A COMPARATIVE EVALUATION OF CARRIED BACTERIAL STRAINS IN SHEEP AND GOATS RAISED IN A MIXED HEARD

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Summary

Most of carried bacterial flora is found in respiratory and digestive tracts of healthy animals or humans and exerts no pathogenic effect under physiological circumstances. When favoring factors induce immune suppression, apparently harmless strains can become highly pathogenic for animals and consumers or people who work in animal sector. The aim of the study was to identify and compare ported digestive and respiratory bacterial strains from goats and sheep cohabiting in a mixed heard. The research was carried out on 30 healthy animals (15 goats and 15 sheep) from the same heard. Nasal and rectal samples were cultured on glucose agar and on special media (McConkey, Chapman) and subsequently the isolates were identified by use of API 20 E and API 20 Staph biochemical tests. Bacterial isolates from sheep respiratory tract were Erwinia spp. (22%), Serratia liquefaciens (8%), Serratia marcescens (13%), Serratia ficaria (9%) Staphylococcus xylosus (22%), and Enterobacter cloacae (26%). The majority of bacterial isolates from goats’ respiratory tract consisted of E. coli strains (54%) while other strains were similarly represented (Serratia liquefaciens 20%, Serratia marcescens 13%, Staphylococcus xylosus 13%), E. coli prevailed in isolates from the digestive system of both sheep (67%) and goats (62%), the rest of bacterial strains being in sheep E. vulneris (17%), Serratia marcescens (11%), and Staphylococcus xylosus (5%) and Staphylococcus xylosus (24%), E. vulneris 5% and Enterobacter cloacae (9%) in goats. Interestingly, bacteria of fecal origin such as E. coli were identified in the respiratory system of goats but not of sheep, while E. cloacae strains were represented in the respiratory tract of sheep but not of the goats. Although the flock was of mixed species, there were differences between the bacterial isolates, suggesting differentiated susceptibility and feeding behavior in these species. Nevertheless, the carried bacterial isolates from clinical healthy goats and sheep could exert pathogenic effects under stressful circumstances, underlining the importance of early identification of pathogens and the accurate sanitary management of the heard.

Key words: bacterial population, sheep, goats, respiratory tract, digestive system

Materials and methods

Most of carried bacterial flora is found in respiratory and digestive tracts of healthy animals or humans and exerts no pathogenic effect under physiological circumstances. When favoring factors induce immune suppression, apparently harmless strains can become highly pathogenic for animals and consumers or people who work in the animal sector.
The aim of the study was to identify and compare ported digestive and respiratory bacterial strains from goats and sheep cohabiting in a mixed heard. The research was carried out on 30 healthy animals (15 goats and 15 sheep) from the same heard. Nasal and rectal swabs were cultured on simple broth for 24 h at 37°C and inseminated on glucose agar plates for another 24h at 37°C, to obtain isolated colonies. For a better identification, the isolated colonies were inoculated also on MacConkey and Chapman agars. The isolates were identified by the use of API 20 E testing system for Enterobacteriaceae family and API 20 Staph. for bacterial colonies raised on Chapman medium. The results of the study were presented graphically with the help of charts and for the statistical interpretation of prevalence differences Fisher exact test (R for Windows) was used.

Results and discussions

The bacterial strains isolated from the digestive and respiratory systems of goats and sheep were shown in fig. no. 1, fig. no. 2, fig. no. 3, and fig.4 respectively.

![Bacterial strains isolates from goat respiratory tract](image)

**Fig. 1.** Bacterial isolates from goats respiratory tract

When the isolates from the respiratory tract of the two species were compared, *Erwinia* spp. (p=0.042) and *Enterobacter cloacae* (p= 0.016) were isolated only from sheep and were absent in goats, while *E. coli* (p= 0.002) was present only in goats and absent in sheep.
Fig. 2 Bacterial isolates from sheep respiratory tract

Erwinia spp. are naturally pathogenic to a wide range of field crops such as maize, rice, sugar beet, and vegetables such as carrots, potatoes, tomatoes, beans and cabbage (4). In this context it is easily to conclude that the presence of this bacterial strain is due to food contamination. Certain species are involved in pathogenic activity especially in patients with immunodeficiency and the fact of pathogenic role of Erwinia spp in animals, was explained by genetic test involving genes sampling from E.coli and other bacteria from Enterobacteriaceae family (1).

Enteroabacter cloacae has emerged in recent studies as a major phytopathogen that infects crops worldwide and affects plants like onions, bean sprouts, melons and lettuce (7). Presence of Enteroabacter cloacae in the respiratory tract of sheep is most probably of vegetal origin the first source of this bacterial strain were the contaminated feed.

E. coli is one of the main inhabitants of the intestinal tract of a large range of animals that includes humans and birds. Most Escherichia coli strains are harmless, but a small proportion is highly pathogen, and is responsible of important cases of disease worldwide (2). Contamination of feed, including pastures or water is common and a result of fecal contamination but the most alarming fact is that some pathogen bacterial strains can persist in farm environment for more than two years (8).
There were no statistically significant differences between sheep and goats concerning the digestive bacterial flora. The variety of bacterial isolates was surprisingly reduced and the presence of normal intestinal flora was surprising considering that the studied animals were clinically healthy.
Serratia marcescens was present in sheep isolates but the number of strains was of no statistical significance. Serratia marcescens bacteria that has a high level of pathogenicity and is involved in various infections affecting humans and animals. Most common infections with Serratia marcescens are urinary infections, endocarditis, miocarditis, and most frequent respiratory infections (3).

Conclusions

Interestingly, bacteria of fecal origin such as E. coli were identified in the respiratory system of goats but not of sheep, while E. cloacae strains were represented in the respiratory tract of sheep but not of the goats. Although the flock consisted of mixed species, there were differences between the bacterial isolates, suggesting differentiated susceptibility and feeding behavior in these species.

Nevertheless, the carried bacterial isolates from clinically healthy goats and sheep could exert pathogenic effects under stressfull circumstances, underlining the importance of early identification of pathogens and the accurate sanitary management of the heard.

References

1. Chatterjee, A. K, Genetic transfer of episomic elements among Erwinis species and other Enterobacteria, Department of Bacteriology, California 1972.
RESPIRATORY AND DIGESTIVE BACTERIA ISOLATED FROM HEALTHY GOATS AND THEIR RESPONSE TO COMMON ANTIBIOTICS

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Summary

Healthy products and production increase at adequate price is the aim of a modern farmer, goat farmers being no exception. For better productive results, breeders use standard prophylactic measures to reduce losses and increase profits. The extensive system of raising goats represents both a tradition and a cheaper farming model. The aim of the study was to identify porting bacterial strains from nasal cavities and rectum (final segment of digestive system) and to evaluate their response to common antibiotics. The experiment was carried out on 10 healthy goats aged 2 to 6 years, randomly chosen from the herd. Samples were collected from both the upper respiratory and from the digestive tract of the animals. Standardized laboratory protocols were used for identification Gram – and Gram + strains. Complete bacterial identification was done by API 20 E and API Staph identification kits. Bacterial isolates from the respiratory tract were Staphylococcus xylosus (40%), Enterobacter cloacae 40%, Pantoea spp. 20% while from the digestive system Staphylococcus xylosus 43% and Escherichia coli 57% were isolated. Antibiotic sensitivity patterns were identified for each of the isolates using ampicillin (AM) 10 mcg, amoxicillin and clavulanic acid (AMC) 30 mcg, cefadroxil (CFR) 30 mcg, gentamicin (GN) 120 mcg, doxycycline (DO) 30 µg, ciprofloxacin (CIP) 5 µg, nalidixic acid (NA) 30 µg, trimethoprim-sulfamethoxazole (SXT) 25 µg, chloramphenicol (C) 30 µg, and methicillin (ME) 5 µg. General resistance to ampicillin, amoxicillin and ciprofloxacin were highly efficient against all isolated strains. Such results could offer valuable information for farmers and veterinary practitioners in choosing adequate treatments to specific bacterial isolates and block irresponsible use of antibiotics.

Key words: microflora, respiratory tract, digestive system, goats, antibiotic sensitivity

Materials and methods

The aim of the study was to isolate and identify bacterial strains that compose the microbial porting flora from apparently healthy goats raised under extensive circumstances in Transylvania. Furthermore, the research intended to evaluate the sensitivity of the bacterial isolates to usual antibiotics. The tested animals were randomly chosen from the herd, as clinically healthy goats aged 2 to
6 years. Samples were collected from both the upper respiratory (n=10) and from the digestive tract of the animals (n=10).

Laboratory standard protocols were used for identification of both Gram – and Gram + bacterial strains. Firstly, the samples were inoculated on meat broth, a culture medium that allows the growth for various bacterial species, and incubated for 24 h at 37°C under aerobic conditions. Subsequently, the bacterial culture mixture was examined microscopically following the Gram stain to establish further appropriate selective growth media. The next stage of identification included inoculation on various selective media: nutritive agar medium with glucose for a better observation of the colonies, MacConkey agar for lactose fermentation of Gram – bacteria (6), Chapman medium for isolation of presumptively pathogenic staphylococci (10). The inoculated plates were incubated for 18-36 h at 37°C in aerobic atmosphere.

The complete bacterial identification was possible with the help of API identification kit. API 20 E for Gram – bacteria regardless of fermentation proprieties and API Staph. for bacterial isolates from Chapman medium were used.

The second stage of the experiment involved the characterizations of the bacterial strains’ sensitivity to usual antibiotics. The antibiotic susceptibility was established using nine antibiotics, namely ampicillin (AM) 10 mcg, amoxacillin and clavulanic acid (AMC) 30 mcg, cefadroxil (CFR) 30 mcg, gentamicin (CN) 120 mcg, doxycycline (DO) 30 µg, ciprofloxacin (CIP) 5 µg, nalidixic acid (NA) 30 µg, trimethoprim-sulfamethoxazole (SXT) 25 µg, chlpranphenicol (C) 30 µg, for both Enterobacteriaceae family and staphylococci. The nalidixic acid was replaced with methicillin (ME) 5 µg in the case of Staphylococcus spp.

Antibiotic sensitivity implied measurements of the inhibition diameters, as approved by Clinical and Laboratory Standards Institute (5)

Results and discussions

The bacterial strains isolated from the digestive and respiratory systems of the goats were shown in fig. no. 1 and fig. no. 2, respectively.

The presence of E. coli in the digestive system is normal for healthy animals and humans considering that this bacteria has numerous non-pathogenic strains which colonize de intestine (Geornaransand co., 2001). Commonly E. coli presence indicates a fecal contamination of feed and is considered a potential lethal foodborne pathogen (19). Most pathogen strains of E. coli may cause a large variety of disease, from diarrhea that can be deadly for newborns, to septicemia a syndrome fatal even for strong adult animals (8). E. coli occurrence in the respiratory tract could be common to goats, taking into account their feeding habits. In the extensive raising system, the feed is most likely composed of grass, shrubs and a large variety of other plants. Similarly, the social behavior of the heard, where the animals stay close to each other (18) explains the presence in the
respiratory system of bacteria normally found in the digestive tract ex. *Enterobacter cloacae*.

**Fig. 1. Bacterial isolates from goats digestive system**

**Bacterial strains isolated from goats digestiv system**

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli</td>
<td>57%</td>
</tr>
<tr>
<td>Staph. xylosus</td>
<td>43%</td>
</tr>
</tbody>
</table>

**Fig. 2. Bacterial isolates from goat respiratory tract**

**Bacterial strains isolated from goats respiratory tract**

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. xylosus</td>
<td>40%</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>40%</td>
</tr>
<tr>
<td>Pantoea spp.</td>
<td>20%</td>
</tr>
</tbody>
</table>

*Staphylococcus xylosus* is a coagulase-positive, Gram-positive coccus that belongs to *Staphylococcus saprophyticus* group. This bacterial group is ubiquitous in nature, persisting in soils and on surfaces. *S. xylosus* is a common commensal bacterium that is generally found inhabiting the skin and mucous membranes of a great variety of mammals and occasionally of humans (13). *S. xylosus* has the
ability to colonizes the skin and surfaces by forming a biofilm (15) Although *S. xylosus* is considered a nonpathogenic *Staphylococcus* and is commonly used as a starter culture for meat products (4), several reports describe opportunistic infections in animals (1) and humans caused by this bacteria.

*Enterobacter cloacae* is an ubiquitous microorganism of the *Enterobacteriaceae* family most commonly found in plants, insects, animals and outer environmental sources like water, residual water (sewage) and soil (14). Although it is a non-specific enteric pathogen, *Enterobacter cloacae* is the most frequent enteric *Enterobacter* identified from intestine besides *E. coli* and it is an opportunistic pathogen in humans and animals. *E. cloacae* is also pathogenic to various plants that are common animal feed like corn (16) and this fact can explain its presence in apparently healthy goats. Recent studies showed that bacteria from *Enterobacter* genus are a major cause of nosocomial infections (11) and are often involved in urinary and respiratory tract infections (12).

*Pantoea* spp. is a commensal bacteria that has been isolated from humans, animals and from environmental sources. Bacteria from this genus were mostly involved in nosocomial infections due to contaminations of various medical consumables (parenteral nutrition tube, transference tube for intravenous hydration) and blood products (2). Most common outbreaks where bacteria from *Pantoea* spp genus were involved, occurred in immune depress patients treated in hospitals, probably due to the microorganism ability to grow in glucose-enriched solutions widely used in hospital environments (9).

Results for the second stage of the study were presented in tables 1 and 2 and consisted in an evaluation of antibiotic susceptibility pattern of bacterial isolates from digestive and respiratory systems.

### Table 1

**Bacterial strains isolated from digestive system of apparently healthy goats and antibiotic sensitivity interpretation according CLSI 2011 zone diameter breakpoints mm**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>AM</th>
<th>C</th>
<th>CFR</th>
<th>AMC</th>
<th>SXT</th>
<th>CN</th>
<th>DO</th>
<th>CIP</th>
<th>NA</th>
<th>ME</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E.coli</em></td>
<td>14 (S)</td>
<td>22 S</td>
<td>18 I</td>
<td>12 R</td>
<td>19 S</td>
<td>18 S</td>
<td>24 S</td>
<td>22 S</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>E.coli</em></td>
<td>13 R</td>
<td>30 S</td>
<td>16 I</td>
<td>15 I</td>
<td>RT</td>
<td>22 S</td>
<td>18 S</td>
<td>24 S</td>
<td>22 S</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>RT</td>
<td>23 S</td>
<td>8 R</td>
<td>RT</td>
<td>RT</td>
<td>26 S</td>
<td>12 I</td>
<td>24 S</td>
<td>16 I</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>RT</td>
<td>24 S</td>
<td>10 R</td>
<td>RT</td>
<td>RT</td>
<td>28 S</td>
<td>22 S</td>
<td>26 S</td>
<td>18 I</td>
<td>-</td>
</tr>
<tr>
<td><em>Staph.xylosus</em></td>
<td>12 R</td>
<td>19 S</td>
<td>36 S</td>
<td>21 S</td>
<td>27 S</td>
<td>32 S</td>
<td>30 S</td>
<td>36 S</td>
<td>-</td>
<td>22 S</td>
</tr>
<tr>
<td><em>Staph.xylosus</em></td>
<td>22 R</td>
<td>20 S</td>
<td>26 S</td>
<td>25 S</td>
<td>36 S</td>
<td>33 S</td>
<td>30 S</td>
<td>33 S</td>
<td>-</td>
<td>22 S</td>
</tr>
<tr>
<td><em>Staph.xylosus</em></td>
<td>20 R</td>
<td>24 S</td>
<td>40 S</td>
<td>27 S</td>
<td>28 S</td>
<td>34 S</td>
<td>24 S</td>
<td>29 S</td>
<td>-</td>
<td>40 S</td>
</tr>
</tbody>
</table>

13
Table 2
Bacterial strains isolated from respiratory tract of apparently healthy goats and antibiotic sensitivity interpretation according CLSI 2011 zone diameter breakpoints mm

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>AM</th>
<th>C</th>
<th>CFR</th>
<th>AMC</th>
<th>SXT</th>
<th>CN</th>
<th>DO</th>
<th>CIP</th>
<th>NA</th>
<th>ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. xylosus</td>
<td>2 R</td>
<td>17</td>
<td>I</td>
<td>4 R</td>
<td>16 S</td>
<td>28 S</td>
<td>18 S</td>
<td>18 I</td>
<td>-</td>
<td>RT</td>
</tr>
<tr>
<td>Staph. xylosus</td>
<td>18 R</td>
<td>24 S</td>
<td>36 S</td>
<td>20 S</td>
<td>22 S</td>
<td>27 S</td>
<td>25 S</td>
<td>26 S</td>
<td>-</td>
<td>26 S</td>
</tr>
<tr>
<td>Ent. cloacae</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
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<td>18 S</td>
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<tr>
<td>Ent. cloacae</td>
<td>RT</td>
<td>8 R</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
<td>24 S</td>
<td>22 S</td>
<td>28 S</td>
<td>12 R</td>
</tr>
<tr>
<td>Pantoea spp.</td>
<td>8 R</td>
<td>25 S</td>
<td>26 S</td>
<td>19 S</td>
<td>RT</td>
<td>28 S</td>
<td>27 S</td>
<td>26 S</td>
<td>12 R</td>
<td>-</td>
</tr>
</tbody>
</table>

Our data showed that the bacterial strains isolated from digestive system were highly sensitivite to Chloramphenicol (100%), Gentamicin (100%), Ciprofloxacin (100%). In addition, all digestive *Staphylococcus xylosus* strains were 100% sensitive to Methicillin. Since resistance to Methicillin was prevalent among human staphylococcal isolates (3), it is most probable that the strains present in investigated goat samples were of animal origin.

Eighty-seven percent of digestive isolates were 100% sensitive to Doxycycline, with only one strain showing intermediary reaction. As opposed, digestive isolates had the lowest sensitivity (14%) towards Ampicillin, which was expressed only by one *E. coli* strain. Furthermore, fifty percent of the strains were sensitive to % Nalidixic acid, 42% to Cefadroxil, 42% to Amoxicillin- clavulanic acid, and 57% to Trimethoprim-sulfamethoxazole. In this case, the disc diffusion test revealed a moderate sensitivity that encouraged further studies on the efficacy of these antibiotics in clinical cases.

Bacterial strains isolated from the respiratory tract showed 100% sensitivity to Gentamicin and Doxycycline, 80% to Ciprofloxacin, while they were resistant to Nalidixic acid and Ampicillin. Moderate reaction was noticed in case of Chloramphenicol (40%), Trimethoprim-sulfamethoxazole (40%), Cefadroxil (40%) and Methicillin (50%). Only the *Enterobacter cloacae* strains were resistant to this antibiotic.

Conclusions

The bacterial strains isolated from goats’ respiratory tract and digestive system were common environmental organisms that could become pathogenic under appropriate circumstances.

General resistance to ampicillin, amoxicillin with clavulanic acid and trimethoprim-sulfamethoxazole was widespread among bacterial isolates from both respiratory and digestive systems. As opposed, doxycycline, gentamicin and ciprofloxacin were highly efficient against all isolated strains.

*Enterobacter cloacae* strains isolated from respiratory tract showed a great resistance to the majority of antibiotics used in the survey except for Gentamicin, Doxycycline and Ciprofloxacin.
Such results could offer valuable information for farmers and veterinary practitioners in choosing adequate treatments to specific bacterial isolates and block irresponsible abuse of antibiotics.

References


ANTIBIOTIC SUSCEPTIBILITY PROFILES AND VIRULENCE FACTORS OF *LISTERIA MONOCYTOGENES* STRAINS ISOLATED FROM MEAT

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Summary

The emerging foodborne pathogen *Listeria monocytogenes* is an ubiquitous bacteria, widely distributed in the natural environment, contaminating numerous food products (raw meat, poultry fish, raw milk, dairy, and vegetables), as well as fast food preparations. *L. monocytogenes* is responsible for listeriosis, an illness characterized by septicemia, meningitis, encephalitis and abortive disease (stillbirth or premature birth of the fetus). It is particularly affecting individuals with great risk, i.e.: immunocompromised, elderly, infants, and pregnant women (and their unborn children). The detection and characterization of *L. monocytogenes* in food may be an effective measure for the prevention of this severe illness. The objective of this study was to evaluate the virulence and antibiotic resistance features of 10 *L. monocytogenes* strains isolated from meat products. Antibiotic susceptibility was evaluated to 20 antibiotics currently used in veterinary and human therapy, and the enzymatic virulence factors were tested on special culture media for pore forming toxins (hemolysins, lecithinase, lipase), exoenzymes (gelatinase, amylase, caseinase, esculinase, DNase), and the adherence to HEp-2 cells. The haemolysins, caseinase and lipase were present in the majority of *L. monocytogenes* strains, which adhered to HEp-2 cells with a predominant diffuse-aggregative mixed pattern. The tested strains isolated from food-processed samples were generally resistant to cephalosporins and nalidixic acid, but remained susceptible to the antibiotics commonly used in veterinary and human listeriosis treatment.

Key words: *L. monocytogenes*, virulence factors, antimicrobial susceptibility.

*Listeria monocytogenes*, one of the most important food-borne pathogens, is an ubiquitous bacterial species, which can lead to listeriosis, with high rates of morbidity and mortality (25-30%), although human listeriosis is very rare, with 0.06-0.33 cases/10⁵ population/year (10). Susceptible population groups include the elderly people, immunocompromised individuals, pregnant women and their fetuses or neonates. Raw meat, met products, fish, raw milk, dairy foods, vegetables, as well as ready-to-eat foods may act as vehicle of transmission of this microorganism. After ingestion, *L. monocytogenes* can cross the gut mucosa and disseminate via the bloodstream; then may invade the central nervous system and the placenta, causing septicemia, meningitis, encephalitis, or fetoplacental infections (2). In pathogenesis of *Listeria monocytogenes* a main role is played by many virulence factors of this pathogen. The primary choice for therapy in
listeriosis is ampicillin or a combination of ampicillin with an aminoglycoside such as gentamycin or streptomycin (3). In the last period, the studies have shown that *L. monocytogenes* strains became multiresistant to antimicrobial substances (1), with significance in public health. The present study evaluates the virulence features and the susceptibility to antibiotics of 10 *L. monocytogenes* strains isolated from raw meat and meat products.

**Materials and methods**

**Bacterial strains.** Ten *L. monocytogenes* strains were isolated from raw meat or meat products, including raw minced meat (pork, beef), pork muscular tissue, beef muscular tissue, smoked bacon, poultry carcass, paste of Romanian sausages, fresh sausages, hot dog (chicken meat), boiled shell snails. These food samples were analyzed according to SR EN ISO 11290 standards part 1 and part 2, (13, 14, 15), and colonies grown on PALCAM agar were identified (Table 1). Additionally, *L. monocytogenes* type 1 ATCC 19111 was used as reference strain. Stock cultures were maintained at -80ºC in Brain Heart Infusion (BHI) broth (Oxoid) with 20% (v/v) glycerol. Before use, pathogenic strains of *L. monocytogenes* were activated in BHI at 37°C for 24 hrs.

**In vitro characterization of enzymatic virulence factors.** After incubation at 37°C for 20 h, on nutrient broth, fresh cultures were spotted on media containing specific substrate for the evaluation of soluble enzymatic factors expression (pore-forming enzymes, proteases, siderophore-like production) (6).

For the detection of *hemolytic* activity, the strains were streaked on 7% blood agar plates. Appearance of a clear zone around the growth area, after incubation at 37°C for 24 hrs was considered a positive reaction.

Lecithinase and lipase, enzymes implicated in pore production and bacterial invasion, were tested by spotting onto 2.5% yolk agar, respectively Tween 80 agar (a final concentration of 1%). After incubation at 37°C for 72 hrs, a clearing zone surrounding the growth area was considered a positive reaction for lecithinase activity, respectively an opaque (precipitation) area for lipase activity.

For the detection of *caseinase* and *gelatinase*, enzymes implicated in the evolution of the infectious process, with tissue damage and rapid bacterial multiplication, the tested strains were spotted onto agar plate with 15% soluble casein, and respectively, gelatin at 1% final concentration. After incubation at 37°C for 72 hrs, the proteolytic activity is indicated by an opaque (precipitation) area for caseinase, and respectively a transparent zone around the culture for gelatinase production.

The *amylase* activity, an enzyme implicated in the polysaccharides hydrolysis, offering a nutritional competitive advantage to producing bacterial strains, was determined using agar with starch as substrate (1% final concentration). After 72 hrs incubation at 37°C, the starch hydrolysis appears as a precipitation zone surrounding the culture spot.
DNase production, an enzyme that catalyses the hydrolytic cleavage of cellular DNA, with lesions in host cell, was studied by spotting the strains onto DNA agar medium. After incubation for 24 hrs at 37°C, a drop of HCl 1N solution was added to each spotted culture and the reactions were examined. A clearing area surrounding the spot area is considered as a positive reaction.

Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>β-hemolysis</th>
<th>CAMP Test</th>
<th>Acid from:</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Staphylococcus aureus</td>
<td>Rodococcus equi</td>
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<td>1</td>
<td>1</td>
<td>1</td>
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<td>Pork muscular tissue</td>
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</tr>
<tr>
<td>Beef muscular tissue</td>
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<td>1</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Poultry carcass</td>
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<td>Hot dog (chicken meat)</td>
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<tr>
<td>Boiled shell snails</td>
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</tbody>
</table>

Characterization of bacterial adherence to HEp-2 cells. Adherence assay was performed on HEp-2 cells, by Cravioto’s adapted method (5, 9). The cells were cultured in Eagle Minimum Essential Medium (EMEM), supplemented with 10% bovine fetal serum. The HEp-2 cells were grown to 70-80% confluent monolayers in tissue culture plates. The cell monolayers were washed 3 times with phosphate buffered saline (PBS) and 1 mL of fresh medium without antibiotics was added to each well. Each well was inoculated with 1 mL suspension from bacterial mid-logarithmic phase cultures grown in nutrient broth, adjusted to 10⁸ CFU/mL. After incubation for 2 hrs at 37°C, the cells were washed 3 times with PBS, fixed.
with 70% methanol for 3 min, and stained with 10% Giemsa solution for 20 min. The plates were washed, dried at room temperature overnight, and examined by light microscopy (x2500). Adherence patterns and the adherence index (percentage of cells with adhered bacteria / 100 numbered cells) were evaluated.

**Antibiotic susceptibility testing by disk diffusion method.** Phenotypic resistance testing was performed by the disk diffusion method. Disk diffusion susceptibility tests were performed according to the procedures recommended by the Clinical and Laboratory Standards Institute (12), using Mueller Hinton Agar (MHA) and Oxoid disks. The antibiotic discs containing 10U Penicillin, 10μg Ampicillin, 30μg Amoxicillin + Clavulanic Ac., 10μg Imipenem, 30μg Cefuroxime, 30μg Ceftriaxone, 30μg Amikacin, 10μg Gentamicin, 10μg Streptomycin, 30μg Neomycin, 30μg Nalidixic Ac., 5μg Enrofloxacin, 5μg Ciprofloxacin, 30μg Oxitetracycline, 15μg Erytromycin, 30μg Chloramphenicol, 30μg Rifampicin, 100μg Sulphamethoxazole, 5μg Trimethoprim, 1,25μg/23,75μg Sulphamethoxazole / Trimethoprim were placed on the surface of the inoculated MHA plate. After incubation for 24 hrs at 37°C, the diameter (expressed in mm) of the growth inhibition zone around each antibiotic disk was measured and interpreted in accordance with the CLSI guidelines, to classify the antibiotic susceptibility of each isolate. *Staphylococcus aureus* ATCC 29213 was used as a control strain.

**Results and discussions**

The evaluation of virulence features in *L. monocytogenes* strains isolated from different meat products could provide useful markers to assess the evolution of *L. monocytogenes* infectious process (table 2).

<table>
<thead>
<tr>
<th>Virulence factors</th>
<th>Positive strains (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase</td>
<td>9</td>
</tr>
<tr>
<td>Lecithinase</td>
<td>0</td>
</tr>
<tr>
<td>Caseinase</td>
<td>10</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>0</td>
</tr>
<tr>
<td>Amylase</td>
<td>0</td>
</tr>
<tr>
<td>DNase</td>
<td>0</td>
</tr>
<tr>
<td>Esculinase</td>
<td>10</td>
</tr>
<tr>
<td>Listeriolysin O</td>
<td>10</td>
</tr>
</tbody>
</table>

On PALCAM agar *L. monocytogenes* has shown green colored colonies surrounded by a black zone, indicating the presence of esculinase, responsible for esculine hydrolysis, resulting esculetin, which could act as an iron chelating agent, providing iron even in limited conditions.
Lipase was present in the majority (9 from 10 strains) of *L. monocytogenes* strains isolated from meat products, while caseinase was revealed in all *L. monocytogenes* tested strains. Lecithinase, gelatinase, amylase and DNase were absent in these food isolates. The expression of the tested soluble virulence factors is shown in Table 2. Hemolysin activity was revealed by a partial hemolysis (*L. monocytogenes* with β - haemolytic colonies) around the growth area on blood agar, and this fact was confirmed by the CAMP test with *S. aureus*.

The microbial adherence to epithelial cells (Fig. 1) is essential for the colonization of the host organism and for the progression of the infectious process. All animals which ingest an infective dose of 10^{10} *L. monocytogenes* develop enteritis (10).

![Fig. 1.](image)

**Fig. 1.** a. Localized adherence - microcolonies on the surface of HEp-2 cells. b. Diffuse adherence – isolated bacterial cells adhered over the whole cell surface. c. Aggregative adherence – bacteria adhered both to the host-cell surface and between them (2500).

The *L. monocytogenes* strains exhibited three distinct adherence patterns to HEp-2 cells, *i.e.*: localized adherence, in which bacteria form characteristic microcolonies adhered on the surface on the host cell; diffuse adherence, in which isolated *L. monocytogenes* cells adhere uniformly to the whole surface of the eukaryotic cell; and aggregative adherence, in which large bacterial aggregates adhere both to the host-cell surface and between them in a compact mass appearance. Some mixed patterns, *i.e.*: diffuse-localized, aggregative-localized and diffuse-aggregative have been also observed. In this study, the majority of the tested strains adhered to HEp-2 cells with a predominant mixed pattern, specially diffuse-aggregative pattern (Table 3).
Table 3

<table>
<thead>
<tr>
<th>Adherence pattern</th>
<th>L. monocytogenes (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized adherence</td>
<td>1</td>
</tr>
<tr>
<td>Diffuse adherence</td>
<td>2</td>
</tr>
<tr>
<td>Aggregative adherence</td>
<td>1</td>
</tr>
<tr>
<td>Mixed pattern</td>
<td></td>
</tr>
<tr>
<td>diffuse-localized</td>
<td>1</td>
</tr>
<tr>
<td>aggregative-localized</td>
<td>1</td>
</tr>
<tr>
<td>diffuse-aggregative</td>
<td>3</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
</tr>
</tbody>
</table>

Adherence patterns of *L. monocytogenes* strains isolated from meat were defined according to the Clinical and Laboratory Standard Institute (CLSI), in order to allow the comparative analysis of the obtained results (1, 8, 12).

Concerning the antibiotic susceptibility profiles (Table 4), all strains, including the reference strain were susceptible to penicillin, ampicillin, amoxicillin+clavulanic acid, amikacin, gentamicin, streptomycin (9 from 10 strains), neomycin, oxitetracycline, erytromycin, chloramphenicol, rifampicin and sulphamethoxazole /trimethoprim.

The standard antibiotic therapy for listeriosis is ampicillin with gentamicin or penicillin alone, while sulphamethoxazole/trimethoprim is used as a secondary agent, especially for patients with allergy to penicillin (8, 11). Rifampin may be also used in the treatment of choice for most clinical forms of listeriosis (4). In the current study, all *L. monocytogenes* isolates were susceptible to these drugs.

All tested *L. monocytogenes* strains exhibited natural resistance to the second and third generation cephalosporins and nalidixic acid, as mentioned by the literature data (1, 7); for this reason, these antibiotics should not be used clinically for treating listeriosis. In particular, resistance to one antibiotic was more common than multiple resistances. One strain was resistant to sulphamethoxazole alone, while to the combination of sulphamethoxazole and trimethoprim all tested strains were susceptible, in accordance with similar data described in literature (1). Two *L. monocytogenes* isolates were intermediary susceptible to ciprofloxacain and one to enrofloxacin. However, the majority of *L. monocytogenes* strains were susceptible to the antibiotics commonly used in the treatment of both veterinary and human listeriosis.
### Table 4

<table>
<thead>
<tr>
<th>Antibiotic / dose (µg/disk)</th>
<th>L. monocytogenes strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td><strong>Beta-lactams</strong></td>
<td></td>
</tr>
<tr>
<td>Penicillins</td>
<td></td>
</tr>
<tr>
<td>Penicillin G 10U</td>
<td>10</td>
</tr>
<tr>
<td>Ampicillin 10µg</td>
<td>10</td>
</tr>
<tr>
<td>Amoxicillin + Clavulanic Ac 30µg</td>
<td>10</td>
</tr>
<tr>
<td>Imipenem 10µg</td>
<td>10</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime 30µg</td>
<td>10</td>
</tr>
<tr>
<td>Ceftriaxone 30µg</td>
<td>10</td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td></td>
</tr>
<tr>
<td>Amikacin 30µg</td>
<td>10</td>
</tr>
<tr>
<td>Gentamycin 10µg</td>
<td>10</td>
</tr>
<tr>
<td>Streptomycin 10µg</td>
<td>9</td>
</tr>
<tr>
<td>Neomycin 30µg</td>
<td>10</td>
</tr>
<tr>
<td><strong>Quinolones</strong></td>
<td></td>
</tr>
<tr>
<td>Nalidixic Ac. 30µg</td>
<td>10</td>
</tr>
<tr>
<td>Enrofloxacin 5µg</td>
<td>9</td>
</tr>
<tr>
<td>Ciprofloxacin 3µg</td>
<td>8</td>
</tr>
<tr>
<td><strong>Tetracyclines</strong></td>
<td></td>
</tr>
<tr>
<td>Oxitetracycline 30µg</td>
<td>10</td>
</tr>
<tr>
<td><strong>Macrolides</strong></td>
<td></td>
</tr>
<tr>
<td>Erythromycin 15µg</td>
<td>10</td>
</tr>
<tr>
<td><strong>Other antibiotics</strong></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol 30µg</td>
<td>10</td>
</tr>
<tr>
<td>Rifampicin 30µg</td>
<td>10</td>
</tr>
<tr>
<td>Sulphamethoxazole 100µg</td>
<td>9</td>
</tr>
<tr>
<td>Trimethoprim 5µg</td>
<td>10</td>
</tr>
<tr>
<td>Sulphamethoxazole / Trimethoprim 1.25µg/23.75µg</td>
<td>10</td>
</tr>
</tbody>
</table>

*S = susceptible; I = intermediary; R = resistant*

### Conclusions

The majority of *L. monocytogenes* strains exhibited as soluble virulence features the production of hemolysin, esculinase, lipase and caseinase.

The adherence assay revealed the relationship between the type of adherence of tested strains and their source of isolation, the majority of them exhibiting predominantly a mixed, diffuse-aggregative pattern.

The features revealed by the virulence assays can be used in the screening of the pathogenic potential of *L. monocytogenes* strains.

The majority of strains were resistant to cephalosporins and nalidixic acid. Ampicillin, or penicillin plus gentamicin remain the treatment of choice for the most forms of listeriosis.

Sulphamethoxazole/trimethoprim may be used as a second-choice therapy.

Most *L. monocytogenes* strains were susceptible to the antibiotics commonly used in the treatment of both veterinary and human listeriosis.
Acknowledgements

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HEMATOLOGICAL CHANGES ASSOCIATED WITH ENZOOTIC BOVINE LEUKOSIS IN CATTLE FROM TIMIS COUNTY

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Summary

The aim of this study was to present hematologic changes associated with enzootic bovine leukosis in animals from Timis County. In this purpose, 82 biological samples (blood), obtained from bovine BLV(+) and BLV(-), were analyzed, and the following hematological parameters were counted: RBC, hematocrit, Hgb, MCH, MCHC, MCV, MPV, WBC, lymphocytes, granulocytes, monocytes and platelets. There were significant differences between BLV(+) and BLV(-) cattle, and also between aleukemic BLV(+) cattle and BLV(+) animals with persistent lymphocytosis regarding WBC and number of lymphocytes. Most BLV(+) cattle assessed were in the early stage of the disease, namely the "carrier" phase, with nonspecific clinical manifestations or hematologic changes.

Key words: enzootic bovine leukemia, hematology, leukocytes, lymphocytes

Enzootic bovine leukemia (EBL) is a viral infection with three stages of evolution. The first one, characteristic to the majority of infected cattle (about 60%), is the "carrier state", not associated with clinical and hematological changes that indicates progression of the disease. About 30% of the BLV(+) animals are classified in the second stage, generically called "hematological", which is characterized by leukocytosis, lymphocytosis and unapparent clinical manifestations or attributable to other diseases. The third stage, the "neoplastic" corresponds viscera tumor development processes, and clinical forms depends on the affected organs (5, 6, 10).

This study, conducted between 2009 and 2010, aimed to reveal hematologic changes associated with enzootic bovine leukemia in animals from Timis County.

Materials and methods

In order to establish hematological changes associated with EBL 82 biological samples (blood), obtained from bovine BLV(+) and BLV(-), were analyzed.

Examined animals (27 BLV(+) cattle and 55 BLV(-) cattle) were from six outbreaks of EBL declared in Timis County between 2009 and 2010. All these animals were female, from non-business growing units (household type), and were declared free from all other diseases monitored under the Program of supervision, prevention, control and eradication of animal diseases, those transmitted from
animals to humans, animal welfare and environmental protection of ANSVSA. Situation about herds was provided by DSVSA Timis.

Blood samples were collected from the jugular vein, in EDTA vacutainers, between July 2009 and November 2010.

Clinical examination was performed both in BLV(+) and BLV(-) cattle, and covered both general and specific issues - physical and functional of the main systems. This exam was the criterion for inclusion/exclusion in the study of BLV(-) cattle, only healthy animals being subsequently subject of the hematological examination.

Quantitative hematological examination was conducted using MB-1830 automated analyzer (Maysun Technology Co., Ltd., R.P. China).

Qualitative hematological examination. Smears were made by the classical method (8), and for staining we used Diff-Quik stain, in standard technique (9). Smears were examined using Optika B-352 microscope (Optika SRL, Italy), and differential white blood cell count was determined by counting of 200 cells (8).

Results and discussions

From the values presented in table 1, we can see that the total number of white blood cell and lymphocyte count were significantly higher (P ≤ 0.05) in BLV(+) cattle compared with the BLV(-) ones. Other hematological parameters monitored suffered some variations depending on status of positivity for EBL, but the differences lacked statistical significance. We mention, however, a slight increase in the number of monocytes and the reduced number of granulocytes in BLV(+) cattle.

Table 1

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Units</th>
<th>BLV(+) cattle</th>
<th>BLV(-) cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \bar{x} \pm Sx )</td>
<td>( \bar{x} \pm Sx )</td>
</tr>
<tr>
<td>RBC ( x10^6/µl )</td>
<td></td>
<td>6.4±1.1</td>
<td>6.7±1.5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td></td>
<td>31.7±6.3</td>
<td>35.2±6.4</td>
</tr>
<tr>
<td>Hgb (g/dl)</td>
<td></td>
<td>10.1±1.5</td>
<td>10.3±2.1</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td></td>
<td>46.8±7.1</td>
<td>49.2±6.8</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td></td>
<td>14±3</td>
<td>15±1</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td></td>
<td>34±2</td>
<td>33±2</td>
</tr>
<tr>
<td>RDW (%)</td>
<td></td>
<td>16±1</td>
<td>17±3</td>
</tr>
<tr>
<td>WBC ( x10^7/µl )</td>
<td></td>
<td>12.9±7.2*</td>
<td>8.3±2.7*</td>
</tr>
<tr>
<td>Lymphocytes ( x10^3/µl )</td>
<td></td>
<td>11.5±6.6*</td>
<td>4.9±1.6*</td>
</tr>
<tr>
<td>Monocytes ( x10^3/µl )</td>
<td></td>
<td>0.9±0.3</td>
<td>0.7±0.3</td>
</tr>
<tr>
<td>Granulocytes ( x10^3/µl )</td>
<td></td>
<td>5.7±2.0</td>
<td>6.1±1.7</td>
</tr>
</tbody>
</table>
Platelets x10^5/µl

<table>
<thead>
<tr>
<th></th>
<th>BLV(+) cattle</th>
<th>BLV(-) cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x ± Sx (%)</td>
<td>Limits (%)</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>67.25±38.72 a</td>
<td>39.5-85</td>
</tr>
<tr>
<td>Monocytes%</td>
<td>4.26±3.51</td>
<td>0.3-10</td>
</tr>
<tr>
<td>Neutrophils%</td>
<td>28.04±17.19 b</td>
<td>13.5-59</td>
</tr>
<tr>
<td>Eosinophils%</td>
<td>3.81±2.44</td>
<td>0.1-8</td>
</tr>
<tr>
<td>Basophils%</td>
<td>0.33±0.12 c</td>
<td>0-0.5</td>
</tr>
</tbody>
</table>


There are statistically significant differences (p \leq 0.05) between values marked with the letters "a, b, c".

These differences are evident after interpretation of the differential white blood cell count: the proportion of lymphocytes is superior in BLV(+) cattle (Table 2).

Analyzing the two blood parameters for which significant differences were obtained between BLV(+) and BLV(-) cattle - the number of white blood cells and the number of lymphocytes - it appears that individual variations were very high (coefficient of variation, CV = 55% for the total number of white blood cells, and 57% for the number of lymphocytes). Based on these individual variations, BLV(+) cattle could be classified into two categories (Fig. 1):

- **aleukemic cattle** (AL), n=17, where the two parameters were under the normal upper limit, specified by the literature: 12,000 leukocytes/µl and 7500 lymphocytes/µl (3, 4);
- **cattle with leukemia / persistent lymphocytosis** (LP), n=10, where the two parameters exceeded the upper physiological limit.
Fig. 1. The number and proportion of aleukemic and with persistent lymphocytosis BLV(+) cattle

As can be seen from the following table, most of the BLV(+) cattle were in the state of "carrier", without leukemia, other hematological changes or clinical manifestation.

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Units</th>
<th>BLV(+) cattle (n=27)</th>
<th>BLV(-) cattle (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LP (37.04%)</td>
<td>AL (62.96%)</td>
</tr>
<tr>
<td>WBC</td>
<td>x10^3/µl</td>
<td>16.3±3.03^a.b</td>
<td>9.4±1.3^c</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>x10^3/µl</td>
<td>9.6±2.1^c.d</td>
<td>5.7±1.09^c</td>
</tr>
</tbody>
</table>

Legend: WBC – white blood cells, AL – aleukemic cattle; LP – cattle with persistent lymphocytosis
There are statistically significant differences (p ≤ 0.05) between values marked with "a, b, c, d"

For the remaining ten BLV(+) animals hematologic stage of EBL manifested, in addition to evident leukocytosis and lymphocytosis, by slightly reducing milk production, mastitis (three animals), breathing problems, manifested by dyspnea (two cows), a slight hypertrophy of superficial lymph nodes (two animals) and microcytic anemia (a cow: 4.9x10^6 RBC/µl and Hgb - 7.8 g/dl). These clinical manifestations are nonspecific. However, correlating the WBC and lymphocytes values (below 20,000 leukocytes /µl and 12,000 lymphocytes / µl) and absence of anemia in most BLV (+) cattle, it can be said that the animals were evaluated in the early stage of the disease. Moreover, the literature reported values higher than those obtained by us, both for cases with persistent lymphocytosis and cattle with neoplasms (between 15,000 and 55,000 leukocytes/µl and 12000-35000 lymphocytes/µl) (1, 2, 6, 7).

The proportion of aleukemic cattle and with persistent lymphocytosis is similar to the data reported in the literature, which emphasizes the predominance of animals without specific hematologic changes (5, 6, 10).
Conclusions

Most BLV(+) cattle assessed were in the early stage of the disease, namely the virus carrier, with nonspecific clinical manifestations or hematologic changes. Hematological parameters investigated reached similar values BLV(+) aleukemic cattle and BLV(-) animals. Among hematological parameters evaluated, the number of leukocytes and lymphocytes in BLV(+) cattle with persistent lymphocytosis was significantly higher compared with the values obtained in aleukemic BLV(+) and BLV(-) cattle. Clinical manifestations of hematologic status of enzootic bovine leukemia are nonspecific.

References

9. ***Diff-Quik stain set*64851
INVESTIGATION ON COMPARATIVE VALUES OF AGID AND ELISA USED IN DIAGNOSIS OF ENZOOTIC BOVINE LEUKOSIS

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Summary

The purpose of this study was to present comparative values of two serological tests, AGID and ELISA, applied in diagnosis of enzootic bovine leukosis in Timis County. 460 biological samples (sera) were assessed using Kit for serological diagnosis of EBL (SN "Pasteur Institute" SA, Bucharest) and ELISA Enzootic Bovine Leukosis Virus (BLV) Antibody Test (IDEXX Laboratories, Inc., Netherlands). For each test, it was determined the proportion of positive and negative results related to the age and breed of animals, type of growing units (households or private farms), locality, and relative to total samples examined. We also determined the quality parameters – sensitivity, specificity, positive predictive value, negative predictive value – and apparent prevalence, true prevalence, Cohen's kappa coefficient, Youden's index, and correlation between the two tests.

ELISA test allowed detection of a higher number of animals with enzootic bovine leukosis, comparing to AGID test, taken as reference. AGID does not allow detection of BLV(+) status in cattle with low titers of anti-gp 51 antibodies.

Key words: enzootic bovine leukosis, AGID, ELISA, Timis County

Enzootic bovine leukosis (EBL) is neoplastic disease produced by a specific virus and, also, is one of the most serious diseases of cattle. Economic losses associated with EBL are particularly important and relate to morbidity, mortality, decreased milk and meat production, costs of prevention and control measures, including compensation, restrictions on the sale and export of milk and meat etc. (8, 11). In addition to these aspects, there is a real or potential risk of transmitting the virus to other species of economic interest, such as sheep and buffalos, and in humans (bovine leukemia virus is related to T-lymphotropic virus that causes leukemia in humans) (2).

Establishing the early diagnosis is essential for an effective control in EBL, for this purpose being applied enzyme linked immunosorbent assay (ELISA on serum samples and milk) and agar gel immunodiffusion test (AGID). These methods led to the eradication of EBL in many European countries, such as Denmark (1990), Spain (1994), Cyprus (1995), Ireland (1997), Luxembourg (1999), Austria (2001), Switzerland (2005), United Kingdom (2006), Slovenia (2006), The Netherlands (2007), Sweden (2007) and Slovakia (2008) (16).
Also in Romania, from the first outbreaks of EBL, were issued several laws that regulate the prevention, control and the official diagnosis methodology.

This study represents a comparative assessment of two serological tests, ELISA and AGID, tests commonly used in the diagnosis of enzootic bovine leukosis.

**Materials and methods**

For this purpose, 460 biological samples (sera), obtained from an equal number of cattle, were analyzed. We assessed bovine population from 11 localities of the Timis County where, in 2009 or earlier, were declared outbreaks of EBL.

All these animals were female, were from private growing units, such as households and private farms, and were aged between two and ten years.

Samples were collected from the following breeds: Romanian Spotted cattle and its half-breeds (RSC), Romanian Black Spotted cattle and its half-breeds (RBSC), Holstein Friesian (HF) and HF-RBSC.

Blood samples were taken from the jugular vein during July-November 2009. After collection, blood samples were maintained at room temperature for one hour, in order to express the serum, which was separated with Pasteur pipettes. The sera were then transferred into labeled microtubes, and were kept in a freezer (-20°C) until processing.

For processing the samples, the following kits were used: *Kit for serological diagnosis of EBL* (SN "Pasteur Institute" SA, Bucharest) and *ELISA Enzootic Bovine Leukosis Virus (BLV) Antibody Test* (IDEXX Laboratories, Inc., Netherlands). Working technique followed the instructions provided by the manufacturers of diagnostic kits.

For each test, it was determined the proportion of positive and negative results related to the age and breed of animals, type of growing units (households or private farms), locality, and relative to total samples examined. The data obtained were processed and interpreted using Excel (Office 2007) and SPSS 7.5 software (SPSS Inc., IBM Corporation, NY, USA), applying the *t test*.

However, due to the fact that the diagnosis of a disease is an imperfect process, which can be expressed in terms of probability rather than in terms of certain, we also determined the quality parameters – sensitivity, specificity, positive predictive value, negative predictive value – of *Enzootic Bovine Leukosis Virus (BLV) Antibody Test* compared with *Kit for serological diagnosis of EBL*, which was considered reference test.

Also, apparent prevalence (% positive samples only by ELISA) and true prevalence (% positive samples by ELISA and AGID), Cohen's kappa coefficient (shows agreement between the two tests: was calculated using SPSS 7.5 software), Youden’s index (shows correlation between sensitivity and specificity; was calculated using the formula: sensitivity+specificity-1), and correlation between AGID and ELISA were determined.
Results and discussions

Comparative assessment of AGID and ELISA tests in terms of their value in the EBL diagnosis showed clear superiority of immunoenzymatic technique in relation to all monitored parameters (type of growing unities, age and breed of cattle). This finding is supported other authors (1, 3, 4, 5, 7, 9, 10, 12, 15).

As shown in Table 1, ELISA test allowed detection of 54 positive samples out of 460 examined (44 in households and 10 in private farms) with 11 more than the number of samples identified as positive by Kit for serological diagnosis of EBL (SN "Pasteur Institute" SA, Bucharest): only 43 positive sample, from which 34 in households and 9 in private farms.

<table>
<thead>
<tr>
<th>Type of farm</th>
<th>No. of assessed cattle</th>
<th>ELISA+ID+</th>
<th>ELISA+ID-</th>
<th>ELISA-ID+</th>
<th>ELISA-ID-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Households</td>
<td>355</td>
<td>34</td>
<td>9.57</td>
<td>10</td>
<td>2.81</td>
</tr>
<tr>
<td>Private farms</td>
<td>105</td>
<td>9</td>
<td>8.57</td>
<td>1</td>
<td>0.95</td>
</tr>
<tr>
<td>Total</td>
<td>460</td>
<td>43</td>
<td>9.34</td>
<td>11</td>
<td>2.39</td>
</tr>
</tbody>
</table>

The difference of 11 samples (2.39%) is explained by the lower level of antibody titer of them (OD=1.801±0.007 and %E/P=100.75±0.34 compared with OD=2.61±0.55 and %E/P=146.52±31.08 for medium and strongly positive sera), which cannot be detected by immunodiffusion test. The lower limit of detection for AGID corresponds to 2.036 OD and 113.8% E/P values obtained by ELISA.

ELISA test superiority is also reflected in relation to age categories of tested cattle, allowing EBL detection, supplementary to the AGID, in 3 animals aged 2-3 years, 4 cattle aged 3-4 years old, 2 animals aged 4-5 years and one aged 5-6 years and 9-10 years. The majority of false negative results by AGID were registered in animals belonging to age groups 3-4 years and 2-3 years. In the category of 9-10 years, AGID did not detect any positive animals. This confirms previous findings that the AGID detect a much smaller number of BLV(+) cattle in the upper age categories, compared to ELISA (13), and that the maximum effectiveness of AGID occurs for age ranges between 4 and 7 years (7, 12, 13).

Regarding breed of cattle from households, a significant difference was recorded in RSC breed (16 animals AGID+ in comparison with 21 ELISA+ cattle), and the smallest difference in RBSC breed (a difference of only two positive samples by ELISA).

Table 2 summarizes results of the two methods, data necessary for the calculation of ELISA quality parameters. The columns refer to the standard test (AGID) and the data on the row, to ELISA.
Table 2

<table>
<thead>
<tr>
<th></th>
<th>AGID +</th>
<th>AGID -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA +</td>
<td>a = 43</td>
<td>b = 11</td>
<td>a+b = 54</td>
</tr>
<tr>
<td>ELISA -</td>
<td>c = 0</td>
<td>d = 406</td>
<td>c+d = 406</td>
</tr>
<tr>
<td>Total</td>
<td>a+c = 43</td>
<td>b+d = 417</td>
<td>N = a+b+c+d = 460</td>
</tr>
</tbody>
</table>

Legend:  
- a = number of positive samples;  
- b = number of false positive samples;  
- c = number of false negative samples;  
- d = number of negative samples;  
- N = total number of assessed samples.

After applying the standard formulas, the following values were obtained:
- Sensibility = 100%
- Specificity = 97.36%
- Positive predictive value (VPP) = 79.62%
- Negative predictive value (VPN) = 100%

The results are comparable to those presented by Sălceanu (14) following the evaluation of the performance of *ELISA Enzootic Bovine Leukosis Virus (BLV) Antibody Test* (IDEXX Laboratories, Inc., Netherlands), compared with AGID, on a number of 79,749 serum samples obtained from Iassi County. Also in our country, Pop (13) determined similar values of the quality parameters for another blocking ELISA kit, based also on the detection anti-gp 51 (ELISA Ingezim BLV Compac).

The apparent prevalence was calculated at 2.39%, the true prevalence at 9.34%, and the correlation between AGID and ELISA was 89.62%, comparable to values reported by other authors (3, 9, 10, 15).

Kappa coefficient, which determines the agreement degree for two tests that classify the results into two or more exclusive categories, such as infected or uninfected animals, was 0.80, value considered adequate according to standard assessment scale (6): strength of agreement <0 = mismatch, 0 to 0.20 = poor, from 0.21 to 0.40 = fair, 0.41 to 0.60 = moderate, 0.61 to 0.80 = good, 0.81 to 1 = very good.

Youden's index (probability of correct classification independent of prevalence) had a value of 0.97, close to the optimal upper limit (Y=1), which demonstrates a high diagnostic value of *ELISA Enzootic Bovine Leukosis Virus (BLV) Antibody Test* (IDEXX Laboratories, Inc., Netherlands).
Conclusions

AGID does not allow detection of BLV(+) status in cattle with low titers of anti-gp 51 antibodies, regardless of growing units type, age or breed of assessed animals.

ELISA test allowed detection of a higher number of animals with enzootic bovine leukosis, comparing to AGID test, taken as reference.

Higher proportion of BLV(+) cattle in the 3-5 years age group suggest a predisposition to natural infection with bovine leukosis virus at this age.

ELISA Enzootic Bovine Leukosis Virus (BLV) Antibody Test (IDEXX Laboratories, Inc., Netherlands) is characterized by high sensitivity and specificity (100%, and 97.36%), showing a high degree of agreement with the reference AGID test (Kappa coefficient = 0.8).

Predictive values of the Enzootic Bovine Leukosis Virus (BLV) Antibody Test (IDEXX Laboratories, Inc., Netherlands) recommends it for use as a diagnostic test for enzootic bovine leukosis in exported cattle and in the eradication program of this disease.

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THE IMPACT OF THE SLAUGHTERING TECHNOLOGICAL’S FLOW STAGES ON THE SALMONELLA SPP. PREVALENCE

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Summary

Salmonelosis is one of the most frequent zoonosis, causing major issues worldwide. In order to manage correctly this zoonosis, related to pork products consumption, control measures must be applied simultaneously, at all production levels, from producer to consumer.

This study intended to highlight the critical stages of the slaughter, in order to reduce carcass contamination with Salmonella spp. Firstly, the study has focused on the microbial load from reception, by performing the microbiological examination of stool samples (collected from the waiting area). Secondly, samples were collected from the surface of the carcass during the seven steps slaughter (bleeding, scalding, plucking, scorching, polishing, evisceration, refrigeration). In order to detect Salmonella, the ISO 6579/2002 technique has been used. The prevalence of Salmonella spp. after examination was 33.3% in the waiting area, after bleeding was 86.6% and 0% after scalding and scorching. During depilation, polish and evisceration, the contamination level increased to 46.6%, 40%, respectively to 80%, in order to decrease again to 33.3% after fast refrigeration.

The results of the study show that on the waiting area, carrier pigs represent a source of infection with Salmonella spp. for non-carrier pigs which are slaughtered. The presence of Salmonella spp. on the carcass may be due to cross-contamination during slaughter.

Key words: microbiological, cross-contamination, Salmonella spp., slaughter.

Salmonelosis is one of the main causes of food poisoning, in most European countries (14, 20). 25% of all Salmonelosis cases diagnosed on humans is caused by pork products consumption (6, 8, 9).

Carrier pigs represent one of the main sources of Salmonella spp. contamination of the carcasses during the technological flow (1, 2, 4, 5). Pigs may test positive for Salmonella organisms found on their skin. Even if strict hygiene standards are applied during the processing of the carcass, cross-contamination with Salmonella positive and Salmonella negative carcasses may occur. The main contamination sources are carrier pigs and environment (surfaces and staff equipment) (5).

The porpouse of the different supervising programmes is to detect the source of Salmonella spp. organisms and the level of contamination. Reducing the presence of Salmonella on the surface of the carcass and on the resulting products
requires monitoring and intervention at farm level (11). Data on the prevalence of different *Salmonella* serotypes on the surface of carcasses can be found in multiple studies.

Hald et. al. (2003) reported a *Salmonella* prevalence varying from 0 to 8.5% out of 1623 carcasses used for the study, which was performed in five different countries. A study carried out by EFSA (26 countries, 5736 samples collected from carcasses) reported a prevalence varying from 0 to 20% (19). The most common serotype in both studies was *S. typhimurium*.

The porpouses of this work is to investigate the dynamics of the *Salmonella* organisms along the slaughter line and to identify possible contamination sources of the carcasses.

**Materials and methods**

The samples were collected in a slaughter house processing 500-600 pigs/hour and 5000-6000 pigs/day. The waiting time before slaughter was as short as possible (not more than 12 h).

150 stool samples were collected, for 6 months, from pig pens around the waiting area (5 from each farm). The samples have been analyzed according to ISO 6579:2002 methods.

Following the same principle, samples have been collected from the surface of the carcasses, in each step of the slaughter (bleeding, scalding, plucking, scorching, polishing, evisceration, refrigeration). The samples prelevation (150 samples) was performed according to ISO 17604:2003, and the processing of the samples, according to ISO 6579:2002.

**Results and discussions**

Analysis of feces from the waiting area revealed a prevalence of 33.3%, caused by the presence of *Salmonella spp.* carrier pigs, which represent a source of contamination to non-carrier pigs.

During the main stages of the technological flow, after analyzing the samples collected from the surface of the carcasses, the prevalence of *Salmonella spp.* varied such as:

- immediately after stunning, the prevalence was of 86.6%, because the pigs were in contact with the hanging tables;
- after scalding, the prevalence was 0%, due to the temperature of 60-62°C of the water;
- after plucking the prevalence increased up to 46.6%, possibly because of the depilation machine, which causes feces to be eliminated from the rectal ampulla;
- after scorching, by respecting the required temperature and time, the prevalence decreased to 0%;
after polishing – 40%;
- after evisceration – 80%.

An increase of the *Salmonella* spp. could be noticed in the last stages, possibly because of cross-contamination related to staff and equipments.

Before delivering the carcass, due to low temperatures during refrigeration (4°C), a decrease of the *Salmonella* spp. prevalence was noticed. The values could be found in figure 1.

![Fig. 1. The presence of Salmonella on various stages of the technological flow](image)

As already stated, lairage is a high risk area for *Salmonella* cross-contamination and it has been suggested that increasing the holding time in lairage will increase this risk (20). In Belgium, slaughter pigs are kept in lairage on average 126 minutes (min 5- max 720 minutes) (7). This time is long enough for animals to become infected with *Salmonella*. However, other published experimental evidence is somehow contradictory on the effect of lairage holding time (3).

After scalding, the immersion of the carcass in a tank of water (60°C for 8 minutes) results the destruction of most bacteria on the surface of the skin (9).

Plucking, the mechanical removal of the hair by rotating drums with scraper blocks which rotate the carcass and remove the hairs, is a source of recontamination by fecal matter (5). It is well known that the plucking steps has a large potential for cross contamination of carcasses (16).
After polishing the carcasses were re-contaminated (8, 12, 17), whereas Gill and Bryant (1992) reported that the bacterial numbers decreased after polishing (8).

The contamination after polishing could be due to contamination from the environment as the polishing equipment is difficult to clean (5) and the water used is mostly cold (7).

Berends (4) estimated that, after scorching, 5-15% from contamination of the carcasses with *Salmonella* occurred during the polishing step, 55-90% during current evisceration practices and 5-35% from further processing.

Botteldoorn et al. (6) found that the levels of *Salmonella* on chilled carcasses varied between three slaughterhouses where chilled carcasses were tested. A single slaughterhouse did not have detectable *Salmonella* on its carcasses whereas at the other end of the scale 36% of carcasses were positive for *Salmonella* post-chill. In two of the abattoirs studied by Botteldoorn et al. (6) the levels of *Salmonella* decreased as a consequence of chilling. However, in the remaining plant, prevalence increased from 6% to 15%.

Mafu et al. (1989) found a high prevalence of *Salmonella* (12.5%) on the chill room floor of a Canadian abattoir, which they attributed to the “coming and going of workers” between the slaughter area (25% prevalence) and the chiller and general manual handling of carcasses.

**Conclusions**

This study shows the importance of the contamination level of the waiting area.

Although pigs can harbor *Salmonella* before stunning, it seems that the slaughterhouse is crucial in the further contamination level along the slaughter line.

During scalding and scorching a decrease of *Salmonella* spp. on the surface of carcasses could be noticed.

Plucking, polishing and evisceration caused an increasing of *Salmonella* spp.

After refrigeration the present of *Salmonella* spp. decreased.

Contamination of the carcasses during the technological flow may be caused by: animals previously slaughtered, carrier pigs, cross-contamination (staff and equipment).

**References**

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THE INFLUENCE OF SLAUGHTERING TECHNOLOGICAL FLOW’S STAGES ON THE MICROBIAL LOAD

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Summary

This study is to highlight the checking points (bleeding, scalding, plucking, scorching, polishing, evisceration and refrigeration) concerning the microbial load of pig carcasses (NTGMA and Enterobacteriaceae) and the staff’s role.

Samples have been collected from the surface of 100 carcasses, along with another 50 samples collected from the staff’s equipment. These samples have been analyzed according to SR ISO 4833/2003, respectively SR ISO 21528-2/2007.

Before scalding, NTGMA varied between 4 and 4.5 log cfu/cm², and Enterobacteriaceae were found on all the carcasses (100%). The presence of NTGMA and of the Enterobacteriaceae reduced after scalding (1.1 log cfu/cm² respectively 0.2 log cfu/cm² normal limits), after singeing (the two organisms’ presence was minimum) and refrigeration ((1.0 log cfu/cm² respectively 0.5 log cfu/cm² normal limits). The prevalence increased after plucking (2.5 log cfu/cm² respectively 1.2 log cfu/cm²), polishing (1.5 log cfu/cm² respectively 0%) and evisceration (1.7 log cfu/cm² respectively 1 log cfu/cm²)

Key words: carcasses, Enterobacteriaceae, pig, prevalence, staff.

The slaughtering process is important to assure meat safety. To prevent microbial contamination, which affects consumer health and causes serious public health problems, appropriate slaughterhouse design and internal control are necessary. The level and type of microbial contamination are monitored for maintaining and improving the hygienic status and quality of meat produced by a slaughterhouse (2).

Aerobic Plate Count (APC) and Enterobacteriaceae Counts (EBC) are generally used as hygienic indicators in the food chain (3, 6, 7, 8). The APC depicts general microbial contamination. The EBC is a marker for possible fecal contamination. Feces are a main source of pathogens, such as Escherichia coli O157:H7, Salmonella or Campylobacter (12).

The specific objectives of this study was to determine the contamination level using NTGMA and Enterobacteriaceae in the surface of pig carcasses after each of seven stages of the slaughter process (bleeding, scalding, plucking, scorching, polishing, evisceration and refrigeration) and the staff’s role.
Materials and methods

Samples were collected from 100 carcasses from stabbing, scalding, plucking, scorching, polish, evisceration to refrigeration. Another 50 samples were collected from equipments (gloves, aprons, knives).

The samples were collected according to ISO 17604/2007 and analyzed according to SR ISO 4833/2003 and SR ISO 21528-2/2007.

Results and discussions

After collection and analysis of samples, the next findings emerged:

- during bleeding, NTGMA varied from 4 to 4.5 log cfu/cm² and Enterobacteriaceae were present on all carcasses. This high microbial load of the carcasses is caused by not respecting procedures regarding hygiene in the waiting area and after stunning;
- during scalding, the load dropped to 1.1 log cfu/cm² respectively to 0.2 log cfu/cm² (within the normal limits) because of the temperature of the water used for scalding (62°C);
- during pluking, NTGMA and Enterobacteriaceae began to increase to 2.5 log cfu/cm² respectively to 1.2 log cfu/cm², possibly because of accumulation of feces in the pluking machine;
- during polishing, NTGMA had a prevalence of 1.5 log cfu/cm² while Enterobacteriaceae were within the normal limits;
- during evisceration, NTGMA had a prevalence of 1.7 log cfu/cm², while Enterobacteriaceae had a prevalence of 1 log cfu/cm² because of the incision in the gastrointestinal mass;
- on the equipments, NTGMA had a prevalence of 2.3 log cfu/cm² and Enterobacteriaceae, of 81.8% (normal limits) (fig.1).

Once the clean area is reached, the carcass may get recontaminated through procedures performed by staff, using inappropriatelly tools (not performing immersion in 82°C water) or by not respecting proper practices (not changing gloves, lack of staff training). The finding of such values was possible since the staff did not respect specific procedures.

Specha et. al. (2006) (5) raported that before scalding, NTGMA ranged from 5.0 to 6.0 log cfu/cm², and Enterobacteriaceae were detected on all carcasses. After scalding (1.9 log cfu/cm² and 12%, respectively), scorching (1.9 log cfu/cm² and 66%, respectively), chilling (2.3 log cfu/cm² and 17%, respectively) and increased (P < 0.05) after dehairing (3.4 log cfu/cm² and 100%, respectively) and polishing (2.9 log cfu/cm²).

The microbial contamination of carcasses and equipment has been studied in an industrial slaughterhouse of Iberian pigs by the Rivas et al. (4). Samples of the surface of carcasses were taken at different stages. It was demonstrated that in scalding and scorching the APC decreased (P < 0.01), while after plucking it’s...
increased (P < 0.01). The Enterobacteriaceae decreased after scalding but increased after evisceration (P < 0.001). The implementation of good manufacturing practices (GMP) in the stages of closure of the anus and evisceration significantly decreased the Enterobacteriaceae. It changed from 61.1% in carcasses without GMP that had counts higher than 1 log cfu/cm² to only 7.4% in GMP carcasses.

In another study, before and after evisceration, Inthavan et.al (1) found mean NTGMA of 4.70 and 4.85 log cfu/cm², respectively. The means of Enterobacteriaceae were 2.81 log cfu/cm² until evisceration, and 2.98 log cfu/cm² before evisceration.

The results highlight the stages of the technological flow which affect the microbial load of the carcasses. The scalding and the scorching causes a decrease of 2 log cfu/cm² compared to bleeding. Plucking, polish and evisceration cause an increase of approximately 1.5 log cfu/cm², and refrigeration causes the microbial load to register a decrease of 0.5 log cfu/cm².

The analysis of the samples taken from the equipments suggested that the values obtained were caused by not respecting hygiene and good practice rules by the staff.

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STUDY OF ANTIMICROBIAL RESISTANCE OF COAGULASE NEGATIVE STAPHYLOCOCCI STRAINS ISOLATED FROM DOGS AND CATS

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Summary

This study aimed to determine the frequency as well as the antimicrobial susceptibility profile of coagulase negative staphylococci isolated from cats and dogs. Of the 52 skin and ear effusion samples were collected isolated 28 (53.84%) strains of coagulase negative staphylococci. The isolated strains were identified by biochemical tests and characterized by their susceptibility to antimicrobial agents. The coagulase negative species identified were as follows: S. epidermidis (23.07%, 12/52), S. haemolyticus (13.46%, 7/52), S. chromogenes (9.61%, 5/52) and S. sciuri (7.69%, 4/52). High rates of resistance to amoxicillin, penicillin G, tetracycline and lincomycin were observed, what is probably due to the frequent use of these antibiotics in veterinary practice. A 39.28% (11/28) percentage of the strains has shown multi-drug resistance. All strains were susceptible to vancomycin, ciprofloxacin, ampicillin with sulbactam, cephalaxin and chloramphenicol. These result indicate the occurrence of resistant staphylococci and point to the need of careful selection of antibiotics based on results of the susceptibility testing, in order to reduce the selection of multiresistant strains.

Key words: staphylococci, coagulase-negative, dog, cat

Coagulase-negative staphylococci have long been regarded as apathogenic but their important role as pathogens and their increasing incidence have been recognized and studied in recent years (5, 6, 10). Members of Staphylococcus genus are often found on healthy and diseased dogs, producing a variety of infections, including bacteraemia, pneumonia, furuncles, abscesses, pyoderma, conjunctivitis and otitis externa (4, 7, 8, 9). Many organisms vary in their susceptibility to antibiotics. Some strains become resistant after wide usage over a period of time (3, 8). Also the overusage of a drug, frequently determined by small animal practitioners without microbiological culturing or antibiotic sensitivity testing may contribute to the emergence of resistant strains of bacteria (2, 3, 7, 9).
Materials and methods

Specimens were obtained from samples of 52 adult dogs of both sexes submitted to the Infectious diseases and Preventive medicine Department of Faculty of Veterinary Medicine Timisoara from veterinary clinics in Timisoara for identification and susceptibility testing between march of 2012 and august 2012. Although detailed historic of clinical cases were not provided, specimens were collected before treatment of the dogs. Animals selected for this study had no known history of prior antibiotic therapy.

The plates were incubated under aerobic conditions for 24 to 48 h at 37°C. Detection of coagulase-negative staphylococci (CoNS) was achieved by conventional methods, including hemolysis patterns on blood agar, the coagulase tube test (Merk, Germany), and detection of clumping factor and protein A by latex agglutination with a Bactident Staph (BioMerieux, France).

After growth, staphylococcal isolates were identified according to their characteristics as outlined in Bergey's Manual of Determinative Bacteriology (1) and the Manual of Clinical Microbiology (10).

After growth on sheep blood agar, coagulase-negative staphylococci was identified on the basis of colony characteristics, Gram stain, pigment production, acid production on D-mannitol, free coagulase and the slide test for detection of clumping factor. Identificarea definitivă a speciilor de stafilococi coagulază negative se poate realiza în diferite moduri.

Similarities were discriminated using the API Staph identification kits.

The antibiotic sensitivity were tested for susceptibility to eighteen commonly used antibiotics through Kirby-Bauer disk diffusion technique. Required to obtain bacterial suspension for antibiogram, four to five identical colonies were incubated in 5 ml of Mueller-Hinton broth (Oxoid) for 2 hours at 37 °C, then with bacterial suspension was plated on Mueller-Hinton agar (Merck, Darmstadt, Germany) by diffusion method, followed by the addition of comercial antibiotic discs: ciprofloxacín, 30 μg; novobiocin, 30 μg; tetracycline, 30 μg; gentamicin, 10 μg; amoxicillin-clavulanic acid, 30 μg; lincomycin, 30 μg; ceftriaxone, 30 μg; cefaclor, 30 μg; polymyxin B, 50 IU; erytromycin, 15 μg; doxycycline, 30 μg; ampicillin/sulbactan, 30 μg; methicillin, 30 μg; vancomycin, 30 μg; cefoxitin, 10 μg; pristinamycin, 15 μg (Oxoid, Ltd., UK). Interpretation of antibiotic resistance were performed through measuring the diameter of the growth inhibition zone and the strains were categorized as sensitive, intermediate or resistant to the drug according to manufacturer’s instructions.

Results and discussions

The present study confirms the occurrence of coagulase negative staphylococcal strains in otitis externa and various skin diseases of dogs and cats, due to the isolation of 28 strains of Staphylococcus from ear discharge and skin
samples of 52 dogs and cats, which represents a rate of 53.84% growth positivity (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Coagulase</th>
<th>n (isolates)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. epidermidis</td>
<td>Negative</td>
<td>12</td>
<td>23.07%</td>
</tr>
<tr>
<td>Staph. haemolyticus</td>
<td>Negative</td>
<td>7</td>
<td>13.46%</td>
</tr>
<tr>
<td>Staph. chromogenes</td>
<td>Negative</td>
<td>5</td>
<td>9.61%</td>
</tr>
<tr>
<td>Staph. sciuri</td>
<td>Negative</td>
<td>4</td>
<td>7.69%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>28 (53.84%)</strong></td>
<td></td>
</tr>
</tbody>
</table>

With regard to the susceptibility to antimicrobial agents of the strains isolated in this study, a high frequency of resistant strains was observed, since 90.9% of the isolates were resistant to at least one drug. Multidrug resistance was a common finding and 11 strains of CoNS (39.28%) showed resistance to two or more different drugs (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Resistant samples</th>
<th>Antimicrobial resistance pattern, n (isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. epidermidis</td>
<td>4/12 (33.33%)</td>
<td>TE, AMC, PB, DO, CN (1), DO, AMC, E, PB, L (1), TE, PB, E (1), PB, L, AMC (1)</td>
</tr>
<tr>
<td>Staph. haemolyticus</td>
<td>3/7 (42.85%)</td>
<td>TE, ME, AMC (1), AMC, PB, DO, E (1), L, AMC, TE, PB, CN (1)</td>
</tr>
<tr>
<td>Staph. chromogenes</td>
<td>2/5 (40.00%)</td>
<td>TE, AMC, CN, PB (1), DO, PB, CN, E (1)</td>
</tr>
<tr>
<td>Staph. sciuri</td>
<td>2/4 (50.00%)</td>
<td>DO, AMC, ME, PB (1), AMC, E, TE, L, CN (1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>11/28 (39.28%)</td>
<td>TE, AMC, PB, DO, CN (1), DO, AMC, E, PB, L (1), TE, PB, E (1), PB, L, AMC (1), TE, ME, AMC (1), AMC, PB, DO, E (1), L, AMC, TE, PB, CN (1), TE, AMC, CN, PB (1), DO, PB, CN, E (1), DO, AMC, ME, PB (1), AMC, E, TE, L, CN (1)</td>
</tr>
</tbody>
</table>


All strains were susceptible to vancomycin, ciprofloxacine, ampicillin with sulbactan, cepahlexin and chloramphenicol.

The present study confirms the occurrence of coagulase negative staphylococcal strains in otitis externa and various skin diseases of dogs and cats, due to the isolation of 28 strains of Staphylococcus from ear discharge and skin samples of 52 dogs and cats, which represents a rate of 53.84% growth positivity.

Coagulase-negative species were most commonly found, and the most frequently isolated staphylococcus species were Staph. epidermidis what agrees
with other researchers (1, 4, 5, 10). Other species, such as *Staph. haemolyticus* were also isolated (7).

Coagulase-negative staphylococci (*S. haemolyticus, S. chromogenes* and *S. sciuri*) were isolated with lower frequency, which is in agreement with other studies that have isolated these species from canine skin diseases (4, 8, 9). Coagulase negative staphylococci species are common inhabitants of the skin endogenous microflora of healthy dogs and can act as opportunistic pathogens (8).

Resistance to antibiotics was frequently observed, with 90.9% of the isolates showing resistance to at least one drug (7). The most active antimicrobial agents against staphylococci isolated from otitis externa of dogs were rifampin and oxacillin. Multidrug resistance was a common pending, and one strain of *Staph. haemolyticus* species, was resistant to all tested antimicrobial agents (2, 6).

A similar percentage of multi-drug resistant strains from canine pyoderma was obtained by Ganiere et al. (3). However, a more recent study has shown a higher rate (94.9%) of multi-drug resistant strains isolated from canine pyoderma (9).

The multi-drug resistant strains emergence is a phenomenon that is occurring worldwide and that has hindered the treatment of human and animal staphylococcal infections.

Conclusions

The coagulase-negative species were the most prevalent in dogs and cats ears and skin.

Coagulase-negative species as a group constitute a major component of the normal microflora of dogs and cats and are considered as important opportunistic pathogens in those species. Nevertheless, in contrast with humans, the presence of these staphylococcal species in animals have received little attention to date are necessary further studies in order to evaluate the real role of those species in the aetiology of the major infections of the dog.

The results indicate the occurrence of resistant staphylococci on dogs with various skin diseases and that canine host may contribute to the maintenance and dissemination of drug resistant staphylococci in our midst. They also warn about the need of a careful selection of antibiotics based on results of susceptibility testing, to reduce the selection of multi-drug resistant staphylococci strains.

This study highlights the emergence of CoNS and once more emphasizes the need for bacterial culture with species identification and susceptibility testing in order to choose appropriate antimicrobial agents.
References


ANTIBIOTIC RESISTANCE OF THE *STREPTOCOCCUS CANIS* STRAINS ISOLATED FROM DOGS

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Summary

This investigation provided data on occurrence of antimicrobial resistance in important pathogenic bacteria from dogs, which may be useful for the small animal practitioner. Resistance was high to the compounds that were most often used, but unfortunately, these compounds were broad-spectrum. Were tested 34 strains of *Streptococcus canis* isolates from dogs with different skin disorders. Bacterial resistance was tested for susceptibility to eight commonly used antibiotics through Kirby-Bauer disk diffusion technique; using commercially available discs. No resistance was observed to ciprofloxacin, novobiocin, ceftriaxone, cefaclor, cefoxitin, pristinamycin and ampicillin/sulbactam. Drug resistance was observed toward polymyxin B (82.35% of the isolates) lincomycin (61.76% of the isolates), erythromycin (52.94%), tetracycline (50.00%), kanamycin (44.11%), gentamycin (35.29%), doxycycline (32.35%), amoxicillin/clavulanic acid (23.52%), methicillin (5.88%), rifampicin (2.94%) and vancomycin (2.94%), respectively.

Data on resistance and usage may form a background for the establishment of a set of recommendations for prudent use of antimicrobials for companion animals.

**Key words:** streptococci, bacteria, antimicrobial, dog

*Streptococcus* spp are gram-positive bacterial cocci that often appear in pairs or chains in routine Gram stains, cytologic preparations, and histologic sections (2, 5, 6, 7, 9). *Streptococcus* spp are easily cultivated, catalase negative, and facultative to strict anaerobes that are categorized on the basis of their hemolytic pattern on blood agar as α-hemolytic, β-hemolytic, or γ-hemolytic (nonhemolytic). Species of β-hemolytic *Streptococcus* are typically pathogenic (5, 9).

*Streptococcus* spp are common opportunistic pathogens of mammals and are associated with a variety of diseases affecting multiple organ systems (1, 4, 10, 12).

*Streptococcus canis* is a Group G beta-hemolytic species of *Streptococcus* and is important to the skin and mucosa health of cats and dogs, however under certain circumstances these bacteria will cause opportunistic infections (9, 10, 12).
Knowledge about the resistance patterns of bacteria to antimicrobial drugs requires constant actualization. Although the impact of veterinary usage of the drugs is unknown, empiric treatments carried out by practitioners without assistance of bacteriological culture and antimicrobial susceptibility test has contributed for the selection of multiresistant strains.

**Materials and methods**

Specimens were obtained from samples of 52 adult dogs of both sexes submitted to the Infectious diseases and Preventive medicine Department of Faculty of Veterinary Medicine Timisoara from veterinary clinics in Timisoara for identification and susceptibility testing between march of 2012 and august 2012. Although detailed historic of clinical cases were not provided, specimens were collected before treatment of the dogs. Animals selected for this study had no known history of prior antibiotic therapy.

Swabs were immediately inoculated on 5% sheep blood agar (Biomedics, Madrid, Spain). The plates were incubated under aerobic conditions for 24 to 48 h at 37°C.

Colonies that showed hemolysis and that were found to be facultatively anaerobic, gram-positive, and catalase-negative cocci (the genera *Streptococcus* and *Enterococcus*) were cultivated on esculin blood agar (Oxoid, Ltd., UK). Esculin-hydrolyzing cultures were further cultivated on kanamycin esculin azide agar (KAA; Merck, Germany) in order to differentiate *Streptococcus* from *Enterococcus* species. The Lancefield group was detected from fresh cultures by a latex agglutination test for the identification of streptococcal groups A, B, C, D, F, and G, according to the instructions of the manufacturer (Streptococcal Grouping kit; Oxoid, Ltd., UK).

All isolates suspected of being *S. canis* were identified by using the API 20 Strep identification system (bio-Mérieux, France).

The antibiotic sensitivity were tested for susceptibility to eighteen commonly used antibiotics through Kirby-Bauer disk diffusion technique. Required to obtain bacterial suspension for antibiogram, four to five identical colonies were incubated in 5 ml of Mueller-Hinton broth (Oxoid) for 2 hours at 37 °C, then with bacterial suspension was plated on Mueller-Hinton agar (Merck, Darmstadt, Germany) by diffusion method, followed by the addition of comercial antibiotic discs: ciprofloxacin, 30 μg; novobiocin, 30 μg; tetracycline, 30 μg; gentamicin, 10 μg; amoxicillin-clavulanic acid, 30 μg; lincomycin, 30 μg; ceftriaxone, 30 μg; cefaclor, 30 μg; polymyxin B, 50 IU; erytromycin, 15 μg; doxycycline, 30 μg; ampicillin/sulbactan, 30 μg; methicilin, 30 μg; vancomycin, 30 μg; cefoxitin, 10 μg; pristinamycin, 15 μg (Oxoid, Ltd., UK). Interpretation of antibiotic resistance were performed through measuring the diameter of the growth inhibition zone and the strains were categorized as sensitive, intermediate or resistant to the drug according to manufacturer’s instructions.
Results and discussions

All 34 presumptive *S. canis* isolates were gram-positive, catalase-negative cocci and showed complete beta-hemolysis. In the first cultivation, all 34 *S. canis* isolates appeared to be esculin negative. However, in the second subculture all isolates exhibited an esculin-positive reaction. None of the 34 isolates grew on KAA. Serotyping showed that all 34 isolates were Lancefield serogroup G. All isolates hydrolyzed glucose, maltose, ribose, and saccharose. They were uniformly negative for inulin, lactose, mannitol, raffinose, salicin, sorbitol, and trehalose. All isolates hydrolyzed arginine, but none of the isolates hydrolyzed sodium hippurate. A total of 61.67% (21 of 34) of the isolates showed α-galactosidase activity. All isolates yielded positive reactions for β-galactosidase and pyrrolidonyl aminopeptidase, whereas all were negative for β-D-glucuronidase as well as pyrrolidonyl arylamidase activities with the API 20 Strep system.

The results of resistance pattern of 34 streptococcal strains are presented in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>Number of strains of <em>Streptococcus canis</em> isolates from dogs (n=34)</th>
<th>Susceptible</th>
<th>Intermediate susceptible</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methicillin – ME – 30µg</td>
<td>28(82.35)</td>
<td>0</td>
<td>2(5.88)</td>
<td>5(14.70)</td>
</tr>
<tr>
<td>Gentamicin – CN – 10µg</td>
<td>20(58.82)</td>
<td>2(5.88)</td>
<td>12(35.29)</td>
<td></td>
</tr>
<tr>
<td>Tetracycline – TE – 30µg</td>
<td>15(44.11)</td>
<td>4(11.76)</td>
<td>17(50.0)</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin – CIP – 30µg</td>
<td>12(35.29)</td>
<td>7(20.58)</td>
<td>15(44.11)</td>
<td></td>
</tr>
<tr>
<td>Kanamycin – K – 30 µg</td>
<td>26(76.47)</td>
<td>8(23.52)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Novobiocin – NV – 30 µg</td>
<td>14(41.17)</td>
<td>2(5.88)</td>
<td>18(52.94)</td>
<td></td>
</tr>
<tr>
<td>Doxycyclin – DO – 30 µg</td>
<td>19(55.88)</td>
<td>4(11.76)</td>
<td>11(32.35)</td>
<td></td>
</tr>
<tr>
<td>Erythromycin – E – 15 µg</td>
<td>13(38.23)</td>
<td>4(11.76)</td>
<td>17(50.0)</td>
<td></td>
</tr>
<tr>
<td>Vancomycin – VA – 30 µg</td>
<td>26(76.47)</td>
<td>7(20.58)</td>
<td>1(2.94)</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone – CRO – 30 µg</td>
<td>29(85.29)</td>
<td>5(14.70)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Polymyxin B – PB – 50UI</td>
<td>0</td>
<td>6(17.64)</td>
<td>28(82.35)</td>
<td></td>
</tr>
<tr>
<td>Rifampicin – RA – 30 µg</td>
<td>30(88.23)</td>
<td>3(8.82)</td>
<td>1(2.94)</td>
<td></td>
</tr>
<tr>
<td>Lincomycin – L – 30 µg</td>
<td>5(14.70)</td>
<td>8(23.52)</td>
<td>21(61.76)</td>
<td></td>
</tr>
<tr>
<td>Cefaclor – CEC – 30 µg</td>
<td>33(97.05)</td>
<td>1(2.94)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pristinamycin – PT – 15 µg</td>
<td>30(88.23)</td>
<td>4(11.76)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ampicillin/sulbactan – SAM – 30 µg</td>
<td>26(76.47)</td>
<td>8(23.52)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin/ clavulanic acid – AMC - 30 µg</td>
<td>21(61.76)</td>
<td>5(14.70)</td>
<td>8(23.52)</td>
<td></td>
</tr>
</tbody>
</table>

*MIC – Minimum inhibitory concentration*

The antibiotic sensitivity test showed that *S. canis* isolates from dogs were sensitive to ciprofloxacin, cefaclor, rifampicin and pristinamycin. All were sensitive or at least of intermediate sensitivity to methicillin, novobiocin, vancomycin, ceftriaxone, and ampicillin/sulbactan. All isolates were resistant or at least of intermediate sensitivity to tetracycline and gentamicin, and all were resistant to...
polymyxin B. In general, only minor differences in antibiotic sensitivities, as estimated from the inhibition zone diameters, were observed between isolates.

The present study provides data on the phenotypic properties and antimicrobial activity of 34 S. canis isolates from 52 dogs with skin disorders and otitis externa. The phenotypic characteristics, based on 18 individual biochemical tests and a commercial identification test (API 20 Strep system), correspond to those reported for S. canis by other investigators (9, 10, 12). Beta-hemolysis on blood agar and carbohydrate fermentation were consistent with earlier descriptions of S. canis (2, 6, 10).

The antibiotic resistance patterns of the S. canis isolates found in this study are generally in agreement with the findings of others researchers (3, 8, 11, 12).

Conclusions

The potential contagiousness and zoonotic risk of this microorganism should be emphasized, considering the high percentage of infected dogs that we studied. Although S. canis is rare isolated, it could present a problem for all pets, even when it is isolated from only one dog.

Data on resistance and usage may form a background for the establishment of a set of recommendations for prudent use of antimicrobials for companion animals.

References


CELLS INVOLVED IN IMMUNE RESPONSE - CIRCULATING MATURE AND IMMATURE FORMS IN CHICKEN EMBRYOS

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Summary

The purpose of this study was to present, in terms of morphometry and cariometry, all cell types, immature and mature ones, present in the blood of chicken embryos (Cobb 500 hybrids). Thus, 140 blood samples were collected daily from the jugular vein and heart of Cobb 500 chicken embryos between days 7 and 20 of incubation. Blood smears were prepared and stained using standard techniques, and cells were morphologically characterized. Also, measurements of the diameter and area of cells and their nucleus were made. Our study demonstrated the presence in the embryos' blood of all immature and mature forms of lymphocytic, monocytic, and granulocytic series. Blood cell picture of 20 days embryos shows qualitative and quantitative aspects comparable to those seen after hatching.

Key words: chicken embryo, hematology, differential blood count

In view of the fact that the chickens is an animal model widely used in various cell biology, developmental biology, immunology, neurology studies, especially in the human stem cell transplantation (7) and because of the lack of reference data in the literature regarding the development of the immune system in Cobb 500 embryos, studying cells involved in immune response become imperative.

The aim of this study was to describe, in terms of morphometry and cariometry, all cell types, immature and mature ones, present in the blood of chicken embryos (Cobb 500 hybrids) between days 7 and 20 days of incubation.

Materials and methods

Biological material for this study was represented by 140 Cobb 500 embryos obtained from S.C. Vis-Avis, Vadul Crişului, Bihor County.

For hematological examination, blood samples were differentially collected, depending on the degree of embryos development, from the jugular vein (days 7-13 of incubation) and heart (days 14-20). Blood smears were prepared (10) and stained by Leichman (2) and May-Grünwald-Giemsa (10) methods, in standard technique.

Smears were examined in terms of types and morphological characteristics of circulating and undergoing maturation cells (using optical microscope Olympus...
CX41), performing measurements of diameter and area of the immature or mature cells, and their nucleus (with BELView 6.2.3.0 software).

In addition, for the last three days of incubation (18-20), differential blood count was made.

**Results and discussions**

*7th day of incubation* can be considered a refractory phase for lymphocyte series, lymphoblasts being identified only very few in smears of the 10 embryos assessed.

Monocytic series was present with the following cell types: monoblasts, early promonocytes, and late promonocytes.

*Between 8th and 13th day* of embryonic development, we recorded a wave of lymphoblasts (especially in the days 8-11 of incubation), the appearance of mature monocytes in blood circulation and release of immature and mature forms of the granulocytic series. Thus, it could be identified, in addition to those described above the following cell types:

- mature monocytes (from the 8th day of incubation) - cells with a diameter of $14.89 \pm 2.02 \mu m$, with oval, indented or hourglass shaped nucleus, and basophilic cytoplasm containing rare eosinophilic granules and vacuoles (fig. 1-a);

![Image](image1.png)

*Fig. 1. Blood smear from eight-days-old embryo: a – monocyte; b – lymphoblasts; c – thrombocytes. Leichman stain, x 1000*

- myeloblasts (identified only between 9th and 12th day of incubation) - spherical cells, with large diameter ($17.38 \pm 1.43 \mu m$), equipped with a spherical nucleus, fine-granular chromatin, and weak basophilic cytoplasm (fig. 2-a);
promyelocytes (strictly identified within 10 to 13 days of incubation) - large cell (16.49 ± 1.03 μm diameter) with eccentric nucleus surrounded by basophilic cytoplasm rich in large, spherical, orange or blue primary granules (fig. 3-b);

metamyelocytes (identified from the 9th day of incubation) - spherical cells with a diameter of 14.21 ± 1.28 μm, indented nucleus with uniformly condensed
chromatin, and cytoplasm containing numerous primary and secondary granules (fig. 2-b);

- mature heterophils (identified from the 9th day of incubation) - spherical or irregular cells with a diameter of 13.71±1.14 μm, bi- or three-lobed nucleus, and cytoplasm containing many rod shaped orange (fig 2-c);

- mature basophils (seen from the 10th day of incubation) - spherical cells (of 11.75±0.98 μm in diameter) with irregular nucleus, partially covered by large, spherical, and intensely basophilic granules (fig. 3-c);

- mature eosinophils (identified from day 13 of embryonic development) - spherical cells (12.40±0.70 μm in diameter), bilobed nucleus and weak basophilic cytoplasm almost completely masked by intense acidophilic granules (orange or bright red) (fig. 4-b);

- macrophages (sporadically observed since the 10th day of incubation) - irregular giant cells with oval or irregular nucleus and basophilic cytoplasm with numerous vacuoles (fig. 3-a).

The presence of macrophages in the circulation of chicken embryos is reported also by other authors, from the 4th day of incubation; in this case the yolk membrane being considered the main source of these cells (5).

The release of immature and mature granulocytes in the embryos circulation (from the 9th day of incubation), corresponds to splenic granulopoiesis which, according to Oláh and Vervelde (6), starts on the 7th day of incubation. Involvement of another lymphoid organ in hematopoiesis (i.e. the spleen) is supported by the reapparition of immature erythrocytes in blood of the 12-13 days old embryos. These immature forms of erythrocytes disappeared or have become
extremely rare since the seventh day of incubation, even if erythropoiesis was taken over by the bone marrow. In addition, the frequency of red blood cell mitosis is superior between 12 and 13 day of incubation, compared with 7-11 days of embryonic development.

Another significant aspect is the presence of mature circulating monocytes, from the 8th day, which contradicts the assertion that these cells appear in the last days of incubation (5) or after hatching (8).

On days 14-17 of incubation were identified almost all cell types described above, except the lymphocytic series. The large lymphoblasts (11-14 μm in diameter) have disappeared from circulation since 15th day, their place being taken, in 16th day, by mature small and medium lymphocytes (the last ones, rare).

For these cells were registered the following parameters: cell diameter 6.91±0.52 μm and 8.78±0.61 μm (range between 6.31 and 7.31 μm, respectively 8.75 and 9.74 μm); cell area - 50.31±6.20 μm² and 67.80±5.02 μm² (range between 42.59 and 58.41 μm² and from 62.75 to 74.94 μm²); nuclear diameter – 6.04±0.64 μm and 7.18±0.37 μm (5.35-6.83 μm and 6.87-7.77 μm); nuclear area - 39.66±7.82 μm² and 53.02±6.13 μm² (between 30.50 and 48.16 μm², respectively 46.05 and 58.19 μm²).

Of immature forms of the granulocytic series, we could see only promyelocytes, until day 15, respectively metamyelocytes, until the 14th day of embryonic development.

The last three days of incubation (18-20) gave the nearest blood cell picture with the situation registered in adult chickens. Thus, a very important aspect which proves preparation for hatching is the disappearance of almost all immature stages of lymphocytic, monocytic and granulocytic series.

Regarding the morphology of blood cells involved in the immune response, it had all the features described in the literature for adult birds’ cells (fig. 5-6) (1, 5, 8, 9, 11).

Fig. 5. Blood smear from 19-days-old embryo: a – mature eosinophil; b – mature heterophil; c – mature basophil. MGG stain, x1000
It also became evident the classification of lymphocyte subpopulation according to the size: small lymphocytes (6-7 μm), medium lymphocytes (8-10 μm) and large ones (over 11 μm) (fig. 6, c and d). Among them, the small lymphocytes, considered thymus independent cells, were best represented especially in day 20 of incubation (87.31% of total lymphocytes, compared with 63.42% in day 19 and 51.9% in day 18). The large lymphocytes were numerous in day 18 (23.36%) compared to the days 19 and 20 (15.04%, respectively 3.87%).

Fig. 6. Blood smear from 20-days-old embryo: a – monocyte; b – thrombocyte; c – medium lymphocytes; d – small lymphocyte. MGG stain, x1000

Cellular and nuclear sizes were similar to those reported in literature (1, 5, 11) and are presented in Table 1.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Diameter (μm)</th>
<th>Area (μm²)</th>
<th>Diameter (μm)</th>
<th>Area (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Limits</td>
<td>Average</td>
<td>Limits</td>
<td>Average</td>
</tr>
<tr>
<td>Mature eosinophils</td>
<td>10.17-13.25</td>
<td>11.79</td>
<td>84.26-164.07</td>
<td>139.81</td>
</tr>
<tr>
<td>Mature basophils</td>
<td>10.45-13.62</td>
<td>11.83</td>
<td>88.11-170.27</td>
<td>121.66</td>
</tr>
<tr>
<td>Small lymphocytes</td>
<td>6.20-7.99</td>
<td>7.05</td>
<td>40.19-60.91</td>
<td>50.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.61-6.98</td>
<td>6.22</td>
</tr>
<tr>
<td>Medium lymphocytes</td>
<td>8.10-10.12</td>
<td>8.92</td>
<td>61.38-82.47</td>
<td>70.23</td>
</tr>
<tr>
<td>Large lymphocytes</td>
<td>12.17-15.55</td>
<td>13.28</td>
<td>115.94-243.9</td>
<td>136.72</td>
</tr>
<tr>
<td>Mature monocytes</td>
<td>11.61-17.87</td>
<td>14.65</td>
<td>94.33-261.84</td>
<td>182.03</td>
</tr>
</tbody>
</table>

| Mature monocytes         | 11.61-17.87   | 14.65      | 94.33-261.84  | 182.03     |

Table 1
Cytometry and cariometry in circulating mature cells of granulocyte, lymphocyte and monocyte series (days 18-20 of embryonic development)
Morphological stability was reflected also in the representation of cell populations involved in the immune response, their mature forms are found in large enough numbers to be possible to determine white blood cell differential count (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Day 18</th>
<th>Day 19</th>
<th>Day 20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x} \pm Sx$ (%)</td>
<td>Limits (%)</td>
<td>$\bar{x} \pm Sx$ (%)</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>37.32±1.86</td>
<td>35.33-40.02</td>
<td>48.50±2.01</td>
</tr>
<tr>
<td>Monocytes %</td>
<td>5.98±1.55</td>
<td>5.16-8.11</td>
<td>4.56±0.84</td>
</tr>
<tr>
<td>Heterophils %</td>
<td>45.74±2.50</td>
<td>42.19-49.04</td>
<td>37.54±1.20</td>
</tr>
<tr>
<td>Eosinophils %</td>
<td>8.48±0.75</td>
<td>7.65-9.71</td>
<td>7.75±1.67</td>
</tr>
<tr>
<td>Basophils %</td>
<td>2.45±0.37</td>
<td>1.94-2.80</td>
<td>1.63±0.44</td>
</tr>
</tbody>
</table>

The values obtained differ considerably from reports of Pârvu and Barna (8) for broiler chickens - 63% lymphocytes, 6.1% monocytes, 27% heterophils, 2.2% eosinophils, and 1.3% basophils - or Lucas and Jamroz (5) in Rhode Island Reds breed - 58.1% lymphocytes, 2.5% monocytes, 35.1% heterophils, 1.2% eosinophils, and 3.1% basophils. You mention that the proportions reported by the above authors correspond to adult birds.

Percentage representation of leukocyte in Cobb 500 embryos is closer, strictly in day 20, to the values obtained by Hussein et al. (3) in Balady chickens aged two weeks - 75±12% lymphocytes, monocytes 1±0.01%, 23.6±0.5% heterophils and 0.6 ± 0.03% eosinophils. Significantly different from our results, Lucas and Jamroz (5) were obtained in six weeks old Rhode Island Reds chickens- 81.5% lymphocytes, 4.5% monocytes, 10.1% heterophils, 1.5% eosinophils and 2.3% basophils - or Lokhande et al. (4) in 15 days aged broiler chickens - 83.5±1.26% lymphocytes, 8.16±1.21% monocytes, 14.5±2.43% heterophils, and 7.66±1.24% eosinophils.

All these data show considerable variation related to age and breed which, of course, even more are pronounced in embryos.

### Conclusions

The results show that Cobb 500 embryos are completely immunological immature up to and including the 7th day of incubation.

Due to the emergence of the morphologically mature cells from granulocyte and monocyte series, we can say that embryos are capable to develop a non-specific cellular immune response, albeit limited, from 8-9 days of incubation.
The presence of mature lymphocytes in the circulation shows that primary lymphoid organs of the Cobb 500 embryos reach a high degree of maturation starting to 15th day of incubation, when the embryos become able to develop a specific immune response.

Blood cell picture of 20 days embryos shows qualitative (the types and cell morphology) and quantitative (particularly the lymphocytes proportion) aspects comparable to those seen after hatching.

References

HEMATOGENIC ROLE OF BONE MARROW IN CHICKEN EMBRYOS

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Summary

The aim of this study was to establish the hematopoietic role of bone marrow in Cobb 500 embryos, and also cytometric and cariometric characterization of mature and immature cells lymphocytic, monocytic and granulocytic series. In this purpose, tibia and femur were daily harvested from 135 Cobb 500 embryos, between 12th and 20th day of incubation, and bone marrow smears were prepared. Cytomorphological features of the cells that belong to lymphocytic, monocytic and granulocytic series showed bone marrow focuses mainly on erythropoiesis, thrombopoiesis, granulopoiesis, and monocytopoiesis, the involvement in lymphopoiesis being substantially lower. Thus, Bone marrow is an important organ for polymorphonuclear and monocytes maturation in chicken embryos, maintaining, even limited compared to mammals, the role of primary lymphoid organ.

Key words: Cobb 500 embryos, bone marrow, hematopoiesis

After first studies at the beginning of the twentieth century, researchers have found that the yolk sac is the only source of hematopoietic cells in chicken embryos. After another half a century, it was claimed that yolk sac generates only once embryonic hematopoietic cells. These cells populate rudimentary lymphoid organs, including bone marrow, which is the definitive hematopoietic organ (2).

Colonization of bone marrow begins on day 10.5 of incubation and continues until hatching. Chicken embryos bone marrow consists of two compartments: the intravascular compartment, responsible for erythropoiesis and thrombopoiesis, and the extravascular one, where myelopoiesis, monopoiesis and lymphopoiesis take place (2).

The aim of this study was the establishing of hematopoietic role of bone marrow in Cobb 500 embryos, and also cytometric and cariometric characterization of mature and immature cells lymphocytic, monocytic and granulocytic series.

Materials and methods

For this study, 135 embryonated eggs of Cobb 500 hybrids were used. The eggs were obtained from S.C. Vis-Avis, Bihor County, Romania.
During the incubation period, the eggs were maintained in commercial incubators (Eggs’ Incubator IO-1P TE, S.C. ELECTROARGES, Romania) at 38°C, and 70% relative humidity, being rocked at an angle of 90° at 6h intervals.

The tibia and femur were harvested during incubation period 12-20 day, using 15 embryos for each incubation day. Bone marrow smears were prepared (8) and stained by Leichman (1) şi May-Grünwald-Giemsa (8) methods, in standard technique. Smears were examined under optical microscope Olympus CX41, in terms of morphological aspects of different types of cells. Cytometry elements - cell diameter, nuclear diameter, cell area and nuclear area - were determined using Belview 6.2.3.0 software. Measurements were made on at least 30 cells of the same type. The results were processed and interpreted using Microsoft Excel software (Office 2007).

Results and discussions

Examination of embryonic bone marrow from the 12th day of the incubation allowed the identification of all immature and mature stages polymorphonuclear and mononuclear leukocytes.

Thus, in myeloid series we identified the following types of cells:

- myeloblasts – round cells with a diameter of 19.17±1.36 μm and a relatively narrow band of weak basophilic cytoplasm, arranged around a large and round nucleus, with fine chromatin and conspicuous nucleoli (fig. 1-d);

![Fig. 1. Bone marrow smear from 16 days-old embryo: a – metamyelocyte; b – promyelocytes; c – rubriblast; d – myeloblast; e – mature heterophil; f – lymphoblast; g – mature eosinophil; h – monoblast; i – mature basophil. Leichman stain, x 1000](image-url)
promyelocytes – cells with a diameter of 17.79±0.97 μm, with eccentric nucleus surrounded by slightly basophilic cytoplasm that contains large primary granules with orange spheres look (granules size were reduced heterophil and basophil promyelocytes) (fig. 1-b and 2-a);

myelocytes – cells with a diameter of 15.13±0.57 μm, condensed nucleus, slightly basophilic cytoplasm containing primary and secondary granules (fig. 2-a);

metamyelocytes – smaller cells with a diameter of 14.8±0.44 μm, slightly indented nucleus and numerous definitive granules (fig. 2-b);

unsegmented heterophils and eosinophils – similar to mature cells, with 14.43±0.61 μm, respectively 14.68±1.02 μm in diameter, but without nuclear lobes (fig. 1-e and 3-e);

mature heterophils – spherical cells with a diameter of 15.13±0.57 μm, clear cytoplasm and 2-3 nuclear lobes partially covered by eosinophilic and rod-shaped granules (fig. 1-a);

mature eosinophils – spherical cells with a diameter of 14.07±0.51 μm, similar with heterophils, but slightly basophilic cytoplasm and bright red circular granules (fig. 1-g);

mature basophils – spherical cells with a diameter of 13.84±0.69 μm, weakly basophilic nucleus, without indentation and mostly central located, which is masked by the intense basophilic granules (fig. 1-i and 2-d).
Fig. 3. Bone marrow smear from 13 days-old embryo: a - early promonocytes; b - mature monocytes; c - lymphoblast; d - mature basophile; e - unsegmented eosinophil. MGG stain, 1000x

Regarding the monocytic series, the following maturation sequence was observed:

- monoblasts – spherical cells with a diameter of 19.17±1.84 μm and a relatively narrow band of slightly basophilic cytoplasm surrounding the circular nucleus, with granular chromatin and apparent nucleoli (similar to myeloblasts) (fig. 1-h);
- early promonocytes – large spherical cells (17.16±1.26 μm in diameter), containing well represented basophilic cytoplasm and circular nucleus with reticular chromatin (fig. 3-a);
- late promonocytes – spherical cells with a diameter of 16.51±0.97 μm and eccentric indented nucleus surrounded by basophilic cytoplasm with rare eosinophilic granules (fig. 2-c);
- mature monocytes – irregular cells (diameter 16.69±1.86 μm) with circular, oval, indented or bilobed nucleus, fine chromatin and abundant blue-gray cytoplasm containing vacuoles and/or fine eosinophilic granules (fig. 2-g - 3-b).

Lymphocytic series was underrepresented compared with granulocytic and monocytic series, being observed strictly immature cells with a diameter of 12.09±1.73 μm, basophilic cytoplasm in the form of a narrow band surrounding a large nucleus, spherical or indented, with coarse chromatin condensed into obvious blocks (fig. 1-f, 2-f and 3-c).

All of these different cell types have been identified depending on the day of incubation and the cell line, mature forms occurring in larger amounts especially in the last five days of development. Lymphoblasts were underrepresented compared to other immature cell types, confirming minimal involvement of bone marrow in lymphopoiesis, described by a number other authors (2, 4, 9).
Cellular and nuclear size corresponding to the sequence of maturation in the bone marrow of cells involved in the immune response is shown in Table 1. Due to the complex shape of the nucleus, and its partial covering by overlapping of primary and secondary granules, cariometry was not entirely practicable for all cell types.

The measurements showed similar values to those reported in the literature for immature forms of the granulocytic, monocytic and lymphocytic series in humans and mammals (5, 6, 7). Also, comparable values were obtained, both in terms of cytometry and cariometry, for mature polymorphonuclear leukocytes and monocytes from bone marrow, identified by Lucas and Jamroz (3) in White Leghorn, New Hampshire and Columbian Plymouth Rocks adult chickens.

Table 1  
Cytometry and cariometry in granulocytic, monocytic and lymphocytic series ongoing maturation in bone marrow

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Cell</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diameter (μm)</td>
<td>Area (μm²)</td>
</tr>
<tr>
<td></td>
<td>Limits</td>
<td>Limits</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>Average</td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>17.4-21.18</td>
<td>19.17</td>
</tr>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Promyelocytes</td>
<td>16.35-19.46</td>
<td>17.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myelocytes</td>
<td>14.35-15.99</td>
<td>15.13</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>Metamyelocytes</td>
<td>14.22-15.45</td>
<td>14.8</td>
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<td></td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Unsegmented</td>
<td>13.25-15.97</td>
<td>14.43</td>
</tr>
<tr>
<td>heterophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsegmented</td>
<td>12.99-15.03</td>
<td>14.68</td>
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<tr>
<td>eosinophils</td>
<td></td>
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</tr>
<tr>
<td>Mature</td>
<td>13.58-16.12</td>
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<tr>
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<tr>
<td>Mature</td>
<td>13.17-14.56</td>
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<tr>
<td>basophils</td>
<td></td>
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</tr>
<tr>
<td>Monoblasts</td>
<td>15.84-20.71</td>
<td>19.17</td>
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<td></td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Early</td>
<td>16.06-19.22</td>
<td>17.16</td>
</tr>
<tr>
<td>promonocytes</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late</td>
<td>15.14-17.72</td>
<td>16.51</td>
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<tr>
<td>promonocytes</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mature</td>
<td>14.49-19.16</td>
<td>16.69</td>
</tr>
<tr>
<td>monocytes</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lymphoblasts</td>
<td>9.7-14.48</td>
<td>12.09</td>
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</table>

As shown in Table 1, the representation of lymphocytic line has been increasingly smaller as the time of hatching approached. This finding is similar to other reports (2, 4, 9) and demonstrate that, in the last week of incubation,
lymphopoiesis occurs in other lymphoid organs, respectively thymus and bursa of Fabricius. The data suggest, however, that bone marrow is an important organ for polymorphonuclear leukocytes and monocytes maturation in chicken embryos, retaining, even limited compared to mammals, the role of primary lymphoid organ.

Conclusions

The role of bone marrow focuses mainly on erythropoiesis, thrombopoiesis, granulopoiesis, and monocytopenesis, the involvement in lymphopoiesis being substantially lower.

Mature cells appeared in larger amounts especially in the last five days of development.

Bone marrow is an important organ for polymorphonuclear and monocytes maturation in chicken embryos, retaining, even limited compared to mammals, the role of primary lymphoid organ.

References

GENERALIZED MYCOBACTERIOSIS IN A PIG RAISED FOR FAMILIAL MEAT CONSUMPTION – A CASE STUDY

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Summary

In Romania the pig breeding in private households constitute an old national tradition and, currently, it is widespread in rural areas. This case report describes a generalized mycobacteriosis in a household slaughtered pig, originating from Timiș County, Romania. Macroscopic examination of pig carcass and organs, such as liver, lungs and lymph nodes, revealed suggestive multifocal white-greyish coloured granulomatous alterations compatible with mycobacterial infections. The presumptive diagnosis was confirmed through microscopic examination of Ziehl-Neelsen stained smear, showing the presence of acid-fast bacilli in tissues. Occurrence of mycobacterial infection in extensively raised pig in rural areas highlighted the possible zoonotic risk for consumers from this region. Taking into account this considerations, further epidemiological studies based on molecular tools aimed to investigate a large number of animals, zoonotic potential of the implicated Mycobacterium species, source of the infection and risk assessment are needed. To author’s knowledge, this is the single published report about the presence of mycobacterial infection in pig in the last three decades from Romania.

Key words: pig, mycobacteriosis, rural area

It is known that the occurrence of mycobacterial infections in pigs can be attributed to bird’s origin Mycobacterium (M.) species, like M. avium or M. intracellulare (1, 4). The occurrence of disease is strongly related by the organic materials consumption of hosts and frequently meets in extensively reared pigs from rural areas. Also, the diagnosis of disorder appears as “slaughterhouse surprise” (2).

In Romania the pig breeding in backyards for household consumption is considered an important economic sector and is estimated at about nine hundred thousand pigs at the end of 2012 (unofficial data). In accordance with old national tradition, pig slaughtering in rural areas is carried out during the cold season, and especially around Christmas and New Year. Commonly, except the trichinoscopic examination for Trichinella larvae, the rigorous carcass examination by veterinary specialists during the slaughtering process is neglected. Therefore, in these areas many zoonotic pathogens can poses significant challenges and possible zoonotic risk for consumers and public health specialists.
This case study describes the presence of mycobacterial infection, as important zoonotic disease, in a private household slaughtered pig during the winter of 2012-2013.

Case presentation

In the beginning of December 2012, one owner originating from rural area of Timiș County was presented with a pig carcass slaughtered in a private household, at the Department of Food Safety and Veterinary Public Health of the Faculty of Veterinary Medicine from Timișoara, Romania for sanitary veterinary examination. Macroscopic inspection of meat and parenchymal organs such as liver, lungs and lymph nodes revealed suggestive multifocal white – greyish coloured granulomatous alterations compatible with mycobacterial infections (Fig. 1, 2 and 3). According to owner report the one-year-old Marele Alb breed female pig weighing 130 kg, was sacrificed at the day of presentation and the visible alterations were observed as “slaughterhouse surprise” at the time of evisceration. The pig was raised in backyards for familial meat consumption together with other two pigs, and was slaughtered due to the progressive weight loss and inappetence in the last period. Likewise, it is important to note that in a private household the owner has grown together with pigs and other animals, like cattle or poultry, favouring contact between livestock’s and birds.

Based on suggestive granulomatous alterations the presence of mycobacterial infection, as presumptive diagnosis, was established. In addition, microscopic examination of the Ziehl-Neelsen stained smear of organ imprint showed the presence of acid-fast bacilli in tissues (Fig. 4) and confirmed the presumptive diagnosis. Following the diagnosis establishment meat and parenchymal organs from the infected animal were confiscated and destroyed.

Materials and methods

In order to identify the presence of possible Mycobacterium pathogens presumed at the time of macroscopic examination of the carcass and parenchymal organs, Ziehl-Neelsen stained smear of meat and organ imprint was prepared and microscopically examined. Briefly, the air dried and heat fixed (10 minutes at 90 °C) slide was stained in carbol fuchsin, rinsed with tap water and decoloured with 3% hydrochloric acid in isopropyl alcohol followed by recoloration with methylene blue, and finally by the new washing. The dried smear was examined under light microscopy using oil immersion lens (magn. x100).

Results and discussions

As expected, microscopic examination of Ziehl – Neelsen coloured smear from meat and organ imprint confirmed the presumptive diagnosis determined at
the time of macroscopic examination. Therefore, the diagnosis of generalized mycobacteriosis was established. Consequently, the destruction of meat carcass and parenchymal organs was recommended.

According to the scientific literature the main causative agents of porcine tuberculosis are recognised the Mycobacterium species belonging to M. avium complex (MAC) including M. intracellulare, M. avium subsp. hominissuis and M. avium subsp. avium (4). The most important reservoir for these species are considered the wild birds, but their isolation from anorganic materials such as sawdust, soil and biofilms has been frequently reported (2, 3). The owner report emphasized the cohabitation of infected animal with other livestock and birds in the private household. Taking into account this considerations, it can be presumed that these animals, and especially birds can be considered as sources of mycobacterial infection. In support of this hypothesis can serve an earlier study carried out by
Schliesser (1967) whereby the author highlighted that species of MAC were not considered to be transmitted to pig to pig.

Occurrence of mycobacterial infection in extensively raised pig in rural areas highlighted the possible zoonotic risk for consumers from this region. Accordingly, further epidemiological studies based on molecular tools aimed to investigate a large number of animals, zoonotic potential of the implicated Mycobacterium species through molecular tools, source of the infection and risk assessment are needed. To author’s knowledge, this is the single published report about the presence of mycobacterial infection in pig in the last three decades from Romania.

Conclusions

The occurrence of mycobacterial infection (regarded as potential zoonosis) in a pig from rural area raised for familial meat consumption offers important data for veterinary authorities.

Therefore, in order to limit the spreading of disease, efficient public health strategies should be implemented by veterinary public health authorities.

References


ANTIBIOTIC SENSITIVITY OF SOME SALMONELLA SEROVARS ISOLATED FROM MEAT AND MEAT PRODUCTS

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Summary

Increasing antibiotic resistance of bacteria, especially of pathogens, including Salmonella, is a subject of global interest. Nowadays, the increasing number of Salmonella strains resistant to antibacterial substances is largely due to the intensive use of antibiotics. Based on these findings, in our research we targeted to highlight the sensitivity of 110 strains of Salmonella to antibacterial substances, strains isolated from chilled meat, minced meat and fresh sausages. Antibacterial substances studied were: amoxicillin, nalidixic acid, clarithromycin, colistin sulphate, furazolidone, gentamicin, nitrofurantoin, polymyxin B, tetracycline and trimethoprim. At the end of the experiment were found differences, most efficient antibiotics were gentamicin, ampicillin / sulbactam, trimethoprim and clarithromycin.

Key words: Salmonella, meat, antibiotic resistance

As a consequence of injudicious use of anti-infective substances in human and veterinary practice, the use of subtherapeutic doses in stimulating and prophylactic treatments, the use of residues from drugs factories in animal feed, the phenomenon of emergence and persistence of Salmonella strains resistant to multiple antimicrobials substances, strains found in meat and meat products has reached alarming levels, thus becoming a major concern of researchers around the world (1, 6, 8).

The multiple resistances to antibiotics in Salmonella serovars may present significant variations, dependent on geographical area and the use of antimicrobials substances in animal husbandry process (3).

It has to be mentioned that values of phenotypic and genotypic resistance, seen in some Salmonella serovars isolated from animals, are not similar to those found in serovars isolated on meat flow on slaughterhouses. Also, both environmental factors and staff hygiene during the meat-processing steps can have a determining effect on phenotypic and genotypic resistance of Salmonella strains isolated from meat and meat products (3, 7).

Problems caused by Salmonella strains with multiple resistance to antibiotics include difficulties in antimicrobial therapy and also allowing the development of serious diseases. For example, a survey conducted in the United States by The National Antimicrobial Resistance Monitoring System shows that the
risk of development of *Salmonella* infections in patients infected with non typhoid *Salmonella*, especially *S. Typhimurium* is two times higher than in patients infected with susceptible strains (10). Similarly, a Danish study concluded that infection with *S. Typhimurium* resistant to quinolone, has been associated with a risk three times higher in an aggressive or even fatal illness during the 90 days of infection, in comparison with the risk observed in infections with susceptible strains (2). In conclusion, we can say that antibiotics resistant *Salmonella* strains would cause infections more severe than susceptible strains, due to the presence of virulence factors on the same mobile genetic elements (9).

**Materials and methods**

*Salmonella* strains were obtained from samples taken from chilled meat, minced meat and fresh sausages, from different commercial units from the Romanian Black Sea coast. The samples were individualized and were sealed, in order to exclude the possibility of germ dissemination into the environment, and were quickly dispatched to the laboratory where they were subjected to bacteriological investigations aimed at isolation, identification and antimicrobial susceptibility according to standards. After being identified as belonging to the genus *Salmonella*, each strain was tested for antibiotic sensitivity by diffusing method on Müller-Hinton agar, according to the CLSI reference standard (4).

The antibiotics used were amoxicillin, nalidixic acid, clarithromycin, colistin sulphate, furazolidone, gentamicin, nitrofurantoin, polymyxin B, tetracycline and trimethoprim.

**Results and discussions**

Research conducted in this experiment aimed the behavior of 110 strains of *Salmonella* isolated from chilled meat, minced meat and fresh sausages to ten different antimicrobials substances.

Regarding the behavior of some *Salmonella* strains to antibacterial substances, Österblada et al. (5) have mentioned that antibiotic resistance of species belonging to the *Enterobacteriaceae* family is high and can cause serious clinical problems.

Following the experiments carried out in this study, it can be said that there were minimal differences in the sensitivity of the antimicrobial activity of the studied strains of *Salmonella*, depending on the sampling site.

Thus, after determination of antimicrobial susceptibility of *Salmonella* strains isolated from chilled meat (Table 1), there is an increased sensitivity to gentamicin (85%), ampicillin / sulbactam (80%), trimethoprim (62.5%) and clarithromycin (57.5%) and a significant resistance to furazolidone (62.5%), nalidixic acid (70%), nitrofurantoin (55%), polymyxin B (55%) and tetracycline (52.5%).
The antimicrobial susceptibility of *Salmonella* strains isolated from chilled meat

<table>
<thead>
<tr>
<th>Tested antibiotics</th>
<th>Total number of tested strains</th>
<th>Susceptible strains</th>
<th>Moderate susceptible strains</th>
<th>Resistant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nr.</td>
<td>%</td>
<td>nr.</td>
<td>%</td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>40</td>
<td>32 80</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>40</td>
<td>4   10</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>40</td>
<td>23 57,5</td>
<td>5</td>
<td>12,5</td>
</tr>
<tr>
<td>Colistin sulphate</td>
<td>40</td>
<td>11 27,5</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>40</td>
<td>3   7,5</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>40</td>
<td>34 85</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>40</td>
<td>5   12,5</td>
<td>13</td>
<td>32,5</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>40</td>
<td>10 25</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>40</td>
<td>6   15</td>
<td>13</td>
<td>32,5</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>40</td>
<td>25 62,5</td>
<td>11</td>
<td>27,5</td>
</tr>
</tbody>
</table>

For *Salmonella* strains isolated from minced meat (Table 2) sensitivity was proven, but with lower values close to those found for strains isolated from chilled meat, noting that maximum sensitivity was recorded to ampicillin / sulbactam (78%), followed by trimethoprim (70%) and gentamicin (68%). Also, a significant resistance to nalidixic acid was found (75%), furazolidone (62.5%) and tetracycline (52.5%).

The antimicrobial susceptibility of *Salmonella* strains isolated from minced meat

<table>
<thead>
<tr>
<th>Tested antibiotics</th>
<th>Total number of tested strains</th>
<th>Susceptible strains</th>
<th>Moderate susceptible strains</th>
<th>Resistant strains</th>
</tr>
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<tr>
<td></td>
<td>nr.</td>
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</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>50</td>
<td>39 78 la</td>
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</tr>
<tr>
<td>Nalidixic acid</td>
<td>50</td>
<td>7   14</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>50</td>
<td>33 66</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Colistin sulphate</td>
<td>50</td>
<td>12 24</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>50</td>
<td>6   12</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>50</td>
<td>34 68</td>
<td>4</td>
<td>8</td>
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<tr>
<td>Nitrofurantoin</td>
<td>50</td>
<td>9   18</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>50</td>
<td>17 34</td>
<td>8</td>
<td>16</td>
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<tr>
<td>Tetracycline</td>
<td>50</td>
<td>6   12</td>
<td>5</td>
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</tr>
<tr>
<td>Trimethoprim</td>
<td>50</td>
<td>35 70</td>
<td>11</td>
<td>22</td>
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</table>

Antimicrobial sensitivity of *Salmonella* strains isolated from fresh sausages presented lower values compared to the strains isolated from chilled meat and minced meat, respectively to gentamicin (70%) and ampicillin/sulbactam (60%), while the resistance phenomenon had values exceeded 60% for four antibiotics from the total of ten substances under study (Table 3).
The antimicrobial susceptibility of *Salmonella* strains isolated from fresh sausages

<table>
<thead>
<tr>
<th>Tested antibiotics</th>
<th>Total number of tested strains</th>
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<th>Moderate susceptible strains</th>
<th>Resistant strains</th>
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<tr>
<td></td>
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<td>nr.</td>
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<tr>
<td>Ampicillin/sulbactam</td>
<td>20</td>
<td>12</td>
<td>60</td>
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<tr>
<td>Nalidixic acid</td>
<td>20</td>
<td>2</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>20</td>
<td>11</td>
<td>55</td>
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</tr>
<tr>
<td>Colistin sulphate</td>
<td>20</td>
<td>4</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>20</td>
<td>3</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>20</td>
<td>14</td>
<td>70</td>
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</tr>
<tr>
<td>Colistin sulphate</td>
<td>20</td>
<td>3</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>20</td>
<td>3</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>20</td>
<td>5</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>20</td>
<td>2</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>20</td>
<td>10</td>
<td>50</td>
<td>7</td>
</tr>
</tbody>
</table>

Conclusions

The antimicrobial substances for which was recorded a maximum sensitivity of *Salmonella* strains were: ampicillin / sulbactam, gentamicin, trimethoprim and clarithromycin.

Research has shown that *Salmonella* strains had a significant resistance to the following antimicrobials: nalidixic acid, furazolidone, nitrofurantoin, polymyxin B and tetracycline.

The analysis of the results, depending on the product from which *Salmonella* strains were isolated has shown that the most conclusive sensitivity was recorded for isolates from minced meat, followed by those from chilled meat and fresh sausages.

References

2. Helms, M., Simonsen, J., Molbak, K., Quinolone resistance is associated with increased risk of invasive illness or death during infection with *Salmonella* serotype Typhimurium. J. Infect. Dis., 2004, 190, 1652–1654.


MODERN METHOD - MICROFLEX LT20 EQUIPMENT - USED FOR THE IDENTIFICATION AND CHARACTERIZATION OF BACTERIA AND FUNGI

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Summary

Microflex LT20 is a MALDI TOF mass spectrometer used for identification and taxonomic classification of biological agents (bacteria, fungi) from samples of air, water, soil and other surfaces after specific processing of samples. The samples to be analyzed are represented by the 16S ribosomal protein, obtained by extraction techniques dedicated to the types of microorganisms taken into study. The basic element is a MALDI TOF mass spectrometer with the mass range between 1.000 - 100.000 Da. Control of the instrument is carried out using a computer that uses Windows operating system. Data processing system controls the purchasing processes, saving and storage of spectral information, and also allows processing of information and access to databases. The external data system is connected to the main component, which allows the image transmission 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 user the acquisition, processing, storage and data evaluation. The spectra obtained are compared with spectra from the database of the device. The software indicates the suitability of the spectrum obtained with the spectrum database, in the order of the scores obtained. The scores are obtained through evaluations and comparisons between spectra and do not represent a percentage.

Key words: identification, MALDI TOF, Microflex LT20, bacteria, fungi

Microflex LT20 is equipped with a database to identify biological agents of various types of samples, produced by Bruker Daltonik Company from Germany. The basic elements is a MALDI TOF mass spectrometer (Matrix Assisted Laser Desorption Ionization - Time of Flight) with the mass range between 1.000 - 100.000 Da used for identification and taxonomic classification of biological agents from samples of air, water, soil and other surfaces after specific processing of samples. Control of the instrument is carried out using a computer that uses the Windows operating system. Microtex LT20 system consists of three major components:
• The basic element is the MALDI TOF mass spectrometer, used for mass spectrometric identification of organic molecules. The basic component is connected to the external system database, which allows transmission of the image of the sample of specific compartment. The enlargement of the sample is essential for the positioning of the laser beam in order to obtain spectra.

• External system database, which allows the user to control the MALDI TOF. It is also used for the acquisition, processing, storage and evaluation of data.

• Vehicle mounting support.

MALDI TOF system can be assembled and prepared to carry out the first analysis, in less than 30 minutes, provided that the last use, the vacuum to have not been evicted. Otherwise, it takes aprox. 4 - 5 hours for the vacuum needed to return to operating parameters.

Materials and methods

For carrying out the tests, Microflex LT20 system had a classical configuration. Other materials used were:

- Laboratory reagents necessary for preparation of the component Matrix.
- Bacterial strains (24 hours) belonging to the species: *Bacillus anthracis, Yersinia enterocolitica, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Brucella suis, Brucella canis.*
- Incubators (at 37°C), media culture, bacteriological loops, etc.

Preparation for work and commissioning were carried out in accordance with the operating manual:

- Power of the mass spectrometer and start up computer.
- Launch the FlexControl program and set up the targeted parameters.
- Launch Bio Profiler program for comparing spectra database.
- Checking the vacuum inside the apparatus required for activation of the laser.
- The last preparatory phase consisted in checking the functioning, through the introduction of standard targets. If the results are correct, the machine is ready for work.

Extraction method with TFA 80%

The micro-organisms identified:

- Bacteria grown on usual medium for 24 hours, or a few days for the slow-growing microorganisms. They can be stored for a few days at room temperature (+20°C) or refrigerated (+4°C), but the spectra quality decreases over time.
- Freeze-dried bacterial cells.
- Inactivated biological material (bacteria and fungi spores).
- Solution Matrix (storage time is a maximum of 2 weeks).

Bacterial strains taken into study were seeded by scarifying using a loop on the culture media recommended for each bacterial species (*Brucella* agar, blood...
agar and nutrient agar) to ensure optimum conditions for development, in order to obtain isolated colonies. The cultures were incubated for 24 hours at 37°C.

**Processing the researched sample**

The colonies were taken with a loop from bacterial or fungal culture and downloaded in an Eppendorf tube (1.5 ml). Then were added 300 µl of sterile distilled water. Over the suspension obtained were added 900 µl ethanol and shake it to mix the bacterial suspension. Eppendorf tube content was mixed by centrifugation for about two minutes up to the sedimentation of the biological material and the supernatant is removed using a pipette. 50 µl formic acid 70% and 50 µl acetonitrile (AN) are added. They are mixed by centrifugation for about 2 minutes and the supernatant is transferred. For the next step, 2 µl of the previous supernatant was displayed in one of the 96 holes (A1, A2...B1, B2...). It needs to air dry and be covered with 2 µl of the matrix solutions. After drying, the samples are ready to be examined with the mass spectrometer Microflex LT20.

**Obtaining the spectra**

At the time of the introduction of steel plate with samples to be examined in camera, the software will indicate the time of commencement of the laser (READY). The system can save the obtained spectra for comparison with existing database.

The samples were analyzed with Microflex LT 20. After two consecutive cultivations selecting significant spectra scores (≥80). From each strain were taken two colonies, after the 16S ribosomal protein extraction, four wells were displayed from the target plate, obtaining 20 spectra for each strain. This spectra were analyzed and compared with the spectra from the device database (if they were any) or other spectra of other examined strains of the same bacterial species, choosing the “cleanest” spectrum, with a minimum score ≥80. The mass range in which the identification was carried out was established between 1000 and 17.000 Da, most strains having “peaks” between 18.000 and 12.000 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.

**Comparison of obtained spectra**

The spectra obtained are compared with the database of the device as follow: in the upper part of the window is shown the spectrum obtained and in the lower part is the reference. The differences between the two spectra are marked with red. The software indicates the suitability of the spectrum obtained with the database, in the order of the scores obtained. The scores are obtained through evaluations and comparisons between spectra and does not represent a percentage.
Finally the mass spectra obtained were processed with the BioProfailer 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.

Results and discussions

Spectra analysis of the biological agents, representing the polypeptide fragments of the 16S ribosome’s revealed a mass range of 1.800 to 12.000 Da with characteristic peaks between 2.000 and 12.000 Da. Average range of characteristic peaks of each bacterial species was: *Bacillus anthracis* 4000 - 6000 Da; *Brucella suis* 3.500 - 6.500 Da; *Escherichia coli* 4.300 - 6.500 Da; *Pseudomonas aeruginosa* 3.300 - 7.500 Da; *Staphylococcus aureus* 3.000 - 5.500 Da, *Yersinia enterocolitica* 4.000 - 6.000 Da, *Candida albicans* 2.800 - 8.700 Da. The average score, taken into consideration to be entered in to the data base of spectra was ≥80, 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, protein configuration is the same, facts that certifies the specificity of the technique for identifying biological and fungal agents studied with Maldi Tof technology.

The different composition of growth medium (blood agar or nutritive agar) had no significant effect on the way the peaks were distributed. However, some differences of spectra are present in the mass range between: 3.000 - 9.000 Da, differences that can be attributed to the various components of culture media tested and their implications in bacterial metabolism. The phase of development (growth) of cells has low impact on the results obtained. Cells in growth phase lag have a very similar pattern with the cells in stationary phase or dead cells.

Below are the mass spectra and the values (Da) of characteristic peaks of studied bacterial species (fig. 1, 2, 3, 4, 5, 6, 7 and 8).

![Fig.1. Mass spectrum of Bacillus anthracis – sample 290-C31](image-url)
Fig. 2. Mass spectrum of *Brucella suis* 31 SV

Fig. 3. Mass spectrum of *Brucella canis* Ciorani 94/2

Fig. 4. Mass spectrum of *E. coli* – sample HB 10101-C31
Fig.5. Mass spectrum of *Pseudomonas aeruginosa* – sample 380-C51

Fig.6. Mass spectrum of *Staphylococcus aureus* – sample 430-C81

Fig.7. Mass spectrum of *Yersinia enterocolitica* – sample IC8710-C61
The Microflex LT20 system, can measure the mass spectra between 1.000 -100.000 Da, so a very wide range of organic molecules from bacteria, fungi and spores.

The equipment includes a standard database for detection of a wide range of biological agents (bacterial and fungal origin).

The Microflex LT20 system, allows quick and accurate identification of biological agents, being an important confirmation or validation means for other methods of microbiological diagnosis.

The Microflex LT20 is a useful laboratory apparatus for microbiological diagnosis, indication of the species of the biological agent, but also for scientific research in the field of medicine in general and microbiology in particular.

References

STAPHYLOCOCCUS SPP. ISOLATED FROM CHEESE COMMERCIALIZED IN MARKETPLACE

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Summary

Fifty-one samples of fresh and ripened cheese, obtained from raw milk were examined for the presence of \textit{Staphylococcus} spp. The variation levels of staphylococci were $1.8 \times 10^{3}/g$ and $2.3 \times 10^{7}/g$, respectively. In about 50\% of samples staph contamination level was between $10^{6}/g$ – $10^{7}/g$. The most commonly species isolated from cheese were: \textit{Staphylococcus aureus} (35.3\%), \textit{Staph. saprophyticus} (21.5\%), \textit{Staph. hominis} (19.6\%), and \textit{Staph. haemolyticus} (13.7\%). \textit{Staph. xylosus} was identified in 3.9\% cheese samples, \textit{Staph. epidermidis}, \textit{Staph. kloosii} and \textit{Staph simulans}, each in 1.9\% of samples. A high proportion of cheese with a large number of \textit{Staphylococcus aureus}, and a great number of cheese samples contaminated with a large percentage of another enterotoxigenic staphylococci highlighting the risk of staphylococcal food poisoning to consumers. The results obtained confirm the improper microbiological quality of raw milk used in the manufacturing of cheese, and also the unsuitable production conditions.

Key words: \textit{Staphylococcus} spp.; cheese; marketplace

Cheeses are a dairy product category appreciated by consumers. On food market in western Romania small producers sold various types of fresh or rippened cheese. These types of cheeses are obtained from unpasteurized sheep, cows and/or goats' milk or mixtures. Raw milk and raw milk products are frequently contaminated with different species of staphylococci of human or animal origin. Is well know that some \textit{S. aureus} strains and also other staphylococci species are able to produce staphylococcal enterotoxins which are involved in staphylococcal food-poisoning. The European Food Safety Authority (EFSA) reported in 2009 that cheese was one of the most important food vehicles in outbreaks of food poisoning caused by staphylococcal toxins (8). For this reason the objectives of the present work were to investigate the occurrence and contamination level of \textit{staphylococcus} species in cheese commercialized in marketplace.

Materials and methods

The research has been carried out on 51 samples of fresh and rippened cheese, obtained from raw milk (cows, sheep or mixtures milk), collected between
June to September 2012. The varieties of manufactured cheese were obtained by different technology and classified into six types: sheep and cow semi soft cheese, ripened and unripened in brine named Telemea, sheep curd – named Urdă, unripened semi soft sheep and cow cheese - named Caş, cottage cheese, and a specific type of sheep cheese grinded and blend with salt - Liptoi.

Samples were collected from marketplace and transported to laboratory. All samples were analyzed for establish the staph contamination level. Ten grams of each sample were placed into a sterile bag, diluted with 90 ml of sterile buffered peptone water and homogenized for 30 second in a stomacher. 0.1 ml samples of $10^{-1}$ to $10^{-4}$ dilutions were plated on the surface of Baird-Parker medium agar (Difco) supplemented with egg yolk and tellurite. The plates were incubated at 37°C for 24 to 48 h. Typical colonies black or dark gray, smooth, convex with an opaque zone were counted. From each sample was selected a colony and transferred to individual plates of TSB agar. The plates were incubated at 37°C for 24 h and then the staphylococci species were identified with automated systems VITEK-2 (BioMérieux, France), using colorimetric cards for Gram-Positive bacteria.

Results and discussions

The contamination level with staphylococci of cheese samples are given in Tables 1. Staphylococci were present in all samples examined, ranging from $1.8 \times 10^3$ to $2.3 \times 10^7$ cfu/g.

Table 1

<table>
<thead>
<tr>
<th>Count of staphylococci (cfu/g)</th>
<th>Number of cheese samples</th>
<th>% of contaminated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^0$ to $10^3$</td>
<td>4</td>
<td>7.84</td>
</tr>
<tr>
<td>$10^4$ to $10^7$</td>
<td>22</td>
<td>43.13</td>
</tr>
<tr>
<td>$10^8$ to $10^9$</td>
<td>25</td>
<td>49.01</td>
</tr>
</tbody>
</table>

Of 51 cheese samples examined about half (25 samples) had highest counts of Staphylococcus ($10^6$ - $10^7$ cfu/g). A similar proportion of samples (43.13%) had a staphylococci contamination level of $10^4$ - $10^5$ cfu/g. Only four cheese samples (7.84%) had a low number of staphylococci ($10^0$ - $10^3$ cfu/g).

Within the coagulase-positive staphylococci two species, S. aureus and S. kloosi, have been recognized. Coagulase-negative staphylococci identified were: S. epidermidis, S. hominis, S. haemolyticus, S. saprophyticus, S. simulans and S. xylosus.

The most recovered species were Staphylococcus aureus (35.3%), S. saprophyticus (21.5%), S. hominis (19.6%), and S. haemolyticus (13.7%). S. xylosus was identified in 3.9% cheese samples, S. epidermidis, S. kloosi and S. simulans, each in 1.9% of samples (Table 2).
In Italy, Normanno et al. (4) found that raw milk was contaminated with coagulase positive staphylococci in high proportion, about 38.4%. This finding is very important considering that many Italian cheeses, both fresh and seasoned, are made with raw milk.

<table>
<thead>
<tr>
<th>Staphylococcus spp.</th>
<th>Number of cheese samples</th>
<th>% contaminated cheeses</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>18</td>
<td>35.29</td>
</tr>
<tr>
<td>S. saprophyticus</td>
<td>11</td>
<td>21.56</td>
</tr>
<tr>
<td>S. hominis</td>
<td>10</td>
<td>19.60</td>
</tr>
<tr>
<td>S. haemolyticus</td>
<td>7</td>
<td>13.72%</td>
</tr>
<tr>
<td>S. xylosus</td>
<td>2</td>
<td>3.92</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>1</td>
<td>1.96</td>
</tr>
<tr>
<td>S. kloosi</td>
<td>1</td>
<td>1.96</td>
</tr>
<tr>
<td>S. simulans</td>
<td>1</td>
<td>1.96</td>
</tr>
</tbody>
</table>

In raw milk cheeses *Staphylococcus aureus* may occur from raw milk produced from animals suffering from clinical or sub-clinical mastitis, from food handlers who are carriers of this pathogen or as the result of inadequate hygienic practices (5). If levels exceed $10^5$/g there is a significant risk that *S. aureus* may produce enterotoxin. Further treatment of the cheese may reduce the concentration of viable cells, but the enterotoxin remained and may cause foodborne illness (9, 10). For these reasons, a limit of $<10^5$ cfu coagulase-positive *S. aureus*/g of cheese has been set for raw milk cheese (7).

In France, among the staphylococcal food poisonings reported in 1999-2000 milk products and especially cheeses were responsible for 32% of the cases (3).

Our research revealed a number of *S. aureus* exceeded $10^5$/g in about 20% of the cheese samples analyzed. More than 50% of the samples had levels higher than $10^5$ coagulase-negative staphylococci.

Although coagulase-positive *S. aureus* is the main agent responsible for food poisoning, some research underlines that coagulase-negative staphylococci (CNS) are able to produce staphylococcal enterotoxin and can be a potential cause of food poisoning (6). Bautista et al. (1) found that some coagulase-negative strains of staphylococci, other than *S. aureus* (*Staphylococcus cohnii*, *S. epidermidis*, *S. haemolyticus*, and *S. xylosus*) produced enterotoxin. On the other hand, Irlinger (2) emphasizes that the risk of poisoning outbreaks caused by coagulase-negative staphylococci in relation to consumption of dairy products should be virtually zero since no coagulase-negative staphylococcus species isolated from milk or dairy products has ever been implicated in a case of food poisoning after eating of dairy products.
Conclusions

A large proportion of cheeses with a high percentages of *Staphylococcus aureus* and other enterotoxigenic staphylococci highlighting the risk of staphylococcal food poisoning to consumers.

The results obtained confirm the improper microbiological quality of raw milk used in the manufacturing of cheese, and also the unsuitable production conditions.

Results also indicate the necessity of improving hygiene measures during milk chain and also in the manufacturing, handling, and storage of cheese commercialized in market place.

Acknowledgments

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References


IN VITRO STUDY OF ANTIMICROBIAL EFFICACY OF SOME HUMAN AND ANIMAL TOOTH PASTE

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Summary
The purpose of this study was to compare the in vitro antimicrobial effect of some toothpastes used for human and animal purpose. The antimicrobial effect was tested by the agar well diffusion method against two Gram negative bacteria species (Escherichia coli - ATCC 25922, Pseudomonas aeruginosa - ATCC 27853), Gram positive bacteria species (Bacillus cereus - ATCC 11778, Staphylococcus aureus ATCC 25923) and also against a yeast specie (Candida albicans - ATCC 10231). All toothpastes taken in study have proved a good antimicrobial effect. Of these, those that are recommended and used for animal had an lower antimicrobial effect than those for human use.

Key words: antimicrobial effect, toothpastes

Dental diseases, both in humans and animals are of particular importance because these will affect whole body health. In the past 10 years, the pet, especially dogs and cats, presented an increased frequency of dental diseases. It is known that by the age of three years, 80% of dogs and 70% of the cats developing dental conditions (4). Periodontal disease is the most common diseases for pets (3). Most of these can be avoided by the use of regular brushing toothpaste, which is currently available in commerce. In cats, there are some breeds that are prone to problems of the mouth; these include Maine Coon, Ragdoll and oriental breeds. Most times, these breeds have more serious dental problems or progressing faster than other races (11).

The number of dog owners who report dental diseases and require specialized care for their prevention and control has increased in recent years and therefore greater diversity of dental diseases and maxillofacial pathology and dental in pets become a topical veterinary practitioners especially those in urban areas (5).

Due to the fact that in recent years there has been an alarming increase in cases of periodontal disease, both in humans and animals, and considering that dental problems are as painful for pet, the purpose of this research was to
determine and compared the antimicrobial effect of some toothpaste products for human and animals, which are commercially available.

**Materials and methods**

The antimicrobial activity of studied tooth-pastes was measured using fresh culture from standard strains of bacteria obtained from Mark Medium Europe Company, France, as follows: Gram-positive bacteria - Bacillus cereus (ATCC 11778) and Staphylococcus aureus (ATCC 25923), Gram-negative bacteria - Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853) and a strain of Candida albicans (ATCC 10231).

Standard microbial cultures were maintained on laboratory condition, at 4 °C, on tubes with Mueller-Hinton broth (Oxoid) for bacteria and Sabouraud dextrose broth for fungi.

Active cultures for experiments were prepared by transferring from the standard strains with the loop in tubes containing Mueller-Hinton broth (Oxoid) for bacteria and Sabouraud dextrose medium for yeast, These were incubated for 24 hours at 37 °C for the bacteria cultures and 48 hours at 25 °C for yeast culture.

The cultures thus obtained were then diluted with Mueller-Hinton broth and Sabouraud dextrose broth or to achieve optical densities of the corresponding test at 2.0 x 10^6 colony forming units (cfu / ml) for bacteria and 2.0 x 10^5 cells / ml yeast strain, density appreciated McFarland scale.

To test the antimicrobial activity in vitro of toothpastes, it was used a adapted method of the diffusion method recommended by the National Committee for Clinical Laboratory Standards (9), namely agar wells method.

Culture media Mueller-Hinton (Oxoid) and Sabouraud dextrose agar were distributed in Petri dishes, and after it solidification was inoculated 1 ml of microbial cultures. The inoculum was spread evenly over the surface of the culture medium. Excess of inoculum was then aspirated.

Then, in sterile conditions, on each Petri dishes were performed four wells with a diameter of 5 mm (one for each toothpaste under study), taking care that they are at approximately 15 mm from the periphery of the plate and respectively 30 mm from each other. Then, was assigned an equal and exact amount of each toothpaste (a cylinder 2-3 mm) in wells without exceeding edge.

As positive control for anti-bacterial and antifungal effect microtablets of Enrofloxacin and that Nystatine has been used. The Petri dishes were kept at rest for 10-15 minutes and then introduced thermostat at 37 °C and 25 °C respectively (for yeasts).

The results were read after 24 hours of incubation for bacteria culture and after 48 hours for Candida albicans. In particular was measured the inhibition zone diameters (mm), including the diameter of wells. The tests were performed three times and the obtained values were used to express the mean and standard deviation.
Results and discussions

Table 1 shows the inhibition zones values measured in millimeters listed as an average of the three measurements and the standard deviation of the four toothpastes that were tested (two for human use: Lakalut and Colgate and two for dogs, Trixie, and Beaphar toothpaste) and for two antimicrobial substances (Enrofloxacin Nistatin respectively) used as a positive control.

<table>
<thead>
<tr>
<th>Toothpaste/ Antimicrobial substances</th>
<th>Bacteria strains</th>
<th>Diameter of inhibition zone (mm)</th>
<th>Average ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Lakalut</td>
<td><em>E. coli</em></td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td><em>Ps. aeruginosa</em></td>
<td>35</td>
<td>34.6</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>28</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td><em>B. cereus</em></td>
<td>29</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td><em>C. albicans</em></td>
<td>24</td>
<td>22.6</td>
</tr>
<tr>
<td>Colgate</td>
<td><em>E. coli</em></td>
<td>34</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td><em>Ps. aeruginosa</em></td>
<td>36</td>
<td>35.6</td>
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<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>56</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td><em>B. cereus</em></td>
<td>48</td>
<td>46.6</td>
</tr>
<tr>
<td></td>
<td><em>C. albicans</em></td>
<td>25</td>
<td>24.3</td>
</tr>
<tr>
<td>Trixie</td>
<td><em>E. coli</em></td>
<td>18</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td><em>Ps. aeruginosa</em></td>
<td>22</td>
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</tr>
<tr>
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<td><em>S. aureus</em></td>
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<td>21.6</td>
</tr>
<tr>
<td></td>
<td><em>B. cereus</em></td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td><em>C. albicans</em></td>
<td>18</td>
<td>17.6</td>
</tr>
<tr>
<td>Beaphar Toothpaste</td>
<td><em>E. coli</em></td>
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</tr>
<tr>
<td></td>
<td><em>Ps. aeruginosa</em></td>
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<td>14.6</td>
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<tr>
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<td><em>S. aureus</em></td>
<td>14</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td><em>B. cereus</em></td>
<td>12</td>
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</tr>
<tr>
<td></td>
<td><em>C. albicans</em></td>
<td>5</td>
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<td>Enrofloxacin</td>
<td><em>E. coli</em></td>
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<td>25</td>
</tr>
<tr>
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<td><em>Ps. aeruginosa</em></td>
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<td>14.6</td>
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<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>11</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td><em>B. cereus</em></td>
<td>12</td>
<td>11.6</td>
</tr>
<tr>
<td>Nistatin</td>
<td><em>C. albicans</em></td>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>

From the obtained results it was found that all tested toothpastes, both for human and dogs use, showed antibacterial and antifungal effect.
One small exception was found, namely, toothpaste Beaphar (recommended for dogs) did not inhibit the development of culture of Candida albicans at all, so its antifungal effect was considered invalid.

The value of the inhibition zones ranged from 12 ± 0 mm, recorded by Beaphar toothpaste (recommended for dogs) against Bacillus cereus and 55 ± 1 mm, induced by the toothpaste Colgate (for human use) against Staphylococcus aureus. In the case of antibiotic, the zone of inhibition ranged from 11 ± 0.57 ± mm against Staphylococcus aureus and Bacillus cereus and 26 ± 1 mm against of Pseudomonas aeruginosa. Antifungal effect of Nystatine, used as positive control, was 14 ± 0.57 mm, against the species Candida albicans.

Of the four toothpastes tested, those for human use showed a good antimicrobial effect, much stronger than toothpaste recommended for dogs. Most strongly antimicrobial effect manifested Colgate paste, which had the largest areas of inhibition of both bacterial species and the species of yeasts tested, namely, of 34 ± 1.15 mm to the species Escherichia coli, of 35 ± 0.57 mm to the species Pseudomonas aeruginosa, of 45 ± 1.52 mm to the species Bacillus cereus, of 55 ± 1 mm to the species Staphylococcus aureus, and of 25 ± 1.15 mm, respectively, on the species of the yeast Candida albicans. It was found that Gram-positive bacteria species (Staphylococcus aureus and Bacillus cereus) are more sensitive to this toothpasta that Gram negative bacterial species (Escherichia coli and Pseudomonas aeruginosa).

A good antimicrobial effect had alo the toothpaste Lakalut. The values of inhibition zones were higher in Gram-negative bacteria species (34.3 ± 0.57 mm at Escherichia coli and 34 ± 0.57 mm at Pseudomonas aeruginosa) combatively with Gram-positive bacteria species tested (28 ± 1.15 at 31 at Staphylococcus aureus and on Bacillus cereus 31±1.15 mm). Also, this toothpaste had a good antifungal effect, the mean value of inhibition zone in Candida albicans was 23 ± 1.52 mm.

Of the two dentifrices suitable for use in oral hygiene in dogs, Trixie has a strong antimicrobial effect compared with Beaphar. Thus, in this case, the values of the inhibition zones were 22 ± 0.57 mm in Pseudomonas aeruginosa and Staphylococcus aureus, and 18 ± 0.57 mm to the species Escherichia coli and of 17 ± 1 mm in Bacillus cereus. To the specie of the yeast, Candida albicans, the inhibition zone achieved by toothpaste Trixie was 17 ± 0.57 mm.

Looking at the results in terms of the composition of each toothpaste tested can find an explanation of the fact that human use toothpastes have a stronger antimicrobial effect than those recommended for oral hygiene in dogs

Colgate toothpaste has triclosan in composition, a substance which has a strong antibacterial effect, especially on the species of the genus Salmonella and coagulase-negative Staphylococcus aureus (6).

Lakalut toothpaste containing chlorhexidine, another well-known antibacterial substances, together with chamomile essential oil with a good anti-inflammatory, antibacterial and antifungal effect, as well as some substances with preservative role.
Toothpastes recommended for dogs (Trixie and Beaphare) containing only substances with preservative role or mint extract and therefore their antibacterial effect is based only on those substances.

Similar results were obtained in other studies, but these have tested only dentifrices for human use. Thus, Prasanth M., 2011 (7) concludes that toothpastes containing triclosan are more effective in the control of oral microbial flora than those without triclosan, and of liquid preparations suitable for cleaning the oral cavity, the most effective are those containing chlorhexidine.

Also, Adwan G. et al., 2012 (1) showed antifungal effect against Candida albicans of the toothpastes containing the plant extract and sodium fluoride.

Conclusions

Human use toothpastes tested exhibited a strong antimicrobial higher than toothpastes recommended for dogs.

The tricolsan presence in the composition of toothpastes provides enhanced antibacterial efficacy against Gram positive bacteria tested.

The clohexidine presence in the composition of toothpastes provides enhanced antibacterial efficacy against Gram-negative bacteria tested.

The toothpastes recommended for dogs that were tested have expressed a moderate antimicrobial activity. Due to the enzyme-rich composition, these toothpastes will act on the formation of biofilm by strong enzymatic mechanisms and not by bacteria controlling.

In vivo studies are needed to highlight the potential of these toothpastes tested in periodontal disease control.

References

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THE WELFARE OF DAIRY COWS IN TWO HOUSING SYSTEMS:
ASSESSMENT OF HEALTH

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Summary

Health is an important indicator of dairy cattle welfare. The aim of this work was the assessment of health in dairy cows kept in two different housing systems. The research was accomplished in 15 dairy farms with tie-stall housing system and in 12 farms with free stalls, in Transylvania, in the period when the cows were housed. The health assessment of the 1120 milking cows (601 kept in tie-stalls and 519 in free stalls) was based on the Welfare Quality® protocol. The obtained data were statistically processed using the SPSS software (descriptive indicators, t test or the Mann-Whitney test). Significant differences (P < 0.05) were found for the mean score of the welfare principle Good health between the two housing types. Significantly higher scores (P < 0.001) were recorded in the free stall farms than in those with tie-stalls for two criteria (Absence of injuries and Absence of diseases) of the three included in this principle. Based on the scores obtained for the criterion Absence of injuries, the farms with tie-stalls were classified in three welfare categories (acceptable, enhanced and excellent) and those with free-stalls in two categories (enhanced and excellent). All the free stall farms obtained excellent scores for the criterion Absence of diseases, while the farms with tethered cows had acceptable, enhanced and excellent scores. In both housing systems unacceptable scores were recorded for the criterion Absence of pain induced by management procedures. The scores for the principle Good health classified the tie-stall farms and those with free housing in only two welfare categories, acceptable and enhanced. This research indicated significant differences between the two housing systems regarding the Good health welfare principle. According to the results obtained in this study, the health status of the dairy cows is better in the farms with free stalls than in those with tie-stalls.

Key words: Welfare Quality® protocol, dairy cow, health assessment

The relevance of health as a measure of animal welfare is well known: a clinically sick animal can not have a good welfare quality. Thus, as Broom stated (2), disease level is of considerable importance in welfare assessment because the welfare of diseased animals is almost always poorer than that of healthy animal. Nowadays the animal welfare concerns and especially the increasing demand for consumer safe and more organic farm products stresses the importance of maintaining, promoting and assessing the health of dairy cows among other farm animals. Maximizing disease prevention (by good hygiene and farming practices); keeping animals in good health and increasing their ability to resist infection,
parasites and metabolic disease (through excellent husbandry practice and health planning); and limiting animal health problems (by selecting appropriate breeds, using high-quality feeds, providing regular exercise and keeping the correct stocking density) are just some of the objectives relevant to health and welfare in organic and also conventional dairy production (5). According to Marley et al. (7), in both conventional and organic dairy herds, the main challenges to health are lameness, mastitis and infertility. Besides the indicators of these problems, many more measures of health are evaluated by the Welfare Quality® Assessment Protocol for Cattle (11) and presented in this study. The aim of this work was the assessment of health in dairy cows kept in two different housing systems.

Materials and methods

This study evaluated 27 Transylvanian dairy farms, 15 with tie-stalls (TS) and 12 with free stalls (FS), in the cold period of the year, when the cows were housed. A minimum number of 30 milking cows were mandatory for a farm to participate in the study. Other requirements were the housing system (tie- and free stalls), easy access during the winter and the consent of the farmer to allow the cows’ evaluation. The mean number of the dairy cows in the farms with tie-stalls (mean ± sd, 70 ± 30.11 dairy cows) and in those with free stalls (mean ± sd, 79 ± 17.98 dairy cows) was similar. The barns were closed in all of the tie-stall farms and in 8 of the free-stall farms. Opened barns were used in 4 of the farms with free stalls. In 14 farms (9 with tie-stalls and 5 with free stalls) the cows had access to pasture. The mean milk production was of 4942.73 kg per cow per year in the tie-stall farms and 5523.83 kg per cow per year in those having free stall barns.

The health assessment of the cows was based on the Welfare Quality® protocol for cattle (11) which includes four welfare principles, 12 criteria and 29 measures. Two trained assessors evaluated the health status of a total number of 1120 milking cows (601 in tie-stalls and 519 in free stalls). The number of the assessed cows in each farm was established according to the Welfare Quality® protocol for dairy cows (11). The Good health principle includes three welfare criteria: Absence of injuries, Absence of diseases and Absence of pain induced by management procedures. Several measures recorded to assess this welfare principle were animal-based but there were also measures based on the farm management and resources. Observation and clinical exam of the animals and also the animal unit manager’s questionnaire were employed to collect the data needed for the evaluation. The description and scoring methodology for the assessed measures are presented in Table 1.

Data collected in the farms was processed using the software program of the Welfare Quality® scoring system (12) for the calculation of the scores for the welfare criteria and principle. Finally, based on the scores obtained for the criteria
and the Good health principle, the farms were classified in a welfare category: not classified, acceptable, enhanced and excellent.

Table 1

<table>
<thead>
<tr>
<th>Welfare criteria and measures</th>
<th>Description</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absence of injuries (AI)</strong></td>
<td>Gait assessment of the animals during walking, observing the timing of steps, temporal rhythm and weight-bearing on feet</td>
<td>Individual level: not lame (score 0), lame (score 1), severely lame (score 2)</td>
</tr>
<tr>
<td>Lameness (loose housed animals)</td>
<td></td>
<td>Herd level: percentage of animals in each of the above categories</td>
</tr>
<tr>
<td>Lameness (tied animals)</td>
<td>Observing the animals regarding foot resting, standing on the edge of a step, weight shifting between feet and reluctance to bear weight on a foot when moving</td>
<td>Individual level: not lame (score 0), lame (score 2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herd level: percentage of animals in each of the two categories</td>
</tr>
<tr>
<td>Integument alterations</td>
<td>Five body regions on one side of the animal are examined with regard to the presence of hairless patches and/or any lesion/swelling</td>
<td>Individual level: number of hairless patches, number of lesions/swellings</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herd level: percentage of animals with no integument alterations, with mild integument alterations and with severe integument alterations</td>
</tr>
<tr>
<td><strong>Absence of diseases (AD)</strong></td>
<td>Observation using continuous behavioural sampling</td>
<td>Herd level: mean number of coughs per animal and per 15 minutes</td>
</tr>
<tr>
<td>Coughing</td>
<td>Observation of the animals, without touching</td>
<td>Individual level: no evidence of the measure (score 0), evidence of the measure (score 2)</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td></td>
<td>Herd level: percentage of animals with score 2</td>
</tr>
<tr>
<td>Ocular discharge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hampered respiration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vulvar discharge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk somatic cell count</td>
<td>Data obtained from milk records for a period of three months prior to the farm visit</td>
<td>Individual level: somatic cell count (SCC) below 400,000 within three months (score 0), SCC of 400,000 or above within three months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herd level: percentage of cows with SCC of 400,000 or above</td>
</tr>
<tr>
<td>Mortality</td>
<td>Data obtained from the animal unit manager or farm</td>
<td>Herd level: percentage of animals dead, euthanized and emergency slaughtered</td>
</tr>
</tbody>
</table>
The obtained data were statistically processed with the SPSS software. Descriptive statistical indicators were determined (mean, standard error of the mean, median, minimum and maximum) for the scores of the criteria: Absence of injuries \( (A_I) \), Absence of diseases \( (A_D) \), Absence of pain induced by management procedures \( (APIM) \), and for the scores of the Good health welfare principle in the farms with FS and TS. The comparison of the data in the two housing types was made using the Mann-Whitney test. The P values less than 0.05 were considered to be significant.

### Results and discussions

Table 2 presents the descriptive statistics for the Good health welfare principle and for the included criteria, comparing the two evaluated housing systems (FS and TS). The difference for the mean scores of this principle differed significantly \( (P < 0.05) \) between the farms with free stall and tie-stall housing (Table 2).

In the tie-stall farms higher scores were found for only one welfare criterion, the Absence of pain induced by management procedures. For this criterion the dehorning / disbudding practices were considered on farm level, because tail docking is not usually performed in Romanian dairy farms. The best score for the measure of disbudding / dehorning is given at herd level, when none of these procedures are performed. Disbudding and dehorning represent routine husbandry procedures in diaries worldwide. For example an extended Italian study (6) shows that dehorning is carried out on 80% of the 639 surveyed farms; 70% of the farmers accomplish the procedure without any specific training or veterinary

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
<th>SF farms</th>
<th>TS farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dystocia</td>
<td>Data obtained from the animal unit manager or farm records; the average number of calvings also recorded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Downer cows</td>
<td>Data obtained from the animal unit manager or farm records; the average number of dairy cows is also recorded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absence of pain induced by management procedures (APIM)</td>
<td>The animal unit manager is asked about procedures used for disbudding / dehorning, use of anaesthetics, use of analgesics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disbudding / dehorning</td>
<td>Herd level: no disbudding / dehorning (score 0), disbudding using thermocautery (score 1), disbudding using caustic paste (score 2), dehorning (score 3) and use of anaesthetics (score 0) no use of anaesthetics (score 2) and use of analgesics (score 0), no use of analgesics (score 2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
help and only 10% of the farmers use local anesthesia. In our study none of the 
free stall farms had excellent scores for this criterion (Fig. 1), meaning that some 
form of disbudding / dehorning was performed in all of these. Only two of the tie-
stall farms avoided completely this procedure, but rather for practical reasons 
(better roping or holding the horned cows’ head, economy as time and money, no 
obvious cause to perform the operation) than avoiding unnecessary suffering of the 
animals. Similarly, in the study performed by Gottardo et al. (6) the farmers favoring 
horned cows have as main reasons aesthetic motivations, lack of time and do not 
have difficulty in handling horned cows. Even if the mean score for this criterion in 
our study was better in the tie-stall farms than in those with free stalls, no 
statistically significant difference was found between the two housing systems. 
Furthermore, as Fig. 1 presents, there were more tie-stall farms (than free stall 
one) not classified and more free stall farms (than tie-stall ones) classified as 
acceptable for this criterion.

Table 2
Descriptive statistics for welfare principle and criteria scores in farms with 
FS vs.TS and the significance of difference between two housing system

<table>
<thead>
<tr>
<th>Principle and criteria</th>
<th>Free stall</th>
<th>Tie-stall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± sem</td>
<td>Median</td>
</tr>
<tr>
<td>Principle: Good health</td>
<td>53.87 ± 2.25</td>
<td>52.70*</td>
</tr>
<tr>
<td>Criterion: AI</td>
<td>80.68 ± 2.30</td>
<td>83.10</td>
</tr>
<tr>
<td>Criterion: AD</td>
<td>100.0 ± 0.00</td>
<td>100.0</td>
</tr>
<tr>
<td>Criterion: APIM</td>
<td>26.50 ± 3.79</td>
<td>22.00</td>
</tr>
</tbody>
</table>

AI - Absence of injuries, AD – Absence of diseases, APIM - Absence of pain induced by 
management procedures, FS – free stall farms, TS – tie-stall farms, sem = standard error of 
the mean
*P < 0.001, P < 0.05

For the other two criteria included in the Good health principle (AI and AD) 
significantly higher mean scores were obtained in the free stall farms than in those 
using tie stalls (P < 0.001, Table 2).

For the Absence of injuries welfare criterion lameness and integument 
alterations were assessed in the cows. Lameness is a painful condition to the 
animal, it is a serious welfare issue as cows suffer and is costly to the dairy farm 
business (4). Bicalho et al. (1) formulate that lameness is a debilitating disease or a 
disease of the debilitated cattle and find a prevalence of lameness (visual 
locomotion score of 3 or more) of 19.8% and 48.2% for parity one and more than
one, respectively, in the 501 lactating Holstein dairy cows enrolled in their study. Even if the welfare implications might not be the same, body lesions have at least the same frequency as lameness worldwide in dairy herds. In a study on 2,335 dairy cows (8) kept in free stall barns the prevalence of skin lesions varied depending of the body region considered. While only 3-9% of the cows had lesions on the hips, fetlock and thighs, the prevalence of body injuries were between 21% on the neck and up to 60.5% on the hocks of the cows. The overall prevalence of skin lesions in a Romanian study was 14.38% in tethered dairy cows (9). Irrespective to the housing system, skin lesions, other body injuries and swellings reflect the impact of the surrounding environment on the animal's body (10). The results of the present study showed that the free stall farms were classified as excellent and enhanced for the Absence of injury criterion, only a few tie-stall farms were found to be excellent, the majority of these being enhanced and acceptable (Fig. 1).

Fig. 1. Classification of the farms (%) with free-stalls and tie-stalls in each welfare category, based on the welfare criteria scores

AI - Absence of injuries, AD – Absence of diseases, APIM - Absence of pain induced by management procedures, FS – free-stalls, TS – tie-stalls

Regarding the Absence of diseases welfare criterion, all of the free stall farms were found to be excellent (Fig. 1) and differ significantly (P < 0.001) from the farms with tie-stalls (Table 2). Many evidences show significant associations between certain specific measures of disease assessed by the Welfare Quality® assessment protocol (11) and cow mortality, culling, lower milk yield, alterations of milk composition, lower pregnancy rates and / or stillbirth and mortality of calves (3). Therefore, the importance of having healthy dairy cows is obvious not only for the welfare of the animals but also for the profitability of the farm and farmer. From this point of view a realistic objective of the farmer would be to advance the dairy
farm owned towards the excellent category for the Good health welfare principle. None of the assessed farms reached this objective, unfortunately (Fig. 2).

![Fig. 2. Classification of the farms (%) with free-stalls and tie-stalls in each welfare category, based on the welfare principle scores GH – Good health, FS – free-stalls, TS – tie-stalls](image)

Conclusions

This research indicated significant differences between the two housing systems regarding the Good health welfare principle. According to the results obtained in this study, the health status of the dairy cows is better in the farms with free stalls than in those with tie-stalls.

References


DETECTION OF STAPHYLOCOCCAL ENTEROTOXINS IN SAMPLES OF CHEESE SOLD ON FREE MARKET

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Summary

Milk products (especially cheeses) are frequently associated with staphylococcal food intoxications. Sometimes, the lack or poor control on free market allow products containing staphylococcal heat stable enterotoxins, to reach the consumer’s table. The aim of this study was to screen different types of cheese sold in three county of free market in order to detect the staphylococcal enterotoxin. 51 samples of cheese (made from raw milk of cow, goat and sheep) were analyzed between March 2012 and January 2013. Enterotoxin production A, B, C, D şi E was determined by enzyme linked fluorescent assay (ELFA) using a MiniVIDAS system. One of the analyzed samples was positive to concentrations of the toxins detected by this technique. It is emphasized that the presence of staphylococcus enterotoxins in cheese sold on free market be regarded as a potential risk for human health.

Key words: staphylococcal enterotoxins, cheese, free market

The tradition of raw milk cheese production and consume still exist in different region of our country. Different type of raw cheeses obtained from sheep and raw cow milk are sold every year on free market. In the last years an increasing of interest to raw milk products was observed, based on increased interest of consumers to consume healthier products. As a consequence of this the preoccupation of this products safety are of interest.

Depending on the obtaining and storage condition these products could contain different pathogens and could be at the origin of foodborne diseases. Raw milk are often contaminated with different strains of Staphylococcus aureus. Contamination level of milk from different species with Staphylococcus aureus was observed by different researcher and varied from 75% in bovine milk to 96% in goat milk (3, 5, 6). For example, staphylococci are frequently isolated also from cheese obtaining from raw milk (1).

A large percent of staphylococi found in milk and milk products are enterotoxigenic. Staphylococcal food intoxication due to milk and milk products consumption is one of the frequent foodborne disease (8). The illness occurred after ingestion of different staphylococcal enterotoxins produced by strains of staphilococi coagulase positive or negative (2). In fact, today five classes of enterotoxins are recognised A, B, C, D, and E (noted SEA to SEE). The most
important staphilococcal entrotoxines responsible of majority foofdborne illness is SEA followed by SED, SEC and SEB (8). The production of enterotoxins is related to staphylococci multiplication, especially in milk and milk products, in favourable conditions.

The purpose of this study was to screen different types of cheese sold in three county of free market in order to detect the staphylococcal enterotoxin and established the safety of this type of cheese.

Materials and methods

For this study 51 samples of domestic cheese (made from raw milk of cow, goat and sheep), collected between June to September 2012, from market place from different counties were analyzed, in order to detect the number of Staphylococcus aureus. From this only 26 samples (kept under freezing conditions -18 °C), when the number of staphylococci was higher than $10^5$ cfu/g, were tested for evidenced the presence of staphylococcal enterotoxins. Domestic cheese were obtained by different technology instead six main category of cheese were analyzed: sheep and cow semi soft cheese ripened and unripened in brine named Telemea, sheep curd – named Urdă, unripened semi soft sheep and cow cheese- named Caş and a specific type of sheep cheese grinded and blend with salt - Liptoi.

Enterotoxins production was determined by MiniVIDAS (using kit SET 2, BioMérieux) system, based on enzyme linked fluorescent assay (ELFA). Vidas SET2 is a rapid and fully automated kit detecting, without differentiation the staphylococcal enterotoxins (SET) types A to E, using a cone coated with antibodies specific for SEA, SEB, SEC1, SEC2, SEC3, SED and SEE. The enterotoxins presence was made directly from cheese samples by concentration, according to the producer protocols. According to the manufacturer, the VIDAS SET 2 method has a sensitivity of at least 0.5 ng enterotoxin g$^{-1}$ food. The system analyzed automatically the results and assigned a test value to each sample and interpreted it as positive or negative. The results are given in a relative fluorescence value (RFV). Estimate that staphylococcal enterotoxins are detected in a 25 g portion if the TV (sample RFV/standard RFV) of the extract is higher or equal to 0.13 and are not detected if the TV of the extract is lower than 0.13 (11).

Results and discussions

The results regarding the level of contamination with Staphylococcus aureus and the presence of staphylococal entrotoxins are presented in table 1. The general level of contamination in analyzed cheese samples was variable depending on type of cheese and month where the samples were taken. Generally, the level of contamination with staphylococci varied $10^2$ to $10^7$. The high levels of Staphylococcus aureus of raw milk could be attributed to high prevalence of the organism in udders of dairy cows and/or due to cross contamination by
hands of milkers. Others researchers found similarly level of cheese from unpasteurized milk contamination with *S. aureus* from 3 – to 8 log cfu/g (2,4,7). The prevalence of pathogenic microorganism, especially *Staphylococcus aureus*, in cheese depended mainly on quality of milk used, general sanitation during obtaining and cheese handling procedures.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sample</th>
<th>Type of cheese</th>
<th>Level of contamination</th>
<th>RFV*</th>
<th>RFV**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Telemea from cow milk</td>
<td>2.3 x 10^7</td>
<td>4</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Caş from sheep milk</td>
<td>1.1 x 10^10</td>
<td>11</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Telemea from sheep milk</td>
<td>1.8 x 10^7</td>
<td>11</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Telemea from cow milk</td>
<td>1.3 x 10^3</td>
<td>3</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Telemea from sheep milk</td>
<td>1.0 x 10^7</td>
<td>24</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Telemea from sheep milk</td>
<td>2.3 x 10^7</td>
<td>766</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Telemea from sheep milk</td>
<td>3.2 x 10^7</td>
<td>6</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Caş from sheep milk</td>
<td>1.0 x 10^6</td>
<td>9</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Telemea from sheep milk</td>
<td>3.2 x 10^6</td>
<td>5</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Caş from sheep milk</td>
<td>4.6 x 10^6</td>
<td>8</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Telemea from sheep milk</td>
<td>1.0 x 10^7</td>
<td>9</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Caş from sheep milk</td>
<td>5.9 x 10^6</td>
<td>10</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Telemea from sheep milk</td>
<td>4.0 x 10^6</td>
<td>4</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Telemea from sheep milk</td>
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<td>3</td>
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<td></td>
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<td>4</td>
<td>3482</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Telemea from sheep milk</td>
<td>1.7 x 10^6</td>
<td>4</td>
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</table>

Note: *Relative Fluorescence Value for sample, ** RFV Relative Fluorescence Value for standard*

Generally, soft cheese made from pasteurized milk there is not much danger having pathogen inside but a concerning could be enterotoxigenic *Staphylococcus aureus*. Production of domestic cheese commonly are poor hygienic conditions associated. In that case the general level of contamination usually and the possibility to having pathogen inside is high. According to the test results (level of *S. aureus* over 10^6 cfu/g), out of 51 samples 26 samples were tested for the presence of staphylococcal enterotoxins SET. The results of testing for the presence of SET using the *VIDAS SET 2* kit showed that 1 (3.84%) samples
were positive and 25 (96.15%) were negative. The sensibility and specificity of Vidas Set 2 analisys method were established by different resercher (10).

Vernozy et al. (9) established that VIDAS SET2 had a great specificity (100%) and sensitivity for Staphylococcal enterotoxins (SET) detection because of the use of new monoclonal antibodies and polyclonal antibodies directed against antigenic sites.

In Brazil, Carmo et al. (2) investigated the presence of staphylococci in Minas fresh raw milk cheese and observed that the isolates were able to produce enterotoxins A, B and C.

The presence of SET was detected in semi soft cheese sample made from sheep milk and ripened in brine. The intrinsec condition (salt concentration, relatively water activity and low pH) from analyzed cheeses did not inhibit the growth of this pathogens and produced heat stable enterotoxins. These products produced in small and medium scale local market highlight a deficit in hygienic measures, lack in cooling facilities and this hand making products may present an additional risk for consumer. According to other researcher calls for better control of cheese contamination sources and taking hygienic measures is necessary for milk products safety.

Conclusions

The results permitted us to concluded that the processing conditions for obtaining domestic cheese sold on free market in these county are variable, based on extremelly variable level of contamination. Cheeses sold on free market posed a certain risk of contamination by toxigenic staphylococci and need of implementing proper hygienic practices.

It is emphasized that the presence of staphylococcus enterotoxins (even in a small percentage) in domestic cheese sold on free market in these counties could be regarded as a potential risk for human health.

Acknowledgments

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BURSA OF FABRICIUS DEVELOPMENT AND COLONIZATION

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Summary

The bursa of Fabricius is a lymphoid organ of all species of birds and it was first
described by Hieronymus Fabricius in 1621. The function of the bursa remained a mystery
until 1954, when Bruce Glick conducted an experiment on normal and bursectomized birds
about production of antibodies against Salmonella, showing the implication of this organ in
humoral immunity. Bursa of Fabricius, originally an ecto-mesodermal rudiment, is colonized
by hematopoietic cells of extrinsic origin. Primordial bursa can be found on the fourth day
of incubation as an epithelial bud in the cloacal region. Colonization of bursa is an ongoing
process which takes place between 8th and 14th day of incubation.

Key words: bursa of Fabricius, development, B cells

History

The bursa of Fabricius is a lymphoid organ of all species of birds and it was
first described by Hieronymus Fabricius ab Aquapendente in 1621. Fabricius was
an anatomy professor at Padova University and built a reputation that attracted
students from all of Europe. The English anatomist William Harvey was his pupil
and Fabricius gave him the first clear description of the semilunar valves of the
veins, which later provided Harvey with a crucial point in his famous argument for
circulation of the blood (25).

Fabricius’ "De Formato Foetu", summarizing his investigations of the fetal
development of many animals, including man, contained the first detailed
description of the placenta and opened the field of comparative embryology. He
also gave the first full account of the larynx as a vocal organ and was first to
demonstrate that the pupil of the eye changes its size (25).

Fabricius described the bursa as following “…I think that this is the place
into which the cock introduces semen so that it may be stored there”. The idea that
the bursa of Fabricius was a semen receptacle was tested by Harvey. Sperm
survived for 20 days after an insemination, providing a temporary support for this
theory (24).

But the true function of the bursa remained a mystery until 1954, when
Bruce Glick conducted an experiment on normal and bursectomized birds about
production of antibodies against Salmonella. This experiment showed that
bursectomized chickens did not produced any agglutinating antibody (24).
After that, a series of experiments with larger numbers of chickens were made to investigate the finding. These experiments confirmed the theory that removal of the bursa of Fabricius would inhibit antibody production (9).

The results were presented in a paper prepared for Science, paper which was rejected with the editor’s suggestion that “further elucidation of the mechanism…should be attempted before publication” (9). The revised paper was published in Poultry Science in 1956 (8).

**Bursa of Fabricius development phases and structure**

Bursa of Fabricius develops in a similar way avian thymus, being originally an ecto-mesodermal rudiment and is colonized by hematopoietic cells of extrinsic origin. Primordial bursa can be found on the fourth day of incubation as an epithelial bud in the cloacal region (4, 7).

The first experiments revealed 3 phases of bursa growth. The rapid growth phase occurs from hatch to 3 week of age. The plateau phase corresponds to period between 4 and 8 week, and the last one, the regression phase, take place after 8 week of age (8). Glick et al. (8) showed that bursal regression occurs before sexual maturity.

The bursa is a sac-like dorsal diverticulum of the proctodeum characterized by tall, thick mucosal folds filled with numerous polyhedral follicles. Each follicle, composed of lymphatic tissue, is divided into a cortex and medulla. A layer of undifferentiated epithelial cells occupies the periphery of the medulla, which is separated from the cortex by a capillary layer. The bursa is lined by a pseudostratified columnar epithelium, except at the apex of each follicle, which is covered by a simple columnar epithelial tuft (2, 3, 16).

**Bursa of Fabricius colonization and B cells**

Unlike the thymus, where colonization takes place in three successive waves, colonization of bursa is an ongoing process which takes place between 8th and 14th day of incubation (7).

Prebursal stem cells originate in the bone marrow and can be found only in the central lymphoid organs during embryonic period. In other words, unlike the thymus for which bone marrow produce progenitor cells also after hatching, the bursa is populated strictly in embryonic period, then taking full bone marrow function in the production of B lymphocytes (7).

Bursectomy conducted in late embryonic stages or soon after hatching results in significant reduction of circulating B lymphocytes and inability to produce antibodies. These phenomena demonstrate the unique role of the bursa of Fabricius, a microenvironment essential for the proliferation and differentiation of B cells (17).

Upon hatching, the bursa contains about 10,000 follicles structured in a cortex with endodermic origin and mesodermal medulla. Follicles contain approximately 100,000-150,000 B lymphocyte. The follicles development is not
synchronous, between 11th and 14th day of incubation being observed the constant emergence of new follicular "buds" (7).

Cortex and medulla are related both structurally and functionally to a follicular epithelium. Structural unit that appears first in ontogeny is medulla (11-12 days of incubation), followed shortly by the formation of follicular epithelium (14-15 days of incubation) (13, 23). Cortical cells first appear around the time of hatching, and cortex structure is finalized completely within two weeks (17, 23).

Medulla can be defined as a classical lymphoepithelial tissue. On days 10-13 of embryonic development, the precursors of bursal secretory dendritic cells (BSDC) penetrate the surface epithelium and induce an embryonic bud in mesenchyme.

Epithelial cells of embryonic bud and BSDC precursors will form a transitional dendro-epithelial tissue that is capable of receiving lymphocyte precursors. Thus, follicular colonization occurs in two stages (12):
1. dendro-epithelial tissue formation;
2. colonization of dendro-epithelial tissue by B cells precursors.

Cell transfer studies focused on the embryonic spleen cells inoculation in immunocompromised embryos have demonstrated the presence of precursors which have the potential to colonize both thymus and spleen. These experiments showed that only prebursal cells express surface antigen chB6, specific to B cells, namely that the "fate" of T and B cells precursors is established before colonization of the two primary lymphoid organs (1, 10). This finding is similar to murine lymphoid precursor's situation: cells that trigger Notch 1 receptor will evolve and be differentiated as T lymphocytes and precursors that do not activate this receptor will become B lymphocyte (20).

Ig gene rearrangement is an essential stage in the development of B lymphocyte line and there is already evidence that this phenomenon occurs in the bone marrow, both for heavy and light chain, before progenitor cells to colonize the bursa. However, these precursors express surface immunoglobulin in the bone marrow (5, 19). Another site for light and heavy chains gene rearrangement is spleen (22).

B cell precursors leave the bone marrow via blood and will constantly colonize the, as already mentioned, between days 8 and 14 of incubation (5). Colonization of bursa involves cell migration through basement membrane that separates the epithelium from mesenchymal tissue (18). Because chicken embryo blood contains also other cell lines, two main explanations were raised about the entering of lymphoid precursors in this organ. First put forward the idea that all lymphoid cells can enter the bursal mesenchyme, being retained only those which will result in mature B cells (5). The second, based on high density B cell progenitors evident on bursal sections, issued the possibility of selective transit, similar to that achieved by naive lymphoid cells through high endothelial venules of lymph nodes and leukocytes through inflamed endothelium to reach the active site of infection (18).
That selective transit could be facilitated by complementary adhesion molecules on vascular endothelium, respectively on B cell precursors, molecules that would allow penetration of bursal mesenchyme scholarships (18). To date there were not characterized adhesion molecules with such a function, but for some of them it was demonstrated the importance in the development and differentiation of B cells. For example, fibronectin localized mainly interfollicular space and at the cortex-medulla junction, would provide support for immature B cells development from day 15 of incubation (15).

The most likely explanation for selective crossing the basement membrane is involvement of a functional Ig receptor-based mechanism. If the majority of the precursors which leave the bone marrow have suffered incomplete (unproductive) rearrangements of Ig genes (17), those who colonize the bursa contain only productive rearrangements and, therefore, express Ig (18). In other words, it is possible that the cells already in bursa emit some signals that are recognized by precursors with functional B cell receptor (BCR) and, therefore, only those precursors will colonize lymphoid organ (14, 18).

The first cells that cross the basement membrane do not have lymphoid nature, being described as secretory dendritic cells. These cells, by their nature, can provide chemokines and other molecules that attract B cell precursors. Thus, it is likely that, after productive Ig gene rearrangements, precursors to express or increase the expression of specific receptors for certain chemokines (18).

Parabiotic experiments (based on blood flow connection between two embryos of different strains) allowed assessment of the number of cells that colonize bursal follicles. This showed that most follicles contain precursors from strain A or B, while others were mixed. This observation demonstrates that each follicle is populated by a small number of progenitor cells, with an estimated average of three and range between two and five cells (17).

Considering an average of 10,000 follicles / bursa, it means that the whole population of chickens B cells derives from about 30,000 precursors that have undergone productive rearrangements in the heavy and light chain loci. Given the fact that only one in three rearrangements is productive for light chain and one of nine for heavy chain, only about 3% of B cells precursors will express functional Ig (10^6 cells) (18).

Immediately after follicles colonization, precursor cells proliferate rapidly. Along with the multiplication, gene conversion also takes place, resulting in heavy and light chains diversification, namely B cell repertoire diversification (18). First mature B cells expressing membrane IgM are identified on 12th day of incubation, and after hatching, 90% of bursal cells are fully functional (7).

In the last week of embryonic development there is an exponential growth of B cells expressing Ig, mitosis taking place at an interval of about ten hours (18). The rapid proliferation is stimulated by growth factors, among which chBAFF, counterpart of B-cell activating factor in mammals, it supposed to be essential (11, 21).
At this point we can speak about an expansion phase in the evolution of the bursa of Fabricius - the cellular compartment evolves from approximately 30,000 cells with a limited repertoire to approximately $10^7$ cells which, following gene conversion, have a highly diversified repertoire (18). In the last 2-3 days before hatching, mature B cells leave the bursa and will populate the secondary lymphoid organs (6).

References


DEVELOPMENT OF SECONDARY LYMPHOID ORGANS IN CHICKEN EMBYOS – SPLEEN AND GUT ASSOCIATED LYMPHOID TISSUES

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Summary

This study aims to systematically assess the histogenesis of spleen and GALT in chicken embryos between 4th and 20th day of incubation. Thus, 85 embryos were histological processed (whole embryos or only spleen and bowel segments), and histological section were made and stained. Microscopic examination aimed daily morphological aspects and changes for spleen and gut associated lymphoid tissues (GALT). Spleen primordium was identified in the 5th day of incubation, and the organ showed a marked growth between 13th and 20th day of incubation, correlated with the embryos development. Splenic parenchyma has uniform structure, without connective trabeculae or obvious demarcation between white and red pulp, up to and including day 20 of incubation. GALT histogenesis begins in the 17th day of incubation.

Key words: Cobb 500 embryos, spleen, gut-associated lymphoid tissues

The spleen is an important peripheral lymphoid organ (secondary lymphoid organ) in which major cell- and antibody-mediated immunes response take place, in addition to other functions (including phagocytosis of foreign structures, elimination of aging blood cells, platelet storage etc.) (7).

Spleen primordium appears, as a mass of mesenchymal cells, in the second day of incubation. Sinusoids with erythrocytes can be identified from the fifth day of chicken embryos development, granulopoiesis begins in the 7th day of incubation, and erythropoiesis from day 11 (4). The spleen plays an important role in embryonic lymphopoiesis, being a site for rearrangement of genes encoding the immunoglobulins of B progenitor cells (3).

The basic structure of the chickens spleen is similar to that described in mammals, but the white pulp is less well represented (55-60% of the total weight of the spleen) than in mammals (1). This, along with the closed type of circulation (capillaries are connected directly with venous sinuses) suggests a smaller contribution in oxygen circulation (4).

Compensating for the absence of encapsulated lymph nodes, birds developed diffuse lymphoid tissues in all regions in which the antigenic stimulation take place. Along the digestive tract, small lymphoid aggregates, solitary nodules or
follicles (gut-associated lymphoid tissues - GALT) can be observed. Also, can be identified several organized lymphoid tissues - cecal tonsils, Peyer plates, Meckel's diverticulum and pyloric tonsil (4).

It is considered that the majority of organized lymphoid tissues begin their development during incubation, thus independent of antigenic stimulation (4).

This study aims to systematically assess the histogenesis of spleen and GALT in chicken embryos between 4th and 20th day of incubation.

**Materials and methods**

To assess the development of the secondary lymphoid organs, 85 fertilized eggs of Cobb 500 hybrids were used. The eggs obtained from Ave Impex Ltd, Satu Mare, Romania, and were incubated in Eggs’ Incubator IO-1P TE (S.C. ELECTROARGES, Romania) according to manufacturer instruction.

The study involved histological processing of whole embryos (5 embryos/ day between 4 and 12 day of incubation) and every day from 13th to 20th day of incubation, five embryos were weighed using analytical balance ALJ 220-4NM (Kern&Sohn GmbH, Balingen, Germany) and from each of them spleen and small and large bowel segments were harvest. Spleens were also weighed and their weights were reported to embryos' weight.

The embryos, spleens and bowel segments were fixed in ethanol 80\(^\circ\), and after that, histological sections were made and stained by hematoxylin-eosin (H&E) method, in standard technique (5).

Histological sections were assessed using optical microscope Olympus CX41 (Olympus America Inc., USA), aiming daily morphological aspects and changes for spleen and gut associated lymphoid tissues (GALT).

The data obtained were processed using SPSS 7.5 software (SPSS Inc., IBM Corporation, NY, USA), \(t\) test and correlation coefficient \((r)\) being calculated.

**Results and discussions**

**Spleen.** Lienal primordium was identified in the 5th day of incubation (fig. 1), similar to report of Oláh and Vervelde (4) and contrary to assertion of Valli and Jacobs (7), which places the beginning of spleen histogenesis in the 8th day of incubation.

In the early stages of histogenesis, it was recorded strictly a reticular structure, without vascular sinusoids. Venous sinuses were formed in the eighth day and, due cells morphology and density, we believe that the spleen colonization by leukocytes starts in the ninth day of incubation. Moreover, Oláh and Vervelde (4) mention granulopoietic and erythropoietic activity from the day 9th, respectively 10th.

Splenic parenchyma has uniform structure, without connective trabeculae or obvious demarcation between white and red pulp, up to and including day 20 of
incubation (fig. 2). Also, the white pulp has diffuse appearance due to the absence germinal centers, which are formed strictly after hatching (4, 8).

Fig. 1. Longitudinal section through a five-days-old Cobb 500 embryo: a – spleen; b – liver; c – mesonephros. H&E stain, x200

Fig. 2. Cross-section through spleen of a 20-days-old Cobb 500 embryo: a – diffuse white pulp; b – sheathed arterioles; c – venule. H&E stain, x400

Sheathed arterioles (surrounded by macrophages and reticular stroma), which are specific for the avian spleen, could be observed from the 12th day of incubation.

The spleen showed a marked growth between 13th and 20th day of incubation, aspect demonstrated by their increase in weight and diameter (Table 1).
Table 1
Evolution of spleen diameter and weight in Cobb 500 embryos between days 13th and 20th of incubation

<table>
<thead>
<tr>
<th>Incubation day</th>
<th>Diameter (mm)</th>
<th>Embryos weight (g)</th>
<th>Spleen weight (g)</th>
<th>% from embryos weight</th>
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<tr>
<td></td>
<td></td>
<td>( \bar{x} \pm Sx ) C.V.%</td>
<td>( \bar{x} \pm Sx ) C.V.%</td>
<td>( \bar{x} \pm Sx ) C.V.%</td>
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<tr>
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<td>0.0141±0.0010</td>
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<tr>
<td>20</td>
<td>4-5</td>
<td>36.55±1.87*</td>
<td>5</td>
<td>0.0152±0.0012</td>
</tr>
</tbody>
</table>

Legend: \( \bar{x} \) – arithmetic mean; \( Sx \) – standard deviation

*embryos were weighed without vitellus

Embryonic spleen size and weight between days 16 and 20 are comparable to those reported by Banga (2) - 2.5 and 4.5 mm, 10.9 and 13.2 mg – in broiler chickens.

Comparing spleen weight values obtained in the successive days, significant difference was obtained only on days 13/14 (\( p < 0.001 \)) 14/15 (\( p < 0.001 \)), 16/17 (\( p < 0.001 \)) and 19/20 (\( p < 0.05 \)). This has been correlated with differences in the proportions of spleen weight from the embryos weight in all phases (\( p < 0.01 \)), except on 19/20 day. In addition, the proportion of spleen weight from the embryos weight registered significant differences (\( p < 0.001 \)) in days 15/16, 17/18, and 18/19.

As can be seen from the above table, the most significant increase in spleen weights were recorded in days 14th and 17. After 17th day of incubation, there is a gradual reduction in the average weight of the organ. The first phase can be explained by the lymphocyte wave (3) and by the high erythropoietic and granulopoietic activity. Weight reduction of the spleen may be due to massive release of granulocytes and erythrocytes.

Spleen weight was directly and significantly correlated with embryo weight, yielding the following values of the correlation coefficient \( r \): day 13 - 0.714 (\( p < 0.01 \)), day 14 - 0.564 (\( p < 0.05 \)), day 15 - 0.387 (\( p < 0.05 \)), day 16 - 0.318 (\( p < 0.05 \)), day 17 - 0.488 (\( p < 0.01 \)) and day 20 - 0.391 (\( p < 0.05 \)).

Regarding GALT, histological investigations did not permit identification of organized structures, like cecal tonsils or Peyer plates, although sections were made through intestinal segments known as the site of these formations in adult birds.
From the 17th day of incubation, were identified only sporadically, small accumulations of basophilic cells (possibly lymphocytes) both in the axis of intestinal villi and adjacent epithelium (Fig. 3).

Fig. 3. Cross-section through duodenum of a 17-days-old Cobb 500 embryo – subepithelial accumulation of lymphoid cells. H&E stain, x400

Weak development of the GALT explains chickens sensitivity regarding pathogens entering the digestive way in the first days of life.

Conclusions

Lienal primordium was identified in the 5th day of incubation, and the spleen showed a marked growth between 13th and 20th day of incubation, correlated with the embryos development.

GALT histogenesis begins in the 17th day of incubation.

The only secondary lymphoid organ that reaches an advanced degree of development during incubation in Cobb 500 hybrids is the spleen.

References


THE IMPACT OF THE TECHNOLOGICAL FLOW ON PIG CARCASS QUALITY

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Summary

The carcass quality of 727,000 pigs butchered during May 2012 until January 2013 was studied, evaluating femur and basin fractures. The study was performed on fat pigs coming from five genetic lines (A - E) and values ranging from 16 % (B) to 31 % (C). Proportional to the weight increase in live pigs (122.56 kg) increased the fracture percent (C), of which 55% were femur fractures.

No connection was established between animals limping or with movement difficulties and animals diagnosed with basin or femur fractures.

Animals coming from the C line were observed to be predisposed to femur and basin fractures, a direct correlation between the large weight of live animals and the femur fracture percentage.

Key words: pig, carcass evaluation

The evaluation of welfare in animals, in general, and particularly, in the swine, represents a significant phase for assessing the carcass and implicitly, the meat. If in the farm and particularly, in the swine shelter, the criteria are correlated to the principles used for evaluating the animals’ welfare (WQ book), in the slaughter house, the criteria used are correlated to the technological flow, seeking, at the same time, to find answers to the unanswered questions in the shelter.

Materials and methods

Subsequent to their arrival to the slaughter house, the following determinations were pursued based on the four principles (feeding, housing, health and behaviour) and taking into account, for the slaughter house, only the third principle: the pigs that have fallen down (irrespective of the causes), the limping pigs and the pigs diagnosed with different fractures, and for the carcasses, the seizures owed to pneumonia, pleurisy, pericarditis and white spot on liver, in 14,840 pigs. The determinations were made from May 2012 to January 2013.

The surprise consisted in the fact that a number of carcasses, which however managed to pass through the initial evaluation, have been seized,
although they have not been subject to the evaluation of animals’ welfare. A significant deformation of the calves (legs) has been found due to the lack of support through the femoral bone and the pelvis bones, meaning a lack of continuance thereof (fracture of the femur and tail bone).

Results and discussions

Subsequent to their arrival to the slaughter house, the following determinations were pursued based on the four principles (feeding, housing, health and behaviour) and taking into account, for the slaughter house, only the third principle: the pigs that have fallen down (irrespective of the causes), the limping pigs and the pigs diagnosed with different fractures, and for the carcasses, the seizures owed to pneumonia, pleurisy, pericarditis and white spot on liver, in 14,840 pigs. The determinations were made from May 2012 to January 2013.

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Results and discussions

Examining the situation in 14,840 animals, the following data, highlighted in table 1, have been found:

<table>
<thead>
<tr>
<th>Farm /no.</th>
<th>Fallen down</th>
<th>Limping</th>
<th>Fractures</th>
<th>Pleurisy</th>
<th>Pneumonia</th>
<th>Liver</th>
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<td>8</td>
<td>92</td>
<td>747</td>
<td>960</td>
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There has been found that, compared to 0.24% pigs with different movement difficulties (fallen) and 0.2% limping pigs, over 13% fractures were recorded; however, the pulmonary diseases reached 25% and the liver was affected in over 40% of the animals that have been examined. The fact that ¼ of animals required the removal of the lungs due to various reasons means that major microclimate problems have occurred.

Analyzing this situation in different farms, we may argue that, following a decreasing order, the situation of pulmonary diseases was as stated below:
in farm 1C, the first position was occupied by 31.98% pulmonary diseases (14 + 17.98), being subject to seizures exceeding the average value by 6.98%;
• in farm 5P, the second position was occupied by 27.96% pulmonary diseases (13.02 + 14.94), exceeding thus the average value by 2.96%;
• the third position came to the farm 2G, i.e. 25% pulmonary diseases (15 + 10);
• in farm 3P, the fourth position came to 15.21% pulmonary diseases (7.9 + 7.31), with 9.79 less than the average value;
• in farm 4B, the fourth position came to 10.8% pulmonary diseases, with 14.2% less than the average value.

Comparing the values to the warning threshold and implicitly, the alarm threshold of the quality-based welfare evaluation, where the following percentages are accepted: 28% (warning) and 55% (alarm) for pleurisy, and 2.7% (warning) and 6% (alarm) for pneumonia, we reach the following conclusion: the animals from four farms, except for the farm 4B, have exceeded both the warning threshold and the alarm threshold with reference to pneumonia but none has exceeded the threshold for pleurisy.

Moving on to the analysis of the data related to the 727.000 carcasses with consistency changes, we found that the femoral bone showed very different structural changes (fig. 1).

a • rupture, however, keeping the bone radius (a),
• a rupture followed by the dislocation of the muscle mass (b),
• a rupture causing the bone fragmentation (c).

Fig 1. Examples of bone ruptures

Attempting to find a proper explanation for this situation, we concluded that these changes are owed to the post-mortem muscular contractions. After elaborating a classification of the farm pigs, taking into consideration the genealogical flow, the existence of five genetic lines (A-E) has been found. A
subsequent classification of the problems involving the femoral fractures (ff) and pelvis fractures (pf) in all five lines emphasized the following data:

- Farm 1C - 11% ff and 19% pf;
- Farm 2G - 55% ff and 36% pf;
- Farm 3P - 11% ff and 16% pf;
- Farm 4B - 13% ff and 15% pf;
- Farm 5P - 10 % ff and 14% pf.

The conclusion has been reached that the large weight of live animals, when arriving, determines a superficial increase of the percentages in the femoral fractures (ff) and pelvis fractures (pf). An increase in the percentage of fractures in November 2012 has also been noted, concomitantly with the weight gain of live animals when delivered (122.56kg). Furthermore, a predisposition to fractures in the pigs coming from the farm 2G has been noticed, because, from the total number of femoral fractures, 55% were identified in animals originating from this genetic line.

However, no relation between the limping animals or animals with different movement difficulties and the animals diagnosed with pelvis or femoral fractures, was found. Similarly, no relation between the limping animals / animals with different movement difficulties and the animals identified with fractures (ff or pf), was found.

Conclusions

The welfare evaluation, based on the quality system, underlined that the warning and alarm thresholds, with reference to pneumonia, have been exceeded; There has been found a predisposition of fractures in animals originating from the farm 2G, animals which benefit from a certain genetic line.

There is a direct correlation between the weight of live animals and the percentage of fractures.

References

INFECTIOUS BURSAL DISEASE VIRUS – A SYSTEMATIC REVIEW

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Summary

Infectious bursal disease virus (IBDV) belongs to Birnaviridae family, Avibirnavirus genus and is the etiological agent of infectious bursal disease. Birds are the only species that develops clinical signs and lesions if are exposed to IBDV. Chicken is the natural host, subclinical infection being recorded in turkey and duck; quail and dove are resistant to experimental infection. Field strains develop different degrees of pathogenicity in chickens and vaccine strains also have infectious potential in chickens. There were isolated many strains that differ from each other in terms of antigenicity and virulence, in Europe and the USA being identified two serotypes of IBDV. The first one, serotype 1 is pathogenic, but serotype 2 is non-pathogenic both for chickens and turkeys.

After infection, humoral immune response against IBDV virus develops normally, but the response to other antigens is significantly reduced. The degree of immunosuppression is proportional to the age of infected birds: poultry older than 21 days do not develop significant lesions in lymphoid tissues and develop a normal immune response after passing through disease, but in birds less than 21 days old, humoral and cellular immunosuppression, and also the damages in lymphoid tissues, are extensive.

Key words: infectious bursal disease virus, pathogenicity, immunogenicity

Infectious bursal disease virus (IBDV) is the etiological agent of infectious bursal disease (IBD, also known as Gumboro disease or Infectious Bursitis). IBD is a disease known and studied for over 40 years, previously being included in the list B of the World Organization for Animal Health (OIE). Also, IBD has been the subject of a special resolution of OIE International Committee in 1995 (21).

The severity of the disease, economic losses, and increasingly large spreading (very virulent strains have been reported in Europe, America, Southeast Asia and Japan) placed IBD in researchers’ attention, along with other avian diseases such as Newcastle disease, avian influenza, avian infectious anemia, Marek's disease, etc.

The disease was first discovered in a farm from Gumboro, Delaware in 1962 (hence the name of Gumboro disease) (2). Subsequently Winterfield (1962) isolated the causative agent of the disease, calling it "infectious bursitis agent" (21). IBDV belongs to Birnaviridae family, Avibirnavirus genus (21).
**IBDV morphology**

IBDV presents itself as an isometric particle with a diameter of 55-60 nm, lacking inner capsid and envelope, as demonstrated by electron microscopy (7, 8). Other researchers say that the birnaviruses lost their characteristic inner capsule during evolution from a common ancestry with reoviruses and have adapted simultaneously to form a tightly closed shell structure (9).

Icosahedral capsid is composed of four polypeptides named: VP1, VP2, VP3 and VP4. Recently was described another viral protein, VP 5 (17 kDa), whose functions are not yet known (9).

IBDV structure is a three dimensional one, and was determined by electron cryomicroscopy. Capsid has a T=13 lattice architecture in which the subunits are arranged like a bunch. The T=13 icosahedral surface of viral particle have trimeric projections similar to those from the second layer of Reoviridae (9, 38).

**Genetic and biochemical structure of IBDV**

From the biochemical point of view, IBVD have a genome comprising of two RNA molecules. First molecule, also known as segment A, encodes the precursor of proteins VP2, VP3, VP4 and a small separate protein VP5, of unknown function; the second segment (B) encodes the viral polymerase VP1 (9, 37).

The largest segment in length, segment A, contains a sequence of 3261 nucleotide and two open reading frames (ORF), partially overlapped. The largest direct reading sequence encodes 1012 amino acids that represent a polyprotein of 110 kDa (pVP2-VP4-VP3), catalytically related to viral protease VP4. This polyprotein produces three mature structural proteins VP2 (40 kDa), VP3 (32 kDa), and VP4 (28 kDa). VP2 and VP3 are the major component of the virus, and VP4 is present in a small amount. The second ORF, corresponding to the nucleotide sequence between 62 and 497 bp encodes synthesis of a 145 amino acids cysteine-rich protein with a molecular weight of 16.5 kDa (30).

The segment B is the smallest one, containing 2827 nucleotides and a single open reading frame (ORF). RNA of B segment have positive polarity and serves as mRNA for the synthesis of a protein called polymerase, named VP1, having a molecular weight of about 94 kDa (4). Polymerase contains 879 amino acids in virulent strains and 878 amino acids attenuated strains. VP1 is a multifunctional protein involved in the replicase and the transferase activity as well as in guanylyl transferase and methyltransferase activity related to the viral pathogenicity (1, 5).

**VP1 protein** is an RNA-dependent polymerase, with a molecular weight of about 94 kDa. In virion is present in low proportions (= 3% of the virion protein mass). VP1 can be free or as protein linked to the genome (VPg). VP 1 protein forms complexes with VP3, having an additional role in the morphogenesis of the virus (28).
VP2 protein has a molecular weight of 40 kDa and contains the antigenic region responsible for the induction of neutralizing antibodies as well as serotype specificity. Single expression of VP2 protein results in the formation of a twisted tubular structure and isometric viral particles (9).

VP2 is divided into three distinct domains arranged radially in viral particles. These domains participate in trimer contacts, each of them burying 4700 Å² of its surface for trimers formation. The cross-section of the trimer in the S domain has the shape of an equilateral triangle, with sides of about 100 Å and 25 Å in thickness. This region insures a tight sealing of the virion through extensive lateral contacts in the T = 13 lattice. The overall height of the molecule, including all three domains, is 85 Å (9). VP2 is able to self-assemble, but the pattern assembly is controlled by a series of compounds, as well as internal protein VP3, viral polymerase VP1 and VP4 protease (27).

The VP3 protein presents group specific antigens which are recognized by non-neutralizing antibody, and a carboxy-terminal region that interact with RNA package, which means that its position is within the capsid (27).

VP4 protein is a non-structural protein related to the appearance of specific microtubules in infected cells (10). This role is different from those shown in previous studies that described VP4 as a minor structural component present in mature virions purified by various methods (19).

VP5 protein was first described for IPNV (12) and subsequently identified in IBDV infected cells (24). This non-structural protein is important for virus pathogenicity, but insignificant in the viral replication and infection. The protein is rich in cysteine (24).

Antigenic structure of IBDV

There were isolated many strains that differ from each other in terms of antigenicity and virulence. In Europe and the U.S. were identified two serotypes of IBDV. Serotype 1 was identified by Cosgrove in 1962 and is pathogenic (16); serotype 2, isolated from turkeys, is non-pathogenic both for chickens and turkeys (15, 16).

The differentiation of the two serotypes is carried out by virus-neutralization tests, being impossible by immunofluorescence antibody test or ELISA. Immunofluorescence reactions have established that the two serotypes of avian infectious bursitis are cross-reactive (23). Immunization against serotype 2 does not protect against serotype 1. The reverse situation cannot be tested because there are not virulent strains in serotype 2.

McFerran et al. (20) were the first to describe IBDV antigenic variation among isolates of European origin. They were demonstrated the existence of two serotypes, 1 and 2, and show a 30% similarity between different strains of serotype 1 and prototype IBDV first described.

Jackwood and Saif (17) conducted a virus-neutralization study on eight serotype 1 commercial vaccine strains, five serotypes 1 field strains and two
serotypes 2 field strains, identifying six different subtypes among the 13 strains of serotype 1 studied. One of the subtypes included all variants isolated. Snyder et al. (33), using monoclonal antibodies, suggest that a major shift occurred in the field of serotype 1 of IBDV.

Following the epidemic in Europe and Asia, there was isolated a group of highly virulent strains (vv = very virulent) and pathogenic belonging to serotype 1. The “vv” strains determine a serious deterioration in bursa of Fabricius, thymus, spleen and bone marrow, with mortality rate over 60% in poultry (36).

In order to identify the genomic region associated with virulence and antigenic variability of IBDV, structural and non-structural genes of different strains belonging to serotype 1 were analyzed. The nucleotide sequence was determined for segment A of 13 strains and segment B of 6 strains.

The 13 strains whose segment A is known are:
- strain 002/73 from Australia (14);
- three strains – In-1, In-1M and P2 – from Germany (25);
- three strains – PBG 98, 52/70 and UK 661 – from UK (3);
- five strains – GLS, DS 326, E/Del, D78 and IM – from USA (34);
- a vv strain, OKYM, from Japan (35).

The 6 strains whose segment B is known are:
- 002/73 from Australia (22);
- Cu-1, Cu-1M and P2 from Germany (25);
- UK 661 from UK (6);
- OKYM from Japan (35).

Pathogenicity of IBDV
Gumboro disease is one of the most damaging infectious diseases that may develop in bird and poultry farms throughout the world. All chicken breeds are affected, but Leghorn develop the most severe reactions and has the highest rate of mortality (11).

The virus has immunosuppressive activity in birds, similar to that induced by HIV in humans. The disease has no public health significance because the virus does not affect humans in any way.

Birds are the only species that develops clinical signs and lesions if are exposed to IBDV. Chicken is the natural host, subclinical infection being recorded in turkey and duck; quail and dove are resistant to experimental infection. Field strains develop different degrees of pathogenicity in chickens and vaccine strains also have infectious potential in chickens.

Immunogenicity of IBDV
Immune system response in birds is subject to a number of factors, little different from those faced by mammals. For this reason, it is necessary the presence and cooperation of organs, effectors and complex mechanisms to achieve a reply to harmful agents.
Distinct differentiation of the two types of immune response, cellular and humoral one, led to the development of experimental bird models to investigate this duality and to elucidate the morphological and functional aspects related to T and B cells.

Contradictory is that the immune response against IBDV virus is normally as the response to other antigens is reduced (26). The explanation could be that birds infected with IBDV are capable of producing anti-IBDV antibodies, as demonstrated even in bursectomized birds, suggesting the major role of the spleen and other lymphoid organs in development of antibodies against IBDV (18).

Immunosuppression is proportional to the age at infection and decreases with increasing age of the bird. Infection, in terms of induced immunosuppression, has no consequences in poultry older than 21 days so that they can develop a normal immune response after passing through disease. In poultry less than 21 days, the infection causes immune function disorders. Infection of one day-old chicks causes severe immune depression and risk of immunological failure against ND, ILT, IB, coccidiosis, Marek’s disease, salmonellosis (32).

There were some studies about differences between some of IBDV, from the immune pathogenicity point of view. The following strains were assessed: a virulent strain, IBDV-IM; one intermediate vaccine strain, IBDV-B2; two mild vaccine strain (IBDV-Lukert, IBDV-BVM) (29). These strains of virus produced the following changes and degrees of protection against IBDV infection:

- IBDV-IM, detectable up to eight days in lymphoid tissues (spleen, cecums) induced an increase in the nitrite level in the blood circulation of 86% of the birds on days 2 and 3 post infection (PI), strongly suppressed the mitogenic response of spleen on days 3-8 PI, and produced the best protection against IBDV infection;
- IBDV-B2 - only a few antigens detected in lymphoid tissues in the first eight days PI; was the most virulent strain, producing a strong suppression of mitogenic response, and caused the most severe damages in bursa of Fabricius and most lesions at three days PI;
- IBDV-Lukert - did not induce any detectable lesions in lymphoid tissue, 67% of inoculated birds had detectable antigens in the bursa of Fabricius at 4 days PI; the induced the lowest protection in comparison with IBDV-IM and IBDV-B2;
- IBDV-BVM - did not induce any detectable lesions in lymphoid tissue, all inoculated birds had detectable antigens in the bursa of Fabricius at 4 days PI, and none of the birds was protected against IBDV challenge.

In chickens infected with IBDV was a slight increase in T cells, suggesting the possibility that the thymus have a compensatory role (31).

After studying the effect of IBDV on bursectomized and normal birds using a very virulent, it was seen a 100% mortality in four weeks old SPF chickens, while the infected bursectomized birds developed a discrete, subclinical disease, and anatomopathological findings were mild – focal necrosis in the lymphoid tissues (13).

The IBDV infection in poultry compromises humoral and local immunity. The effect is more pronounced when the infection affect young birds. Cellular
immune response is also compromised, and immunosuppression seems to be the direct result of B cell or their precursor’s lysis.

References

RESEARCH ON IMPLICATION OF SOME CYTOKINES IN ENZOOTIC BOVINE LEUKOSIS

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Summary

The aim of this study was the assessment of interferon γ (IFN-γ) and interleukin 10 (IL-10) levels in cattle with enzootic bovine leukosis from Timis County households. To determine serum concentration and dynamics of the two cytokines, were analyzed 82 biological samples (sera) obtained from EBL+ (n = 27) and healthy cattle (n = 55). Sera were assessed by applying quantitative immunoenzymatic techniques, using Bovine Interferon γ (IFN-γ) ELISA Kit and Bovine Interleukin 10 (IL-10) ELISA Kit (Cusabio Biotech, P.R. China).

Concentration of the two cytokines were significantly higher (p ≤ 0.001) in BLV(+) cattle compared with BLV(-) animals. Given IFN-γ concentration, we could distinguish three categories of BLV(+) cattle: cattle with low IFN-γ level, below 20 pg/ml (5.75±2.06 pg/ml, n = 10); cattle with average IFN-γ values, between 20 and 40 pg/ml (33.13±5.67 pg/ml, n = 6) and cattle with high IFN-γ level, 60 pg/ml (86.90±16.85 pg/ml, n = 11). These groups of cattle have inversely proportional values of IL-10, respectively IFN-γ low values were correlated with high IL-10 concentrations (67.25±19.36 pg/ml) and high values of interferon with reduced concentrations of interleukin (10.67±6.62 pg/ml).

Concentration of assessed cytokines is influenced by the enzootic bovine leukosis stage. IL-10 can inhibit the expression of other proinflammatory cytokines with antiviral activity, favoring the transition to persistent lymphocytosis stage.

Key words: enzootic bovine leukosis, IFN-γ, IL-10, ELISA

Enzootic bovine leukosis (EBL) is a malignant neoplastic disease of the reticuloendothelial system, characteristic of adult animals, manifested by an extremely complex symptomatic evolution, caused by the various locations of the neoplastic aggregations of B cells (13).

In the last decades, a series of in vitro and in vivo experimental studies have focused on the analysis of human and mouse cytokines, demonstrating the major implications of these molecules in inflammatory processes, activation or inhibition of effector cells and the elimination of pathogens or tumor cells (5, 6, 7, 11, 17). Thus, determining the level and dynamics of cytokines in enzootic bovine leukosis (EBL) is important both for elucidating the pathogenesis and evolution of
viral disease, and for determining molecular methods to stop bovine leucosis virus (BLV) replication.

In this purpose, in our experiments we chose to assess interferon γ (IFN-γ) and interleukin 10 (IL-10), cytokines with essential role in cellular and humoral immune response (17), in cattle with enzootic bovine leukosis from Timis County households.

IFN-γ, lymphokine produced by activated NK and T cells has antiviral activity and regulates cell growth, being the main activator of macrophages and promotor of cytotoxic cell differentiation (17). Its properties underlying various methods of early diagnosis, of which the most important test is in vitro detection of bovine tuberculosis, applied both in the USA (2) and in some European Union countries (20).

Biological activity of interleukin-10 is mainly focused on inhibition of the inflammatory response. Also, IL-10 is involved in regulation of T and B cells, NK cells and antigen presenting cells differentiation and proliferation (3, 14, 18).

Materials and methods

To determine serum concentration and dynamics of the two cytokines, were analyzed 82 biological samples (sera) obtained from EBL+ (n = 27) and healthy cattle (n = 55). Sera were collected from six outbreaks of enzootic bovine leukosis from Timis County.

All the assessed cattle were female and were from non-professional growing units, such households.

Sera were assessed by applying quantitative immunoenzymatic techniques, using Bovine Interferon γ (IFN-γ) ELISA Kit (Cusabio Biotech, P.R. China) and Bovine Interleukin 10 (IL-10) ELISA Kit (Cusabio Biotech, P.R. China). The technique respected manufacturer instruction manual provided by the kit.

Briefly, antibody specific for IFN-γ has been pre-coated onto microplate provided by Bovine Interferon γ (IFN-γ) ELISA Kit (Cusabio Biotech, China). Standards and samples were pipetted into the wells and any IFN-γ present was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for IFN-γ was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash, to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of IFN-γ bound in the initial step. The color development was stopped and the intensity of the color was measured.

The microtiter plate provided in Bovine Interleukin 10 (IL-10) ELISA Kit (Cusabio Biotech, China) has been pre-coated with goat-anti-rabbit antibody. Standards and samples were added to the appropriate microtiter plate wells with an antibody specific for IL-10 and Horseradish Peroxidase (HRP) conjugated IL-10. The competitive inhibition reaction was launched between with HRP labeled IL-10 and unlabeled IL-10 with the antibody. A substrate solution was added to the wells.
and the color developed in opposite to the amount of IL-10 in the sample. The color development was stopped and the intensity of the color is measured.

Calculation of results involved the construction of a standard curve based on the average OD of standard sera, after subtraction of average OD corresponding to blank wells. Cytokine concentration in the samples was extrapolated from values of standard sera using a four-parameter logistic curve, performed with MasterPlexReaderFit 2010 software (Hitachi Solution America, Ltd., SUA).

All the data obtained were processed using Excel (Office 2007) and SPSS 7.5 software (SPSS Inc., IBM Corporation, NY, USA), applying the t test. Also, the Pearson’s correlation coefficient (r) and determination coefficient (r²) were calculated.

Results and discussions

As shown in Table 1 and Figures 1-2, the serum concentrations of the two cytokines were significantly higher (p ≤ 0.001) in BLV(+) cattle compared with BLV(-) animals.

<table>
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<th>BLV(+)</th>
<th>BLV(-)</th>
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<tr>
<td>IFN-γ</td>
<td>44.90±38.53</td>
<td>7.22±4.84</td>
</tr>
<tr>
<td>IL-10</td>
<td>36.46±28.28</td>
<td>9.04±8.49</td>
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Legend: $\bar{x}$ – arithmetic mean; $S_x$ – standard deviation

These results are similar to those reported by Azedo (4) in experimental infection with BLV in Holstein cows: 34.48±30.25pg/ml for IFN-γ and 83.86±78.51 pg/ml for IL-10. We mention that the author used and ELISA kit for human IL-10, not for bovine interleukin.

Increased serum levels of the two cytokines or increased expression of mRNA encoding IFN-γ and IL-10 in enzootic bovine leukosis has been reported by other authors (1, 9, 10, 12, 15, 21).

Coefficients of variation corresponding to IFN-γ and IL-10 levels were very high (85%, respectively 77%) (figs. 1 and 2). Similar variations have been observed in healthy cattle. We mention that five healthy cattle had undetectable IFN-γ concentration, and these results were removed from the statistical analysis.

These evident individual variations can be attributed to genetic characteristics, type of farm (conditions are very different from household to household), physiological condition, infections or other diseases prior / current / clinically unapparent etc.
Fig. 1. Serum concentration of IFN-γ in BLV (+) cattle

Fig. 2. Serum concentration of IL-10 in BLV (+) cattle
Literature indicates very low or very high concentration, for example 300 pg/ml for IL-10 in clinically healthy cattle (16). Similarly, Hisaeda et al. (8) reported high serum IFN-γ in cows suffering from coliform mastitis, even after the period of convalescence. Also Azedo (4) obtained some very high values and coefficients of variation of 58% and 86% for serum IFN-γ and IL-10 in healthy Holstein cattle.

For BLV(+) cattle, the most important factors influencing the serum concentration of both cytokines were, most likely, manifestation of the disease (aleukemic or leukemia with persistent lymphocytosis) and evolution stage. Thus, given IFN-γ concentration, we could distinguish three categories of BLV(+) cattle:

- Cattle with low IFN-γ level, below 20 pg/ml (5.75±2.06 pg/ml, n = 10);
- Cattle with average IFN-γ values, between 20 and 40 pg/ml (33.13±5.67 pg/ml, n = 6);
- Cattle with high IFN-γ level, 60 pg/ml (86.90±16.85 pg/ml, n = 11).

These groups of cattle have inversely proportional values of IL-10, respectively IFN-γ low values were correlated with high IL-10 concentrations (67.25±19.36 pg/ml) and high values of interferon with reduced concentrations of interleukin (10.67±6.62 pg/ml) (fig. 3).

Corroborating these results with hematological profile of the BLV(+) cattle (unpublished data), we could see that the 11 cattle with high IFN-γ and low IL-10 titer were aleukemic. Also, the 16 cattle with low and medium concentrations of IFN-γ (i.e. high or medium levels of IL-10) had values above the upper limit of the lymphocyte number.

These variations in IFN-γ concentration, according to the stage of the EBL, are reported by other authors. Thus, Konnai et al. (9), comparing aleukemic cattle and with persistent lymphocytosis, showed that the first group of animals expressed significantly higher levels of IFN-γ.
Another study, focused on the immune response developed by sheep experimentally infected with BLV, shows that the spreading virus is poor in animals that had initially high levels of mRNA encoding IFN-γ, even if the authors used the same infective dose (19).

Also, Yakobson et al. (21) reported in experimental infections in cattle, that IFN-γ is detectable at high levels after the first 2-3 weeks after exposure to BLV and its level decreases in 12-weeks, after which remained constant.

High concentration of IFN-γ in aleukemic cattle demonstrates once again the importance of their antiviral action, which enhances the cellular immune response, as well as the humoral immune response by antibodies, and opens the possibility of its use as a therapeutic factor in enzootic bovine leukemia.

Returning to the correlation between IL-10 and IFN-γ serum concentrations, we can say that the apparent association of these data is confirmed by statistical analysis: the correlation coefficient r -0.8289 indicate a high negative correlation between the two cytokines (p ≤ 0.001). The coefficient of determination r² 0.68 shows that 68% of the IL-10 concentration variation determined variation of IFN-γ level, which corresponds to a significant effect.

Given the frequency of our national and local screening for enzootic bovine leukemia, the cattle were assessed in the first months of BLV infection.

Conclusions

Serum levels of IFN-γ and IL-10 in cattle with enzootic bovine leukemia were significant higher than those of healthy cattle.

Concentration of assessed cytokines is influenced by the enzootic bovine leukemia stage.

Serum concentration of IFN-γ is negatively highly correlated with IL-10 level.

IL-10 can inhibit the expression of other proinflammatory cytokines with antiviral activity, favoring the transition to persistent lymphocytosis stage.

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ANTIMICROBIAL SUSCEPTIBILITY OF BIOFILM FORMING BACTERIA FROM ORAL CAVITY IN DOGS

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Summary

The occurrence and antimicrobial susceptibility of Staphylococcus, Streptococcus, Pasteurella and Neisseria strains collected from supragingival sites in dogs were evaluated. From 33 dogs with dental plaque were identified 10 strains of Staphylococcus, 22 of Streptococcus, 24 of Pasteurella and 14 of Neisseria. We have selected 10 strains of Staphylococcus, 10 of Streptococcus and 11 of Pasteurella and Neisseria in order to determine the antimicrobial susceptibility. All strains were susceptible to most of the antibiotic tested, however different resistance rates to different antibiotics among strains were observed. The role of Staphylococcus, Streptococcus, Pasteurella and Neisseria strains from oral cavity of dogs in biofilm formation is needed to be defined in order to prevent the appearance and evolution of other dental diseases of oral cavity such as dental calculus, dental caries and periodontitis.

Key words: antimicrobial susceptibility, biofilm, dental diseases, plaque

Dental plaque can be defined as a diverse community of microorganisms, with greater than 10^{10} bacteria per milligram found on the tooth surface, as a biofilm embedded in an extracellular matrix of polymers of microbial and host origin. It has been estimated that as many as 400 distinct bacterial species could be found in dental plaque. In addition to the bacterial cells, plaque contains a small number of epithelial cells, leukocytes, and macrophages.

The extracellular matrix is formed from bacterial products and saliva and contains proteins, polysaccharide and lipids. Today, there is a large interest on the role of biofilm as microbial communities in all sectors of medicine, industrial and environmental microbiology (1).

Many researches from last years have provided a solid foundation for current studies of oral biofilm. In these studies there were a lot of reports about the diversity of the resident oral microflora, both at genus and species level in health and disease (6).

Dental biofilm gathers preferentially on stagnant sites that afford protection from the removal forces that react in the mouth. Dental plaque can develop in distinct phases including:
(a) adsorption of host and bacterial molecules to the tooth surface;
(b) passive transport of oral bacteria to the tooth surface;
(c) co-adhesion of later colonizers to already attached early colonizers;
(d) multiplication of the attached micro-organisms;
(e) active detachment.
In biofilm communities there are biochemical and metabolic interactions but cells have also been shown to communicate with one another via diffusible molecules. This communication involves cell-cell signalling systems (quorum sensing) that help them to adapt and survive various environmental factors and regulate the expression of genes that can influence their ability to cause disease (4).

Benefits of a microorganism community lifestyle that can form biofilm include: (a) a larger habitat for growth, (oxygen-consuming species create favorable environmental conditions for obligate anaerobes); (b) metabolism of these bacteria is more efficient; (c) resistance to stress and antimicrobial agents, and (d) enhanced virulence (2, 5, 7).

Dental plaque biofilm has direct implications on periodontal disease and is essentially a succession of microbial colonization of oral bacterial flora that can induce the appearance of an imbalance between the pathogenic and resident microbial flora favoring the transition from health to disease (3).

The exact definition of microbial biofilm was a real problem faced by scientists over time due to the fact that its structure and composition is highly variable and is influenced by the action of several external and internal factors. Because microbial biofilm was very hard to be identified in recent decades attention has focused on the study of microbiologists on visualization, formation and strength of microbial communities involved in its composition as planktonic bacteria and as their capacity of biofilm forming bacteria and direct implications in disease.

Materials and methods

The research was made on 33 dogs from Budapest-Hungary. Samples were collected from dogs with plaque as well as from dogs with other diseases of hard tissues of the mouth. Sampling was performed in dogs under anesthesia with or without intubation. Anesthesia was done according to the animal weight with: dormitum, calysol, fentanyl, propofol.

To highlight recent and most mature plaque we used Miradent dye (10 ml containing water, sodium benzoate, potassium sorbate, CI 45410, CI 42090), which reveals young plaque in pink and mature plaque in dark blue. After intubation and general anesthesia samples were collected from dogs with chronic oral diseases or dental plaque from different breeds, age, sex, nutrition. At the laboratory of microbiology the samples were processed for identification of four genera that we have chosen for identification to reveal the antimicrobial susceptibility and the capacity of biofilm formation in vitro: Staphylococcus spp., Streptococcus spp., Pasteurella spp. and Neisseria spp.
Antimicrobial susceptibility was done by Vitek 2 (bioMérieux, France) for *Staphylococcus*, *Streptococcus* strains and by disc diffusion method for *Pasteurella* and *Neisseria* strains.

From 33 dogs with dental plaque were identified 10 strains of *Staphylococcus*, 22 of *Streptococcus*, 24 of *Pasteurella* and 14 of *Neisseria*. Antimicrobial susceptibility was done for 10 strains of *Staphylococcus*, *Streptococcus* and 11 strains of *Pasteurella* and *Neisseria*.

For *Staphylococcus* species we tested the antimicrobial susceptibility for: benzyl penicillin, ampicillin/sulbactam, oxacillin, imipenem, gentamicin, kanamycin, enrofloxacin, marbofloxacin, erythromycin, clindamycin, vancomycin, tetracycline, nitrofurantoin, fusidic acid, mupirocin, chloramphenicol, rifampicin, trimethoprim/sulfamethoxazole.

For *Streptococcus* species we tested the antimicrobial susceptibility for: ampicillin, ampicillin/sulbactam, oxacillin, imipenem, gentamicin high level, gentamicin, enrofloxacin, marbofloxacin, erythromycin, clindamycin, vancomycin, tetracycline, nitrofurantoin, chloramphenicol and trimethoprim/sulfamethoxazole.

For *Pasteurella* and *Neisseria* species we tested the antimicrobial susceptibility for: gentamicin, kanamycin, amoxycillin, doxycycline, ampicillin, norfloxacin, ciprofloxacin, cepazolin, polymyxin B, penicillin G, flumequinorom and tetracycline.

**Results and discussions**

All 10 strains of *Staphylococcus* species were susceptible to most of the antibiotic tested, however different resistance rates to different antibiotics among strains were observed. Antimicrobial susceptibility was tested by Vitek 2 bioMérieux, France.

All strains were resistant to benzyl penicillin, 8 strains were sensitive to ampicillin/sulbactam, 2 strains were resistant, 8 strains were sensitive to oxacillin and 2 strains were resistant, 9 strains were sensitive to imipenem and 1 was resistant, 8 strains were sensitive to gentamicin and 2 strains were resistant, 7 strains were sensitive to kanamycin and 3 strains were resistant, 6 strains were sensitive to enrofloxacin, 4 were resistant, 9 strains were sensitive to marbofloxacin, 1 strain was resistant, 7 strains were sensitive to erythromycin, 3 strains were resistant, 7 strains were sensitive to clindamycin, 3 were resistant, 5 strains were sensitive to tetracycline and 5 were resistant.

All strains were sensitive to nitrofurantoin, fusidic acid, mupirocin and chloramphenicol, 9 strains were sensitive to rifampicin, 1 result was intermediate, 8 strains were sensitive to trimethoprim/sulfamethoxazole and 2 were resistant (Fig. 1).
Antimicrobial susceptibility for *Streptococcus* spp. was done for 10 strains. From 10 strains, 10 were resistant to ampicillin, 7 strains were sensitive to gentamicin high level, 3 were resistant, 7 strains were sensitive to gentamicin, 3 were resistant, for enrofloxacin the results were intermediate for all strains, for marbofloxacin 3 strains were sensitive, 7 intermediate, for erythromycin 6 strains were sensitive, 4 resistant, for clindamycin 4 were resistant, 6 intermediate, for vancomycin 7 were sensitive, 3 resistant, for chloramphenicol 5 were sensitive, 5 resistant, for trimethoprim/sulfamethoxazole all strains were resistant. All strains were sensitive to ampicillin/sulbactam, oxacillin, imipenem, tetracycline and nitrofurantoin (Fig. 2).

Antimicrobial susceptibility for 11 strains of *Pasteurella* spp. and *Neisseria* spp. was done by classic disk diffusion method. From 5 strains of *Pasteurella* 4 were sensitive to gentamicin and 1 intermediate, 4 strains were sensitive to kanamycin, 1 was resistant, 4 strains were sensitive to amoxycillin, 1 was resistant, 4 strains were sensitive to ampicillin, and 1 was intermediate, 4 strains were
sensitive to cephazolin, 1 was intermediate, 4 were sensitive to polymyxin B, 1 was intermediate, 2 strains were sensitive to penicillin G, 1 was intermediate and 2 were resistant. All strains were sensitive to doxycycline, norfloxacin, ciprofloxacin, flumequinorom and tetracycline (Fig. 3).

Fig. 3. Antibiotic susceptibility of *Pasteurella* spp. strains

From 6 strains of *Neisseria* spp. 1 was sensitive to gentamicin, 5 were intermediate, 3 were intermediate to kanamycin, 3 were resistant, 4 were sensitive to amoxycillin, 2 intermediate, 4 were sensitive to ampicillin, 2 were intermediate, 5 were sensitive to ciprofloxacin, 1 was intermediate, 4 were sensitive to cephazolin, 2 were intermediate, 1 was sensitive to polymyxin B, 5 were intermediate. All strains were resistant to penicillin G and sensitive to doxycycline, flumequinorom and tetracycline (Fig. 4).

Fig. 4. Antibiotic susceptibility of *Neisseria* spp. strains
Conclusions

Staphylococcus spp. are sensitive to penicillins but our result have shown resistance to: benzylpenicillin 100%, ampicillin/sulbactam 20%, oxacillin 20%. To aminoglycosides most bacterial strains were sensitive but we had resistance to gentamicin 20%, kanamycin 30%. To fluoroquinolones Staphylococcus spp. are sensitive but to enrofloxacin 60% were sensitive and 40% resistant. To benzylpenicillin, nitrofurantoin, fusidic acid, chloramphenicol and mupirocin Staphylococcus spp. are sensitive and in our study all strains were 100% sensitive to this antibiotic.

Streptococcus spp. are sensitive to penicillins but we had resistance 100% to ampicillin. Clindamycin is an effective antibiotic against Streptococcus spp. but we had 100% resistance, to tetracycline and nitrofurantoin sensitivity was 100% and to trimethoprim/sulfamethoxazole resistance was 100%.

Penicillins are effective antibiotics against some species of Pasteurella. In our study 20% of strains were intermediary to amoxycillin and ampicillin. To penicillin G. 40% of strains were resistant. Very efficient antibiotics against Pasteurella spp. were: doxycycline, tetracycline norfloxacin, ciprofloxacin, flumequinorom and the sensitivity was 100%.

Neisseria spp. are sensitive to penicillins but we had resistance to amoxycillin 33%, ampicillin 33% and to penicillin all results were intermediary 100%. High sensitivity was seen for: doxycycline, tetracycline, norfloxacin and flumequinorom.

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IDENTIFICATION AND CHARACTERIZATION OF BIOFILM FORMING BACTERIA IN ORAL CAVITY OF DOGS

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Summary

Research over the past decade has led to the recognition of dental plaque as a biofilm, a well organized accumulation of microbial communities attached to an environmental surface. The oral cavity may act as a reservoir for several pathogens related to local and systemic infections. Therefore, the aim of this study was to identify the microorganisms found in oral cavity of dogs and to determine the prevalence of pathogenic isolates. The study reveals the prevalence of 2 bacterial strains that can form supragingival biofilm in oral cavity of 33 dogs: \textit{Staphylococcus} and \textit{Streptococcus}. The characterization of these strains was made by using standard procedures of bacterial culture and identification was made by Vitek 2 (BioMérieux, France).

Key words: bacterial strains, biofilm, dental plaque, microorganisms.

Oral diseases are common in dogs, as they are in humans, often leading to tooth decay and systemic diseases. From extensive research with humans, it has become clear that understanding the microbial ecology of the mouth is fundamental to elucidate the etiology of most oral diseases, yet the oral ecology and microbiology of the dog remains largely uncharacterized (2).

Oral ecosystem is very diverse, estimating about 500 commensals bacterial species. These organisms are disseminated in various oral niches, so biofilms can be localized on tonsil crypts, tongue, dental plaque and gingival crevices where there is low redox potential (Fig. 1) (3).

Biofilms can be quartered on soft tissues of the oral cavity or on buccal and palatal mucosa. However the number of microorganisms residing mouth is small due to the ability of its turn over. In this case, microorganisms that can adhere to the surface of epithelial cells of the oral mucosa will be removed when the epithelial cells are removed from this level. Microorganisms are found in large numbers on the surface of the tongue because they are protected by lingual papillae and by the direct action of saliva.

On hard tissues of oral cavity microorganisms are found in large numbers and can form dental plaque (biofilm) which is the main cause of periodontal disease and tooth decay.
Genus *Staphylococcus* (gr. staphyle=cluster; cocc=beans), originally included in the family *Micrococcaceae*, currently includes 31 species. These bacteria are spherical shaped cocci that are grouped in characteristic piles. *Staphylococci* are Gram positive, non spor forming, nonmotile, and non capsulated, aerobic, facultative anaerobic bacteria. *Staphylococcus* spp. colonies are large, round type S, pigmented with white gray, white to yellow citrine, golden yellow or orange pigment (5).

Among staphylococci, the most common species isolated from oral cavity in dogs are: *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus warneri*, *Staphylococcus intermedius* (1,4).

Genus *Streptococcus* has a large number of bacterial species of which the most important are: *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactie*, *Streptococcus equi*, *Streptococcus suis*, *Streptococcus porcinus*, *Streptococcus bovis*, *Streptococcus canis*, *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus caprinus*, *Streptococcus ovis*, *Streptococcus pneumoniae*, *Streptococcus gallinarum*, *Streptococcus uberis*. Germs are spherical or oval, symmetrical or asymmetrical grouped in pairs or chains of variable length. Are Gram positive bacteria, nonmotile (except serological group D), without capsule (except *Streptococcus pneumoniae*), non forming spores, aerobes, facultative anaerobes. They form small colonies, punctiform, gray-yellow, translucent, type S, R, M or G (5).

In oral cavity of dogs alpha-hemolytic streptococci were isolated more frequently than beta- and gamma hemolytic streptococci (4).

**Materials and methods**

Samples were collected from supragingival sites of 33 dogs under anesthesia with oral dental plaque and other diseases of oral hard tissues. After
highlighting recent and mature dental plaque identification was made for 10 strains of *Staphylococcus* spp. and 10 strains of *Streptococcus* spp.

The characterization of this strains was made by standard procedures of bacterial culture and identification was made by Vitek 2 (BioMérieux, France). The databases of the VITEK 2 identification products are constructed with large strain sets of well-characterized microorganisms tested under various culture conditions. These strains are derived from a variety of clinical and industrial sources as well as from public (e.g., ATCC) and university culture collections.

Isolation was made on blood agar for *Staphylococcus* spp. and Edwards for *Streptococcus* spp. (Fig. 2). Cultures were incubated at 37°C for 24 hours.

![Fig. 2. Staphylococcus and Streptococcus from oral cavity in dogs - colonies on blood agar and Edwards](image)

After 24 hours Gram staining, catalase and oxidase test were performed to confirm bacterial genera. *Staphylococcus* spp. are catalase (+) and oxidase (-), Gram positive, *Streptococcus* spp. are catalase-oxidase (-), Gram positive bacteria. *Staphylococcus* and *Streptococcus* strains were stored at -50°C in BHI broth and glycerol. For identification these strains were passed in BHI agar and incubated for 24 hours at 37°C.

**Results and discussions**

For identification there were performed several biochemical tests with Vitek 2 system. The unknown bio-pattern was compared to the database of reactions for each taxon, and a numerical probability calculation was performed. Various qualitative levels of identification were assigned based on the numerical probability calculation. For *Staphylococcus* spp. probability was 94-99%, for most strains 99% and for *Streptococcus* spp. probability was 90-98%.
From 10 strains of *Staphylococcus* collected from supragingival sites of tooth we have identified: 7 strains of *Staphylococcus intermedius*, 2 strains of *Staphylococcus warneri*, 1 strain of *Staphylococcus epidermidis* (Fig. 4).

**Fig. 3. Percent and number of *Staphylococcus* spp. in oral cavity of dogs**

From 10 strains of *Streptococcus* spp. we have identified: 3 strains of *Streptococcus canis*, 3 strains of *Streptococcus suis*, 2 strains of *Streptococcus sanguinis*, and 2 strains of *Streptococcus ovis* (Fig. 4).

**Fig. 4. Percent and number of *Streptococcus* spp. in oral cavity of dogs**

**Conclusions**

Plaque is the result of these bacteria providing a substrate for other oral bacteria to accumulate on the dogs teeth forming layers, microcolonies of various bacteria.
The process of dental diseases begins with the accumulation of supragingival, aerobic bacteria on teeth surfaces. This biofilm leads to dental diseases and may be accompanied with halitosis. The presence of staphylococci and streptococci in the oral cavity is directly associated with oral diseases.

In our study *Staphylococcus intermedius* was the most frequent species 70%, *Staphylococcus warneri* 20% and *Staphylococcus epidermidis* 10%.

The prevalence of *Streptococcus* species was: *Streptococcus canis* and *Streptococcus suis* 30%, *Streptococcus sanguinis* and *Streptococcus ovis* 20%.

These bacteria are important etiological substances of the dental biofilm as well as the free bacteria in saliva.

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