IN VIVO VIRAL TITRATION OF 2 INFLUENZA A SUBTYPES IN C57/BL6J LABORATORY MOUSE

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Summary

Influenza research offers major directions on the study of appropriate animal models. The approach of an 'in vivo' viral titration may be extremely delicate in a context of animal models that can't be naturally infected. In this case we are talking about influenza A virus and the titration of 2 mouse-adapted strains (Puerto Rico H1N1 and Scotland H3N2) in C57/Bl6j laboratory mice. As we already know the mouse is not naturally infected by Influenza A viruses. Some cases of natural infection have been described after experimental intranasal infection of mice with H5N1 HPAI subtype. In the present study mice were intranasally infected with increasing serial tenfold dilutions/strain of pure virus and the lower lethal dose MLD100 (one hundred percent mouse lethal dose) required to kill all the mice in 6-7 days (peak of infection) was determined. The interest to conduct this study derives from the need to evaluate an influenza vaccine on the same animal model. The protection assured by the chosen vaccine will be evaluated in future through an experimental challenge infection (with the same strains of influenza A virus) of immunized mice but this will make the object of another study.

Key words: Influenza A virus, H1N1, H3N2, *'in vivo'* titration, MLD10

The pathogenesis, therapy and prophylaxis of influenza A viruses is a major challenge from a zoonotic and pandemic point of view (1). To elucidate all aspects relating to the thorough understanding of this complex virus *'in vitro'* and 'in vivo' models are available to researchers. Despite the late evolution of *'in vitro'* research, animal models will always be necessary in deciphering the mechanisms of pathology related to a living virus. Therapy and vaccinology are also two other directions that cannot be developed without the existence of a suitable animal model. Main appropriate models for the studies regarding epidemiological evolution are the main natural hosts: the birds and the pigs. (3). Common mammalian species acceptable in a proof of concept for vaccine efficacy and immunogenicity are: mice, ferrets and macaques. As for safety testing (toxicity and local testing) rabbits appears to be species of choice for this approach (tolerance of influenza and different vaccines) (1, 2, 3).

We have chosen mice as a main model in a proof of concept strategy on the development of influenza A vaccine. C57Bl/6j mice are an extremely precious tool because of their known genome which can facilitate cellular immunological researches in a vaccination approach. The use of pure virus to determine a clinical development in an influenza experimental infection in mice is a method

inappropriate for a further study of virus challenge designed to reproduce natural symptoms of influenza in mouse. In the present study we proposed to determine MLD100 (mouse lethal dose 100) meaning establishing the lowest dilution of H1N1 and H3N2 pure influenza virus lethal to 100% of a flock of C57/Bl6j mice (4). These 2 strains of influenza A virus are mouse adapted being well known that only H5N1 HPAI virus may be naturally infective in mice. Basically these 2 laboratory strains are human but after repeated passages on mouse they lost their patogenicity in human becoming pathogenic, even lethal in mouse. Here, we titrated "in-vivo", these 2 Influenza A mouse-adapted strains (Puerto Rico/8/34 H1N1 and Scotland H3N2). The 2 virus stocks (A/Scotland H3N2 and A/PR8 H1N1) were kindly provided by the CIBU (La Cellule d'intervention biologique d'urgence) laboratory from Pasteur Institute. The two mouse-adapted infectious agents were exclusively manipulated under type II PSM in a biosafety level 2 laboratory at Pasteur Institute. These viruses are pathogen for mice but they are not very contagious inside their species. The 2 strains are low virulent and low or not contagious for human being.

Materials and methods

Serial tenfold dilution of the 2 viruses H3N2 and H1N1 were prepared. Six dilutions, 10^{-1} to 10^{-6} were inoculated in C57Bl/6j mice (20 µL/nostril in each nostril, under Rompun/Imalgene low anesthesia) and the lowest lethal dose required to kill all the mice in 6-7 days (peak of infection), was determined. Survival rate was established following daily observation for 13 days. At the end of the study, the percent of lung lesion score was determined for some of the surviving mice. The lowest lethal dose required to kill all the mice in 6-7 days (peak of infection), was determined.

Physical examination

A physical examination was conducted before infection and during experimental days. Mice were firstly observed on their cages in order to notice any possible major changes. The general individual examination contained the observation of following elements: signs of illness, general mood, skin, external mucosae (oral, nostrils, vaginal, anal), whiskers, hair, claws, facial appearance, eyes. Body weight of surviving mice was taken the last day of experiment and it was compared with the weight of healthy mice of the same age that were not implicated on this study.

The clinical signs of influenza infection in mice include ruffled fur (piloerection), loss of appetite, weight loss, neurological symptoms (hind-limb paralysis), decrease of physical activity, tendencies to hunch-up and huddle together or completely isolate themselves from the rest of the group, increase of the respiratory rate, hypothermia and finally death. Development of influenza

infection's signs, weight on the last day of experiment and above all survival were monitored for 13 days.

Organs macroscopic examination

For an internal examination, animals were preliminary anesthetized by Xylasine/Ketamine. The dissection was conducted (approach of abdominal, thoracic and cranial cavities) in order to notice any pathological changes. In influenza macroscopic lungs aspect is relevant. In this study, the lung hepatization is a reachable indication of influenza infection and we quantified it through a lung lesion scoring (percentage of affected pulmonary lobes from the entire mass of the lung) at the end of our study, at 13 days after infection on some of surviving mice.

Table 1

Mice groups' designation

	Group	*Virus/control*	*Dilution of pure virus*	*N° of animals/group*	*Volume of viral dilution/nostril*
	Group 1	H1N1	10^{-1}	8F	20µL
	Group 2		10^{-2}		
	Group 3		10^{-3}		
	Group 4		10^{-4}		
	Group 5		10^{-5}		
	Group 6		10^{-6}		
	Group 7	H3N2	10^{-1}		
	Group 8		10^{-2}		
	Group 9		10^{-3}		
	Group 10		10^{-4}		
	Group 11		10^{-5}		
	Group 12		10^{-6}		
Total	**12groups**	**NA**	**NA**	**96 mice**	**NA**

C57Bl/6j mice from Janvier provider, H2b haplotype, n (number of infected animals) = 96, 6 weeks old (held in husbandry 2 weeks before infection). The infection was reproduced trough intranasal instillation of **40µL** per mouse under light anesthesia (Imalgene/Rompun). A mild anesthesia was recommended to ensure an infection as close to natural infection with influenza (in order to allow the entrance of the virus on the primary respiratory airways in animals that are not

completely anesthetized). A mouse of the group infected with 10^{-6} H1N1 viral dilution died after the i.n. instillation (delicate procedure), so he was not the subject of the final analyze.

Results and discussions

Lethality and disease evolution

On the day 5 post - infection 6/8 mice from the first two groups of mice infected with H1N1 mouse adapted virus were already dead demonstrating the high virulence of this strain. The mice from the groups infected with the next dilutions: 10^{-2} – 10^{-5} showed signs of cyhosis, dehydration, prostration, paresis (fig. 1 and 2). Animals infected with the 10^{-6} dilution of H1N1 virus didn't show any clinical signs. The symptomatology was milder following H3N2 experimental infection a casual cyphosis being recorded for the mice infected by the first 4 viral dilutions.

On the following day (day 6) the two mice remaining alive belonging to the two groups of mice infected with the first 2 dilutions of H1N1 virus were found dead in their cages. Mice infected with 10^{-3} dilution were found all dead. Last three groups of mice infected with doses of 10^{-4}, 10^{-5} and 10^{-6} of the pure H1N1 virus were alive, but the state of the group infected with 10^{-4} dilution of virus was poor. H3N2 virus progression was slower, 10^{-1} dilution was lethal for only 2 mice in this group. Mice infected with the following dilution of virus had a loose state of prostration and they had a hunched posture. The other mice showed no signs of disease.

Day 7 post-infection: H1N1 continues to be the most virulent virus, 2 of 8 mice infected with the 10^{-4} dilution being found dead on their cages. As for the H3N2 virus the last 6 living mice of the 10^{-1} dilution died. For 2 mice from the next group the virus was lethal.

The infection is progressive on the next days: day 8 post-infection: 5 of 6 leaving mice were dead for the H3N2 10^{-2} dilution and 1/6 and 1/7 mice of the H1N1 10^{-4} and 10^{-5} viral dilutions. 3/8 mice (H3N2 virus, 10^{-3} dilution) and 3/5 (H1N1 virus, 10^{-4} dilution) died on the day 9 following the infection.

Influenza's evolution is becoming slower with mortality stopping on the 13th day after infection when a total of 38/96 the total number of mice survived. Mice were euthanized this same day because of their important body weight lost.

The most common clinical signs were: ruffled fur, tendency of isolation, neurological symptoms (hind-limb paresis), inappetence, eyelids half closed, abdominal breathing, and dehydration.

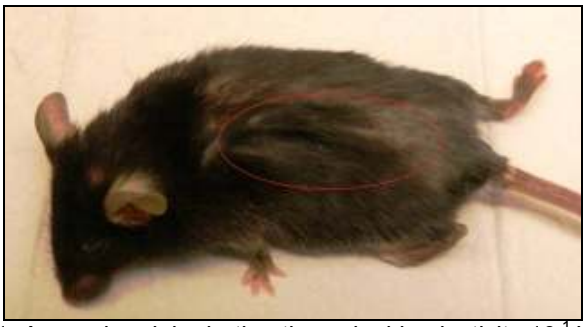

Fig. 1. Assessing dehydration through skin elasticity 10^{-1} H1N1

Fig. 2. Hunched posture, closed eyes, hind limbs (paresis), piloerection, 10^{-1} H1N1

Different results have been obtained for the 2 viruses, fact that it was already expected because of the well known pathogenicity of H1N1 against H3N2 virus. As we can see below (fig. 3) for the first four dilutions of H1N1 virus (10^{-1} - 10^{-4}) we obtain a mortality of 100% after 12 days following infection. Despite the fact that 10^{-3} dose seems to be the most homogeneous one, capable to assure the lethality of 100% of the group after 6 days from the infection we decided to consider as the lowest dose the 10^{-4} viral dilution with a peak of infection on day 6 after intranasal instillation.

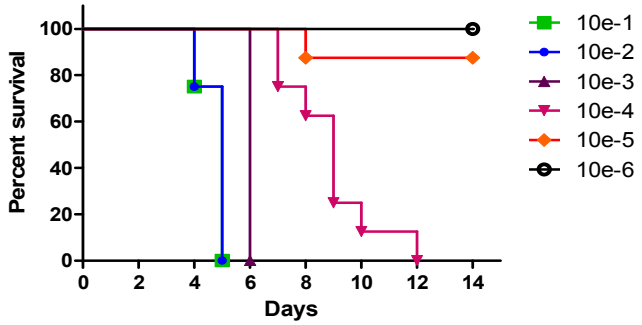

Fig. 3. Survival after H1N1 challenge

On the other hand, for H3N2 virus only the 2 strongest doses 10^{-1} - 10^{-2} assured the mortality of 100% of the mice belonging to the same group (fig. 4). Using 10^{-3} dose we didn't assured the mortality of 100% of the mice that's why we decided to consider the peak of infection for day 7 at an intermediary dose of 5.10^{-3} as being the lowest MLD100.

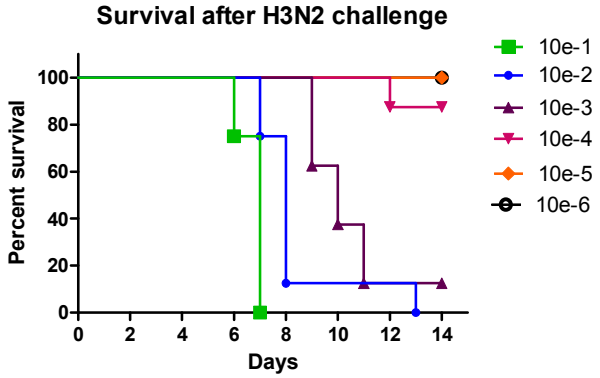

Fig. 4. Survival adter H3N2 challenge

Body weight

On day 13 surviving mice have been weighted and the values of their weight were compared with the ones of a negative control group – this group of animals is not mentioned on the beginning because he didn't make the object of our analyze (obtaining a mean of 22 grams). An equal body weight lost mean of 13% was registered for the surviving mice infected with 10^{-3} and 10^{-4} doses of H3N2 virus. After that, a linear decreasing trajectory can be noticed for the next two

dilutions of H3N2 virus: 10^{-5} and 10^{-6}: 11% and 9%. For H1N1 challenged mice the body weight was significant, with a mean of 18% for the last 2 doses: 10^{-5} and 10^{-6}.

Table 2

Body weight (grams) on day 13 of infection for the surviving mice compared to the uninfected mice

H3N2 10^{-3}	H3N2 10^{-4}	H3N2 10^{-5}	H3N2 10^{-6}	H1N1 10^{-5}	H1N1 10^{-6}	Uninfected
19	19	21	20	16	18	22
	19	19	19	19	17	23
	19	20	19	18	19	20
	20	20	21	17	20	22
	18	19	20	18	18	24
	19	20	21	18	18	23
	19	18	20	19	17	21
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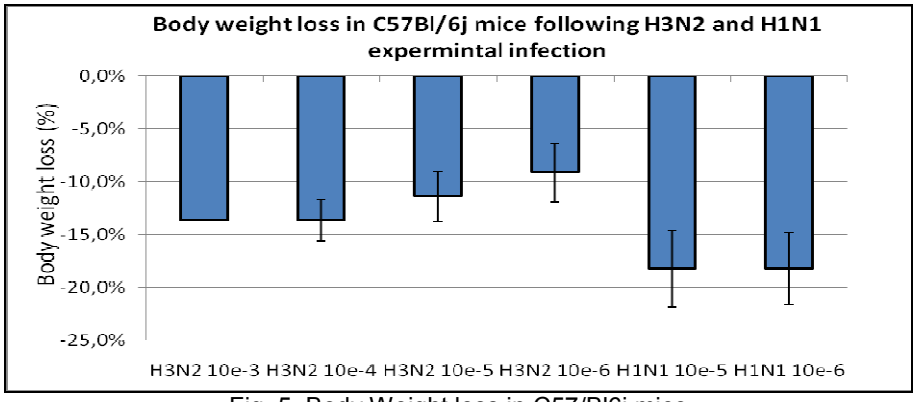

Fig. 5. Body Weight loss in C57/Bl6j mice

For a statistical interpretation of the weight lost, Kruskall-Wallis test have been used being an appropriate test for analyze of small samples (groups of 8 animals may be difficult to statistically analyze with common statistical test as ANOVA, Student test, etc). We considered as significant the differences between the group of uninfected mice and the last two groups of mice infected with H1N1 flu which confirms once again the high virulence of this specific viral strain.

Lung scoring

13 days following the infection only some of the remaining animals (the ones infected with the highest dilutions) have been euthanized and a lung lesions score has been conducted/lobe/mouse. The dissection was conducted (dorsal approach of thoracic cavity firstly, abdominal and cranial cavities) in order to notice any pathological changes.

Table 3

Score of pulmonary lesions in C57/Bl6j mice/lobe

N° of surviving mouse	Group	Left lung		Right lung			Median/ mouse
		Left lobe	Right lobe	Superior lobe	Middle lobe	Inferior lobe	
S1	H3N2 10^{-3}	10%	0%	0%	0%	0%	0%
S2	H3N2 10^{-4}	0%	0%	0%	0%	0%	0%
S3	H3N2 10^{-4}	30%	0%	20%	0%	100%	20%
S4	H3N2 10^{-4}	20%	70%	20%	40%	50%	40%
S5	H3N2 10^{-4}	20%	30%	20%	30%	40%	30%
S6	H3N2 10^{-4}	20%	0%	40%	40%	0%	20%
S7	H3N2 10^{-4}	20%	0%	20%	40%	40%	20%
S8	H3N2 10^{-4}	10%	30%	100%	30%	0%	30%
S9	H1N1 10^{-5}	20%	10%	10%	30%	30%	20%
S10	H1N1 10^{-5}	20%	100%	100%	10%	100%	100%
S11	H1N1 10^{-5}	30%	100%	20%	20%	20%	20%
S12	H1N1 10^{-5}	30%	30%	20%	30%	10%	30%
S13	H1N1 10^{-5}	20%	100%	5%	60%	20%	20%
S14	H1N1 10^{-5}	100%	20%	20%	100%	20%	20%
S15	H1N1 10^{-5}	30%	30%	10%	100%	90%	30%

Macroscopically no pathological changes were observed except for the pulmonary ones of lung hepatization. For the mice infected with 10^{-3} dilution of

H3N2 virus insignificant lesions were noticed. As for the next dose of 10^{-4} the values ranged between 10 and 40%. Big differences were noticed for 10^{-5} dose of H1N1 virus, 5 – 70% (fig. 6) which supports once again the highest patogenicity of H1N1 virus despite the fact that we analyzed one of the lowest doses.

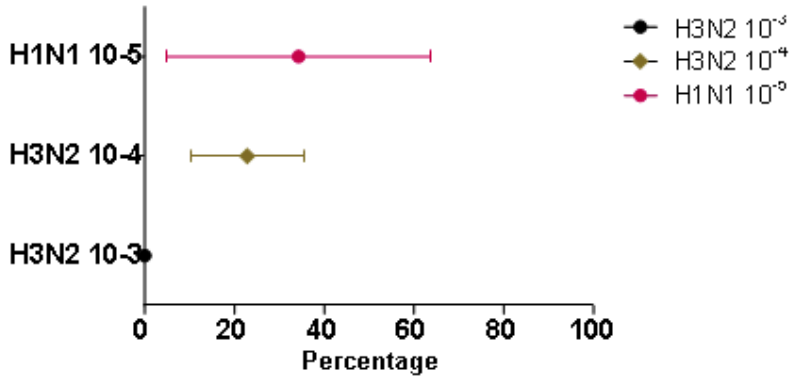

Fig. 6. Scoring pulmonary lesions – Median/group

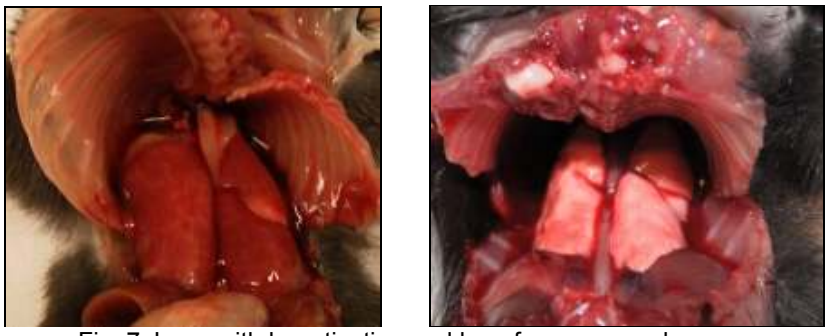

Fig. 7. Lung with hepatization and lung from a normal mouse

Conclusions

Titrating '*in vivo'* 2 specific influenza A viruses allows us to know the pathogenicity of 6 tenfold serial dilutions of pure virus and to determine the lowest mouse lethal dose 100% on groups of animals of same age. The reproduction of an experimental infection on the study of vaccinolgy is always necessary, the *'in vitro'* titration not being enough. We should think of choosing the lost pathogenic dilution extrapolating to the fact that for natural infection usually very low viral titers are normally enough to produce a high infection. In mouse the subject is even more sensitive knowing that the virus has been specially adapted to this species in order to finally reproduce after repeated passages on it a highly pathogenic disease in an animal model that cannot be infected naturally.

The lowest MLD100 of the H1N1 virus (10^{-4} viral dilution with a peak of infection on day 6 after intranasal instillation) was different of H3N2 MLD100 virus dilution (5.10^{-3} with a lately peak of infection at day 7 following experimental infection), a more important dilution of the H1N1 virus is required to reproduce the severe illness in C57/Bl6j mouse. This comes to confirm that this strain is more pathogenic than the H3N2 virus strain.

Except the '*in vivo'* viral titer we also determined the infection peak which is a critical endpoint for a study of vaccinology with viral challenge.

The results obtained following *in vivo* titration of mice-adapted influenza A strains: Puerto Rico/8/34 H1N1 and Scotland H3N2 can be considered as satisfactory in order to design a study of prime-boost-challenge. As an important factor age of mice should be taking in consideration (6 weeks old for the present study) on the future studies.

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IN VITRO COMPARATIVE SUSCEPTIBILITY TO ANTIBIOTICS OF *E. COLI* STRAINS ISOLATED FROM DOMESTIC AND WILD RABBITS

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Summary

Bacteria and other microorganisms that cause infections are remarkably resilient and can develop ways to survive drugs meant to kill or weaken them. This antibiotic resistance, also known as antimicrobial resistance or drug resistance is due largely to the increasing use of antibiotics.

The study aimed to identify *E. coli* strains from wild and domestic rabbits, and to see if these strains developed antimicrobial resistance.

The *E. coli* strains were collected from the digestive tract of hares and rabbits. After being characterized as *E. coli*, each strain was tested for sensitivity to antibiotics in Muller-Hinton agar by radial diffusion method, and inhibition diameters (ID) were measured. The antibiotics used were: Ciprofloxacine (CIP), Polimixine B (PB), Sulfamethoxazole-Trimethoprim (SXT), Nalidixic Acid (NA), Gentamicine (CN), Oxytetracicline (TE), Chloramphenicole (C), and Streptomycine (S).

There were differences in the antimicrobial resistance of *E. coli* isolated from digestive tract of haires and domestic rabbits, such as effects of the antimicrobials used in this study was better for the *E. coli* strains isolated from hares compare to those for the *E. coli* strains isolated from domestic rabbits. The least effective antibiotic for *E. coli* strains from both types of sources was SXT (average inhibition diameter= 7.12 mm for *E. coli* strains from domestic rabbits and 11.45 mm for the *E. coli* strains isolated from hares).

The most effective antibiotics for the studied *E. coli* strains from domestic rabbits and hares rendered as: CIP (average ID= 28.51 mm), CN (average ID= 21.55 mm), C (average ID= 20.95 mm).

Key words: *E. coli,* antibiotic resistance, rabbits

Escherichia coli is an enteric bacteria found in virtually all mammals, and birds throughout the world. *E. coli* infections are generally a problem with young animals and rabbits are no exception (5).

In veterinary medicine, antibiotics represent an important part of the chemical contaminants, due to their frequent use to control infectious diseases. The use of antibiotics in animal production has lead to a substantial decline of the infectious diseases of the animals and a subsequent diminishment of the infectious agent transmission risk to consumers. Therefore, the use of antibiotics has lead to

an increase in the safety of the food chain. However, the presence of these residues in meat could be harmful to consumers because antibiotic residues in low concentrations favor the development of microbial resistance, which could have serious consequences (1).

The emergence of antimicrobial-resistant microorganisms, in both humans and animals, calls for the development of new strategies for administering antibiotics in order to bring these microorganisms under control and, most importantly, to prevent the risk of resistant bacteria being transmitted to animals and humans.

Antibiotic treatment should be removed in time to ensure their elimination from the tissues. The legislation for pharmaceuticals and veterinary products established a withdrawal period of 28 days for antibiotics in fattening rabbits, limiting the addition of antibiotics to the first days of fattening (4).

There are no provisions concerning the microbial load of game carcasses and data on the antibiotic resistance encountered in bacteria isolated from game species. Some researchers reported susceptibility to ampicillin, chloramphenicol, ciprofloxacin, kanamycin, streptomycin and sulphamethoxazole in all 61 strains of *Enterobacteriaceae* isolated from 13 domestic rabbits (6).

Although rabbit *E. coli* showed general sensitivity to antimicrobials, some exhibited antimicrobial resistance. Presence of pathotype marker genes, serotypes and resistance in rabbit *E. coli* indicated that it could constitute potential source of transmission to human (3).

This paper aimed to investigate and compare the antimicrobial activity of some antimicrobial agents against *E. coli* strains isolated from wild and domestic rabbits.

Materials and methods

The *E. coli* strains were obtained from samples collected from the digestive tract of wild and domestic rabbits. After being identified as *E. coli*, each strain was tested for sensitivity to antibiotics in Muller-Hinton agar by radial diffusion method, and inhibition diameters (ID) were measured. The antibiotics used were: Ciprofloxacine (CIP), Polimixine B (PB), Sulfamethoxazole-Trimethoprim (SXT), Nalidixic Acid (NA), Gentamicine (CN), Oxytetracicline (TE), Chloramphenicole (C) and Streptomycine (S).

Results and discussions

Regarding the presence of E. coli in the digestive tract of rabbits, Davies R. (2), states that hares are reservoirs of some *E. coli* strains with zoonotic potential. Regarding the sensitivity to antibiotics, Österblada et al.,2006, found that antibiotic resistance among *Enterobacteriaceae* family members is high and can cause serious clinical problems.

There were differences in the antimicrobial resistance of *E. coli* isolated from digestive tract of haires and domestic rabbits, since the antimicrobials used in this study were more efficient against the *E. coli* strains isolated from hares when compared to those isolated from domestic rabbits.

Pisoni et al. also observed that a biotype of *E. coli* isolated from the same rabbits farm twice has usually the same behavior to antibiotics but is easily changed when the same biotype is isolated from other farmed rabbits.

The least effective antibiotic for *E. coli* strains from both sources was SXT (average inhibition diameter = 7.12 mm for *E. coli* strains from domestic rabbits and 11.45 mm for the *E. coli* strains isolated from hares).

Table 1

Inhibition diameters to antimicrobials for *E. coli* strains isolated from domestic rabbits

Sample	TE	S	CIP	C	NA	CN	PB	SXT
1	22	16	27	26	11RC	25	14	14
2	17	17	30	28	21	17	14	24
3	17	12	32	25	21	13	11	21RC
4	TR	TR	16	23	TR	20	11	TR
5	15	14	31	25	21	22	14	19
6	18	15	31	28	22	20	13	TR
7	TR	15	25	26	TR	22	13	TR
8	15	14	30	TR	TR	24	13	TR
X	13	12.87	27.75	22.62	10.62	20.37	12.87	7.12

Legend: TR=total resistence; RC=resistent colonies

Table 2

Inhibition diameters to antimicrobials for *E. coli* strains isolated from hares

Sample	TE	S	CIP	C	NA	CN	PB	SXT
1	20	11	25	22	25	23	15	23
2	20	16	30	23	25	23	15	19
3	RT	15	35	RT	24	23	14	25
4	20	17	30	29CR	26CR	25	14	RT
5	20	16	30	25	23	23	13	RT
6	20	17	30	20	25CR	23	15	29CR
7	22	18	30	23	20	23	12	RT
8	20	16	28	27	25	26	16	RT
9	15	14	25	23	21	22	11	25
10	19	12	29	25	19	20	11	16
11	17	14	30	24	20	19	11	18
X	17.5	15.1	29.3	19.27	18.36	22.72	13.36	11.45

The most effective antibiotics for the studied *E. coli* strains from domestic rabbits and hares rendered as: CIP (average ID= 28.51 mm), CN (average ID= 21.55mm), C (average ID= 20.95 mm).

Table 1 summarizes the *in vitro* susceptibility profile of *E.coli* strains, from domestic rabbits, to antimicrobial agents. Table 2 summarizes the *in vitro* susceptibility profile of *E. coli* strains, from wild rabbits, to antimicrobial agents.

Antibiotic resistant colonies and total resistance were encountered for numerous of the *E. coli* strains isolated from domestic rabbits to some of the tested antibiotics in this study. Meanwhile, resistant colonies and total resistance was observed only for a few strains of *E. coli* isolated from wild rabbits.

Conclusions

The widespread use of some antibiotics in domestic rabbits therapy has lead to presence of total resistance and resistant colonies to some of those antibiotics (Sulfamethoxazole-Trimethoprim, Nalidixic acid, Streptomycine, Oxytetracicline) unlike hares were total resistance and resistant colonies were observed in a very few cases probably due to the proximity to domestic animals.

Low efficiency of Polimixine B against *E. coli* strains isolated from the digestive tract of hares and domestic rabbits impose that this antibiotic should not be used in colibacillosis treatment of these species.

The differences found in this study regardind the antibiotics sensitivity among the strains isolated from domestic rabbits and hares can be due to the fact that the domestic rabbits had access to antibiotic feed supplement unlike hares that are unlikely to have come into contact with antibiotics of any kind.

Increased sensitivity of *E. coli* strains to CIP, CN and C indicates that the germs have not yet acquired resistance to these antibiotics and these antibiotics can be used to combat diseases caused by *E. coli* in rabbits.

Since the emergence of antimicrobial resistance in colibacillosis can complicate treatment options is required continuous surveillance of resistance zoonotic pathogens, including *E. coli*, to ensure public health.

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SALMONELLA GENUS – MONOGRAPHIC STUDY

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Summary

In livestock salmonellosis causes significant losses due to mortality (which, sometimes, can be considerable), abortion, growth delays, and outgoings on treatment and prevention measures. Also, species from *Salmonella* genus pose a major importance for public food. Possibility of human infection is continually increasing, in the context of the circulation of food contaminated by raw materials or during the technological flow, storage and / or distribution. Due to high resistance of *Salmonella* in the environment and food, the disease can be triggered even by eating food products in powder form and with a best before date for years.

Key words: *Salmonella,* monograph, epidemiology, nomenclature

Salmonella is a genus of rod-shaped, Gram negative bacteria, non-spore-forming, with diameters around 0.7 to 1.5 μm, lengths from 2.0 to 5.0 μm, predominantly motile enterobacteria, due to the presence of peritrichous flagella (exception *Salmonella gallinarum* and *Salmonella pullorum*), pathogenic to humans and animals with the following main characters: they ferment glucose with gas production, producing hydrogen sulfide, may use citrate as the sole carbon source, does not ferment lactose and sucrose and do not produce indole and urease.

There are over 2,500 serotypes of *Salmonella*, all of which are included in the *Salmonella* genus. Within the genre there are also serotypes that do not present some of those general characters mentioned above, such as lactose fermentation (slow - late fermentation) and hydrogen sulfide production (4, 7, 11).

Salmonella infections have increased incidence in all animal species because of the germs ubiquitous presence and the existence of germ bearers, but also in humans. Salmonella is one of the most important sanitary veterinary problem because of economic losses and their implications in human health by triggering food poisoning due to food consumption (3).

Taxonomy and nomenclature of *Salmonella* genus

The *Enterobacteriaceae* nomenclature is a controversial issue, over time various attempts of systematization have been done. In literature there are several

approaches regarding the taxonomy and structure of *Enterobacteriaceae* family (20, 23, 31).

A first approach regarding the *enterobacteriaceae* taxonomy is consistent with Bergey's Manual of Systematic Bacteriology and contains 30 genera, including the *Salmonella* genus (20). Based on the antigenic findings of Andrews between 1921-1924, White proposes the first antigenic identification scheme, later extended by Kauffmann and used nowadays as Kauffmann - White scheme of *Salmonella* identification and classification. The scheme was subsequently extended in antigen typing practice in bacteriology and virology.

Back in 1939, using a series of specific bacteriophages, Craigie and Yen compile the first *Salmonella typhi* typing scheme (which was later extended), its usefulness is still undeniable in epidemiological investigation of typhoid fever. Based on the model of this scheme, using sets of phage with different origins, there were developed lisotyping schemes for most *Salmonella* serotypes of local, regional or global major epidemiological interest, such as *paratyphi B*, *typhimurium*, e*nteritidis*, *anatum*, *panama*, *weltewreden* or other serotypes (41).

The instability of *Salmonella* genus classification reflects the many possibilities of variation and selection, characteristic for the members of this genus, due to their habitat, mainly intestinal (11). In this extremely complex and variable biotope, which provides *Salmonella* optimal conditions for an active metabolism and in which phages are present and disintegration to free DNA may occur, all genetic variation mechanisms are actively working (11). Thus, numerous species, serogroups and serotypes of *Salmonella* subdivide themselves in biotypes, phage types (lysotypes), bacteriocinotypes and resistance types. On the other hand, cross serological reactions with species from others genera are numerous, which suggests that boundaries are taxonomic only, recombination being the rule (11, 15)

In this context, in the last edition of Bergey's Manual of Systematic Bacteriology, the *Salmonella* genus includes eight species: *S. enterica, S. bongori, S. choleraesuis, S. arizonae, S. enteritidis, S. paratyphi, S. typhi, S. typhimurium*. *S enterica* is the type specie, which metabolic corresponds to *S. cholerae suis* (18).

Last reconsideration of the taxonomy of *Salmonella* genus revealed that, initially, in an incorrect manner, the antigenic structure of *Salmonella* species was considered as defining for the species, thus creating a precedent with unpredictable taxonomic consequences, the existence of a genus with more than 2000 species (7, 10).

Ewing's et al comprehensive studies regarding the phenotype and Le Minor and Popoff genotypic studies (DNA – DNA hybridization) quoted by Neguţ and Buiuc (7), as well as many other investigations led to significant changes of *Salmonella* genus nomenclature. Popoff and Le Minor proposed the existence of three species of *Salmonella* genus (7): *Salmonella enterica* including six subspecies (*S. enterica*, *S. salamae*, *S. arizonae*, *S. diarizonae*, *S. houtenae* and *S. indica*), *Salmonella bongori* with one specie and *Salmonella subterranea*.

Even nowadays, the taxonomy of this genus remains uncertain. In practice is still used the classification proposed by Le Minor and Popoff who admit the existence of two species: *S. enterica* and *S. bongori*. *S. enterica* specie includes, based on exoenzymatic criteria, six subspecies: *S. enterica*, *S. salamae*, *S. arizonae*, *S. diarizonae*, *S. houtenae* and *S. indica*. *S. bongori* recently defined as a specie has no subspecies. To all of these species, in 18th of March, 2005, a new one is added, discovered by Shelobolina et al. (30) and published in the validation list. Biochemical differentiation of these groups is presented in Table 1.

Table 1

Differential characters of species and subspecies from *Salmonella* genus

Nr. crt.	Test	*Salmonella enterica* - subspecies						*Salmonella bongori*
		enterica	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
1	Beta galactosidase	-	-	+	+	-	D	+
2	Gelatinase	-	+	+	+	+	+	-
3	Galacturonate	-	+	-	+	+	+	+
4	Growth in KCN broth	-	-	-	-	+	-	+
5	Malonate	-	+	+	+	-	-	-
6	d-Tartrat	+	-	-	-			
7	Gamma glutamyl transferase	+	+	-	+	-	-	-
8	Beta glucuronidase	D	D	-	+	+	+	+
9	Dulcitol	+	+	-	-	-	D	-
10	Salicin	-	-	-	-	-	D	-
11	Sorbitol	+	+	+	+	+	-	-
12	Lactase	-	-	+75%	-75%	+	-	-
13	Bacteriophage lysis	+	+	-	-	-	+	D
14	Habitat	Warm blooded animals	Cold blooded animals			-	+	D

Salmonella enterica subspecies *enterica* is the largest one, including 1504 serotypes from a total of 2541 described till in 2007 (19). Serotypes numerical weighting, according to Popoff, is presented in Table 2 (27). Based on antigenic criteria, subspecies are divided into serotypes, taxonomically designates using the Kauffmann – White scheme nomenclature, respectively *Salmonella enterica* subspecies *enterica* serotype *Typhimurium*, serotype *Enteritidis*, serotype *Choleraesuis*, serotype *London* and others (Table 2) (20).

For an easy work, in practice is used terminology like *Salmonella* (written in italics), followed by serotype name, written with the first letter capitalized (eg. *Salmonella* serotype Anatum). This nomenclature, as a result of biochemical and genetic studies from last decades, represents a compromise between taxonomic

exigencies and conservative practical needs, derived from the use of Kauffmann – White scheme.

Table 2

***Salmonella* genus nomenclature**

Specie	Subspecies	Serotype (serovar)	Serotypes number
Enteric	*enterica* (subspecies I)	Choleraesuis, Enteritidis, Paratyphi, Typhi, Typhimurium	1.504
	salamae (subspecies II)	9,46:z:z39	502
	arizonae (subspecies IIIa)	43:z29:-	95
	diarizonae (subspecies IIIb)	6,7:l,v:1,5,7	333
	houtenae subspecies IV)	21:m,t:-	72
	indica (subspecies V)	59:z36:-	13
Bongori	subspecies VI	13,22:z39:-	20
subterranea	-	-	-
	TOTAL	-	2.539

Epidemiological aspects

Salmonellosis are widely spread, being specific zoonoses, some strains are ubiquitous, others having only a regional character. Most strains are highly pathogenic, for infection occurrence only 15 – 20 bacteria are required, but this is influenced by subject age, physiological state, certain preexisting infections and the strain involved (6, 13, 21).

Back in 1880, Eberth and Koch discovered in the necrotic lymph follicles of typhoid fever corpses a bacillus incriminated in the disease etiology. Later, Gartner managed to growth this bacillus (29, 31).

In 1885, Salmon and Smith had isolated *S. choleraesuis* (initially named *Bacillus choleraesuis*) from pigs with swine pest, in 1888 Gartner had described in Germany the first episode of food poisoning, with 58 cases of ill people from consumption of beef meat, slaughtered for necessity reasons and had assigned to this microorganism the name of *Bacterium enteritidis*. In 1899 in Belgium an outbreak was caused by a bacillus previously isolated from mice in a laboratory animal facility and described by Loeffler, called *Bacterium aertrycke* (9, 29, 31).

Actually, Salmonellosis is the generic name for all kinds of infections produced by more than 2000 bacteria species from *Salmonella* genus from *Enterobacteriaceae* family. These are divided into several groups, according to their antigenic structure, but most common are *Salmonella typhimurium* (52,6%), *S. panama* (15,7%), *S. enteritidis* (9,4%) and *S. derby* (3,8%). Anyway, the number of isolated species varies from one country to another (121 in Romania, 140 in England, 188 in the Czech Republic, 37 in Bulgaria). In 1905, Babeş had described

the first case of salmonellosis, a food poisoning caused by lamb roast consumption (12, 24, 32).

Salmonella enterica subspecies *enterica* is the most common subspecies, including 1504 serotypes from a total of 2541 serotypes described up in 2007. It represents 90% of isolated from humans and warm blooded animals. Others subspecies and *Salmonella bongori* represent a small percentage and are isolated mainly from poultry, from cold blooded animals and from the environment, where they rarely can contaminate man and mammals (19).

Besides this great diversification of *Salmonella* serotypes, currently there can be distinguished also large differences in their geographical distribution, as a result of numerous and intense international and intercontinental trades of contaminated food (powdered eggs, poultry meat, canned and others), thus salmonellosis could be considered as the most common infectious diseases. In most countries of the world, salmonellosis incidence has increased over tenfold in the last five years (in our country, the morbidity rate, due to *Salmonella* infections, increased from 20.9% in 2003 to over 40.3% in 2007) (7).

The basic characteristic of *Salmonella* contamination is that there are no organoleptic changes that could highlight the possible presence of germs, food products remain unchanged in terms of appearance, color, smell and taste. As regards the processing and storage conditions of food, should be noted that the minimum temperature for the development of *Salmonella* is 7°C, refrigeration temperature only prevent their multiplication, but not leading to their destruction. For improper storage conditions, should be considered that the necessary time for a tenfold multiplication of a *Salmonella* population is 24 hours at 15.6°C and only nine hours at 21°C (2, 4).

Salmonella is one of the most common causes of human and animals gastroenteritis. Worldwide, infections are primarily associated with consumption of contaminated food or feed. Food-producing animals, including cattle, poultry, pigs are recognized as reservoirs of *Salmonella* (12, 14, 26).

In recent years an increase was observed in the prevalence of antimicrobial resistance of *Salmonella* strains (26). New *Salmonella* serovars have emerged in recent years. In late 2007, were notified more than 2,500 *Salmonella* serovars. During the last decade, a clone of *Salmonella enterica* multidrug-resistant from *Salmonella typhimurium* serovar, with DT_{104} final phagic rank has worldwide spread and has been evaluated as one of the most common causes of food poisoning in many countries (26).

Surveillance based on laboratory tests is essential in order to support intervention efforts, including surveillance and response efforts to reduce the prevalence of zoonotic salmonellosis (26).

In Europe were annually processed a number of 1786 isolates of *Salmonella* strains (with variation from 30 to 26.095, average 313), these being isolated in the participating laboratories or received for characterization from other laboratories. There were serotyped 916 (between 0-7, 120, average 300)

Salmonella strains in participating laboratories and 341 strains were annually tested for antimicrobial susceptibility of these strains (6, 26, 29, 32).

In our country, identified *Salmonella* serovars were: *Salmonella enteritidis*, *S. typhimurium*, *S. braenderup*, *S. heidelberg*, *S. modar*, *S. montevideo*, *S. saintpaul*, *S. seftenberg*, *S. tenneessee*, *S. concord*, *S. glostrup*, *S. lomita*, *S. agona*, *S. menden*, *S. bredeney*. Among these, *S. enteritidis* and *S. typhimurium* were the two *Salmonella* serovars most frequently isolated both from mammals and birds and also from their products. There is a direct correlation between the identification frequency of these two *Salmonella* serovars and the incidence of food poisoning outbreaks registered in Romania (7, 44).

Pathogenic aspects

Recent evidences had demonstrated the aggressive capabilities of *Salmonella* through both recognized bacterial pathogenic mechanisms: virulence and toxicity (3).

Germs enter through the digestive track and if the number of ingested microorganisms is greater than 10^5, they can trigger disease in healthy adults. Gastric acidity is part of the body defense mechanism against these bacteria, being responsible for the destruction of a great amount of ingested germs. A massive contamination is achieved through ingestion of food in which germs had multiplied themselves as in a culture medium (25).

In our country, the majority of Salmonella capable to trigger some forms of gastroenteritis are represented by Typhimurium and Enteritidis serovars Typhi *s*erovar and Paratyphi serovar of *Salmonella* are the etiologic agents of enteric fevers (28).

The virulence represents the major mechanism of *Salmonella* aggression, through the virulence factors (Table 3) and consists in their capacity of attach, multiply and invade the intestinal wall

Table 3

Virulence factors of *Salmonella* species

The virulence factor	The biologic effect
Enterotoxins	- Salmonella strains can produce heat labile enterotoxins and heat stable enterotoxins, their effects are not yet elucidated.
Verotoxin (Shiga–like toxin)	- Citotoxin involved in tissue invasion and destruction - Related but distinct from those produced by *E. coli* and *Shigella* spp.
Vi antigen	- Adhesins produced by three serovar: Typhi, Paratyphi C and Dublin; - Associated with microorganism's virulence.
Fimbrial	- Produced by *Salmonella* genus strains

Release of endotoxin by intravacuolar phagocytosis leads to fever and a series of chain reactions caused by the release of TNF (tumor necrosis factor) by macrophages.

Virulence plasmids from several Salmonella serotypes (*Typhimurium*, *Enteritidis*, *Choleraesuis*, *Gallinarum*, *Pullorum* and others) stimulates a rapid multiplication of bacteria containing them, beyond the host defense possibilities (17).

Siderophores, compounds with a relatively low molecular weight, have the ability to bind ferric ions, interfere due to enterochelin in the internalization process, thus increasing the virulence (1, 2). Enterochelin (enterobactin) is an aromatic isomer of dihydroxybenzoic acid cupled with L-serine.

The "Vi" antigen is another virulence factor, only present in Typhi and Paratyphi serovar and sometimes in Dublin serovar. It inhibits bacterial opsonization by the third component of the complement (C_3), thus preventing phagocytosis through macrophages.

Toxigenicity is the second important component of *Salmonella* pathogenicity. It was demonstrated that *Salmonella* possess both type of bacterial toxins: exo and endotoxins (1, 2, 5). Genetic analysis proved that there are some differences between sequences of enterotoxin coding genes in *Salmonella* and *Vibrio cholera*. It appears that genetic encoding is not limited only to the bacterial chromosome; evidence of enterotoxigenic transfer mediated through plasmid was proved (24).

Between the highly pathogenic *Salmonella* species for human, *S. typhi*, that produces typhoid fever, has to be mentioned. Between pathogenic species, the most representative are: *Salmonella paratyphi* A, B, C, (produces paratyphoid fever), *S. typhimurium*, *S. enteritidis*, *S. choleraesuis*, *S. anatum* (produce food poisoning) (25). In animals, salmonellosis can occur in cattle (enteric salmonellosis of young calves, enteric salmonellosis of cattle), sheep and goats (salmonella dysentery, sheep and goat frank dysentery, sheep salmonella abortion), equine (colt salmonella septicemia, soliped salmonella dysentery, and mare salmonella abortion), swine (enteric salmonellosis), domestic carnivores (salmonelic toxemia), fur animals (salmonelic toxemia), rodents (typhosalmonellosis). In birds, there are described both kinds of infections produce by immobile salmonella: fowl typhoid (*Salmonella gallinarum*) and pullorum disease (*Salmonella pullorum*) and mobile salmonella – fowl paratyphoid (8, 22).

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PHENOTYPICAL DETECTION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCTION IN *ESCHERICHIA COLI* ISOLATED FROM BROILERS CHICKENS

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Summary

ESBLs are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins and monobactams. The presence of an ESBL-producing organism in a clinical infection can result in treatment failure if one of the above classes of drugs is used (1). This paper presents the results at double disk test of *Escherichia coli* strains isolated from broiler chickens reared in intensive unit and sacrificed in a poultry slaughtering unit in order to identify the ESBL-producing strains.

Key words: *Escherichia coli*, ESBL, antimicrobial sensitivity

Emergence of resistance to β-lactam antibiotics began even before the first β-lactam, penicillin, was developed. The first β-lactamase was identified in *Escherichia coli* prior to the release of penicillin for use in medical practice (1).

Many genera of gram-negative bacteria possess a naturally occurring chrosomally mediated β-lactamase. These enzymes are thought to have evolved from penicillin-binding proteins, with which they show some sequence homology. This development was likely due to the selective pressure exerted by β-lactam-producing soil organisms found in the environment (1).

Zoonotic bacteria which develop resistance to antimicrobials are of special concern since they might compromise the effective treatment of infections (6).

Materials and methods

In order to identify the ESBL-producing strains of *E.coli,* caecal isolated from broilers were collected and analysed.

During November 2011 to February 2012 the sampling was done through monthly visits in the abattoir where samples were collected.

In the laboratory caecal samples were enriched overnight in buffered peptone water. This enrichment stage ensured that minority components of the *E. coli* flora were detected. From buffered peptone water, samples were plated onto two Petri plates with EMBA media supplemented with cefotaxime and ceftazidime 1µg/ml.

All *Escherichia coli* strains, recovered from these agars, fermented lactose on culture media, the biochemical character that is considered constant at *Escherichia coli* strains. All lactozo-positive strains were subjected to further testing by the minimum set MIU (aiming at mobility, hydrolysis of urea, production of indole by decay of tryptophan) and TSI (following the fermentation of glucose, lactose and production H2S).

To detect ESBL-producing strains we used double-disk test.

This is one of the easiest and most cost-effective methods for use by clinical laboratories (1).

Isolates recovered from EMBA plates were tested for antimicrobial susceptibility with cefotaxime, cefotaxime/clavulanic acid, ceftazidime, ceftazidime/clavulanic acid and cefpodoxime and cefpodoxime/clavulanic acid.

Working protocol has been followed step by step:

-turbidity of the suspension was controlled and adjusted to 0.5 McFarland (1.5×10^8CFU / ml) using sterile saline(4,7);

- a sterile non-toxic swab was inserted into the adjusted suspension, and then the swab was rotated several times, pressing firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab;

- the dried surface of an agar plate was inoculated by streaking the swab over the entire sterile agar surface; this procedure was repeated two more times, to ensure an even distribution of inoculums (4,7);

- after 15 min. of waiting, time for any excess surface moisture to be absorbed, the antibiotic disks were applied using an automated dispencer (4);

- the plates were placed in an incubator at 37°C, after 16-18 hrs. of incubation, each plate was examined and the diameters of the zones of complete inhibition measured, including the diameter of the disk (4,7);

- interpretation of the zone sizes was made by referring to BSAC Methods for Antimicrobial Susceptibility Testing Version 10.2 May 2011 and the strains were reported to be either susceptible, or resistant (2).

Results and discussions

On EMB, the presence of *E. coli* species was confirmed by emergence of colonies slightly raised, dark purple color with metallic luster and golden-green reflections.

TSI- glucose fermentation with gas production +, lactose fermentation +, production of H_2S -

MIU- mobility +, indole +, urea –

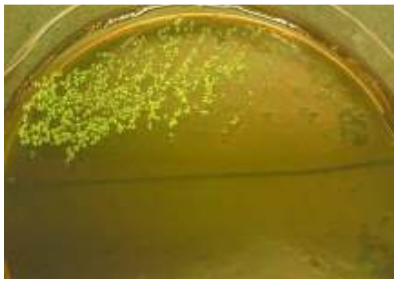

Fig.1. *Escherichia coli* on EMBA

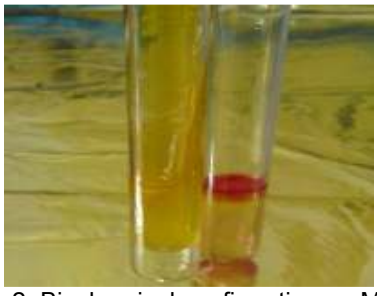

Fig.2. Biochemical confirmation on MIU and TSI

To detect ESBL-producing strains we used double-disk test.

Comparison of the zones given by cefotaxime 30 µg versus cefotaxime + clavulanate 30 + 10 µg and ceftazidime 30 µg versus ceftazidime + clavulanate 30 + 10 µg discs were made.

Another combination double-disk method compares the zones given by cefpodoxime 10 µg and cefpodoxime + clavulanate 10 + 1 µg discs (5).

Table 1

The β-lactams used to reveal the ESBL-producing *Escherichia coli* were:

Nr. crt.	***β-lactam***	***Disk content (µg)***	***Abbreviations***
1.	*Cefotaxime*	*30*	*CTX*
2.	*Cefotaxime/clavulanic acid*	*30/10*	*CTC*
3.	*Ceftazidime*	*30*	*CAZ*
4.	*Ceftazidime./clavulanic acid*	*30/10*	*CZC*
5.	*Cefpodoxime*	*10*	*CPD*
6.	*Cefpodoxime/clavulanic acid*	*10/1*	*CPC*

ESBL production is inferred if the zones given by the discs with clavulanate are ≥5 mm larger than those without the inhibitor (2).

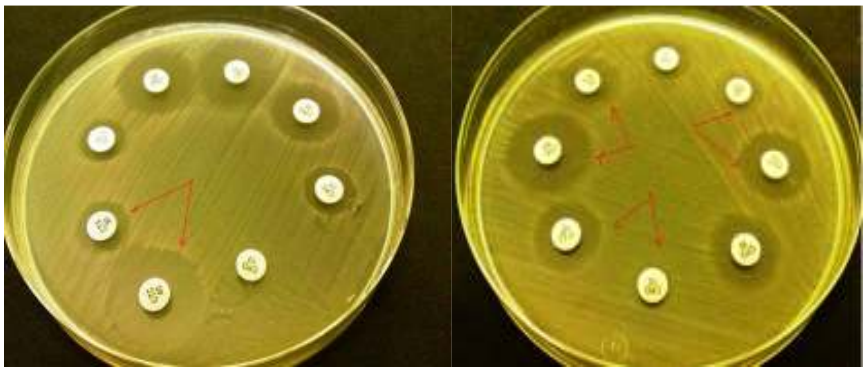

Fig.3 Double disk test

Table 2.

The results obtained by using double disk test

Number total of samples	*Nr. of E. coli strains obtained on EMBA/ CAZ and EMBA/CTX*	*Number of ESBL positive strains identified using CTX-CTC and CAZ-CZC or CPD-CPC*	*% of ESBL positive strains identified*
40	*9*	*6*	*15*

As shown in Table 2 a percent of 15% from the samples collected were positive at phenotypic double disk test.

BSAC recommends that organisms inferred to have ESBLs should be reported resistant to all penicillins (except temocillin) and cephalosporins, including the fourth-generation cephalosporins cefepime and cefpirome (2).

Escherichia coli strains isolated from broilers that proves to be ESBL positive represent a risk factor for public health. According to a rapport published by EFSA in 2011, recent findings indicate transmission of ESBL genes, plasmid and clones from poultry to humans is most likely to occur thought the food chain (5, 3).

Conclusions

From 40 caecal samples harvested from broilers sacrificed in a slaughterhouse, we isolated 9 strains of *Escherichia coli*.

Escherichia coli strains were subject to a double disk test using: Cefotaxime-Cefotaxime/clavulanic acid, Ceftazidime- Ceftazidime/clavulanic acid and Cefpodoxime-Cefpodoxime/Clavulanic acid.

After performing double disk phenotypic tests, 6 of *Escherichia coli* strains proved to be ESBL-producing strains.

ESBL-producing strains isolated from animals may represent a threat for public health because there are limited therapeutic options left for some of these organisms.

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MODERN METHOD - MALDI-TOF MASS SPECTROMETRY - USED FOR THE IDENTIFICATION AND CHARACTERIZATION OF BIOLOGICAL AGENTS FROM *BRUCELLA* GENUS

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Summary

The present research had the aim of developing and optimizing a method for identifying biological agents from *Brucella* genus, by mass spectrometry, with the Microflex LT 20 equipment. Microflex LT 20 is a MALDI-TOF mass spectrometer (Matrix Assisted Laser Desorption Ionization - Time of Flight) equipped with a databases to identify biological agents of various types of samples, prepared with specific methods. The studied strains were cultured in a specific culture medium using classical bacteriological techniques to obtain isolated colonies and to identify them. The results were later confirmed by microbiological analysis of samples with the Microflex LT 20 equipment. The comparison of mass spectra of the tested strains, with the reference spectra of the strains from the database revealed a set of constant proteins regardless of growing conditions, which may represent the proteic signature of biological agents from *Brucella* genus. Proteic spectra of the species of biological agents from the genus, with scores ≥ 75, were stored and incorporated into the database of the Microflex LT 20 equipment for detection and identification of biological agents.

Key words: *Brucella spp*., MALDI-TOF, identification

Brucellosis is an infectious disease with subacute or chronic evolution, found in several species of animals and also humans. It is primarily characterized by abortion and proliferative-necrotic lesions that mainly affects genitals, and affects reproduction function in both females and males. Similar clinical aspects of this disease have been described in humans, before the bacteriological era, by Hippocrates and much later in the eighteenth century, episodes of epizootic bovine abortion have been reported in some northern European countries (Denmark, England, etc.). In l86l Marston describes such a disease occurring in humans, in the island of Malta, which he calls "Mediterranean fever" or "remitting fever." The first scientific data that clarified the causes of disease, were obtained by the British physician David Bruce, who together with his team, in l687 highlights the etiologic agent of the disease for the first time, in smears from the spleens of five people that died because of an infectious disease known on the island by many names (Malta

fever, intermittent fever, tifomalaric fever, pseudotifos). Other discoveries concerning the etiology of brucellosis in animals are made by Bang (1897), Traum (1914), Thomsen (1926), Buddle (1956), Stonner and Lackman (1957), Carmichael and Brunner (1968) (Table 1).

Brucella are aerobic organisms, many strains require the presence of CO_2 in the atmosphere of growth. They are relatively slow-growing and demanding bacteria, compared to other aerobic germs. To grow they require thiamin, biotin and nicotinamide, but not X and V growth factors. Their growth can be stimulated by the addition of normal serum in 5-10%. Growth inhibition occurs in the presence of bile salts, tellurite and selenite. The optimal temperature is 36-37^{0}C and a pH around 7. After 48-72 hours of growth, smooth colonies (S) are round, with a 1-2 mm diameter, with smooth edges, translucent, honey-colored. The rough colonies (R), *Brucella ovis* and *Brucella canis*, have a dry appearance, they are grainy, and have a yellow to golden yellow color. Species of *Brucella* (*Brucella abortus. Brucella melitensis, Brucella suis*) can turn into R forms during faulty cultivation or in degraded or old cultures, which makes it difficult to correctly identify them.

Table 1

Species of *Brucella spp*. in the order of their discovery and their current names

Date and author	Name of the strain	Isolated from	Name of the species
1987 – Bruce	*Micrococcus melitensis*	Spline – sick human	*Brucella melitensis*
1897 – Bang	*Baccillus abortus infectiosi bovis*	Cow – uterin exudate	*Brucella abortus*
1914 – Traum	*Baccillus abortus suis*	Sow	*Brucella suis american (biotip 1)*
1926 – Thomsen	*Baccillus abortus suis*	Pig (Denmark) Rabbit (*Lepus europeus*)	*Brucella suis* european or danish (biotype 2)
1956 – Buddle	*Brucella ovis*	Rams – epidimitis	*Brucella ovis*
1957 – Stonner – Lackman	*Brucella neotomae*	Forest rats – SUA (*Neotoma lepida*)	*Brucella neotomae*
1962 – Pinigin – Petuhova	*Brucella rangiferi*	Raindeers – *Rangiferi tarandi*	*Brucella suis* (biotip 4) (*Brucella rangiferi*)
1967 – Korol – Parnas	*Brucella murium*	Rodents (Mus, Apod – mus) in natural outbreak	*Brucella suis* (biotip 3) (*Brucella murium*)
1968 – Carmichael – Brunner	*Brucella canis*	Bitches with epizootic abortion	*Brucella canis*

MALDI TOF spectrometer is the main component used for the identification of biological agents threw mass spectrometry. The external data system is

connected to the main component, which allows the transmission of the image of the sample inside the specific compartment, zooming in and viewing the sample being essential for positioning the laser beam to obtain spectra. The external data system, allows the user the acquisition, processing, storage and data evaluation.

The analysis software incorporates features for processing, as well as identification and classification of spectra. Processing parameters can be defined by the user (if data reduction or base corrections are required, etc.) the result being a list of clearly defined peaks. The identification of unknown microorganisms is achieved by comparing lists of individual peaks with the database. It generates a score based on identified masses compared with the intensity of correlation between them, this comparative score will be used to finalize the results.

Materials and methods

Obtaining cultures of *Brucella spp.*

The conditions to grow these microorganisms, in order to obtain single bacterial colonies, used to generate the spectra are very important in the economy of Maldi TOF identification technique. For cultivation a special medium was used, for germs belonging to genus Brucella spp (trypticase-soy-agar, tryptose agar-agar and blood). To obtain a good reproducibility and comparability between strains and database, it is necessary to meet the optimal cultivation temperature of analyzed species. It is also recommended to use the same culture medium for all strains that are studied, previous experience showing that very small variations in almost all of reproducible peaks appear in spectra obtained in these conditions. To obtain accurate final results the use of fresh cultures (24 hours cultures), or bacterial colonies grown slowly over the span of several days, is recommended.

Preparation of inoculums

The inoculums was obtained from three strains, *Brucella abortus, Brucella melitensis* and *Brucella suis* that were sown on blood agar culture medium, afterwards being put on the agar trypticase-soy or agar-tryptose. From the obtained culture a suspension of bacteria was prepared, which was verified by the fast Pampana test, with aqueous 0.1% tripaflavin, for the identification of S-R dissociation and then threw microscopic examination to assess the purity of the cultures.

Plates insemination

Each Petri dish with culture medium is controlled before sowing to check bacterial and fungal sterility, using the thermostat for 24 hours at temperature of 37-38°C. In Petri dishes, declared fit, the inoculums is seeded by scarifying using a loop on agar-trypticase-soy, tryptose agar, in order to obtain isolated colonies. Seeded Petri dishes are kept at room temperature ½ - 1 hour, then they are placed into the thermostat (37-38°C) with the agar layer facing upward and they are incubated for 24 hours. After incubation, the plates are removed from the thermostat and are examined with care, macroscopic, removing the ones found to be contaminated. Petri plates with pure culture (isolated colonies) of *Brucella spp*,

are then used for extracting the culture out of which 16S ribosomal proteins are extracted. Out of each Petri dish three colonies were extracted, each colony being a double sample in the end.

16S ribosomal protein extraction microorganisms profiling procedure "extraction of tifluoracetic acid (TFA) 80%"

The procedure is used for the extraction of ribosomal proteins in bacteria in vegetative form but it works very well on sporulate biological material also. To prevent oxidation reactions it is very important to work quickly especially after the extracts have dried and α-cyano-right 4-hydroxycinnamic acid (HCCA) matrix solution has to be immediately added. About 10 mg of biologic material is extracted with a loom from the Petri dish and is inserted into the Eppendorf tube. 50 µl 80% TFA are added. The suspension is pipetted until complete dissolution. After 10-30 minutes are passed 150 ml H2O and 200 ml acetonitrile (AN) are added. They are mixed by centrifugation at, 6000-8000 r/min, for about 2 minutes and the supernatant is transferred to a new Eppendorf tube. For the next step,1 µl (up to 2 µl) of the previous supernatant (biological material) are extracted and applied as a thin film on the steel target plate (the well), it needs to air dry and be covered with 2 µl of the matrix solution. After drying the samples are ready to be examined with the mass spectrometer Microflex LT 20.

The sample test analyze

The studied *Brucella* spp strains were analyzed with the mass spectrometer Microflex LT 20 at least once (after a cultivation, when satisfactory scores of ≥75 were obtained) or after two consecutive plantings. For each strain two colonies were taken, after the 16S ribosomal protein extraction, four wells were displayed from the target plate. On each sample (well) there were at least three to 5 shootings that resulted in about 20 spectra for each strain. These spectra were analyzed and compared with spectra from the device database (if they were any) or other spectra of other examined strains of the same bacterial species, choosing the “cleanest” (without noise) spectrum, with a minimum score ≥ 75 to be entered into the database.

The mass range in which the identification was carried out was established between 1000 and 17.000 Da, most strains having "peaks" between 2000 and 10.000 Da. The parameters established in the identification program Flex Control - m/z - molecular weight (mass), SN - Noise (Signal Noise), Quality Fac - quality factor (Quality Factor), Res - resolution (Resolution), Intens - Intensity (Intensity) , area - the area of defined area (area), were introduced according to working data for the bacteria section. Finally the mass spectra obtained were processed with the BioProfiler Expert program. All examined spectra were identified as belonging to tested bacterial species, actually the results confirmed the results obtained with the conventional bacteriological techniques. A graphic spectrum (mass spectra) was made for each *Brucella spp*. strain, in which the characteristic peaks of polypeptide fragments analyzed can be viewed and their molecular weight can be quantified in Daltons. Also using Flex Analysis software mass spectra of bacterial strains of the

same species were compared with each other to grasp the similarities and differences between spectra obtained.

Results and discussions

Spectra analysis of the genus *Brucella spp*. biological agents, representing the polypeptide fragments of the 16S ribosome's revealed a mass range of 2.000 to 10.000 Da with characteristic peaks between 1.000 and 12.000 Da. Average range of characteristic peaks of the genus was: 2.700-3.600 Da, 3.600 - 4.300; .4.800 – 6.200 Da, 7.300 – 9.700 Da. The average score, taken into consideration to be entered into the data base of spectra was ≥75, a representative score that gives the certainty of a correct identification. We can conclude that strains are similar in terms of 16S ribosomal protein structure, proving a degree of kingship, by comparing the results of the identification tests of strains studied with Maldi Tof technology.

The different composition of growth medium had no significant effect on the way the peaks were distributed. The phase of development (growth) of cells has a low impact on the results obtained. Cells in growth phase lag have a very similar pattern with cells in stationary phase or dead cells. The mass range in which similarities have been observed in studied species of *Brucella spp*. was between 6.000 – 8.000 Da. An analysis of this range shows characteristic peaks of mass species between 6.800 and 7.300, ±100 Da. Below are the mass spectra and the values (Da) of characteristic peaks of studied bacterial species of the genus *Brucella spp*. (figures 1- 4).

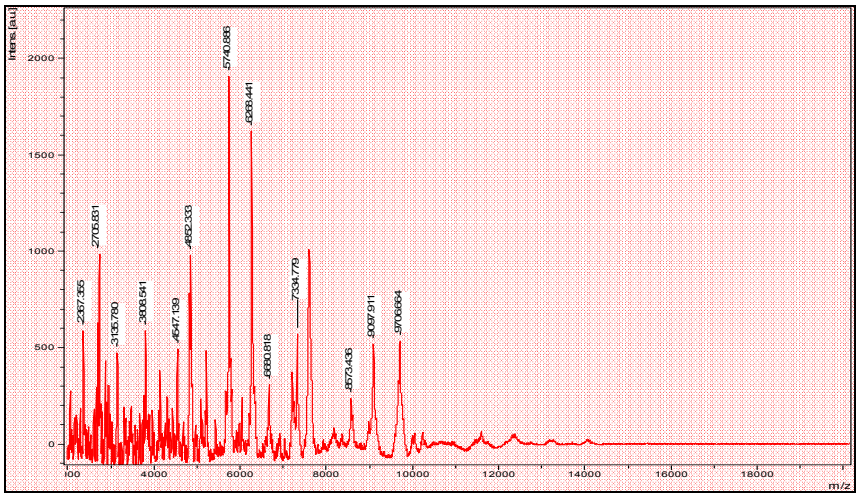

Fig. 1. Graphic representation of *Brucella melitensis* Iasi 54 spectrum

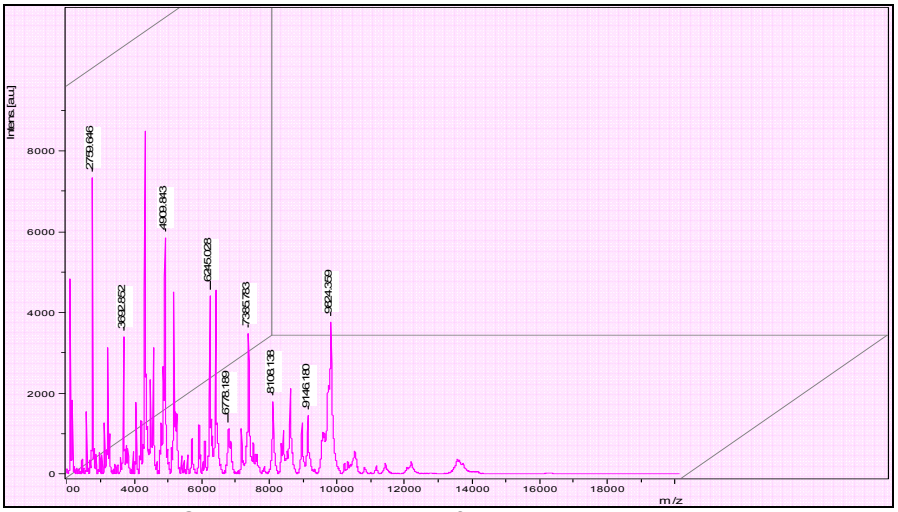

Fig. 2. Graphic representation of *Brucella abortus* 99 spectrum

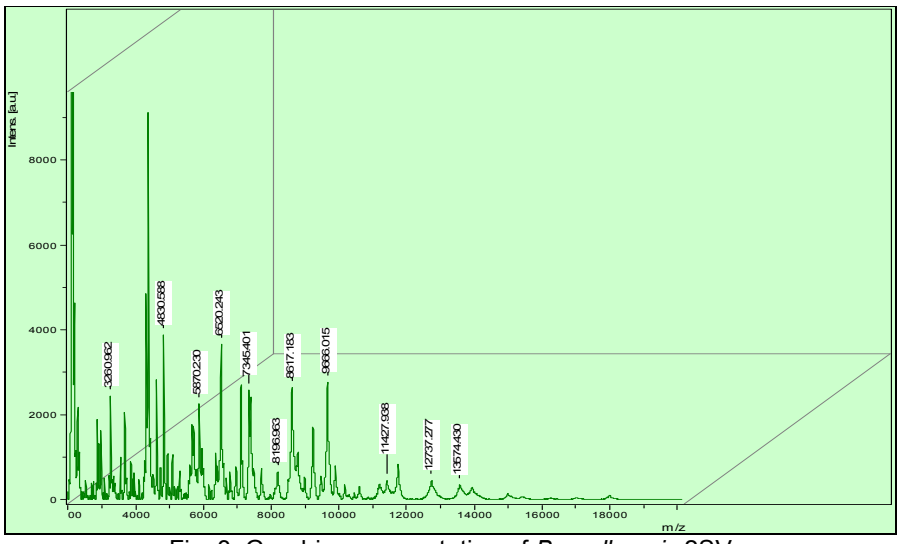

Fig. 3. Graphic representation of *Brucella suis* 2SV

Brucella suis strain 2SV, isolated from an outbreak of swine brucellosis in Mehedinti county, was compared with another strain of *Brucella suis* 5 SV, isolated in the same period out of the same outbreak. The degree of kinship of the two strains was obvious, the characteristic peaks of proteic spectra had very similar

values. For better data processing and evaluation, the spectra obtained were analyzed in groups of 4, by overlapping and individual comparison, a way which allowed more accurate assessment of the degree of correlation between *Brucella spp*. studied strains (figure 5). It is highly important that characteristic peaks are reproducible, a quantifiable result being the frequency in which the same peak would appear in successive measurements.

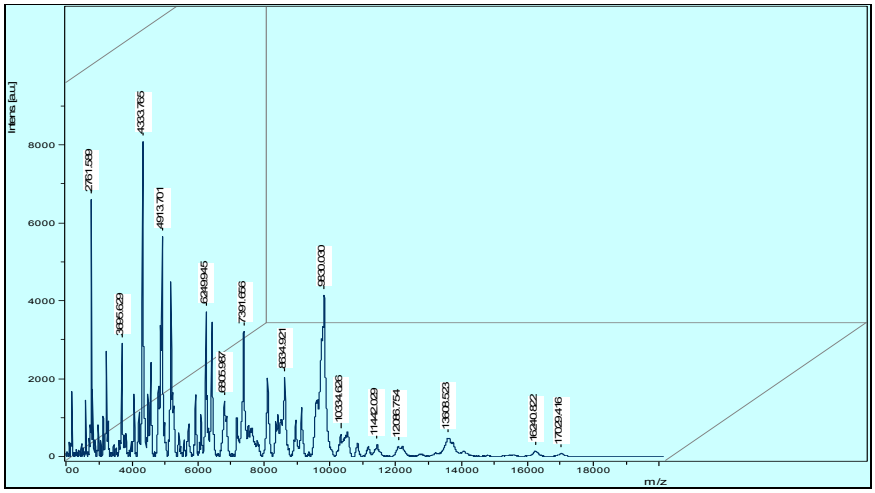

Fig. 4. Graphic representation of *Brucella suis* 5 SV spectrum

Through the analysis of mass spectra presented in the molecular weight figure of characteristic polypeptidic fragments, it can be seen that the characteristic peak values for *Brucella spp.* Strains are ranged between 2.705-13.600 Da. As a result of the fact that the strains belong to the same microbial species (*Brucella spp*), characteristic polypeptidic fragments were also found in other studied strains of the same species.

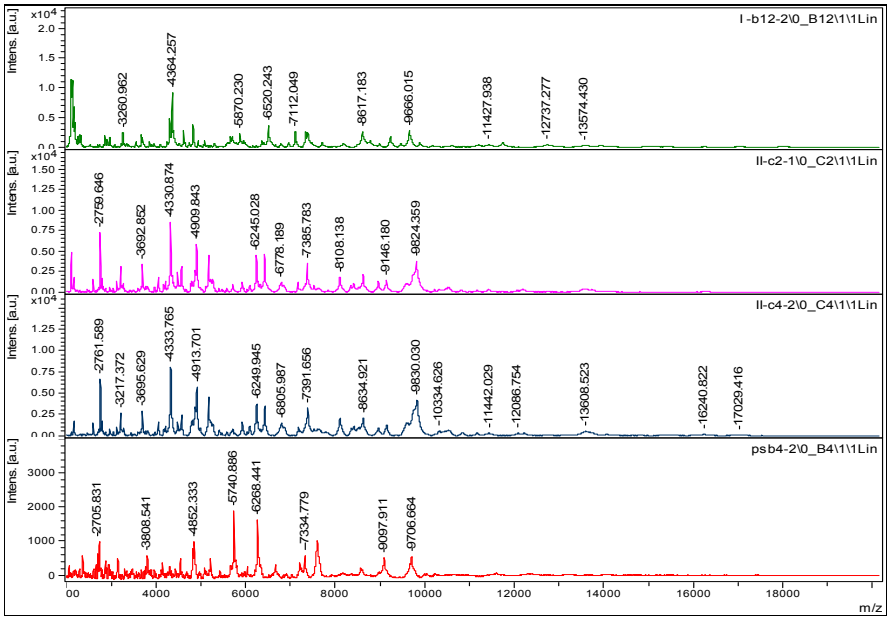

Fig. 5. *Brucella spp*. spectrum and molecular weight comparison (*Brucella melitensis* Iasi 54, *Brucella abortus* 99, *Brucella suis* 2SV, *Brucella suis* 5 SV)

Conclusions

The Microflex LT 20 system, allows quick and accurate identification of biological agents, being an important confirmation/validation means for other methods of microbiological diagnosis.

Average characteristic peak range for *Brucella spp*. genus was between the fallowing mass domains: 12.700-3.600 Da, 3.600-4.300 Da, 4.800-6.200 Da, 7.300-9.700 Da.

The average score of spectra taken into account for the introduction in the database was ≥75, a representative score that gives the certainty of a correct identification. By comparing the results of the tests to identify the strains studied with Maldi TOF technology, we can conclude that most are similar in terms of 16S ribosomal protein structure, thus showing a degree of kinship.

Comparison of mass spectra of the genus *Brucella spp*. strains studied, and spectra of reference strains from the database revealed a high degree of association, represented by a significant correlation of polypeptidic fragments measured, graphics of overlapping spectra being extremely relevant.

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DKcré CELL LINE TRANSFECTION USING CANINE ADENOVIRAL VECTOR CAV2

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Summary

DKcré cell line is widely used in veterinary medical research because the cells support growth of many viruses including adenovirus, canine hepatitis, influenza, vaccinia and others. In this paper we evaluated the efficiency of DKcré cell line transfection using a canine adenovirale vector derived from CAV2. This vector was first deleted E1 gene and has replaced by a fluorescent protein.The plasmide pRecCAV2dlE1CMVGFP was exposed to AscI enzyme action and then we made transfection using two different kits producers with two different concentrations of DNA for each kit and canine cell confluence was 60-80%. After 48 hours of transfection we have seen the appearance of characteristic adenovirus cytopatic effect (ECP) and using fluorescence microscopy we detect ewpression of green fluorescence protein (EGFP).

Key words: Transfection, CAV2, vectors, ECP, GFP.

The process of introducing nucleic acids into-eukaryotic cells by non-viral methods is defined as transfection. Using various chemical, lipid or physical methods, this gene transfer technology is a powerful tool to study gene function and protein expression in the context of a cell.

Development of reporter gene systems and selection methods for stable maintenance and expression of transferred DNA have greatly expanded the applications for transfection. Assay-based reporter technology, together with the availability of transfection reagents, provides the foundation to study mammalian promoter and enhancer sequences, *trans*-acting proteins such as transcription factors, mRNA processing, protein-protein interactions, translation and recombination events (5).

Transfection is a method that neutralizes or obviates the issue of introducing negatively charged molecules (phosphate backbones of DNA and RNA) into cells with a negatively charged membrane. Chemicals like calcium phosphate and DEAE-dextran (8) or cationic lipid-based reagents (3, 12) coat the DNA, neutralizing or even creating an overall positive charge to the molecule. This make site easier for the DNA transfection reagent complex to cross the membrane, especially for lipids that have a “fusogenic” component, which enhances fusion with the lipid bilayer. Physical methods like microinjection (9) or electroporation simply punch through the membrane and introduce DNA directly into the cytoplasm (2).

Replicative vectors expressing the rabies virus glycoprotein, derived from canine adenovirus have been reported to be promising vaccines in various animal models (1). Adenoviral-based vaccines derived from canine adenovirus type 2 (CAV-2) have been successfully developed in domestic mammals by insertion of the foreign gene into the E3 region, a non-essential region for virus replication (4) or into E1 region leading to production of non-replicative vectors (6).

The object of this process was to introduce DNA of interest, in our case the vector recombinant CAV-2 E1 gene deleted and expresses GFP protein, in DKcré canine cell line and see if recombinant virus can replicate the canine line that was transfected.

In our research we used two commercial transfection kits available (*Lipofectamine*-**Invitrogen** and *jetPrime*-**PolyPlus transfection**), that come from different manufacturers to see if there are large variations in transfection process. I also use features two different concentrations of DNA for each transfection kit showing the efficiency of transfection process and if we have a small amount of genetic material of interest. To evaluate the effectiveness of transfection process i watched appearance of citopatogen effect characteristic in adenovirale infection in DK cells and the GFP transient expression by fluorescence microscopy.

Materials and methods

2.1. Cells and viruses

We used dog kidney cells (DK) which expressing the CAV2 E1 region vector (DK-E1) (10), were grown in MW6 plates as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal bovine serum, 1% natrium – pyruvate, 1% antibiotics (penicillin and streptomycin) at 37°C in 5% CO_2. Construction of CAV-GFP non-replicatif vectors has been described by other researchers (7, 11).

2.2. Transfection assays

Before transfection process in canine line DK we did MidiPrep (plasmid purification) then did enzymatic digestion of the plasmide pRecCAV2dlE1CMVGFP using enzyme AscI – BioLabs. Electrophoresis performed after digestion of plasmid revealed the presence of DNA interest submitted to gel.For transfection we used two different kits: Lipofectamine manufactured by Invitrogen and jetPrime manufactured by PolyPlus transfection.

2.3. Transfection analyses

Transfection analyses were carried out using fluorescence microscopy by Olympus U-RFL-T for transient expresion of GFP and optical microscopy for appearance of citopatogen effect characteristic in CAV2 cells infection.

Results and discussions

Before transfection in Dkcre canine cell line was made purification of plasmid *pRecCAV2dlE1CMVGFP* and was obtained at spectrophotometer Eppendorf a concentration 12 ng/ml of double-stranded DNA. O.D. at A260 nm was 0,233 and A 280 nm was 0,129 with ratio 260/280 of 1.80.

For transfection were chosen two different amounts of DNA (3 mg and 5 mg of DNA). To achieve enzymatic digestion with AscI we calculate the required amount of DNA 3 mg DNA ~ 12 ml and 5 mg DNA ~ 21 ml. After digesting plasmid, electrophoresis revealed the presence of two bands migrating corresponding quantities of 3 mg DNA (12 ml) and 5 mg DNA (21 ml) deposited on agar gel.

Regardless of transfection kit used in addition to the two volumes of DNA, was used as negative control cells Dkcré un-transfected and to see the transient expression was used plasmid pRecCAV2dlE1CMVGFP (fig. 1).

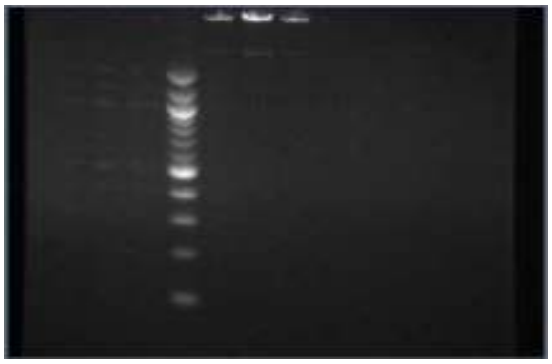
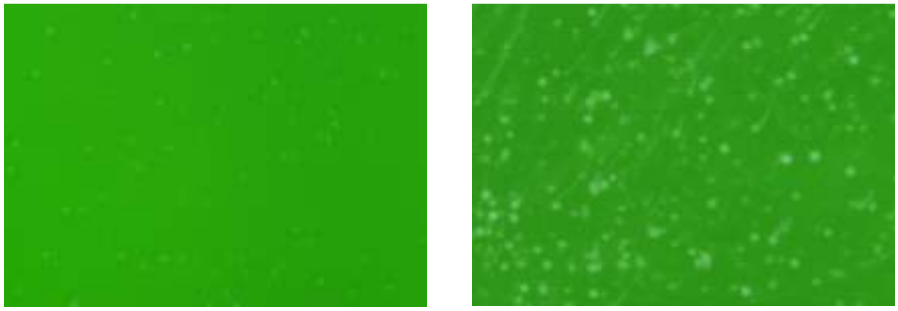


Fig.1. DNA electrophoresis

Transient expresion GFP

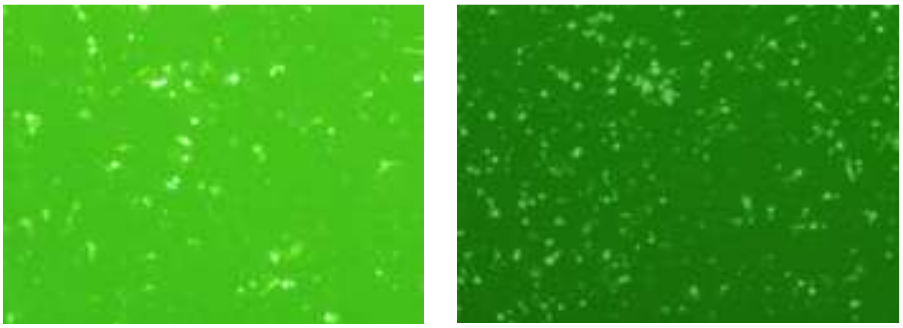
For transfection DK cells we used two different kits (*JetPrime*–PolyPlus transfection and *Lipofectamine* - Invitrogen). After transfection DK line using Lipofectamine kit with two concentrations of DNA (3µg and 5 µg) we observed appearance of transient expression GFP at 48 and 72 hours using fluorescence microscopy (fig 2).



A **B**

Fig.2. Transient expression GFP in DK cells after 48h (A) and 72h (B) post-transfection using Liopofectamine kit

We observed appearance of transient expression GFP after transfection of DK cells using JetPrime kit with two different concentrations of DNA (fig.3).

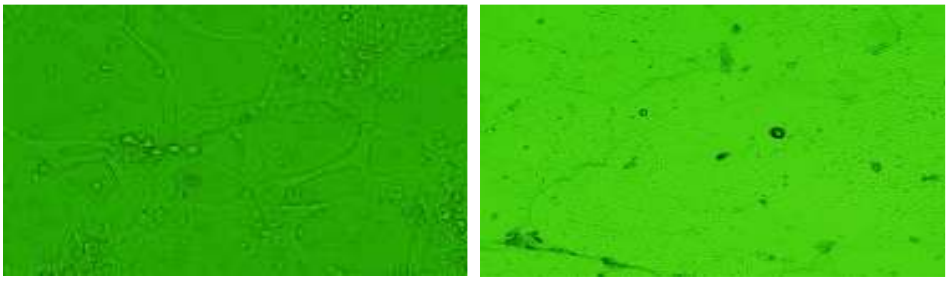


A **B**

Fig.3. Transient expression in DK line with 3µg (A) and 5µg (B) of DNA using JetPrime transfection kit

Citopatogen effect (ECP)

After 48 hours of transfection, using the optical microscope we observe the appearance of cytopathic effect characteristic in adenoviral infection (fig. 4). Between the two transfection methods used have not noticed major differences in terms of cytophatic effect, but differences in the intensity of CPE were observed between the two amounts of DNA used for transfection. The plates transfected with 5 mg DNA citopatogen effect was more pronounced than in those transfected with 3 g DNA.



A B

Fig.4. Cytophatic effect after 72 hours post-transfection using 3µg DNA (A) and 5µg DNA (B)

We used two different transfection kits manufacturer to make a comparison between them in terms of effectiveness, but if used for transfection of Dkcré line 3 mg DNA and 5 mg DNA differences were not observed. It is possible that the use of higher concentrations of DNA for transfection either of these two methods are more effective or possible both transfection methods are inefficient when using DNA concentrations increased cellular toxicity may occur through the use of quantities enhanced reagents of kits used.

Conclusions

Number of vectors and techniques for transfection of cells have been developed over the last decades, each having advantages and disadvantages in their applicability on culture cells.

In this study, canine adenovirus type 2 vectors were used to transfect DKcré cells. Have demonstrated that Cav-2 vectors can transfect dog cells because after two days we observed transient expression of GFP (that prouve Cav-vectors are inside DK cells) and the appearance of cytopathic effect wich is characteristic for adenoviral infections.

Both transfection kits (Lipofectamine and JetPrime) that we used work fain in different concentrations of DNA, without appearing cytotoxicity.

In future we propose to transfect and others type of cells (human, bovine, etc) for different medical aplications using canine adenovirus type 2 vectors.

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THE ALLERGENIC POTENTIAL OF AIRBORNE PARTICLES AND MICROORGANISM ON ANIMAL AND PERSONNEL FROM ONE LABORATORY ANIMAL FACILITY

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Summary

In order to establish the potential exposure of animal and human workers to airborne particles and microorganisms from a laboratory animal facility, two rooms from such a facility were examined. In one room (A room) mice were housed and in the other room (B room) guinea pigs were housed. Total particle count, mesophilic aerobic total count (MATC), staphylococci, coli forms and fungi from a known air volume were assessed. The mean value of particle with an aerodynamic diameter of 1 µm was 22.225, for 2 µm diameter particle the value was 9.988, for 5 µm diameter particle the value was 1.624, for 10 µm diameter was 499, for 15 µm diameter was 167, and for 20 µm diameter was 129. The mean value of MATC was 7.880 CFU / cubic meter, for staphylococci was 4.345 CFU / cubic meter, coli forms were 1.095 CFU / cubic meter, and fungi were 1.510 CFU / cubic meter. No major differences between the rooms were recorded. Bedding change procedure increase the number of particle dust and airborne microorganisms in the air of the facility.

Key words: airborne microorganisms**,** laboratory animal facility, particle.

The occupational risks in laboratory animal facilities include exposure to airborne dust particles and airborne microorganism via the respiratory tract or skin contact with animal allergens. Patients with allergies to guinea pigs, rats, rabbits and mice were reported in 1961. Since then, allergy to laboratory animals has become a well-know occupational disease in personnel exposed to them. The major allergens from the mouse and rat are lipocalin proteins produced by the liver and excreted in the urine. The allergens are found on particle over a considerable size range, including those >10µm in aerodynamic diameter (5, 12). Contact with contaminated bedding seems to be a major source of allergen exposure (dust particles and microorganism). Besides the fecal and urine excretions, the soiled bedding also contains fur, hairs and dander from the animals, as well as food particles. This provides a growth environment for bacteria and fungi.

The contaminants in the bedding may be spread with the dust particles to the breathing zone of the personnel when various care or experimental procedures are carried out. Hence, the dustiness of bedding is a critical determinant of exposure to the risk factors which the bedding contains (1, 7).

The microbial growth in soiled bedding is natural: animals have their own microbial flora and their fecal wastes contain high amounts of intestinal bacteria, which may or may not grow in the bedding material. These, or the microbes originating from the environment, may include species with risk factors for the health of humans or animals. Fungal-induced rhinitis, for example, been reported in rats grown on corncob contact bedding with a high fungal spore load (2, 4).

In this study, the value of particle with an aerodynamic diameter of 1, 2, 5, 10, 15 and respectively 20 µm and the concentrations of mesophylic bacteria, staphylococci, coli forms and fungi were measured in one laboratory animal facility. Besides the bedding analyses, airborne concentrations of inhalable and breathing dust were measured during the work activities in the animal rooms and bedding store to evaluate the levels of these contaminants in the workplace air.

Materials and methods

The animals (mice and guinea pigs) were housed in conventional animal rooms of size 2.50 x 2.00, height 3.00 m, and natural ventilation, temperature 20.2°C for mice and 18.5°C for guinea pigs. The relative humidity in the animal rooms was 50±10%. The animals in their cages were situated on racks near the walls. The animals were maintained in compliance with the *European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes*.

The animals were under a circadian rhythm of 12:12, the lights on at 07:00 h. Their diet was a normal diet for mice and guinea pigs. The bedding used was aspen chips.

Measures were made in front of cages. Dust particles were determined with *Laser Particle Counter* device. The contamination level of air with bacteria and fungi was determined by suction method of a known air volume and impact on culture media, in order to retain germs, with a *Sas Super 100* device. Among microbiological indicators of air contamination, MATC, staphylococci, coli bacterias and fungi were assessed. The culture media used were *nutritive agar* for MATC, *Chapman agar* for staphylococci, *Levine agar* for coli forms and *malt extract agar* for fungi. The aspirated air volume was 50 liters.

Results and discussions

During February - March of 2011, multiple samples were carried; in order to a proper evaluate of the biological factors from the micro-environment. In the animal laboratory facility, were housed and cared 180 mice and 40 guinea pigs. During determinations, in both rooms particles with an aerodynamic diameter of 1 and 2 micrometers prevailed. Thus, before bed changing, the mean values were 22.255 for 1 micrometer diameter particles and 9.988 for 2 micrometers diameter particles.

Although these particles reach the lung alveoli, a significant amount of them are immediately eliminated within the next exhalation, another part is eliminated over several hours. While, *Thulin* et al. (12) state that particles with an aerodynamic diameter bigger or equal with 10 micrometers have an allergenic role, others researchers (8) suggests that particles with 5 micrometers diameter could became allergenic factors, because those particles could reach the bronchiolar level and could enter into the blood stream.

The mean number of particles with a diameter between 5 and 20 micrometers is 605 per air cubic meter. The bed changing moment generates a five time higher level of airborne particles.

The detailed values of particle count per air cubic meter are listed in table 1 and table 2. *Kowalski* et al. (8) found a mean of 62 particles / air cubic meter in a laboratory animal facility, this value increase at 158 particles / air cubic meter during bedding change.

Considering ISO 14644-1:1999 classification of environment contamination according to airborne particles diameter, the animal laboratory facility belongs to level 7 (on a scale ranging from 1 to 9). This is equivalent with an environment well contaminated with particles, with allergenic potential to human and animals (13).

Table 1

Dynamic diameter of airborne particle depending on bedding in A room

Airborne particle diameter	Particle number before bedding change	Particle number in moment of bedding change	Particle number two hours after bedding change
1µm	22.6×10^3	113.2×10^3	27.3×10^3
2 µm	10.2×10^3	48.7×10^3	14.5×10^3
5 µm	1.8×10^3	5.3×10^3	2.2×10^3
10 µm	527	2.8×10^3	754
15 µm	183	499	328
20 µm	130	368	248

Table 2

Dynamic diameter of airborne particle depending on bedding in B room

Airborne particle diameter	Particle number before bedding change	Particle number in moment of bedding change	Particle number two hours after change bedding
1µm	21.8×10^3	110×10^3	29×10^3
2 µm	9.7×10^3	48×10^3	12×10^3
5 µm	1.4×10^3	7×10^3	4×10^3
10 µm	471	2×10^3	837
15 µm	150	724	382
20 µm	128	660	196

All types of contaminants sought were found in the mice and guinea pigs rooms. Regarding MATC, in room A, the mean value was 5.860 CFU/air cubic meter, while in room B the mean value was 9.900 CFU/air cubic meter.

The staphylococci prevail, in room A there were 3.200 CFU/cubic meter, in room B there were 5.440 CFU/cubic meter, followed by coli forms (930 CFU/cubic meter in room A and 1.260 CFU/cubic meter).

The total count of fungi on unchanged bedding was 1.160 CFU/cubic meter in room A and 1.860 CFU/cubic meter in room B. The background concentrations of total airborne bacteria, 7.880 F.C.U/m^3, were at high level as generally found in ordinary indoor environments.

The recoded values are comparable with those obtained by *Kaliste* and all (7) during bedding change. However, these values are well below those reported from other animal facilities, such as poultry confinement buildings (36.000±7.400 F.C.U./m^3) or swine buildings (20.000±60.000 F.C.U./m^3, (3).The concentration of viable fungi, 1.510 F.C.U,/cubic meter was higher than those reported in recent studies (706 F.C.U./cubic meter), these values are comparable with those obtained from poultry or pig farming. For example, in chicken production the fungal concentrations were reported to be 2.500± 24.000 F.C.U/m^3 (6). In swine production, levels of 200±9.000 F.C.U/m^3 were found (3).

Conclusions

All types of contaminants sought were present in the mice and guinea pigs rooms, but the concentrations were well below hazardous levels. The work activities increased the concentrations, the levels returned to background levels two hours after the work finished.

The duration of increased concentrations showed the ventilation effectiveness, poor ventilation and hygiene, increased particulates and aero-microflora concentrations in the environment.

The obtained values were not higher due to the small number of animals.

Airborne contaminants should be considered as potential occupational health risks for all working with laboratory animals and appropriate monitoring and preventive measures adopted.

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TUMOURS OF THE AVIAN IMMUNE SYSTEM

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Summary

The immune system of birds, as in the case of mammals, consists of a complex network of a large number of cell types and cascades of soluble factors, all of which work in concert to induce protective immune responses against the continuous challenge from a wide range of pathogens. A properly functioning immune system is vital to the survival of poultry flocks kept in intensive conditions, where they are constantly challenged by a plethora of rapidly spreading infectious pathogens. The avian immune system plays a crucial role in preventing disease and maintaining health. The immune system is also vulnerable to different types of diseases, and many pathogens specifically target this system with devasting effects. The three neoplastic diseases mainly the avian immune system are (1) Marek's disease (MD)-associated T-cell lymphomas, (2) avian leukosis tumors of B-cells and other hematopoietic cells, and, (3) rethiculoendotheliosis virus (REV)- induced tumors characterized by a variety of syndromes including lymphoid neoplasia.

Key words: tumors, rethiculoendotheliosis, Marek's disease, avian leukosis

Marek's disease

MD, named after the Hungarian pathologist Joseph Marek, is a lymphoid neoplasia of domestic chickens and, less commonly, turkeys, quails and geese. A recent survey has indicated that MD still remains a major problem in many countries (2).

The most recent estimates of economic impact of MD on the world poultry industry is in the range of US$ 1-2 billion annually (16).

MD is not vertically transmitted through infected eggs. However, chicks become infected with the virus almost immediately after hatching from heavily contamined poultry houses.

Cytolytic infection occurs between 3 and 6 days after infection leading to extensive atrophic changes in the bursa of Fabricius and thymus causing some early mortality. After this early cytolytic period, the virus becomes latent, and the clinical expression of paralysis or tumors can occur anytime after 3-4 weeks after infection. Under field conditions, most of the cases begin after 8-9 weeks. Marek's disease (MD) is a prevalent T-cell lymphoma of chickens caused by a cell-associated alphaherpesvirus, Marek's disease virus (MDV).

Lymphoma genesis by MDV requires the expression of Meq (Marek's EcoRI-Q-encoded protein), a basic leucine zipper (bZIP) protein expressed during lytic infection, latency, in MDV- induced lymphomas, and derived cell lines (7).

Clinical signs can vary from bird to bird. The most common clinical sign is the partial or complete paralysis of the legs and wings. When the nerve controlling the neck muscles are affected, symptoms such as torticollis are observed. Similarly, the involvement of the vagus nerve can result in the paralysis and dilation of the corp. Such birds could also show symptoms of gasping and respiratory distress. Apart from generalized manifestations such as depression, weight loss, anorexia and diarrhoea, the clinical signs are less marked. MD is characterized by neurological signs, chronic wasting, and T cell lymphomas that predominate in the visceral organs (5).

Mortality can increase rapidly over a few weeks, or can continue at a steady rate or decline over several months. Acute cytolitic disease observed with some of the recent very virulent MD virus (vvMDV) strains shows a severe atrophy of the lymphoid organs and a very high mortality usually between 10 and 14 days of age. The affected organs show lymphoid infiltrations, with the degree of infiltration correlating with the disease manifestations.

The control of the disease is essentially based on preventive vaccination, although improved biosecurity and genetic resistance can contribute to the control. Vaccination with live attenuated vaccines represents the main strategy for the prevention and control of MD. These are usually administered as cell-associated vaccines to day-old chicks to provide protection against the natural challenge the chicks are exposed after leaving the clean hatchery environment and being placed in the infected poultry house environment.

With the introduction of *in ovo* immunization methods, an increasing number of birds are vaccinated by this route. MD vaccines, derived from all the three MDV serotypes.

The most widely used serotype 1 vaccine is derived from the CVI988/Rispens strain and is effective against most of the vvMDV and vv+MDV pathotypes.

Antigenically related serotype 2 strains such as SB1 and 301B/1 are also used widely in many countries.

The serotype 3 FC-126 strains of herpesvirus of turkey (HVT) are available as cell-free and cell-associated forms of vaccines. Many of these vaccines are effective individually, the concept of protective synergism (6) has led to widespread use of polyvalent vaccines with two or more strains administered simultaneously (11).

Immune Responses to MDV. MDV has major immunosuppressive effects on the hosts. The immune responses developing during the early cytolitic phase are crucial for the outcome of infection, since any impairment of immune responses during this phase could delay the establishment of latency prolonging the cytolytic destruction of immune cells by virus-induced apoptosis. The immune response is important during the latency phase for preventing onset of MD lymphomas.

The vaccine-induced immunity is thought to be primarily anti-tumor responses, since MDV vaccines do not prevent super infection with the virus. Vaccines do reduce cytolytic infection thereby preventing extensive damage to the immune system through the continued destruction of immune cells.

Innate immune responses against MDV include changes in cytokine expression as well as NK and macrophages responses. These include the up regulation of a number of pro-inflammatory cytokines driving a Th-1-type response, the increased transcription of inducible nitric oxide synthase II (iNOS), as well as the enhanced NK cell and macrophage activity (16).

Humoral responses and cell mediated responses have been described after natural infection or vaccination with MDV. The importance of humoral immune responses is considered to be relatively minor due to the highly cell-associated character of the virus. The presence of maternal antibodies may delay virus replication and interfere with vaccine-induced immunity especially when cell-free vaccines are used. Virus-neutralizing (VN) antibodies can be induced by MD vaccination/infection, and these are mostly thought to be directed against the antigens such as the glycoprotein B (16).

Avian Leukosis. Avian leukosis embraces several different leukemia-like neoplastic diseases of the hematopoietic system. These tumors are induced by avian leukosis virus (ALV), members of the *Alpha retrovirus* genus of the family *Retroviridae* (15).

The group of closely related avian sarcoma and leukosis viruses (ASLVs) evolved from a common ancestor into multiple subgroups, A to J, with differential host range among galliform species and chicken lines. These subgroups differ in variable parts of their envelope glycoproteins, the major determinants of virus interaction with specific receptor molecules. Three genetic loci, *tva*, *tvb*, and *tvc*, code for single membrane-spanning receptors from diverse protein families that confer susceptibility to the ASLV subgroups (8).

Avian leukosis can be grouped into different types of tumors: lymphoid leukosis (LL), tumors of B-lymphocytes, is one of the commonest forms of leukosis. LL occurs in chickens from about 4 months of age and is most commonly caused by ALV of subgroups A and B. Gross pathological changes include diffuser nodular enlargement of the bursa of Fabricius, liver, spleen and other organs due to coalescing foci of extravascular immature lymphoid cells. Erythroid leukosis or erythroblastosis is an uncommon, usually sporadic, tumor of the erythroid cells occurring mainly in adult chickens.

The disease is an intravascular erythroblastic leukemia. In the affected birds, the liver and spleen, and sometimes the kidneys, are moderately and diffusely enlarged and often of bright cherry-red color. Microscopically the liver shows intra-sinusoidal accumulations of rather uniform, round, erythroblasts, the spleen shows accumulations of erythroblasts in the red pulp, with bone marrow showing enlarged sinusoids filled with erythroblasts. Myeloid leukosis broadly involves both myeloblastic myeloid leukosis (myeloblastosis) and myelocytic myeloid leukosis (myelocytomatosis).

The disease has become particularly prevalent in broiler breeders infected with subgroup J ALV in many countries. ALV are also associated with a variety of

solid tumors, including fibrosarcoma, chondroma, haemangioma, histiocytic sarcoma, mesothelioma, myxoma, nephroblastoma, osteoma and osteoporosis (9).

ALV induce tumors by two main types of mechanisms:

- by the activation of a cellular proto-oncogene after ALV integration. This leads to the activation of cellular oncogenes leading to neoplastic transformation, the mechanism being described as "insertional mutagenesis". Examples of such oncogene activation include the *c- myc* in LL and *c-erb*B in erythroblastosis (4). As this mechanism of induction usually takes several weeks or months these viruses are termed "slowly transforming" viruses.
- by the activation of a transduced oncogene carried by the ALV genome. Such viruses are able to induce tumors rapidly and are termed "acutely transforming" viruses. Examples of such viruses include MC29 that carry *v-myc* and myeloblastosis viruses that carry *v-myb* oncogenes.

ALV can be transmitted either by vertical (congenital or egg) or by horizontal spread through contact.

In the vertical transmission, the eggs become contaminated with the virus within the oviduct leading to the infection of chick embryos during incubation. This route of congenital infection leads to strong associations between the presence of virus in vaginal swabs, egg albumen and embryos, which provide the basis for ALV eradication programmes in breeding stock. Congenitally infected chicks are an important source of contact infection in the hatchery and during the brooding period, and meconium and faeces from congenitally infected chicks contain high concentrations of ALV.

The horizontal mode of spread is responsible for the high incidence of infection in flocks. Sources of virus from infected birds include faeces, saliva and desquamated skin. Unlike MDV, the survival of ALV outside the body is relatively short and hence eradication of the virus is feasible in many farms.

Eradication of ALV from a flock depends on breaking the vertical transmission of virus from dam to progeny and preventing re-infection of the progeny. The procedures for eradication depend on the identification and elimination of hens that shed ALV to their egg albumen and hence to their embryos and chicks. Such hens, which are viraemic and shedders of the virus, are usually identified by testing their cloacal/vaginal swabs or egg albumen by enzyme-linked immunosorbent assay (ELISA) for presence of high levels of ALV gs-antigen. Continuous monitoring and elimination of infected birds will break the spreading life cycle of the virus and lead to ALV eradication.

Immune Responses to Leukosis Sarcoma Viruses. Humoral responses mediated through specific neutralizing antibodies are the essential components of immunity against retroviruses. This can be demonstrated in congenital egg transmitted ALV infections, where absence of neutralizing antibodies is usually associated with high levels of viremia and virus shedding (1).

Major histocompatibility complex (MCH)-restricted CTL-mediated immune responses have also been identified against retroviral infections. The difference in

susceptibility to Rous sarcoma virus (RSV)-induced tumors by different haplotypes of chicken lines (17). While some genetic lines of chickens inoculated with the RSV develop tumors that progress inducing death, RSV-induced tumors show regression in other lines (13, 14). The regression of tumors in the resistant lines is determined by a dominant gene, R-RS-1, that lies within the MHC locus (17). Conserved peptide motifs of RSV proteins that bind to the MHC have been shown to be protective against RSV tumor growth in chicken with the B-F12 haplotype (3). Recently, the peptides motifs of the single dominantly expressed class I molecule associated with the MHC-determined responses to RSV have been identified (18).

NK cells and macrophages also may play a role in the immune responses against RSV tumors. For example, it has been demonstrated that ALV-transformed cell lines such as LSCC-RP9 could be lyses by NK cells. Similarly, macrophages from RSV tumors regress or lines of chicken showed more cytotoxicity on these cell lines demonstrating the role of these cell types in immunity against RSV tumors.

Rethiculoendotheliosis. Rethiculoendotheliosis refers to a group of syndromes in poultry and game birds associated with REV (19).

REV, belonging to the family *Retroviridae*, shows no serological relationship to ALV. One of the syndromes associated with REV is the runting disease syndrome characterized by runting, bursal and thymic atrophy, enlarged peripheral nerves, abnormal feather development, proventriculitis, enteritis, anaemia, and liver and spleen necrosis. The abnormal feathering, in which the barbule of wing feathers are adhered to the feather shaft, is termed “nakanuke” in Japanese, and has been seen in chicken flocks vaccinated with REV-contaminated vaccines. REV also induces chronic lymphoid neoplasm in the bursa of Fabricius and other organs. These tumors are of B-cell origin and are caused by REV proviral insertion activation of the cellular *myc* oncogene. Non-bursal lymphomas of T-cell origin have also been induced experimentally with latent periods as short as 6 weeks involving the thymus, liver, heart and spleen.

Acute reticulum cell neoplasia (rethiculoendotheliosis) is induced by the defective T-strain of REV that carries the *v-rel* oncogene. When injected into young chicks, the T-strain, originally isolated from a turkey with leukosis lesions, induces rapid proliferation of primitive mesenchymal or reticuloendothelial cells causing death within1–3 weeks.REV is transmitted both horizontally by contact with infected chickens and turkeys, and vertically from tolerantly infected chicken and turkey dams. Vertical transmission can also occur from infected male chickens and turkeys. REV can be detected in faeces and cloacae swabs. REV gs-antigen, and sometimes infectious virus, can be detected in albumen of eggs from tolerantly infected hens. Because of the usually sporadic and subclinical nature of REV infections in chickens and turkeys, large-scale control procedures have generally not been considered necessary in commercial poultry production.

However, freedom of infection in breeding flocks that produce progeny for export is required by some importing countries. It is considered probable that REV eradication could be achieved by prevention of vertical transmission by detecting

shedding dams and sires for REV gs-antigen by ELISA, and rearing progeny in isolation, using methods similar to those for control of leukosis.

Immune Responses to REV. Neutralizing antibodies play an important role in immunity to REV. In addition to the humoral immune responses, cell-mediated immunity is considered to be a major element in REV infection, ad REV has been used as an important model for studying virus-specific, MHC-restricted CTL responses in chickens. Neonatal thymectomy increased REV-induced mortality in birds challenged with the T-strain virus, suggesting that cells-mediated responses are important for protection. Cell-mediated immune responses and NK cells may also influence anti-REV immune responses.

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HUMORAL IMMUNE RESPONSE IN SHEEP AFTER VACCINATION ASSOCIATED WITH AN IMMUNOMODULATOR PRODUCT

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Summary

Complex network of the interactions between immune system and mycoplasmas involves specific and non-specific immune responses. The specific defense mechanisms include production of anti-mycoplasmas antibody of different classes and sub-classes at both systemic and local level, stimulation of cell mediated immunity, and opsonization and phagocytosis of the bacteria. The specific relations triggered by pathogenic mycoplasmas plays an important role in the lesions occurrence and in enhancing of clinical manifestations caused by mycoplasmas.

The purpose of this study was to assess the effect of the anti-*Mycoplasma agalactiae* vaccine over the immune response in sheep. We assessed the intensity and duration of humoral immune response in two groups of animals: one immunized only with the vaccine, and the other with vaccine in conjunction with an immunomodulator product (Bio-Mos).

Key words: sheeps, antibodies, immunomodulation

Immunological reactivity of the organisms is conditioned by the degree of morphological and functional development of humoral and cellular factors involved in immune reactions.

Mycoplasmas have a wide series of non-specific immunomodulator effects over the immune system cells by inducing suppression, by polyclonal stimulation of the B and T cells, by inducing the synthesis of different cytokines, by amplifying the cytotoxicity of macrophages, NK cells, and T cells, by stimulating the expression of cellular receptors, and by activating the complement cascade (5, 11).

Even though mycoplasmas induces both a cellular and humoral immune response, the latter is usually the most studied. Thus, the indirect diagnosis is made by using serological tests, which reveal specific antibodies present in the infected animal's blood (1, 2, 8).

In this study we assessed the effect of the anti-*Mycoplasma agalactiae* vaccine over the sheep's immune response, and the intensity and duration of humoral immune response in two groups of animals, both vaccinated, one of them being feed with fodder supplemented with Bio-Mos (Alltech), 1kg/tone of fodder.

Materials and methods

The research was made on a group of 30 adult sheep, in dry (nonlactating) period, divided in two groups: A (control) and B (experimental). They were kept in the same conditions and fed the same concentrated fodder. The fodder administered to lot B was supplemented with Bio-Mos (Alltech), 1 kg/tone of fodder.

In order to stimulate immunity, the sheep were injected with *Mycoplasma agalactiae* (vaccine against sheep's contagious agalactia. The dose used was 1 ml administered s. in the flank region, at the beginning of the experiment and after 21 days.

The immunomodulator effect of the Bio-Mos probiotic was assessed based on specific immune response.

In order to carry out serological tests, blood samples were taken from all sheep studied, as follows: S1 – in the day of vaccination, S2 - 21 days after the vaccination; S3 - 42 days after the vaccination; S4 – 63 days after the vaccination; S5 – 84 days after the vaccination; and S6 – 105 days after the vaccination. Biological samples were processed in the laboratory of Microbiology and Immunology Department, Faculty of Veterinary Medicine Timisoara.

Results and discussions

Induction of a rapid immune response is the key to stimulate the animals' development and performance. Improving the capacity of organism to defend against pathogens by increasing the antibodies titer and activity indicates a greater ability to combat diseases, and will result primarily in improved health and growth performance (6).

Among strategies to reduce exposure to pathogens are strict biosecurity programs, use of feed additives, such as organic acids or probiotics, and vaccination. Vaccines that stimulate production of specific antibodies reduce immune stress and, therefore, have a positive effect on animals' health. However, depending on the type of vaccine, vaccination may have a negative impact on growth performance due to acute phase proteins secretion (9).

The Bio-Mos supplement in the sheep's diet had a strong effect over the humoral immune response, our results being comparable with those obtained by other researchers in poultry and turkeys (3, 4, 10). The presence and levels of specific antibodies, measured by indirect ELISA, are presented in Tables 1 and 2.

ELISA technoque allowed, because the sensitivity, highlithing the exact titer of specific antibodies against the vaccine main antigens.

The antibody titre, expressed as optical density units using a multichannel spectrophotometer, has increased gradually in both control and experimental group. The highest values were registered in experimental group (vaccinated + Bio-Mos (Table 2).

Table 1

Humoral immune response in control group

No.	Time between sampling					
	Day of vaccination	**At 21 days**	**At 42 days**	**At 63 days**	**At 84 days**	**At 105 days**
1	124	277	409	432	197	174
2	122	190	485	462	195	187
3	198	372	421	1321	504	160
4	124	311	593	1116	391	404
5	270	187	545	166	269	208
6	272	251	445	1159	225	364
7	210	248	298	1440	360	166
8	242	148	659	534	376	226
9	335	215	438	597	375	388
10	253	419	502	577	554	323
11	268	413	540	785	578	215
12	216	193	462	762	278	179
13	294	444	544	815	206	355
14	171	247	657	876	225	245
15	360	272	362	349	215	243
$\bar{x}$ ± Sx	**230.6 ± 19.11**	**279.1± 24.13**	**490.6± 26.34**	**759.4 ± 95.36**	**329.9 ± 34.06**	**255.8 ± 22.38**
C.V.	**32.10**	**33.48**	**20.79**	**48.63**	**39.97**	**33.88**

Legend: $\bar{x}$ = arithmetic mean; Sx = standard deviation; C.V. = coeffiecient of variability

The secondary immune response is more intense, being generated by the renewal of the immunological memory, consecutive to the second inoculation of the vaccine ("booster effect"). After 14 days from the second vaccination, the level of anti-*Mycoplasma agalactiae* antibodies was approximately 7 times higher in the experimental group than in the control group ($\bar{x}_{Pr/P}$ = 6.83±1.06). These results are similar with other literature data, which claim that the antibodies induced by an inactivated vaccine against sheep's contagious agalactia, with sodium hydroxide as adjuvant, are detectable even after three months post-vaccination (7).

Table 2

Humoral immune response in experimental group

No.	Time between sampling					
	Day of vaccination	**At 21 days**	**At 42 days**	**At 63 days**	**At 84 days**	**At 105 days**
1	123	411	1150	938	850	752
2	122	485	1278	1755	1412	753
3	171	874	1288	1090	1432	946
4	124	444	1545	1580	1320	720
5	372	413	1604	1598	1376	903

6	419	419	1124	1811	844	1207
7	413	911	1379	1768	1212	1231
8	343	372	1356	1648	1398	913
9	270	890	1232	1824	1522	879
10	84	874	1564	1644	1476	938
11	177	575	1347	1698	1322	712
12	137	272	1404	1757	1751	663
13	173	911	989	1099	1623	629
14	196	545	1288	1824	1293	1091
15	132	870	921	1814	1412	521
$\bar{x}$ ± Sx	**217.1 ± 29.73**	**617.7 ± 61.72**	**1297.9 ± 50.88**	**1589.9 ± 76.52**	**1349.5 ± 62.79**	**857.2 ± 53.51**
C.V.	**53.03**	**38.69**	**15.18**	**18.64**	**18.02**	**24.18**

Legend: $\bar{x}$ = arithmetic mean; Sx = standard deviation; C.V. = coeffiecient of variability

The highest average value for antibody titer (1589±76.72 unities of optical density) was recorded in the experimental group at 42 days after the second vaccination, consecutive to the secondary antigenic stimulation.

In comparison with experimental group, for control group, where no probiotics were administrated, the values were lower: at 42 days after the second vaccination, the difference between the optical density units recorded in the control and experimental group was of 830.46.

Considering the better results obtained in experimental group (vaccinated + Bio-Mos) and because the significant differences between experimental and control group, we consider that the Bio-Mos probiotic stimulate the immunological reactivity in sheep. Thus, improved immune status of animals can control sub-clinical infections and improve health.

Conclusions

Vaccine against sheep's contagious agalactia induces a primary and secondary immune response of vaccinated sheep, expressed as antibody production that lasts for at least three and a half months post-vaccination.

The antibody titer increases after the first immunization (primary immune response), amplifies after the second immunization (secondary immune response), reaches a maximum level at 45 days, and then gradually decreases until the end of the experiment (day 108), to values that are comparable to those following the primary response.

In comparison with the control group, the mean values of antibodies concentration were higher in experimental group, regardless the moments of biological sampling.

Based on the obtained data, we recommend revaccination to stimulate immune response at four months after last vaccination against sheep's contagious agalactia

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IN VITRO EVALUATION OF CANINE LYMPHOCYTES RESPONSE TO HERBAL EXTRACTS

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Summary

The ability to modulate the immune system functions is reported in case of several herbal-derived products (1,2,6,13) and based on this capacity such products could prove beneficial in the treatment of immune-related diseases and also in the prevention of infectious diseases. The purpose of this study was to determine the effects of eleven commercial herbal ethanolic extracts on *in vitro* canine lymphocyte proliferation. The immunomodulatory response was determined by lymphocyte proliferation assay using concanavalin A, lipopolysaccharide and phytohaemagglutinin as a control for mitogen stimulation. The experimental conditions of the present study pointed out inhibitory effects on the *in vitro* blast transformation capacity of canine lymphocytes, with the most intense inhibitory effect induced by the alcoholic extract derived from *Calendula officinalis* L.

Key words: immunomodulation, canine lymphocytes, herbal extracts

In recent years, a growing interest on herbal-derived products therapeutic potential was noted as it is supported by the results of a large number of experimental and clinical studies (2, 3, 5). Along other complex biological properties, the ability to modulate the immune system functions is reported in case of several herbal-derived products (1, 4, 6, 7, 8, 9, 10, 14) and based on this capacity such products could prove beneficial in the treatment of immune-related diseases and also in the prevention of infectious diseases.

The aim of this research was to investigate the effects of certain herbal extracts on canine leukocytes *in vitro* reactivity by performing the leukocyte blast transformation test. This method allows the measurement of the mononuclear cells response towards sensitizing antigens.

Materials and methods

The experiment included eleven commercial ethanolic extracts derived from the following herbal species: *Ocimum basilicum* L., *Thymus vulgaris* L., *Echinacea purpurea* Moench, *Salvia officinalis* L., *Lavandula angustifolia* Mill., *Vaccinium myrtillus* L., *Calendula officinalis* L., *Hippophae rhamnoides* L., *Rosmarinus officinalis* L., *Mellisa officinalis* L. and *Mentha piperita* L.

This study was preceded by a selection of herbal extracts and concentrations based on the results of their cytotoxic potential screening on canine T cells isolated using the Accuspin ^{TM} –Histopaque®-1077 (SigmaAldrich) system. Cell viability after a 24 hours contact with tested herbal extracts was measured using trypan blue exclusion method.

Blood samples (n=10) were collected on heparine from clinically healthy dogs (n=10) and subjected to leukocyte blast transformation test, the whole blood culture micromethod as described previously (9,11). Leukocytes were cultured in RPMI 1640 culture medium supplemented with 5% fetal calf serum (FCS), antibiotics (penicillin 1000 UI/mL and streptomycin 1000 μg/mL) and medium pH adjusted to 7.2. Cells were plated in duplicate in 96-well microtiter plates.

Sixteen experimental variants for each blood sample were considered, namely (1) control (untreated blood culture), (2) lippopolysaccharides (LPS) (1μL per well) treated culture, (3) phytohaemagglutinin-M (PHA) (1μL per well) treated culture, (4) Concanavalin A (ConA) (1μL per well) treated culture, (5) alcohol (2.5 μL per well) treated culture, (6–16) alcoholic extracts of *Ocimum basilicum* L., *Thymus vulgaris* L., *Echinaceea purpurea*, *Salvia officinalis* L., *Lavandula angustifolia* Mill., *Vaccinium myrtillus* L., *Calendula officinalis* L., *Hippophae rhamnoides* L., *Rosmarinus officinalis* L., *Mellisa officinalis* L. and *Mentha piperita* L. (2.5μL per well) treated cultures. The specified volums of LPS, PHA, Con A and alcoholic extracts were established when using the same technique during preliminary studies as being the most effective *in vitro* for the tested species (8,9,10,11). The cultures were incubated for 48h at 37.5°C and 5% CO_2. Cell growth was quantified by means of the glucose consumption technique, with glucose concentrations measured in the initial medium and in all variants at the end of the incubation period, using a standard (100·mg/dL) glucose solution, by means of an orto-toluidine colorimetric test, as follows: 12.5mL of the cultural supernatant were transferred to 0.5mL of orto-toluidine reagent, boiled for 8min, cooled suddenly in cold water and read in a spectrophotometer at 610nm wavelength (Unico 2100, United Products Instruments, Inc., Dayton, NJ, USA), using the reagent as a blank.

The stimulation index (SI) was calculated as follows: SI % = [(MG–SG)/MG]*100, where SI = blast transformation index, MG = glucose concentration in the initial culture medium and SG = glucose concentration of the sample after incubation. All experiments were performed in duplicates and the obtained data were statistically processed using ANOVA post hoc followed by Dunnett test.

Results and discussions

Stimulation indices (SI) established in case of the tested herbal extracts presented values comparable to that of the control or to those corresponding to standardized variants stimulated with mitogens (Con A, LPS, PHA) (fig. 1).

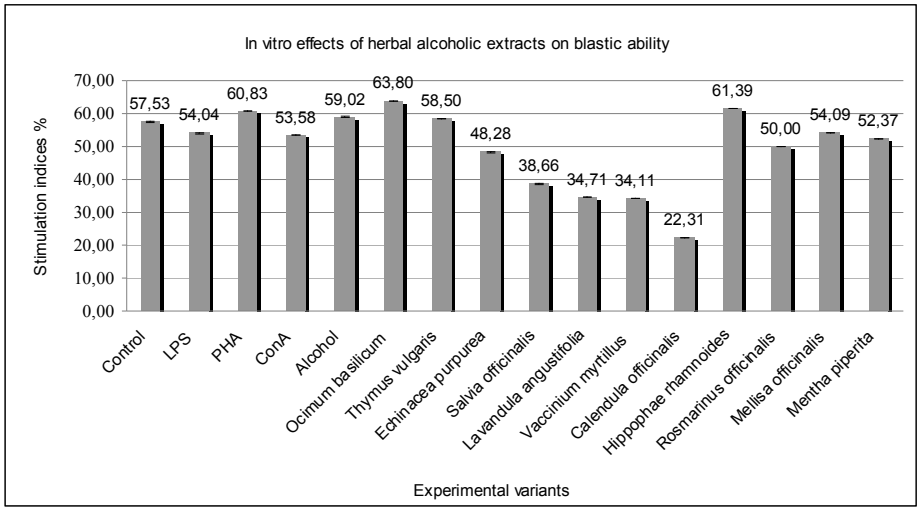

Fig. 1. Immunomodulatory ability of tested herbal extracts

For most of these herbal products, the stimulation indices (SI) possessed values ranging between 48.28% - 63.80%, with no statistically significant differences ($p > 0.05$).

For four of the studied alcoholic extracts, namely those derived from *Salvia officinalis* L., *Lavandula angustifolia* Mill., *Vaccinium myrtillus* L and *Calendula officinalis* L. stimulation indices statistically significant lower compared to those observed for other experimental variants (SI values ranging between 22.31% and 38.66% and $p<0.05$) were determined. There was no statistically significant difference between these four extracts in inducing *in vitro* blast inhibitory effects ($p>0.05$). The lowest value of the stimulation index was calculated for the alcoholic extract of *Calendula officinalis* L. (SI=22.31%) with a statistically significant difference when compared with the control ($p = 0.0010$). This result suggested the above mentioned herbal product expresses an intense inhibitory activity on canine lymphocytes.

Another herbal product displaying similar effects was represented by the alcoholic extract of *Echinacea purpurea*, whose immunomodulating properties are well documented in the literature (1,2,3,4,5,8,9). In this case, only a relatively modest stimulation index (48.28%) was noted, slightly decreased compared to untreated control ($p > 0.05$).

The highest values of stimulation index as determined during this experiment are those observed in case of alcoholic extracts obtained from *Ocimum basilicum* L. (63.80%) and *Hippophae rhamnoides* L. (61.39%). Still, considering the results of ANOVA statistical analysis and standard mitogen substances, an intense or strong immune-enhancer activity can not suggested ($p > 0.05$).

Regarding the immunomodulatory potential of plant derived-products numerous bibliographic references demonstrate herbal active compounds ability to influence major components and functions of cell-mediated immune response in different animal species (1,7,8,9,10,11,13).

Preparations obtained from different species of Echinacea are among the most commonly used immunomodulatory products of plant origin, often recommended as supportive therapy in viral upper respiratory infections (2,3,5). Although Echinacea extracts are used as immuno-enhancers, scientific evidences to justify and sustain the potential therapeutic are still controversial.

Conclusions

The experimental conditions of the present study pointed out inhibitory effects on the *in vitro* blast transformation capacity of canine lymphocytes, with the most intense inhibitory effect induced by the alcoholic extract derived from *Calendula officinalis* L. (the immunosuppressive potential is statistically supported by the value of p, $p = 0.0010$).

Further *in vitro* and *in vivo* studies regarding the optimal concentrations, mechanisms of action, bioavailability, potency, or synergistic effects between the bioactive compounds are required to characterize and validate the therapeutic potential and consider this herbal product as immune modulating agent in dogs

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WATTLE TEST RESPONSE IN CHICKENS TREATED WITH VEGETAL EXTRACTS FROM TAXONOMICALLY DIFFRENT PLANTS

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Summary

The immunomodulatory effects of several vegetal extractions are well known. The reasearch was carried out on Rock x Cornish, 47 days old chickens sc injected with 0.5 ml of alcoholic extracts of *Calendula officinalis* (n=7), *Echinacea angustifolia* and *Urtica dioica*, as well as alcohol (solvent control and saline (environment control). On days 0 and 7 the birds were primed with a 5% SRBC suspension and the wattle test with 0.1 ml of the antigen lysate was performed on day 14. The wattle was measured before and 48 h after the injection and differences were expressed in mm. The results indicated that there was a hyposensitizing rather than stimulating effect of the *C. officinalis* and *U. dioica* extracts on the delayed type hypersensitivity reaction, taxonomically different plants acting similarly. Nevertheless, there was a positive effect for the *E. angustifolia* extract, suggesting its stimulating or pro-inflamatory effect.

Key words: chickens, hypersensitivity test, *Calendula officinalis, Echinacea angustifolia, Urtica dioica*

The interest raised lately by the vegetal preparations as drugs used on scientific basis is being connected with major health problems their use in topical or general administrations could solve. The associations of vegetal extracts and classical medicine are not unusual, since many of the plants' active principles were showed to have immunostimulating capacities (4, 5).

Variables related to immune function such as flow cytometry, and identification lymphocyte markers could be used but require specialized training or laboratory facilities. An inexpensive, easily applied skin test using phytohaemagglutinin (PHA) or antigens as mitogens, has become a popular, effective tests that can be applied with minimal training and without specialized equipment.

The skin test provides a measure of the proliferative response potential of circulating T lymphocytes to an injected mitogen or antigen. Phytohaemagglutinin as well as antigens have long been recognized for their mitogenic and blastogenic properties. These tests have been routinely applied, with some variation in the body sites used, according to a long-established protocol developed in poultry science (8).

Calendula officinalis and *Echinacea angustifolia* have been renown for their immune stimulating effects besides general therapeutic effects (4*). Urtica dioica* or stinging nettle is a Eurasian weed with the unusual feature that different parts of the plant are used fairly distinctly, less know for immune efficacy. Contact with the hairs or spines on the stems and leaves of the stinging nettle causes the release of several biologically active substances and cause dermatites. The leaves and seeds are used both as mild diuretics, for nonspecific support of the urinary tract, and as topical and internal anti-inflammatory while the root has been investigated as a treatment for symptoms of benign prostatic hyperplasia (7, 9, 11). Extracts from the stinging nettle may provide therapeutic value for some inflammatory medical conditions (3).

The *Urtica dioica* lectin (UDA) was reisolated and found to stimulate the proliferation of human lymphocytes (10); however, little is known about whether treatment with this plant can enhance antigen-specific immunity. From the water extract of the roots of *Urtica dioica* (stinging nettle) a polysaccharide fraction was isolated which revealed activity in the carrageenan rat paw edema model and lymphocyte transformation test.

The experiment was carried out to establish the influence of an in vivo treatment with three different extracts of taxonomically different plants (*Calendula officinalis, Echinacea angustifolia* and *Urtica dioica*) on the delayed type hypersensitivity in antigen (SRBC) primed chickens.

Materials and methods

The hypersensitivity to a SRBC lysate as a measure of adaptive cell mediated response was tested in vegetal extract treated chickens. Two experiments (A and B) were carried out on immunologicaly mature, 47 days old, Rock x Cornish broiler chickens, coming from different farms.

A. Three groups of chickens, of 14 birds each, were subjected to sc injection of 0.5 ml of: alcoholic nettle plant extract (group I); 70º alcohol (group II) and saline (group III). The treatment was done daily, for 7 days.

B. Four equal groups of chickens (n=7), were sc injected for seven days with 0.5 ml/bird/day of: an alcoholic *Calendula officinalis* extract (group I); an alcoholic *Echinacea angustifolia* extract (group II); 70º alcohol (group III) and saline (environmental control) (group IV).

All the groups were injected on days 0 and 7 with a 5% suspension of SRBC in saline (0.5 ml/administration/ bird) and a wattle test using a lysate of the 5% SRBC suspension, obtained by repeated freezing-thawing was performed on day 14. The lysate was injected in a 0.1 ml/bird dose, in a single wattle injection. Two measurements of the wattle were performed, before and 48 hours after the injection.

Mean values, standard deviations and the significance of the differences between the groups were estimated.

Results and discussions

The skin test is being routinely applied in many avian studies, according to a long-established, standard protocol, which was conducted efficiently, and without compromising precision of the test. Briefly, the immune response is considered to be the difference in swelling in wing webs injected with PHA or antigen and phosphate buffered saline as a control (8). A diversity of results and including birds exposed to a variety of treatments, could be attained by applications of this test.

The most studied the effects of taxonomically different plants, *C. officinalis, E. angustifolia* and *Urtica dioica*. The nettle plant extract presumably differed from the other two by its agglutinin (UDA), an unusual plant lectin, different from all other known plant lectins with respect to its molecular structure and its extremely low specific agglutination activity. This small lectin (8.5 kDa) is a T cell mitogen distinguishable from classical T cell lectin mitogens by its ability to discriminate a particular population of CD4+ and CD8+ T cells as well as its capacity to induce an original pattern of T cell activation and cytokine production (1, 2, 6).

In the nettle extractions treated groups the the wattle test gave the lowest values, when compared to the alcohol treated and control groups. The differences, were significantly increased ($p<0.05$) in group II, treated with alcohol, when compared to all the other groups. The nettle extract acted in this case rather inhibiting in the expression of hypersensitivity, than stimulating (Table 1). These observations were discordant with those mentioned in the literature, where the nettle *Urtica dioica* agglutinin exerts a slow stimulating effect on the T lymphocytes, presumably the major cell population involved in the local reaction.

Table 1

Differences in wattle test values in the antigen primed *Urtica dioica* treated experimental group (mm)

Group/reading	Day 0	after 48 h	difference
I	1.36±0.45	1.44±0.19	0.20±0.13
II	1.16±0.36	1.41±0.37	0.46±0.25
III	1.45±0.040	1.74±0.40	0.23±0.16

In experiment B, the *C. officinalis* extract, known in the literature for its stimulating effect (4, 9) acted inhibiting ($p<0.05$) when compared to the *E. angustifolia* extract.

Table 2

Differences in wattle test values in the antigen primed *C. officinalis* and *E. angustifolia* treated experimental groups (mm)

Group/reading	Day 0	after 48 h	difference
I	1.271±0.228	1.414±0.219	0.17±0.11
II	1.028±0.149	1.371±0.125	0.34±0.19
III	1.043±0.230	1.257±0.382	0.23±0.19
IV	0.929±0.214	1.157±0.223	0.24±0.17

The encountered values were even lower than those exerted by the U. dioica extract, suggesting a stronger negative influence on the *in vivo* adaptive cell mediated immunity (table 2).

Without looking at the cell populations involved in the local response, these results supported the hypothesis that the *C. officinalis* and *U. dioica* extracts contained principles active in inhibiting the migration of T cells to the injection site, while that of *E. angustifolia* induced stimulation or a more pronounced inflammatory *in situ* response, when compared to the alcohol.

Conclusions

We concluded that there was a hyposensitizing rather than stimulating effect of the *C. officinalis* and *U. dioica* extracts on the delayed type hypersensitivity reaction, taxonomically different plants acting similarly. Nevertheless, there was a positive effect for the *E. angustifolia* extract, suggesting its stimulating or pro-inflammatory effect.

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ASSESSMENT OF THE BIOLOGICAL EFFECTS OF SEVERAL NANOSTRUCTURED SILVER COMPOUNDS IN CELL CULTURES FROM DOGS WITH DERMATITIS

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Summary

Monitoring the changes of the immune system in dogs with dermatitis, following a treatment protocol with a nanostructured silver compound, could provide useful information for general practitioners as an alternative to the classic antimicrobial treatment. The study was carried out on a group of for dogs that with staphylococcal-like dermatitis, during a period of four weeks, in which the treatment, with the cream containing a nanostructured-based silver compound, was applied during in the first week (days 0 to 7). Three blood samples were obtained from each dog: one before the treatment (day 0), the second after one week following the treatment (day 15), and the third after another two weeks (day 29). The study was based on *in vitro* leukocyte transformation test, providing clues on the existence of a cell-mediated immune response in dogs with staphylococcal-like dermatitis. Aero gels and aged gels, which contain nanostructured silver compounds in different concentration, inhibited blastogenesis of lymphocytes in whole blood cultures.

Keywords: leukocyte blast transformation test (LBTT), dogs, dermatitis, silver, nano-structured compound

During an immune response, the activation of T cells to a specific antigen results in a cascade of intracellular signaling events, and to the differentiation of naive T cells into memory cells. Once memory cells have formed, they can become activated to proliferate much more readily upon subsequent exposure to the original antigen (3). Researchers indicated that nanoparticles can stimulate and/or suppress the immune responses, and that their compatibility with the immune system is largely determined by their surface chemistry (1). T and B Lymphocytes are more resistant to nanoparticle toxicity compared to monocytes and NK cells (3). Modifying these factors can significantly reduce the immunotoxicity of nanoparticles and make them useful platforms for drug delivery (1).

The nanoparticle−cell interactions are also modulated by surface size and surface chemistry (4). Mitogen substances form a diverse group in terms of origin (the bacterial, plant or animal) and chemical structure (proteins, glycoproteins, etc. lipozaharide.). They can stimulate both major nonspecific lymphocytes. Lymphocyte proliferative response to mitogen, can be measured by lymphocyte transformation test, the test that measures immune system cell reactivity. Cell growth is achieved by consumption of existing glucose in medium, so at the end of the incubation period, the determination of residual glucose allows calculation of

stimulation indexes (2). Understanding the nanoparticle−cell interaction is critical for the safe design and development of nano-materials (4).

Material and methods

The target group of the study was represented by a group of four dogs with staphylococcal-like dermatitis. An ointment containing a nanostructured silver compound, prepared at the Department of Physics at the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca was used for the local treatment of the lesions. The therapy was applied daily, for seven days, from day 0 to day seven according to the clinical signs and laboratory isolates. The total experimental period was of four weeks, blood and microbiological samples being taken on days 0, 15 (one week after the end of local treatment) and 29 (end of the experimental period).

On days 15 and 29 the patients were also re-evaluated by clinical examination.

Leukocyte blast transformation test (adapted from 2). The leukocyte blast transformation test measures the *in vitro* reactivity of mononuclear cells to sensitizing (*in vivo* encountered) antigens. Cell growth was quantified by means of the glucose consumption technique. Blood sampled on heparine (50 UI/ml) was diluted with four times the amount of RPMI 1640. The mixture was distributed in 96-sterile-wellplates (200·ml per well). Ten compound-treated variants were tested in duplicate for each individual animal, namely M, A to D and A1 to E1 (1.5μ l/well). The concentration of silver in the nanostructured compounds that were used in cell cultures were: A – 0.0 % (matrix), B - 0.1%, C - 0.15%, D - 0.2%; A1 - 0.01%, B1 - 0.05%, C1 - 0.1%, D1 - 0.15% și E1 - 0.2%. Meantime, the first set of compounds A - D were aero gels, while A1 - E1 were aged gels. The cultures were incubated for 48·h at 37.5°C and 5% CO2. Glucose concentrations were measured in the initial medium and in all variants at the end of the incubation period, using a standard (100·mg/dl) glucose solution, by means of an orto-toluidine colorimetric test. To do this, 12.5·μl of the cultural supernatant were transferred to 0.5·ml of orto-toluidine reagent, boiled for 8·min, cooled suddenly in cold water and read in a spectrophotometer (SUMAL PE2, Karl Zeiss, Jena) at 610·nm wavelength, using the reagent as a blank. The transformation index (TI) was calculated as follows: TI%=[(MG–SG)/MG]´100, where TI=blast transformation index, MG=glucose concentration in the initial culture medium and SG=glucose concentration in the sample after incubation.

Results and discussions

Despite considerable progress in the field of metal nanoparticles synthesis, major challenges remain in many practical applications of nanoparticles which require their immobilization on solid substrates, presenting additional difficulty in separation and processing. Bibliographic surveys shows the high viability of basic chemical and physical sciences that are promoting the

applicability of nanostructured silver compounds in a number of demanding areas. Just in the most recent years a real interface of existing technologies has taken place, and moreover new technologies are emerging in a few scattered articles (4)

Lymphocyte transformation indices were shown in tables 1 to 3.

Table 1

Initial leukocyte transformation indices (day 0)(%)

Case No.	M	A	B	C	D	A1	B1	C1	D1	E1
1	80.43	78.26	64.86	38.77	61.96	73.55	77.17	67.39	75.36	56.52
2	80.07	80.80	68.12	72.10	39.49	73.55	71.38	59.78	60.87	57.97
3	63.41	53.26	52.17	27.90	19.57	48.91	46.74	26.45	33.33	17.75
4	54.71	43.48	32.97	31.52	0.00	35.14	14.13	12.32	5.80	26.09
X	**69.66**	**63.95**	**54.53**	**42.57**	**30.25**	**57.79**	**52.36**	**41.49**	**43.84**	**39.58**
S	**12.74**	**18.46**	**15.93**	**20.20**	**26.58**	**19.05**	**28.70**	**26.35**	**30.78**	**20.69**

The average indices induced by the studied compounds for the first sampling was below the control value, which indicates that all nanostructured compounds acted inhibiting. Compound D had the highest while compound A has the lowest rate of inhibition at the initial sampling. There was no activity pattern when aerogels versus aged gels were compared, but a pattern could be identified in relation to the silver content of the gels. The increase in the silver content decreased the blastogenic response, more pronouncedly in aerogel treated cultures than in aged gel treated ones (56.57% and 31.64%, respectively). These results suggest a reactivity of the immune cells to both structure type and silver content of the tested gels.

Table 2

Leukocyte transformation indices on day 15 (%)

Case No.	M	A	B	C	D	A1	B1	C1	D1	E1
1	48.82	44.49	55.90	47.64	48.03	47.64	26.77	0.00	30.71	38.98
2	60.63	57.09	35.43	12.60	42.52	50.39	30.31	32.68	23.23	68.11
3	72.83	71.65	58.27	66.53	34.25	68.50	68.50	55.90	48.82	58.27
4	58.66	51.57	27.16	30.31	1.57	53.15	40.94	23.62	16.93	7.08
X	**60.24**	**56.20**	**44.19**	**39.27**	**31.59**	**54.92**	**41.63**	**28.05**	**29.92**	**43.11**
S	**9.86**	**11.52**	**15.30**	**23.13**	**20.80**	**9.33**	**18.90**	**23.12**	**13.80**	**26.89**

Leukocyte transformation test results for the second sampling (day 15) were shown in Table 2.

Following the seven day *in vivo* treatment of dermatitis lesions, all transformation indices were further decreased, for both types of gels, indicating a sensitization of the immune cells by the local treatment. Nevertheless, the decrease was non significantly lower with the increase of the silver concentration, but still more pronounced in aerogels than aged gels (47.55% and 21.53%, respectively). The two compound of both categories, with the highest silver content showed a different, increasing stimulation index pattern, which persisted in time (table 3). While in aerogel treated cultures the growth index followed strictly the initial (day 0) decreasing pattern, the value for the aged gel containing 0.2% silver was almost as high as in the compounds with 0.1 and 0.15% of silver. These data could indicate that increasing the amount of silver in the nanostructured compound will not bring supplementary benefit for the *in vitro* stimulation of the immune cells.

Table 3

Leukocyte transformation indices on day 15 (%)

Case No.	M	A	B	C	D	A1	B1	C1	D1	E1
1	46.38	50.60	73.49	59.94	61.14	64.16	35.84	54.22	61.14	62.95
2	65.66	53.61	40.96	49.10	23.49	34.94	52.71	48.49	22.29	56.02
3	65.66	53.01	39.46	38.55	11.44	45.78	44.28	42.77	0.00	26.50
4	83.13	78.31	68.98	68.98	43.97	80.72	79.82	70.18	45.78	57.53
X	**65.21**	**58.88**	**55.72**	**54.14**	**35.01**	**56.40**	**53.16**	**53.91**	**32.30**	**50.75**
S	**15.01**	**13.02**	**18.02**	**13.19**	**22.00**	**20.21**	**19.06**	**11.81**	**26.82**	**16.44**

Conclusions

All studied compounds acted inhibiting *in vitro* on leukocyte blastogenesis, at all times, the effect being more pronounced with the increase of the silver content and also the tertiary structure.

High porosity (aero gels) combined with lower amounts of silver induced a higher stimulation index while for a smaller contact surface (aged gels) a beneficial effect was observed also with the highest concentration of silver.

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CHANGES OF THE INNATE CELL MEDIATED IMMUNITY IN DOGS WITH DERMATITIS FOLLOWING A NANOSTRUCTURED COMPOUND TREATMENT

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Summary

Monitoring the changes of the immune system in dogs with dermatitis, following a treatment protocol with a nanostructured silver compound could provide useful information for general practitioners as an alternative treatment to the classic antimicrobial treatment. The study was carried on a group of for dogs with staphylococcal-like dermatitis, in a period of four weeks, of which the treatment with the cream containing a nanostructured silver compound, was applied during the first week (days 0 to 7). The protocol included three blood samplings, on days 0 (before the treatment), day 15 (one week after the closing of the treatment) and day 29 (at the end of the experimental period). The study was based on an *in vitro* carbon particle inclusion test, monitoring the functional capacity of circulating phagocytes. Phagocytic properties were augmented during the four weeks of the study, suggesting the involvement of phagocytic subsystem in healing wounds and the possible interaction with the local nanostructured silver ointment treatment.

Key words: nanostructured silver compound, phagocytosis, dermatitis, dogs

Silver nanoparticles are among the most commercialized nanoparticles worldwide. They can be found in many diverse products, mostly because of their antibacterial properties. Despite its widespread use only little data on possible adverse health effects exist. It is difficult to compare biological data from different studies due to the great variety in sizes, coatings or shapes of the particles (4).

As it is well known phagocytes are a group of immune cells specialized in finding and inactivating bacteria, viruses, dead or injured body cells, but also inert particles. There are three main types, the granulocyte, the macrophage, and the dendritic cell.

The ability of ingestion can give information about the potential of the organism to defend itself from exterior attacks.

Cellular uptake of micro- and nano- sized particles is an expected phenomenon for phagocytic cells (macrophages) and cells that function as a barrier and/or transport for (large) compounds. Except for macrophages, the health effects of cellular uptake of nanoparticles have not been studied in depth (2).

Few tools and methods exist that can adequately track nanomaterial properties and reactivity in biological or physiological systems. Importantly, these methods are primarily *in vitro* assays to date (3).

Many materials testing methods well-established for macroscale

biomaterials and for soluble pharmaceutical products are simply adapted in a variety of ways to discover correlations between nanomaterial properties and the biological responses *in vitro* to cells and *in vivo* to animal preclinical models (3).

Materials and methods

The study was carried on a group of four dogs with staphylococcal-like dermatitis. An ointment containing a nanostructured silver compound, which was prepared at the Department of Physics at the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca was used for the local treatment of the patients.

The therapy was applied daily, for seven days, as soon as the patients were diagnosed with dermatitis (day 0), by case history, clinical signs and laboratory isolates. The total experimental period was of four weeks, blood and microbiological samples being taken on days 0, 15 (one week after the closing of the local treatment) and 29 (end of the experimental period).

On days 15 and 29 the patients were also re-evaluated by clinical examination.

Carbon particle inclusion test (adapted from Ghergariu et al. (1)). Blood samples collected on heparin (50 IU/ml) were distributed in 0.5 ml aliquots in sterile tubes and were mixed with two μl of an India ink supernatant, obtained by centrifugation at 1308 g for 40 min (Hettich Mikro 22 R, Germany). 0.15 ml of the mixture were transferred immediately to 2 ml of saline and the rest was incubated at 37°C for 20 min. Another 0.15 ml of the sample were transferred to saline and the incubation was continued to 40 min, repeating the operation. All tubes containing saline, blood and ink were centrifuged at 419 g and the supernatants were read spectrophotometrically (SUMAL PE2, Karl Zeiss, Jena) at λ=535 nm and *d*=0.5 cm. There was a decrease in absorbance with time as carbon was phagocytized. Phagocytic activity index was calculated as the difference between the natural logarithms of the optical densities of the phagocytosis at 0–20 min and 20–40 min, as well as for the entire period (0 to 40 min) divided by time (20 min).

Results and discussions

The readings of phagocytosis were presented in tables 1 to 3.

As indicated in table 1, the phagocytosis was weaker during the first and higher during the second period of incubation. The average phagocytosis per period as well as for the entire incubation time was less pronounced than that of healthy dogs (0.52±0.12 and 0.49±0.63, respectively) (1). This indicated an impeded phagocytosis in dogs with staphylococcal-like dermatitis.

One week after the treatment, the clinical examination indicated a healing in progress of the skin lesions as well as a decreased number of bacteria in the microbiological isolates.

Table 1

Phagocytiosis during the first sampling – day 0

	Optical density			Interpretation			Periods		
Case No.	**0 min**	**20 min**	**40 min**	**0 min**	**20 min**	**40 min**	**ln0 min-ln20 min**	**ln20 min-ln40 min**	**ln0 min-ln40 min**
1	0.099	0.074	0.077	-2.31	-2.60	-2.56	0.29	-0.04	0.25
2	0.077	0.159	0.123	-2.56	-1.84	-2.10	-0.73	0.26	-0.47
3	0.046	0.075	0.058	-3.08	-2.59	-2.85	-0.49	0.26	-0.23
4	0.010	0.024	0.034	-4.61	-3.73	-3.38	-0.88	-0.35	-1.22
X	**0.058**	**0.083**	**0.073**	**-3.14**	**-2.69**	**-2.72**	**-0.45**	**0.033**	**-0.42**
S	**0.039**	**0.056**	**0.038**	**1.02**	**0.77**	**0.53**	**0.52**	**0.29**	**0.61**

This was concordant with a slight increase in the phagocytosis, during the first, second and overall incubation periods (Table 2). Considering these facts, the progress of the healing process seemed to be paralleled by a restauration of the physiological activity of the innate cell-mediated immunity. Such an outcome could also be the result of the use of a nanostructured silver compound, given the documented beneficial activities of silver ions in skin lesion therapy (4).

Table 2

Phagocytiosis during the second sampling – day 15

	Optical density			Interpretation			Periods		
Case No.	**0 min**	**20 min**	**40 min**	**0 min**	**20 min**	**40 min**	**ln0 min-ln20 min**	**ln20 min-ln40 min**	**ln0 min-ln40 min**
1	0.035	0.016	0.006	-3.35	-4.14	-5.12	0.78	0.98	1.76
2	0.03	0.032	0.054	-3.51	-3.44	-2.92	-0.06	-0.52	-0.59
3	0.031	0.067	0.094	-3.47	-2.70	-2.36	-0.77	-0.34	-1.11
4	0.018	0.045	0.045	-4.02	-3.10	-3.10	-0.92	0.001	-0.92
X	**0.029**	**0.040**	**0.050**	**-3.58**	**-3.34**	**-3.37**	**-0.24**	**0.031**	**-0.21**
S	**0.007**	**0.022**	**0.036**	**0.29**	**0.60**	**1.20**	**0.78**	**0.67**	**1.33**

The initial and overall phagocytosis increased further, being concordant with complete healing of the lesions in two of the four patients by the end of the experiment, reaching levels close to the physiological ones. Such an effect of the ointment supplemented with silver containing nanostructured compound supported

its further use in staphylococcal-like dermatitis treatment, with further establishment of a dose-dependent effect and optimal duration of the treatment.

Table 3

Phagocytiosis during the third sampling – day 29

	Optical density			Interpretation			Periods		
Case No.	**0 min**	**20 min**	**40 min**	**0 min**	**20 min**	**40 min**	**ln0 min-ln20 min**	**ln20 min-ln40 min**	**ln0 min-ln40 min**
1	0.029	0.021	0.029	-3.54	-3.86	-3.54	0.32	-0.32	0.00
2	0.055	0.021	0.058	-2.90	-3.86	-2.85	0.96	-1.02	-0.05
3	0.024	0.064	0.072	-3.73	-2.75	-2.63	-0.98	-0.12	-1.10
4	0.048	0.011	0.026	-3.04	-4.51	-3.65	1.47	-0.86	0.61
X	**0.039**	**0.029**	**0.046**	**-3.30**	**-3.74**	**-3.16**	**0.44**	**-0.58**	**-0.13**
S	**0.015**	**0.024**	**0.022**	**0.39**	**0.73**	**0.50**	**1.06**	**0.43**	**0.71**

Conclusions

During the treatment of staphylococcal like dermatitis in dogs with a silver containing nanostructured compound, as lesions healed, the phagocytic index increased, suggesting both the involvement of the phagocyte subsystem in healing wounds and the beneficiall efect of the used nanostructured compound on phagocytic activity in dogs.

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ROLE OF MHC MOLECULES IN ANTIGENS RECOGNITION AND PRESENTATION

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Summary

MHC molecules provide the context in which different antigenic peptides (viral, bacterial, helminthic origin) are presented to T cells by antigen presenting cells (APC). Peptides presented by MHC molecules are not only foreign antigens, derived from internalized antigens in APC and other cells or viral antigens, it can be self antigens synthesized within cells or internalized by endocytosis from extracellular fluid. Antigen presentation is the most important function of MHC molecules, which determines T cells activation and expression of their functions. Ultimately, cooperation mechanisms involved in cellular adaptive immune responses (cell and antibody mediated immune response) are dependent on major histocompatibility complex, and thus genetically restricted.

Key words: MHC, antigen, receptors

Major histocompatibility complex (MHC) is a distinct genomic region consists of genes characterized by a high degree of polymorphism. MHC was shown in all animal species included in infraphylum Gnathostomata - vertebrates with mobile jaws; MHC genes have not been identified in vertebrates lacking such jaws (agnathas) and invertebrates included in phylum Chordata (21).

The existence of MHC molecules was observed initially by Gorer, a British researcher who described, in 1936, the first molecule of histocompatibility, respectively a blood group antigen in mice.

Benacerraf, Dausset, and Snell (Nobel Prize in 1980) findings were essential in this area, indicating genetically determined structures on the surface of cells that regulate a number of immunological mechanisms (21).

Nature and origin of peptides associated with MHC molecules

Antigens presented by MHC molecules are not fully native structure. Before connecting the MHC molecules, proteins are processed (degraded). Generally, this process takes place in intracellular environment by the action of some enzymes. The association between peptides and MHC molecules occurs at the level of some organelles, the interaction of these molecules being done in different sites within cells (1, 7).

In relation to the antigen presenting cells, antigenic peptides bound to and presented by MHC molecules have exogenous or endogenous origin. It is established that antigen receptors (TCR) of cytotoxic T cells (Tc), restricted by MHC class I,

recognize and interact with antigens of endogenous origin, synthesized in target cells, while helper T cells (Th), restricted by MHC class II, recognize and associate, through TCR, exogenous antigenic peptides. Therefore, antigens introduced and processed in the cytoplasm of target cells determine the response of lymphocytes Tc, whereas exogenous antigens generate exclusively Th cell activation (7, 11).

Experimental changing of the peptides origin can determine the class of T cells that receive information, respectively the type of immune response restriction (class I or class II). This is the case of hemagglutinin (HA), a major antigen of influenza viruses, associated with host cell membrane, which induces a mild response of Tc cells. However, HA generated in the cytoplasm induces an intense response from the same cells (6, 22).

Peptides associated with MHC class I derive from endogenous cytosolic proteins, association taking place in rough endoplasmic reticulum (RER). In the first stage of this process is involved proteasome, an organelle responsible for majority of the enzymatic cleavage of proteins. Proteasome has a complex structure, consisting of 12-15 subunits. By endopeptidases action, antigens are degraded in proteasome, resulting small peptides of 5-15 amino acids. Denatured proteins are marked with ubiquitin. Proteasome components are encoded by genes *LPM7* and *LPM2* of the *MHC* region, and expression of these genes is induced by IFN-γ. The products of these genes are involved in the processing of peptides presented by MHC class I molecules (8, 9, 18).

The resulting peptides cross the RER with two carrier molecules (TAP). The carriers have a heterodimeric structure and are encoded by *TAP1* and *TAP2* genes, located in mouse and humans, near LPM genes, in the region of the *MHC* class II. *TAP* products belong to ABC transporters superfamily and are localized in RER. They moved peptides in the RER, especially those consisting of 8-15 amino acids (12, 16, 17, 18).

Various stages of antigenic proteins processing are correlated. Thus, complexes formed by MHC class Iα and β 2-m molecules are associated with TAP in RER, the subsequent dissociation of TAP occurring during transport in *cis* part of the Golgi complex (3, 16, 17).

After entering in the RER, peptides are associated with MHC class I molecules. MHC class I molecules formation is achieved by complex mechsnisms, with the participation of accompanying molecules, as calnexin. These molecules transport the complex, composed of heavy chain α, β2-m light chain, and peptide (complex formed in RER) to the cell surface. MHC class I molecules not associated with a peptide are unstable, so that only complexes including a peptide interact with TCR (5, 19).

MHC class II molecules are associated with exogenous antigenic peptides in an endosomal compartment. α and β chains are associated with a molecule called invariant chain (Ii) in RER. The latter is encoded by a gene that is not part of the *MHC* region. Complex of MHC class II molecule and Ii chain is transported through the Golgi apparatus to an acid endosomal or lisosomal compartment,

where the polypeptide is detached, allowing the binding of antigenic peptide. Then, $\alpha\beta$ complex reaches the cell surface (1, 15, 19).

Antigenic peptides, obtained by cleavage of exogenous peptide, are associated with MHC class II molecules in appropriate compartment, which is explained by intracellular circular paths of the latter. It was found that peptides derived from exogenous proteins processing and MHC molecules are synthesized in the RER and then are transported through the Golgi apparatus. At this stage, MHC class I molecules are associated with antigenic peptide and the MHC class II molecules with Ii invariant chain. Invariant chain contains sequences that provide release of MHC class II molecules from the RER. After passing through the Golgi apparatus, segregation of the two classes MHC molecules occurs. Further, molecules of both classes pass through endosomal / lisosomal compartment and then reach the cell surface. MHC class I molecules go directly to the cell membrane (22). Endosomal / lisosomal structures are different from ordinary endosomes and lysosomes, being directly involved in transport and storage of MHC class II molecules. In acidic compartments, named MIIC, are stored MHC class II molecules associated with peptides derived from exogenous antigens. These structures have endosomes and lysosomes features, including several membrane structures (1, 10, 19).

In the process of binding antigenic peptide to MHC class II molecules in humans, molecule HLA-DM has a major role. HLA-DM is like MHC class II molecule, consisting of two chains (α and β), encoded by two genes (*DMA* and *DMB*) located in region II of the *MHC* (10, 19).

Formation of the MHC molecule-antigenic peptide-antigen receptor complex

Binding of antigenic peptides to MHC molecules can be both dependent and independent of their sequential structures (6).

(1) Independent binding of the primary structure (amino acid sequence of antigenic peptide). MHC molecules ability to bind to many epitopes underlies cell-mediated immunity to a practically unlimited number of antigens (10). The binding capacity, expressed through the interaction of conserved residues of the MHC molecule and peptide, is independent of amino acid sequences. In class I molecules, the binding of peptide main chain involves amino acid residues clustered only at both ends of the combination site (PBC) that interact with free N-terminal and C-terminal regions. Participation of both ends to binding of antigenic peptide and that they are closed, explains the tendency of short peptides to make stable association with PBC. Appearance of the two molecules in contact is uneven, with irregularities. The MHC class II molecules has, unlike class I molecules, conserved residues that interact with the peptide main chain are distributed over the entire site of the combination. Therefore, antigenic peptide main chain is in close contact with PBC and has a flat conformation. This way of interaction, achieved by forming hydrogen bonds, provides to MHC molecules a larger, but not unlimited, capacity of binding peptides (1, 19, 23).

(2) Dependent binding. Independent type of interaction between MHC molecules and antigenic peptides (shown above) is not achieved in all cases

because there are natural peptides that have the characteristics necessary for binding to MHC molecules. Some of the peptide side chains get in contact with residues present in the PBC; trough these anchor residues increase affinity and specificity of interactions between peptides and MHC molecules. The peptide contains also inhibitor residues that interfere with PBC and causes a reduction in binding. These interactions are dependent to amino acid sequences, in their existence being involved PBC irregular surface, consisting of pockets, pockets in which is achieved the interaction with certain side chains of peptides. Most pockets of PBC include polymorphic residues, presenting structural aspects and dimensions characteristic for MHC molecules alleles (1, 10, 19, 23).

Whatever type of interaction between MHC and peptide molecule we speaking, this binding is crucial for recognition to foreign antigens on the APC surface by T cells. Antigen (bacterial, viral, etc.) and MHC molecule will form a complex, which, in turn, binds the antigen receptor (TCR) of T cells, resulting, finally, the so-called trimolecular complex. In this complex, the antigen is located in the middle, between the combining sites of the MHC molecule and the receptor molecule (19, 21).

Trimolecular complexes are formed in two cases, differentiated by cells and molecules:

- complexes composed of MHC class I molecules, antigenic peptides, and $CD8^+$ T cells receptors, in this situation are involved cells infected with viruses or tumoral cells, respectively cytotoxic T cells;

- complexes composed of MHC class II molecules, antigenic peptides and $CD4^+$ T cells receptors; are involved antigen presenting cells and helper T cells.

Antigenic peptide is placed in a cavity formed by the NH_2 terminal domains, that are in the form of α helix, and β sheet structure of MHC molecule. This cavitary conformation ensures a proper binding of antigenic peptide to MHC antigen, by expressing the forces of attraction between the two molecules (2, 6). Polymorphism of MHC molecule, obvious in walls and the lower areas of the formed cavity, explains the differential binding of peptides to MHC antigens from different alleles and, ultimately, interaction "specificity" between the two molecules. In other words, the interaction between MHC molecule and antigenic peptide has a degree of specificity, so that each antigenic determinant does not bind to all MHC molecules. This specificity is not as strict as that expressed in interaction between antigens and TCR or immunoglobulin receptors of B cells (BCR). Explanation lies in the difference in the number of different binding sites for potential antigenic determinants which is 10^6 - 10^9 in TCR or BCR and only a few dozen in MHC molecules. Increased diversity of binding sites on receptor molecules is due to rearrangements of genes that encode them, a process that is not found in genes encoding MHC molecules (15, 19).

Characteristics of peptides bounded to MHC

Antigenic peptides bounded to PBC of MHC molecules have different sizes, depending on the two classes of MHC. Thus, antigens presented by class I molecules are composed of 8-10 amino acids, whereas peptides presented by

class II molecules include 12-24 (mean 14 amino acids). This is explained by the different conformation of PBC, depending on MHC class (2, 6).

Interaction between MHC molecule and peptide (the contact between agretop and desetop) was confirmed by equilibrium dialysis and by demonstrating the covalent binding of radioactive labeled peptide. With this experimental model is possible to identify structure involved in binding to the MHC molecule (6, 13).

Between antigenic peptides and MHC molecules are established hydrogen bonds. From this point of view, there are also differences between the two classes of molecules: hydrogen bonds are concentrated at the two ends of the peptide chain that binds to MHC class I molecules, while in the antigenic peptides that interact with MHC class II molecules are distributed along the length of chain (21).

A series of data about the characteristics of peptides that interact with MHC molecules of combining sites were obtained by analyzing their eluted, purified, and sequenced antigens (19).

MHC molecules interact and only with peptide antigens, not with the polysaccharides. Therefore, the latter cannot be presented to T cells or induce cell-mediated immune responses.

MHC antigens involved in the formation of trimolecular complex have a constant structure in each individual. Antigenic peptides have, of course, the most significant variability. The complementarity determining regions (CDRs) of antigen receptor has a different variation, depending on the molecule that is combined. Thus, the CDR1 and CDR2 of restitope that links to MHC molecule histotope (forming α-helix areas) have limited variability. In contrast, CDR3 that binds to the antigenic peptide is in paratop and have a high structural variability (19, 24).

In developing immunological synapses during the antigen presentation are involved only the trimolecular complex molecules. Indeed, according to data summarized by Gao and Jakobsen (5), essentially to this immune mechanism are three classes of interactions:

- between TCR ($\alpha\beta$ heterodimers)-CD3 and their ligands represented by polymorphic MHC molecules and antigenic peptides (specific interactions) (4, 14);

- between CD8 ($\alpha\alpha$ or $\alpha\beta$ heterodimers) or CD4 molecules (monomeric or, according to recent data, dimeric molecules), which are coreceptor of T cells, and their ligands on the surface of cells presenting the same molecular complex, peptides and MHC class I or class II molecules (20);

- between coreceptor molecules (for example, CD28 and CD152) and adhesion molecules (for example, CD2), on the one hand, and their ligands such as CD80 and CD86 for CD28, respectively CD152 and CD58/48 for CD2, on the other hand; other molecules involved in these interactions are, for example, the recently described DC-SIGN, present on dendritic cells, which binds to molecules ICAM-3 (dendritic cell-specific ICAM-3 grabbing nonintegrin) (5, 24).

Genetic control of the immune response

MHC linked genetic control is achieved through participation *Ir* genes, and would involve a co-recognition mechanism. In the immune response to soluble protein antigens, a significant role in initiating humoral mechanisms belongs to auxiliary T cells (Ta). Ta cells recognize the antigen presented by MHC class II molecules. The ability to develop a response to an antigen depends on the association between MHC molecules and antigenic peptides and also on cell repertoire, able or not able to recognize this association (4, 18).

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COMPARATIVE STUDY REGARDING PERFORMANCE OF SOME COMMERCIALLY AVAILABLE SILICA MEMBRANE RNA ISOLATION KITS

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Summary

This paper describes the results obtained by testing three of the most used commercially available RNA extraction kits based on spin columns and silica membrane technology and one low-cost kit with the same principle. Comparative studies were performed using Avian Influenza (AI) inactivated strain from National Refference Laboratory for Avian Influenza and Newcastle Disease and AI matrix protein detection real time RT-PCR protocol for cuantification and comparison. Results obtained showed good performance of the most used kits with some interesting differences and poor performance of the low-cost kit, emphasizing the need for rigurous testing of the newly introduced kits (reagents) in order to maintain the performance/cost efficiency balance.

Key words: RNA, extraction, silica membrane, performance

Nowadays, molecular biology tests (including PCR and RT-PCR) rely mostly on using commercially available kits for nucleic acids extraction as well as revers-transcription and amplification. This approach provide the means of speeding up the required steps for biological samples preparation, nucleic acids extraction and amplification, ensuring fast detection and consequently rapid intervention in limiting the effects at individual and/or flocks level for contagious diseases (1,4).

One of the most widely used technology for nucleic acids extraction is based on silica membrane selective binding ability (1,3,5,6) – in the presence of high chaotropic salts concentration, the membrane allows binding of the nucleic acids, whereas proteins and other contaminants are washed away (2,4); by changing the salt concentration to a minimum, the nucleic acids are eluted and purified from contaminants. Such technology is fast (no alcohol precipitation required), inexpensive, delivers high-purity nucleic acids for use in most downstream applications and no silica-slurry carry over.

Materials and methods

Testing of extraction kits was performed in order to assess the binding capacity of the membrane as well as small volume RNA elution efficiency (50µl). For each extraction kit used, protocols were established following manufacturer recommendations.

Biological material subjected for testing was represented by inactivated AI strain supplied by National Reference Laboratory for Avian Influenza and Newcastle Disease.

Test design: in order to avoid concentration variations, aliquots of 200µl viral strain were prepared in 1,5ml Eppendorf tubes and stored at -80ºC until processing; moreover, serial tenfold dilutions of the standard were performed immediately before extraction. After each extraction, the RNA was stored at -80ºC until complete extraction with all the kits tested – therefore, all extracted standards and the extracted RNA benefited from the same numbers of freezing/thawing cycles.

Performance parameters were selected according with laboratory standard ISO 17025 and included extraction efficiency by repeatability and reproducibility.

1. *Reproducibility*: for each tested kit, a total number of five samples were selected as follows – first sample was represented by standardized strain and the following four by serial tenfold dilutions of the standard (10^{-1} to 10^{-4}). All four kits were tested by two skilled analysts, using different aliquots and recommended protocol. After each extraction, the RNA was stored at -80ºC.

In order to avoid possible variations from the amplification step, highly diluted samples (10^{-3} and 10^{-4}) were analyzed in duplicates.

2. *Repeatability*: due to the low number of reaction/kit supplied by manufacturer (a total number of ten extractions allowed), this parameter was not tested for kit No3. Also, for kit No4 (the low cost kit), due to the inconsistent results obtained in reproducibility experiments, this parameter was also not tested, since the kit was considered inappropriate and disqualified.

For each of the remaining kits, a total number of ten samples were selected as follows: first three samples were represented by 10^{-3} standard dilutions, the next three by 10^{-4} and the last four by 10^{-5} standard dilutions.

Results and discussions

1. *Reproducibility:* results analysis was performed by simultaneous amplification of the extracted RNA from each of the four kits and the same analyst by using Real Time RT-PCR AI matrix protein gene detection on LightCycler 2.0 instrument and interpretation of the obtained Ct (crossing threshold) values. Results are shown in table 1.

Table 1

Ct values obtained for reproducibility parameter

	Analist 1							
	Kit No1		Kit No2		Kit No3		Kit No4	
Standard	20.14		23.58		22.90		20.91	
10^{-1}	25.06		25.96		25.79		34.77	
10^{-2}	28.04		28.98		29.60		39.07	
10^{-3}	31.51	31.87	32.13	32.18	33.19	32.49	36.73	≥40
10^{-4}	35.13	34.31	36.21	35.80	34.50	36.69	Neg	39.32
Analist 2								
Standard	20.17		22.94		21.02		24.48	
10^{-1}	25.49		25.47		26.83		34.95	
10^{-2}	28.49		27.77		28.81		39.64	
10^{-3}	31.12	30.36	33.38	33.21	33.61	34.06	Neg	Neg
10^{-4}	34.66	33.76	35.50	35.87	35.02	34.91	Neg	Neg

Based on the Ct values obtained, standard curves for each tested kit was obtained. For higher dilutions (10^{-3} and 10^{-4}), the best Ct value for slope/linearity coefficient was selected (Figures 1 to 4).

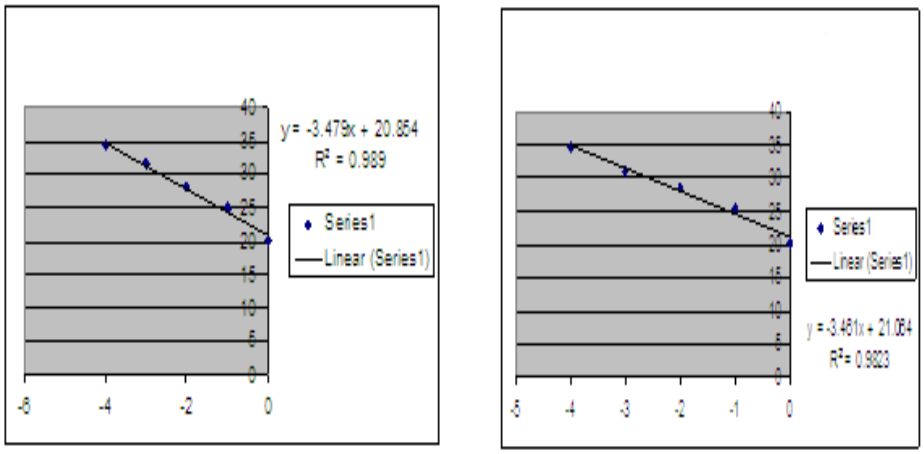

Fig. 1. Standard curve, slope and linearity coefficient for kit No1 for analyst 1 (left) and analyst 2 (right)

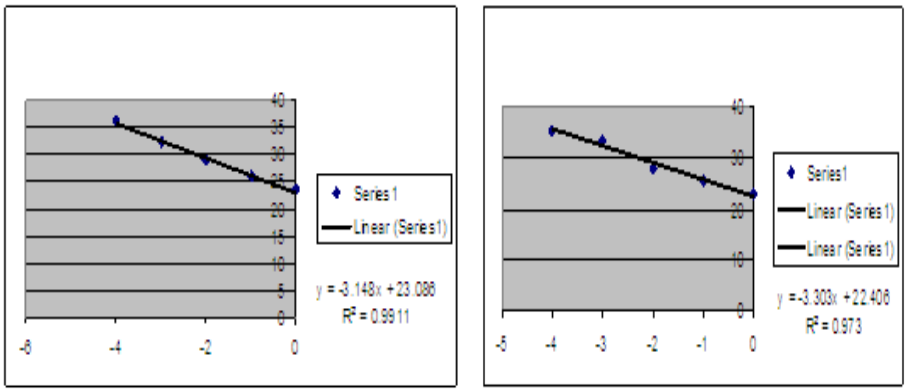

Fig. 2. Standard curve, slope and linearity coefficient for kit No2 for analyst 1 (left) and analyst 2 (right)

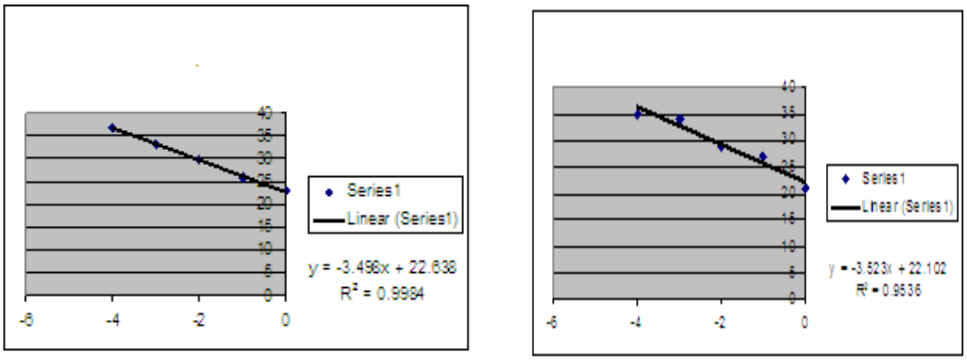

Fig. 3. Standard curve, slope and linearity coefficient for kit No3 for analyst 1 (left) and analyst 2 (right)

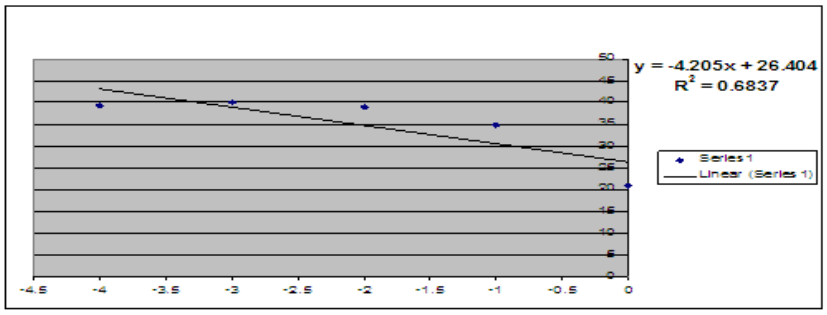

Fig. 4. Standard curve, slope and linearity coefficient for kit No4 for analyst 1

Results interpretation:

Kit No1 – obtained Ct values were overall the smallest (best extraction sensitivity), especially for standard strain; standard curve analysis showed a slope situated in the proper interval (-3.1÷ -3.6) and R^2 coefficient ≥ 0.98;

Kit No2 – adequate Ct values (higher than kit No1, probably due to the supplementary DNase incubation time of 15 minutes); standard curve analysis showed a slope situated in the proper interval (-3.1÷ -3.6) and R^2 coefficient ≥ 0.98;

Kit No3 – adequate Ct values; standard curve analysis showed a slope situated in the proper interval (-3.1÷ -3.6) and R^2 coefficient ≥ 0.98;

Kit No4 – Ct values obtained for standard strain and serial dilutions showed inconsistent results in binding/elution capacity; standard curve analysis showed a slope situated outside the proper interval (-4,205) and poor R^2 coefficient of 0.68.

2. Repeatability: results analysis was performed through simultaneous amplification of the RNA extracted with each kit and by the same analyst, using Real Time RT-PCR AI matrix protein gene detection on Smart Cycler instrument and interpretation of the obtained Ct (crossing threshold) values (table 2).

Table 2

Ct values obtained for repeatability parameter

	Kit No1	**Kit No2**
10^{-3}	27.29	29.27
10^{-3}	28.65	29.23
10^{-3}	26.54	29.10
10^{-4}	32.16	32.95
10^{-4}	30.64	32.97
10^{-4}	33.32	32.48
10^{-5}	35.04	36.75
10^{-5}	34.15	35.37
10^{-5}	33.87	36.17
10^{-5}	33.21	36.35

Based on the Ct values obtained, standard curves for each tested kit was obtained (figure 5).

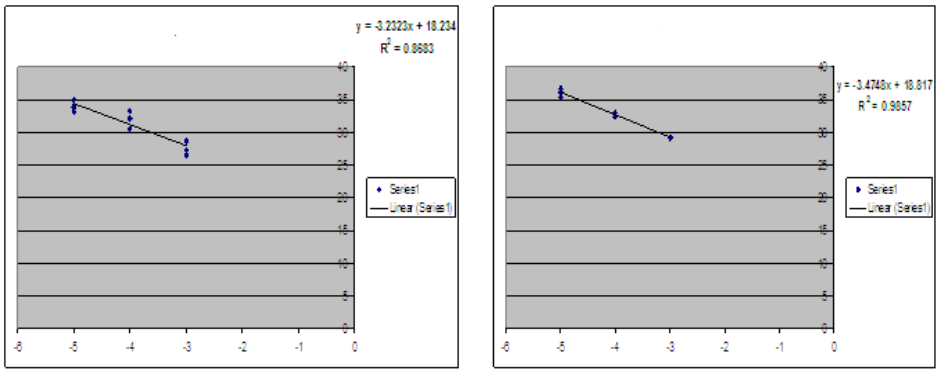

Fig. 5. Standard curve, slope and linearity coefficient for kit No1 (left image) and kit No2 (right image) repeatability

Results interpretation:

- Kit No1 – Ct values obtained for each dilution showed an adequate behavior of the kit, with acceptable variations between same dilutions samples;
- Kit No2 – Ct values obtained were overall higher than for kit No1 (probably due to the supplementary DNase incubation time of 15 minutes), but adequate. Moreover, Ct values obtained especially for 10^{-3} and 10^{-4} dilutions showed superior repeatability than kit No1.

Conclusions

Even though the silica membrane technology remains one of the most widely used techniques in nucleic acids extraction, there are sometimes significant differences between manufacturers in terms of efficiency that users should be aware of. Moreover, for comparable performance of two or more commercial kits, some minors but sometimes important differences still occurs that might be of influence especially if quantification (relative and/or absolute) is required. Therefore, every protocol that will be designed for RNA detection should be “fit for purpose” in terms of cost/efficiency.

Acknowledgements

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food biotechnology based on the eco-economy and the bio-economy required by eco-san-genesis".

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WATER DISINFECTION USING AN ELECTROCHEMICAL REACTOR WITH ASYMMETRIC CURRENT DENSITIES

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Summary

The present experiment was aimed to assess the possibility of water disinfection using an electrochemical reactor with asymmetric current densities and a saline solution as support electrolyte. The built reactor favourises anodic oxidation reactions with generation of bactericide chlorine derivates (natrium hypochlorite, hypochloric acid and free dissolved chlorine) and also direct anodic oxidation of organic matter which acts synergistic with free chlorine for an increased bactericidal effect. Total free chlorine was determined by a titrimetric method, with methyl-orange and bacterial counts were determined by the plate count method. Bacterial counts decreased progressively throughout the treatment and after three minutes all the bacteria were killed. Chlorine levels increased gradually to 8 mg/dl after 20 minutes of electrolysis. Water disinfection using an electrochemical reactor with asymmetric current densities is an efficient, cost effective alternative to classic water disinfection treatment.

Key words: microbial contaminated water, free chlorine, electrochemical reactor.

Bacterial contamination of surface or underground waters is one serious environmental problem, especially in areas where there are industrial animal breeding farms. The presence high bacterial loads in these waters have detrimental economic and social effects and pose serious risks for human health.

The purpose of this experiment was to determine the possibility of water disinfection by in situ generation of biocides using an electrochemical reactor with asymmetric current densities. Also there was made a rough estimation of the electric energy costs of this method.

Materials and methods

The experiment is centered on an electrochemical reactor with asymmetric current densities. The experimental setup (fig. 1) includes: a power source with ampermeter and voltmeter, an electrochemical reactor and a system for continuous measurement of anodic potential against a saturated calomel reference electrode with a high impedance electronic voltmeter.

Fig. 1. The experimental device

The reactor was designed and built using a central stainless steel electrode and several peripheral graphite electrodes. The device has a steerer driven by an electric engine for continuous homogenization of the contents (fig. 2).

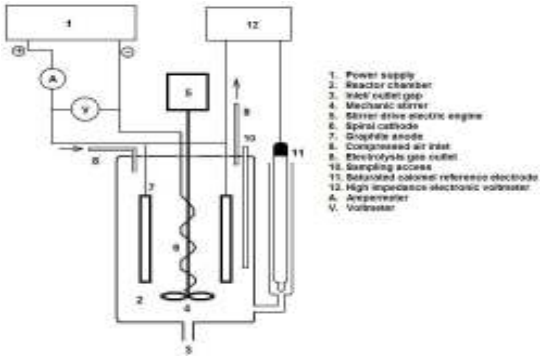

Fig. 2. Schematic of the experimental device

Constructive characteristics of the reactor:

- Total working capacity 100ml,
- Eight 6mm diameter graphite anodes, with a total surface of 45.21 cm^2,
- Anodic current density of 110 A/m^2,
- A stainless steel cathode with total surface of 3,61 cm^2,
- Cathodic current density of 1385 A/m^2,
- Working current 500 mA.

The working principle of the electrochemical reactor is very well suited for the proposed purpose: to favourise the anodic oxidation reactions with free chlorine generation (such as natrium hypochlorite, hypochloric acid and free dissolved

chlorine) in a short time and with minimum electricity use. The substances generated are known bactericides.

For this experiment there was used a standardized *Staphilococcus aureus* strain (ACTC 25923). The bacteria were cultured on solid media (tryptic soy agar-Oxoid) for 16 hours at 37°C, suspended in 0.9% NaCl to a density of $3x10^{10}$ cells/ml. Ten ml of the suspension were loaded into the asymmetric electrochemical reactor together with 90 ml of saline to a final bacterial density of $3x10^{9}$ cells/ml.

Total free chlorine was determined by a titrimetric method, with methyl-orange in sulphuric acid solution. The anodic over potential was measured throughout the experiment to ensure the current is sufficient for chlorine generation. Bacterial counts were determined by plate count method, after 24 hours incubation at 37°C on plate count agar (Oxoid). Bacterial density and free chlorine in the working solution were determined after loading the reactor and then after 30 seconds, 1, 2, 3, 4, 5, 10, 15 and 20 minutes.

Results and discussions

The initial bacterial density in the reactor was determined to be $3x10^{9}$ bacteria per milliliter. Bacterial counts decreased progressively throughout the treatment and after three minutes all the bacteria were killed. Chlorine levels increased gradually to 8mg/dl after 20 minutes of electrolysis (fig. 3).

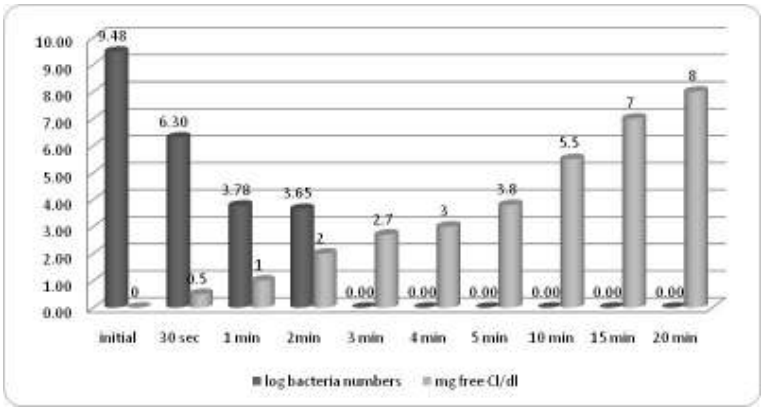

Fig. 3. Bacterial counts and free chlorine concentration evolution during electrolysis

Chlorine levels generated through this procedure reached 27 ppm after three minutes of electrolysis, a level comparable to the chlorine levels used for drinking water disinfection. The initial very high bacterial water contamination of $3x10^{9}$ UFC/ml was neutralized after just three minutes of electrolysis. These results

confirm the assumption that the direct anodic oxidation of organic matter acts synergistic with the free chlorine generated for an increased bactericidal effect. The summation of these two mechanisms reduces the free chlorine levels necessary for water disinfection. The reduced chlorine levels in waters after disinfection may have a benefic environmental effect for water treatment procedures. The up scaling of this method for industrial use would lead to an energy use of 2 KWh/m^3 of highly contaminated waters.

Conclusions

Water disinfection using an electrochemical reactor with asymmetric current densities is an efficient, cost effective alternative to classic water disinfection treatment.

Acknowledgements

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ASPECTS OF MILK CYTOGRAM IN THE CASE OF CATTLE STERILE MASTITIS

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Summary

Our previous studies regarding the somatic cell distribution in milk from cattle with monobacterial mastitis produced by bacteria of genus *Staphylococcus spp.* and *Streptococcus spp.* have shown the existence of a correlation between it and the causative pathogen agents. Thus, indirectly, according with the aspect of milk cytogram we can indicate, with some certainty, the causative agents of cattle monobacterial mastitis. In this study we tried to establish the blood and epithelial cells (known as milk somatic cells) distribution in milk from cattle with sterile mastitis and if there is a correlation between them.

Key words: cattle, mastitis, somatic cells, milk

Mastitis still continues to be a problem for dairy farms all over the world, because the clinical forms cause economic loss due to treatment costs, lost quarters, perhaps dying cows and most importantly, discarded milk, and the subclinical forms silently reduce milk production and quality until detected with a somatic cell count. Somatic cell counts (SCC) have long been used as a way of measuring milk quality. Most dairy companies base their milk pricing policy, among other things, on SCC values of the milk. The somatic cells consist mainly of immune cells that enter the milk compartment of the udder. Only a minority of these cells are dead cells from the udder tissue. There are always small quantities of immune cells in the cow's milk, and their function is to protect the udder against infection by bacteria. When bacteria do enter the udder, the number of immune cells increases rapidly, as the immune system attempts to overcome the infection. Once the infection has been cleared, the SCC levels gradually drop to normal. The measurement of SCC has become one of the most reliable indicators for determining milk quality and the price of raw milk within the dairy industry (3, 4, 5). Several methods such as the direct microscopic somatic cell counting (DMSCC) method, electronic particle counting method, and fluoro-optic electronic cell counting method by disk cytometry or flow cytometry have been used to determine the SCC. Among them, DMSCC with methylene blue staining is the reference method for making a direct estimation of the cells, but this method is slow and requires trained staff (1). In addition, the lack of specificity between the cells and cytoplasmic particles, as well as the time and labor requirements are negative aspects of DMSCC (2).

While mastitis remains a complex disease, applying the basics of mastitis prevention and control will minimize its deleterious effects on a dairy herd.

Materials and methods

From the 145 raw milk samples processed at Cell Biology-Histology-Embryology Department, ten milk samples came from cows with sterile mastitis, which, after the appearance of milk secretion, were divided into:

- 1 mastitic milk sample with organoleptic normal appearance (ONA)
- 3 mastitic milk samples with organoleptic changes (OC);
- 6 samples with purulent mammary secretion (PMS).

The tests that were applied on milk samples included indirect mastitis tests (R-Mastitest, Mast-O-Test), bacteriological (milk culture on Petri dishes and Api System) and cytological (Leishmann-Romanovski stain and microscopic differentiated cells count) exams.

Milk cytogram, in compare with leukogram, represents a qualitatively and quantitatively exam of leukocytes and epithelial cells (which determines the percentage of each type of white cells and epithelial cells from the total number of cells counted).

Although the milk samples were positive at mastitis indirect tests and presented organoleptic changes, we considered to be sterilized because the bacteriological exam was negative; respectively we ascertained the absence of bacterial colonies on grown agar plates.

Results and discussions

We considered that the examined milk samples were taken from cows with mastitis which became sterile either through the application of a successful antibiotic treatment in the near past, whether in mammary gland toke place a self-sterilization process by bacterial antagonism. However, we could not excluded the possibility that cows have suffered a monomicrobial infections of the mammary gland, but produced by fastidious bacteria with culture medium demands, making it more difficult to identify.

The most important events, deserving to be taken into account, which produce self-sterilized mammary gland are: recognition and rapid destruction of bacteria by white cells (for this reason, by example, streptococcal mastitis and those produced by faecal coliforms are often bacteriological negative); low pathogenity of the causative microbial agents, and/or competition between different species of bacteria for nutrients (the decline stage of the bacterial multiplication) that will induce numerical decline and death of them.

The data were summarized in the following table, presenting individually each case (table 1).

After analyzing the data it shows that, from the cytological point of view, milk from cows with untreated sterile mastitis contained very well represented polymorphonuclear neutrophils, both numeric and percentage. The average proportion of mentioned cells was 71.38%. So, even without the presence of bacterial colonies in Petri dishes, the neutrophilia that we noticed in sterile mastitic milk represent a response to an infection.

Table 1

Milk cytogram for sterile mastitic milk

SAMPLE	MILK ASPECT*	TREATMENT	***NE %***	***EO %***	***BA %***	***MF %***	***LM %***	***EC %***
1.	LAN	X	65.54	2.16	0	8.91	19.27	4.09
2.	LMO	X	77.59	0.43	0	8.62	13.14	0.21
3.	SMPP	X	92.40	0	0	5.23	2.37	0
4.	SMPP	X	62.80	0	0	8.30	26.20	0
5.	SMPP	X	68.20	0	0	6.10	25.57	0
6.	SMPP	X	66.33	0.49	0	13.36	19.80	0
7.	SMPP	X	64.72	0	0	17.80	13.63	3.03
8.	SMPP	X	73.40	0	0	2.80	21.50	2.30
9.	LMO	Synulox	7.90	0	0	31.86	59.06	1.16
10.	LMO	Synulox	13.54	0	0	27.72	52.90	5.08

* - see the meaning of abbreviations which have been mentioned above; X – without treatment; Ne – neutrophils; Eo – Eosinophils; Ba – basophils; Mf – macrophages; Lm – lymphocytes; EC – epithelial cells

Instead, the examination of raw milk smears from sterile mastitic cows, after antibiotic treatment, has shown drastic reduction of polymorphonuclear neutrophils number, in this case, the better represented cell population was lymphocytes, followed closely by macrophages.

From the immunological point of view, the absolute reduction in the number of neutrophils (neutropenia) is found especially in poisoning. So, we can assume that the intramammary administering of antibiotics causes death of bacterial agents, but to the same extent, it seems to reduce the effectiveness of natural defense mechanisms, if the polymorphonuclear neutrophils being the first cells that enter the mammary gland parenchyma. In the mammary gland, polymorphonuclear neutrophils are considered the second line of defense after the physical barriers posed by teat cistern and streak canal.

The data could not be interpreted in a statistic way because the number of reported sterile mastitis cases was quite small.

Conclusions

Distribution of the somatic cells in milk samples from cows with sterile mastitis was very different depending on the application or not of a treatment with antibiotics.

Differential microscopic cells count applied on milk smears from cattle with untreated sterile mastitis shown that the major cell population was represented by neutrophils.

Differential microscopic cells count applied on milk smears from cattle with sterile mastitis treated with antibiotics shown that the major cell population was represented by lymphocytes.

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THE PREVALENCE OF THE *YERSINIA ENTEROCOLITICA* SPECIES ON THE SLAUGHTERING FLOW

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Summary

Having in view that pigs are the only animals from which *Yersinia enterocolitica* has been frequently isolated, considered to be O3 serotype, biotype 4, the variety implicated the most in human diseases, in the researches that we have performed, we proposed the isolation and identification of this species on the slaughtering flow. For this we have collected and analyzed 800 samples from two units for pig rising, aiming to verify 16 groups made up of 10 animals (8 groups from one unity and 8 from the other) on the technological flow of slaughtering.

Key words: pig, *Yersinia enterocolitica*, slaughtering, isolation, identification

From the species of the *Yersinia* gender, the *Yersinia enterocolitica* species is frequently isolated from both man and domestic and wild animals as well as from the products that come from them (1, 3, 4, 5). The bacterium is also widely spread in freshwater, soil and plants.

Mead et al. (6), in a study conducted in 1999, show that the prevalence of the *Yersinia enterocolitica* in food in the U.S.A is of approximately 90%. Food of animal origins which is most frequently contaminated with *Yersinia enterocolitica* is: pork, poultry, beef and lamb, milk, ice-cream, sea food, etc. Pork and milk are the causes of most human food poisonings in different regions of the globe (4, 5).

The bacterium causes infections with localization in the digestive tube in numerous animal species and man with the most important symptoms being diarrhea, pneumonia, abortion and lymphadenitis (3).

Materials and methods

In order to evaluate the incidence of the *Yersinia enterocolitica* species in the swine species, there were collected 800 samples from two units: A and B, aiming to verify 16 groups made up of 10 animals (8 groups from unit A and 8 from unit B) on the technological flow of slaughtering.

For the isolation and identification of the presence of *Yersinia enterocolitica* species, we have used enriching mediums, initially proposed by Toora (soya

trypticase 20 g, glucose 1g, NaCl 5 g, agar 13g and distilled water 1000 ml), and modified subsequently by Bhaduri et al. (2) by supplementing the formula with 2.5 g of yeast and 2 g of bile salts.

In order to avoid contamination we used the antibiotic formula proposed by Bhaduri et al. (2), an alcoholic solution which contains irgasan (inhibits the multiplication of Gram-positive bacteria) 1mg/ml. The solution is prepared at the moment of use and can be kept for four weeks at a temperature of -20°C.

According to the affirmations of Bhaduri et al. (2), confirmed by ourselves during the period of this study, irgasan should be added to the main medium after 24 hours of incubating the samples at 12°C, to reduce the inhibitive effect of the antibiotic on the *Yersinia enterocolitica* species, especially of the extremely virulent strains which posses virulence plasmids.

For the selective isolation we used, as main medium, deoxicolate-citrate-manitol agar (DCMA). The composition of the main medium was supplemented with 3g of yeast extract (rich source of B vitamins) because it has been taken account of aspects related to the metabolism of the *Yersinia enterocolitica* species and of the fact that the "adapted enriching broth" inhibits the development of germs due to the antibiotic formula we used. The yeast extract, alongside meat extract peptone and tryptone provides the nutritional substances necessary for the bacterial growth.

Results and discussions

Study of the portage before slaughtering

In order to isolate *Yersinia enterocolitica* germs, of intestinal origin, 160 samples were harvested from the rectum, with the help of cotton tampons, from the two units A and B aiming to verify 16 groups made up of 10 animals (8 groups from unit A - 80 samples and 8 groups from unit B - 80 samples).

The sampling was effected after the classification in quality classes (according to the age, sex, body weight, conformation, stage of weight gain) both from groups of pigs in a poor maintenance stage, with diarrhea (generally watery, without being hemorrhagic) as well as from animals which, at the veterinary exam before slaughter, presented themselves in good shape.

After the harvest, the cotton tampons were introduced in tubes with PSB medium (phosphates-sorbitol-bile salts) in a quantity of 5 ml. After that they were seeded on isolation medium (CIN and SSDC).

From 160 preservations (rectal tampons), through direct seeding, after the bacteriological exam for the identification of *Yersinia enterocolitica* species, 5 positive samples have been identified: three from unit A and three from unit B (table 1).

The data obtained emphasize a higher incidence in unit A which was determined by the different origin of the animals. The positive samples were submitted to some biochemical test for confirmation and identification of the *Yersinia enterocolitica* species. The biochemical test results outlined that the five isolated strains belong to the *Yersinia enterocolitica* species (table 2).

Table 1

Isolation of the *Yersinia enterocolitica* species of intestinal origin before slaughtering

Unit	No. groups	No. analysed samples	Of which positve			
			Groups		Samples	
			No.	%	No.	%
A	8	80	3	37.50	3	3.75
B	8	80	2	25.00	2	2.50
A+B	16	160	5	31.25	5	3.125

Table 2

Results of the biochemical exam

Test	Strain				
	1	**2**	**3**	**4**	**5**
Ornitin-decarboxylase	+	+	+	+	+
Urease	+	+	+	+	+
Acid glucose	+	+	+	+	+
Oxydase	-	-	-	-	-
Lizin decarboxylase	-	-	-	-	-
Rhamnose	-	-	+	-	-
Citrat	-	-	-	-	-
Sucrose	+	+	+	+	+
Sulfurated Hydrogen	-	-	-	-	-

Study of the incidence of *Yersinia enterocolitica* after slaughtering

The technological process of slaughtering starts with the electrical stunning of the animals, followed by complete bleeding and scalding in a warm water basin, for approximately 2 minutes, after which the resulted carcasses are suspended on the conveyer belt.

In the two abattoirs taken into study, the carcasses were brought by the conveyer belts, after singing and washing, in front of the evisceration band, provided with fixed trays in which the organs are put after evisceration. The evisceration process is performed through a circumanal incision and an abdominal incision after which the gastrointestinal mass is extracted manually. The spleen is detached from the gastrointestinal mass and is processed in a separate compartment. Then the section of the sternum is performed with the help of an electric chainsaw. The evisceration technique for pigs involves the removal in common piece of a series of organs as: tongue, esophagus, trachea, heart and liver. The kidneys remain in their natural adherence and are removed alongside with the perirenal fat tissue. The sectioning of the carcass in two halves is made with the mechanical chainsaw. We mention that, for the research, samples have been collected after evisceration from the intestinal content and from organs (liver,

kidneys, tongue and carcass). They were seeded on selective isolation mediums (CIN, SSTC and modified medium).

The results obtained at the bacteriological exam of the 160 samples collected from the intestinal content of animals coming from the 16 groups, showed an incidence of *Yersinia enterocolitica* of 8.125%. There were different according to the selective medium used. Thus the highest number of positive samples consecutive to the bacteriological exam, were found in the case in which the modified medium was used. If on the selective medium CIN and SSTC only 11 positive animals were discovered, on the modified medium the presence of the *Yersinia enterocolitica* was discovered in 13 animals with mention that the positive samples came from animals belonging to two groups of ten animals from each unit.

Table 3

Isolation of the *Yersinia enterocolitica* species after the evisceration process

Growth medium	Unit						Total		
	A			B			No. analysed samples	Of which positive	
	No. analysed samples	Of which positive		No. analysed samples	Of which positive			No.	%
		No.	%		No.	%			
CIN	80	7	8.75	80	4	5.00	160	11	6.875
SSTC	80	7	8.75	80	4	5.00	160	11	6.875
modified	80	8	10.00	80	5	6.25	160	13	8.125

A different incidence of the presence of positive samples from one unit to another as well as depending on the used medium has also been detected.

After testing of the biochemical activity of the 13 strains which were isolated from the intestinal content, it has been found that they all belong to the *Yersinia enterocolitica* species (table 4).

From the animals belonging to the groups which were identified as positive, at the exam of the intestinal content, 160 samples were collected: 40 from the liver, 40 from the tongue, 40 from the kidneys and 40 sanitation samples harvested by wiping a 10 cm^2 marked surface from the carcass. The obtained results showed a low incidence of the presence of *Yersinia enterocolitica* species in the analyzed organs (0.83%). We mention that from the analyzed organs the exams made on the kidneys and liver were totally negative, while from the tongue only one strain was isolated. From the carcass 4 samples (10%) were positive (table 5).

Table 4

The results of the biochemical exam for the *Yersinia enterocolitica* strains isolated after evisceration process

Strain	Test								
	Ornitin-decarbo-xylase	Ure-ase	Acid from glucose	Oxy-dase	Lizin decarbo-xylase	Rham-nose	Citrate	Mobility at 25°C	Esculine
1	+	+	+	-	-	-	-	+	-
2	+	+	+	-	-	-	-	+	-
3	+	+	+	-	-	-	-	+	-
4	+	+	+	-	-	-	-	+	-
5	+	+	+	-	-	-	-	+	-
6	+	+	+	-	-	-	-	+	-
7	+	+	+	-	-	+	-	+	-
8	+	+	+	-	-	-	-	+	-
9	+	+	+	-	-	+	-	+	-
10	+	+	+	-	-	-	-	+	-
11	+	+	+	-	-	-	-	+	-
12	+	+	+	-	-	-	-	+	-
13	+	+	+	-	-	-	-	+	-

Table 5

Isolation of the *Yersinia enterocolitica* species from organs after direct seeding on selective media

Organ	Unit						Total		
	A			B			No. analysed samples	Of which positive	
	No. analysed samples	Of which positive		No. analysed samples	Of which positive			No.	%
		No.	%		No.	%			
Liver	20	-	-	20	-	-	40	-	-
Tongue	20	1	5	20	-	-	40	1	2.5
Kidney	20	-	-	20	-	-	40	-	-
Carcass	20	3	10	20	1	5	40	4	10.0
TOTAL	80	4	5.0	80	2	1.25	160	5	3.12

The study of the *Yersinia enterocolitica* species prevalence on harvested samples at refrigeration and after the deposit of the refrigerated meat

In order to make a quantitative evaluation of the contamination of pig carcasses which come from animals from the 4 groups, 40 samples have been prelevated before refrigeration and 40 after refrigeration. The obtained results showed that refrigeration had negatively influenced the viability of the strains of *Yersinia enterocolitica*. Thus, the incidence in carcasses identified as positive reduced from 5.00% before refrigeration to 3.75% after refrigeration (table 6).

Table 6

Direct isolation of *Yersinia enterocolitica* germs after refrigeration and deposit of the refrigerated meat

Unit	Prelevation period of the samples					
	Before refrigeration			After refrigeration		
	Carcass No.	Of which positive		Carcass No.	Of which positive	
		No.	%		No.	%
A	40	1	2.50	40	1	2.50
B	40	3	7.50	40	2	5.00
Total	80	4	5.00	80	3	3.75

Study of the incidence of *Yersinia enterocolitica* species on the samples obtained after freezing

The effect of low temperatures on the viability of the *Yersinia enterocolitica* species has been outlined also in the freezing process of the carcasses. Research regarding isolation, identification and determination of the pathogenic microflora from the pig carcasses has revealed a diverse microflora with possible implications in food-poisoning in people. The presence of Yersinia Enterocolitica species on the surface of fresh carcasses was of 5% and after 72 hours from the moment in which the freezing process was started, the percentage of the positive strains was of 1.25% (table 7).

Table 7

Evaluation of the quantitative contamination with *Yersinia enterocolitica* during freezing after direct isolation

Unit	Prelevation period of the samples					
	After 24 hours from freezing			After 72 hours from freezing		
	Carcass No.	Of which positive		Carcass No.	Of which positive	
		No.	%		No.	%
A	40	1	2,50	40	-	-
B	40	2	2.50	40	1	2,50
Total	80	3	2.50	80	1	1.25

Conclusions

During slaughtering, a decrease in the presence of *Yersinia enterocolitica* germs can be observed in the first links.

From our researches, according to those worldwide, it results that, presently, the slaughtering process allows the exigent compliance to the conditions of hygiene and disinfection in order to limit the spreading of the *Yersinia enterocolitica* which favors the intercontamination phenomenon.

Pig carcasses have presented, consequently to the quantitative evaluations, a low contamination after direct isolation and after preliminary enrichment.

Pig carcasses have presented through direct isolation a contamination of 10% after 24 hours of refrigeration and 7.5% after 72 hours of refrigeration.

During the operation of evisceration, the pig organs have presented, through enrichment a preliminary low rate of contamination (groups which were found contaminated after the isolation of *Yersinia enterocolitica* from rectal prelevated) for liver samples, as well as spleen samples, kidney and tongue samples.

It has been observed that when the evisceration is made, contamination can be made accidentally through intestinal rupturing, favoring thus the presence of the germ on the flow. In our case though, the slaughtering g mistakes were punished with total confiscation.

The analyzed meat preparations have presented a zero grade of contamination through direct isolation and preliminary enrichment.

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MICROBIAL COLONIZATION OF SWINE ADENOIDS ("PHARYNGEAL TONSILS") TARGET CONTROL FOR MEAT SAFETY

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Summary

The purpose of our research is to evaluate the microbiological configuration from swine adenoids microbiota. A number of 81 probes were examined directly from the swine adenoids and 56 probes underwent bacteriologic examination. Direct microscopic examination and *Gram staining* of adenoids samples harvested directly from abattoirs sacrificed swine revealed microbial polymorphism.

The most common species were Gram negative bacilli in 26 probes (63.41%) followed by diplococci in 16 probes (39.02%), *Streptococcus species* in 13 probes (31.70%), *small Gram positive* in 11 probes (26.82%), *pleomorphic Gram*-positive non-spore-formers in 7 probes (17.07%) yeasts of Candida strains in 7 samples(17,07%)and Gram-positive spore-formers in a single probe (2.43%). The bacteriologic exam for bacterial identification using WALKAWEY System (automated system for bacterial identification) performed on 56 swine adenoids followed by Bacterial subtypes identifications using API microtest systems revealed the following configuration: *Staphylococcus aureus* (8 strains), *Streptococcus hemolyticus* (7 strains), *Staphylococcus xylosus* (3 strains), *Staphylococcus chromogenes*, *Staphylococcus hyicus, Staphylococcus simulans and Staphylococcus lentus* with one species each*, Streptococcus suis II* (3 strains), *Streptococcus suis I* (1 strain), *Streptococcus porcinus (2 strains), Streptococcus pyogenes* (1 strain), *Pseudomonas aeruginosa* (7 strains), *Pseudomonas fluorescens* (4 strains), *Klebsiella pneumoniae* (3 strains), *Escherichia coli* (3 strains), *Proteus mirabilis* (3 strains), *Aerococcus viridans* (2 strains), *Acinetobacter baumannii* (1 strain), and *Hafnia alvei* (1 strain).

Further study is needed to determine the link between occupationally exposed group, general population and diagnostic evaluation in suspected outbreak detection.

Key words: microbial colonization, adenoids, swine, meat safety

Zoonoses have a major impact on health in developing countries. The area known as tonsillar is known as an area located at the junction of the two anatomical structures: alimentary canal and respiratory structures. Those pathways are used by all most pathogenic microbes and viruses to invaded the organism and an important element in the pig's immune system.

Tonsils are the portal for major pathogens: *Acinobacillus pleuropneumoniae* (9), *Pasteurella infections multocida*, *Streptococcus suis II* (1).

Many of the potential pathogenic micro-organisms in group of *Streptococcus*, Staphylococcus, *Entrobacteriaceae*, inhabits almost normally in this area due to the anatomical configuration that determined itself to the survival and development of micro-organisms(3). Tonsils are masses of lymphoid tissue which functions as the secondary Lymphoid organs and they are initiates the immune response against pathogens. By their structure tonsils represents a filter that captures, focuses and try to annihilate the microbes. Slaughterhouse of the exam at swine tonsils frequently put in evidence the inflammation of exudation produced and maintained by dangerous germs for the health of humans and animals. In slaughtering this zone may be a risk zone for meat and by-products if handling is not adequate, the digestive tract by tonsils can disseminate on housings. Tonsils are, therefore, a reservoir for pathogen and nonpathogenic bacteria .For this reason the tonsilar area is of high standards of care in terms of handling and management to ensure a high degree of security possible for carcasses microbiological and obtained. Bacteriological test carried out on pig pharyngeal tonsils signaled that they were proven to be colonized with a large number of bacteria: *Staphilococus. aureus, S. haemoliticus, S. xylosus, S. cromogenes, S. hycus, S. simulans and S. lentus, Str. suis. II, Str. suis I, Str. pyogenes, Str. porcinus and Enterobacteriaceae, E. coli,* which is the most important indicator of fecal contamination, *Klebsiela, Candida* yeast. The study shows that the pharyngeal tonsills are highly contaminated with microorganisms and can be an important source of contamination in slaughterhouse.

Materials and methods

The methodology involved was harvesting from the pharyngeal tonsil pigs slaughtered for consumption in normal slaughtering and processing them through microscopic examinations directly on fingerprint (81) and 56 sampling for bacteriological exam completed with identification of isolated strains using microtest API systems. Pharyngeal tonsils were collected from two slaughterhouses around of the Baia-Mare town from which one is approved for intra-Community trade and another with low-capacity slaughtering. Have tonsils samples from different batches of swine. Tonsils sampling was done immediately after evisceration with sterile instruments, and were packed in sterile plastic bags with a sticky label. In order to preserve microbial load of tonsil area, after sampling they were transported to the laboratory in the shortest time ensuring refrigeration temperature 4 °C. In the laboratory, from each tonsil were prepared glass slides by fingerprinting method. Those slides were stained by the GRAM method, and then were microscopically examined. For bacteriological exams have been processed 56 samples of pharyngeal tonsils. The tonsils were grouped so that some of them were passed on pre-enrichment culture media and another part was used for dilution method (10^2). For pre-enrichment culture media it been have used tomato sauce, haf-fraser and peptonic water.

Incubation was done according to the standard method for determining the bacteria of the genus *Listeria* - 30 degrees, the 37 degrees respectively *Salmonella*. Later they were on average pre-enrichment for Listeria-Fraser tomato sauce with Vasiliadis Rappaport and tomato sauce MKTT for *Salmonella,* then were inseminated agar plates with ALOA and Oxford for *Listeria Cromagar*, Rambach and *Salmonella Meitert Istrate*. For the rest of the samples as a diluent was used physiological serum and since the two dilutions were inseminated Petri plates with different culture media: Slanetz, Cromagar for coliform bacteria (e.coli) Chapman and Baird Parker medium, Myp.

On isolation culture media depending on the characteristics of the colonies and selectivity culture media were chosen by randomization colonies characteristics of each species developed and proceed to identify them with the help of WALKAWAY (automatic system of microbial identification) used along with plates containing Microscan dilution medium, antigerms in reagents and biochemical dilutions seriate selected. Bacteriological test was completed with the identification of strains isolated systems using microtest API which is a standardized system for identification of Enterobacteriaceae and other Gram negative, bacteria which are using 21 biochemical tests and a specific database.

Results and discussions

In the case of samples collected and analyzed by bacteriological examinations from pharyngeal tonsils were isolated 8 strains of *Staphylococcus aureus*, which represents 14.28%, 7 samples of *S. hemoliticus*, 3 strains of *S. xilosus* which is representing 5.03% and a strain of *S. cronogeus*, 1.78% *S. hycus* 1.78%, *S. simulans* and s. 1.78% *S. lentus* 1.78%, and Str. pyogenes strain, which mean 1.78% of each one. It is important for public health to report the presence other bacterial species as: *Pseudomonas aeruginosa, Klebbsiella pneumoniae, Pseudomona fluorescens.*

This study shows that pharyngeal tonsils assure a carry state and multiplication place for numerous bacteria species. In tonsils the bacteria polymorphism persists and so this organs are an important source of contamination for meat and by-products which are produced in the slaughterhouse. Tonsils colonized with pathogens could play an important role in the contamination of meat in the slaughterhouse during manipulation. Research on microbial tonsils load derived from domestic pigs intended for human consumption have pointed out the important aspects.

The results from the 81 pharyngeal tonsils sampled from slaughtered domestic pigs that were Gram stained and examined with microscope may outline the presence of Gram negative bacilli in 26 of samples, Gram positive in 19 samples, diplo and streptococcus in 29 samples and also in 4 samples the presence of yeasts from the *Candida* genus.

The results from the 56 tonsils sampled from swine and analyzed by bacteriological using WALKAWEY system, complete with identification of isolated strains using Microtest API systems are presented in table 1. From all samples were isolated 8 strains of S*taphylococcus aureus*, representing 14,28%, 7 strains of *S. hemoliticus* i.e. 12,5%, 3 strains of *S. xylosus*, which mean 5,03%, one strain of *S. cromogenes*, *S. hycus, S. simulans, S. lentus* 1.78% of each.

Table 1

The results of bacteriological exams from 56 tonsils

No of samples	Positive sample							Negative sample
	S. aureus	*S. hemoliticus*	*S. xylosus*	*S. chromogenes*	*S. hycus*	*S. lentus*	*S. simulans*	
56	8 (14.2%)	7 (12.5%)	3 (5.3%)	1 (1.7%)	1 (1.7%)	1 (1.7%)	1. (78%)	34 (62%)

The results of isolation and identification of bacteria from the *Streptococcus* genus in tonsils samples are presented in table 2. These results have been shown the following species: *Str. suis II* (3 strains), i.e. 5.03%, Str. *suis I* one strain (1.78%) *Str. pyogenes* -one strain (1.78%) and *Str. porcinus* - 2 strains that mean 3 2%.

Table 2

The results of *Streptococcus* species isolated from tonsil samples

No of samples	Positive sample				Negative sample
	Str. suis II	Str. suis I	Str. pyogenes	Str. porcinus	
56	3 (5.03%)	1 (1.78%)	1 (1.78%)	2 (3.57%)	49 (87.84%)

From Enterobacteria's family (table 3) have been isolated one by one 3 strains (*Klebsiela pneumoniae, E.coli, Proteus mirabilis*), which mean 15.09% and a stem of *Hafnia alvei* (1.78%).

Table 3

The results of Enterobacteriaceae strains isolated from tonsil samples

No of samples	Positive sample				Negative sample
	Klebsiella pneumoniae	E. coli	Proteus mirabilis	Hafina alvei	
56	3 (5.03%)	3 (5.03%)	3 (5.03%)	1 (1.78%)	46 (82.13%)

It is important to public reveal the epidemiological hazard for public health through the caries and eliminators of some bacterial strains like: *Pseudomonas aeruginosa* (7 strains) i.e. 12.5% of samples examined, *Pseudomonas fluorescens* (4 strains) 7.14%, *Aerococcus viridans* (2strains) 3.57% and 1 strain of *Acinetobacter baumanii*, respectively 1.78% (table 4).

Table 4

The results of other species isolated from tonsils samples

No of samples	Positive sample				Negative sample
	Ps. aeruginosa	Ps. fluorescens	Aerococcus viridans	Acinetobacter baumani	
56	7 (12.05%)	4 (7.14%)	2 (3.57%)	1 (1.78%)	42 (75.01%)

Tonsils are a well-known portal entry and a perdurable multiplier for more micro-organisms, both in humans and in animals, including pigs Salles and Midleton (13). Pigs are mostly asymptomatic shedding of *Salmonella enterica*, *Listeria monocytogenes* and *Yersinia enterocolitica. Yersinia enterocolitica* has proved to be a more frequent inhabitant in pig tonsils Bucher et al. (4). In 2005 the infection incidens of *Campylobacter* spp., *Salmonella* spp., *Yersinia* spp., reported in humans was 51,6%,38,2% and 2,6% respectively,according to a study done by Norrung and Buncic in 2008(10).

Listeria monocytogenes was isolated from 46% of the farms, which could indicate that some risk factors from farms can have an influence on the result. Among the risk factors described in the literature has been shown to increase the risk of infection with *Listeria monocytogenes* in humans (7).

Antio et al. (2) show that Listeria monocytogenes is most frequently encountered at pig male (22%) than sows (6%).

Esteban, Oporto, Aduriz, Juste and Hurtado (6) studied samples of feces from 30 pigs from Spain 17 and actual reported results negative for *Listeria monocytogenes* to all the pigs.

Campylobacter spp. has been rare in pork tonsils only 6% were colonized with pathogens. On the other hand, **Campylobacter** spp. has been identified in 62% of the fecal samples collected from pigs in the slaughterhouse. Has been identified a single case with the presence of *Campylobacter* spp. in the tonsils, but absent in the feces.

Conclusions

The study provides information on the configuration of common bacteriological load of the pharyngeal tonsils of pigs slaughtered for human consumption.

The isolation in high frequencies of *Streptococcus suis* serotype II underline it importance for pig and human health knowing that it is associated with invasive disease at pigs and humans.

The most common bacterial strains found in this study which represent a possible biological hazard for contamination of the meat was: *Klebsiella pneumonial, Ps. aeruginosa, S. aureus, Proteus mirabilis (3 strains), Candida.*

The most efficient method for detecting these bacteria strains proved to be WALKAWAY system used in a rapid and safe manner.

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THE STUDY OF AEROBIC MESOPHILIC GERMS AND *SALMONELLA* GENUS FOUND ON THE SURFACE OF PORK CARCASSES

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Summary

The study focuses on the novelty evaluation of the hygienic processing of pork meat obtained in a small scale slaughterhouse found in the Maramures county. Our main objective was to asses the following microbiological criteria involving the presence of *Salmonella* spp., mesophilic germs and *Enterobacteriaceae*. The microbial load was evaluated from the surface of 20 pork carcasses obtained in that particular small scale slaughterhouse which produces pork meat for public consumption. Following the investigations we revealed that the last two parameters are in accordance with the community legislation for pork carcasses, but for the first one (identification of *Salmonella* spp.) there was a sample identified as contaminated with *Salmonella* spp. serological group C.

Key words: aerobic mesophilic bacteria, *Salmonella*, pork carcasses

This study focuses on the hygiene status assessment of swine carcasses processed in the slaughterhouses found in Maramures County, through microbiological determination applied in concordance with the European standards of microbiological criteria for slaughtering establishments

The main microbiological criteria determined were; the Salmonella spp. Isolation, the aerobic mesophilic germs count and the *Enterobacteriaceae* number evaluation.

In the slaughtering establishments from the areal, the pork carcasses might be slightly contaminated with microorganisms from various sources (processing machineries, workers, water, air, feces etc.), which would lead to economic losses in case the presence of pathogens or toxins that may cause humans diseases is confirmed. The microbiological criteria are useful in validation and verification of the HACCP procedures, among other hygiene control measures.

In order to obtain an accurate examination result following the Community rules, it is necessary to harvest samples from multiple areas of the carcasses.

The microbial contamination of pig carcasses during the slaughtering process involves a security issue which the pork meat industry is facing. The *Enterobaceriaceae* count, N.T.G. and *Salmonella* spp. Isolation from pork

carcasses surfaces shall comply with the national legislation in line with the Community law.

Materials and methods

The investigations were made on a number of **40 samples**, collected in small-scale sluaghterhouse (20 pork carcasses destined for public consumption). The sample harvesting was made from 20 pigs, from 4 different areas: shoulder area, goitre, thigh, chest.

The methods and working procedures used were in accordance with National and Comunity legislation, respectively Regulation (CE) no.2073/2005 regarding the microbiological criteria for food products modified with Regulation. (CE) no.1441/2007. The *Salmonella* spp. isolation was made in accordance with the working protocol established in SR EN ISO 6579/2003 updated in 2006. For the determination of NTG the working method is described in SR EN ISO 4833/2003 and for *Entrobacteriaceae* in the SR EN ISO 21528/2004 standards.

For the isolation of *Salmonella* spp. the technique of abrasives sponges containing peptonised water was performed, and the harvesting made from 4 test ed areas of each carcass ; goitre, thigh, chest neck. For each area the abrasives sponges were used to colllect the samples from the surface at the end of the carcass processing and also before the refrigeration step. After the harvesting, the sponges were packed into a sterile bag, identified and transported to an microbiology laboratory and refrigerated at a temperature of 0 - 4°C until their examination. The harvesting for NTG and *Enterobacteriaceae* spp. was made after the carcasses were processed and before the starting of the cooling process from the same areas as in the case of Salmonella spp. tests, using also sterile device.The 4 tissue samples from each carcass were taken within an area of 20 cm, and the fragments obtained by cutting a muscle area of 2,5/2 cm. The sample flaps were introduced in aseptic conditions in to sterile bag, identified and sent to the laboratory under refrigeration conditions (4 degrees C). In the microbiological laboratory following procesing was made by adding at the four flaps collected from each carcass 100 ml physiological saline peptone and then equlised in a Stomacher at least two hours at 250 rev./minute.

The blender performed a decimal dilution in diluent (saline peptone). From each dilution 1 ml was taken and included in the cooled medium to 45ºC (average PAC) for N.T.G. and VRBG (*Enterobacteriaceae*), for Total Number of Germs (NTG), and violet red bile agar (VRBA) for Enterobacteriaceae.

For every bag with 4 sponges, 400 ml were add of physiological saline peptonat and shaken, then incubated for 24 hours at 37º C. Afterwards it was proceeded to the selective enriching by taking 0.1 ml of the culture obtained in the previous phase, added 1 ml into the Rappaport Vasiliadis enviroment for 24 hours at 41.5º C.Then 1 ml was added to the KTTN medium which was incubated for 24 hours at 37ºC. A culture of the

environment is passed on to the XLD agar or Rambach incubated for 24 hours at 37° C.The, an isolation on selective culture is made, one takes a culture wire from the RVS medium, scratches on agar with XLD and a wire on agar Rambach, than incubates 24 hours at 37° C. The *Salmonella* confirmation was done by testing on biochemical characteristics as colonies of insulation from each of the 5 plate. The confirmation showed a single positive colony of Salmonella Group C.

Results and discussions

The assessment of the hygiene parameters in the 20 carcasses taken into study, based on the NTG parameter stated by the national legislation harmonized with the Comunitary one is shown in Fig. 1:

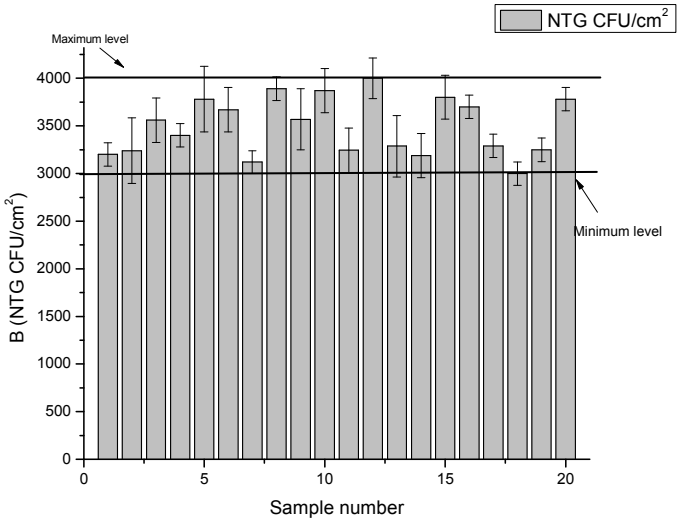

Fig. 1 Representation of the NTG CFU/cm^2 values obtained at the analyzed pork carcasses

Total number of mesophilic aerobic bacteria in the samples examined varied between 3000 and 4000 /CFU cm^2 at each samples examined. Within these values all the samples analyzed are in accepted for the pork meat obtained in authorized slaughtering units.

The *Enterobacteriaceae* spp. have been found in less than 10% of the samples examined with values ranging between 35-50 cm being in accordance with the accepted values for this microbiological parameter.

The Salmonella group C was present on a single sample, which means 5% of the total number. At the morphological exam of the selective media for the identification of *Salmonella* genus there were revealed red colored colonies with black center due to H_2S production on the XLD environment and some red colored colonies characteristic for *Salmonella* spp. on Rambach environment (Fig. 2 and 3).

Fig. 2 and 3 **.** *Salmonella spp.* colonies on XLD culture media (original), respectively on Rambach selective media (original photo).

Regarding other reseraches made by romanian researchers, the level of bacteria contamination at swine carcasses varies from 10^{-2} to 10^{-3} CFU/cm^2 and the Enterobacteriaceae load was satisfactory at 2 log. CFU/cm^2 (1).

Some foreign researchers have identified Enterobacteriaceae in 76% from the total number of samples (7) and others in 56% from the samples harvested from the pork carcasses' surface (9). In a comparative study made in swedish slaughterhouses following the same protocol as our study, in order to asses the NTG and Enterobacteriaceae load (E.coli), the positive sample percentage were higher for E.coli (52-84%) than those obtained at the samples collected by other methods (3-14%)(6). Similar results are reported also by other researchers in which the Enterobacteriaceae load is higher when the samples are taken by our method of harvesting (68-100% vs. 15-24%) (2, 3, 8).

A study made in order to asses the presence of *Salmonella* spp. by harvesting samples from three slaughtering processes (after bleeding, hygiene step and refrigeration) show that from 182 positive sample, 24% were contamined with *Salmonella* spp. after bleeding and 3% after refrigeration (5). The most frequently met subspecies were *S. typhimurium* (27%) and S. dersy (40.5%).

Conclusions

The pork carcasses examined comply with the sanitary conditions requested by the national legislation and also the community legislation in what concerns the NTG parameter and the Enterobacteriaceae load. Regarding the presence of *Salmonella* spp. there is a contamination noticed at 5% of the carcasses which might cause health problems for the consumers.

The obtained results force the slaughtering units to continue the microbiological surveillance on the production flow and to permanently improve the sanitary measures in order to increase meat quality and public health.

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RESEARCH REGARDING THE ISOLATION OF *SALMONELLA* SPP. FROM MEAT AND MEAT PRODUCTS

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Summary

Although in the last decades, important progress has been registered, worldwide and nationally, in what regards the general life level, hygiene conditions, the population's sanitary knowledge, methods of stopping the multiplication of microorganisms (refrigeration, freezing, high temperature treatment, conserving substances, washing and disinfecting agents with high efficiency), food poisonings continue to occur with high frequency in all the countries in the world, independent of their level of development. Having in view all these aspects in our research, performed on a number of 4941 samples of meat and meat products which came from processing units spread in the area of the Constanta County, we had in view the isolation and identification of germs belonging to the *Salmonella* gender, with implications in the appearance of food poisonings in people.

Key words: *Salmonella*, isolation, identification, meat, meat products.

Salmonella spp. are ubiquitary microorganisms, present in the intestine of birds and mammals, reptiles and insects. The circle of salmonellas in nature includes as hosts both animals and humans (1, 6, 7).

Non-typhoid *Salmonella* spp. (those which don't cause typhoid and paratyphoid fever) are the most important etiological agents of food poisonings in the world (2, 7).

The *Salmonella* Typhimurium serovar has been, for many years, the most important preoccupation of specialists in public health, with special references at the DT104 clone, with a multiple resistance towards antibiotics (4, 5) but it has been gradually replaced in the last decade both in the European Union and in other regions with the *Salmonella* Enteritidis serovar (3). Presently, worldwide, the most frequent serovars isolated from the focuses of food poisonings with *Salmonella* are *Salmonella* Enteritidis, *Salmonella* Typhymurium and *Salmonella* Newport (in decreasing order) (6).

In the European Union, the *Salmonella* Infantis serovar represents the third most important microorganism for human *Salmonella* food poisonings. These data, referring to the classification of serovars and the number of cases of *Salmonella* infections in humans are include in The estimative report of the relative contribution

of different foods and animal sources for human salmonellosis in the EU (reported before the European Authority for food safety) (8).

If the infection with the *Salmonella* Enteritidis serovar is associated especially with eggs consumption (in 95% of cases), poultry (meat chicken and turkey), and products derived from these (in 56.9%, respectively 30.4% of cases), *Salmonella* Typhimurium is involved especially in food poisonings caused by the consumption of pork and pork products (in 63% of cases) (8).

Materials and methods

The researches were conducted in the period 2009-2011, taking into study a number of 4941 samples of meat and meat products, which came from processing units from the area of the Constanta County (table 1). Laboratory investigations regarding the isolation and identification of the germs from the *Salmonella* gender have been made in the bacteriologic diagnosis laboratory from the Veterinary and food safety laboratory Constanta.

Table 1

The origin of the analyzed samples for isolation of salmonellas

Product	Total samples	Of which		
		2009	2010	2011
Frozen meat	1330	180	610	540
Minced meat	2356	291	1195	870
Fresh Sausages	567	168	290	109
Minced meat paste	449	140	170	139
Liver	52	52	-	-
Leberwurst (liver sausage)	24	-	-	24
Head cheese	36			36
Sanitation	127	34	39	54
TOTAL	**4941**	**865**	**2304**	**1772**

Harvesting of Samples

The harvesting of the samples has been made in strict conditions of asepsis, avoiding as much as possible the exterior contaminations, an extremely important aspect because deceleration of germs imposes an enrichment stage and the contamination with only one germ is sufficient to condemn the product. Little manipulation, limitation of sectioning and shredding of the food product, has been the main principle in harvesting such samples.

Samples of meat and mat products have been exposed to examination. The samples which were harvested and individualised using serial numbers were sealed to exclude the dissemination of germs in the environment and were subjected to bacteriological investigation targeting the isolation, identification and serotyping of germs according to present standards. The samples were analysed using the methodology provided in SR.EN.ISO 6579/2003.

Examination techniques

The principle of the classic methods consists of the isolation from pathological materials and other products of bacteria and their identification based on their cultural, morphological, biochemical and serological characteristics. In the case of widely spread serovars which have an epidemiological importance, their phage sensitivity, their antibiotype and bacteriocinotype are set. The initial identification of the gender stage is important, because it also implies the differentiation from the other members of the *Enterobacteriaceae* family. The study of the biological particularities (experimental pathogenicity) presents importance for some serovars. These methods target the complex classical bacteriological exam which implies a bacterioscopic examination, isolation, cultivation and identification of salmonellas from the suspected pathogenic products. Salmonellas can be isolated through a variety of techniques which can appeal or not to pre-enrichment for the resuscitation of the salmonellas which have a reduced viability, enrichment media which contain inhibitive substances for contaminating germs, selective media and for differentiated diagnosis which allow the differentiation of salmonellas from other enteric species.

Results and discussions

From the 4941 analysed samples we have isolated and identified 139 strains, belonging to the *Salmonella* gender (table 2). After the examination of the smears, we have highlighted Gram negative germs, with a bacillar and cocobacillar form, with measures between 2-4/0.5-0.7µm, unsporulated, uncapsuled, but with cilia.

Table 2

The prevalence of the *Salmonella* strains

Year	Analysed samples (No.)	Isolated strains	
		No.	%
2009	865	38	4.39
2010	2304	59	2.56
2011	1772	42	2.37
Total	**4941**	**139**	**2.81**

Due to the fact that the bacteriological exam, which targets the isolation, identification and serotyping, has the highest diagnosis value, it was done as soon as the samples reached the laboratory. We used simple media and enrichment media in the same time for isolation. We made re-seedings from these on selective media for the isolation and identification of the cultures.

To obtain a number as high as possible of isolated colonies, the performing of the dispersions was done carefully, watching that the surface of the plates with

media was dry, without condensation drops avoiding, thus, the usage of plates with a dehydrated medium.

Analyzing the obtained results after the examination of the 4941 samples, we see a different incidence from one year to another but with a descending evolution.

A different incidence of strains was found dependent on the analyzed product (table 3). Thus, the highest medium incidence during the entire period has been registered in the case of meat products: fresh sausages (3.70%) and minced meat paste (3.34%)

Table 3

Incidence of strains according to the nature of the samples

Product	Total analyzed samples	Isolated strains (No.)	%	Perioad					
				2009		2010		2011	
				No.	%	No.	%	No.	%
Frozen meat	1330	40	3.00	7	0.53	18	1.35	14	1.05
Minced meat	2356	51	2.65	14	0.59	21	0.89	13	0.55
Fresh sausages	567	21	3.70	8	1.41	10	1.76	5	0.88
Minced meat pasta	449	15	3.34	6	1.34	6	1.34	5	1.11
liver	52	1	1.92	1	1.92	-	-	-	-
Leberwurst	24	1	4.17	-	-	-	-	1	4.17
Head cheese	36	1	2.78	-	-	-	-	1	2.78
Sanitation	127	9	1.09	2	1.57	4	3.15	3	2.36
TOTAL	**4941**	**139**	**2.81**	**38**	**0.77**	**59**	**1.19**	**42**	**0.85**

The obtained data show that the main source of contamination of the fresh products is represented by refrigerated meat which shows an incidence of *Salmonella* strains of 3.00%. This aspect also results from the prevalence of *Salmonella* strains in the sanitation tests (1.09%) as a result of the cutting stages of refrigerated meat. Analyzing the obtained results regarding the classification of the strains in serogroups, we have come to the conclusion that in all the cases of the analyzed products there was a high incidence of strains from the serogroups B (65.83%) and C (23.02%) (table 4).

Also, the presence of strains belonging to the serogroups E and G in the refrigerated meat has determined their highlighting during the initial technologic process in minced meat and subsequently in the obtained products (sausages and minced meat paste).

Table 4

Assignation of isolated strains in serogroups

Product	Isolated strains	Serogroup									
		B		C		E		G		I	
		no.	%	no.	%	no.	%	no.	%	no.	%
Refrigerated meat	40	28	70.00	7	17.50	3	7.50	2	5.0	-	-
Minced meat	51	28	54.90	8	15.69	9	17.65	5	9.8	1	1.96
Fresh sausages	22	11	50.00	6	27.27	3	13.64	1	4.54	1	4.54
Minced meat paste	14	7	60.00	5	35.71	2	14.29	-	-	-	-
Liver	1	-	-	1	100	-	-	-		-	-
Leberwurst	1	-	-	1	100	-	-	-		-	-
Tobă	1	-	-	1	100		-	-	-	-	-
Sanitation	9	5	55.56	3	33.33	1	11.11	-	-	-	-
TOTAL	139	79	56.83	32	23.02	18	12.95	8	5.76	2	1.44

The results obtained after typing the 139 strains of *Salmonella* also show a high incidence of serovars from the groups B and C and a much lower incidence of serovars G and I.

Conclusions

The incidence of the *Salmonella* strains in the analyzed samples was of 2.81% with a high prevalence of serogroups B (56.83%) and C (23.02%).

The high frequency of strains isolated from refrigerated meat shows that it represents the main source of contamination of the meat products.

The *Salmonella* spp. isolated were classified in 5 serogroups B, C, E, G, and I, and 29 serovars were identified.

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STUDY OF DYNAMICS AND INFLUENCE OF DIFFERENT ENVIRONMENTAL FACTORS ON FORMATION OF MICROBIAL BIOFILMS

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Summary

Ability of 19 bacteria strains isolated from biofilms developed on various surfaces (inert and meat surfaces) from food industry plants to form biofilm, under various conditions of pH (5; 7.5 and 8.5) and temperature (10; 22 and 37° C), were tested. Estimation of quantitative production of the biofilm has done using the crystal-violet test. The greater biofilm formation has been observed in *P. aeruginosa* isolated from pig carcasses, maintained at 10º C, pH 7.5, for 10 days, which was 2.36 times higher in comparison with the biofilm produced by the same strain maintained 72 hours, at 22° C, pH 7.5 and 1.56 times more abundant than the biofilm formed at 37° C, pH 7.5 after 72 hours. In alkaline medium (8.5) all bacterial strains and their associations, isolated from the carcasses surface and 50% of the strains from inert surfaces had a higher biofilm production in comparison with those maintained to pH 5, both 24 and 72 hours. *Pseudomonas* strain showed an increase of biofilm mass of 196.11-218.25% and *Escherichia*, between 145.89 and 212.01%, after three days. The acidic pH of the media (pH 5) is unfavorable for biofilm development by normal bacterial flora of meat. However, some bacterial strains isolated from inert surfaces were stimulated to produce biofilm in acidic conditions (eg. *Salmonella* and *P. fluorescens*). Mesophilic conditions (37° C) and pH 7.5 favored biofilm formation within 24 hours.

Key words: microbial biofilm, food industry, temperature, pH effect;

Environment from food processing plant offer a variety of conditions that favor biofilm formation, such as high humidity, nutrients and a diversity of microbial species from raw materials (6). Such biofilms are potential sources of food contamination with undesirable spoilage and pathogenic bacteria. Moreover, when parts of the biofilm detaches, these microorganisms can spread easily (10). Constituents of biofilm microflora, can include species with health significance, such as: *Salmonella* spp, *Klebsiella* spp, *Pseudomonas* spp, *Campylobacter* spp, *Escherichia coli, Listeria* spp. Bacterial colonization and biofilm development on the surfaces from food processing equipment leading to degradation of the metal surfaces and plastics, contribute to clogging pipes and reducing heat transfer, with negative impact on some processes, notably thermal treatment apply to foods (7,

8). For control microbial biofilms of in food industry need a better understanding of biofilm physiology, the interaction of organisms within the biofilm and the impact of environmental factors on biofilm forming species.

The purpose of this research was to assess the effect of environmental conditions on dynamic of biofilm development produced by species isolated from food industry units.

Materials and methods

Bacteria strains. For testing 19 bacteria strains and three associations of them were selected. Of these, 10 strains were isolated from biofilms developed on various inert surfaces (*E. coli* - three strains, *E. cloacae, Salmonella* - two strains, *P. aeruginosa*, *P. fluorescens*, *E. sakazakii*, *S. epidermidis*) from three processing units: a slaughterhouse, a meat processing plant and a milk processing plant. Biofilm was sampled from different surfaces most frequently find in food industry: steel, plastic, rubber, epoxy, mosaic and cement. Other nine strains (*E. coli* - three strains, *E. cloacae, S. liquefaciens* - two strains, *S. marcescens*, *P. aeruginosa*, *P. putida*), and bacterial associations (*E. coli - P. aeruginosa; S. liquefaciens - E. cloacae*; *S. liquefaciens - E. coli*) were selected from the biofilm developed on the surface of meat from different species (pork, beef, chicken).

Growth conditions. Strains isolated from biofilm (stored at -80° C), was incubated for 24 hours with 9 mL Brain Hearth Infusion (BHI - Oxoid), at 37° C. From these, a 24 h cultures where prepared on Brain Hearth Agar (Oxoid) and sub-cultured into 1% sterile peptone water to give an inoculum concentration of 0.5 McFarland standards in turbidity. This solution was then diluted 1:30 in BHI with different pH values: 5, 7.5 and 8.5. After vortexing, 150-µl volumes were transferred into eight PVC micro titer plate wells per strain, for each strain or associations strains test. Micro titre plates were incubated at 10˚ C, 22˚ C and 37˚ C for 24; 72 and 240 hours. After incubation period, the content of each well was removed and their washing was performed twice, with 160 ml of 0.9% sterile saline solution, to remove any planktonic cells. Then each well was stained with 160 µl of 0.1% crystal violet solution in water for 10 minutes, then dye was removed. After staining, plates were washed twice with 170 ml of sterile saline solution 0.9%. The quantitative analysis of biofilm production was performed by adding 170 µl of 96% ethanol, with a contact time of 30 minutes, to destain the wells. The level of optical density (OD) was measured at 540 nm with an ELISA reader (2).

Data were statistically analyzed by Wilcoxon test, comparison of pairs, using *SPSS* program.

Results and discussions

Effect of temperature. The greater biofilm formation has been observed to strains maintained in optimal conditions of pH (7.5), for 10 days, at low temperature

(10º C). As expected, *P. aeruginosa* (strain 20 isolated from the surface of pig carcasses) developed the most abundant biofilm (fig. 1). The amount of biofilm formed by this bacteria was 2.36 times higher in comparison with the biofilm produced by the same strain maintained 72 hours, in optimal conditions for development of *Pseudomonas* spp. temperature (22° C) and pH (7.5) and 1,56 times more abundant than the biofilm formed at 37° C, pH 7.5 after 72 hours. Moreover, the ability of *P. aeruginosa* to adhere to surfaces is associated with extensive production of exopolysaccharide (EPS) (9).

From research conducted by *Chen and Stewart* (2002) cit. by *Myszka and Czaczyk* (9) that exopolysaccharides are mainly responsible for maintaining the structural integrity of biofilms produced by *P. aeruginosa*. EPS can promote a preconditioning of surface, making then adhesion process more favorable.

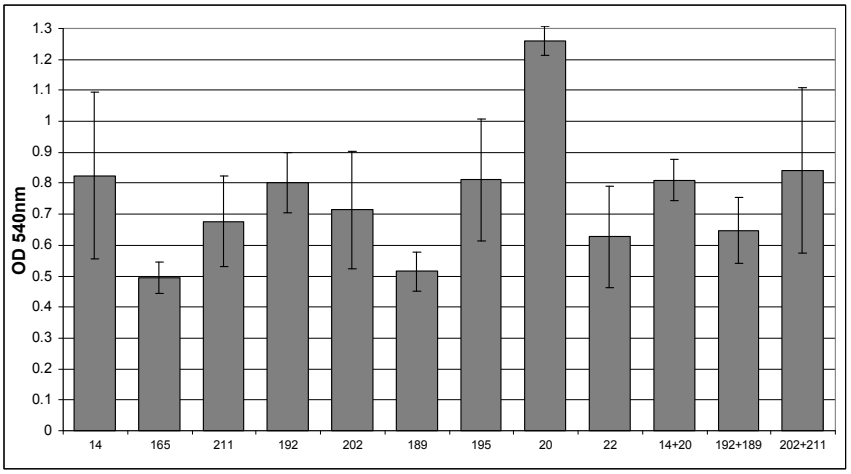

Fig. 1. Biofilm development by bacterial strains isolated from meat surface at pH 7.5, 10 ° C, for 240 h

14 *E. coli/* pig carcasses **165** *E. coli/* pig carcasses **211** *E. coli/* chicken carcasses**192** *E. cloacae/* cattle carcasses **202** *S. liquefaciens/* chicken carcasses **189***S. liquefaciens/* cattle carcasses **195***S. marcescens/* cattle carcasses **20** *P. aeruginosa/* cattle carcasses **22** *P. putida/* pig carcasses **14+20** *E. coli+P. aeruginosa/* pig carcasses **189+192** *S. liquefaciens+E. cloacae/* cattle carcasses **202+211** *S. liquefaciens+E. coli /* chicken carcasses

Biofilms produced by strains of *E. coli* isolated from meat surface, under the same conditions (pH 7.5, 10° C, 240 h) was, on average, 1.5 times higher than of that seen in case of incubation at 37° C, for three days (table 1).

It is surprising that, at a temperature of 22° C and pH 7.5 from all bacterial strains, isolated from meat and inert surfaces, and associations of them, about half of them (11strains) produced a lower amount of biofilm after 72 hours, representing

between 2.2 and 9.3 of the mass of biofilm formed at 37° C and pH 7.5. There were also exceptions. For example, *E. coli* isolated from the cutting table (plastic) produced a quantity of biofilm to 3.25 times higher than is that seen at 37° C, pH 7.5 after 72 hours.

Effect of pH. An increased production of biofilm was observed with increasing pH.

In alkaline medium (8.5) all bacterial strains and their association, isolated from the carcasses surface and 50% of the strains from inert surfaces had a higher biofilm production in comparison with those maintained to pH 5, both 24 and 72 hours. For example, *Pseudomonas* strain showed an increase of biofilm mass of 196.11-218.25% and *Escherichia*, between 145.89 and 212.01%, after three days. Similar results were obtained *Hoštack et al.* (5) studying biofilms produced by four isolates of *P. aeruginosa* isolated from hospital environment. This study has shown that biofilm mass produced at pH 8.5 was 139-244% compared with biofilm produced at pH 5.5 and at pH 7.5, 136-164% from baseline.

Similarly behavior was observed to bacteria at pH close to neutral (7.5). Two exceptions were noted: biofilm formed by *S. liquefaciens* on beef and association of this strain with *E. cloacae*.

Table 1

Optical density of bacterial biofilms formed by strains isolated from meat surface at pH 7.5 and different incubation temperatures

Strain	Growth condition			% differences between biofilms develop at 22°C and 37°C	% differences between biofilms develop at 37°C and 10°C	% differences between biofilms develop at 22°C and 10°C
	22° C 72 h	37° C 72 h	10° C 240 h			
E. coli[1]	0.545	0.496	0.824	91.00	166.12*	151.19*
E. coli[1]	0.429	0.344	0.496	80.18	144.18*	115.61
E. coli[2]	0.515	0.482	0.675	93.59	140.04*	131.06
E. cloacae[3]	0.647	0.574	0.800	88.71	139.37*	123.64*
S. liquefaciens[2]	0.759	0.528	0.713	69.56*	135.03*	93.93
S. liquefaciens[3]	0.856	0.334	0.514	39.01	153.89*	60.04*
S. marcescens[3]	0.716	0.760	0.812	106.14	106.84	113.40
P. aeruginosa[1]	0.532	0.807	1.259	151.69*	156.00*	236.65*
P. putida[1]	0.582	0.587	0.626	100.85	106.64	107.56
E. coli + P. ae.[1]	0.744	0.513	0.810	68.95*	157.89*	108.87
S. liq+E. cloacae[3]	0.946	0.488	0.648	51.585*	132.78*	68.49
S. liq.+E. coli[2]	0.752	0.699	0.842	92.95	120.45	111.96

Note: [1]pig carcass, [2]chicken carcass, [3]cattle carcass, optical density at 540 nm; *p ≤0.05

Harjai et al. (3) described biofilm production in *P. aeruginosa* higher at pH 8 than at pH 5–6, which they explained by higher alginate production. It seems that the alkaline environment contributes to the accumulation of carbon in the cell,

which stimulates increased synthesis of alginate (anionic polysaccharide with a high water binding capacity giving viscous consistence of biofilm). At high pH porins (channels of communication with the external environment) from the wall structure are more numerous compared to cells maintained in other media, which explains the presence of increasing amounts of alginate in biofilm structure formed by *Pseudomonas* under these conditions (4). Similar association between alkaline pH and biofilm production were also reported in other organisms, eg. *Stenotrophomonas maltophilia* (1).

Overall, the acidic environment (pH 5) is unfavorable for biofilm development by normal bacterial flora of meat. However, some bacterial strains were stimulated to produce biofilm in acidic conditions. For example, *Salmonella* strain isolated from surface of dehairing machinery, maintained for 72 hours at pH acid produced double amount of biofilm in comparison with the same strain exposed to the same conditions for 24 hours (table 2). Similar results were obtained for *P. fluorescens* strain isolated from the surface of plastic box (an increase in biofilm mass of 1.62 times within 48 hours). Some strains of *E. coli, Salmonella, P. aeruginosa* and their associations (*S. liquefaciens* and *E. coli*) maintained in mesophilic conditions (37° C, three days), at pH 7.5 lead to a higher biofilm production (2-10 times), compared to biofilm production at pH 5, in three days.

Table 2

Development of bacterial biofilms formed by strains isolated from inert surfaces at 37 ° C and pH 5

Strain tested/ source	**OD at 37° C, pH 5**		**% differences between biofilms develop at pH 5 in 72 h and 24 h**
	24 h	**72 h**	
P. fluorescens/box (plastic)	0.109	0.176	162.15*
E. coli / cutting table (plastic)	0.227	0.245	108.31
E. coli/ table (stainless steel)	0.135	0.156	115.64
E. cloacae/ carcass hook (stainless steel)	1.009	0.763	75.64*
E. coli/ floor (epoxy)	1.281	0.775	60.49*
Salmonella/ dehairing machinery (stainless steel)	0.170	0.336	198.16*
P. aeruginosa/ milk pump (rubber+stainless steel)	1.107	0.741	67.01*
S. epidermidis/ milk pipe (stainless steel)	1.184	0.880	74.36*
E. sakazakii/ floor (mozaic + ciment)	0.597	0.307	51.52*
Salmonella/milk pipe (stainless steel)	0.296	0.303	102.61

Note: OD = optical density at 540 nm; *p ≤0.05

Effect of temperature and pH. Mesophilic conditions (37° C) and pH 7.5 favored biofilm formation within 24 hours. Exposure of microbial strains at 37° C for 72 hours is not reflected proportionally in bacterial biomass film formed by most bacteria isolated from meat and inert surfaces. Only three strains isolated from meat surface (*E. coli* from pig and poultry meat and *P. aeruginosa* from pig carcass) produced a slightly higher amount of biofilm (representing between 1.2 and 1.4% from biofilm formed in 24 hours). 80% of strains isolated from inert surfaces (8 of 10 strains) produced a smaller amount of biofilm after three days of maintenance at 37° C, representing around 50% of 24 hours biofilm production.

Conclusions

Maintenance of bacteria isolated from meat surface (*Enterobacteriaceae*, *Pseudomonas* and association of them) in low temperature conditions (10° C), for 10 days, at pH 7.5 stimulates the biofilm production;

The acidic environment (pH 5) is unfavorable for biofilm production over time by normal bacteria of meat;

Some bacterial strains are stimulated to produce biofilm in acidic conditions (*Salmonella*) or alkaline (*E. coli, P. aeruginosa*), reaching to double the amount of biofilm, after three days of incubation at 37° C;

Mesophilic conditions (37° C) and optimum pH (7,5) favors biofilm formation within 24 hours;

Exposure of microbial strains at 37° C for 72 hours is not reflected proportionally in the biomass of bacterial film formed by most bacteria isolated from meat and inert surfaces. Conversely, for some strains of *E. coli, Salmonella, P. aeruginosa* and their associations (*S. liquefaciens* and *E. coli*) in mesophilic conditions, at pH 7.5, the biofilm production were two to 10 times higher, in comparison with maintaining at pH 5, for three days;

At pH 7.5, 50% of strains isolated and their associations produce a smaller amount of biofilm after three days of incubation at 22° C, compared with those exposed to 37° C.

Alkaline pH (8.5) stimulates the biofilm production by some strains of *E. coli*, *Salmonella* and *Pseudomonas*, producing two or three times more biofilm in a period of 72 hours, compared with the same strains grown in acidic medium.

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STUDY OF BACTERIAL BIOFILM STAGE FORMATION ON STAINLESS STEEL SURFACES WITH VARYING DEGREES OF ROUGHNESS

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Summary

Functional properties of biofilms are closely related with its spatial architecture, the antimicrobial resistance is a very relevant example of the matrix form importance and especially the of three-dimensional cellular organization. The purpose of this research was to highlight the structure of the biofilm produced by various species or groups of microorganisms by using current methods used to study microbial biofilm structure (fluorescence microscopy, confocal microscopy).

The materials tested were 304 and 316 polished and unpolished stainless steel. Strains used for testing were ATCC strains: *Pseudomonas aeruginosa* 27853, *E. coli*- 25922, *Listeria monocytogenes* 25923 and *Staphylococcus aureus* - 19114.

Biofilm growing was performed in culture media with addition of meat extract (4% o) or reconstituted milk powder (4% o). At various time intervals (6, 24, 48 and 78h) the plates were taked, washed with saline and stained with acridine orange (2 minutes) and then washed again with saline to remove excess dye . Examination of these plates was made with a microscope Leica DM 2500 model in fluorescence and biofilm of 72 h for examination was performed with laser scanning microscope. The most suitable stainless steel surface for biofilm formation was 304 unpolished stainless steel type 304, followed by polished stainless steel type 304.Speed and appearance of biofilm formation was influenced by surface roughness (surface of 304 stainless unpolished) the presence of nutrient medium (addition of reconstituted milk) and the tested strain (ATCC *Pseudomonas aeruginosa* biofilm formed most rapidly presenting the large surface area to 72 hours).Cell surface components (flagella or fimbria) and presence of compounds in the culture medium (eg lactic acid) favorably affect the initial attachment of microorganism to the substrate.

Key words: biofilm arhitecture, stainless steel, roughness

Microbial biofilm is a complex structure of microorganisms aggregated and integrated into a highly hydrated matrix composed of extracellular polymeric substances attached to the interface between the liquid-solid (8).

Functional properties of biofilms are closely related with its spatial architecture. In this context, antimicrobial resistance is a very relevant example of the matrix form importance and especially the of three-dimensional cellular organization. This organization (dimensional) determines the existence of

heterogeneous conditions within the biofilm due to different chemical gradient. Knowing the structure of biofilms is the first element in understanding the behavior and survival strategies of microorganisms within these structures, which allows you to choose the most effective control measures.

The purpose of this research was to highlight the structure of the biofilm produced by various species or groups of microorganisms by using current methods used to study microbial biofilm structure (fluorescence microscopy, confocal microscopy).

Materials and methods

The materials tested were 304 and 316 polished and unpolished stainless steel. Strains used for testing were ATCC strains: *Pseudomonas aeruginosa* 27853, 25922 *E. coli*, *Listeria monocytogenes* 25923 and *Staphylococcus aureus* 19114.

Isolation of these strains was done on the isolation medium specific for each group separately. Identification of strains was performed using API 20E galleries and device Vitek-2 (bioMérieux).

Stock cultures were stored at -80 ° C in brain-heart broth (Brain Heart Infusion-- BHI broth, Oxoid) and glycerol. Before each experiment to obtain fresh cultures, strains were seeded on the surface of BHI agar (Oxoid), poured into petri dishes and incubated at 37 ° C for 24 hours.

Biofilm growing was performed in culture media with addition of meat extract (4% o) or reconstituted milk powder (4% o). On the surface of stainless steel plates was spread a quantity of 300 ml reconstituted milk (4g praf/1000ml milk) or meat extract (4g carne/1000ml extract) previously plated with the bacterial strains mentioned above. At various time intervals (6, 24, 48 and 78h) the plates were taked, washed with saline and stained with acridine orange (2 minutes) and then washed again with saline to remove excess dye . Examination of these plates was made with a microscope Leica DM 2500 model in fluorescence and biofilm of 72 h for examination was performed with laser scanning microscope.

Results and discussions

Biofilm formed by *Pseudomonas aeruginosa* ATCC 27853 passes through several stages of development (from 6 to 72 hours), from initial attachment to the substrate followed by division to form microcolonies and biofilm maturation phase matrix corresponding exopolisacharides matrix formation. There is some irregularities in terms of biofilm formation, depending on the nature of the surface that was formed and the nutrients present in the growth medium. On the stainless type 304 unpolished *P. aeruginosa* formed biofilm in different phases, depending on its roughness and especially the richness of nutrients in the growing medium. Thus, after 6 hours of growth, both in the presence of meat extract and reconstituted milk, there were observed irreversibly attached of micro-

organisms to the surface. Cells are linked together and to substrate forming clusters. In this phase has already begun active communication between attached microorganisms, a phenomenon called *Quorum Sensing*. Emphasize the initiation phase of this process, irreversible substrate attachment, was reported by Sauer et al (8). There was a difference in the number of irreversibly attached microorganisms on surface, which appears to be higher in the experiment with reconstituted milk addition in the growth medium.

Type 304 satin stainless steel surface in the presence of meat extract, after 6 hours of incubation was observed that cell attachment is still reversible. Instead, in the experiment with reconstituted milk addition as a source of nutrients, cell surface attachment is irreversible. Attaching to the substrate (corresponding to the first phases of microbial biofilm formation) is a process favored mainly by the presence of cilia in this specie.

Numerous studies have highlighted the importance of the flagella in the initial attachment of *P. aeruginosa* to different types of surfaces, highlighting the importance of flagella motility in this process. It was also shown that *P. aeruginosa* lost this motility during other phases of biofilm formation (probably due to loss of flagellum) (8).

Further development of the biofilm (24, 48 and 72 hours), 304 on stainless steel surface unpolished was different, depending on the availability of nutrients in the environment. In the presence of meat extract, there was a progressive development of the biomass of cells embedded in exopolysaccharides (EPS**)** matrix from 6 hours to 72 hours gaining the penultimate stage of development (the second stage of ripening) at which cells attached get an appreciable thickness. At this stage of biofilm development genes responsible for intra-and interspecific communication seem to be activated. In the experiment with reconstituted milk after 72 hours of growth, could be seen passed away to a new biofilm phase development, the dispersion, where the cells are released from the biofilm, gaps were seen and the stainless steel surface remained uncovered.

On stainless steel Type 304 polished surface, both in the presence of meat extract and reconstituted milk, *P. aeruginosa* formed biofilm after 72 hours achieved only to the first stage of maturation, represented by clusters of cells embedded in matrix EPS. Even in this case there was a visible difference in the biofilm formed on the surface, between the two experiments. In the presence of meat extract biofilm formed looks like continuous layer of cells attached thinner compared to biofilm formed in the experiment where the reconstituted milk was added to the environment (in which its thickness is greater).

Speed attachment of *P. aeruginosa on* stainless steel surfaces type 316 was dependent primarily on the degree of roughness of the surface and secondly on the presence of nutrients in the environment. Thus, on unpolished stainless steel, in the experiment with beef extract and reconstituted milk after six hours of growth at 37°C, was found the presence of large numbers of cells closely linked together, cemented in a layer uniform, forming piles, at this stage the attachment is

irreversible. *P. aeruginosa* biofilm, in the experiment with meat extract, was formed in less time, so at 24 hours was found the presence of cells linked together through EPS matrix. After 48 hours the biofilm is present as thin, but continuous over the entire stainless steel and after 72 hours the thickness of this layer is greatly increased. In the presence of reconstituted milk *P. aeruginosa* form, starting from 24 hours of growth, clusters of cells embedded in EPS matrix and only after 72 hours of growth was found forming a thin, uniform, the cells attached to stainless steel surface.

On 316 unpolished stainless steel surfaces was observed (after 6 hours of growth under optimal conditions), the presence of cells reversibly attached to surfaces, in both experiments. On 24 and 48 hours cells adhere to each other in the continuous layer (in the presence of meat extract) and adhere to each other, but form and piles (in the presence of reconstituted milk) thus initiating the irreversible attachment. In both experiments, after 48 hours, the presence of un uniformly thin layer of cells attached to the surface was found. Clusters of cells formed at this stage will remain attached to the substrate until the last stage of biofilm development (72 hours)

Other researchers have found that *P. aeruginosa* form specific clusters of cell in the second phase of biofilm formation after 8 h of incubation under appropriate conditions. (8).

E. coli developed on stainless steel unpolish surface Type 304 rough form of clusters biofilm. After 6 hours the attachment was irreversible, observing a fairly uniform distribution of germs all over. In the presence of meat extract after 24 hours of growth, microcolonies attached together as a network gill were observed. The distance between microcolonies change over time, mesh network becoming smaller, biofilm development carried out both on the surface, but especially in height. In the medium with the addition of milk reconstituted E. coli biofilm formed rough form of clusters of different thickness after 72 hours of growth.

The *E. coli* biofilm formation on stainless steel polish surface Type 304 was being influenced by different elements present in the environment. In the experiment with meat extract, after 48 hours the biofilm looks like a thin layer covering the entire surface, giving a thickness change over time from 6 hours to 72 hours. In the medium with the addition of milk reconstituted *E. coli* formed biofilm rough form of clusters, resembling like a network.

Biofilm formed by *E. coli* on stainless steel type 316 unpolished surface was quite similar to those formed on stainless steel unpolished surfaces type 304, with some differences. Cells are irreversibly attached to the substrate, spreaded uniformly on the surface and shows a tendency to cluster after 6 hours of cultivation in the presence of meat extract and only after 24 hours of cultivation in the presence of reconstituted milk. On stainless steel surface Type 304 the tendency for development of biofilm with formation of clusters with network aspect, with different thicknesses and more dense in the presence of meat extract, was observed.

The same difference in the type of biofilm dependent on the nutrients in the environment was maintained for the *E. coli* biofilm formed on stainless steel polished 316 surface on the environment with meat extract added looks like thin biofilm covering the whole surface, giving a thickness change over time from 6 h to 72 hours. In the medium with the addition of milk reconstituted *E. coli* formed rough clusters biofilm, resembling like a network.

Many researchers have established that *E. coli's* ability to form biofilm will vary greatly dependent isolates and environmental growth conditions (presence or absence of nutrients) (2, 5).

Listeria monocytogenes strain ATCC 19114, formed on stainless steel surfaces biofilm as a braided uniform layer developed on the surface in environments with beef extract and clusters with network aspect in environments with the addition of reconstituted milk. In all experimental variants to 6 hours after cultivation, regardless of surface, or nutrients in the environment, bacteria irreversibly attach to these surfaces, the tendency of agglomeration, more evident in the growth media plus meat extract, were found

After 24 hours of growth, on unpolish stainless steel surface 304 (with the addition of meat extract) clusters of cells embedded in the EPS matrix were found. Differences in the biofilm form on of this type of steel surface were more evident after 48 hours of cultivation. Thus, in the presence of meat extract biofilm reaches a certain thickness, it becomes denser and looks like knitting matrix, and biofilm formation is faster. The biofilm formed in the presence of reconstituted milk has the network aspect and the development was evident only after 72 hours.

On 304 polished stainless steel surface is found that the cells attached to the surface after 24 hours begin to clutter and piles embedded in the matrix, especially in the presence of meat extract, were observed. This type of structure was observed in the experiment the reconstituted milk was added only after 72 hours of growth.

Listeria monocytogenes on 316 stainless steel surface formed later biofilm, knitting specific form throughout the experiment with addition of meat extract, but only after 72 hours of growth was observed. On 316 polished stainless steel clusters cells embedded in the matrix were formed after 24 hours of growth in the presence of meat extract, which demonstrates that surface roughness influences the speed of formation of biofilm. Using confocal microscopy allowed us later to state that the structures observed was biofilm structure and not only not only a simple cells adherence to the substrate compared to others researchers observations (3). The differences observed by different researchers in terms of *L. monocytogenes* substrate attachment ability could be to diversity of strains used in experiments (1,4,6).

Staphylococcus aureus ATCC 25923 formed on the surface of unpolished stainless 304, after 6 hours of growth formed cell clusters in the presence of meat extract and reconstituted milk. After 24 hours due to the formation EPS matrix areas appear irregular surface coating, in which microorganisms are arranged in clusters alternating with areas of confluent growth. Biofilm formed in medium with

reconstituted milk added was much thicker, and after 24 hours was almost confluent layer observed . Biofilm formed on stainless steel surface 304 with meat extract is much thinner and only after 72 hours of development covering the whole surface.

On 304 polish stainless steel 304 biofilm formation was very different from growth on the surface of stainless unpolished. Thus, in the first 6 hours in the presence of reconstituted milk and after 24 hours in the presence of meat extract clusters cells were observed only along the existing striations on the surface followed by irreversible attachment (at 48 and 24 hours). The biofilm development was as uniform layer.

Staphylococcus aureus strain attached in the presence of meat extract was delayed on stainless steel unpolished surface 316. After six hours of cultivation only rare cells that shows a tendency to cluster and after 24 hours the initiation of adesion to the surface were observed. The formed biofilm after 72 hours is much denser and cover more area. Supplement of medium with reconstituted milk seems to influence favorably the initial cell attachment and biofilm formation. After 6 hours area is almost entirely covered with cells attached to each other and after 24 hours the cells are embedded in a EPS matrix, after 72 hours is observed with mature biofilm surface coating uneven areas alternating with areas of confluent growth. On polish stainless steel 316surface was not observed significant growth differences from growth on polished stainless 304. The biofilm development was made after 24-48 hours and attachment was carried along on its streaks surface. Certain components such as proteins associated surface attachment and biofilm formation have a great impact on biofilm formation than other adezin *S. aureus*. Thus, stafilococcii presenting this antigen on their surface produce significantly higher biofilm than not having that antigen (2).

Conclusions

The biofilm formed by ATCC strains used in the experiment was different depending of the type.

The most suitable stainless steel surface for biofilm formation was 304 unpolished stainless steel type 304, followed by polished stainless steel type 304.

Speed and appearance of biofilm formation was influenced by surface roughness (surface of 304 stainless unpolished) the presence of nutrient medium (addition of reconstituted milk) and the tested strain (ATCC *Pseudomonas aeruginosa* biofilm formed most rapidly presenting the large surface area to 72 hours).

Cell surface components (flagella or fimbria) and presence of compounds in the culture medium (eg lactic acid) favorably affect the initial attachment of microorganism to the substrate

Using confocal microscopy and image analysis programs offer considerable potential obtained detailed quantitative and qualitative analysis of biofilm especially its architecture.

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ELECTROCHEMICAL BYPASS OF BACTERIAL BIOFILMS' DEFENCES

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Summary

The present experiment was aimed to assess the *S. aureus* biofilm removal efficiency from stainless steel by electrochemical gas generation onto the metal surface. There was used a sodium citrate electrolyte solution and the metal coupons were placed at the either the anode or cathode. Following acridine orange staining, fluorescence images were acquired and the biofilm attached to the surface was estimated by dedicated software. Best results were obtained when hydrogen was generated, with 99.99 removal of biomass and 98.92 removal of attached bacteria. The proposed method is very cheap, readily available and has no corrosive effects on the metal.

Key words: biofilm, electrolysis, removal efficiency.

Biofilms are surface-associated bacterial communities that predominate in natural and pathogenic ecosystems. The matrix-enclosed bacterial cells in these communities assume a phenotype that differs profoundly from that of their planktonic counterparts (1).

The EPS matrix covering the bacterial cells acts as a shield, protecting the bacteria from harmful environmental factors or cleaning methods, making hygiene removal measures extremely difficult. The cleaning methods presently used are costly, involving the use of detergents and enzymes, mechanic or physical (ultrasound) which in time destroy the stainless steel surface.

The purpose of this research was to evaluate the possibility of detaching the bacteria from the stainless steel support by bypassing the EPS matrix. For this purpose we used an electrochemical method for generating gaseous hydrogen or oxygen right on the stainless steel surface, below the bacteria and the matrix, aiming to physically detach the biofilm.

Materials and methods

For this experiment a standardized *Staphylococcus aureus* strain was used (ATCC 25923). Bacteria were cultured in Petri dishes using 20 ml of tryptic soy broth for 48 hours at 37°C. In the culture media there were inserted seven steam sterilized 3x1 cm stainless steel (AISI 316) coupons. Biofilm formation was

evaluated by confocal laser scanning microscopy (CLSM) (*Leica* DM 2500) using an acridine orange (AO) stain.

For generating the gaseous discharge on the stainless steel coupons, a PVC electrolysis chamber with platinum electrodes was designed and constructed. Individual stainless steel coupons were connected to either the anode (for oxygen release) or cathode (for hydrogen release) and immersed in a sodium citrate (1.76 %) electrolyte solution. The current intensity was set arbitrary to 200 mA and 7.2 V. After electrolysis the coupons were stained with AO for 2 minutes and examined by fluorescence microscopy (*Leica* DM 2500). The images taken were processed in *Leica* application suite for baseline normalization and for removing auto fluorescence and analyzed by specialized software: *Comstat*, *BioImageL* and *CellC* (2, 3).

Results and discussions

Biofilm formation visualized by CLSM followed by image processing revealed the biofilm parameters: biovolume – 1.32 $\mu m^3/\mu m^2$, maximum thickness 3.77 µm, average thickness 1.84 µm, and surface covered 56%. These parameters suggest a young, developing biofilm, which rapidly colonizes the stainless steel surface.

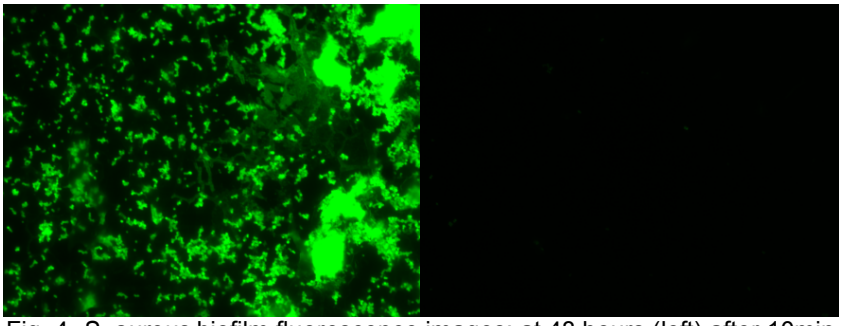

Fig. 4. *S. aureus* biofilm fluorescence images: at 48 hours (left) after 10min electrolysis at the cathode (right) (AO-staining 63X)

Biofilm total biomass, bacterial counts and removal efficiency (table 1) were different for the tested experimental conditions. Results showed the best removal efficiency was attained when the coupons were connected at the cathode.

Table 1

Biofilm removal efficiency

			Oxygen		Hydrogen	
		Control	5 min	10 min	5 min	10 min
Bacteria	number	895.82± 46.12	818.64± 46.12	736.00± 86.59	34.82± 76.79	9.64± 7.64
	% reduction		8.61	17.84	96.11	98.92
Biomass	μm^2	206344.77	18618.84	45922.57	332.18	12.32
	% reduction		90.98	92.28	99.84	99.99

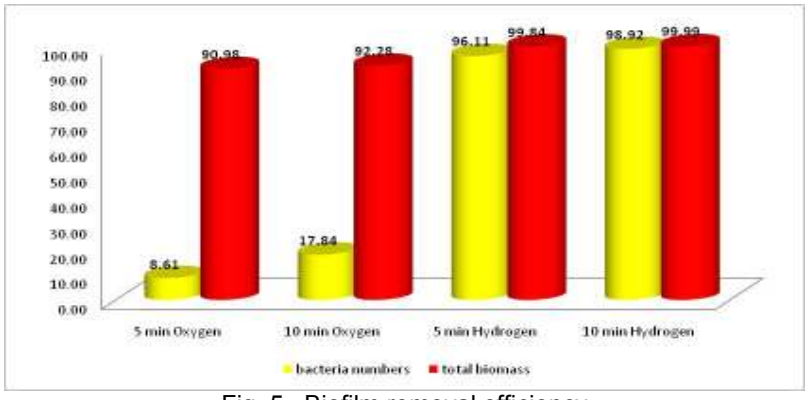

Fig. 5. Biofilm removal efficiency

The differences observed between the experimental variants may be explained by a lower volume of oxygen release compared to hydrogen for the same current intensity. Also, the release of oxygen beneath the bacteria and the EPS may alter, due to its chemical reactivity, the chemical structure of the biofilm components, perhaps also coagulating proteins adhered to the stainless steel surface and keeping them in place. The lower removal of bacteria compared to biomass, as well as the presence of scattered bacteria remained attached to the surface may be explained by the formation of preferential ionic channels during electrolysis around the bacteria which act as insulators on the conductive surface of the metal.

As expected, the longer exposure time improves the efficiency of the method. The presented method's efficiency is well above mechanical cleaning, of enzymatic detergents and ultrasound. Hydrogen generation on the stainless steel surface has absolutely no corrosive effects. Method costs are all extremely low, making it a viable solution for biofilm removal from stainless steel surfaces.

Conclusions

Electrochemical hydrogen generation in a sodium citrate electrolyte removes 99.99% of total biomass and 98.92 % of bacteria from a 48 hours *S. aureus* biofilm.

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THE PREVALENCE OF *YERSINIA* SPECIES IN SLAUGHTERED PIGS

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Summary

This study follows the isolation frequency of *Yersinia* germs from different organs and carcasses samples collected from slaughtered pigs. The study presents a high importance because it reveals the probability of contamination with bacterial species of *Yersinia* genus, which are pathogen for human beings (especially for infants) through alimentary toxic infections, manifested by austere diarrhea syndrome.

To determine the isolation prevalence of *Yersinia* bacteria, a total number of 3.427 organs and muscle samples were harvested and processed through microbiological assays; the study was developed during a period of 2 years (2010-2011).

The statistical analysis of the data obtained showed a total prevalence of 0.321 with a yearly fluctuation of frequency isolation situated between short limits (from 0.25 % to 0.54%).

Key words: *Yersinia*, prevalence, pig carcasses

The epidemiological studies performed by groups of scientists from all over the world concluded that the bacterial species comprised in *Yersinia* genus are pathogen only for humans and primates. Many epidemical episodes registered in countries with a developed sanitary system and elevated hygiene level, captivated the attention by an exalted number of clinical cases and the severe morphological and clinical signs. However, the actual real rate of morbidity by toxic infection with *Yersinia* is higher than the official one, the increasing number of episodes all over the world generating a serious reason of concern for public health.

The origin of food borne diseases with *Yersinia* are a series of vegetal food products (salads, fruits, green vegetables) or mixed products (salad with meat or cheese), the animal products being rarely incriminated (especially milk and milk products are considered risk products). Habitually the animal products are contaminated while non-hygienically processing stages or through their manipulation by infected persons or chronic wearers. A series of studies demonstrated even the possibility of *Yersinia* germs' multiplication on contaminated food products with a minimal germs' content, the multiplication taking place even at 10^{o}C (13, 14).

Taking into account the deficient hygiene conditions assured while evisceration and transport of game (slaughtered pigs), we considered propitious

the microbiological investigations in order to statistically determine the isolation frequency of *Yersinia* germs, a motivation being the habit of this species to frequent forests with still water sources (swamps, marshlands) which are constantly contaminated with faecal germs.

Materials and methods

In order to establish the isolation prevalence of *Yersinia* germs, there have been collected and bacteriological analyzed samples of muscle from different anatomical parts of slaughtered pigs' carcasses, sanitary swabs loaded on peritoneal surfaces and parietal pleura and also different portions of organs (liver, spleen, kidney, mesenteric lymphatic centers, intestinal portions).

The collection of the samples was performed mainly from recently hunted slaughtered pigs (maximum 60 minutes after the blast), obtained by authorized hunting in small groups or collective hunting.

The area comprised in this survey is the hunting areas from the southern and south-eastern part of the country, including the region of the Danube Delta. The sample collection was done often by the authors themselves, yet, taking into consideration the restrictions of slaughtered pigs hunting, some of the samples have been collected by the hunters, especially prepared to perform this operation.

The sample collection was executed between 2010 and 2011 and gathered a total number of 3427 samples. Each sample was transmitted to laboratory and processed followed also the work stages comprised in the ISO techniques (SR ISO 10.273, SR 12925, STAS 2356-82), and also the work techniques used actually by different microbiology laboratories in order to establish a diagnosis of this bacterial species. The work method used in this case comprised several stages:

- the sampling from animal is realized in strict aseptic conditions. From each sample a quantity of 10 g of muscle tissue is taken, for meat samples or, depending on the situation, which are introduces in the tow enrichment media, obtaining a percentage of 1/10 or 1/100 (percentage mass/volume or volume/volume).

- the enrichment is realized in 2 different selective liquid media: 10 g (ml) of sample are introduced in 90 ml of PSB medium (peptone-sorbitol-biliar salts broth), obtaining a percentage of 1/10. The incubation is realized at 25^{o}C for 5 days. Another 0.1 g (ml) of sample are introduced in 9 ml of ITC medium (irgasan-ticarcylin-potassium chlorate broth), obtaining a percentage of 1/100. The incubation is realized 25^{o}C for 2 days.

- for isolation and identification, a culture loop from the enrichment broth PSB is passed directly on the surface of CIN agar and 0,5 ml of culture from the same medium is transferred in 4.5 ml of KOH saline solution 0.25 % and is homogenized for 10-20 seconds. After the homogenization a culture loop is passed on the CIN agar surface.

In parallel, a loop from the culture obtained in the enrichment broth ITC is passed on the surface of SSDC gelose (Salmonella-Shigella, with sodium desoxycholate and calcium chlorine). The incubation of the dishes is realized at 30°C, in aerobiosis, for 24 h. After 24 hours, the dishes are examined with a magnifying glass or using an oblique light to recognize the characteristic colonies of *Yersinia enterocolitica*. On the surface of CIN gelose, the characteristic colonies are small (1 mm), flat, with a red spot in the center, surrounded by a pink area, semitransparent (having the aspect of an "ox's eye"), and bile precipitate, and, after examining on oblique light, they are tiny granulated and not iridescent. On Wauters (SSDC) gelose, the characteristic colonies are small (1 mm) and grey, with a thin margin, and by examining on oblique light, they are tiny granulated and not iridescent.

For confirmation, a number of 5 characteristic and/or suspect colonies are selected from the selective culture media dishes, which were reintroduced on the surface of some dishes with nutritive gelose in order to obtain pure cultures to perform the confirmation through biochemical assays. The dishes with sowed nutritive agar are incubated at 30°C for 24 h.

The identification-confirmation stage was realized by using API 20E galleries: the API 20E system offers the possibility of identification of *Yersinia* germs in 24 h, as well as other enteric bacteria. The gallery presents 20 microtubes which contain dehydrated substrates for ascertaining of enzymes, carbohydrates' assimilation or inhibition assays. The gallery comprises 2 parts. The first one contain the enzymatic tests and the conventional ones. The dehydrated substrates were inoculated with a suspension of germs which helps rehydrating these ones. The manifested reactions during the incubation period (at 37°C in aerobiosis) were spontaneous color modifications or the ascertaining of the color after adding some specific reagents. The second part of the gallery comprises the assimilation or inhibition tests. After incubation at 37°C for 24 h, the reactions were read visually, using the table of reading and identification or the identification catalogue API 20E.

The gallery can offer and realize: enzymatic reaction which ascertains the presence of beta-galactosidase (ONPG); 5 classical chemical reactions: urease (URE); the reduction of nitrates in nitrites (NO_2); the production of H_2S (sulphuric hydrogen); the production of indole (IND), the production of acetoin (VP); 10 assimilation reactions: the assimilation of glucose (GLU); the assimilation of mannitol (MAN); the assimilation of inositole (INO); the assimilation of sorbitol (SOR); the assimilation of rhamnose (RHA); the assimilation of saccharose (SAC); the assimilation of melibiose (MEL); the assimilation of amygdaline (AMY); the assimilation of arabinose (ARA); the assimilation of citrate; an enzymatic reaction which ascertains the gelitinase (GEL); an enzymatic reaction which ascertains the dehydrolase: arginin-dehydrolase (ADH); 2 enzymatic reaction which ascertain the decarboxylase: lysine-decarboxylase (LDC); ornithin-decarboxylase (ODC); an enzymatic reaction for ascertaining cytochrome-oxidase (OX); an enzymatic reaction for ascertaining tryptophane-desaminase (TDA). The API 20E Gallery, BIO

MERIEUX S.A. comprise: 25 API 20E galleries; 25 incubation dishes; 25 result sheets; 1 technical sheet; TDA reagent; IND reagent; VP1 and VP2 reagents; NIT1 and NIT2 reagents; OX reagent; paraffin oil; pipettes. Initially a Gram coloration is performed on every analyzed stems and, afterwards, the reactions for betagalactosidase, urease and H_2S, in order to examine the inherence of the germ to *Yersinia enterocolitica* species. A dish with CIN agar is prepared; the dishes are incubated at 30^{o}C for 24-48 hours; it is verified the presence on the slide of Gram-negative cocobacilli, bipolar dyed; a culture from the selective isolation media dish is collected using a sterile swab (carefully, in order to avoid the penetrating of the medium) and a suspension in 3 ml of NaOH 0,85% is realized, bringing the suspension at a turbidity equal to 6 on McFarland scale. The dehydrated substrates of the gallery are inoculated with the bacterial suspension, and the incubation is realized at 37^{o}C, in aerobiosis. At this moment, the interpretation of the results is done, using the reading table of the gallery.

Results and discussions

The statistical analysis of the performed research on a total number of 3427 samples (1893 in 2010 and 1534 in 2011) permitted the conclusion of an annual incidence at relatively low values, with variations from a year to another. The number of positive samples and the isolation frequency of *Yersinia* germs are synthetically presented in table 1.

Table 1

Identification of the number of positive samples and the isolation frequency of bacteria belonging to *Yersinia* genus, from analyzed samples

The type of samples	The number of positive samples and the isolation frequency (%) / period					
	2010		2011		Entire period	
	no	%	no	%	no	%
Samples of muscle from carcass	1	0.05	0	0	**1**	**0.03**
Samples of liver portions	0	0	0	0	**0**	**0**
Samples of spleen portions	0	0	0	0	**0**	**0**
Samples of intestinal portions	4	0.25	2	0.14	**6**	**0.19**
Samples of mesenteric lymphatic centers	2	0.11	2	0.14	**4**	**0.12**
Samples of kidney	0	0	0	0	**0**	**0**
Sanitary swabs	2	0.11	1	0.07	**3**	**0.09**
Total samples	**9**	**0.54**	**5**	**0.25**	**14**	**0.321**

The results obtained demonstrated a low incidence at portage of bacteria belonging to *Yersinia* genus (maximum 0.22 %), but these low values can be determined by the inhibition of *Yersinia* bacteria by preferential development of other bacteria which populate the intestine. The highest isolation frequency was observed at the level of serous surfaces (pleura and peritoneum), this specific observation demonstrating the carcass post-mortem contamination either because of slaughtered pigs evisceration in improper conditions (evisceration in improper conditions and minimal techniques) or because of the carcass faecal contamination.

There haven't been observed isolations from parenchimal organs, and from the muscle samples there has been made a single isolation (which we can consider accidental), the *Yersinia* germs which accede to the organism through contamination by oral way, arrive at the mesenteric lymphatic center as maximum level.

Although the total prevalence, in the survey period, demonstrates low values (0.398), it can be concluded the possibility of these bacteria to contaminate the slaughtered pigs' carcasses, these constituting contamination sources for consumers. Following this, a series of alimentary products obtained through thermal processing by using methods which involve low values of temperature applied on short periods of time, or alimentary products consumed without being processed (raw), can be involved in alimentary toxic infections episodes.

Conclusions

After collection and processing through techniques and bacteriological assays a total number of 3427 samples of muscle, different organ parts and sanitary swabs pleura and peritoneum, there have been isolated a number of 14 bacterial stems which were identified as belonging to the species included in *Yersinia* genus.

The statistical analysis of the obtained results on a 2 years period permitted the establishing of a total prevalence of 0.321. The annual variations of the isolation frequency were situated between relatively low values (between 0.25% and 0.54%).

From the total number of analyzed sample types, the highest value of isolation frequency was observed for the sanitary swabs of pleura and peritoneum (0.09% for the total period), the other isolations being considered accidental.

The bacterial species form *Yersinia* genus can contaminate the slaughtered pigs carcasses, the products and by-products obtained after processing, these carcasses being considered the origin of food toxic infections.

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THE *YERSINIA ENTEROCOLITICA* ISOLATION AND IDENTIFICATION METHOD FROM FOOD PRODUCTS

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Summary

The research was performed to optimize the methodology diagnosis of *Yersinia enterocolitica* from different categories of samples. Following this objective, in the first stage the general technique of collecting and processing the samples was adapted and optimized, raising the diagnosis rapidity. In a second stage of research there have been artificially contaminated different types of samples with *Yersinia enterocolitica* stems, checking the efficiency and accuracy of optimized method.

The last stage of research was directed to constituting a methodological scheme to assure rapidity and identification diagnosis for *Yersinia enterocolitica* species.

Key words: *Yersinia enterocolitica*, food products, isolation, identification

The actuality and the interest exercised by the *Yersinias* are determined by the fact that these bacterial species generate a series of morbid entities which are under the attention of many bacteriologists, epidemiologists and doctors. On the theme of the infections produces by these bacteria in the latest years have been published numerous studies, and the research in this domain acquired a special amplitude. In the last decade of the past millennium, numerous researches pulled the attention that the bacterial species in *Yersinia* genus are frequently involved, in case of human beings, in production of alimentary toxic infections manifested by diarrheas acute syndrome.

From a clinical point of view these yersinioses do not present special complications and are efficiently treated, the epidemiological risk is important, being observed episodes that comprised hundreds of cases (3, 8, 11, and 12). Initially, *Yersinia pestis* species was considered to have the most important epidemiological risk. Afterwards, many groups of research observed that they are involved in food toxic infections in humans and other species of animals. In present days, although there have been discovered and comprised taxonomically many species of *Yersinia*, are considered principal pathogenic for humans 3 species: *Yersinia pestis, Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. Taking into account that the contamination sources, for human beings, are represented generally by food products which are not thermally processed or subjected for short periods of time to thermic processing and at values of temperature of maximum 70^{o}C, we considered adequate the performing of several investigation which could

reveal the optimal values of temperatures for conservation and growing of *Yersinia enterocolitica stems*, the period of surviving of this bacterial species at low values of temperatures (in refrigerated or deep-frozen food products) or at raised values of temperature (in food products subjected to a thermal treatment).

Materials and methods

The adapted method that we used to collect and primary processing the samples include a series of differential elements concerning the biological type of products which is subjected to complex bacteriological investigations in order to diagnose the presence of bacterial species from *Yersinia* genus.

In case of the samples constituted of intestinal mass, integrally collected, there must be performed the aseptically opening of the caecum of swine and fowl like this: it is collected caecal content using the culture loop and it is seeded directly on the surface of CIN medium and SSDC medium (Wauters). The incubation is realized in a thermostat at the temperature of 30^{o}C for 24-48 hours. The same samples are diluted in 10 ml of physiological serum (the cecum content left on the culture loop is introduced in test tubes with diluent's), and after the dilution and homogenizing 1 ml in this suspension it is introduced in 9 ml KOH solution 0,25 % in 0.85 % NaCl and it is agitated for 10 seconds. The content of a loop (0,1) ml is passed over the surface of the selective isolation media (CIN, SSDC). The seeded dishes are incubated for 24-48 hours at 30^{o}C in aerobic conditions.

In case of organs collected integrally (liver, kidney, gizzards and fowl hearts) and samples of muscle tissue: 10 g of product are weighed aseptically, introduced in sterile bags; over the sample are introduced 10 ml of diluent (physiological peptonate serum); it is realized the grinding and the homogenization in a stomacher model 400 for 2-3 minutes; from the homogenized are taken 10 ml and introduced in a vial with 90 ml of enrichment broth PSB (percentage of 1/10). The incubation of the seeded enrichment broths is realized at 25^{o}C, for 5 days, and after the addition of the selective agents (irgasan and ticarcylin) at the used medium, the incubation is continued at 12^{o}C for another 24 hours. For the selective isolation media directly seeded or after decontamination with KOH, the incubation is realized in aerobiosis, at 30^{o}C, for 24-48 hours. At the end of specific incubation periods of each enrichment medium used a loop full of culture obtained is passed over the CIN and SSDC gelose, and the dishes are incubated in the same temperature conditions.

In case of samples collected through stippling of the surface and interior of the carcasses: the swabs are introduced aseptically in test tubes with 5 ml PSB medium, for homogenizing and movement of the germs by hitting with energy the test tube against the palm for 30 times, until the swab is deteriorated; the content of a loop from the homogenized preparates is passed over the dishes with selective media (CIN, SSDC), incubating 24-48 hours at 30^{o}C. Another technique used by the authors is the enrichment of the same swabs for 5 days for the suspensioned

ones in PSB medium, respectively for 24 hours, adding the selective supplements, followed by a reincubation for 24 hours at 12°C, in case of using ITC medium. After enrichment, a loop with culture is passed over the surface of selective isolation media. The seeded dishes are incubated at 30°C for 24-48 hours. After 48 hours incubation the dishes are examined in order to establish the presence of characteristic colonies. The Gram dyeing method is realized from the characteristic colonies (using 1 ml of physiological serum for dilution); from the dilutions retained it is sectorially seeded the ITC medium. After incubation in the conditions described earlier, the pure cultures serving for performing of some biochemical tests, as it follows: after 24-48 hours from the incubation it is done the test for marking out the urease and ornithin-decarboxylase, the absence test for lysin-decarboxylase and oxidase, the absence test of citrate utilization and for producing H_2S, the test of esculin hydrolyze, the test for using some saccharides (glucose) and the absence of using others (lactose) on Kligler medium and the test of using saccharose, salicin and rhamnose.

For milk samples: after collecting the product in strict aseptically conditions, the pH is adjusted at 7,4 units; the samples are introduced in a centrifuge, at 2500 rpm, for 15 minutes (centrifugation is realized at refrigeration temperatures, in centrifuge tubes of 25 ml capacity, in which are introduced 10 ml of sample); the supernatant is separated from the deposit and this last one is used for seeding directly on the selective isolation media, but especially in case of thermal or chemical treated milk, besides the direct seeding on selective media is realized also the enrichment in percentage of 1/10, afterwards the other stages to follow in the way described earlier.

Results and discussions

In case of using the method ISO 10273/1994, the collecting of the samples from animal food products is realized in strictly aseptic conditions. Form each sample to examine are taken 10 g of muscle tissue for the samples of meat, or, in case of any other situation, 10 ml of milk or milk product which are introduced in the two enrichment media, obtaining this way a percentage of 1/10 or 1/100 (percentage mass/volume or volume/volume).

The enrichment is realized on two selective liquid media:

- 10 g (ml) of sample are introduced in 90 ml of PSB medium (peptone-sorbitol-biliar salts broth), obtaining a percentage of 1/10. The incubation is performed at 25°C for 5 days.

- 0,1 g (ml) of sample are introduced in 90 ml of ITC medium (irgasan-ticarcylin-potassium chlorate broth), obtaining a percentage of 1/100. The incubation is performed at 25°C for 2 days.

In order to isolate and identify, a culture loop from the enrichment broth PSB is seeded directly on the surface of CIN agar and 0,5 ml of culture from the same medium is transferred in 4,5 ml KOH, saline solution 0,25 % and it is homogenized

for 10-20 seconds. After homogenization a culture loop is passed over the surface of CIN agar. In parallel a loop with culture obtained in the enrichment broth ITC is passed over an SSDC gelose (Salmonella-Shigella, with sodium desoxycholate and calcium chloride). The dishes incubation is realized at 30^{o}C, in aerobiosisi, for 24 hours. After 24 hours the dishes are examined with a magnifying glass or in oblique light for examining the presence of characteristic colonies of *Yersinia enterocolitica*. On the surface of CIN gelose, the characteristic colonies are small (1 mm), flat, with a red center, surrounded by a pink margin, semitransparent (characteristic aspect of an "ox eye") and bile precipitate, and, at examination under oblique light, are tiny granulated and noniridescent. On the surface of Wauters gelose (SSDC), the characteristic colonies are small (1 mm), grey, with a thin margin, and by examining in oblique light, are tiny granulated and noniridescent.

For confirmation, it is selected a number of 5 characteristic colonies and/or suspect ones from the dishes with selective culture media, which were reintroduced on the surface of some dishes containing nutritive gelose in order to obtain a pure culture for confirmation through biochemical tests. The dishes with seeded nutritive agar are incubated at 30^{o}C for 24 hours.

In case of using the Identification System API 20E, it is permitted the identification in 24 hours of germs from *Yersinia g*enus, and also of other enteric bacteria, but it is necessary the initial obtaining of a purified subculture, the methodology comprising a period of minimum 48 hours. The gallery presents 20 microtubes which contain dehydrated substrates for marking out enzymes, assimilation of carbohydrates or inhibitions tests. The gallery comprises 2 parts: the first one contains the enzymatic tests and the conventional ones and the second part contains the assimilation and inhibitions tests.

The dehydrated substrates were inoculated with a germ suspension which helped rehydrating these ones. The reactions showed during incubation period (at 37^{o}C, in aerobiosis) manifested spontaneous modifications of color, or marking out color by adding some reagents. After 24 hours incubation at 37^{o}C, the reading of reactions was performed visually, using the reading and identifying table or the identification catalogue API 20E. The gallery can offer and realize: an enzymatic reaction that marks out the beta-galactosidase (ONPG); 5 classical chemical reactions: urease (URE); the reduction of nitrates in nitrites (NO_2); the production of H_2S; the indole production (IND); the acetoin production (VP); 10 assimilation reactions: glucose assimilation (GLU); mannitol assimilation (MAN); inositol assimilation (INO); sorbitol assimilation (SOR); rhamnose assimilation (RHA); saccharose assimilation (SAC); melibiose assimilation (MEL); amygdaline assimilation (AMY); arabinose assimilation (ARA); citrate assimilation (CIT); an enzymatic reaction which marks out the gelitinase (GEL); an enzymatic reaction which marks out dehydrolase: arginin-dehydrolase (ADH); 2 enzymatic reactions which mark out decarboxylase: lysin-decarboxylase (LDC); ornitin-decarboxylase (ODC); an enzymatic reaction which marks out cytoxhrome-oxidase (OX); an enzymatic reaction for marking out tryptophane-desaminase (TDA).

It is initially realized a Gram dyeing technique on all the analyzed stems, and afterwards, reactions for betagalactosidase, urease, H_2S, for verifying the aparthenence of the germ to *Yersinia enterocolitica* species. A culture is prepared on a dish with agar CIN; the dishes are incubated at 30^{o}C for 24-48 hours; it is verified the presence on the slideof Gram-negative cocobacilli, bipolar dyed; it is collected culture from the dish with an isolated selective medium, using a sterile swab (carefully, in order to avoid the penetrating of the medium) and it is realized a suspension in 3 ml of medium with NaOH 0.85 %, bringing the suspension at a turbidity equal to 6 on McFarland scale. The dehydrated substrates of the gallery are inoculated with the bacterial suspension, and the incubation is realized at 37^{o}C in aerobiosis. Afterwards, the results are interpreted, using the reading table of the gallery.

The differences between the 2 methods presented initially and our optimized method can be remarked from the way of collecting and processing of samples.

In comparison with ISO 273/1994 method, in the optimized method is observed rapidity in obtaining a presumptive diagnosis, in 48 hours from the collection of the sample being determined the aparthenence to *Yersinia* genus. In case of using the identification galleries API 20E is necessary the obtaining in the first phase of several primary pure subcultures, this needing a minimum period of 48 hours (frequently 72-96 hours), in which it is realized the primary processing of the sample, the incubation in a liquid enrichment medium, the passing over on a solid medium surface, the forming of subcultures from isolated colonies, considered presumptively as pertaining to *Yersinia* genus. In case of the optimized method, the species confirmation is realized by using a battery of biochemical tests which include a series of stages and in which it is revealed also the pathogenic traits of the isolated stem.

For the presumptive confirmation tests are chosen a number of 5 colonies obtained on the surface of nutritive gelose and are tested for: morphology (a fragment was taken from each isolated colony on every dish and a Gram dyeing was performed; from each separate colony was realized a suspension in 1 ml of nutritive broth; for later observation, all the suspensions in which the Gram negative germs were observed, bacilli or cocobacilli, with round heads bipolar dyed); the indole production (a part of the isolated colonies are seeded by using a loop in a test tube containing triptone broth; the test tubes are incubated at 30oC for 24 hours, afterwards several drops of Erlich-Kovacs are added; a red color signifies the presence of a positive reaction); the urease production (by using the seeding loop or needle, a fraction from the isolated colony is taken and seeded through passing over on the slope of a Christensen agar; the incubation is realized at 30oC for 24 hours; a pink-red color of the medium after incubation for 24 hours at 30oC signifies the presence of a positive reaction); oxidase elaboration (by using a platinum loop or a glass wand a fraction of the isolated colony is taken and deposited or passed over a filter paper imbibed in oxidase reagent. the apparition of a violet-purple color or intense blue one in 10 seconds is interpreted as a positive reaction); the utilization of saccharides on Kligler medium (Kligler gelose is seeded with each of the colonies selected until now,

by puncturing the column and striking the slope). The incubation is realized at 30oC for 24 hours and it is prolonged until 48 hours in aerophilic atmosphere; the interpretation of the reaction is performed concerning the color of the column and slope of the incubated medium, and also by the presence or absence of gases on Kligler agar the column is yellow (fermented glucose), without H2S or gases from glucose, and the slope remains unchanged (lactose negative).

For biochemical confirmation tests, there were selected only the colonies which behaved as it follows: urease positive, indole production negative, acid from glucose positive; gas from glucose negative; acid from lactose negative; H_2S production negative; oxidase negative. Lysin decarboxylation - using a loop, a fragment of a colony is seeded on each dish, with lysin liquid medium distributed in test tubes. The incubation is performed at 30^{o}C for 24 hours. The apparition of a violet color indicates a positive reaction, meanwhile a yellow one indicated a negative reaction. Ornitin decarboxylation - by using a loop, a fraction is taken from an isolated colony and seeded in a test tube with liquid medium containing ornitin; the apparition of a violet color after 24 hours incubation at 30^{o}C is considered a positive reaction. The utilization of the citrate as unique source of carbon - the slope of Simmons gelose is striked with a fragment of the isolated colony. The incubation is performed at 30^{o}C for 24 hours. The changing of the color in blue is considered a positive reaction. The utilization of carbohydrates - the liquid medium is seeded (peptonated water with phenol red or bromtimole blue), supplemented with saccharose and rhamnose. The test tubes containinh the medium are incubated at 30^{o}C, 24 hours. The apparition of a yellow color after the incubation indicated a positive reaction; meanwhile the red color is considered a negative reaction.

For the presumptive test of pathogenity are retained only the colonies which showed the following reactions: lysin-decarboxylase negative; ornitin-decarboxylase positive; rhamnose negative; saccharose negative; citrate hydrolyze negative and the following issues are verified: the fermentation of salicin (the liquid medium is seeded - peptonated water with salicin, with a fragment of an isolated colony; the test tube with the liquid medium is incubated at 30oC for 24 hours; the apparition of a yellow color indicates a positive reaction, meanwhile a red color is considered a negative reaction); the esculin hydrolyze - using a loop, the slope of a bile-esculine agar is striked with a fragment of an isolated colony. The incubation is performed at 30oC for 24 hours; when the medium become black around the colonies, the reaction is a positive one; the Yersinia enterocolitica stems are virulent, possess plasmydes, esculin-negative, but not the biotype 1A (american), which respond in a positive way to this test; the production of pyrazinamidase - a fragment of an isolated colony is taken with a loop and passed over the slope of a cassein-soya gelose, containing pyrazinamid; after 48 hours of incubation at 30oC, 1 ml of sulphate fero-amoniacal 1 % is added; the apparition of a red-brown color indicates a positive reaction; the calcium dependence at 37oC - by using a loop it is seeded a quantity of 0,1 ml from a suspension of 103 cells/ml on the surface of 2 Petri dishes with cassein-soya gelose, with magnesium and oxalate; the incubation is performed at 25oC for 48 hours (a dish with cassein-soy gelose and a

dish with cassein-soya gelose, with magnesium and oxalate) respectively at 37oC for 48 hours, in a similar way for the other 2 dishes; the result is considered positive, if, at 25oC, the colonies present a homogenous waist and if, at 37oC, in the presence of magnesium and oxalate, it is observed an inhibition of the culture, obtaining in the end 2 types of colonies: ones tiny, with a diameter of 0,1 mm and others large, with a diameter of 0.5-1 mm, with a percentage of the small colonies of 20 %; the point colonies are calcium-dependent and virulent, supposedly pathogens).

Conclusions

The collecting and primary processing of the samples for presumptive diagnosis of bacterial species pertaining to *Yersinia* genus, is of 48 hours maximum in case of using the optimized method, comparative with the classical methods which need a period of time relatively larger, of over 72 hours.

For a presumptive diagnosis of confirmation for the species, the utilization of the optimized methods offers a higher accuracy of the results in comparison with the ISO 10273 method and the revealing of pathogenity traits, in comparison to API 20E.

The rapid scheme for identifying *Yersinia enterocolitica* species from different types of samples, by using the obtained results after the research made by the authors, assures a rapid diagnosis and offers the possibility of therapeutical intervention in an optimal period of time or the withdrawing in real time from commerce, processing or direct consume of the products contaminated.

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STUDIES ON *CANDIDA* SPP. PRESENCE IN ORAL CAVITY OF DOGS

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Summary

The aim of this study was to monitor the incidence of *Candida* spp. on different sites in the mouths of healthy dogs and dogs with periodontal diseases.

Samples were collected from supragingival plague and from dorsal face of tongue of 15 healthy dogs and in 15 dogs with oral diseases. The fungi from *Candida* spp. were present in a proportion between 20% and 33.3% in healthy dogs and between 46.6% and 53.33% in dogs with oral diseases. Microscopic fungi of the genus *Candida* were isolated from both healthy dogs and from those with periodontal disease but in different proportions. In dogs with dental diseases the presence of *Candida* was increased

Key words: *Candida* spp., oral cavity, dogs

There are only few studies on the oral microbiology of the dogs, because it was considered that the microorganisms from dog oral cavity it is similar to that in humans (2; 3; 6).

Candida species are single-celled organism that naturally occurs in the digestive and genital tracts of warm-blooded animals including humans. In healthy bodies it is kept in low level by beneficial bacteria. If the beneficial bacteria is damaged or destroyed because of use of broad-spectrum antibiotics, antihistamines, chemotherapy or radiotherapy the organism causes candidiosis. The ability of *C. albicans* to change from its yeast form to hyphal forms (morphogenesis), adherence, and enzyme production contribute to the virulence of fungal infections (5).

Also, in human medicine *Candida* was frequently associated with the formation of biofilms on implantable medical devices, being reported that the occurrence rate of oral *Candida albicans* in patients with dentures was higher than in patients without dentures (1, 4).

The aim of this study was to monitor the incidence of Candida spp. at different sites in the mouths of both healthy dogs and periodontal diseases dogs.

Materials and methods

There were taken samples from 30 dogs with different ages and sexes treated for different pathology in veterinary clinics. These dogs are grouped in two lots: the first with oral diseases (gingivitis and periodontitis) and the second, the healthy dogs.

Samples were taken from the supragingival plaque and from dorsal face of the tongue. The sterile swabs in duplicate were used to collect the samples from the mentioned oral surfaces of the dogs.

One swab was used to prepare wet mount by using lactophenol cotton blue stain for direct microscope examination. The other swab was used for inoculation onto Sabouraud dextrose agar with cloramfenicol (SDA Plates) that were incubated at 32 ºC for one week. For the fungal growth observation the plates were examined daily. Each colony on these plates was microscopic examined and subcultured on Sabouraud medium to obtain pure culture that ulterior was subjected to biochemical test for *Candida albicans* confirmation.

Results and discussions

From all 30 studied dogs were taken a number of 60 samples, in duplicate, one for direct microscopy examination and the other for culturing and identification. Presence of *Candida* spp. in healthy dogs is presented in tables 1 and for the dogs with gingivitis and periodontitis in table 2.

Table 1

The presence of the Candida spp. in healthy dogs

Sample	Supragingivally plaque		Dorsal face of tongue	
	Microscopic examination	Culturing examination	Microscopic examination	Culturing examination
1	-	+	+	+
2	+	+	+	+
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	+	+
7	-	-	-	-
8	+	+	-	-
9	-	-	-	-
10	-	-	-	+
11	-	-	-	-
12	-	-	+	+
13	-	-	-	-
14	-	-	-	-

15	**-**	**-**	**-**	**-**
TOTAL / proportion of positive samples	**2 / 13.3%**	**3 / 20.0%**	**4 / 26.6%**	**5 /33.3%%**

Of the 15 healthy dogs examined, only three (20%) were able to reveal the presence of the genus yeast species in cultivation from samples taken from supragingivally plaque, and if samples taken from the back of the tongue were positive five (33.3%) of the 15 samples processed.

The species of the genus *Candida* were present only in three (20%) of the 15 examined dogs, after cultivation on Sabouraud medium of samples taken from supragingivally plaque, and in case of samples taken from dorsal face of tongue if samples *Candida* spp were present in five (33.3%) of the 15 processed samples.

There were observed a little difference between the results of both examination methods. Number of cases with positive results for *Candida* spp. was a little bit higher in case of culturing method than those that were direct microscopically examined.

The results obtained on examinations of samples from dogs with gingivitis and periodontitis (table 2) revealed that 7 dogs from 15 had *Candida spp.* on supragingivally plaque (40%) and 8 (53.3%) dogs were positive on samples from dorsal face of tongue.

In both examined groups, there were few samples in which the direct microscopic examination revealed no presence of *Candida* spp., but which proved to be positive after cultivation on culture media.

Table 2

The presence of the *Candida* spp. in dogs with gingivitis and periodontitis

Sample	Supragingivally plaque		Dorsal face of tongue	
	Microscopic examination	**Culturing examination**	**Microscopic examination**	**Culturing examination**
1	+	+	+	+
2	+	+	+	+
3	+	-	+	+
4	-	-	-	-
5	-	+	-	+
6	-	+	+	+
7	-	-	-	-
8	-	-	-	-
9	-	-	+	+
10	+	+	-	-
11	+	+	+	+

12	+	+	+	+
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
TOTAL / proportion of positive samples	**6 / 40.0%**	**7 / 46.6%**	**7 / 44.6**	**8 / 53.3**

In a study realized by Kobayashi (4), eleven of the 15 dogs from Animal Center and 9 of 23 of family dogs had the fungi in their oral cavity. In this study the *Candida* was found only in 3 dogs of Animals Center and in 1 dog from family dog group, the other yeast form fungi were represented by *Malassesia* spp. In other study done by Vijay and Mahendra (7), was concluded that out of thirty four samples subjected to mycological examination *C. albicans* was isolated in low proportion of 11.76% (four dogs).

Conclusions

In most samples, the presence of *Candida spp*. were confirmed both direct microscopic examination, and cultural examination from all most of the samples from dog oral cavity.

Microscopic fungi of the genus *Candida* were isolated from both healthy dogs and from those with periodontal disease but in different proportions.

In dogs with dental diseases the presence of *Candida* was increased.

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