection or from the populating moment (7, 14, 18).

The results of quantitative determinations prove both the existence of a very great number of microorganisms per cubic meter of air and a tight correlation between the dust and the number of these microorganisms. Thus, by the aging of the bedding, the bacteria number increase, by mean, from 191,000 to more than 3,000,000/m^3 air (this observation is also valid for the fungi and molds spores) (2, 6, 9, 13).

The situation is incomparably better in houses with birds reared in cages, where the highest mean values are around 87,000 bacteria / m^3 air (1, 17).

It is very well known the fact that through the shelter air quality the production performance can be influenced. The effect on animals and birds, although important, represents at least an economic problem, if not also an ethical one. The

most expensive costs associated with a poor air quality are transposed and paid through threatening the workers health (3, 8, 11, 15).

Till nowadays, a hygienic norm considering the airborne microorganisms is not yet set, most probably cause of the great differences between determinations, dependent of the used method (gravitational sedimentation or aspiration, numerous variants being available for each one of them). Some authors consider that a value of 500,000 – 700,000 germs/m^3 air in hen house is acceptable, while others consider that at a value over 250,000 germs/m^3 air the microbial stress appears (4, 5, 10, 12).

Knowing these aspects, the research conducted in this study had aimed to underline the airborne mesophilic bacteria and their dynamic in a shelter with birds reared in cages, during an exploitation cycle.

Materials and methods

The research was conducted in a laying hen's farm, reared in enriched cages, localized in the west part of Romania, in Arad County. The farm is equipped at European standards, respecting the EU-BAT (European Union best available techniques) requirements.

The farm is composed by one building, with a single hall, on a single level, one building for egg sorting and depositing and one administrative building.

The production hall is rectangular, 55 meters long, 14 meters deep and 4.20 meters high, with a total surface of 770 m^2.

In the hall there are five lines of enriched cages, each of them with four levels. Both, the distance between the lines, and the distance between the lines and the walls is 100 cm. The hall is equipped with air cooling systems, ventilation systems, artificial lightening, automatic feeding and drinking systems, egg collecting and transport automatic system, automatic system for feces collecting and removal.

The determination and quantification of microclimate biological factors were made weekly, at each visit, from five points as follows: from the four corners of the hall and its center, a total of 240 samples.

Total number of aerobic mesophilic bacteria (TNAMB) determination was done by suction method, using a PBI Air Sampler SAS Super 100 device (International PBI SpA Milano, Italy) and nutrient agar plates. The device was set to aspirate a volume of one liter of air. Samples were collected from the second level of cages.

After aspiration, each Petri plate was identified by marking the date and place of sampling and was repackaged with the same paper used for sterilization of the plates. In maximum three hours after the sampling, the Petri plates were brought to the microbiology laboratory, where they were incubated in the thermostat at 37°C for 24 hours.

After incubation, the Petri plates were examined and the colonies developed on the surface of the culture medium were counted. The number of

colonies identified from each plate was equalized to the actual values, using table data, standardized by the manufacturer PBI Air Sampler SAS Super 100.

TNAMB values, expressed in CFU / m^3 air, were statistically analyzed by calculating the arithmetic average, standard deviation and the range of registred values by showing the minimum and maximum determined concentrations. The arithmetic averages were calculated for each of five sampling points at each visit. Also arithmetic averages were calculated for the values obtained in the course of a month, for each point of sampling. Raw data obtained from the investigations were processied through biostatistical methods, by using the Microsoft Excel spreadsheet.

For testing the statistical significance of differences between the averagesof the studied indicators, we used analysis of variance ANOVA Single Factor test, included with Microsoft Excel software. Along with the ANOVA test we also used the MANN WHITNEY (Wilcoxon) test from the MINITAB 14 program.

Results and discussions

The farm was populated in September. The mesophilic airborne bacterial load is shown in table 1.

Table 1

Total number of aerobic mesophilic bacteria load (CFU/m^3), from a laying hens house reared on cage system, during, one year of exploitation

No	Month	$\bar{x}$ ± Sx / month	$\bar{x}$ ± Sx / trimester
1	**September**	$3.61 \times 10^4 \pm 0.54 \times 10^4$	$4.24 \times 10^4 \pm 0.66 \times 10^4$
2	**October**	$4.94 \times 10^4 \pm 1.47 \times 10^4$	
3	**November**	$4.17 \times 10^4 \pm 0.65 \times 10^4$	
4	**December**	$8.77 \times 10^4 \pm 3.96 \times 10^4$	$1.36 \times 10^5 \pm 5.41 \times 10^5$
5	**January**	$1.25 \times 10^5 \pm 0.23 \times 10^5$	
6	**February**	$1.94 \times 10^5 \pm 0.34 \times 10^5$	
7	**March**	$1.83 \times 10^5 \pm 0.34 \times 10^5$	$1.06 \times 10^5 \pm 0.21 \times 10^5$
8	**April**	$1.09 \times 10^5 \pm 0.21 \times 10^5$	
9	**May**	$1.06 \times 10^5 \pm 0.21 \times 10^5$	
10	**June**	$1.61 \times 10^5 \pm 0.29 \times 10^5$	$1.53 \times 10^5 \pm 0.96 \times 10^5$
11	**July**	$1.57 \times 10^5 \pm 0.26 \times 10^5$	
12	**August**	$1.39 \times 10^5 \pm 0.18 \times 10^5$	

In the first month of exploitation, the total count of airborne mesophilic bacteria had a mean value of $3.62 \times 10^4 \pm 0.54 \times 10^4$ CFU / m^3 air, but in the next two months a slow increase was recorded, thus the mean values were $4.94 \times 10^4 \pm 1.47 \times 10^4$ CFU /m^3 air in October and $4.17 \times 10^4 \pm 0.65 \times 10^4$ CFU / m^3 air in November. During the first trimester, the mean value of total count of airborne mesophilic bacteria was $4.24 \times 10^4 \pm 0.66 \times 10^4$ UFC / m^3 air.

Krysztofik, cited by Woijcik (19) consider that the airborne bacteria load from shelters for laying hens reared on cages should not exceed 100,000 CFU / m^3 air. Considering the above recommended values, we can affirm that during the first exploitation trimester, the airborne mesophilic bacteria load was maintained to a very low level.

Analyzing the results for the second exploitation trimester, the airborne mesophilic bacteria mean value, in the first month (December) was 8.77 x 10^4 ± 3.96 x 10^4 CFU / m^3 air, higher than the last month of the previous trimester. Thus, in the first two weeks of the month, the average values of this index were lower, namely 5.82 x 10^4 ± 3.96 x 10^4 CFU / m^3 air and respectively 5.10 x 10^4 ± 1.15 x 10^4 CFU / m^3 air, but in the next two weeks a sudden increase of the airborne bacteria level was observed, recording ten times higher values, in the order of 10^5.

Also, in the second month of the second trimester, an increase of the mean value of total number of mesophilic aerobic bacteria was recorded: 1.25 x 10^5 ± 0.23 x 10^5 CFU / m^3 air, with higher differences, strongly significant, compared to previous month ($p<0.001$).

In the third month of the second trimester, the mean value of airborne bacteria load was 1.94 x 10^5 ± 0.34 x 10^5 CFU / m^3 air, with strongly significant differences compared both with the first month ($p<0.001$) and with the second month ($p<0.001$).

Analyzing the obtained data, during the whole trimester, we can underline the fact that the total number of mesophilic aerobic bacteria from the poultry house reared on enriched cages recorded a mean value of 1.36 x 10^5 ± 5.41 x 10^5 CFU / m^3 air, with a significant difference compared to the previous trimester ($p<0.001$), slightly overcoming the recommended value of Krysztofik, cited by Woijcik (19), but still respecting the recommended value for animal shelters specified by Decun (5), which is 2.5 x 10^5 CFU / m^3 air.

In the third trimester, the aerobic mesophilic bacterial load presented significant variances from one month to another. Thus, in the first month of this trimester (March) the total number of mesophilic aerobic bacteria recorded a mean value of 1.83 x 10^5 ± 0.34 x 10^5 CFU / m^3 air. In the second month of the third trimester (April) the value recorded had an evident decrease, with a mean value of 1.09 x 10^5 ± 0.21 x 10^5 CFU / m^3 air, significantly lower compared to the previous month (March). In the last month of this trimester (May), the airborne total number of mesophilic aerobic bacteria had, again, a reduced value, with a mean value of 1.06 x 10^5 ± 0.21 x 10^5 CFU / m^3 air, significantly lower than the first month (March), but very close to the second month (April).

The mean value of total aerobic mesophilic bacterial load for the last trimester was 1.53 x 10^5 ± 0.96 x 10^5 CFU / m^3 air, a value slightly higher compared with the previous trimester. The value, for each month oscillated between 1.61 x 10^5 ± 0.29 x 10^5 CFU / m^3 air in the first month of this trimester (June), 1.57 x 10^5 ± 0.26 x 10^5 CFU / m^3 air for the second month (July) and 1.39 x 10^5 ± 0.18 x 10^5 CFU / m^3 air for the third month (August).

The dynamics of airborne mesophilic bacterial load from the air of the laying hen house, reared on enriched cages iss presented in figure 1.

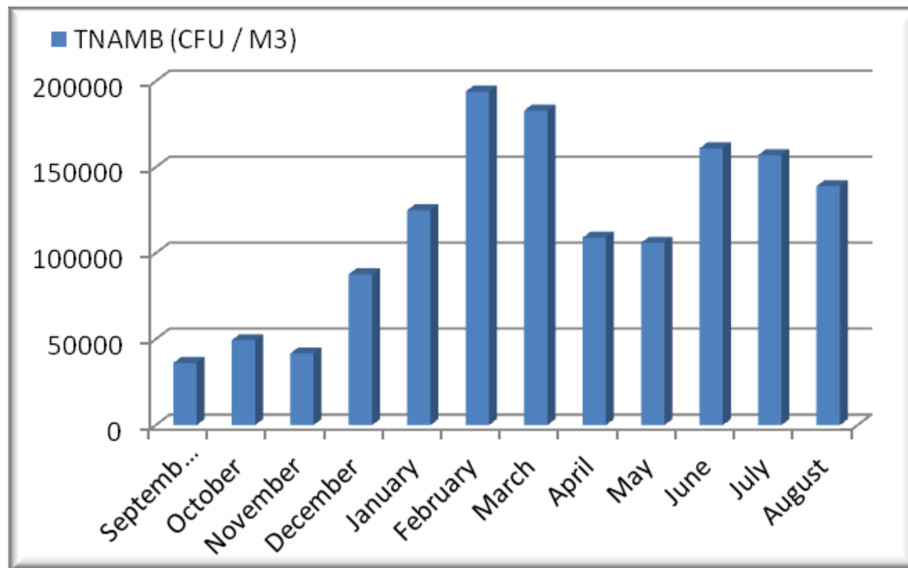

Fig. 1 Dynamic of total number of aerobic mesophilic bacteria (TNAMB), expressed in CFU / m^3 air, in the air of laying hen house reared on enriched cage system, during one year of exploitation

Analyzing the evolution of the air mesophilic bacterial load for the first three month of exploitation it observes that in the first month (September) this indicator kept relative uniform values. In the second month (October) the total aerobic mesophilic bacteria value had raise and presented an extremely high oscillation and in the last month of the first trimester (November), the number of airborne mesophilic bacteria had significantly decreased.

During the second trimester, the total aerobic mesophilic bacteria dynamic indicates a suddenly relative increase in the first month (December), a relative constant level during the second month (January), and again a significantly increase in the third month (February).

Is it to underline the fact that the total number of aerobic mesophilic bacteria ranged, during the first two months of this trimester around 10^5 CFU / m^3, which is a recommended value as a reference by some researchers (16). If take into considerations some other recommendations (2.5 x 10^5 CFU / m^3), it can be stated that, during the whole quater, the total number of aerobic mesophilic bacteria had corresponding values.

The dynamics of total number of aerobic mesophilic bacteria from the laying hens hall, during the last trimester, indicates the maintenance at an acceptable level, if is to be reported to Decun's recommendations (5), which is 2.5 x 10^5 CFU / m^3 air.

Thus, in the first month of this last trimester, the total number of aerobic mesophilic bacteria registered an insignificant decrease, but became obvious starting with the hall depopulation.

Conclusions

In the in cage system for laying hens the total number of aerobic mesophilic bacteria continuously rise from population till at the end of exploitation cycle.

The monthly mean value of TNAMB from the hall of laying hens presented major oscillation during the exploitation cycle, rising, from 3.61 x 10^4 CFU / m^3 of air in the first month, to 1.94 x 10^5 CFU / m^3 air in the sixth month, when it was recorded the maximum value, just to slightly decrease after, but still remaining at values of around 10^5.

Mainly, the total number of aerobic mesophilic bacteria ranged, for a long period of time, under the recommended values as references by some researchers.

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MOLECULAR DETECTION OF TICK BORNE ENCEPHALITIS VIRUS IN FIELD COLLECTED TICKS FROM ROMANIA

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Summary

Tick-borne encephalitis virus (TBEv) is an member of the family *Flaviviridae* and is transmitted by the bite of infected ticks or occasionally by ingestion of unpasteurized milk. In Romania the geoclimate and ecological conditions allow the maintenance and circulation of TBEv in natural foci, situation that caused the outbreak of 1999 in Sibiu county.Ticks are of considerable medical and veterinary importance and tick-borne encephalitis infections are emerging diseases in humans.

We are analyzed pools of the adult ticks (*Ixodes spp.*, collected by flaging technique from the vegetation and the *Hyalomma spp.*, collected from spur tortoise *(Testudo greaca)*. The sites were randomly selected and the ticks were preserved in alcohol or RNAlater® (Ambion®, Applied Biosystems) solution until identification.The RNA extraction was performed with Trizol methods and amplification reaction was done after quantification of genetic material with specific nanodrop system.The molecular detection of TBEv was achieved by reverse transcription PCR, using specific primers for 3'UnTranslated Region (UTR) of the RNA genome of the virus (included in the TicKitqPCR kit concept).

The positive results have demonstrated the potential implication of ixodide species (*Ixodes ricinus* and *Hyalomma aegypti*) in transmision of Tick Borne Encephalitis virus in Romania.

Key words: 3'UnTranslated Region (UTR),TBE virus, *Ixodes spp., Hyalomma spp.*

Tick borne encephalitis virus (TBEv) is an arbovirus, member of the *Flaviviridae* family, *Flavivirus* genus.There are three genetic lineages clearly distinguished: the European or Western tick-borne encephalitis virus, the Far Eastern tick-borne encephalitis virus and the Siberian tick-borne encephalitis virus subtype (5). TBEv is transmitted by the bite of infected ticks or occasionally by ingestion of unpasteurized milk. In Romania the geoclimate and ecological conditions allow the maintenance and circulation of TBEv in natural foci, situation that caused the outbreak of 1999 in Sibiu county (11). Hard ticks (Acari: Ixodidae)

comprises 25 species in Romania (1,6, 8), 13 of these belonging to the genus Ixodes. *Ixodes ricinus* and *Hyalomma aegypti*, is vectors for TBEv in Romania.Ticks are of considerable medical and veterinary importance because many of species are important vectors for different human and animal diseases (4).

TBE virus is about 50 nm total diameter and has an infectious genomic single-stranded RNA. The length of the genome is about 11 000 nucleotides (10 927 - 11141 depending on the strain). TBEv has three main structural proteins: immunogenic envelope glycoprotein E, membrane-associated protein M and capsid protein C. Protein C, together with genomic RNA, forms the nucleocapsid; the protein coat consists mainly of glycoprotein E, and in mature virus particles – of protein M. Gene sequences coding structural proteins (E, M and C) are located in the first fourth of the TBEv genome, the rest is occupied by nonstructural gene sequences (NS). Non-coding regions limit the TBEv genome at the 5'- and 3'-terminations. Genetic analysis of E glycoprotein coding gene sequences has been used to determine TBEv subtypes (2) (Fig. 1).

The 3'UTR region of the genome of TBE virus, contains the RNA secondary structure elements responsible for cyclization genome. 3'UTR region shows significant heterogenity at the nucleotide sequence level and contains various structures and conserved structures responsible for maintaining the viability of the virus (7, 9) (Fig. 2).

Te most rapid and sensitive assay for TBEv has been based on reverse-transcriptase-polymerase chain reaction for amplification the 3'UTR of the RNA genome of the virus. The method is widespread in research and surveillance laboratories and was designed by Schwaiger & Cassinotti (10).

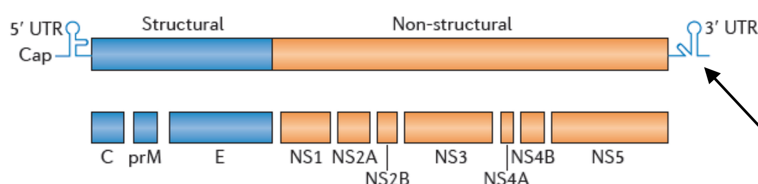

Fig. 1. Flavivirus genome (www.mdpi.com)

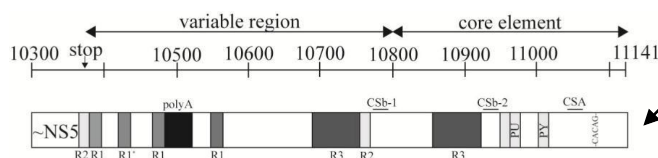

Fig. 2. 3'UnTranslated Region (UTR) of the RNA genome of the TBEv

Materials and methods

The ixodid ticks were collected from Sibiu County (Brateiu, Cristian, Sadu, Sibiu Dumbrava), between June 24, 2006 and October 29, 2008 and from Tulcea County (Tutuiatul, Suluc, Parc Macin) between June 3 and 1st July 2006. A total number of 72 adult *Ixodes ricinus* ticks were collected by flaging technique from the vegetation (Sibiu County) and 117 adult *Hyalomma aegypti* ticks were collected from spur tortoise (*Testudo greaca)* (Tulcea County). Until identification under stereomicroscop were preserved in 95% alcohol or RNAlater® (Ambion®, Applied Biosystems) solution. For established the presence of TBEv we are tested 11 pools of ixodes ticks. RNA extraction was performed with TRIzol® method (Ambion®) and quantification of genetic material with specific nanodrop system (NanoDrop One^{C}, Thermo Scientific). For the identification of TBEv, samples were processed using the TBEv real-time RT-PCR method (adapted by us after the Schwaiger and Cassinotti, 2003) in the presence of the positive control represented by reference TBE Hypr strain. The molecular detection of TBEv was achieved, using specific primers for 3'UTR of the RNA genome of the virus.

Results and discussions

Extracted RNA (Fig. 2) from the reference TBE Hypr strain and total RNA extracted from Ixodes ticks, was used for cDNA syntesis with SuperScript III One-Step RT-PCR System with platinium Taq DNA Polymerase (Invitrogen) in the presence of gene-specific primers for 3'UTR.

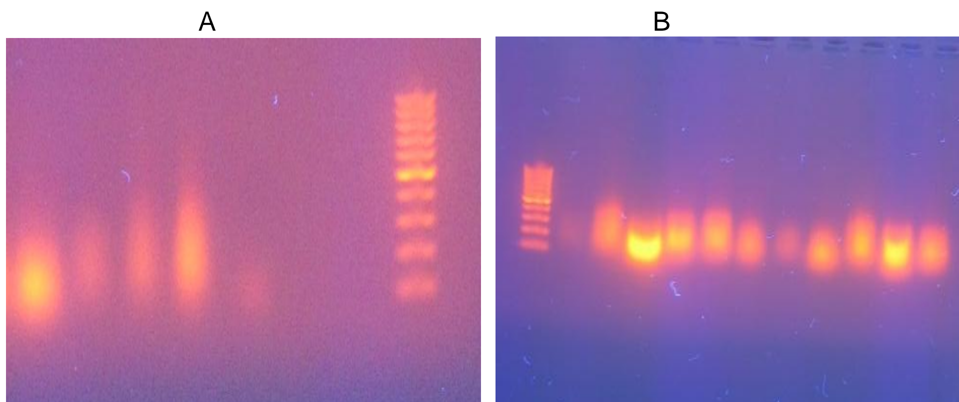

Fig. 2. Total RNA isolation from ticks using TRIzol® method (Ambion®) (M=1kb ladder) (1,5% agarose without formaldehide)
A: *Ixodes ricinus*; B: *Hyalomma aegypti*

After extraction the RNA was quantificated by spectrophotometric analysis and absorbance values at A260/ A230 were in the range of 1.8 – 2.0, which demonstrates that is pure enough to be used in molecular amplification reactions. For detection of TBEv-specific RNA, a quantitative real-time RT-PCR protocol, according to Schwaiger and Cassinotti method were used. TBEv-RNA was amplified in an optimized 25 µl reaction mixture with 2X Greenstar Master mix and specific primers (F-TBE 1, R-TBE 1, 100uM). Real-time RT-PCR was carried out in a Cepheid Smart Cycler Real Time system.

Specific RNAs from TBEv were detected (3' UTR-genomic region) in 2 *Ixodes ricinus* pools, comes from Sibiu County (Cristian, Sadu) and 8 *Hyalomma aegypti* pools comes from Tulcea County (Tutuiatul, Suluc, Parc Macin). No specific TBEv RNAs were detected in one pool of *Ixodes ricinus* comes from Sibiu County (Bratei) (Fig. 3).

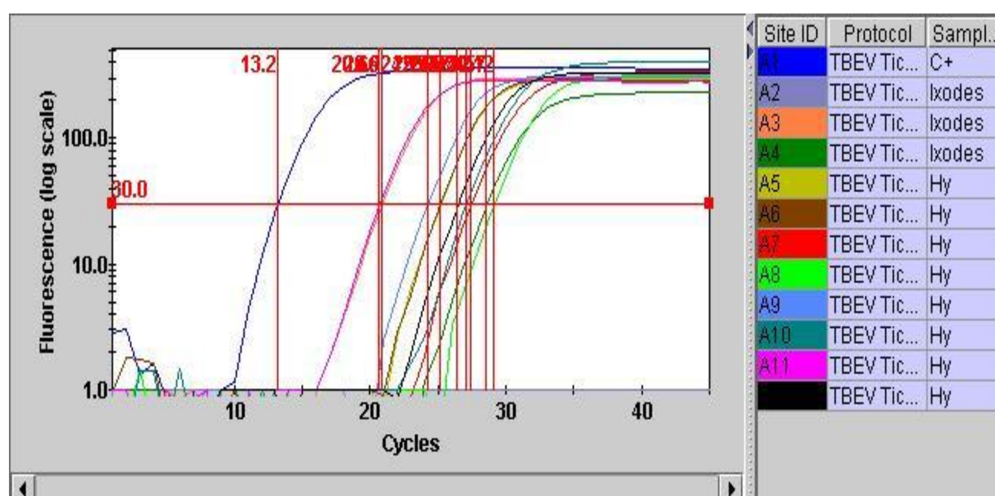

Fig. 3. Positive samples (FAM channel) for *TBEv,* from *Ixodes ricinus and Hyalomma aegypti*; C+ (TBEv Hypr strain); (3' UTR region)

The Ct values for the positive control was 13.2 and for analyzed samples were < 29, which means strong positive reactions and presence of nucleic acid target in the sample (Table 1).

Table 1

Real Time PCR 3' UTR region of the TBEv - Ct value

Sample	C+	Ix.1	Ix.2	Ix.3	Hy.4	Hy.5	Hy 6	Hy.7	Hy.8	Hy.9	Hy.10	Hy.11
3' UTR Ct values	13.2	0.00	20.9	28.57	25.14	25.1	27.42	29.12	24.19	27.1	20.66	26.44

C+ (TBEv Hypr strain); Ix.1, Ix.2, Ix.3 (RNA *Ixodes ricinus* pools);
Hy.4, Hy.5, Hy 6, Hy.7, Hy.8, Hy.9, Hy.10, Hy.11(RNA *Hyalomma aegypti pools*)

Conclusions

The positive results have demonstrated the potential implication of both ixodide species (*Ixodes ricinus and Hyalomma aegypti*) in transmision of Tick Borne Encephalitis virus in Romania.

This study also draws up a model of analysis and identification of TBEv by Real Time PCR in *Ixodes ricinus and Hyalomma aegypti* vectors collected from Romania TBE risk area, using the in house of the Real Time TickitqPCR concept.

Acknowledgments

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DETERMINATION OF SERUM IMMUNOGLOBULINS IN NONSPECIFIC STIMULATED DAIRY COWS

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Summary

Determination of serum immunoglobulins (IgA, IgG, IgM) concentration allows the assessment of the health status of lactating cows, including in experimental conditions. In the present experiment we used a group of seven lactating cows, clinically healthy, in the first 3-4 months of lactation with high milk production, to determine the level of IgA, IgG and IgM in their serum before and after inoculation with an immunomodulatory phytoextract (patent pending).

According to the experimental protocol, cows' blood was collected before s.c. administration of the phytoextract, then at 24 hours, 4 days, 7 days and 14 days from the time of inoculation. Blood samples were processed for hematological and serological analysis. Hematological examination showed a significant hyper-leukocytosis even at 24 hours after phytoextract inoculation which lasted up to 14 days; increased neutrophils level at 24 hours and significant decrease in lymphocytes that rise gradually to normal level after 14 days.

For the determination of circulating immunoglobulins, serological tests using ELISA kits were carried out. Comparing to the mean values (expressed in mg / ml) registered prior to the experiment, at 24 hours after phytoextract inoculation, a marked decrease was recorded for all parameters, after which each type of immunoglobulin turned to a different direction. The main values of IgA, with special role in mucosal protection and IgM, the main immunoglobulinic opsonin in serum were slightly varying during the 14 days experimental period (plateau level); IgG showed a significant gradual decline even at 14 days after experiment (probably because the body's defense response was taken by phagocytes).

Key words: hyperleukocytosis, immunomodulation, neutrophilia, immunoglobulins

Immunoglobulins (Ig) represent a group of related proteins with antibody functions (Ac), which exist as membrane receptors on lymphocytes or B-effector free molecules. At electrophoresis, most of Ig migrate in the γglobuline zone and less in β globuline zone. Their synthesis is carried out by B lymphocytes reached the final maturing phase (plasma cells).

Some molecules are cytophilic fixed on the Fc receptor on the membrane of macrophages, B lymphocytes, granulocytes, neutrophils, mast cells, basophils,

etc. Ig molecules fix complement (C), activate phagocytosis mechanisms and can cross various barriers of the body (8, 9).

Structurally, the Ig monomer molecule contains two heavy chains H (Heavy) and two light (Light), connected by disulfide and non-covalent bonds.

Ig molecule are heterogeneous in terms of their specificity:

-isotypic- present in all individuals of a species and expressed in classes and subclasses of Ig

-alotypic- define their particular antigenic character ,appeared to a group of individuals within the same species, as a result of minor changes in amino acid sequence from the chain constant region H or L (Gm, I, INV, ISF, etc).

-idiotypic- expresses an enormous variety of specificities by an individual.

Class A Immunoglobulins (IgA) represents 15-20% of total Ig in the blood stream. They are present in blood serum (in monomeric, serum) and mucosal secretions (secretory, the dimer and trimer). Secretory component is a glycoprotein secreted by the epithelial cells in the epithelium who transports IgA and provides resistance to proteolytic enzymes (2). Secretory IgA (SIGA) provides local immunity, blocks the adhesion of the microorganisms to the mucosal epithelium, neutralizes some toxins, enhances phagocytosis, agglutinates microbes (bacteria, fungus hindering adsorption and reproduction ofvirions into epithelial cells).

G class immunoglobulins (IgG) appear in large quantities in the secondary immune response and constitute 70-85% of the total immunoglobulins (7). It is the only Ac who can cross the placenta and guards the fetus from infection and protects the newborn (questionable in cattle) (4).

Immunoglobulin class M (IgM) is about 5-10% of all circulating immunoglobulins (serum macroglobulin). IgM activate complement via the classical pathway, is 100 times more effective than the IgG cytolysis of the cell. IgM is the first class of Ig produced by plasma cells and the main class that appears in the primary immune response. In the monomeric form in serum IgM are in very low concentrations but can be secreted in the mucus, which ensures their protection (5).

In literature, the amount of immunoglobulin in the blood serum of cattle varies significantly depending on the season and reaches the highest concentration in milk in spring and summer. Kociņa et al., (2012) (6) states that the average parameters of immunoglobulin G, A and M in the blood serum of investigated cows differ depending on the non-specific udder infection ($p > 0.05$).

There are studies where concentrations of IgA, IgG, IgM from blood samples increases considerably with the incidence of mammary gland infections (3, 5). In clinical mastitis, immunoglobulin from cow's milk obviously increase (1,5).

Materials and methods

The experiment was carried out at the farm of the Institute of Research and Development for cattle breeding Balotesti on 7 lactating cows, clinically healthy, in

their first 3-4 months of lactation with high milk production, to determine the IgA, IgG and IgM in their serum before and after they were inoculated with a phytoextract with immunomodulatory properties (patent pending). The cows' age was from two to eight years, and the average was 4. Lactating cows were bred in stabulation system, grouped and fed evenly with rations appropriate to their physiological state. The experimental protocol consisted in the creation of a batch of seven cows from which blood was taken for hematology and biochemical analysis prior to s.c administration of the phytoextract and after 24 hours, 4 days, 7 days and 14 days from the start of the experiment.

The concentrations of IgG, IgA and IgM in serum were determined by ELISA in the laboratory of Biochemistry of the Faculty of Biology of the University of Bucharest and interpreted by the laboratory of Immunology of the Faculty of Veterinary Medicine of the University of Agricultural Sciences and Veterinary Medicine from Bucharest.

Results and discussions

The purpose of this study was to determine the immune status of cows during lactation, characterized by concentrations of IgA, IgG and IgM of the peripheral blood samples, and to assess whether changes in levels of immunoglobulins can be associated to the state of productivity and the specific history of lactating cows after administration of the herbal extract with immunomodulatory activity.

For this study were selected seven clinically healthy lactating cows, which were in the first 3-4 months of lactation with high milk production.

We considered the baseline levels of circulating immunoglobulins as benchmarks, thus we compared the average values of the samples with them, and also with references from the literature for the 3 classes of immunoglobulins considered (table 1).

Table 1

Serum immunoglobulin physiological values in cows compared with reference values from literature

	Ig	Average mg/ml		Reference Values g L-1		
Baselines	Ig A	2.40	10 %	3.30	7 %	by Liu, G. L. et al., 2009 (7)
	Ig G	23.6	89 %	42	92 %	by Zagorska, J., et al., 2007 (10)
	Ig M	0.25	1 %	0.30	1 %	by Zagorska, J. et al., 2007 (10)

In Figure 1 is presented the % concentration of the three immunoglobulin classes.

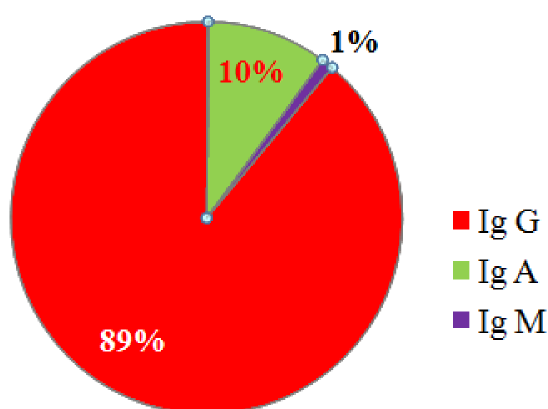

Fig. 1. Graphical representation of immunoglobulins determined on average sample

Compared to the serum level reported by some authors as reference points, the results have shown a significant decrease of the three classes of immunoglobulins. A very significant decrease was noticed in IgG and IgA. In the experimental protocol, the history showed that one blood sample came from a cow with a history of subclinical mastitis diagnosed and treated 2 weeks ago.

After administration of the extract, blood samples were taken at 24 hours, 4 days, 7 days and 14 days. The result of the determination of these three classes of immunologlobulines is shown in table 2.

The interpretation of the results at intervals set by the Protocol revealed a suggestive graphic showing that at 24 hours after administration of our extract the values of immunoglobulins decreased in all 3 classes (Figure 2), and the downward trend was found in IgG and IgA and little in IgM class. At the IgG class, after 7 days of the experiment, there was a slight increase in the level of its reactivity explained by general starts. In parallel it was made a blood test (hematology), which revealed hyperleukocytosis at 24 hours after inoculation of the extract and also a decrease of neutrophils. A significant decrease in lymphocytes started to rise gradually to return to 14 days, which would explain the variation in the level of Ig M (a sharp decrease in 24 hours and a gradual recovery) and running parallel to the circulating lymphocytes in the case of touch by cell-receptor class B.

Table 2

The results of changes in the level of circulating immunoglobulins in the established protocol

Sample		1	2	3	4	5	6	7	Media	
U/M	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	%
Initial	Ig A	2.70	2.20	2.20	2.40	2.10	2.30	2.70	**2.40**	10
	Ig G	29	22	21	24	23	25	21	**23.6**	89
	Ig M	0.20	0.23	0.22	0.28	0.28	0.27	0.29	**0.25**	1
24 h	Ig A	2.50	2.80	1.90	1.60	1.80	2.10	2.30	**2.14**	9.6
	Ig G	20	24	19	20	21	19	18	**20.1**	89.6
	Ig M	0.18	0.16	0.13	0.21	0.23	0.21	0.20	**0.19**	0.8
4 days	Ig A	2.50	1.90	1.10	1.40	1.30	2.60	2.30	**1.87**	9.5
	Ig G	22	20	19	21	20	18	22	**17.5**	89.3
	Ig M	0.18	0.25	0.20	0.19	0.23	0.27	0.29	**0.23**	1.2
7 days	Ig A	2.10	1.20	1.70	1.80	1.50	2.30	2.10	**1.81**	12.3
	Ig G	16	10	14	15	14	10	10	**12.7**	86.2
	Ig M	0.21	0.24	0.23	0.22	0.21	0.25	0.20	**0.22**	1.5
14 days	Ig A	2.00	1.60	2.30	2.10	2.00	2.10	1.90	**2.00**	11.8
	Ig G	10	15	11	13	14	15	14	**14.7**	86.9
	Ig M	0.25	0.21	2.22	0.21	0. 23	0.21	0.22	**0.22**	1.3

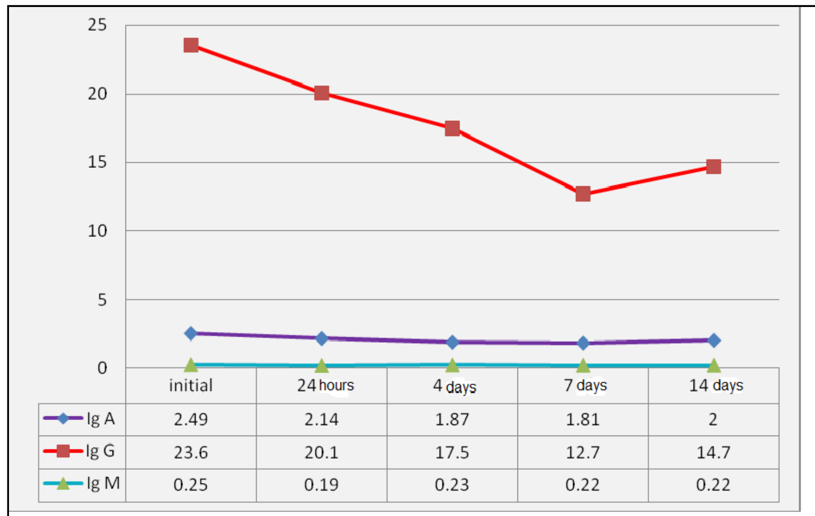

Fig. 2. The results of changes in the level of circulating immunoglobulins in the established intervals

Conclusions

Determination of IgA, IgG and IgM was performed on a group of seven clinically healthy cows in the first 3-4 months of lactation. The protocol used an injection with a plant extract with immunomodulating effect. Benchmark against other scientific works, the values of the 3 classes of immunoglobulins was found significantly decreased.

Determination of circulating immunoglobulin classes at 24 hours, 4, 7 and 14 days showed that at 24 hours following administration of extract, immunoglobulins values were significantly decreased in all 3 classes. After 24 hours the downward trend was seen in IgG and IgA and little in IgM class.

Further research is required to establish the variation of immunoglobulin classes after nonspecific stimulation in others cattle types.

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METABOLIC STATUS OF PEKIN AND DOMESTIC DUCKS GROWN IN MICRO FARMS

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Summary

Web-footed poultry are not the solution for the “worlds hunger”, they are an important niche în meat production chain, being the only poultry that use food mostly ignored, such as: the grass growing aside the roads, moats, swamps. From ducks people use: meat, eggs, pouf, grease, eventually inferior members (flippers-for gelatin aș well aș carpenters clay); ducks are household poultry, resistant to disease, temperature variations etc. The biological material for this research is represented by the ducks from two micro farms (Tăticu- Hărman Farm which grow domestic ducks, Brașov County; Luisa Savu Frumușani Micro farm, Călărași County which grow pekin ducks). În our country, în 2016, it is not known the existence of any intensive growing farm for web-footed. The evaluation of the heard ducks health was achieved by assessment of the metabolic status based on biochemical and hematological determinations.
Key words: domestic ducks, pekin ducks, metabolic status

Unlike chickens, ducks grow very quickly. In four months, the ducks reach 2.5 kilograms weight, thus they can be slaughtered; generally antibiotics or growth hormones are not used for their growth (1). Ducks are bred with minimal investment, they need shelter in winter, a pool or a pond (optional); access to water etc. (2, 4).

Materials and methods

The ducks were reared in two microfarms (Ferma lu' Tăticu - Harman, Braşov County; Frumuşani, Calarasi County). To assess the state of health of the herd, we harvested five blood samples from Pekin ducks and 5 blood samples from Muscovy ducks; metabolic status was assessed by biochemical and hematological determinations.

Results and discussions

The advantages of growing ducks in semi-intensive and extensively systems are presented below:

Table 1

The advantages of growing ducks in semi-intensive and extensively systems

The advantages of growing Pekin ducks	The advantages of growing Muscovy ducks
1. Rapid growth – in 56 days they reach 4 kg (even 4.5 kg in males) 2. The meat is tasty 3. They are resistant to diseases 4. Annually they produce up to 150 eggs per female, weighing 80-90 g 5. Quiet temperament	1. They eat less, the maintenance costs are lower 2. They grow easy even without access to a continuous source of water 3. The mute ducks are not noisy and do not need special attention and care 4. They have a good tolerance of frost 5. Very resistant to diseases 6. The meat is highly appreciated by consumers

1. Case study no. 1 – Biochemical status of Pekin and Muscovy ducks is presented in tables 2 and 3

Table 2

Biochemical status of Pekin ducks, aged over 1 year

No.	Protein g/dL	Albumin g/dL	Globulins g/dL	Cholest. mg/dL	ASAT IU/L	ALAT IU/L	P mg/dL	Ca mg/dL	Mg mg/dL
Ref.	**3.63 +/- 0.4**	**1.19 +/- 0.1**	**1.44 +/- 0.3**	**-**	**-**	**-**	**14.3 +/-0.8**	**11.6 +/-0.5**	**7.0 +/-0.3**
1	5.57	1.83	3.74	125	25	33	12.00	13.00	4.30
2	5.14	1.23	3.91	153	27	27	13.20	12.50	4.56
3	5.04	1.11	3.93	128	19	45	13.00	12.00	5.34
4	4.78	2.02	2.76	136	20	38	12.80	11.57	4.32
5	5.10	1.73	3.37	150	29	30	13.00	12.45	4.23
B	**5.12**	**1.58**	**3.54**	**138**	**24**	**34.6**	**12.60**	**12.30**	**4.55**

The values presented above show hypoproteinemia, hypoalbuminemia, hypercholesterolemia (compared to reference values in hen); hyper-gammaglobulinemia; hypophosphoremia, hypercalcemia, hypomagnesemia.

Pekin ducks, came out of winter "emaciated", they rich plumage mislead us, but after slaughtering the ducks had only 1.400- 1.700 grams - very low value for ducks older than 1 year (5).

Table 3

Biochemical status of Muscovy ducks aged over 1 year

No.	Protein g/dL	Albumin g/dL	Globulins g/dL	Cholest. mg/dL	ASAT IU/L	ALAT IU/L	P mg/dL	Ca mg/dL	Mg mg/dL
1	3.66	0.92	2.74	149	16	19	2.70	9.60	4.30
2	3.04	0.98	2.16	156	20	24	2.90	10.11	5.40
3	2.96	1.02	1.94	160	23	15	1.78	9.56	4.23
4	3.12	1.16	1.86	140	32	45	2.30	8.90	5.13
5	2.78	0.80	1.98	186	35	56	1.90	10.0	4.60
B	**3.11**	**0.98**	**2.13**	**158**	**25**	**29**	**2.31**	**9.63**	**4.73**

The values presented above show hypoproteinemia, hypoalbuminemia, hypergammaglobulinemia; increased hypophosphoremia, hypocalcemia, hypomagnesemia.

Although from biochemically point of view, the mineral elements value is low, there are no clinical signs of phosphorus-calcium demineralization that would have occurred in such conditions in broilers and laying hens. We conclude that in correlation with ducks age (12- 14 months) occurred an adaptation of the body to the minimum necessary minerals to ensure physiological functionality (3).

The average results of biochemical analysis are showed comparatively for the two breeds of ducks in table 4.

Table 4

Biochemical status – comparison between Pekin and Muscovy ducks

No.	Protein g/dL	Albumin g/dL	Globulins g/dL	Cholest. mg/dL	Glucose mg/dL	ASAT IU/ L	ALAT UI/L	P mg/dL	Ca mg/dL	Mg mg/dL
1	3.11	0.98	2.13	158	198	25	29	2.31	9.63	4.73
2	5.12	1.58	3.54	138	108	24	34.6	2.60	12.30	4.55

Note: 1- Muscovy ducks; 2 – Pekin ducks

The results presented in Table 4 lead to the following conclusions:

-Total protein and protein fractions are higher in Pekin ducks;

-Cholesterol and glucose are lower in Pekin ducks;

-The enzymatic activity is similar in the two breeds, with a small enzyme disorder (alanine aminotransferase (ALAT) in Pekin ducks

-Hypophosphoremia in both breeds;

-Decreased serum calcium in Muscovy ducks and normal in Pekin ducks.

Muscovy ducks cholesterol in relation to the cholesterol level in the adult hen (125 +/- 30) is close to normal of an adult hen; in fact, the fat is deposited subcutaneously in ducks and occur on organs, especially liver, only in fattening ducks.

2. Case study no. 2 – Haematological status of Pekin and Muscovy ducks is presented în tables 5 and 6

Table 5

Variation of erythrocyte series in Muscovy ducks aged 1 year

No.	Erythrocytes (mil/mm3)	Hb (g /dL)	Ht (%)	MCV µ3	MCH (pg Hb/ E)	MCHC (gHb/ dL E)
1	4.28	13.8	65	151.86	32.24	21.23
2	3.13	11.2	45	143.70	35.78	24.88
3	4.03	13.7	55	136.40	33.90	24.90
4	2.78	12.8	35	125.80	46.00	36.57
5	3.18	11.3	31	97.40	35.50	36.45
B	**3.48**	**12.56**	**46.2**	**131.03**	**36.68**	**28.80**

The values presented above show normal levels for erythrocytes and, hemoglobin, increased hematocrit and MCV, decreased MCHC. The increased hematocrit is clinically correlated with dehydration and low water consumption.

Increased MCV denotes macrocytosis, but normally this is accompanied by hyperchromia (increased MCHC) which is not this case. To elucidate this situation we calculated the globular value (Vg) or color index showing the relative load of erythrocytes with Hb compared to the normal reference for this species. It is calculated using Gowers formula, being equal to the ratio of hemoglobin concentration in grams and the number of erythrocytes per mm3 of the same blood (N) multiplied by the ratio of reference normal values of erythrocytes of species (n) and normal quantity of Hb (hb).

Interpretation is :

-normal globular value = 1 (0.85 to 1.15)

-globular value under 0.85 = hypochromia

-globular value over 1.15 = hyperchromia

Our results show that globular value (compared to averages obtained) is 1.03 = normal.

Table 6

Variation of erythrocyte series in Pekin ducks aged 1 year

No.	Erythrocytes (mil/mm3)	Hb (g /dL)	Ht (%)	MCV µ3	MCH (pg Hb/E)	MCHC (gHb/ dL E)
1	2.91	11.00	39	134.02	34.36	28.20
2	2.78	10.50	41	147.48	37.76	25.60
3	2.90	11.23	43	148.27	38.72	26.11
4	2.34	9.76	39	166.6	41.70	25.02
5	2.55	12.03	34	133.3	47.17	35.38
B	**2.69**	**10.90**	**39.2**	**145.93**	**39.94**	**28.06**

The values presented above show erytropenia, decreased hemoglobin, hematocrit, and MCHC, increased MCV.

Since erythremia and hemoglobin level are low, we can conclude that there is anemia; to specify the type of anemia, the blood constants indicate a macrocytic anemia (increased MCH denotes macrocytosis and decreased MCHC denotes hypochromia), which is not this case. To elucidate such confusing situations we calculate also globular value here:

Vg = 1.16; values above 1.15 indicate hyperchromia; we conclude that is a case of hyperchromic macrocytic anemia.

The average results of hematological analysis are showed comparatively for the two breeds of ducks in Table 7.

Table 7

Hematological status – comparison between Pekin and Muscovy ducks

No.	Er (mil/mm3)	Hb (g Hb/dL)	Ht (%)	MCV µ3	MCH (pg Hb/E)	MCHC (gHb/ dL E)
1	3.48	12.56	46.2	131.03	36.68	28.80
2	2.69	10.90	39.2	145.93	39.94	28.06
Note: 1- Muscovy ducks; 2 – Pekin ducks						

The results presented in Table 7 lead to the following conclusions:

-number of erythrocytes in Peking ducks is low compared to Muscovy ducks erythremia and also to reference values;

-Hemoglobin and hematocrit are lower in Pekin ducks

-MCV is increased in Pekin ducks and in both breeds the value is increased compared to the reference values,

-MCHC is decreased in both breeds compared to the reference value (32 gHb / dL E).

Conclusions

In Muscovy ducks there is a deprivation of mineral macroelements and consecutively of microelements.

Low levels of cholesterol, blood glucose and increased protein level is considered a "metabolic advantage" that determines the high quality of Pekin ducks meat and certifies the increasing consumer demands for this breed.

The enzymatic activity is not impaired in both races, which shows that only in ducks undergo fattening hepatic steatosis occur, accompanied by disruption of enzyme activity.

Gowers formula is a biomathematical calculation method that helps accurate determination of morphological and chromic type of anemia.

From hematological point of view, Peking ducks are "hematologic deficient" as the main erythrocyte constants are low compared to references for this species and also to Muscovy ducks.

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IDENTIFICATION OF ORAL MICROFLORA IN DOGS WITH DIAGNOSED DENTAL DISORDERS

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Summary

Out of over 300 species of bacteria present in the oral cavity in companion carnivores, some are present only at certain times (transient, nomadic), but most of them are permanent residents of the dental plaque and may induce severe periodontal diseases.

Gingival, parodontal and dental plaque samples were examined by classical microbiological techniques and identified by API chromogenic test.

The results indicated that *Staphylococcus spp.* was found in all patients, but was present only in gingival isolates (32.25%). Microorganisms of the genus *Streptococcus* accounted for 8% of the bacteria isolated from the dental biofilm.

The genus *Porphyromonas* held the highest overall prevalence (18.18%), followed by *Corynebacterium spp*. (17%), *Eikenella (Bacteroides) corrodens* (12.12%), *Actinomyces canorus* (7.7%) and *Prevotella intermedia* (7.57%) of the entire bacterial population. *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* were found in 7% and 6.06% of the total number of oral microorganisms, respectively.

According to this study, the plaque in companion dogs was not a homobacterian aggregate, but a biofilm composed of several bacterial species. The larger the number of dental biofilm bacterial species, the more serious should be the clinical development of the dental disease.

Key words: dental plaque, microflora, *Porphyromonas spp.*, *Corynebacterium spp.*

For veterinary practitioners, diseases of the oral cavity in dogs are both a medical challenge and a possibly consistent financial source, untapped by most veterinary offices. Research conducted in the USA shows that 83% of urban dogs are suffering from one or more oral diseases (7). Out of the oral morbid states in dogs, the maximum frequency and highest importance belong to periodontal diseases (5, 4, 9, 7).

Materials and methods

In order to investigate the involvement and the importance of the cultivable microorganisms in shaping the clinical picture of gum and/or periodontal disease, complex microbiological examinations were carried out in a homogenous group of dogs (n=6) previously diagnosed with dental disorders.

Microbiological sampling from the six animals included in the study was performed by the following protocol (Table 1):

Table 1

Microbiological sampling protocol for the dogs in study

Case no.	Gingival sample (G)	Periodontal pockets sample (PP)	Dental plaque sample (P)
1	+	+	+
2	+	-	+
3	+	+	+
4	+	-	+
5	+	+	+
6	+	+	+

Sampling from the base of the periodontal pockets was completed using periodontal probes or subgingival sterile scoops. Cases 2 and 4 were not sampled from the periodontal pockets, because these had a depth that could be considered normal (1 mm). Microbiological samples from the gums corresponding to the sick tooth were taken using sterile swabs and placed into Amies transport medium. From all the 6 cases microbiological samples were taken from the dental plaque of the affected teeth. Immediately after sampling, the harvesting tools (probes, scoops) were immersed and washed in 1 ml phosphate-buffered saline (PBS) distributed in 1 ml sterile Eppendorf tubes. The samples were maintained at -20° C until processing.

From each sample, two Columbia agar plates with the addition of 5% defibrinated sheep blood (DifcoTM) and a plate of Columbia anaerobic (DifcoTM) were seeded. Subsequently, one plate was incubated under normal conditions of aeration at 37°0 C for 48h, when a first check was carried out, after which incubation was extended for seven days with daily checks. The second seeded Columbia agar plate with the addition of 5% defibrinated sheep blood (DifcoTM) was introduced in Anaerocult C mini bags (Merck KGaA, Darmstadt, Germany) to create optimal conditions for capnofile or microaerophilic microorganisms (8-10% carbon dioxide, 5-6% oxygen). The samples seeded on anaerobic Columbia agar (Difco) were incubated at 35°0C for 48 hours under anaerobic conditions as previously described, and, in case of negative results were further incubated up to 7 days. Bacterioscopy was performed after Gram staining of isolated colonies. Gram negative bacteria were further investigated on other chromogenic culture media (Drigalski and MacConkey agar, bioMerieux, Inc., Lyon, France) and API 20E and API 20NE biochemical stripes.

Gram positive bacteria were tested by API Staph, API Strepto or API Coryne, choice based on the bacterioscopy results. Colonies which developed on Columbia anaerobic agar (DifcoTM) were tested using API 20A (bioMérieux Inc., Lyon, France). The specific API software was used for classification of the results.

Results and discussions

Following the complex microbiological examination, 66 (n = 66) bacterial strains belonging to 14 different genera were isolated and identified (Table 2).

Table 2

The most prevalent bacteria found in combined sampling sites

Bacterial species	Prevalence
Actinomyces canis	8%
Porphyromonas canoris	8%
Prevotella intermedia	8%
Corynebacterium spp.	17%
Porphyromonas gingivalis	8%
Eikenella corrodens	12%

The results indicated that *Porphyromonas* spp. isolated from the dental plaque and from the periodontal pockets, accounted for 18.18% of all isolates. Within the genus *Porphyromonas*, *P. gingivalis and P. canoris* were isolated in equal proportions (41.66%), while *P. canigingivalis* represented 16.66%. These results sustain, on the one hand, the involvement of this microbial genus in the shaping of the dental bacterial biofilm and, on the other hand, its participation in causing inflammation of the periodontal structures. Interesting to note that *P. gingivalis* was only isolated from the contents of two periodontal pockets, in two patients, while this species is constantly isolated from human patients with chronic marginal periodontitis (8, 11, 12, 1, 10).

Eikenella (Bacteroides) corrodens represented 12.12% of all strains and was isolated from all cases of periodontal inflammation. The bacterium was absent, though, in cases of gingivitis. The absence of the genus *Eikenella* from the gingival samples is due to its strict adherence to the gingival sulcus (6).

Prevotella intermedius was present in the dental biofilm in 50% of cases, accounting for 7.57% of all isolates. In light of these results, *Prevotella* genus may be granted an important role in periodontal pathology of pets, similar to the role it holds in human pathology.

Fusobacterium nucleatum, isolated in half of the cases of periodontitis, but absent in cases of gingivitis, represented 6.06% of all oral isolates. Cases in which *Fusobacteria* were identified had the most severe clinical course.

Actinomyces canis proved, according to the results of the study, an important agent in the dental pathology of companion dogs, as it was identified in 33.3% of the samples from the dental biofilm. Similarly, *A. canis* was present in 75% of the periodontal pockets samples, but was absent in gingival samples and represented 7.57% of the total oral isolates.

As opposed to literature data, *Actinobacillus actinomycetemcomitans* represented only 5% of the bacteria isolated from the periodontal pockets and only 7% of all dental bacteria (Table 3).

Table 3

The distribution percentage of bacterial species isolated from the periodontal pockets

Bacterial species	**Prevalence**
Actinomyces canis	14%
A actinomycetemcomitans	5%
Porphyromonas canoris	10%
Prevotella intermedia	10%
Corynebacterium spp.	18%
Fusobacterium nucleatum	10%
Porphyromonas gingivalis	10%
Porphyromonas canigingivalis	5%
Eikenella corrodens	18%

Corroborating the clinical examination with the microbiological results, the authors have observed that the cases in which *A. actinomicetemcomitans* was identified, the degree of inflammation and gingival retraction was very severe.

Staphylococcus spp. was identified in all cases, but only at gingival level, representing a significant proportion (35.25%) of the gum isolates (Table 4). *S. intermedius* and *S. epidermidis* were isolated in equal proportion. They accounted jointly for 66.6% of all identified staphylococci. Their presence on the gums may be connected to the grooming behavior.

Table 4

Bacterial species isolated at gingival level

Bacterial species	**Prevalence**
Pasteurella canis	6%
Escherichia coli	11%
Staplycoccus spp.	35%
Streptococcus spp.	18%
Corynebacterium spp.	12%
Lactobacillus salivosus salivosus	6%
Micrococcus luteus	6%
Pseudomonas spp	6%

Microorganisms of the genus *Streptococcus* were identified only twice in the dental biofilm, accounting for only 8% of all bacteria isolated from this level (Table 5).

Table 5

Distribution of the bacterial species isolated from the dental biofilm

Bacterial species	**Prevalence**
Actinomyces canis	7%
A. actinomycetemcomitans	7%
Porphyromonas spp.	26%
Prevotella intermedia	11%
Streptococcus spp.	8%
Corynebacterium spp.	20%
Fusobacterium nucleatum	7%
Eikenella corrodens	14%

No streptococci were identified in the periodontal pocket, but they were present quite frequently (66.66%, four of the six cases) at the gingival level. The results of this study on streptococci confirm the findings of some authors (2), stating that canine streptococci do not play a very important role in periodontology. As opposed to this, streptococci are considered "base bricks" of human dental plaque, where they intervene mainly as "pioneers" of colonization. It seems that their role in canine dentistry was assumed by corynebacteria (2, 3,13).

Corynebacterium spp. was identified in all 6 cases, both in the dental biofilm and in the periodontal pocket, accounting for 17% of all isolates. Similar to *A. actinomycetemcomitans*, *Corynebacterium spp.* is able to coaggregate with the majority of the dental biofilm bacteria (3). As for the genera *Micrococcus*, *Escherichia*, *Lactobacillus*, *Pasteurella*, the authors consider that the strains isolated from the gingival level do not hold an important pathogenic role and are part of the normal oral flora in dogs.

Conclusions

The bacterial plaque investigated in this study showed to be rather a biofilm composed of several coaggregated bacterial species than a homobacterian aggregate. The severeness of the clinical course was highly dependant on the number of isolated bacterial species. Different oral sampling sites showed similarity concerning not only the frequency but also the number of isolated species. These findings highlight the importance of the dental biofilm in the pathogenesis of periodontal inflammation.

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THE IMMUNOLOGICAL VALUE OF DIFFERENT INACTIVATION METHODS IN OBTAINING DESENSITIZING PRODUCTS FROM STAPHYLOCOCCAL STRAINS

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Summary

Clinical observations carried out on some canine patients suffering from atopic dermatitis with concurrent staphylococcal infection led to the conclusion that the bacteria could cause hypersensitivity. Intensely pruritic inflammatory lesions tending to expand suggested an allergic reaction. In these cases, immunotherapy is carried out with gradually increasing doses of allergen over a period of time. The study aimed to compare the value of different preparatory techniques to obtain desensitizing allergens for dogs with staphylococcal dermatitis.

Different inactivation methods (heating to 80ºC for 10, 20 and 30 min, repeated freezing-thawing) were tested to prepare various antigenic extracts from two staphylococcal strains, *S. aureus* and *S. intermedius*, isolated from dogs with bacterial dermatitis. Sera sampled from similarly diseased dogs were tested for the presence of anti-staphylococcal antibodies and possibilities for further use in describing the antigenic properties of the staphylococcal preparations, by rapid agglutination and agar gel diffusion against a *S. aureus* strain. Rapid and slow agglutination, as well as double immune diffusion tests were then used to define the antigenic properties of the staphylococcal extracts.

The diagnostic value of the preparation and usefulness of different inactivation techniques in obtaining antigenic products are discussed.

Key words: *Staphylococcus aureus, Staphylococcus intermedius*, antigenic structure, inactivation

Dermatites which rely on destructive interference of microorganisms of the genus *Staphylococcus* with the skin hold an extreme importance both in human dermatology and pets medical practice (5). The incidence of dermatites, where staphylococci are certainly incriminated, is increasing within the canine population worldwide. Moreover, these diseases represent a major public health problem when transmitted to humans and are classified as zoonoses (6, 4).

Currently, the basic treatment protocol for the prompt control of germs belonging to the genus *Staphylococcus* is based on the use of antibiotics. However, the special bio-genetical plasticity of the *Staphylococcus* allowed the installment of antibio-resistance phenomena. Antibioresistance arisen on a permissive field of genetic flexibility led directly and inevitably to therapeutic failures. These issues stress the need for immunotherapeutic intervention and

bring to a rethinking of therapeutic guidelines as an absolute necessity in the future.

Considering these aspects, this study aimed to comparatively evaluate two original methods of mixed physical-enzymatic inactivation of isolated staphylococci by considering the lysates' antigenicity, immunogenicity, lack of pathogenicity and therapeutic efficacy, in order to obtain a suitable immunotherapeutic product.

Material and methods

The study was conducted on a non-homogeneous group of 6 dogs (n=6) of the following breeds: German Shepherd (n=3), Doberman (n=1), Akita (n=1) and Shar Pei (n=1).

The 11 staphylococcal isolates (n=11) obtained, representing the strains of *Staphylococcus intermedius* (n=10) and *Staphylococcus aureus* (n=1) were subjected to two types of inactivation:

Procedure I – with the following major steps:

• Step 1. Treating with pure standard lysozyme, in an amount of 1 mg /ml of saline (3 mg/tube), pH 7, incubation for 24h at 37°C.

• Step 2. Monophasic ultrasonic treatment for 10 minutes, using BANDELIN ELECTRONIC ultrasonicator (power 1 of the device).

Procedure II - with the following major steps:

• Step 1. Treating with pure standard lysozyme in an amount of 2 mg /ml of saline (6 mg lysozyme/tube), pH 7, followed by 24 hours incubation at 37°C.

• Step 2. Ultrasonic treating for 5 minutes, using BANDELIN ELECTRONIC ultrasonicator (power 1 of the device)..

Two types of antigenic mass were obtained after the application of these two inactivation - lysis methods.

Results and discussions

Using procedure I of inactivation the authors obtained, for all strains, slightly coarse, greyish liquids. Allowed to sediment, the liquids formed a very dark deposit. These changes were supposingly due to the disintegration of the ultrasonication probe during the operation, when microscopic pieces of aluminium came off. To avoid adverse effects that might occur *in vivo* after injection of such impure products, staphylococcal bacterins obtained after applying procedure I of inactivation were excluded from subsequent tests.

Application procedure II resulted in obtaining slightly coarse, white lactescente, opaque liquids for all strains. These were subjected to further testing.

The 11 staphylococcal bacterins (n = 11) obtained using procedure II underwent total protein dosage using Lowry method. The total protein concentration values ranged from 0.76 g/dl to 1.36 g/dl. The protein concentration was adequate, when compared to that of other similar products (1 g protein/dl)(2).

The lysates were tested for the efficacy of the inactivation by seeding on usual culture media. Their multiplication capacitiy was evaluated after 24 hours. Microscopic changes were assessed by analyzing Gram stained smears of each lysate.

Staphylococcal lysates pretreated with lysozyme (24h) and subsequently subjected to the action of ultrasound showed approximately 1% morphologically intact cells/microscope field. Other aspects observed consisted of fragments, "shadows" and "domed" cells, as well as viability loss.

In order to perform immunological tests, the authors used sera collected from 5 dogs (other than those in the study), 4 of which presented clinical symptoms of chronic relapsing staphylococcal dermatitis, diagnosis later confirmed by microbiological examinations. Serum from a clinically healthy dog, with no history of skin disease, considered as "negative control" was also applied.

The rapid slide agglutination test was employed for immunological investigation. Sera from the five animals were used to assess the antigenicity and thus the immunogenicity of the lysates (bacterins) obtained.

All four sera from dogs with staphylococcal dermatitis tested positive in rapid slide agglutination reaction, both to *Staphylococcus aureus* and to *Staphylococcus intermedius* strains. The serum taken from the animal with no history of skin disease tested negative in the rapid agglutination reaction, both to *S. aureus* and *S. intermedius*. According to these results, the serum could be considered as a negative control.

Positive results were obtained from rapid agglutination assay for all lysates, with all four positive sera, in all 11 staphylococcal bacterins.

Ultrasonication for only 5 minutes further enhanced the lytic activity of the lysozym. Ultrasounic treatment was milder than to alter antigenic structures. Lysates obtained during the latter protocol satisfied the requirements of an immunomodulatory product: they showed unchanged antigenicity, were immunogenic and apatogenic (9).

Table 1

List of antigenic valences of the autologous lyzates in all cases studied

Case No.	**No. of *Staphylococcus spp.* strains isolated**	**Antigenicity of the lyzate**
1	1	mono
2	3	tri
3	3	tri
4	1	mono
5	1	mono
6	2	bi

Lysates prepared from strains isolated from each animal were mixed, thus obtaining bi- or tri-antigenic/bi- or trivalent products depending on the number of isolates in each case. Mono-antigenic lysates were prepared for cases 1, 4 and 5,

from which only one strain of *Staphylococcus spp.* was isolated. In each case, the authors administered mono-, bi- or trivalent staphylococcal bacterins prepared from autologous staphylococcal strains (Table 1).

The attempt of immunomodule by specific antigenic stimulation was performed on all six dogs with piodermatitis.

Inoculation of lysates could be achieved in various ways. In order to assure the success of the immunomodulatory therapy, the most suitable route of administration must be chosen. The dosage, the pace of performing the antigen injection and the duration of the procedure must be determined individually (1, 3, 8). These variables are dependent on the status of the "biological field" that is to be treated, therefore the immunostimulating schemes cannot be universal (4, 9).

The subcutaneous route is considered available, less painful for the animal and favorable to the formation of large quantities of antibodies (2). Hence, the subcutaneous route was chosen in the present experiment. The staphylococcal bacterin was administered for 4-5 times in an amount of 1 ml, subcutaneously, at 7 to 10 days intervals, to 4 of the 6 cases (case 1, 2, 4 and 5). In cases 3 and 6, only two inoculations were carried out due to the adverse effects observed.

After the inoculation of the lysates, the lesional score dropped by half or by even more than half at the end of therapy in 4 of the six cases studied, which represents a therapeutic efficacy of 66.66%.

Over pruritus, immunomodulatory therapy had beneficial effects in 4 (66.66%) of the six cases, a decrease was noted starting with the second inoculation. By the end of the immunomodulatory therapy, the pruritus disappeared completely.

Evidence shows that this immunomodulatory act has a maximum therapeutic efficacy in cases with superficial skin lesions, allegation also supported by Scott et al. (8). The authors believe that unfavorable results obtained in cases 3 and 6 are due to the inoculation of staphylococcal antigens on biological terrains with hypersensitivity to staphylococcal haptens.

Conculsions

The lysates obtained by procedure II satisfied all the requirements of an immunomodulatory product: they have unchanged antigenicity, are immunogenic and apathogenic. The immunomodulatory therapy has found maximum success in cases with superficial pyoderma. No relapses were observed in the 4 cases which have responded favorably to immunomodulatory therapy. Considering the adverse effects noticed in two of the six cases, we do not recommend this treatment protocol in dermatitis caused by hypersensitivity to staphylococci.

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COMPARATIVE ANALYSIS OF FAT GLOBULES OF MILK FROM ALPINE AND CARPATHIAN GOAT

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Summary

Fats represent the basic morphological characteristics of milk. It is the most variable component present as globules or microspherules with diameter between 1,6 μm and 10 μm. They are different from one species to another, within the same species can vary depending on breed, the animal's health and stage of lactation. The purpose of the study is the comparative analysis of spherules of fat and of the conglomerates between them in goat milk and colostrum originated from two different races. Therefore morphophysiological investigations were conducted on samples of milk and colostrum obtained from clinically healthy Alpine (n = 12) and Carpathian goats (n = 12) during March to July 2015, using the Squash technique on vital preparations colored panoptic (Dia-Quik-Panoptic) and (Gill's Haematoxilin Romvachrom testing). Microscopic examination revealed the presence of small spherules of fat and a more evident agglomeration in both compared races' colostrum. At Alpines, quantitatively, there is an obvious agglomeration of microspherules fat, which leads us to argue that in terms of percentage this breed's milk is more concentrated in fat. It is not recommended the staining technique (Gill's Hematoxylin Romvac Rom) because after examination preparations are deteriorating rapidly. We recommend using Dia Quick Panoptic because through this technique preparations are of better quality and highlight much better the morphological and physiological characteristics of the spherules' fat.

Key words: goat milk, colostrum, fat spherules

Fats represent the basic morphological characteristics of milk, being the most fluctuating component of milk, present as globules or microspherules, with their diameter between 1.6-10 μm. These differ from one species to another and within the same species they can vary depending on breed, the animal's health and its lactation stage (5). They are mainly represented by triglycerides, these being formed of glycerol and fat acids synthesized "de novo" in mammary cells in the alveoli, or taken directly from the blood circulation under the action of lipase (3). The other components of the lipid complex are phospholipids (0.8%), which are associated with membrane proteins and cholesterol (0.3%), located mainly in the coating of fat cells (15).

Goat milk differs from cow and human milk by some particularities of composition, which implies either advantages (17) (a better digestion of fats, a lower rate of allergy compared to cow milk), either disadvantages (inappropriate

content in some vitamins and minerals). The disadvantages explain why raw goat milk is not proper before the first year of life (similar to cow milk) and explain why in industry it is necessary to add vitamins in order to compensate for the natural deficiency of this type of milk (6). Furthermore, goat milk is digested easier and does not frequently create the allergies specific to the consumption of cow's milk (9,14). One of the main aspects of the composition of goat's milk refers to the nature of the fat in which exist triglycerides with medium chain, consisting of fat acids whose carbon chain has from 6 to 14 carbon atoms and which usually reaches 30%, compared to cow's milk which usually reaches only 20%. For this reason, goat's milk receives a particular interest through its use as therapy for some metabolic diseases (2,7,8,9). The goat's milk fat is more easily digested compared to that of the sheep, because of the dimensions of the fat globules, of goat milk, which are smaller but with a larger specific surface, and the lipases in the intestine can attack the lipids faster (13). Fat acids with short chains were used in treating several metabolic disorders and coronary diseasses (1,12). The fat globule contains a drop of the milk's lipids, surrounded by a membrane made of proteins and phospholipids. The nutritional importance of this component is supplying energy, being a good source of fat acids essential for all organisms, especially for those growing. They also transport the liposoluble vitamins A, D, E, K (15). The milk fat globules (MFG) are formed in the mammary gland's epithelium, during lactation (10). The weight ratio between fat globules and globules' membrane is not yet known, but is considered to be between 2 and 6% of the total weight of fat globules (10,11). The globules' diameter varies between 1 and 12 µm, depending on the species. In the case of goat milk the small and medium size spherules of 6-8 µm prevail (4).

Materials and methods

In this study were performed microscopic examinations and vital cytological preparations obtained from samples of milk and colostrum freshly collected from goats belonging to Alpine French breed (n = 12) and Carpathian Romanian breed (n = 12). During March - July 2015 were collected two samples of fresh milk monthly and one sample per day during colostral period (the first 5 days of lactation). The goats were clinically healthy at the second and third lactation, with similar body weight for the same breeds. For the determining were utilized: Squash technique, panoptic colored (Dia-Quick-Panoptic); The coloring is based on a mixture of eosine, methylene blue and Azure II, but the solution is bought already prepared from the manufacturer. The preparations are dried at ambiental temperature after which they are ready to be examined. The examination between blade and lamella emphasizes the configuration of fat microspherules from milk and also gives the possibility of identifying milk depending on species or cathegory, and of identifying the freshness of the product as an aliment. As a novelty was tested Gill's Hematoxylin Romvachrom which is based on three successive coloring

solutions Romeozin, Hematoxylin and Romvachrom, bought already-made from the manufacturer and which is normally used for obtaining histological preparations subsequently included in paraffin. This coloring emphasizes very well the abundance and the morphology of fat microspherules.

Results and discussions

The examination of microscopic preparations spotlights the small size of the spherules of fat from goats milk compared with data from the literature, which increases digestibility property of goat milk toward the cow milk. Also highlighted microspherules of goat milk on the smears have smooth spherical membrane, which supports and demonstrates the freshness of milk. Globules diameter varies predominant being microspherules of small and medium sized between 6-8 µm. (Fig. 1 and 2).

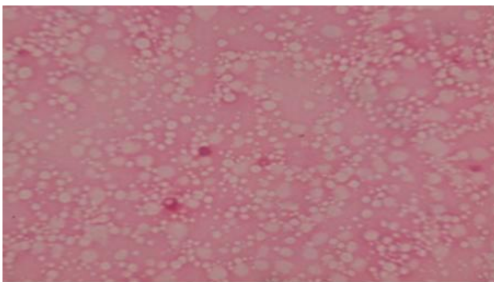

Fig.1. Appearance of fat spherules/ immersion 100x /milk of Alpine

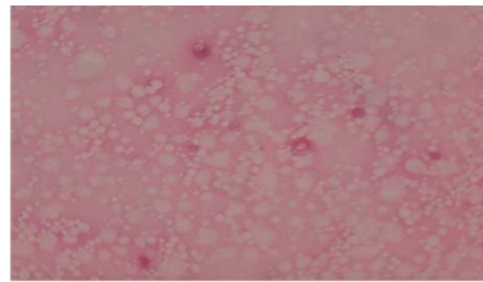

Fig.2. Appearance of fat spherules/ immersion 100x /milk of Carphatine

It is noticed an abundance and a cluster more obvious of fat spherules in colostrum milk (Fig. 3 and 4), day one and two, both in Alpine goats' milk and in the Carpathian goats, which is an important characteristic in the early days of lactation, after that the aggregation of spherules decreases slightly but the abundance remains specific.

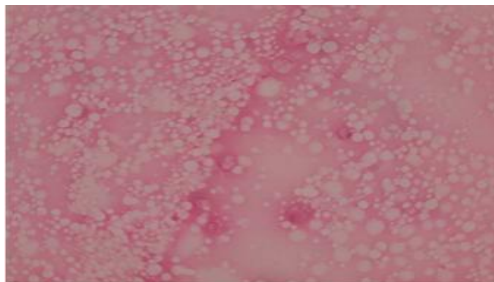

Fig.3. Appearance of fat spherules/ immersion 100x /colostrum milk of Alpine

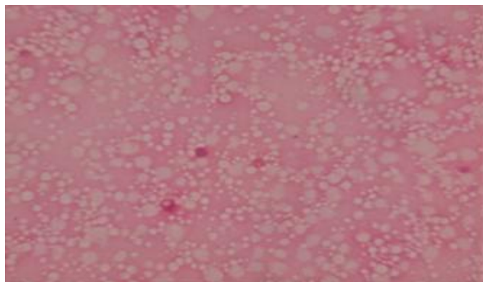

Fig.4. Appearance of fat spherules/ immersion 100x /colostrum milk of Carphatine

Exam between blade and lamella, most of the smears looks almost identical, making it difficult to reveal characteristic differences, specific to milk of those two goats breeds studied. However, it can show here abundance of fat spherules, small size globules, spherical, without cytolysis, which confirme the freshness of milk (Fig. 5 and 6).

Fig.5.Vital preparation / immersion / milk of Alpine

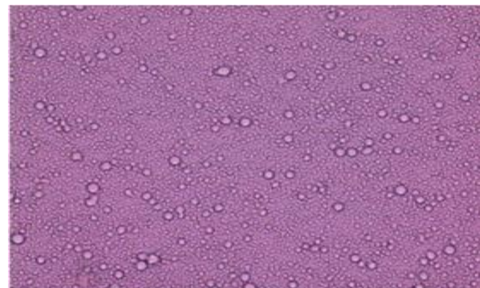

Fig.6.Vital preparation / immersion / milk of Carphatine

Technique of colored Gill's Hematoxyling Romvachrom (Testing) (Fig.7 and 8) customizes very good the shape, the size and the abundance of fat spherules, as well as for exam with the blade and lamella, but it is difficult to reveal characteristic differences between those two breeds since the smears looks almost identical.

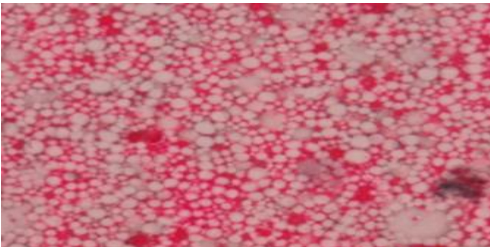

Fig.7. Gill's Hematoxilin Romvachrom immersion/milk of Alpine

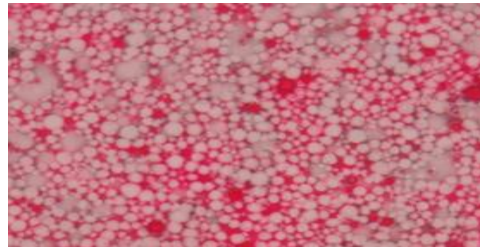

Fig.8.Gill's Hematoxilin Romvachrom immersion/ milk of Carphatine

Conclusions

Fat milk has lower density than water. Because it contains a lot of lipids, these take the form of an emulsion which is observed in aqueous continuous phase and a discontinuous lipid, these two phases are not miscible, surface tension cause spherical globules. Their membranes contain proteins, neutral and polar lipids, compounds carbohydrate nature and ribonucleic acids (16).

Microscopic examinations revealed the abundance of small spherules of fat

in milk of those two breeds compared to those found in the milk of other species. This trait was considered the main morphological element through which it can microscopic differentiate the origin of milk, depending on species, and also correlates with the increased degree of digestibility of goat milk. At Alpine, in terms of quantity, there is an obvious microspherules accumulation of fat, which leads us to argue that this breed milk is more fat concentrated in terms of percentage.

In the colostrum of both breeds, the microscopic examinations comparatively revealed the abundance of little spherules of fat and a more obvious agglomeration but quantitatively, at Alpines, there is an obvious abundance in fat microspherules.

The exam between blade and lamella, most smears looks almost identical, making it difficult to reveal characteristic differences in morphology for the two races, through this method

The examination through the Gill's Hematoxylin Romvachrom method greatly emphasized the abundance and morphology of fat globules, but there is an inconvenience because after the examination with immersion objective the smears are deleted and can not be subsequently reassessed.

We recommend using DiaQuick Panoptic method because by this technique the preparations have a much better quality and highlight morphological and physiological characteristics of the spherules fat.

It is not recommended this staining technique - (Gill's Hematoxylin Romvachrom) just as a faster alternative because the coloring time is very short and after examination the preparations will deteriorates easily. This technique is specific for emphasizing the histological preparations.

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STUDIES ON FUNGI AIR CONTAMINATION IN POULTRY HOUSE REARED ON ENRICHED CAGES

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Summary

The results of a study done on the fungi load and dynamics in the air from a house for hens reared on enriched cages, over the cycle and exploitation are presented.

The mean of TNF increased from $14.47 \times 10^3 \pm 1.63 \times 10^3$ in the first quarter of operation cycle to $1.93 \times 10^4 \pm 0.36 \times 10^4$ in the last of breeding.

The increase of fungi in air load hall caged hens was very slow in the first three months of the operating cycle. The concentration of fungi found in hen's house taken in this study may present a health risk factor for people working in this environment

Key words: fungi, dynamics, hens' house, deep litter

Air quality in shelters animals and hens can influence the performance of their production. The effects on living organisms of fungi from the air of these shelters represent not only an economic problem, in the case of animals and birds, but also a medical and health problem for the workers that are working there (7, 20).

Farmer's lung disease, asthma, low production performance and reduced resistance to disease of poultry and farm animals are certainly aspects that may be related to the presence of dust, harmful gases and microorganisms in the air shelters (2, 3, 5).

Many diseases are caused by inhaling fungal spores during agricultural activities and livestock production: aspergillosis, histoplasmosis, blastomycosis, and coccidioidomycosis, adiaspiromicosis (1).

Fungal spores may cause allergic conditions. Large spores of *Puccinia* spp., or *Alternari*a spp., are being held in the nasal passages, those of *Cladosporium* spp., are being held in large bronchi and produce immediate type sensitivity. Spores less than 5 micrometers, from *Actinomyces* spp. (1 micrometer diameter), *Aspergillus* spp. and *Penicillium* spp. (3-5 micrometers in diameter), induce delayed hypersensitivity (6, 18, 21).

Exposure to high levels of organic dusts contaminated with various kinds of fungi can cause pneumonia hypersensitivity, but it is not known with certainty if the disease is due to toxic or allergic effects of the agents (14, 15, 16, 25).

The highest concentrations of fungi were detected in the houses of poultry reared on permanent litter, compared with their growth in batteries (12). The poultry houses, levels of aerosolized fungi, determined in various studies, vary within wide limits, depending on the technology of growing season, the size and age of the flock, the selected sampling method and so on (9, 10, 11, 13, 17, 19).

The concentrations of fungi in poultry houses reared in cages (2.700 CFU / m^3 of air) were significantly lower than in other systems of rearing (who had averages between 7.500 and 320.000 CFU / m^3 air) (7).

Knowing these aspects, which are usually neglected, was considered as a necessity a research in this field, to highlight the load of fungi in the air of the poultry house. The air was sampled by aspiration method with the device SAS 100 and the dynamics of the fungi concentration over a year of operation.

Materials and methods

The research was conducted in a farm of laying hens reared in improved battery cages, over a year, from September 2014 to August 2015. The farm is located in the Western part of Romania, the county of Arad. It is equipped to European standards, respecting the requirements of BAT - EU (Best Available Techniques European Union).

The farm is composed of a block, with one hall, on one level, building sorting station and egg storage and administrative building.

The production hall is rectangular, 55 meters long, 14 meters wide and 4.20 meters high and a total area of 770 m^2.

In the hall there are five rows of improved batteries, each with four levels.

The hall is equipped with facilities for: air conditioning (cooling) air ventilation, artificial lighting, automated feeding, watering, collection and transport of eggs, collection and disposal of manure.

At each visit, determination and quantification of total number of fungi were performed. The air samples were collected from five points as follows: from the four corners of the hall and in its center (a total of 240 samples for the all period of experiment).

For air sampling was used the aspiration method with the device PBI Air Sampler SAS Super 100 (International PBI SpA Milano, Italy) and Petri dishes with Sabouraud medium. For each sample, the device was set to aspirate five air liters.

After sampling, Petri plates were incubated in the thermostat at 35 °C for 2-3 days when were examined and developed colonies were counted. The number of colonies obtained on each plate was established to the real values, using the tabular data of the device, standardized by the manufacturer of PBI Air Sampler SAS Super 100.

The obtained data were processed through biostatistical methods, using Microsoft Excel application by calculating the arithmetic mean and standard deviation. The arithmetic means were calculated for each of five sampling points at

each visit. Also arithmetic averages were calculated for the values obtained in the course of a month and for the semester. For the statistical significance of differences between the averages of data was used analysis of variance test with ANOVA Single Factor, included with Microsoft Excel. Along with ANOVA test also Whitney Mann test was used (Wilcoxon) from Minitab 14 program.

Results and discussions

The results on total airborne fungi in caged hens hall where experiments were conducted during a year of operation are shown in Table 1.

Te mean of total number of fungi in the air (TNF / m^3 of air) in the first month of operation was $2.62 \times 10^3 \pm 0.51 \times 10^3$ CFU / m^3, and in the second month of the quarter these value increased significantly, recording an average of $5.10 \times 10^3 \pm 0.64 \times 10^3$ CFU / m^3. Compared with the previous month difference was highly significant ($p < 0.001$). It was noted that, in November, however, there were no major differences between weeks so, the mean of total number of fungi was maintained around 5×10^3 CFU / m3.

Also, in the last month of the first study quarter, the mean of TNF was similar to that observed in the previous month, respectively $5.72 \pm 0.95 \times 10^3 \times 10^3$ CFU / m^3, and it was observed that this increase is insignificant, versus the previous month.

During this quarter, the total number of fungi in the air had a mean of $4.47 \times 10^3 \pm 1.63 \times 10^3$ CFU / m^3. Taking into account the values recommended by Interdepartmental Commission for Maximum Permissible Concentrations of Harmful Agents on Human Health, that recommends for workplaces, a maximum permissible concentration of fungi below 50.000 CFU /m,^{3} could be say that during first quarter of operation, this indicator remained below recommended value (8).

In the second quarter, in the hall, the load of airborne fungi ranged from one month to another (Table 1). In the first month (December) was found a mean of $6.65 \times 10^3 \pm 0.86 \times 10^3$ CFU /m^3 significantly higher compared to the previous month (November) in the first quarter. In the second month of this quarter (January), the fungi load in air the hall fell, the mean of total number of fungi was $5.15 \times 10^3 \pm 1.12 \times 10^3$ CFU /m^3. Instead, in the third month of the quarter (February), the total number of fungi was significantly increased; the mean value obtained being $9.38 \times 10^3 \pm 9.48 \times 10^3$ CFU /m^3, significantly higher compared with the previous month.

Analyzing the values obtained during of this quarter the average load of fungi in air of hall was $7.05 \times 10^3 \pm 2.24 \times 10^3$ CFU / m^3, significantly higher than the average for the previous quarter.

In the third quarter, total number of fungi (CFU / m3) in air of the hall for hens reared on enriched cages had an average value of $1.15 \times 10^4 \pm 0.89 \times 10^4$ CFU /m^3 in the first month (March) and $1.24 \times 10^4 \pm 0.06 \times 10^4$ CFU / m^3 in the second month (April), value significantly higher than in the previous month

($p<0.05$). The same average value ($1.24 \times 10^4 \pm 0.06 \times 10^4$ CFU /m^3), identical to that recorded in the previous month of this quarter (April), was found in the third month (May), without significantly differences between these.

At the level of the second quarter, the average value of the concentration of fungi in the hall was $1.21 \times 10^4 \pm 0.05 \times 10^4$ CFU / m^3. Comparing the results obtained during this quarter with the previous quarter (second quarter), we can say that the differences are significantly higher ($p<0.05$).

In the last quarter of the study, the total number of fungi recorded a mean value of $1.58 \times 10^4 \pm 0.14 \times 10^4$ CFU / m^3 in the first month (June). In the second month (July), the load of fungi in the air of the hall has increased significantly, reaching an average of $2.31 \times 10^4 \pm 0.27 \times 10^4$ CFU / m^3, which is a highly significant difference compared with the previous month ($p<0.001$).

In the third month of the quarter (August), the concentration of fungi in the air of the hall had a mean value of $1.89 \times 10^4 \pm 0.18 \times 10^4$ CFU / m^3, that represent a significant decrease from the previous month ($p <0.01$).

Table 1

Total fungi load (CFU/m3), from a house of laying hens reared on cage system

No	Month	$\bar{X} \pm Sx$ / month	$\bar{X} \pm Sx$ / semester
1	**September**	$2.62 \times 10^3 \pm 0.51 \times 10^3$	$4.47 \times 10^3 \pm 1.63 \times 10^3$
2	**October**	$5.10 \times 10^3 \pm 0.64 \times 10^3$	
3	**November**	$5.72 \times 10^3 \pm 0.95 \times 10^3$	
4	**December**	$6.65 \times 10^3 \pm 0.86 \times 10^3$	$7.05 \times 10^3 \pm 2.24 \times 10^3$
5	**January**	$5.15 \times 10^3 \pm 1.12 \times 10^3$	
6	**February**	$9.38 \times 10^3 \pm 9.48 \times 10^3$	
7	**March**	$1.15 \times 10^4 \pm 0.89 \times 10^4$	$1.21 \times 10^4 \pm 0.05 \times 10^4$
8	**April**	$1.24 \times 10^4 \pm 0.06 \times 10^4$	
9	**May**	$1.24 \times 10^4 \pm 0.06 \times 10^4$	
10	**June**	$1.58 \times 10^4 \pm 0.14 \times 10^4$	$1.93 \times 10^4 \pm 0.36 \times 10^4$
11	**July**	$2.31 \times 10^4 \pm 0.27 \times 10^4$	
12	**August**	$1,89 \times 10^4 \pm 0, 18 \times 10^4$	

Dynamics of total fungi in air of hall for hens reared in enrichment cages is shown in figure 1. It can be seen an increase of this indicator, in the first quarter of operation. In the second month after the hens were introduced in the hall, the fungi starts to increase. In the second month values have remained relatively at the same level for the third month. In the second quarter, was observed a slow decline, in the second month, but it increase at levels on the order of 10^4 CFU / m^3 in the last month.

These values are comparable to values reported by other authors. For example, *Matković et al.* (10) reported for the same type of hens reared system in

cages, a minimum value of 0.075 x 10^4 CFU /m^3 air, determined in September and a maximum values of 8.56 x 10^4 CFU /m3 air, determined in June. *Karwowski* quoted by *Lonc and Pleva* (9) determines in the air of hens house a concentration fungi between 1.7 x 10^2 to 2.4 x 10^4 CFU / m^3 air.

Examining the dynamic load of fungi from caged hen's hall, in the third quarter, it can be seen that only in the second month was uniformly increasing trends. In the first month there was a marked increase comparatively with the last month of the previous quarter. In the third month of this quarter the fungi concentration from the hens hall tended to decrease.

The dynamics of the concentration of fungi in the air hall, registered in the last experimentally quarter (Fig. 1), has been an increasing trend in the first two months, more evident in the second month. Instead, in the last month, the concentration of fungi in air hall was low compared to previous months but still high comparatively with the last month of the previous quarter.

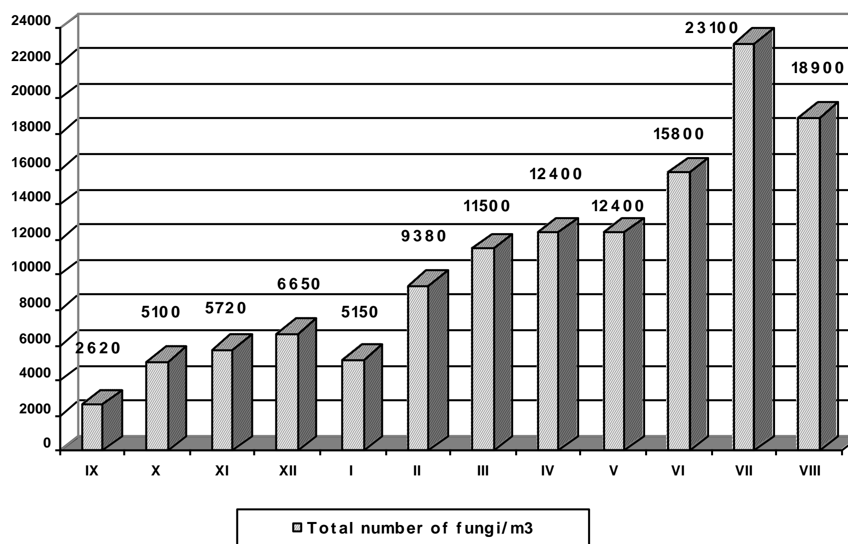

Fig. 1. The dynamic of fungi concentration in the hall of hens reared on enriched cages

In practice, in animal shelters, the value of the microbial load found in the air often are exceeding by 10 to 20 times comparatively with admitted values by some authors, 25.000 germs / m^3 (4). As permanent bedding aging, the number of fungal spores and molds increase from an average of 8000 /m^3 air and reach over 50000 /m^3 air (17). The situation is incomparably better in the halls of hens kept in cages, were the highest mean value are 14000 fungi and mold spores / m_3 air (13,

17). The highest concentrations of active fungi and total fungi have been detected in poultry houses, compared to shelters of other categories of farm animals. (17).

Conclusions

Fungal load in the air from the house for the hens reared in enriched cages has achieved high values during the operating cycle.

Mean value of total number of fungi in the air of studied hall showed a significant increase during the experiment, starting with the first month of operation, from the 1.43 x 104 CFU / m^3 in the first month of operation at 5.28 x 104 CFU / m_3 last month

The increase of fungi in air load hall caged hens was very slow in the first three months of the operating cycle.

The concentration of fungi found in hen's house taken in this study may present a health risk factor for people working in this environment

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HIGHLIGHTING THE CROSS-REACTION BETWEEN WEST NILE VIRUS AND OTHER FLAVIVIRUSES BY IMMUNOFLUORESCENCE

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Summary

West Nile virus (WNV) belongs to the genus *Flavivirus*, family *Flaviviridae*. Flaviviridae family include a large number of pathogenic viruses to humans and animals. Due to their morphological, structural (common polypeptides) and genetic similarity and physicochemical properties, the different species of *Flaviviridae* express common antigenic and serological reactions. Since 1996, the major outbreak in Romania, the virus has become a problem of public health and veterinary surveillance in Romania, Europe, the Mediterranean basin and later in the US.

Yellow fever virus (YFV) is highly pathogenic to humans, it is considered as a type-species of the genus *Flavivirus*.

Using an indirect immunofluorescence (IF) assay (BIOCHIP® Technology, EUROIMMUN) we observed the existence of cross-reactions between YFV, WNV and Tick Borne Encephalitis virus (TBEV). To demonstrate the presence of antibodies specific for every virus we used ELISA (IgM antibody capture ELISA and ELISA IgG).

Despite the cross-reaction wich occur IF is an analytical method recommended when it is too difficult or expensive to prepare specific reagents required by other methods (eg. ELISA). The IF test can contribute as a valuable screening test for flaviviruses detection.

Key words: *Flaviviridae,* West Nile virus, yellow fever virus, immunofluorescence reaction, enzyme immunoassay reactions

West Nile virus (WNV) is a widespread re-emerging pathogen that belongs to the *Flaviviridae* family, *Flavivirus* genus and is one of the most threatening flaviviruses in Europe (3). This arbovirus is transmitted by mosquitoes in a cycle in which different species of birds act as reservoir hosts, amplifying the virus. Spillover from this cycle occasionally occurs and may cause *West Nile* disease in

mammalian hosts. Horses and humans may be particularly affected, which is a matter of great concern to the veterinary and public health authorities of countries with *West Nile* cases. Although mammals are susceptible to WNV infection, most species are regarded as dead-end hosts; WNV does not efficiently replicate within their cells and they cannot transmit WNV to new vectors (4).

The geographical area of WNV circulation encompasses most of Africa, Israel, North America and South America, Australia and scattered areas in southern and central Europe, including Russia, Czech Republic, Hungary, Greece, Romania, Italy, Southern France, Portugal, Turkey and Spain (7,10,15).

Most WNV infections are asymptomatic in horses and humans or are associated with an influenza-like illness (characterized by moderate to high fever, weakness, and myalgia). Only infrequently, in less than 1% infections in humans and 10% of infections in horses, do acute meningitis, encephalitis, or flaccid paralysis develop (the latter has only been reported in humans); neurological symptoms and lesions are not specific to WNV infections (14). Consequently, laboratory tests are essential to confirm or exclude WNV infection. Because of the virus' low-level and short-term viremia in humans and horses as well as the late appearance of clinical signs when the viremic phase is over, the primary tools used to diagnose WNV consist of indirect or serological tests that aim to detect WNV antibodies. Rapid and high-throughput assays that do not require the use of infectious virus, such as ELISAs, hemagglutination-inhibition tests (HITs) or immunofluorescence assays (IFAs), are usually preferred. However, seropositivity has to be interpreted with care because of the frequent cross-reactions among flaviviruses observed in these tests; results should be systematically confirmed by comparative virus neutralization tests (VNTs) that use a panel of viruses known to circulate in the area under investigation (4,5). Accordingly, serological tools have to be adapted to specific epidemiological situations involving WNV.

Yellow fever (YF) is one of the well-known diseases in the areas of Africa and South America where it is endemic. Even though the live attenuated 17D vaccine strain provides very efficient and long-lasting protection against the disease, missing vaccination coverage causes regular outbreaks with high numbers of cases and deaths (16). Because little attention is paid to this deadly disease, several cases of infections in unvaccinated travelers visiting areas of endemicity occurred, some of them with fatal outcome (1,2). Additionally, in recent years serious side effects after YF vaccination became apparent, which require further thorough analysis (6,12).

Immunofluorescence assays use slides coated with flavivirus-infected cells. Serum samples are deposited on the slides, and the attachment of antibodies is revealed using fluorophore-conjugated immunoglobulins that demonstrate specificity to anti-species IgGs or IgMs. This method is fast and easy to perform and may be used to differentiate between IgM and IgG antibody responses. However, it is not adapted for screening purposes. IFA slides coated with WNV, JEV, YFV, and DENV are commercially available for the diagnosis of human

infections (9). BSL-3 facilities are not required because inactivated virus is bound to the slides. When dealing with antibodies against viruses within the JEV serocomplex, IFAs demonstrate greater specificity in detecting IgMs than do ELISAs; in contrast, IFAs show the same level of specificity as competitive and IgG indirect ELISAs when detecting IgGs (9,13).

ELISAs are preferred screening tools because of their rapidity, sensitivity, reproducibility, and affordability. Three different assays are commonly used: The competitive ELISA, the indirect IgG ELISA, and the Ig**M** **A**ntibody-**C**apture (MAC) ELISA. Ready-to-use diagnostic kits for veterinary and human purposes are commercially available.

Specificity problems associated with flavivirus cross-reactivity, have been reduced through the application of algorithms (11). The sensitivity and specificity of commercially available ELISAs has been reviewed by Zhang et al. in 2009 (17). A survey of public health and commercial diagnostic reference laboratories in 2008 revealed that ELISA or microsphere immunoassay-based IgM and IgG assays were most commonly used for the diagnosis of WNV infections, with significantly fewer laboratories using PCR, PRNT, and culture isolation (8).

Materials and methods

The tests were performed in order to achieve a calibration curve for checking the immunization values obtained from use of STAMARIL (yellow fever vaccine), Sanofi Pasteur.

For this study we used a serum from a immunized person with the STAMARIL vaccine two years ago that was diluted (1/10, 1/50, 1/100, 1/500) in order to achieve of the calibration curve and a negative control serum from a nonimmunized person with STAMARIL vaccine. We also used a human positive serum for *West Nile* virus and a human positive serum for Tick Borne Encephalitis virus from persons with neuroinfections. Detection of IgG and IgM antibodies for this samples was made with ELISA method.

Yellow fever virus IIFT assay Anti IgG (Euroimmun, Germany) is an immunofluorescence test that use biochip technology to detect YFV IgG antibodies. The test is simple, has a quick recovery and proves especially useful for screening serum when samples are numerous, such as would be necessary in epidemiological research on the spot (in focus).

Immunofluorescence technique was performed using a Zeiss 510 confocal microscope (Germany) maintaining laser scanning parameters for each sample evaluated: pinhole 210 micrometers Objectives Plan-Nofluor 20 x 0.5, 30% intensity, Argon Laser filter eg 488nm/em: 505nm (green light). Cytoplasmic counterstaining was performed with EVB and was revealed using a laser source of He/We ex: 543nm/em: 585nm, intensity 12% (Red signal).

Results and discussions

Using an indirect immunofluorescence (IF) assay (BIOCHIP® Technology, EUROIMMUN) we observed the existence of cross-reactions between YFV, WNV and TBEV.

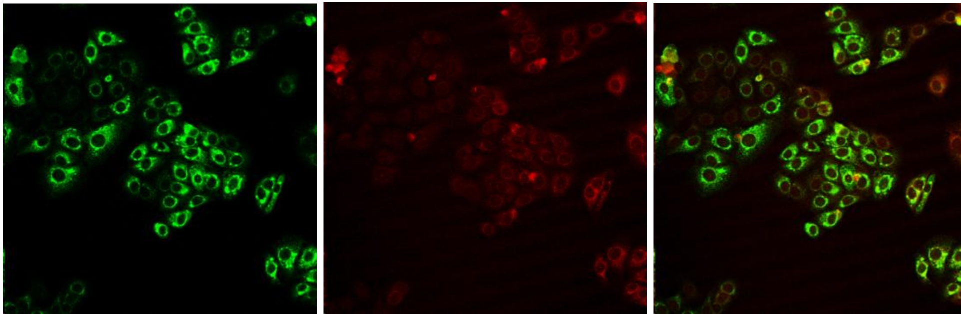

Fig.1. Anti-Yellow fever virus IIFT, sample immunized person, dilution 1:10 (strong fluorescent-green signal)

Antibodies against Yellow Fever virus by binding with specific secondary antibodies labeled with a fluorochrome, induce the fluorescent signal occurrence in the infected cells. Cytoplasm contains fine and granular structures or fluorescent granular inclusions. Some of the cells are not infected and do not show any specific fluorescence. If the sample contains antibodies against yellow fever virus, image obtained at fluorescence microscope must to be similar to the positive control. (Fig. 1) The titre is defined as the sample dilution factor for that the specific fluorescence is poorly visible.

A B

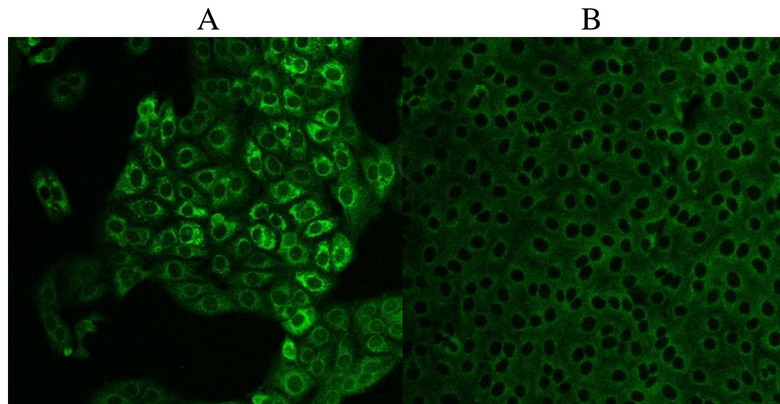

Fig. 2. Anti-Yellow fever virus IIFT, sample nonimmunized person, dilution 1:1, A positive antigen YF (fluorescent signal strong-green) and B antigen negative YF

Surprisingly enough, the nonimmunized person has submitted Yellow Fever antibodies, although yellow fever is not endemic in Romania (Fig 2). We suspected that it could be of other flavivirus that is endemic in Romania (WNV and TBEV) with which yellow fever virus can give cross-reactions. For this reasons we made a second tests in which we introduce positive human serum against WNV and positive human serum against TBEV. The fluorescent microscopy capture revealed the presence of specific structure in the infected cells with WN and TBE viruses. (Fig 3)

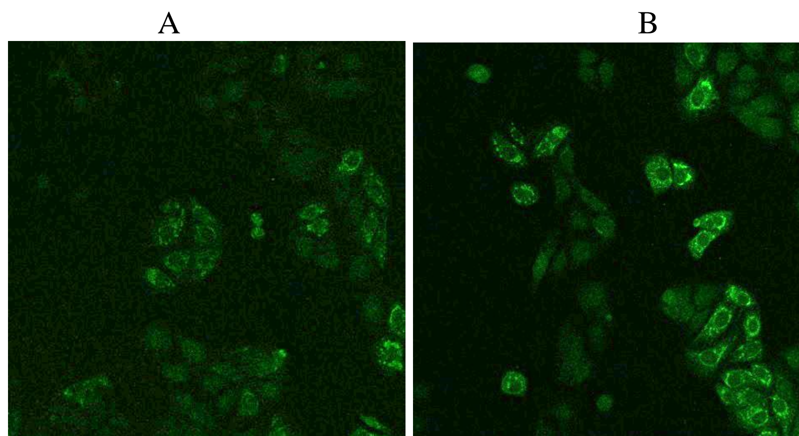

Fig. 3. Anti-Yellow fever virus IIFT, A: positive human serum for West Nile virus and B: positive human serum for Tick-Borne Encephalitis virus, dilution 1:1 (moderately-green fluorescent signal)

Conclusions

Using an indirect immunofluorescence (IF) assay (BIOCHIP® Technology, EUROIMMUN) we observed the existence of cross-reactions between YFV, WNV and TBEV.

The IF test can contribute as a valuable screening test for flaviviruses detection.

IF is an analytical method that can be recommended when it is too difficult or expensive to prepare specific reagents required by other in house methods (eg. ELISA) or in the field rapid indentification.

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HISTOMORPHOMETRICAL AND HISTOARHITECTURAL ASPECTS OF THYMUS DEVELOPMENT IN COBB 500 EMBRYOS

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Summary

The aim of this study was the histomorphometric assessment and characterization of embryonic thymus development in Cobb 500 hybrids. 45 fertilized eggs of Cobb 500 hybrids were used, and five embryos or cervical region of 5 embryos were collected in the day 4, 6, 8, 10, 12, 14, 16, 18 and 20 of incubation and were fixed in ethanol 80°. Histological sections were stained by hematoxylin-eosin method and were assessed aiming the morphological aspects and the following parameters: the area and perimeter of the lobules, the thickness of the cortex and the perimeter and area of the medulla. The earliest hystological record of the lymphoepithelial structure of the thymus in Cobb 500 embryos was obtained in the 8th day of incubation. This appearance of the thymus was preserved until day 10 of embryonic development, when it was also notices the formation lobules. Thymus of 18-20 days old Cobb 500 embryos resembles the thymus of young birds, showing that this organ is fully developed before hatching.

Key words: Cobb 500, embryo, thymus

The avian thymus is located bilateral and parallel to the vagus nerve and internal jugular vein. Each thymic "body" consists of 7-8 bean- or button-shaped lobes which extend from the third cervical vertebra to the cranial thoracic region. Thymic lobes structure is similar to that described in mammals (4).

The first thymic rudiments derived from pharyngeal pouches 3 and 4 appear in the third day of incubation and after only 24 hours separate from the pharynx and turn into two compact cords of epithelial cells. The further development of the thymus is dependent on signals from adjacent mesenchymal cells, which are responsible for the transformation of thymic epithelium in a lymphoid organ (4).

Based on embryonic quail-chick chimeras, it was possible to show that the avian thymus is colonized by cells derived from all three germ layers (endo-, meso and ectoderm). Epithelial stromal cells form a network, incorporating lymphoid

component both in cortiex and medulla, having their origin in the endoderm of the puches already mentioned. Connective tissue, which is separating the thymic parenchyma in lobes and lobules, pericytes and smooth muscle cells of blood vessels derive from neural crest ectoderm. Hematopoietic component that differentiates in T lymphocytes, macrophages and medullary dendritic cells originates in the mesoderm. Also, the endoderm is the source of endothelial cells (4).

The development of the avian thymus was described in various species and breeds, but currently currently there are no data on thymic histogenesis in Cobb 500 hybrids. The aim of this study was the histomorphometric assessment and characterization of embryonic thymus development in this hybrid.

Materials and methods

In order describe the development of the embryonic thymus, 45 fertilized eggs of Cobb 500 hybrids were used. The eggs were purchased from Ave Impex Ltd, Satu Mare, and were incubated in a commercial incubator IO-1P TE (S.C. ELECTROARGES, Romania).

Five embryos or cervical region of 5 embryos were collected in the day 4, 6, 8, 10, 12, 14, 16, 18 and 20 of incubation, depending on their size (the whole embryos between day 4 and 8 of development, and the thymus with the cervical skin between day 10 and 20).

The embryos and tissue samples were fixed in ethanol 80°. Histological sections were made and stained by hematoxylin-eosin (H&E) method, in standard technique (2).

Histological sections were assessed using optical microscope Olympus CX41 (Olympus America Inc., USA), aiming the morphological aspects and the following parameters: the area and perimeter of the lobules, the thickness of the cortex and the perimeter and area of the medulla.

The data obtained were processed using SPSS 21 software (SPSS Inc., IBM Corporation, NY, USA), *t test* being calculated.

Results and discussions

The earliest hystological record of the lymphoepithelial structure of the thymus in Cobb 500 embryos was obtained in the 8th day of incubation (Fig. 1).

As shown in the picture above, the typical histoarchitecture of this primary lymphoid organ is not yet established, the embryonic lobes being unstructured, with an area measuring 4239.76±12435.07 μm^2 and containing basophilic lymphoblasts with an average diameter of 7.5 µm, and epithelial cells of 6-9 µm diameter.

This appearance of the thymus was preserved until day 10 of embryonic development, when it was also notices the formation lobules (2-4 lobules / lobe), separated by thin connective tissue septa. There was no clear demarcation

between the cortical and medullary areas. The measurements – the perimeter and area of cross-sections through lobules of 631,8±287,1 μm, respectively 36522,2±15147.2 μm^2 – showed a significant increase compared to the body eighth day of incubation ($p < 0.001$).

The 12th day of incubation provided the first significant evidence of morphological changes in embryonic thymus. Thus, the medulla was apparent in the large lobes as an area more or less regular, colored less intense compared to the adjacent cortical (Fig. 2). At the same time, it has been observed an increase in the number of lobules/thymic lobe (4-8 lobules).

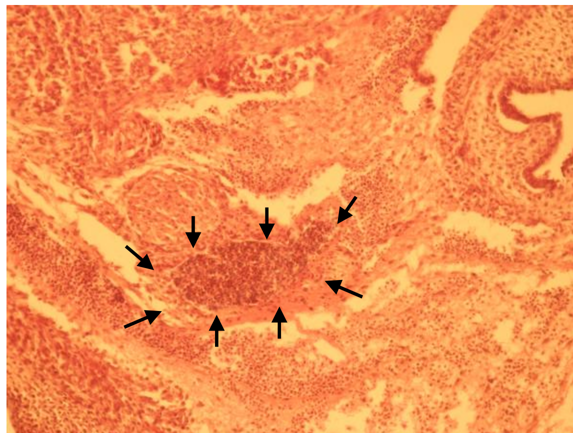

Fig. 1. Cross section through the cervical region of a eight-days-old embryo: thymic lobe is marked by arrows. H&E stain, 100x

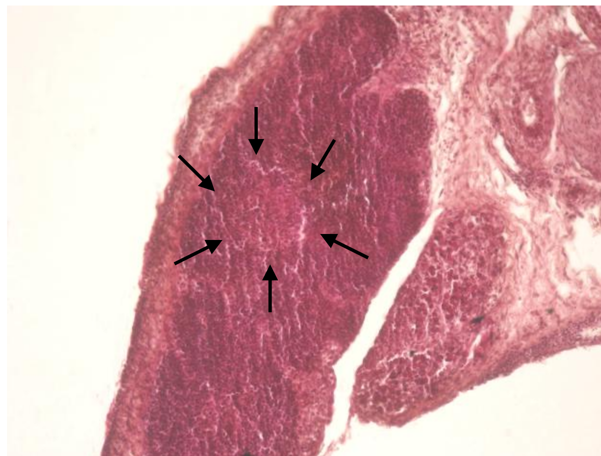

Fig. 2. Cross section through the thymus of a 12-days-old embryo: medullary area is demarcated by arrows. H&E stain, 200x

The development of the cortex and medulla in this stage of embryonic development has been established also by Banga et al. (1) in meat breeds, and it is slightly delayed compared with the data provided for Leghorn breed by Yang et al. (7) - days 9-10 of incubation. These authors also reported the first rudimentary Hassall corpuscles in day 12 of incubation, which could not be found in Cobb 500 embryos on cross-sections stained using the the same method (Hematoxylin-Eosin) (7).

The interval between day 14 and 16 of incubation showed histological aspects relatively similar to those described above, the only differences being offered by histometric values (Table 1), which were significantly higher than in the 12th day ($p < 0.001$), revealing the continuous accelerated development of the lymphoid organ.

Table 1

Histometrical aspects of thymus in Cobb 500 embryos aged 12-20 days

Incubation day	No. lobules /lobe	Lobules perimeter (μm)	Lobules area (μm^2)	Cortex thickness (μm)	Medulla perimeter (μm)	Medulla area (μm^2)
12	4-8	1117±230	76183±29150	84.8±17.8	342.3±88.5	9635.3±866.7
14	3-9	1504.2±805.6	162171.6±71349.3	146.12±63.93	1114.67±480.50	60988.3±26131.9
16	6-16	1627.1±831.7	194489±106320	190.33±101.57	926.34±428.14	70576.2±3692.8
18	6-17	2098.3±964.1	289397±116430	235.68±149.76	1205.52±582.91	80146.7±3891.2
20	8-22	2454.5±1228.5	476290±304834	327.5±150.4	1270.23±505.65	101858.9±87335.4

Legend: The values are shown as average ± standard deviation. The differences between days are significant for all paramenters monitored ($p<0.01$)

In the last four days of embryonic development it has been possible to establish the number of thymic lobe: 4-6 in the right cervical region and 4-7 in the left one, similar to data reported in the literature for other breeds and hybrids (1, 4, 5, 7).

During this interval we can note a stabilization of the thymus structure, the organ increasingly resembling the thymus described in young birds (Fig. 3-5): increase of the number and size of thymic lobules, tendency of some lobules to merge in a larger medullary area, a clear demarcation of cortex and medulla and the presence of reticular structures in the medulla, respectively of Hassall corpuscles in cortex and medulla, in rudimentary form, on the 18th day of incubation (Fig. 3, detail).

Also at day 18 of incubation, the blood vessels are visible at the junction of cortex with medulla (Fig. 4). The low number of lymphocytes in the medulla and their presence in the blood vessels indicates the “export” of these cells. These findings confirm reports in the literature relating to the fact that T lymphocytes start leaving the embryonic thymus to colonize secondary lymphoid organs (3, 4, 6).

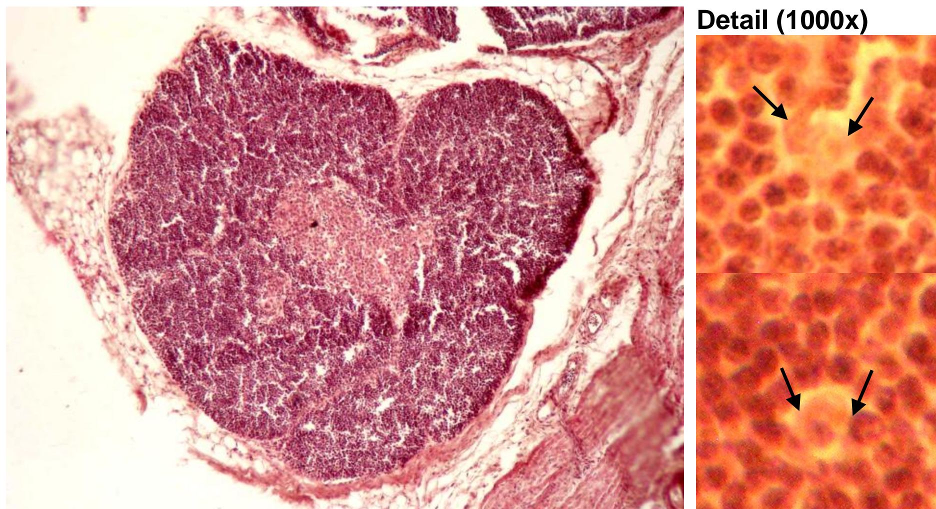

Fig. 3. Histological structure of a thymic lobule in 18- days-old Cobb 500 embryo; detail – Hassall's corpuscules. H&E stain, 100x

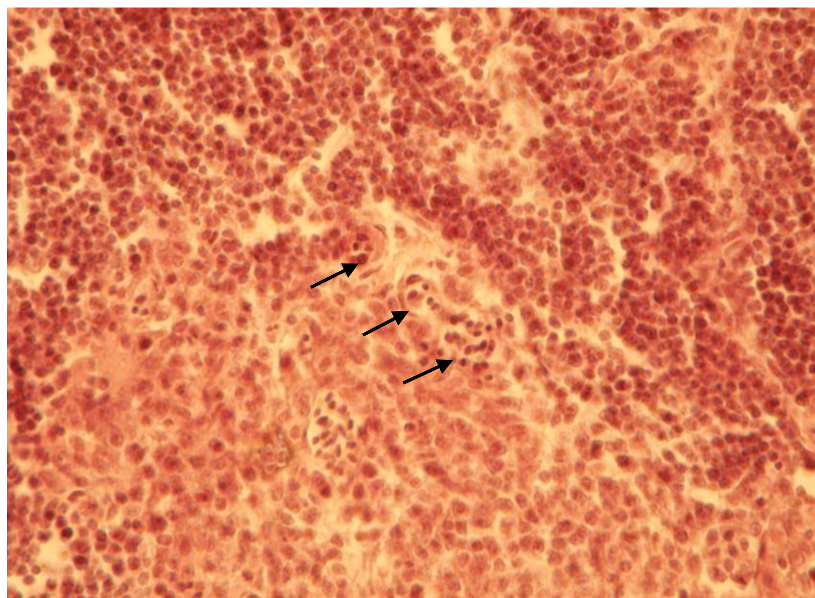

Fig. 4. Blood vessel with intraluminal lymphocytes at corticomedullary junction; 18-days-old chicken embryo. H&E stain, 400x

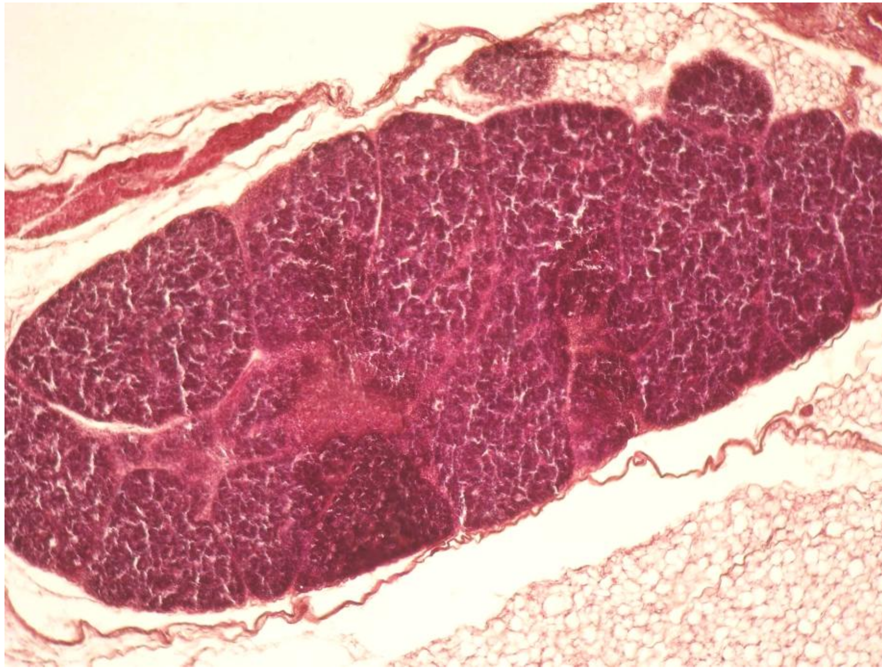

Fig. 5. Cross section through a thymic lobe in 20-days-old Cobb 500 embryo. H&E stain, 100x

Thymus development maintained at a high level during the entire study, all histometric parameters monitored registering significant differences from day to day (Table 1).

Conclusions

Embryonic thymus is colonized by immature lymphoid cells from 7th day of incubation.

Aparition of thymus' typical histoarchitecture, with medulla and cortex, shows that 12-days-old Cobb 500 embryos possess mature and naive T lymphocytes.

Thymus of 18-20 days old Cobb 500 embryos resembles the thymus of young birds, showing that this organ is fully developed before hatching.

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PHENOTYPE OF THE T CELLS INVOLVED IN IMMUNOLOGICAL TOLERANCE IN POULTY

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Summary

Immunological tolerance represents a complex of phenomena characterized by the absence of the specific responses to antigens. It is one of the fundamental properties of the immune system and is associated with its ability to discriminate between own antigens (self antigens) and foreign antigens (non-self antigens). The purpose of this study was to establish the major T cell populations involved in aquired immunological tolerance to xenogeneic antigens. The experiment was carried out on 90 embrionated eggs of Cobb 500, to which the immunological tolerance was induced by inoculation of xenogeneic blood and bone marrow mononuclear cells at 6th day of incubation. At the age of three weeks, the compatibility assessment and the analisys of the T cells phenotype was performed. The result showed that the memory T cells and particually CD25^{+} memory T cells are present in a higher proportion in experimental groups.

Key words: imunological tolerance, poultry, CD25^{+} T cells

Hundred years ago, Paul Ehrlich found that allogeneic red blood cells inoculated in animals induce the synthesis of hemolytic antibodies, but the inoculation of the own cells does not result in the development of the humoral immune response. According to Erlich "... the organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism own elements and so giving rise to autotoxins … so that we might be justified in speaking of a 'horror autotoxicus' of the organism" (5). This *horror autotoxicus* state is currently know as immunologica tolerance.

Immunological tolerance represents a complex of phenomena characterized by the absence of the specific responses to antigens. It is one of the fundamental properties of the immune system and is associated with its ability to discriminate between own antigens (self antigens) and foreign antigens (non-self

antigens), resulting a protective status against exogenous antigens (pathogens), together with the absence of reactions against own antigens. State of tolerance is an ongoing process that begins during fetal development and lasts throughout life (13).

Immunological tolerance can be induced both by self or non-self antigens, and the condition for tolerance innitiation is the early contact with such antigens, during the maturation of the lymphocytes in central lymphoid organs or after these cells have been exported from the central lymphoid organs to secondary lymphoid organs. Therefore, in order to be initiated, exposure to antigen is necessary, but for its persistence, continued presence of antigen is required (11, 13).

Depending on the origin of the antigen, immunological tolerance occurs in two forms: natural tolerance (to self antigens) and acquired tolerance (to non-self antigens) (12).

Natural tolerance of T and B cells to own antigens is acomplished through several central and peripheral mechanisms that have complementary and synergistic effects, so imperfections of the one mechanism are corrected by the the second one (13). The mechanisms involved are: deletion of self reactive clones, clonal anergy, cell-mediated suppression, and ignorance by the immune system of the antigens from some organs (2, 9, 11, 13).

The concept of acquired immunological tolerance was founded by Sir Frank Macfarlane Burnet and Peter Brian Medawar (Nobel Laureates in Physiology or Medicine in 1960 for this discovery) (534). Based on the clonal selection theory (1), the two researchers have found that mammals that come in contact with non-self antigens during immunological immaturity do not develop immune responses against them.

The purpose of this study was to establish the major T cell populations involved in aquired immunological tolerance to xenogeneic antigens.

Materials and methods

Biologic material. 90 embryonated eggs of Cobb 500 hybrids (S.C. AVE IMPEX SRL, Romania) were used as recipients, and as donors, three ducks.

Antigenic material. Blood sample (5 ml / donor 1) were taken on heparin from the brachial vein. Bone marrow samples (5 ml / donor 2 and 3) were aspirated also on heparin from the medullary cavity of the femur and tibia of the same leg, according to the method previously desribed (10).

Blood and bone marrow samples were processed by centrifugation on Ficoll-density gradient (StemCell Technologies Inc, Vancouver, BC, Canada) (8). Mononuclear cells were labeled with PKH2 dye, according to PKH2 Green fluorescent cell linker mini kit protocol (Sigma Aldrich®, St. Louis, MO, USA).

Embryonated eggs have been subjected to inoculation of xenogeneic cells (blood mononuclear cells and bone marrow mononuclear cells) of donor birds in allantoic sac, at 6th day of incubation (Table 1).

After hatching, the chicks were divided into three experimental groups whose indicative corresponded to that of donor docks, and each experimental group was matched to a control group consisting of an equal number of birds not subject to inoculation of xenogeneic cells during incubation (Table 1).

Table 1

Experimental scheme in concordant xenotransplant (duck to hen)

Embryo annex	Antigenic material used	Donor birds	No. of embrionated eggs	No. of viable chicks	Groups
Allantoic sack	blood mononuclear cells	D1	30	8	E1
	bone marrow mononuclear cells	D2	30	12	E2
		D3	30	14	E3

Mixed chimerism was assessed by flow cytometry, using BD Influx flow cytometer (Becton Dickinson, USA); 14-days-old birds from experimental groups obtained after inoculation of xenogeneic mononuclear cells stained with PKH2 were assessed for the persistence of donor's cells used to induce immunological tolerance;

Donor-recipient compatibility was checked according to Sigma PK Linker protocol for mixed lymphocyte reaction; the test covered all experimental birds and 15 randomly choosed control birds (one week before skin grafts transplant);

Distribution of T cells subsets. Five birds/experimental and control group were assessed using flow cytometry at the age of three weeks. Cells were labeled with monoclonal antibodies as follows: antibodies anti-CD3 to label T lymphocytes, antibodies anti-CD4 for T helper lymphocytes, antibodies anti-CD8 for cytotoxic T cells, antibodies anti-CD45RO for differentiating between memory and naive T cells, antibodies anti-CD28 for differentiating between memory and effector T lymphocytes, and antibodies anti-CD25 for label the eventual activate subset of T cells from subpopulation Treg - regulatory T cells. This labeling served in determining the lymphocytes T profile in experimental and control groups.

Results and discussions

The assessment of the mixed chimerism revealed 1.14-1.33% xenogeneic cells in pheripheral blood of 12.5% of E1 group birds and 21.42% of E3 group birds. Values between 0.21 and 0.75% were obtained in 20% of E1 group birds and, 25% of E2 birds and 21.42% of E5 group birds.

Compatibility test performed on mixed lymphocyte cultures revealed 100% compatibility between donor ducks the recipient birds with more than 0.75% xenogeneic cells in pheripheral blood and complete donor – recipient incompatibility for all the other experimental and control individuals.

After lymphocyte profile analysis using flow cytometry, the lymphocytes subsets were defined as follows: naive $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^-CD28^+$; memory $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^+CD28^+$; effector $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^+CD28^-$; effector $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^-CD28^-$; naive $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^-CD28^+$; memory $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^+CD28^+$; effector $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^+CD28^-$; effector $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^-CD28^-$; regulatory T cells with phenotype $CD3^+CD4^+CD25^+$; and regulatory T cells with phenotype $CD3^+CD8^+CD25^+$.

Naive T cells were represented in a superior proportion in all the individuals of the experimental E1 and control groups the differences being statistically insignificant (Table 2).

Table 2

The average percentage distribution of T cells subsets in birds subjected to inoculation of concordant xenogeneic cells into allantoic sac (6th day of incubation)

Group		Naïve T cells (%)	Effector T cells (%)	Memory T cells (%)	$CD25^+$ T cell (%)*
E1	**X**	**40.66**	**28.54**	**30.79**	**8.65**
	ST	17.62	9.75	7.96	2.24
	CV%	43	34	25	25
C1	**X**	**61.29**	**19.43**	**19.27**	**5.96**
	ST	3.61	1.80	2.05	1.03
	CV%	5	9	10	17
E2	**X**	**20.34**	**42.73**	**36.93**	**11.98**
	ST	3.06	4.65	4.24	1.79
	CV%	15	10	11	14
C2	**X**	**58.82**	**18.26**	**22.92**	**6.43**
	ST	3.42	1.63	2.87	1.54
	CV%	5	8	12	23
E3	**X**	**19.88**	**41.25**	**38.86**	**12.03**
	ST	2.59	5.09	3.97	1.51
	CV%	13	12	10	12
C3	**X**	**62.01**	**17.51**	**20.48**	**6.17**
	ST	5.44	1.37	3.82	1.20
	CV%	7	7	18	19

As shown in Table 2, the birds of experimental groups subjected to the inoculation of xenogeneic cells have significant higher number of memory T cells and $CD25^+$ T cell. Also, the naïve T cells are less represented in experimental birds.

The assessment of lymphocyte subsets mainly aimed to identify the memory T cells (CD25+) with the potential implications in immunological tolerance. This subpopulation, called T regs (regulatory T lymphocytes), is recognized by all

researchers as being involved in delayed rejection of allografts in humans and laboratory animals and the mechanisms of tolerance towards them (3, 4, 6, 7, 14).

The statement of “potential implications in immunological tolerance” associated to CD25+ avian lymphocytes suggests that we cannot fully consider them T regs because they don’t express the FOXP3 marker as their mammalian counterparts. So far, no simmilar molecule was described in birds, and genetic studies have not identified any sequence with sufficient identity to state that it is the FOXP3. Because of this, using the acronym T regs is questionable.

Conclusions

Presence of xenogeneic cells in the peripheral blood of recipient birds, after immunological tolerance induction, is accompanied by a lower proportion of naïve T cells and a higher percentage of memory T cells, especially CD25+ memory T cells

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CLIMATE FACTORS EFFECTS ON THE EVOLUTION OF THE MAIN PHYSICAL PARAMETERS OF RAW MILK OBTAINED ON THE BACKGROUND OF A SUBCARPATHIAN MOUNTAIN AREA

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Summary

The biodiversity of the mountain pastures is in good part insured by the specifics of the climate factors and it is the ground for obtaining traditional dairy products, with a high quality flavor and texture. These qualities significantly increase the opportunities of reevaluating milk and dairy products obtained in mountain side regions. In this study we intend to evaluate the influence of climate factors on the seasonal evolution of the main physical parameters (freezing point, density and pH) of raw milk produced in the climate conditions of the sub-Carpathian mountain region of Valea Gurghiului. The studies were performed on samples of raw milk collected and processed by a commercial society in the south-western part of the Eastern Carpathians. The research consisted in monitoring the seasonal dynamics of temperature, humidity, atmospheric pressure and precipitations levels, in collaboration with a meteorological station in the area, in order to evaluate the influence of these climatic factors on the physical properties of freshness of raw milk. Sample testing was peformed with the Ekomilk M semiautomatic analyzer and the statistic analysis of the obtained data was made using the MedCalc program and the Pearson r. correlation coefficient. The overall analysis of the obtained data underlied the evaluation of the freshness of raw milk, by quantifying the influences the climatic factors had on the freezing point, density and pH of the milk. The statistic analysis of the data values obtained from evaluating the variables in the climatic factors – freezing point/density/pH of the milk relation has provided relevant correlations for the purpose of this study. These correlations have revealed a high degree of freshness of the raw milk, confirmed by medium levels of the freezing point (-0.566°C), density (28.24-28.92 g/L) and pH (6.68), obtained in average conditions of a temperature of 11.5-21°C, humidity of 66-83% and atmospheric pressure of 962.9 mb. We consider that the air's temperature and relative humidity and their evolution are environmental factors that have a major impact on the biodiversity of the mountain pastures and that favorably influence the overall composition of raw milk, especially the evolution of the specific physical characteristics for determining the degree of freshness of the milk.

Key words: freezing point, density, pH, raw milk, mountain biodiversity

In the case of raw milk, the physical properties are indications to assess the freshness for the milk and for the traditional dairy products. Freezing point, density and pH are the main physical indices, reflecting the freshness of raw milk. They are influenced largely by the biodiversity of mountain pastures, which has a major impact on capitalizing traditional cheeses, giving them superior flavor and texture (9). Concerning the quality of milk produced in mountainous areas, it is well known that this is influenced by the evolution of climatic factors with significant impact on the expression of the productive potential of lactating cows (12).

According to current trends the cheese production from raw milk is growing and the consumers' requirements are increasingly high regarding food with traditional character. As is well known, the geo-climatic conditions in mountain areas provide in general a higher level of animal health and welfare, which is reflected in the composition and quality characteristics of obtained productions, including milk has major share (12).

Currently, obtaining sustainable livestock production must be a major challenge for any farmer, where dairy products provide the main link between primary production and the final product. In this regard they have been developed specific practices to this segment of production that aim at supporting farmers to maximize production without affecting the animals' health and welfare (10).

In our country, more and more farmers begin to understand that in order to achieve these desiderates, we need to raise livestock from indigenous breeds which are better adapted to environmental conditions, namely climatic factors, which in the case of lactating cows exert a major impact on their health and thereby the milk produced. They exert their effects by direct action on the animal organism, or indirectly through feed biodiversity, producing significant seasonal variations and individual on the quantity and composition of milk produced (10, 6).

Among climatic factors, significant effects on lactation cow exert temperature, humidity, atmospheric pressure, light, rainfall and altitude. Farmers frequently monitor environmental temperature and humidity because these factors exert a major impact on indices of freshness milk thereof respectively composition and quality (3).

The aim of this paper was to evaluate the influence of temperature, humidity, atmospheric pressure and precipitation as basic climatic factors, the main indices of physical health and freshness of milk obtained in an area of sub-Carpathian mountain biodiversity.

Materials and methods

Conducted research has consisted of monitoring and analysis of the main physical parameters of raw milk (freezing point, density and pH), processed in a processing unit. They were studied samples of raw milk coming from traditional households and commercial dairy farms, predominantly indigenous breeds, kept in conditions of sub-Carpathian mountain areas.

The study began with the identification of milk suppliers. Which have a milk classified in product standards, taken from legislation, supplemented with requirements set by the processing unit. Following assessments carried out in the area of activity of the unit under study, we reported three sources providing milk commodity: small producers (n= 650), micro farms (n = 11) and large farms (n = 2). The milk from small producers has been taken over by the six collecting points, which are organized and managed by processing unit. Concerning micro farms and large farms, using its own cooling tanks, they allowed receiving milk directly from the farm. During a period of 20 months were investigated milk samples, grouped in three samples corresponding to the 3 sources: source first (milk from small producers), second (milk derived from micro) and third (milk from of large farms). Monthly from each source were collected up to 16 milk samples that were subjected to laboratory analysis, totaling finally as 320 tests per source, having a total of 960 samples analyzed. The analysis consisted of physico-chemical testing of milk samples with the analyzer Ekomilk M, including the basic compositional parameters with the determination of the following the physical indicators of milk commodity: freezing point, density and pH. In parallel, it is been resorted also to using the usual methods to verify the acidity (titrimetric method) and the density (the method with thermo-milk-densimeter) of milk. Data from testing milk samples were centralized on each parameter and then processed as average values of three sources of milk commodity. Monitoring climatic parameters have collaborated with weather stations in the area, from which we obtained data on seasonal dynamics of the temperature, humidity, atmospheric pressure and rainfall. These parameters were monitored daily by Meteorological Service Targu-Mures, from which we took over the centralized data and we have statistically processed using MedCalc program for statistics in biomedical research. Data analysis included finally quantify the influence of the main climatic factors on the development freezing point, density and pH of milk commodity. With this purpose we resorted to using Pearson r - correlation coefficient for analysis of the connection between the two variables and the intensity (climatic factor and indicator of milk). Graphical representation of this connection was achieved using dispersion diagram (scatter) which the orientation and dispersion of the points cloud provide the picture of the connection between the two variables.

Results and discussions

Seasonal fluctuations of freezing point depends on climatic factors. Correlations between the freezing point (-0.566^{O}C) and air temperature (21°C), were positive in summer (p = 0.047), showing that the increase of the air temperatures was in the same time with the increase of the freezing point values (Tab.1).

By analyzing the correlation between the freezing point of milk and the humidity of air it showed that the evolution of humidity (66%) had a negative impact

(r=-0.358) and statistically significant (p=0.0001) freezing point (-0.566^{O}C) in summer. In this regard dominants summer period have been decreasing humidity and increasing the freezing point (Fig.1). Regarding the dynamics of pressure and precipitation is noted that these parameters did not influence the freezing point statistically significant in any of the four seasons.

Table 1

The statistical correlations between the freezing point and the climatic parameters in summer season

Correlation freezing point-climatic parameters		
Average air temperature (°C)	Pearson Correlation	**0.161**
	Sig. (2-tailed)	**0.047**
Air moisture (%)	Pearson Correlation	**-0.358**
	Sig. (2-tailed)	**0.0001**
Precipitations (L/m^2)	Pearson Correlation	**-0.265**
	Sig. (2-tailed)	**0.060**
Air pressure (mb)	Pearson Correlation	**-0.024**
	Sig. (2-tailed)	**0.769**

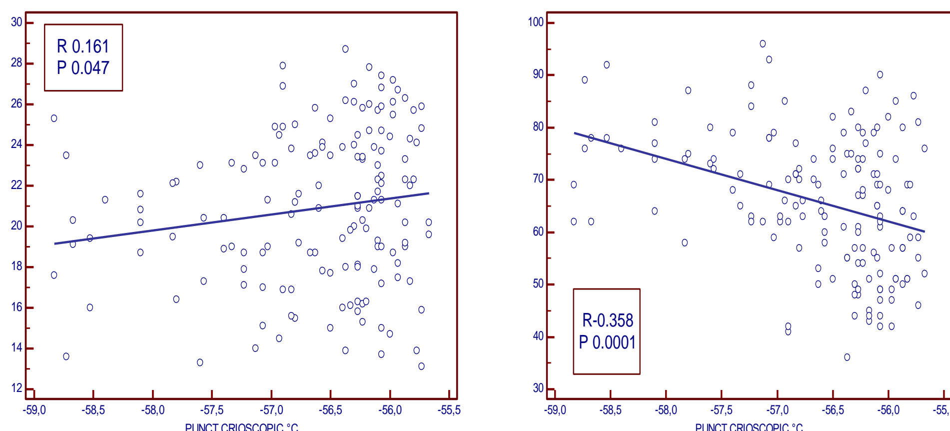

Fig. 1. Correlation between temperature, air humidity and freezing point of milk

Seasonal fluctuations of density is dependent on climatic factors. Statistical analysis of data on milk density and air temperature, revealed positive correlations in autumn (p=0.023), which showed growth of both variables (Fig.2). Comparative in the summer season this correlation was negative (p=0.002) and indicated that the drop in temperature can increase milk density. On the other hand, the evolution of humidity (66-74%) had a positive effect density (28.24-28.92 g/L) in summer (p=0.0001) and autumn (p=0.012), indicating growth of these variables. In the

same context, it was placed and evolution of the winter season, the correlation between these parameters was negative (p = 0.026), indicating that lowering humidity can increase milk density (Fig.3).

Negative influences on milk density (28.91 g/L) has exercised and dynamic air pressure (962.9 mb) in the winter season (p=0.006), indicating that the drop in air pressure can increase this parameter. Unlike in seasons of spring, summer and autumn density was not statistically influenced by the evolution of air humidity (Fig.3). Regarding precipitation should be noted that this factor did not exert relevant influence on the density of the milk in any of the four seasons of the year.

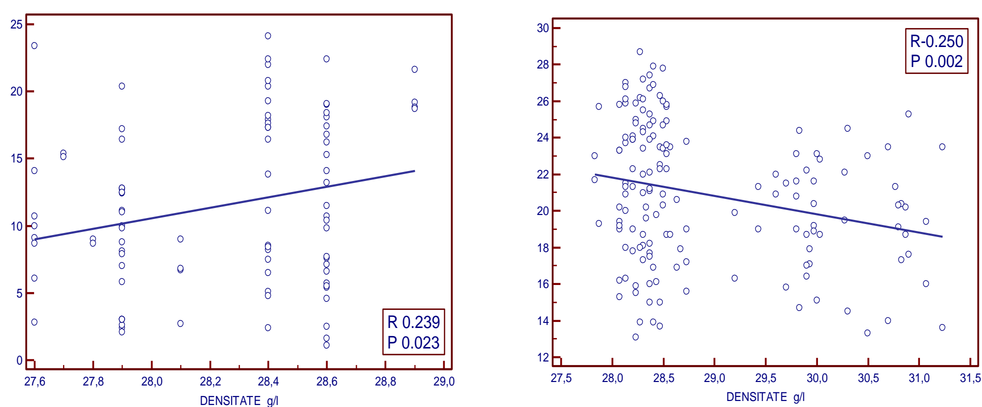

Fig. 2. The correlation between air temperature and milk density

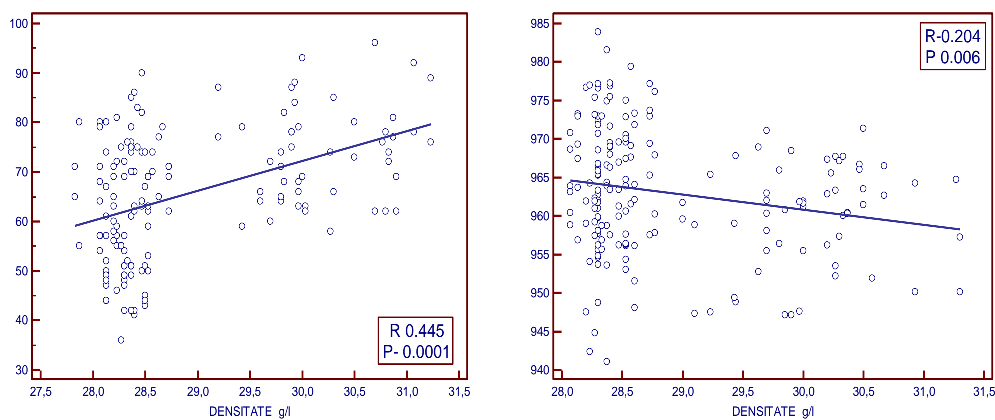

Fig. 3. The correlation between humidity, atmospheric pressure and milk density

Seasonal fluctuations in pH is dependent on climatic factors. Statistical

analysis of data recorded for this parameter revealed negative correlations between air temperature (11.5^{O}C) and pH of milk (6.68) in the autumn season (p=0.042). Following the interpretation of this correlation we concluded that the lowering the temperature determinated the increase of the pH of milk (Fig.4). Regarding the increasing of humidity (66-74%) we found that this climate factor negatively influenced the milk pH (6.68) in summer (p = 0.004) and autumn (p = 0.009), which showed that the decrease of humidity caused an increase of pH (Fig. 4).

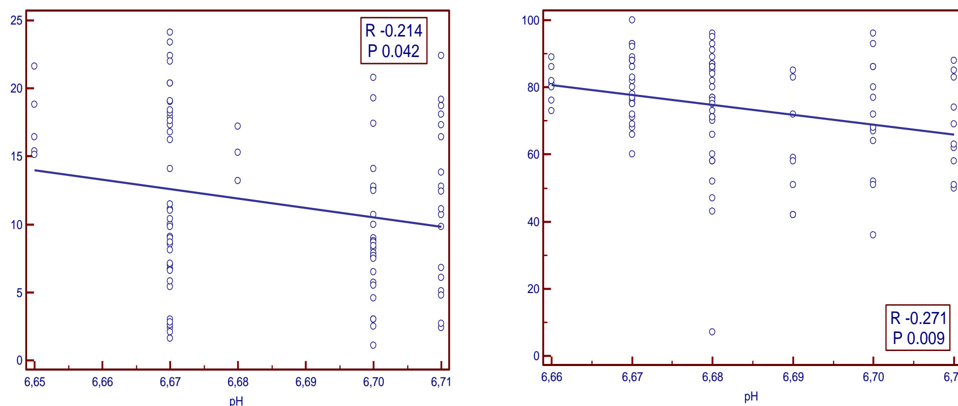

Fig. 4. The correlation between temperature-humidity of air-milk pH

Comparative the temperature evolution has exerted the same influence over pH and density, but only in autumn. In contrast, changes in humidity exerted more important influences that were interested in summer and autumn seasons. Regarding the dynamic air pressure, to mention it is that it exercised significant influence on density only in winter season On the other hand, the dynamic of precipitation did not significantly affect the density developments, the freezing point and pH of milk. Thus, average levels for the freezing point (-0.566^{O}C) density (28.24-28.92 g/L) and pH (6.68) were obtained in terms of average values of temperature 11.5-21°C, of humidity 66-83 % and atmospheric pressure 962.9 mb. Together with the results of other studies in the field, the correlations established by us indicate that the evolution of the content of fat, protein, not fat dry substance and default the whole composition of the milk varies depending on the environmental conditions, the major impact falls on temperature, followed by humidity and atmospheric pressure (13, 6, 8)

In this regard, the latest research focused on the effects of environmental conditions on milk composition, indicated that cows with high milk production are more susceptible to heat stress than cows with low milk production; parity have an important role(7,2). The results obtained to quantify thermal effect on milk production, by investigating climatic factors (temperature, humidity, wind speed and

brightness), revealed decreased milk production due to heat stress (14, 4, 5).

We note also that modern procedures currently used in this segment of production, aimed at reducing the negative effects of climatic factors on the health and performance of animals, although in the intensive systems, respectively in the large farms can not be implemented (1). Therefore, to step up research in this area and implement effective strategies for ensuring the welfare and productive performance of lactating cows (10, 11).

Conclusions

Statistical analysis of the data obtained revealed that oscillations of temperature during the summer-autumn seasons, negatively influenced the evolution of density ($p = 0.002$) and pH ($p = 0.042$) and positively the evolution of freezing point ($p = 0.047$) milk commodity samples investigated. In contrast, humidity dynamics exerted negative influences on both: the freezing point ($p = 0.0001$) and the pH ($p = 0.009$) in the summer season. Negative influences were also from air pressure, who exerted on the evolution of milk density ($p = 0.006$) in the winter season. The dynamics of precipitations and air pressure exerted no significant effect on the evolution of the freezing point and pH of the milk in any of the four seasons.

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ANTIFUNGAL INDICES OF SOME STRAINSOF MICROMYCETES AGAINST MYCOTIC INFECTIONS OF *APIS MELIFERA*

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Summary

The propose of study had served 21 strains of micromycetes from the National Collection of Non-pathogenic Microorganisms from Institute of Microbiology, Academy of science of Moldova and two pathogenic strains – *Aspergillus flavus* and *Aspergillus niger*. The tested strains were isolated from soil samples taken from the central zone of Moldova. As nutrient medium for isolation, of micromycetes served malt agar, Czapek, Sabouraud-Amidono and ammonium. Isolation of strains was performed in thermostat at temperature 28°C, during 14 days. As a result of screening were selected two strains SP.62 and SP. 97 with potential antifungal increased against *Aspergillus flavus* pathogen with higher antifungal actions against petrified offspring of bees. The diameter of inhibition zone *of Aspergillus flavus* was on the strain SP. 62 - 30 mm, and of the strain p. SP. 97 - 35 mm.

Key words starins, micograma, însemination, screening, micromycetes.

Micromycetes are microscopic fungus. They are widespread in environment, live in air, water, soil, in habitation of people, etc. Separately each fungus is invisible with the naked eye, but on surfaces we can observe them in the form of a mold – spots or raids with a certain smell and color. These are the same microorganisms, as well as bacteria, they only differ in more difficult structure (1,4).

There are different classifications of micromycetes. Some scientists refer to them yeast, which are widely used in the food industry, others consider them a separate type of microorganisms. The famous representative of micromycetes is mold fungus – penicillium. Thus, many types of microscopic fungus are widely used by the person in the food industry, economy and medicine. But there are also other micromycetes which do a lot of harm to environment and with the person tries to fight in every possible way.

According to many authors filamentous fungi are very common ones in nature, with a major concentration in soil, especially in the top layer of the soil which ensures conditions for growth and survival. Fungal spores are found frequently in surface of plants, in the digestive tract, especially the herbivores in the food industry bio decomposition activity is unwelcome because the fungal causing loss of seed foods.

As a side effect is the formation of mycotoxins by some molds that foods become unusable. The molds are the aerobic microorganisms therefore require the presence of oxygen for growth in air or dissolved oxygen in liquid medium. Molds

processes are possible if there is empty and are produced by species of the genus *Penicillium, Aspergillus, Phyalophora*, etc. Molds can be formed on the surface of the juice and may release coloring substances or degraded natural pigments the juice or other natural products. *Micromycetes* supports the great amounts of tanning substances, often associated with woody vegetation, realizing their debris degradation. For this reason, bring their mushrooms in the formation of organic soils of the party at the same time, having a special role within trophic chains (3).

Currently *Micromycetes* are widely used in producing of biologically active substances used in the production of additives for stimulating the immune system, and some of them as products with antibacterial effect. The importance of pharmaceutical products obtained with the help of microorganisms can mention: antibiotics, amino acids and enzymes with therapeutic role, hormones, vaccines, interferon, interleukins, bacteriocine, alkaloids, and vitamins. Of substances derived from filamentous fungal are vitamin B and ergosterol-provitamine D.

Taking into account the above mentioned the purpose of study served 21 strains of *Micromycetes* from the National Collection of Non-pathogenic Microorganisms from Institute of Microbiology, Republic of Moldova. The objectives of the investigations was a screening of some strains of micromycetes from different regions of the soil with antifungal action against pathogenic fungals which causes mycotic diseases in bees; appreciation of inhibition properties of some fungal strains producers of pathogenic strains toward 2-*Aspergillus flavus, Aspergillus niger*; establishing the possibility of use of fungal strains with antifungal action in some diseases in honey bees (2, 5).

Materials and methods

The study materials served 21 strains of *Micromycetes* from the National Collection of Nonpathogenic Microorganisms. Tested strains were isolated from soil samples taken from the central zone of Moldova. As a test crop were used 2 strains of fungi: *Aspergillus flavus* and *Aspergillus niger*, pathogens of Asspergilosis (petrified offspring) to bees, which were isolated from samples taken from a hive of bees. Antimicrobial properties of micromycetes have been studied according to the diffusion method by using Agar blocks (2, 3). The method is based on the ability of diffusion of metabolites produced microorganisms studied in depth of agar and of the action of the active substance diffusion area on the test-crop. To perform the experiment of fungal strains to be tested were grown for 4 days on malt agar medium at a temperature of 28-30°C. The diameter of the zone of inhibition of infection agent was measured after 4 days of cultivation at a temperature of 28-30 °C on the malt-agar strains pathogenic on Petri plates in volume were introduced with agar blocks of tested micromycetes. Morpho-cultural study of fungal strains with antifungal activity increased potential has been conducted examining the visual and microscopic strains grown on four nutrient medium: Malt agar; Czapek;

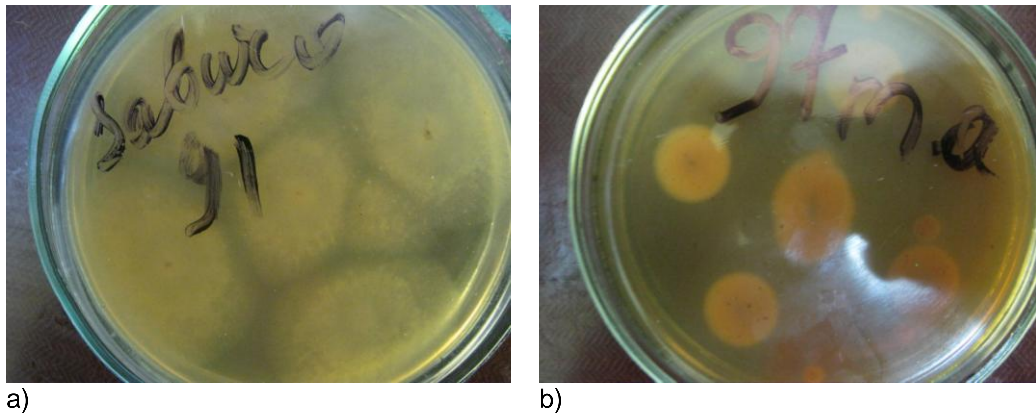
Sabouraud - amidono and ammonium (4). The investigations were carried out after 4; 7 and 14 days of cultivation at temperature 28-30 ° C (5).

Results and discussions

This study was conducted in the laboratory of non-pathogenic micro-organisms of Academy of science of Moldova. In the images below are presented the stages of research (fig. 1 a, b).

a) b)

Fig.1 a) Collection of the pathological material from bee family, b) pleasing of the blokes of micromycetes on environmental mediums

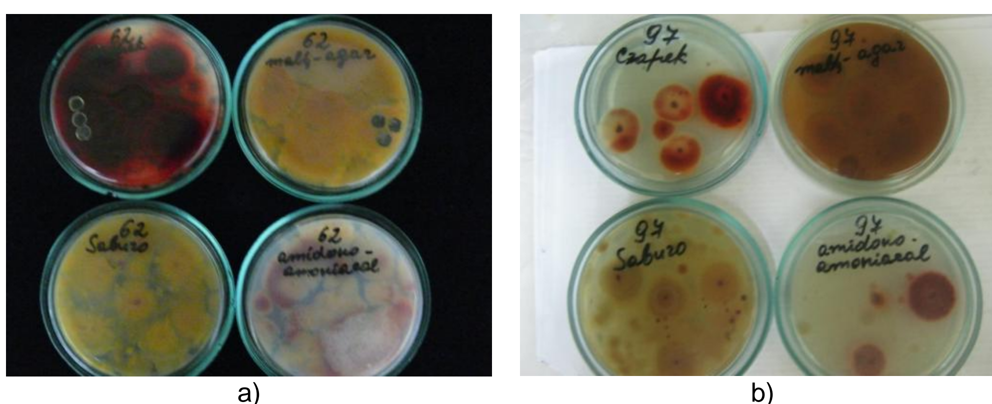


a) b)

Fig. 2 a) Fungal colonies on Saburo environment;
b) fungal colonies on malt agar environment

The pathologic material for laboratory investigation was collected from bee family of the pandan of the Academy of Sciences of Moldova (fig.1, a) and sended in laboratory for investigation (fig. 1, b). For isolation of micromyceters was used the malt agar; Czapek; Sabouraud-amidono and ammonium nutrient mediums.

The diameter of fungal colony (fig.2 a) has -1.4 – 1.5 cm, with green-dark color and irregular shape, wavy profile, with a white border up to 3 cm. In the situation of inoculation on the malt agart, the diameter of the colony was 2.4 – 2.5 cm, with dark green color, very smooth and with round or free-form mycelium and flat profile, greenish pink toward to the center.



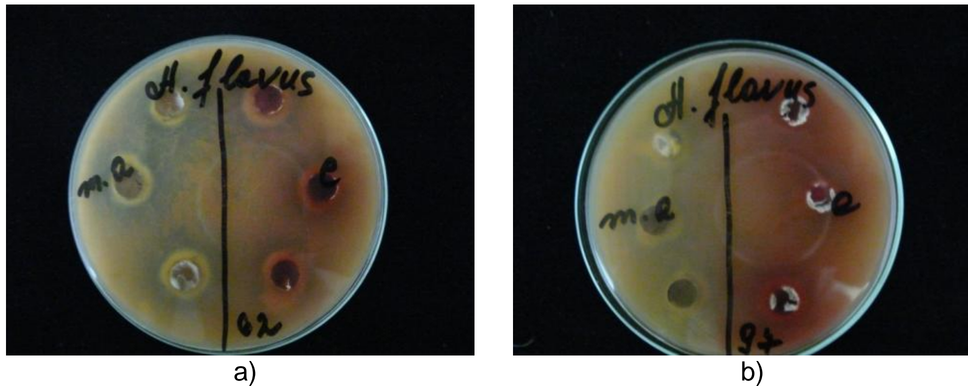
a) b)

Fig. 3. Colony appearance of p. SP. 62 and p. SP 97 after 14 days of cultivation in different environments: a) p. SP. 62; b) p. SP 97

Diameter size of the colony was 2.5 – 3.0 cm, with dark – green color, very sporulated placement, with round or oval shape. On rivers side, more intense color is pink. The morphological structure of colonies are presented with fluffy colonies that differ by type of nutrient medium and period if incubation.

The strain p.sp.62 is growing and developing very well on all four tested cultivation medium. For this strain, malt-agar and Czapek environments are more favorable for cultivation as it grows, with faster sporulation. On these cultivatiing media the maximum diameter of the colony was 3.5 – 4.0 cm, as well as an increased sporulation. Taking into account that the malt-agar and Czapek media are more conducive to growth these experiments were carried out in order to determine the antifungal activity of the strain grown on these culture media. The strain p. SP 97 colony diameter was - 2,3 -2,5 cm, with dark green, rough, irregular shape, flat profile, wavy edge. Border yellow - red with diameter 1cm.

As shown in fig. 3 a) and b) it can confirm that, in order to obtain exzomethabolitics proprieties to combat the pathogen *Aspergillus flavus*, antifungal strains p.sp. 62 and p.sp. 97 have grown better on malt-agar medium.



a) b)

Fig. 4. Areas of inhibition zones to the pathogen *Aspergillus flavus* of the action of exomethabolits of micromycetes: a) - p.sp.62 and b) - p.sp.97.

In fig. 4 a) and b) there are the results on the inhibition of the growth of colonies of pathogen *Aspergillus flavus* development under the action of blocks of micromycetes. The diameter of the blocks of culture of micromycetes had the varies of inhibition zones of micromycete strain p. SP 62 up 25.8 to 4.2 cm, and of the micromycete strain p. SP 97 up 22, 0 to 3,2cm .

Conclusions

Following the screening performed in 21 fungal strains were selected two cultures of micromicetes (p.sp.62 and p.sp. 97) which demonstrated the increasing antifungal potential on Aspergillus flavus, pathogens of aspergillosis (petrified brood) in bee.

Diameter of inhibition area on pathogen *Aspergillus flavus* under the action of micromycete exometabolits of strain p.SP. 62 was around 30 mm and of strain p. SP.97 – 35 mm.

The optimal cultivating media for growing strains p.SP.62 and p. SP. 97 in order to obtain enhance exometabolits with antifungal properties is malt-agar medium.

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RESEARCH REGARDING THE EPIDEMIOLOGY OF BLUETONGUE DISEASE IN TIMIS COUNTY

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Summary

The purpose of this study was to establish the evolution, spreading and prevalence of Bluetongue disease in sheep and cattle in the Timis County during 2014. A total of 1390 susceptible animals (134 sheep and 1256 cattle) from 42 localities of Timis County were examined using the competitive ELISA. The immunoenzymatic assay confirmed the presence of antibodies against VP7 protein of Bluetongue virus for 9 cattle and 74 sheep. The prevalence of Bluetongue disease in Timis County, for a total of 44,046 cattle and 826,603 sheep was 0.02%, respectively 0.008%. Bluetongue disease evolution was one centered on two major outbreaks, on the Eastern and Southern area of the County, noting that most of the positive cases were recorded in Southern outbreak (6 cattle and 51 sheep).
Key words: Bluetongue disease, ELISA, prevalence, Timis County

Bluetongue disease is caused by Bluetongue virus (BTV), a member of *Orbivirus* genus and *Reoviridae* family.

Bluetongue was first described in the mid-nineteenth century by French biologist Francois Le Vaillant, during a trip to the Cape of Good Hope. After 40 years, Duncan Hutcheon, the official veterinarian at Cape of Good Hope, has noted that the disease is transmited by insect vectors (11).

In 1905, Spreull describes a typical case of disease, which at that time was known as "fever", but the clinical manifestation in this case started with hyperthermia, followed in 7-10 days by characteristic mouth lesions and tongue cyanosis. After this episode, the disease was known as Bluetonguedisease (6, 11). In 1906, Theiller succeeds in his attemps to isolate the virus and prepares an inactivated vaccine that has been used for a long time prevent the disease (9).

Bluetongue has been considered a disease that develops only in Africa until 1943, when it was reported the first outbreak in Cyprus. Subsequently, the disease was reported in Turkey, Israel, U.S.A., Portugal (1950), Spain (1950), Pakistan, Australia (1978). In 1996 there were reported cases of the disease in sheep and cows in Belgium, Germany, the Netherlands and Luxembourg (5).

It is now accepted that the disease affects domestic (sheep, goats, cattle) and wild ruminants (buffalo, deer, several species of African antelope) and some species of the order Artiodactyla ie camelids (camel, alpaca, guano and vicuna). The horses or pigs are resistant. Although the sheep are most severely affected, cattle are the main reservoir of the virus and are very important in the epidemiology of the disease (1, 12).

Culicoides imicola is one of vector species involved in the transmission of BTV in Africa, Asia and Southern Europe, and this species moved gradually toward the Northern regions of Europe, possibly due to climate changes (7, 10), which could have contributed to the increased incidence Bluetongue disease in these areas. However, some recent outbreaks of Bluetongue disease were reported in areas where *C. imicola* is not present, suggesting the involvement of other vector species. *C. obsoletus* and *C. pulicaris* species are found in large numbers in Northern and Central Europe, and are considered vectors of BTV too (2-4, 7, 8). In August 2006 Bluetongue disease outbreaks have occurred in the Netherlands, Belgium, Germany, Luxembourg and North-eastern regions of France, with 5 degrees further north, confirming again that BTV can be transmitted in the absence of *C. imicola* (11).

In 2014, Bluetongue diseasse was reported for the first time in our country in a number of Counties, including Timis County. This paper aims to establish the evolution, spreading and prevalence of Bluetongue disease in sheep and cattle in the Timis County during 2014.

Materials and methods

Animals examined. According to the Methodological regulation of the *Program for surveillance, prevention, control and eradication of animal diseases, those transmitted from animals to humans, animal and environmental protection, identification and registration of cattle, swine, sheep, goats and horses* of ANSVSA approved by Order no. 29/2014 (13), a total of 1390 susceptible animals (134 sheep and 1256 cattle) were examined in 42 localities of Timis County.

ELISA. Serum samples were assessed by competitive ELISA, using the Bluetongue Virus (BTV) Antibody Test (IDEXX Laboratories, Inc., Netherlands). This kit facilitates the detection of specific antibodies directed against VP7 of BTV in individual serum and plasma samples from cattle, sheep and goats.

Briefly, in the microplate wells coated with recombinant protein VP7 the dilluted srum samples were added and incubated. Immediately after the incubation the conjugate was added. In the presence of the immune complexes of VP7-serum antibodies, the conjugate is prevented from binding the VP7epitopes. In the absence of anti-VP7 antibodies in the sample, the conjugate is free to bind the corresponding epitopes. Unbound conjugate was washed and the enzyme substrate was added. In the presence of the enzyme substrate was oxidized and a blue compound developed, which turned yellow after blocking. The color of the

compound is inversely proportional to the amount of anti-VP7 of the test sample. The microplates were read at 450 nm using Tecan Sunrise microplate reader (Tecan Group Ltd., Austria).

Calculating the average OD of negative control serum

$$\text{Average OD of negative control} = \frac{OD1_{450} + OD2_{450}}{2}$$

Calculating the OD for tested smples

$$S/N\% = \frac{\text{OD Sample}_{450}}{\text{average OD of negative control}}$$

Interpretation of the results. Samples with S/N% greater than or equal to 80% are considered negative for the presence of antibodies against Bluetongue virus. Samples with S/N% greater than 70% but less than 80% are considered doubtful and must be retested. Samples with S/N% less than or equal to 70% are considered positive.

Results and discussions

The competitive ELISA test on serum samples obtained from 1,256 cattle confirmed the presence of antibodies against Bluetongue virus VP7 protein for nine animals, in five localities of Timis County (Fig. 1). They represent 0.71% of all cattle assessed.

Compared to the cattle situation, ELISA technique allowed the detection of a greater number of positive sheep (n = 74) of 134 suspected animals (56.92%) (Fig. 2).

The prevalence of Bluetongue disease in Timis County in 2014, for a total of 44,046 cattle and 826,603 sheep was 0.02%, respectively 0.008% (Fig. 3).

Bluetongue disease evolution in Timis County was one centered on two major outbreaks, on the Eastern and Southern area of the County (Fig. 4), noting that most positive cases were recorded in Southern outbreak (6 cattle and 51 sheep).

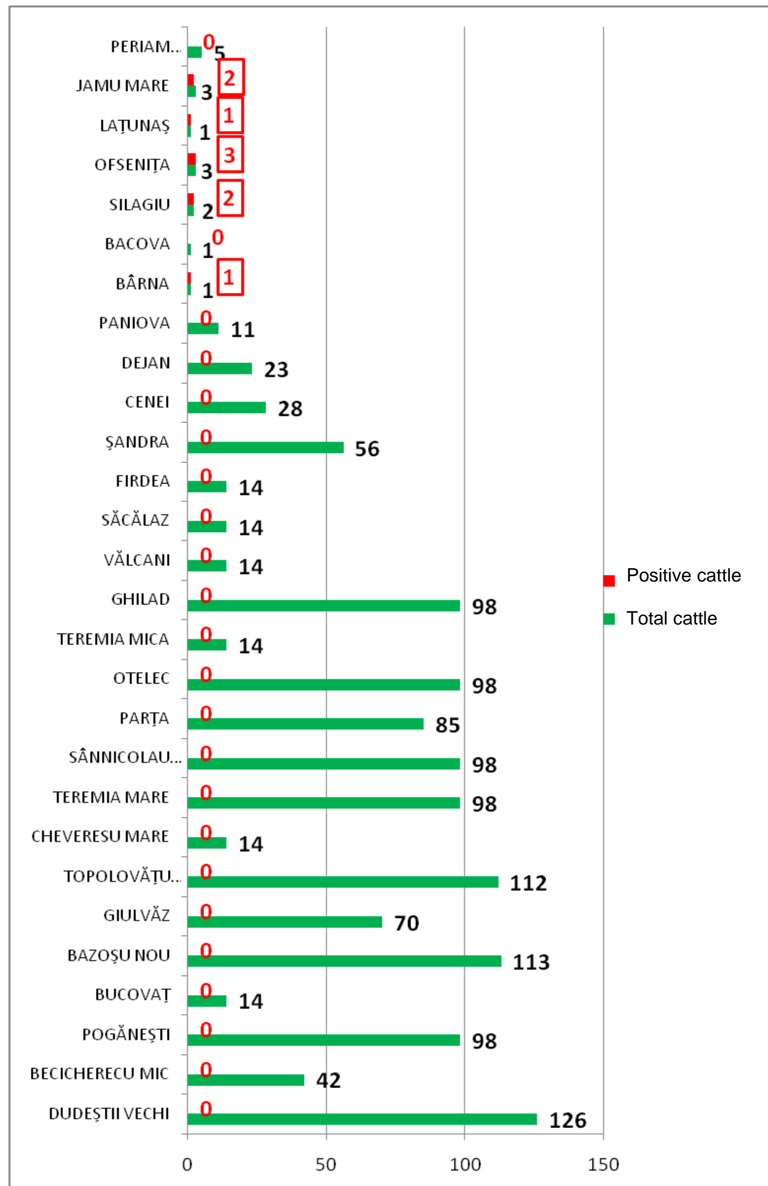

Fig. 1. The number of cattle positive for BTV detected by ELISA in correlation with the total number of cattle examined in Timis County

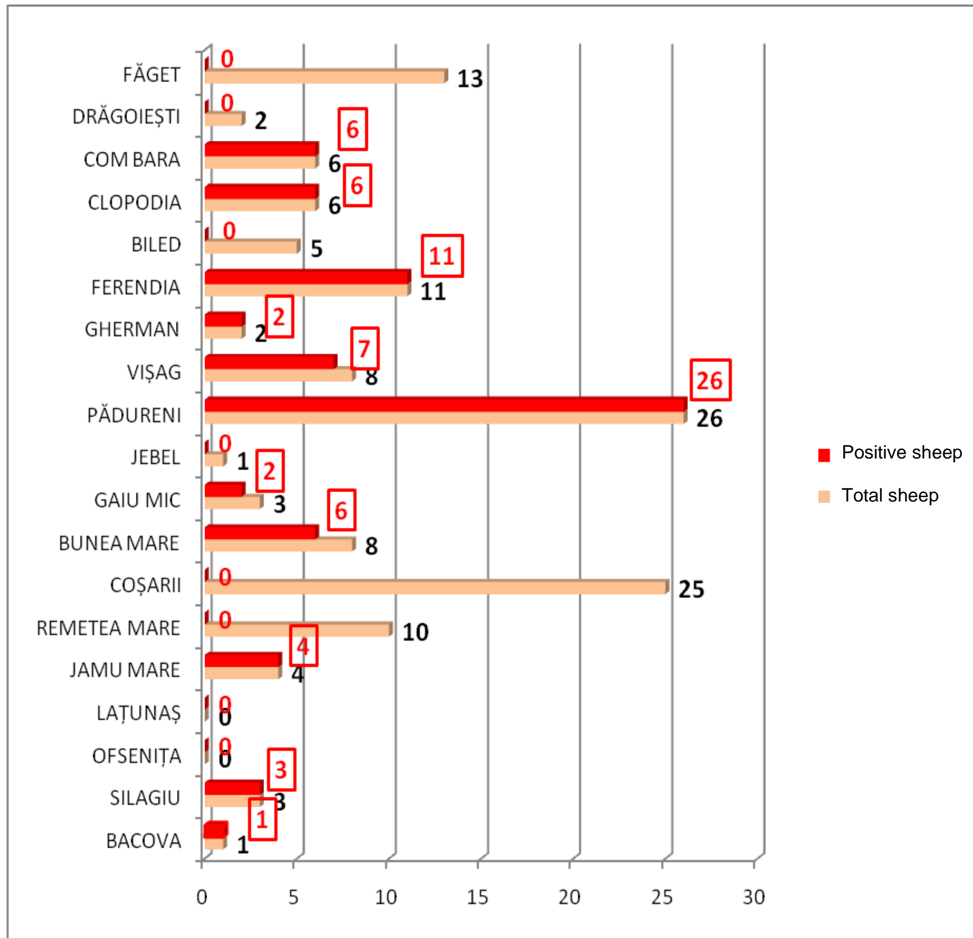

Fig. 2. The number of sheep positive for BTV detected by ELISA in correlation with the total number of sheep examined in Timis County

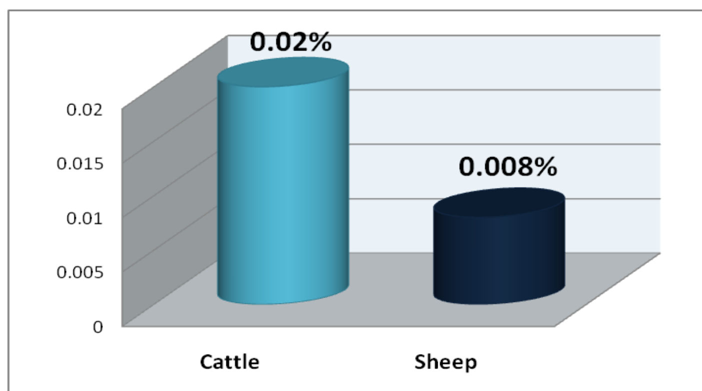

Fig. 3. Prevalence of Bluetongue disease in Timis County

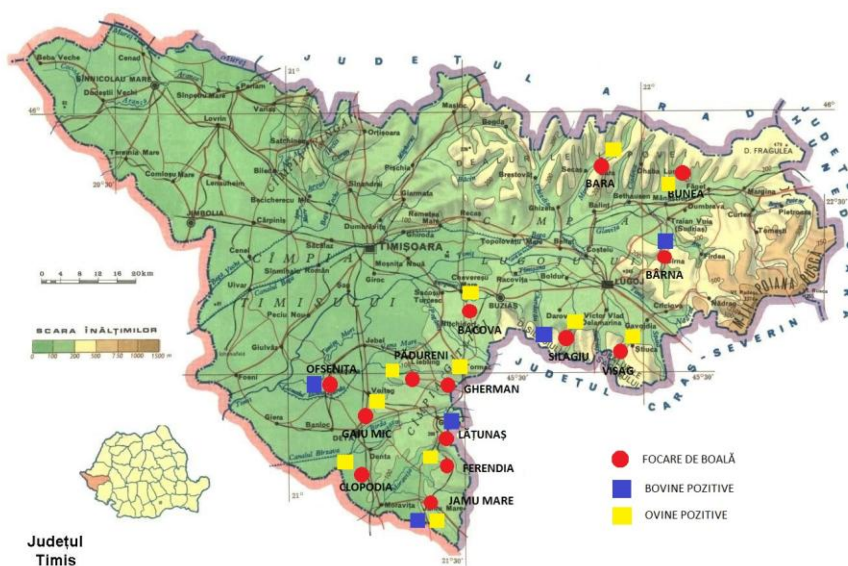

Fig. 4. Bluetongue disease outbreaks in Timis County during 2014

It should also be noted that most of the localities affected by this disease were located near the border with Caras-Severin County (7 out of 13 localities with positive animals), County where, according to ANSVSA reports published on "Weekly Disease Information" of OIE, were detected as positive for BTV a very large number of sheep (fig. 5).

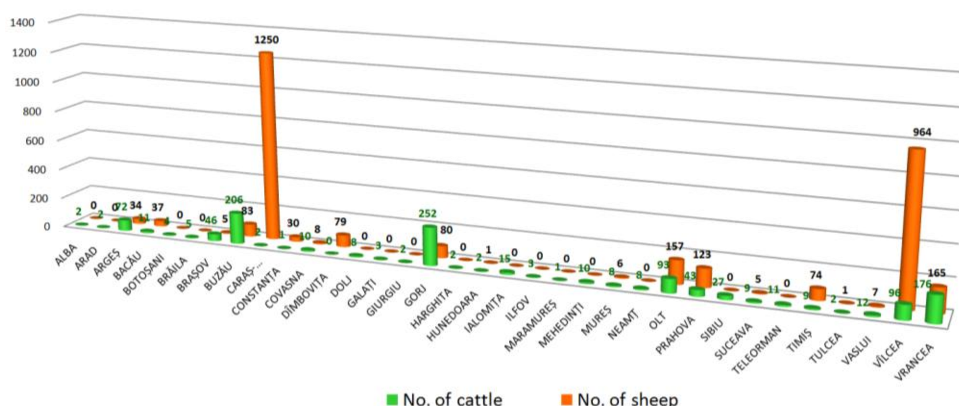

Fig. 5. The Counties and the number of Bluetongue disease cases reported in our country during 2014 (14)

Geographic distribution of the outbreaks in Timis County, and of the majority of outbreaks in neighboring countries - Hungary at the border with Romania (positive animals - 10 sheep and 18 cattle) and Serbia at the border with Bulgaria and the Caras-Severin and Timis Countie of our country (positive animals - 984 sheep and 167 cattle) - confirms Bluetongue disease spread from South to North and from East to West.

Conclusions

During the first outbreak of Bluetongue disease in Timis County were reported 9 positive cattle and 74 positive sheep out of 1390 susceptible animals tested in 2014.

The prevalence of Bluetongue disease in Timis County in 2014, for a total of 44,046 cattle and 826,603 sheep was 0.02%, respectively 0.008%.

Bluetongue disease evolution in Timis County was one centered on two major outbreaks, on the Eastern and Southern area of the County.

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IMMUNOMODULATORY EFFECT OF A *PLANTAGO MAJOR* WATER EXTRACT IN MICE

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Summary

Plantago major L. is a species in the family *Plantaginaceae*, well known for its wound healing, antiinflamatory, antioxidant, antibiotic, antitumoral, modulator of the cell-mediated immunity, hematopoietic effects, being used in traditional medicine for centuries. The aim of this study was to demonstrate the immunomodulatory effect of *Plantago major* water extract after vaccination against *Pasteurella multocida* and the experimental infection with a *Pasteurella* strain pathogenic for mice. Inoculation of the Plantago major water extract before experimental infection led to a reduced mortality and stimulation of the leukocyte proliferation, reversing the neutrophils-lymphocytes ratio.

Key-words: *Plantago major*, immunomodulation, mice

Common plantain or Broadleaf plantain (*Plantago major* L.) is a species of flowering plant in the family *Plantaginaceae.* It is a common plant that grows worldwide in temperate zones, thought to be of Eurasian origin and now naturalized throughout the world.

The Common plantain has been used for centuries in traditional medicine to treat diseases of the digestive tract, blood, inflammations, wounds, tumors. The active ingredients in the *Plantago major* leaves and seeds are polysaccharides, fat, caffein acid derivatives, flavonoids, glycosides, irinoid and terpenoids (6), and their main effects are wound healing (6, 7, 9), antiinflamatory (3), antioxidant (2), antibiotic (4), antitumoral (5), modulator of the cell-mediated immunity (1), hematopoietic (8), etc. Also, total leaves extracts and phenolic compounds of them possess antiviral activities, showing anti-herpes and anti-adenoviral activities (1).

The purpose of this study was to demonstrate the immunomodulatory effect of *Plantago major* water extract after vaccination against *Pasteurella*

multocida and the experimental infection with a *Pasteurella* strain pathogenic for mice.

Materials and methods

Plantago major extract was obtained from fresh plants collected in Timis County. The water extract was prepared mixing 1 g of leaves powder with 10 ml of boiling bidistiled water. The extract was filtered and the solvent was removed using a vacuum evaporator, then before the inoculation to animals, was disolved in phosphate buffer saline obtaining the working concentration of 1000 µg in 0.2 ml PBS.

The experiment was carried out on 80 white mice (20-22 g), aged 30 days, divided into 3 groups and treated with (Table 1):

- *Plantago major* extract – 1 mg / animal, inoculated intraperitoneally in a volume of 0.2 ml, twice, in day 1 and 14 of the experiment;
- *Pasteurella multocida* vaccine - 0.2 ml / animal, administered twice, in day 1 and 14 of the experiment (Table 1).

Table 1

Experimental scheme

Group	No. of animals	*Plantago major* extract	*Pasteurella* vaccine
C (control)	10	-	-
E1 (experimental)	10	-	+
E2 (experimental)	10	+	+

At the 21st day of the experiment, the mice were challenged with *Pasteurella multocida* (strain T-611, serotype B, concentration of 10^8 bacteria/ml), in a volume of 0.2 ml/animal.

At the 28th day of the experiment and before that for the dead animals, the blood samples were collected and the mice were killed by cervical dislocation. The spleen, thymus and mesenteric lymph nodes were collected and and weighed.

The assessment of immunomodulatory effect of *Plantago major* extract has considered the following:

- resistance to experimental infection with *Pasteurella multocida*;
- changes in white blood cells count;
- quantitative changes of primary and secondary lymphoid organs.

Results and discussions

The results of the resistance to *Pasteurella multocida* challenge experiment are presented in Table 2.

Table 2

The results of resistance to infection test

Group	No. of animals	Mortality	
		No.	%
C	20	20	100
E1	20	1	5
E2	20	0	0

The mortality was 100% in control group (untreated), only 5% in the vaccinated group (E1 – *Pasteurella vaccine*) and 0% in animals of E2 group.

Comparison of the average weight of central and pheripheral lymphoid organs (Table 3) has shown a significant difference ($p \leq 0.05$) between the E2 group (vaccination + *Plantago major* extract) and the other groups only in spleen case. The mesenteric lymph nodes weight was lower in animals of goup E2 than in the animals of group C and E1 (vaccinated), and the thymus weight of E2 group was lower than in E1 group.

Table 3

Average weight of central and peripheral lymphoid organs

Group		Thymus (g)	Spleen (g)	Mesenteric lymph nodes (g)
C	**X**	0.2067	0.1673	0.0721
	SD	0.022	0.012	0.005
	CV	10.74	7.34	8.23
E1	**X**	0.2508	0.1980	0.0789
	SD	0.071	0.021	0.009
	CV	28.53	10.8	11.40
E2	**X**	0.2445	0.2213	0.0656
	SD	0.042	0.048	0.0013
	CV	17.17	22.11	20.45

As shown in Table 4, the *Plantago major* extract has a significant effect on the total number of white cells and lymphocytes ($p \leq 0.05$). The extract stimulates the proliferation of the cells involved in specific immune response, characterized by the reversing the ratio of neutrophils and lymphocytes.

The immunomodulatory effect of various *Plantago major* extracts was previously reported in literature both in vitro and in vivo. Chiang et al. (1) demonstrated the stimulative effect of hot water *Plantago major* extract on lymphocites proliferation and interferon-gamma production. Velasco-Lezama et al. (8) showed the hematopoietic activity of methanol extracts *in vitro*.

Table 4

White blood cells count

Group		Leukocytes mm^3	Of which				
			Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
C	X	8301	4163	79.6	88.2	3589.4	288.6
	SD	944.8	1138	29.6	20.2	327	135.4
	CV	11.38	27.34	16.51	22.93	9.11	46.90
E1	X	8875	2982	200.3	89.7	5241	359.3
	SD	1293.9	583.1	35.6	13.6	670.9	120.1
	CV	14.57	19.55	17.81	15.20	12.80	33.54
E2	X	9995	3363	287	85.1	5881	365
	SD	1035.7	618.9	41.9	19.6	417.5	50.8
	CV	10.40	18.40	14.62	23.11	7.10	13.92

The immunomodulatory effect of various *Plantago major* extracts was previously reported in literature both in vitro and in vivo. Chiang et al. (1) demonstrated the stimulative effect of hot water *Plantago major* extract on lymphocites proliferation and interferon-gamma production. Velasco-Lezama et al. (8) showed the hematopoietic activity of methanol extracts *in vitro*.

Conclusions

Plantago major water extract stimulates the immune response after vaccination against *Pasteurella multocida* in mice, enhancing also the protective effect of inactivated vaccine.

The water extract stimulates the proliferation of lymphocytes, reversing the neutrophils – lymphocytes ratio.

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IN VITRO BLAST TRANSFORMATION AND LEUKOCYTE MIGRATION INHIBITION TESTS AND THEIR DIAGNIOSTIC VALUE IN BOVINE TUBERCULOSIS

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Summary

Rapid and accurate diagnosis of bovine tuberculosis (BT)represents one of the most important feaatures of the disease allowing epidemiological control and elimination of zoonotic risk. As well known, cell-mediated immunity is the background of the immune response, therefore one would expect alterations of blast transformation and leukocyte migration in infected animals.

The investigations were carried out on a BT positive farm, classified based on tuberculinic skin test, on blood samples collected on heparine (50 IU/ml), from both positive and negative animals. The blood was subjected to the *in vitro* leukocyte blast transformation and leukocyte migration inhibition tests. The leukocyte blast transformation involved the dilution of the blood sample 1:4 with RPMI 1640 and subsequent cultivation in 96 well plates, using aditionally PHA M, bovine and avian tuberculin and incubation time of 62 h at 37^0 C. The cell growth was monitored by orto-toluidine glucose consumption test. For the leukocyte migration inhibition, the blood was centifuged in density gradient at 1500 rpm, the leukocytes were washed in RPMI 1640 three times by centrifugation (800 rpm) and the final deposit was harvested, diluted with RPMI and placed in capilary tubes. The leukocytes were allowed to migrate for 18h at 37^0 C in migration chambers against RPMI with addition of bovine and avian tuberculin and migration area diameters were measured.

The spontaneous blast transformation index (%) was significantly ($p<0.05$) increased (20.23±11.08) in the positive group, when compared to both bovine (0.886±13.62) and avian (3.12±10.86) tuberculins, with a very high variability of the individual values. There were no statistically significant differences between the BT positive (1.06 ±0.881mm) and negative (1.65±1.36) groups for the leukocyte migration inhibition test.

These results rather suggest the complementary value of the two laboratory tests then their certainty diagnostic value.

Key words: bovine tuberculosis, blast transformation, leukocyte migration inhibition diagnosis

Increasing of worldwide interest to infection with *Mycobacterium bovis* is due to the imminent danger of disease transmission to humans and to the adverse consequences in health and productivity of animals, in breeding and meat animal trades from areas where the etiologic agent of TB is present (18.21). In addition to bovines, tuberculosis affects other domestic animals and wildlife worldwide with annual losses to agriculture estimated at $ 3 billion (1, 10, 25).

Efforts that included milk pasteurization, intradermal testing and subsequent removal of reagent animals led to a significant reduction in bovine tuberculosis and to the recognition of a number of countries in the European Union (EU) as free of the disease, in accordance with Directive 64/432/EEC of the Council (7). It is believed that the proportion of human tuberculosis due to *M. bovis* is less than 2% in countries with official TB control program (12, 13).

Today, the standard method for detection of tuberculosis and its eradication in several regions of the world is represented by the tuberculin test (single and comparative test) (4, 17). These are based on the interpretation of local reaction emerging in infected animals following intradermal inoculation of PPD and reading at 72 hours (when the maximum intensity of specific response is recorded). In spite of the wide use, there is clear evidence of reduced sensitivity of this test, registering false negative reactions (when testing was performed during pre-alergic period in states of chronic malnutrition, especially with deficiencies of protein, in case of conditions that induce temporary or lasting eclipse of cutaneous response to PPD, in the presence of different circumstances that induce patent depression and sustainable cell-mediated immunity such as HIV in humans, in treatment with corticosteroids or other immunosuppressive drugs) (19, 23) or false positives (in advanced pregnancy, in case of endoparasitism: fasciolosis, hydatidosis, linguatulosis). False positive results can have an important economic impact in the field of the eradication programs, due to the unnecessary slaughter of healthy animals. These may undermine farmers' confidence in the program, while false negative results will maintain the infection in the herd (2). Thus, their detection is of paramount importance, especially as it seeks to reduce global prevalence of tuberculosis in the herd of cattle (5,14). Aside from these drawbacks, the costs for control and eradication programs should be considered (in 2008 and 2009 UK has allocated more than £ 100 million annually for this operation) (11).

On the other hand, the intradermal skin test with tuberculin interferes with the immunological status of the animal, requiring a period of 60 days for the repeating of the test. Therefore, in recent decades, significant efforts have been made to improve the means of tracing cattle infected with *M. bovis* and immunological tests fall into this context (6).

Material and methods

Investigations were carried out in a unit with declared bovine tuberculosis, on a total of 23 animals. All animals underwent simultaneous comparative testing and were then divided as follows: group positive -15 and group negative -8.

Laboratory examinations were performed on blood samples collected on heparin in quantities of 50 IU/ml.

The test of blast transformation was conducted in RPMI 1640 medium, in 96-well plates, using the mitogen PHA M, bovine tuberculin (TB) and avian

tuberculin (TA). Incubation lasted for 62 hours and the assessment of cell growth was performed by determination of residual glucose (o-toluidine test).

For inhibiting the migration of leukocytes, the blood centrifuged in combination with RPMI medium was incubated for 18 hours at 37°C, after which the diameters of the zones of migration were measured. Inhibition parameters were calculated reporting the diameter of the migration zone to that of the control.

Results and discussions

TB is monitored by Directive 2003/99/EC (8), as *M. bovis* is a zoonotic agent (24). Tuberculosis is considered a "re-emerging disease" in several EU countries which are considered officially free (22), leading to the discussion on reintroducing skin tests as part of an effective control (20). Although OIE identifies intradermal tuberculin test as a primary screening test for tuberculosis in cattle (15), the frequent cases of errors in skin allergic response led to identifying and comparing diagnostic tests with immunological background in cattle and humans which may bring to the development of scientific support for the implementation of an effective program to eradicate the disease. Understanding the immune system as a whole determines the understanding of the normal function or alteration in the complex web of immunity.

The literature reports the intensity of lymphocytic blast transformation in animals experimentally infected with mycobacteria (typical and atypical tuberculosis) using stimulants. The intensity of blast transformation decreased 2 to 3.5 times if comparing typical mycobacterium inoculation with the result obtained by inoculating the atypical mycobacteria using PPD as a stimulating agent. These results are superimposable over that obtained during the monitoring of the activity of T-lymphocytes in humans and bovine tuberculosis (3). The identification of lymphocytes subpopulations that are involved in a strong cell-mediated response is not secondary. In this context, classical mitogen-induced suppression by mitogens such as concanavalin A, in the case of individuals infected with *M. tuberculosis* (9) or in reduced activity interpretable using rosetting tests E may be highlighted.

Lymphocyte proliferative response to mitogens can be assessed by blast transformation test, implied for measuring cell reactivity. T_H cells (CD + 4), sensitized to antigen, can directly activate the suppressor cell precursors, antigen specific (16). The process is accompanied by metabolic activation and increase in protein synthesis reflected by the increased use of amino acids and reducing sugar in the medium. A measurable response to mitogens in the lymphocyte culture appreciated by various methods signifies the presence of competent immune cells, namely the existence of a cell-mediated immune response.

Analyzing the data obtained, it was found that for the positive group the migration diameters of control samples not treated with tuberculin were situated between 0 and 7.5 (mm), while the treated samples belong to the range 0 - 5.75

(mm). Calculations indicate that the positive group lies between the values 0 and 4.5.

For the negative group, mean diameters for untreated samples ranged between 0.6 and 5.3 and of those treated with tuberculin between 1.85 and 7. Inhibition indices for this group were comprised between 0.74 and 3.75 (Table 1).

Table 1

Mean and standard deviation for the two groups in leukocyte migration inhibition test

	Group I (+) n=15	**Group II (-) n = 7**
X	1.06	1.65
S	0.881	1.36

It has been demonstrated by laboratory techniques that in sensitized macrophages from infected animals the ability to migrate in the liquid medium is inhibited in the presence of trace amounts of tuberculin. Lymphokines secreted by lymphocytes do not act directly, but through effector cells, usually macrophages. Some of these mediators, such as macrophage migration inhibition factor (MIF) and leukocyte migration inhibitory factor (LIF) stop the migration of macrophages or white blood cells to capillary tubes. Using the technique of migration inhibition, sensitized cells or the antigen itself can be detected.

In the experimental groups it was observed that the lymphocytes of the control group migrated over a larger area when compared to the sensitized group. Although inhibition indices were increased, actually representing stimulation indices, the action of tuberculin over sensitized leukocytes was obvious. Values obtained are inconsistent with the allergy test results. It is possible, however, that the absence of functional Th lymphocyte from the leukocyte concentrate production to have determined the production of very low quantity or even absence (LIF), migration no longer stopped. On the contrary, tuberculin could exert an attractant role, mobilizing cells out of the capillary tube.

Excessive variability of values obtained in this test makes it less accurate for the diagnosis of tuberculosis. Development of particular conditions for the test execution to provide better reproducibility, the accurate assessment of the quantities of tuberculin used, the optimal composition of the migration medium and the optimal time are all useful parameters in improving the technique. Nonetheless, the concentration of cell suspension should be taken into account, ensuring sufficient living space for each cell. The viability test with trypan blau avoids misinterpretation due to lack of migration because of the high percentage of dead cells.

In leukocyte migration inhibition assay there is a larger migration area of leukocytes in the experimental control group compared to the sensitized group.

It is possible, however, that the absence of Th functional lymphocyte from the leukocyte concentrate to have determined the production in very low quantity or even absence of LIF, thus migration no longer stopped.

The analysis of data presented in the blast transformation test shows an extremely broad variation of stimulation indices for both control culture, for which, however, all values remain positive and for the variants treated with PHA P bovine tuberculin (BT) or avian tuberculin (AT). Many of these values are in fact proving negative inhibition of cell growth. This could suggest poor efficiency in the case of PHA phytohemagglutinin-P as compared to PHA-M (with demonstrated efficiency) in mammals, while it is clear that in the case of tuberculins the development of lymphocytes (T and B) is inhibited in the presence of the mycobacterial extracts.

Perhaps inhibitory factors of the PPD tuberculin extracts are stimulants for the avian and bovine tuberculin. A correlation between the stimulation indices for one extract or another could not be established.

Examining mean values and standard deviations it was observed that adding the individual values obtained for the positive group, spontaneous blast index was increased (20.23 ± 11.08) while PHA P caused a slight inhibition (-0.53 ± 10.79). Comparing the average values of blast indices for the two types of tuberculin, stimulation was very weak compared to culture induced by bovine tuberculin (13.62 ± 0.886) and approximately 4 times higher for the one induced by the avian tuberculin (3.12 ± 10, 86) (Table 2).

Table 2

Mean and standard deviation of blast index values for the two groups

Group	Control	PHA	BT	AT
I(+)	20.23±11.08	-0.53±10.79	0.886±13.62	3.12±10.86
II(-)	13.35±5.21	6.24±5.39	0.030±0.004	0.0380±0.012

Response to phytohemagglutinin is significantly higher in the control group (6.24 ± 5.39), suggesting that mitogenic stimulation fails to induce normal responses to positive group. However, the blastogenetic index of the negative group is quite low, confirming the inefficiency in using a single dose, which may not be optimal.

Starting from the state of activation of the lymphocytes present in tuberculosis, it was noticed that the amino acids transport in lymphocyte cultures sensitized after treatment with tuberculin intensified.

Applying the quantification of residual glucose to assess cell growth it was found that both types of tuberculin used determine positive indices even higher than in the case of lecithin treatment, a more pronounced response to avian tuberculin type than to the bovine type.

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THE DIAGNOSTIC VALUE OF THE SIMULTANEOUS USE OF DELAYED TYPE HYPERSENSITIVITY AND GAMMA INTERFERON QUANTIFICATION TESTS IN BOVINE TUBERCULOSIS

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Summary

The main agent of tuberculosis (TB) in mammals is *M. bovis*. Its broad host range also includes humans, and *M. bovis* caused TB in the latter cannot be differentiated from the M. tuberculosis induced disease. Thus, the importance of the accurate differentiation by laboratory methods of the causative agent is utmost important for the epidemiology of human cases. The study aimed to comparatively investigate the diagnostic value of cutaneous cell-mediated reactivity (ST) versus *in vitro* gamma interferon response (GIFN) towards avian and bovine tuberculin (APPD and BPPD, respectively). Out of a total of 29 Romanian spotted dairy cows, aged from five to nine years, ten tested positive for both techniques, 11 were ST pozitive, GIFN negative, three responded doubtful to ST and were negative for GIFN while negative for both tests were five animals. The GIFN optical density values (OD) recorded in GIFN test were higher for the ST positive animals than for the doubtfull and negative ones. Similarly, there were statistically significant ($p<0.05$) differences between the OD values red for the specific BPPD (0.362±0.355 units) versus the non/specific APPD (0.186 ±0.103 units) in the ST –GIFN positive animals, which were higher than those recorded in ST positive – GIFN negative animals (BPPD – 0.121±0.029 units and APPD - 0.111±0.019 units). In both ST doubtful and ST negative-GIFN negative categories, the values recorded for the specific BPPD were lower (0.150±0.06 units) than those recorded for the non-specific APPD (0.170± 0.075 units). In ST negative GIFN negative animals the BPPD and APPD values were close to each other (0.125± 0.017 and 0.127± 0.016 units, respectively). These results suggested that there was a parallel disgnostic course of the two tests, with high values for specific and low values for non specific sensitisation, respectively.

Key words: *M. bovis*, tuberculosis, diagnosis, delayed type hypersensitivity, gamma interferon

Tuberculosis, a bacterial disease caused by *Mycobacterium tuberculosis,* is currently one of the major infectious diseases with worldwide impact. In 2010, there was an estimated number of 8.8 million new cases (13% in co-infection with HIV), and 1.5 million deaths were attributed to TB (16). According to the study published by Jenkins et al (2010), it is estimated that about 1 million children have contracted tuberculosis and of these 32,000 with multidrug resistance strains (10).

The main causative agents of bovine tuberculosis are *M. bovis* and, to a lesser extent, *M. capraise*. Globally, most zoonotic TB cases are caused by *M. bovis* and cattle are the main reservoir (5). Zoonotic transmission of these pathogens is well described and occurs primarily through close contact with infected cattle or trough consumption of contaminated animal products such as unpasteurized milk (6, 1). However, there is evidence of significant influences that predisposing factors exert, such as inadequate technology conditions (in animals) or unsuitable habitat (for people), poor nutrition and multiple deficiencies in causing and worsening disease progression.

TB problem is still not resolved, even in countries where the standard of living is considered adequate for human existence. Thus, in 2014, at least 31,700 cattle were slaughtered in Britain due to intradermal testing results (9). EU legislative documents require drastic measures with regard to bovine tuberculosis control. These actions are implemented in order to control the spread of this disease, for achieving eradication and elimination of the risk factor which diseased cattle represent for people involved in the activities related to livestock sector.

Out of the diagnostic tests in current use in tuberculosis, classic examinations which have already demonstrated their utility can be reviewed. Such are hypersensitivity skin tests, cultural exams, direct bacterioscopy on pathological material and histopathological examinations (3,4,8). In the case of cattle herds, hypersensitivity skin tests hold the widest applicability, considering the execution facility and ease in reading the results. However, due to multiple possible errors of diagnosis (pseudo or paraalergic) owed to subjectivity in reading local reactions and the need for retesting in case of doubt, which extends waiting times and keep potential source of infection in the herd, researchers are constantly seeking more efficient alternatives and faster ways of diagnosis.

Determination of gamma interferon concentration is a modern technique recently applied for the diagnosis of bovine tuberculosis. Evaluation was performed comparative to the intradermal test, which has the broadest application in the world today. The differences between the two tests were not proven to be statistically significant. However, the authors recommend the use of the test in current practice with immune competent individuals.

Materials and methods

Investigations were conducted on a total of 29 cattle aged between 5 and 9 years, originating from private households. For skin allergic response, the comparative test was performed simultaneously by inoculating bovine and avian PPD tuberculin, concomitant, in dose of 0.1 ml, on the side of the neck, in two separate points (7). The interpretation of the reaction was carried out at 72 hours, by evaluating the local reaction and by measuring the skin fold thickness for each injection site in part. Afterward, the animals were classified in categories positive, negative and retest, according to the table for skin test interpretation of the results.

For the EIAs – GIFN test, blood was sampled into tubes containing lithium heparin (Hep - Li) as an anticoagulant. Lymphocytes stimulating and culturing from whole blood was done by using 2 antigens (bovine and avian PPD). The protein concentration used for optimal stimulation of the sample was 300 micrograms/ml. Therefore, commercial tuberculins were diluted with sterile phosphate buffered saline (PBS), pH 7.2 in a ratio of 1: 3. After the specified amount of blood was distributed into wells, 0.1 ml/well of avian tuberculin diluted 1: 3 was dispensed into the rows of wells 2 and 5 and the same amount of bovine tuberculin in wells 3 and 6, using another dropper. In rows 1 and 4 was assigned a volume of 0.1 ml/well of sterile PBS 0.01 M, pH 7.2. The plates with stimulated blood cultures were incubated at 37 ° C for a period of 16-24 hours (2). The period was calculated starting from the distribution of the reagents for the last sample. The absorption of each well was read within 20 minutes from the completion of the reaction. Absorption values were used to calculate the results. To interpret the results, the average value of absorption for avian and bovine PPD and for each sample without antigen is calculated. The mean values of bovine PPD are compared with those of the negative control. All bovine PPD samples which had an average absorption> 0.050 than the average absorption of the negative control required the repeating of the exam.

Considering the results of the comparative simultaneous test (CST) and Bovigam test, animals were divided into 4 categories:

- TCS positive and Bovigam positive - 10 animals
- TCS positive and Bovigam negative - 11 animals
- TCS recontrol and Bovigam negative - 3 animals
- Bovigam negative and TCS negative - 5 animals

Results and discussions

Subsequent to viral antigenic stimulus, the cells synthesize a molecule with antiviral effect, called interferon (alpha and beta interferon). Lymphoid cells activated by the antigen are capable of integrating a biochemical substance similar to interferon, but with a distinct chemical and antigenic structure (12). This was called the immune interferon or gamma interferon. The main source of gamma interferon is represented by the activated T lymphocyte, directly linked to antigenic information flow (13). The property of sensitized T lymphocytes to secrete gamma interferon *in vitro* was also exploited by developing test for interferon dosage.

It has been demonstrated that, generally, γinterferon rises above the threshold of positivity since the 30th day after the infection, but not all animals respond equally to antigenic stimulus and in the same interval from infection, suggesting genetic variability in cell mediated immune response and in determining disease status (11). It is possible that some factors such as care state, stress, pregnancy, parturition decrease the level of interferon γ in the analyzed samples (14,15).

Table 1

Results of the γ interferon dosage in categories of tested animals (mean and standard deviation)

Animal category	Bovine PPD (ODU)	Avian tuberculin (ODU)
Skin test positive, Bovigam positive	0.362±0.355	0.186±0.103
Skin test positive, Bovigam negative	0.121±0.029	0.111±0.019
Skin test retest, Bovigam negative	0.15±0.06	0.17±0.075
Skin test negative, Bovigam negative	0.127±0.017	0.127±0.016

In the TCS positive, Bovigam positive category, ODU were the highest for both tuberculins. Mean was of 0.362 and standard deviation of 0.355 in stimulation with bovine PPD, whereas with avian PPD the mean was 0.186 and the standard deviation 0.103. In the category TCS recontrol but negative Bovigam, lowest average values of optical densities were observed for both tuberculins (0.121 for bovine PPD and 0.111 for avian PPD). A more intense response to stimulation with bovine PPD was noted in comparison with avian PPD stimulation, for both categories (Table 1).

In the last two categories, in which Bovigam test was negative and TCS was dubious and negative, results of γ interferon dosage were relatively close. The mean and the standard deviation were 0,150±0,060 for bovine PPD and 0,170±0,075 for avian PPD (in category TCS dubious). In category TCS negative, Bovigam negative, values were identical (0,127±0,017) in the stimulation with both tuberculins. Comparing responses to stimulation with bovine PPD and avian PPD of the same category, it was observed that in the TCS retest group, stimulation with avian PPD was a little more intense than the one with bovine PPD and showed an approximate equality of the response to both tuberculins in the case of negative TCS.

Conclusions

Considering the values obtained, it can be stated that there are very large differences in intradermal bovine tuberculin test reading (averages over 7 mm), which are or are not reflected in the Bovigam test values. The intervention of other causes which explain these results is of course possible. Such is the chronic parasitosis (e.g.:linguatulosis.), which doesn't remit with antiparasitic treatments.

In all categories other than the positive, it was found that optical density values for the Bovigam test are maintained below 0,150 on average. If individual values instead of mean values are measured, it is noted that in other categories

than the positive, values are found above the mean limit. It seems, therefore, that Bovigam test has rather a group diagnostic value than an individual diagnostic value.

BOVIGAM does not affect the immune status of the animal, because the PPD is not injected to animals. This means that the gamma interferon test can be repeated as often as is necessary. Moreover, Bovigam test is not influenced by the allergic test recently performed. As in the case of any test, Bovigam may provide false - positive or false - negative results, depending on the local conditions. The test must be interpreted in the context of obtaining clinical, anamnestic and epidemiological information of the animals tested.

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FLUORESCENT ANTIBODY TEST IN RABIES DIAGNOSIS: TIPS, PITFALLS AND TROUBLESHOOTING

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Summary

Rabies is a fatal zoonotic viral infection of the central nervous system with the causative agent Rabies virus capable of infecting all mammal species. The most widely used test for rabies diagnosis is Fluorescent Antibody Test (FAT), which is recommended by both WHO and OIE. The FAT is sensitive, specific and cheap. The sensitivity of FAT depends on the specimens, equipments, reagents and on the proficiency of the diagnostic staff. In this paper we would like to review the literature and to highlight the major critical points that occur when the method is performed in order to get reliable results.
Key words Rabies, diagnosis, fluorescent antibody test, microscope.

Rabies is an acute viral disease that affects humans and other mammals causing a lethal form of encephalitis. The diseases is induced by neurotropic viruses belongs to the *Mononegavirale* order, the *Rhabdoviridae* family and the *Lyssavirus* genus which causes between 37,000 and 87,000 human deaths annually, worldwide (5, 11). In most laboratories it is usually diagnosed by the detection of viral antigen in the brain by using fluorescent antibody test (FAT), the gold standard test for rabies diagnosis (6, 8). The aim of this paper is to update the date from literature regarding critical points of FAT in order to release reliable results.

Materials and methods

The direct fluorescent antibody test is carried out on brain smears by the application of fluorescein isothio- cyanate-labelled anti-rabies antibodies to the smear or impressions followed by incubation and washing steps. Examination of the smears or impressions under ultra-violet microscopy reveals fluorescence associated with particulate antigen located in the cytoplasm of infected neurons (Fig. 1) (9).

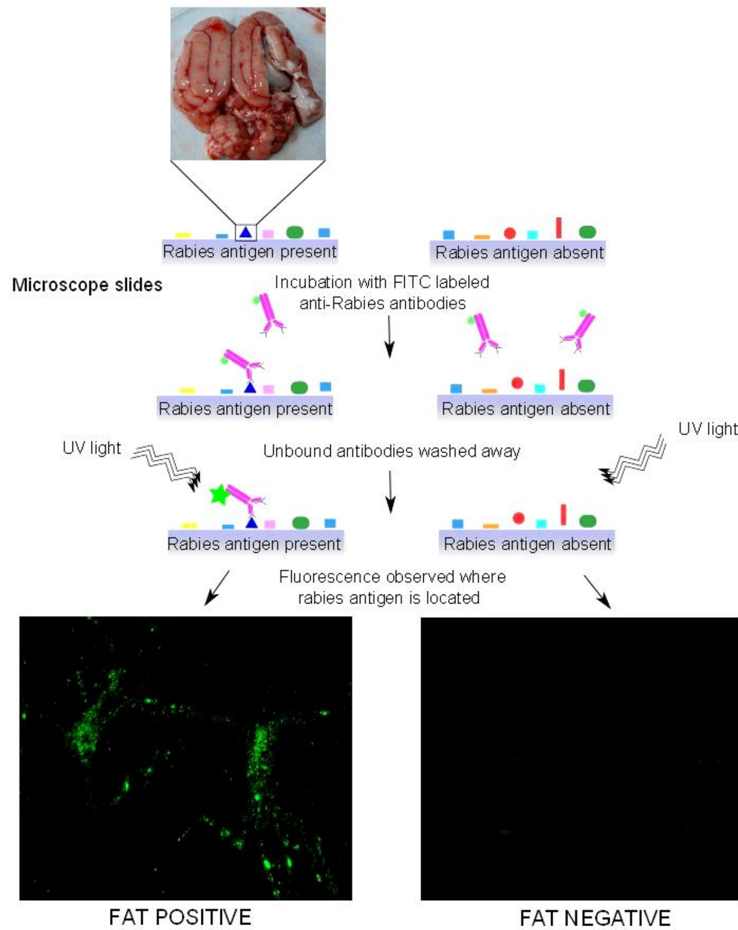

Fig. 1. Schematic fluorescent antibody test for Rabies

The fluorescent antibody test is the most favored test as it is reliable, rapid and cheap. These qualities of the diagnostic test for rabies diagnosis are critical for the correct medical management of human contacts, for the management of animal contacts and for the implementation of rabies control measures. The sensitivity and specificity of the FAT is very high but may be influenced by the quality of the specimen, conjugate and reagents, equipment and the skills and experience of the people involved in rabies diagnosis.

In this paper a review of study related FAT technique that have been performed at the National Reference Laboratories for Rabies from Institute for Diagnosis and Animal Health Bucharest is presented.

Results and discussions

Sample collection. Rabies virus does not infect all structures of the brain equally. It was found that there was frequent variation in the quantity of antigen between regions of the brain. Current authorities recommend that hippocampus and brain stem are the most reliable structures for detecting viral antigen (6, 11).

According to CDC, viral antigen is widespread in the brain of most animals positive for rabies, but because spread may also be unilateral, especially in larger animals, a negative finding for rabies can be made only if a complete cross-section of the brain stem is examined (2).

The results of the study performed by Bingham indicate that composite sampling of the brain should be practiced when the fluorescent antibody test is used (1). But where composite sampling is not possible then the brain stem and the thalamus should be used.

Positive and negative controls. Control brain material for slide preparation should be obtained from naturally or experimentally infected animals with wild local rabies virus and a fixed strain (CVS) and must be included in each test (4).

Buffer mounting medium should be used at pH 8.5 or higher. A lower pH can result in a weak fluorescence and can disconnect conjugate from antigen (3). High concentration of glycerol may provide weak staining (10).

FITC-conjugated anti-rabies antibodies. There is no need for using negative and positive FITC Conjugates. Suspensions of Normal Mouse Brain (NMB) and Rabid Mouse Brain (RMB) are not used as specificity controls anymore. RMB and NMB were used as diluents for FITC-labeled policlonal serum conjugates to control the presence in the reagent of extraneous antibodies present in animals used for the production of rabies hyperimmune serum as a result of a natural infection. The next discussion specifies two types of undesired staining (specific staining related to extraneous antibodies and shared epitopes; non-specific staining related to mechanisms other than antigen recognition). Extraneous antibodies are present in animals used for the production of rabies policlonal serum as a result of a natural exposure. If the same agent is present in the sample we have to test, a false positive result of rabies may obtain. The problem of extraneous antibodies is only for hyperimmune serum reagents, because monoclonal antibody contains only antibodies reactive with antigens on rabies virus. Therefore, using a monoclonal antibody reagent as one of the two reagents in every test is a very good control for recognition of extraneous antibodies. Non-specific staining may also occur because of antibody recognition of a shared epitope between rabies virus and another organism. Although both policlonal serum and monoclonal antibody are susceptible to this type of problem, shared epitopes are uncommon and unlikely to be present in the antibodies comprising two different reagent sources. Like extraneous antibody, the inclusion of two different reagents in every session is an effective control for recognition of shared epitopes. The binding of antibody to a tissue by mechanisms unrelated to antigen recognition is the most often cause of undesired staining. For example, charged protein attraction increases with increasing

fluorocrom: protein ratios which may vary between different reagents and between different batches of the same reagent. Some tissues are more sensitive than others to undesired staining. For instance, the myelin basic protein content and antibody binding capacity of an animal's brain is higher if animal is older; altereted tissue becomes "sticky" to different degrees. Antibodies may also be unspecifically bound to tissue by Fc receptors in the sample we test (2).

*Fluorescence microscope***.** The quality of the fluorescence microscope is a critical point and it is very close related to the sensitivity of the DFA test. Fluorescent microscopy makes use of the property of molecules to absorb light of a specific wavelength and then to emit the light at longer wavelengths (fluorescence). The exciting light is separated from the emitted light by filters in the microscope. The different elements of the fluorescent microscope which are involved in the sensitivity of the instrument are very important.

Immersion oil objectives prevents the loss of light that occurs in the airspace between coverslip and dry objective, although both dry and oil objectives can be used for rabies diagnosis. If oil objectives are used, immersion oil with the same refractive index (1.515) as glass should be used. The immersion oil should be produced specifically for fluorescence applications and evaluated for autofluorescence and best contrast between FITC and tissue.

Different light sources for routine rabies diagnosis are mercury (Hg) or xenon (Xe) gas arc lamps, with the short arc (HBO 100W) Hg lamp being most commonly used. Both light sources provide suitable amounts of light at wavelengths appropriate for FITC excitation. Mercury and Xenon arc lamps require caution during operation because of the danger of explosion due to very high internal gas pressures and extreme heat generated during use. Never ignite a lamp outside of its housing or observe the lamp directly when it is burning. Neither mercury nor xenon lamps should be handled with bare fingers in order to avoid inadvertent etching of the quartz envelope. Change bulbs only after the lamp has had sufficient time to cool. You have to store lamps in their shipping containers to avoid accidents (7).

*Experience of the people***.** In addition to the technical aspects previously detailed, each testing laboratory must maintain the competency of its employees. All new employees should be theoretically and practically trained, and competency should be evaluated by the head of department on a routine basis. Competency can be assessed by notification of all procedural aspects on a routine basis, as well as performance on proficiency test samples organized every year by National References Laboratory for Rabies, and testing on internal blind samples (2).

Conclusions

Every laboratory should have two different anti-rabies conjugates. The two sources should be one monoclonal antibody conjugate and one polyclonal (hyperimmune) serum conjugate.

Laboratories involved in Rabies diagnosis have to attend every year to the proficiency test organized for county sanitary veterinary laboratories at the national level by NRL for Rabies and NRL to attend to the test organized by European Reference Laboratory for Rabies.

There is no substitute for constant practice and experience in performing DFA testing.

As with any mechanical device, the microscope will function only as well as it is maintained. Periodically maintenance should be performed by a certified technician. Routine maintenance should be performed according to the manufacturer's specifications.

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