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SEROLOGICAL STUDY OF SELECTED VIRAL PATHOGENS IN WILD BOAR FROM EASTERN ROMANIA

ADRIANA ANITA, DANIELA POREA, DRAGOS ANITA, GHEORGHE SAVUTA
University of Agricultural Sciences and Veterinary Medicine Iași,
Faculty of Veterinary Medicine
aeanita@uaiasi.ro

Abstract: *There are expanding populations of wild boars (Sus scrofa) in almost all European countries including Romania. Free-living domestic, feral and wild swine have been implicated in the epidemiology of diseases affecting humans, livestock and wildlife. However, little information on prevalence and distribution of the viral infectious agents among wild boar populations in Romania is available.*
The objective of this study was to evaluate the seroprevalence of swine hepatitis E virus (HEV), Aujeszky's disease virus (ADV) and swine influenza virus (SIV) in selected wild boar populations in four Counties: Iași, Vrancea, Galați and Buzău. Serum samples were collected from wild boars (Sus scrofa) harvested during the 2014 - 2015 hunting season in Eastern Romania. Sera were tested by tree different enzyme-linked immunosorbent assay for the presence of specific antibodies against each viral pathogen. Of the 184 serum samples tested, 21 (11.41%) and 5 (2.71%) were seropositive for HEV and SIV antibodies, respectively. Antibodies against gE of pseudorabies virus were detected in 83 out of 184 (45.1%) wild boar samples. Based on data regarding prevention measurers of these viral diseases, it must be assumed that the antibodies of serologically positive wild boars are induced by natural infection.
These results support the hypothesis that wild boars may act as a reservoir for certain infectious pathogens and a source of infection for domestic pigs and humans. Furthermore effective and science-based disease monitoring programs should continuously be carried out in wild boar populations.

Keywords*: wild boar, seroprevalence, hepatitis E virus, Aujeszky's disease virus, swine influenza virus.*

INTRODUCTION

Controlling infectious diseases in wildlife is often difficult because the ecological processes driving transmission between wildlife reservoirs and sympatric livestock populations are poorly understood. Knowledge of the diseases circulating in wildlife populations is significant not only for conservation and livestock production but also to ensure public health. The risk of transmission of pathogens from free-ranging wild boars (*Sus scrofa scrofa*) to domestic pigs (*S. scrofa domesticus*) is of increasing concern in many European countries. Wild boars can act as reservoirs for many important infectious diseases in domestic animals, such as classical swine fever, pseudorabies and brucellosis, and in humans, diseases such as hepatitis E, tuberculosis and trichinellosis. Wild boars could potentially reintroduce disease by contaminating food and the environment surrounding indoor commercial swine. Feral pigs can also contact domestic pigs that are raised outdoors (backyard pigs). Diseases of concern to domestic pigs include swine influenza, pseudorabies and hepatitis E. Aujeszky's disease (AD) is an economically important disease of domestic pigs, for which several European countries have implemented national eradication programs. The causative agent, Aujeszky's disease virus (ADV; Suid herpesvirus 1) is a neuroinvasive virus with a wide host range that excludes only higher primates, that belongs to the genus *Varicellovirus* in family *Herpesviridae*. Infections in domestic swine can result in fatal encephalitis in newborn pigs, mild respiratory signs or subclinical infections in older animals (Romero et al, 2001) and abortion in pregnant sows, survivors being latently

infected. Feral swine are a recognized reservoir of ADV (Van der Leek et al, 1993; Lipowski, 2003) and thus represent a possible source for infection of domestic swine.

Influenza viruses are segmented RNA viruses belonging to the *Orthomyxoviridae* family, which includes three different Influenza virus genera: A, B, C. Among these, influenza A viruses (IAV) are the most common and can infect humans and several animal species, both wild and domestic, such as pigs, dogs, horses, and birds. Due to their viral genome segmentation, all IAVs, including pig influenza viruses, undergo continuous genetic evolution. Swine has very important role in influenza virus ecology, being sensitive to both avian and mammalian (including human) influenza viruses (Payne et al, 2011)

Hepatitis E virus (HEV) is a member of the family *Hepevirida*e. Hepatitis E is recognized as a zoonosis, and swine and wild boars (*Sus scrofa*) are known reservoirs of HEV infection. HEV is a small, non-enveloped virus with a RNA genome. Hepeviruses that infect humans are classified into species *Orthohepevirus* A (Smith et al, 2014) which consists of at least six genotypes (Gt1-6). Gt3 and Gt4 are zoonotic and are infecting animals and cause sporadic human infections.

The aim of this study is to provide preliminary information on the seroprevalence of swine hepatitis E, Aujeszky's disease and swine influenza in the selected wild boar population from Eastern region of Romania, where this species is widespread.

MATERIAL AND METHOD

The serological investigation were made in four Counties: Iași, Vrancea, Galați and Buzău, on wild boars samples collected during the 2014 - 2015 hunting season. A total of 184 serum samples were tested for the presence of antibodies against swine hepatitis E virus (HEV), Aujeszky's disease virus (ADV) and swine influenza virus (SIV). Detection of anti-HEV and anti-SIV antibodies was made using ID Screen® Hepatitis E indirect multi-species, respectively ID Screen® Influenza A antibody competition multi-species (ID.Vet, France). For anti-ADV antibodies it was used the available commercial kit SVANOVIR® PRV gE-Ab (Boehringer Ingelheim Svanova, Sweden). All assay procedures were carried out following the manufacturer's instructions and the optical density (OD) was measured with Tecan Sunrise (Tecan Group Ltd, Switzerland).

RESULTS AND DISCUSSIONS

Of the 184 serum samples tested, 21 (11.41%) and 5 (2.71%) were seropositive for HEV and SIV antibodies, respectively. Antibodies against gE of pseudorabies virus were detected in 83 out of 184 (45.1%) wild boar samples (Table 1). Based on data regarding prevention measurers of these viral diseases, it must be assumed that the antibodies of serologically positive wild boars are induced by natural infection.

Table 1

Results of the serologic assays on wild boar serums

County	No. of tested samples	HEV Ab		SIV Ab		ADV Ab	
		Positive	Negative	Positive	Negative	Positive	Negative
Iași	91	13	78	0	91	37	54
Galați	47	4	43	3	44	28	19

Vrancea	16	1	15	2	14	5	11
Buzău	30	3	27	0	30	13	17
Total	184	21 (11.41%)	163	5 (2.71%)	179	83 (45.1%)	101

The result of our study demonstrate the suidherpesvirus-1 circulation in wild boar population in Eastern Romania. The overall prevalence of anti- ADV antibodies in wild boar was 45.1%, the highest value being registered in Galați County (59.57%).

In Europe efforts are being carried out to control ADV in domestic pigs. Most countries have implemented strict national eradication programs based on initial large scale vaccination of pigs with attenuated glycoprotein E (gE)-deleted vaccines. Despite these efforts for Aujeszky disease eradication in domestic pigs, the disease is being continuously reported in wild boar populations. The high seropositivity rate for ADV antibodies is compatible with data from other European countries. In Germany was observed an increasing seroprevalence (from 0.4% in 1985 to 16.5% in 2008) and widespread AD distribution in wild boar (Pannwitz et al, 2012). Moreover Aujeszky disease prevalence in wild boar has been recorded in other European countries such as Italy (30-51%; Montagnaro et al, 2012), Croatia (55% ; Zupancic et al, 2002), Slovenia (31%; Vengust et al, 2006) and Russia (32%; Kukushkin et al, 2009) suggesting that ADV may be endemic in most of these wild boar populations. Wild boar are considered a limiting factor for the eradication of infectious diseases with significant economic impact in swine industry such as Aujeszky's disease virus (ADV) or porcine circovirus type 2 (PCV-2).

In this study it is shown that HEV infection is present in wild boars from all four Counties where investigations were made. The overall prevalence of anti- HEV antibodies in wild boar was 11.41%, the highest value being registered in Iași County (14.28%).

The complete dynamics of HEV infection in wildlife is still unknown, but the previous fact facilitates the maintenance and circulation of the virus, posing a risk to human health in the case of meat consumption from susceptible animals. Prior investigations undertaken in Romania highlighted the presence of anti-HEV antibodies in wild boars in Eastern Romania (Porea et al, 2015) in three counties: Iași (11,1%), Bacău (11,1%) and Vrancea (6,25%). In Spain was observed a high seroprevalence (57.40%) and HEV RNA detection by real-time RT-PCR (10.12%) in wild boar (Kukielka et al, 2015). The presence of HEV antibodies in wild boars was detected in several countries such as Estonia (17.2 %; Ivanova et al, 2015), Italy (10.2%; Martinelli et al, 2015), Poland (44.4%; Larska et al, 2015) and Switzerland (12.5%; Burri et al, 2014). Studies achieved in European Countries has shown how domestic pigs and wildlife (wild boar) may interact in hunting founds, generating a complex epidemiological situation in terms of interspecies pathogen transmission.

Our investigation on wild boar also revealed the presence of SIV-antibodies in animals from two Counties: Galați (6.38%) and Vrancea (12.5%). The overall seroprevalence was estimated at 2.71% (5 out of 184 serums). There are indications that wild boar can play a role in the epidemiology of IAV, as antibodies against the three subtypes of swine influenza virus (SIV), H1N1, H3N2 and H1N2 have been detected in European wild boars (Ruiz-Fons et al, 2008). Similar prevalence results were obtained in Germany (2.1%; Sattler et al, 2012) and Spain (4%; Vicente et al, 2002) highlighting the presence of influenza viruses among

wildlife. In Romania Pascu C et al (2012) detected the presence of swine influenza virus subtypes H1N1 and H3N2 in different age groups in 45 herds in Western Romania. The levels of seroprevalence varied between 0.6%in nursery pigs to 22.1% in sows.

As the number of European wild boar increases, the interaction with domestic livestock also increases, and this raises concerns of direct and indirect human exposure to zoonotic agents. Wild boar is one of the most popular game species in Romania with an increasing interest from hunters and can be found in many habitat types. The results of our study highlight the possible role in the circulation of some pathogens in wild boar populations in Eastern Romania.

CONCLUSIONS

This article is the first one to evaluate the prevalence and risk of infectious agents in wild boars from Eastern Romania and their potential transmission to livestock and humans. The present serologic study suggests a widespread exposure of wild boars to ADV and HEV. With the presented scenario, where wildlife populations represent a potential sanitary risk for livestock, wildlife disease research may provide an opportunity for stakeholders to reconsider the current approach of disease eradication in livestock towards a less severe but more sustainable concept of disease control.

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SEROLOGICAL STUDY ON BVD AND IBR INFECTION ON CATTLE FROM BOTOȘANI COUNTY

DRAGOS ANITA, ADRIANA ANITA, GHEORGHE SAVUTA
University of Agricultural Sciences and Veterinary Medicine Iași,
Faculty of Veterinary Medicine
danita@uaiasi.ro

Abstract: *BVDV (bovine viral diarrhea virus) and IBRV (infectious bovine rhinotracheitis virus) are significant viral conditions of cattle in Romania, with widespread distributions and the ability to cause significant economic losses. They cause a variety of clinical outcomes that range from the inapparent (sub-clinical) to the more severe including abortion, infertility and immuno-suppression that underlies calf respiratory and enteric diseases.*

*Our study consisted in testing 100 bovine serums for specific antibodies against BVDV and IBRV, using two commercial ELISAs (HerdChek*BVDV Ab and HerdChek*IBRgB, IDEXX Laboratories, Switzerland). All samples were collected during May 2015 from extensively reared bovines in East of Botoșani County.*

Out of one hundred cattle tested 18 were found positive for IBR antibodies and 29 were identified as positive for BVDV antibodies. From all seropositive animals, 12 were detected as positive for both type of antibodies (11 females and one male). This can be explained by a possible vaccination with a polyvalent vaccine, all twelve animals being adults aged between 3 and 8 years. The rest of seropositive animals may be due to infection with circulating wild viruses.

Many cattle breeders do not realize that their herds are infected, and those that aren't infected may be at significant risk of becoming so, with potentially disastrous consequences. For the two viral disease systematic approach and strict implementation of control measures are essential.

Keywords**:** bovine, IBR, BVD, antibody

INTRODUCTION

Bovine Respiratory Disease (BRD) is costly to beef production due to effects on weight gain, production efficiency, antibiotic therapy, and in some cases animal death. In spite of improved efforts for prevention and treatment, there is also evidence that morbidity manifests as sub-clinical illness and can result in irritations of the lungs and, in severe cases, pulmonary tissue damage in animals never identified for illness (Apley, 2006). Bovine viral diarrhoea/mucosal disease (BVD-MD) and infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR-IPV) infections are one of the important viral agents causing also reproductive problems in cattle. These pathogens, along with stress and other environmental factors, have been shown to have a synergistic effect on each other so that the severity of the disease is worse with concurrent infections than with an individual pathogen (Solis-Calderon et al, 2005). In addition, viral pathogens have been show to weaken the host's immune response making the host more susceptible to opportunistic pathogens (*Haemophilus somnus* and *Pasteurella multocida*).

Bovine viral diarrhea virus can have a profound affect on BRD incidence within a population. The prevalence of BVDV within the cattle industry has been reported as ranging from 0.3% to 0.4% and 2.6% to 2.5% within chronically ill and dead cattle, respectively (Fulton et al., 2005). Persistently BVDV infected cattle (BVD-PI) can be associated as a

main cause of the increased BRD epidemic. Therefore, BVD-PI cattle are affective in initiating outbreaks of BRD in otherwise healthy cattle within a population.

IBR occurs in all continents, although there are differences in prevalence and incidence (Ackermann and Engels, 2006). Cattle populations of many countries are endemically infected with BHV-1. The infection is infrequently life threatening, the introduction of BHV-1 into a cattle farm can cause severe economic losses due to production losses and restrictions in the international trade of livestock (Nandi et al., 2009).

BHV-1 comes in contact with herds mainly through the introduction of new animals, either in the acute phase of a primary disease or when latently infected. Even semen from infected bulls and infected embryos are suitable virus carriers. In latent infected cattle, periodically BHV-1 reactivates, the virus is shed, and consequent virus transmission occurs (Jones and Chowdhury, 2010). Therefore, the IBR antibody carrier should always be considered as a potential source of infection to other animals.

The aim of this study was to establish the seroprevalence for BVD and IBR in dairy cattle population of the Western region of Botoşani County, by means of comparing two seroepidemiologic investigations.

MATERIAL AND METHOD

A total of 100 cattle, reared in household system were surveyed in this study, during July 2015.

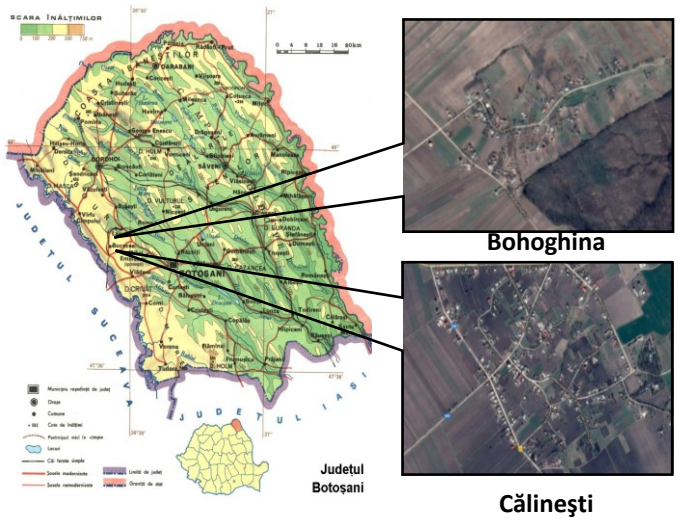

Fig. 1 Collection sites from Botoşani County

Blood samples were collected from each of the examined animal by puncture of the jugular vein into sterile vacutainer tubes without anticoagulant. All samples were centrifuged in the laboratory at 1500 x g for 20 min and sera were separated and stored at -20°C until analyzed. Sera were examined for IBR and BVD antibodies using two commercially available ELISAs (HerdChek*BVDV Ab and HerdChek*IBRgB, IDEXX Laboratories, Switzerland). All samples were tested according to the manufacturer's instructions.

RESULTS AND DISCUSSIONS

Out of one hundred cattle tested 18% were found positive for IBR antibodies and 29% were identified as positive for BVDV antibodies (fig. 2). From all seropositive animals, 12% were detected as positive for both type of antibodies (11 females and one male). This can be explained by a possible vaccination with a polyvalent vaccine, all twelve animals being adults aged between 3 and 8 years. The rest of seropositive animals may be due to infection with circulating wild viruses.

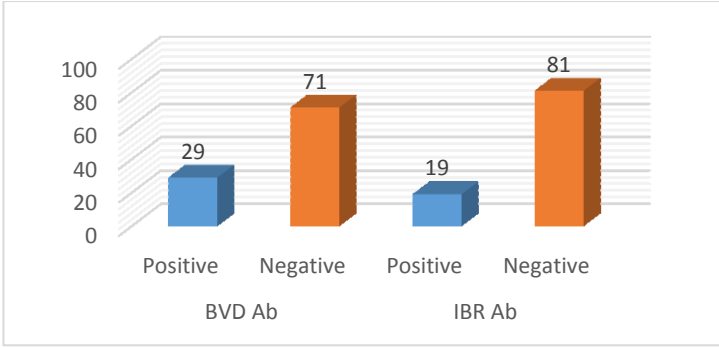

Fig. 2 Results of serologic tests for BVD and IBR antibodies in cattle from Botoşani County

Of the 100 blood samples, 59 were collected from Bohoghina village and 41 from Călineşti, respectivelly. The percentage of seropositive animals was different between the two localities. In Bohoghina village were identified 7 cattle positives for IBR Ab and 15 positives for BVD Ab, four cattle being identified as positives for both types of antibodies. In Călineşti village were identified 11 positive animals for IBR Ab and 13 positives for BVD Ab, in eight bovine being positives for both types of antibodies (table 1).

Table 1

Results of serologic tests for BVD and IBR antibodies by cattle origin

Locality	No. of samples tested	No. of samples positive for IBR Ab	% of positivity	No. of samples positive for BVD Ab	% of positivity	No. of samples positives for both Ab	% of positivity
Bohoghina	59	7	11,86	15	24,42	4	6,77
Călineşti	41	11	26,82	13	31,70	8	19,51

The seroprevalence for the animal population under study was mild, with similar values found in the majority of the studies carried out in bovines worldwide. This prevalence value contrasts with the lack of clinical evidence indicating that in most cases the disease appears in a sub-clinical way or is not considered by the farmer and/or the professionals in charge.

The seroprevalence tendency for both diseases has experienced a constant increase. The strategy adopted conscious or unconsciously by farmers of ignoring the presence of such agents in the population has enabled these diseases to reach levels where it is difficult to

identify an animal that has had no contact with the virus thus making the planning of control strategies particularly complex. In the case of IBR the disease control is difficult to apply due to the latency state the virus adopts in the organism that keeps the animal infected throughout its life.

Due to the potential impact of these agents, we presume that the only option is to develop systematic investigations in order to outline recommendations for farmers. In other countries this type of sanitary problems are part of the Health Programs of national investigation institutions allowing the development and coordination of control sketches for these diseases.

CONCLUSIONS

Results of this study clearly established that IBR and BVD are subclinically prevalent in cattle reared in household system from Botoșani County. In order to reduce the incidence of BRD in dairy livestock, on biosecurity measures are important in preventing human mediated spread of the disease by relocating positive animals. As diagnosis tool, iImmunoenzymatic assays are advantageous because they are cheap, reliable and quik to perform. Thus, ELISA may be a useful tool in large scale screening and eradication programms giving insight to the local IBR and BVD infection status.

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ASPECTS OF NONTUBERCULOUS MYCOBACTERIA ZOONOTIC RISK

IOANA ALEXANDRA ANTON, ADRIANA ANITA, DRAGOS ANITA, LUANDA ELENA OSLOBANU (LUDU), GHEORGHE SAVUTA
University of Agricultural Sciences and Veterinary Medicine of Iasi,
Faculty of Veterinary Medicine
ioana.anton10@yahoo.com

Abstract: *Latest developments in molecular techniques have led to characterization of over 150 members of the Mycobacterium genus. In addition to the obligate pathogens, Mycobacterium leprae and those of the Mycobacterium tuberculosis complex, the genus also includes the nontuberculous mycobacteria (NTM). These environmental bacilli, also referred as atypical or MOTT (Mycobacteria other than tuberculosis), have a global distribution and can be isolated from many sources, as water systems (household plumbing, tap water), aerosols, soil or animals.*

Worldwide, the emergence of NTM lung disease has been increasing constantly, beginning with 1950s, when they were reported as potential pathogens in humans. Moreover, many different other manifestations were described, especially in immunosuppressed susceptible hosts. In animals, these acid fast microbes have the ability to cause pulmonary and extrapulmonary granulomatous disorders.

The most frequently isolated species from clinical specimens are Mycobacterium avium complex, M. intracellulare, M. kansasii, M. xenopi, M. gordonae and M. fortuitum. These are also the species with the widest geographical distribution.

In this review, we highlight the salient aspects concerning nontuberculous mycobacteria clinical implications and laboratory diagnosis procedures, considering the bacilli involvement in both human and animal pathology, and their zoonotic risk.

Keywords: *Nontuberculous mycobacteria, humans, animals*

INTRODUCTION

The nontuberculous mycobacteria (NTM) refers to all the species in the *Mycobacterium* family that have the potential to cause disease in humans and animals, but do not cause tuberculosis (TB) or leprosy. The increased attention that the NTM group has gained, results from the multitude of human and animal case reports and the wide range of clinical manifestations but also from the economic and productivity losses due to confounding diagnosis with major animal mycobacteriosis, as tuberculosis and paratuberculosis, in cattle. In this paper, we try to review the most important aspects concerning nontuberculous mycobacteria, its clinical implications and the latest laboratory diagnosis procedures, in consideration to the bacilli involvement in both human and animal pathology, and their zoonotic risk.

A view over the many isolated mycobacterial species described in different case reports from various countries worldwide suggests clearly that NTM distribution differs by region. Geographical and climatic differences are illustrated by the various clinical aspects

encountered in animals and humans and their treatment outcome.

Among the factors that contribute to the increasing prevalence of human NTM mycobacterial disease, the most important may be considered the following: immunosuppression (in HIV/AIDS patients), frequently used chemoterapeutics medication in cancer patients, improvement of molecular techniques, for isolation and identification of different disease causative agents, the continuous change of human environments, occupancy of niches colonized by mycobacteria, and creating propitious conditions, like water systems (tap water, bath systems with central heating), remnant infection surveillance programs in countries where bovine tuberculosis programs are applied (Kazda et al., 2009).

Non-specific skin reactions in animals, to mycobacterial antigens, remnant infection surveillance pressure and the wide range of clinical syndromes caused by mycobacteria, in both humans an animals, stimulated the research regarding the ecology and epidemiology of NTM, in order to establish the environmental niches that contribute to their multiplication and spread.

Classification

Depending on their ecology and pathogenicity, mycobacteria can be divided in three groups, according to J Kazda et al.:

- the Obligate Pathogenic Mycobacteria (OPM),
- the Potentially Pathogenic Mycobacteria (PPM),
- the Environmental Saprophytic Mycobacteria (ESM)

The Obligate Pathogenic Mycobacteria comprises the most specialized species, from *Mycobacterium tuberculosis* group and *Mycobacterium leprae*, with ability to cause mycobacteriosis in humans and animals. These species are not usually found in environmental niches.

Airborne transmission is characteristic, in particles smaller than 5µ. The Potentially Pathogenic Mycobacteria group includes species that can be isolated from environmental sources but can also thrive in susceptible animal organisms: *Mycobacterium kansasii, Mycobacterium ulcerans, Mycobacterium xenopi, Mycobacterium marinum, Mycobacterium haemophilum, Mycobacterium avium complex* (*MAC*), *Mycobacterium abscessus, Mycobacterium chelonae, Mycobacterium fortuitum*. Environmental Mycobacteria is the largest group, with slow growing species like *Mycobacterium cookii* or *Mycobacterium hiberniae* that can determine non-specific reactions to tuberculin. These species are commonly isolated from many environmental niches (water, soil, sphagnum vegetation) and are usually considered contaminants. (Kazda et al., 2009).

The Mycobacterium genus was initially classified according to the growing rate and pigment production (Helou et al., 2013; Jarzembowski and Young, 2008), by Ernest Runyon (Table 1).

Table1. NTM classification according to Runyon

Slow growing (more than 7 days)	I PHOTOCHROMOGENS	Yellow-orange pigment production when exposed to light	M.kansasii M. marinum
	II SCOTOCHROMOGENS	Yellow-orange pigment production with or without light	M. scrofulaceum M. gordonae M. syulgai
	III NONCHROMOGENS	No pigmentation	M. avium M. intracellulare M. xenopi
Rapid growing (mature colonies in agar in less than 7 days)	IV	No pigmentation	M. fortuitum M. chelonae M. abcessus M. mucogenicum

The latest developments in molecular techniques showed that *MAC* consists in more than the four already recognized species *Mycobacterium avium, Mycobacterium hominissuis, Mycobacterium silvaticum and Mycobacterium paratuberculosis.* The other species included in this complex are *Mycobacterium intracellulare, Mycobacterium colombiense, Mycobacterium bouchedurhonense, Mycobacterium timonense, Mycobacterium arosiense, Mycobacterium marseillense (*Mirsaeidi et al., 2014).

Occurrence

NTM are free-living organisms, ubiquitous in the environment that has a strong impact over the virulence of mycobacteria (through temperature, pH, natural inhibitors, UV light). The main sources mentioned in the reviewed literature were: water (waste or surface water, swimming pools, hot tubs, tap water) especially from developed areas, sphagnum vegetation, soil, air (aerosolized NTM), biofilms, food products, medical equipment (Kazda et al., 2009, Thomson et al., 2013, Kankya et al., 2011).

Many NTM species were isolated from a variety of aquatic systems: natural surface water, lake, swamps, waste water, industrial and tap water, swimming pools, hot tubs, fish tanks, as they resist to decontamination methods or structure in biofilms. Hospital water distribution is mentioned as contaminated and *M. gordonae* and *M. avium* were isolated from intake and endpoint samples (Crago et al., 2014). Furthermore, *Mycobacterium chelonae* and *Mycobacterium avium* were isolated from surface water, along with other rare species of mycobacteria (*M. psychrotolerans, M. setense, M. insubricum, M. porcinum, M. llatzerense, M. austroafricanum, and M. arupense*), using special media (Middlebrook 7H11j, Middlebrook 7H11j-PANTA, or Middlebrook 7H11j-PANTAV) and special decontamination methods (Radomski et al., 2010). In a study realized in the metropolitan area of Tehran, from damp water, tap water, running water from race way and soil samples, *M. Farcinogens, M.*

Kansasii, M. Simiae, M. Gordonae, M. Fortuitum, M. Chelonae were isolated. It was demonstrated that *M. farcinogenes and M. fortuitum* were commonly encountered in water and soil and after comparison with isolates from human patients it was discovered that *M. simiae* and *M. chelonae* are frequent in inhabitants patients.(Akbar-Velayati et al, 2014). *M. abscessus* was isolated from ten different sources of drinking water in Brisbane, Australia (Thomson et al., 2013).

Aquatic plants, amoebae, aquatic vertebrates and invertebrates, biofilms may be NTM reservoirs in aquatic ecosystems(Radomski et al., 2010). NTM share habitats with free-living amoebae (FLA) and can grow inside them as endosymbionts. In this manner, mycobacteria is protected from disinfectants and antibiotics. In a study regarding the co-presence of free-living amoebae and NTM in hospital water networks, in America, NTM and FLA were found in water and biofilm samples collected, particularly *Acanthamoeba spp, Hartmannella vermiformis* (Ovrutsky et al., 2013). In addition, *Mycobacterium gordonae, Mycobacterium peregrinum, Mycobacterium chelonae, Mycobacterium mucogenicum and Mycobacterium avium* were isolated from the taken samples. The study concluded the fact that *M. avium* can not only survive, but also replicate in various FLA, with a high replication rate in *Acanthamoeba lenticulata*, frequently recovered from environmental samples including drinking water samples (Ovrutsky et al., 2013).

NTM were isolated from different types of soil, such as surface soil, forest soil, household and arable soil. *M. lentiflavum, M. heidelbergense, M. Austroafricanum* were isolated by PCR amplification of mycobacterial 16S rRNA gene (Mendum et al, 2000; Kopecky et al 2011, Chilima et al 2006, Fyfe et al 2007). Stable floors, peat, shower aerosols may also be niches for various NTM species as *M. a. Hominissuis, M. fortuitum, M. gordonae, M. chelonae, M. terrae, M. xenopi, M. flavescens, M. phlei*. (Eisenberg et al., 2009; Matlova et al., 2012; Perkins et al., 2009).

The wide global distribution of NTM is sustained by the reports from different countries and continents, regarding isolation from environmental sources or, more important, from clinical samples originating from human or animal patients with different mycobacterial disease or asymptomatic. An NTM-NET (Nontuberculous Mycobacteria Network European Trials Group) collaborative study illustrated the geographic diversity of nontuberculous mycobacteria in pulmonary specimens, in 2008. There were 91 different NTM species isolated in the 20182 samples considered and 30 countries around the globe participated in this study. With variations in some territories, *Mycobacterium avium complex* was the most isolated species in the majority of the countries, followed by *Mycobacterium gordonae* and *Mycobacterium xenopi.* In South Africa and Australia, the most prevalent species was *Mycobacterium intracellulare*, unlike South and North America and Europe, where *MAC* predominated (W. Hoefsloot, J. Ingen et al, 2013).

Clinical implication in animal and human hosts

Various NTM species have many implications in the veterinary field of infections disease and can affect a wide range of host, wild and domestic animals, birds and reptiles.

MAC members are recognized as causative agents of important animal diseases that can determine important economic losses through impact factors, as morbidity or mortality.

Mycobacterium avium subsp. paratuberculosis (Map) is the causative agent of paratuberculosis, also known as Johne's disease, a chronic granulomatous enteritis, that affects often domestic and wild ruminants (cattle, sheep, goats, camelids and buffaloes, cervids) and has a global distribution. (OIE a, 2015). With a negative economic impact on livestocks worldwide, paratuberculosis is recognized since late nineteenth century. This bacteria can also infect and produce disease in non-ruminant species: wild boars, bears, rodents, foxes, raccoon, monkeys (with similar lesions to Crohn's disease in humans).

Mycobacterium avium subsp. a*vium* is the causative agent of avian tuberculosis that affects companion, captive exotic, wild and domestic birds (OIE b, 2015). *Mycobacterium avium* has also been isolated from cattle, pigs, horses (Coelho et al, 2013).

A study on human and animals origin isolates, that was conducted in Norway, revealed *Mycobacterium avium subsp. homminissuis* as cause for porcine and human disease. Clinical forms are rarely found in pigs but reproductive disorders may be present or subclinical infection affecting lymph nodes and lymphatic tissues, at digestive level, with visible lesions may be observed at slaughter (Johansenet al., 2007). In a study undertaken in two pig herds, with sporadic cases of *M. avium* infection *Mycobacterium avium subsp. homminissuis* was detected using 16S rDNA sequencing and PCR in faeces, peat and lymph nodes samples collected from the herd that used peat. From the same herd, other NTM species were isolated: *M. bohemicum, M. palustre, M. celatum* all with implication in human pathology, lymphadenitis, disseminated or pulmonary infection (Agdestein et al., 2014). Pig carcasses represent a source for human infection. Infected faeces might facilitate the transmission and maintenance the infection in herds. Also it was discovered that bedding materials such as peat or sawdust represent an important source for *Mycobacterium avium subsp. homminissuis (*Matlova et al., 2012).

In Finland, Tirkkonen et al. performed a study on porcine isolates and identified by 16S rRNA sequencing *M. avium* strains. The results were compared to human isolates, by IS 1245 restriction fragment length polymorphism (RFLP) method. A close genetic relatedness between the human and porcine isolates was identified. Other similar studies were conducted in Netherlands, Sweden or Germany (Tirkkonen et al., 2007).

There are other studies in which other NTM species are incriminated as etiological agents. *M. fortuitum, M. smegmatis* can cause mastitis or nodular dermatitis in sheep and cattle (Perianu et al. 2011). *M. Fortuitum* was isolated from reptiles, amphibians, wild boar, swamp buffalo or seals (Bercovier ,Vincent, 2001). *M. szulgai* and *M. kansasii, M. avium* can cause cutaneous lesions in dogs and cats (Gross et al., 2005). Related to *M. tilburgii, M. simiae, M. genavense* it has been described the canine leproid granuloma which is a nodular cutaneous or subcutaneous disease, with a self-limiting character of the lesions, determined by a single causative mycobacterial organism, yet to be characterized (Gross et al., 2005; Malik et al, 2013). The syndrome has a wide geographic distribution, commonly encountered in Australia and Brazil, but cases were also reported in New Zealand, Zimbabwe, Columbia

and different areas of United States (Craig E. Greene, 2011). Buruli ulcer, a chronic localized infection caused by *M.ulcerans,* affects both animals and humans. The diagnosis in dogs that present unexplained ulcerative dermal lesions may easily be missed, thus the delays in treatment can lead to the establishment of extensive wounds that can be difficult and expensive to treat (O'Brien et al., 2011). *M. marinum* can affect fish populations and cause high morbidity, mortality and economic losses. *M. avium subsp. silvaticum* is isolated from tuberculous lesions in birds, especially wood pigeons (Bercovier and Vincent, 2011). *M. kansasii* a rare animal pathogen was isolated from birds, domestic and feral pigs, deer, monkeys with pneumonic lesions and inflamed lymph nodes, cattle and unpasteurized cow milk (Bercovier and Vincent, 2011).

Wild animals are also susceptible to NTM. Circulation of mycobacteria in wildlife can lead to environmental contamination and maintenance of the disease as it was observed in the control an eradication programs for bovine tuberculosis in cattle embarrassed by brushtail possums, badgers, red deer or indian buffalo.

There are various NTM implications in human clinical pathology. As stated by the American Thoracic Society in the last ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases, in 2007, the broad categories of clinical forms are: pulmonary disease, disseminated disease, lymphatic disease, skin, soft tissue and bone disease, hypersensitivity-like disease (Hot tub lung) and catheter-associated infection (Griffith, Aksamit and Brown-Elliott et al., 2007).

Many human patients have predisposing factors, such as: traumatic skin breaches, underlying lung pathology or architectural defects (bronchiectasis, prior infections with *M. tuberculosis,* cystic fibrosis, chronic obstructive pulmonary disease, body habiti), generalized congenital or acquired immunosupressive disorders (such as HIV). These aspects cumber the diagnosis.

Susceptible patients, especially for *MAC* are immunocompromised persons, children, elderly patients, mostly males, over fifty years old, with a smoking history or underlying lung pathology, previously mentioned, but also postmenopausal female patients that present nodular or bronchiectatic *MAC* disease (Griffith, Aksamit and Brown-Elliott et al., 2007).

Pulmonary disease is the most common clinical NTM manifestation (Jarzembowski and Young, 2008). The frequently NTM species associated with pulmonary disease are *MAC*, *M. kansasii, M. gordonae, M. Xenopi* (Jarzembowski and Young, 2008).The symptoms are nonspecific, cough (recurring or chronic), fatigue, malaise, dyspnea, fever, weight losses or chest pains. Radiographic may be observed either fibrocavitary, tuberculous-like or nodular bronchiectatic aspects.It has been described also Lady Windemere syndrome, a less common form, diagnosed in elderly women with voluntary suppression of cough, presenting interstitial pulmonary infiltrates, with no cavitation or hilar lymphadenopaty (Bhatt et al., 2009).

In children, most common NTM presentation is lymphadenitis in the head and neck area, caused predominantly by *MAC*, followed by *M. scrofulaceum* and *M. Kansasii.* (F. Baquero-Artigao, 2005).

Another described form is the disseminated disease, especially in patients with HIV, with $CD4^+$ less than 50 cells per µl is determined by *MAC, M. chelonae, M. abscessus, M. Xenopi* but other nontuberculous mycobacterias may be isolated. Common symptoms are fever, night sweats, weight loss, diarrhea and abdominal pain. Furthermore hypersensitivity-like disease (hot tub lung) is determined by *MAC* and *M. abscessus, M. fortuitum, M. chelonae, M. Mucogenicum* are isolated in patients with Catheter-associated infection (Jarzembowski and Young, 2008).

Diagnosis

There is a variety of specimens that can be collected for microbiologic analysis and other diagnostic techniques and procedures: body fluids, tissue samples (lymph node, skin, biopsy material), aspirated pus, abscess contents, wound specimens sputum, bronchoalveolar lavage fluid, bronchial washings, gastric lavage fluid, blood, urine, stool, water, soil. Processing is necessary before the media inoculation. If the specimens are aseptically collected (from sterile sites), then can be directly inoculated into appropriate medium. Potentially contaminated samples are subjected to chemical and enzymatic treatment (sodium hydroxide, dilute sodium hypochlorite), vortex mixing or physical disruption for tissue maceration (Weissfeld et al., 1994). Staining procedures are used as diagnostic methods. NTM are acid-fast bacilli, due to their cell wall complex structure and may appear pleomorphic, long filaments or cocoid forms, with uniform staining properties. Standard used procedures are Ziehl-Neelsen or Kinyoun that use carbolfuchsin as primary stain and fluorescence stainings Auramine-Rhodamine and Auramine O., which are more sensitive (Weissfeld et al., 1994). Minimum 300 fields need to be investigated, at a high resolution (x1000), in carbolfuchsin smears in order to be declared negative. In fluorescence microscopy a lower magnification (x250) is used and mycobacteria appears as orange-yellow rods, on a black background (Jarzembowski and Young, 2008). For cultivation, a variety of media may be used. For primary isolation, broth, egg based and agar based media are suitable: Middlebrook 7H9 and Dubos Tween albumin broth, Lowenstein-Jensen media (egg-based medium with malachite green dye), Middlebrook 7H10 and 7H11 or Lowenstein-Jensen agar-based media. Selective media enhance the recovery of mycobacteria: Gruft modification of Lowenstein-Jensen, selective 7H11. Radiometric detection systems (BACTEC) or MGIT (Mycobacteria Growth Indicator Tubes) may be used for a faster detection (Jarzembowski and Young, 2008; Weissfeld et al., 1994). Optim grow temperature is between 35^{o}C and 37^{o}C, in 5%-10% CO2, except *M. marinum, M. ulcerans, M. chelonae* and *M. haemophilum* that thrive between 25-33^{o}C.

Intradermal Tuberculine Test (ITT) may be used for cattle screening. Immunological methodes such as ELISA, in paratuberculosis, based on humoral immune response, IFN-γ assay or Lymphocyte Proliferation Assay based on cell-mediated immune responses are used too (OIE a, 2015). The immunochromatographic assay based on MPT64 antigen identification is appropriate for discrimination of *Mycobacterium tuberculosis Complex* from NTM (Bostănaru et al., 2014).

Genetic methods, polymerase chain reaction amplification-based (PCR-based) assays can rapidly detected NTM species from specimens: BDProbeTec ET system, Amplified Mycobacterium Tuberculosis Direct Test (MTD) (Gen-Probe, Inc, San Diego, Calif) and the AMPLICOR Mycobacterium tuberculosis Test (Roche Diagnostic Systems, Inc, Indianapolis, Ind.) (Jarzembowski and Young, 2008; Woods, 2011). Methods that use the variability of nucleic sequences, such as 16S rDNA, rpoB, gyrB: PCR, PCR-REA, sequencing analysis (IS 900, IS 901, IS 1245), spoligotyping, MIRU-VNTR are now currently used in research and diagnostic laboratories.

Discussion

Despite these important advances and latest developments in the epidemiology and molecular diagnostic methods of NTM strains, susceptibility to disease is not yet fully clarified. These bacterial organisms are now considered significant pathogens, with implications in a variety of clinical disorders (WHO, 2008). A cumulative research effort is necessary in order to identify and completely understand and establish the possible sources and populations at risk, adequate treatment protocols, development and implementation of prevention and eradication measures. Thus, appears the need for establishing the NTM environmental niches, as it can interfere with the detection of the tuberculous mycobacteria or even cause economic losses, because of their opportunistic infective capacity.

CONCLUSIONS

NTM represent a heterogeneous group, with a wide environmental distribution and potential of causing disease in humans and animals. Development in genomic techniques revealed an increased number of NTM species with clinical impact and eased the diagnosis procedure, unlike the classic methods based on biochemical properties that are time consuming and are not able to offer an etiological diagnosis. Zoonotic potential should not be neglected, particularly in immunocompromised patients, whose number increases more and more, in our country. *Mycobacterium* species transmitted by environment and wildlife should be considered public health concerns. We consider that the importance of NTM is underestimated in România or at least underdiagnosed and it is necessary to perform studies focusing on NTM species, environmental niches and the implications in human and animal health in accordance with the One Health Concept.

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PRELIMINARY STUDIES ON THE INCIDENCE OF SWINE EPERYTHROZOONOSIS IN MOLDAVIA (ROMANIA)

SONIA CARAGEA,
HOJBOTA GEORGIANA, MANOLE MIHAILESCU
SC LABORVET SERV SRL
mihailescu_manole@yahoo.com

Abstract: *The aim of the work is to establish the incidence of Eperythrozoonosis swine in farms from northeastern Romania by correlating clinical picture and histopathological examinations of the bacteria Mycoplasma suis highlighting Laboratory (Eperythrozoon suis) in the blood of sick animals and the diagnosis of infectious hemolytic anemia.*

Methods: The research was conducted throughtout:

I. Recovery of data collected over a period of 5 years by epidemiological investigation, clinical and pathologic examination in a total of nine pig farms in the region of Moldavia, that imported youth pigs from U E countries ; data showed a clinical picture, and a dynamic lesion identical in all farms with epizootic investigation: transgression, adynamia, accentuated pale skin , afebrile and death in 1-2 days or 1-2 weeks after the beginning of clinical symptoms. The pathological exam revealed: pallor accentuated of the skin, appearance of the corpse at the opening with diathes(bleeding generalized, blood aqueous hemolysis) internal organs and muscles pale and looking degenerated, gastrointestinal content with blood , clot and the appearance like "grounded cofee". Morbidity was between 1 and 3% ,and the mortalitty beeing 100% in the individuals with clinical symptoms.

II. Laboratory exams for the diagnosis of hemolytic anemia related with the presence of Mycoplasma Suis in the blood of animals with clinical signs. Examinations were made during September 2015 in Bacau at SC Laborvet Serv. 4 sets of blood samples were analyzed from individuals with clinical signs and from clinically healthy ones of the farm. There were performed whole blood smears colored with May Grunwald Giemsa stain and were examined under a microscope lens with immersion x 1000. Exploration of haematological and biochemical profile (CBC) was performed on biochemical analyzers IDEXX Vet Test and IDEXX Vet Auto Read.

Results and discussion: pathological lesions justify the characteristic symptomatology of the category of fattening piglets. Blood smears from pigs with clinical signs of disease revealed the presence of erythrocyte surface disposed with cocoid corpuscles that were identified as Mycoplasma Suis (Eperythrozoon suis), erythrocyte starred in various stages of cytolysis; in samples from clinically healthy individuals Mycoplasma suis was not detected and no red cells with altered forms were discovered , and complete blood count and biochemical examination were placed in the reference range of the species and categories. The sick pigs did show increases in total leukocytes (up to 54.10 x 109 / L) based on granulocyte growth (to 96.9%) mononuclear leukocytes were below normal for growing pigs (1.5 x 109 / L) hematocrit values were between 4% and 7% and hemoglobin between 2.2 g / dl - 3.2 g / dl. The average hemoglobin concentration (MCHC) showed no significant decrease (from 29.5 to 30.1 g / dl); Platelets were elevated between 210 and 461 K / ml compared to 126 K / ml in those clinically healthy. The analysis of biochemical parameters showed growth in all affected individuals ALT, GGT variations with values below the reference range but above the limit, increasing total serum protein ,albumin and globulins due to growth.

Conclusions:

1. The clinical and pathological views were almost identical in all investigated farms throughout the observation period reported.

2. Characteristic clinical signs related to the presence of bacteria in red cells offers a positive diagnosis for swine Eperythrozoonosis .

3. In favor of the diagnosis of infectious hemolytic anemia advocates very low hematocrit and hemoglobin, mean hemoglobin concentration showed no significant declines which excludes deficiency anemia;

4. Platelets have raised values at some individuals but most were below the limit, which confirms the state of diathesis, vascular endothelial damage (3) and the infected state with neutrophilic ,granulocytic and lymphocytic depletion.

5. Clinically healthy individuals of the same herd was not confirmed the healthy carrier state (absence M. suis in red blood cells).

Keywords: *Mycoplasma suis, incidence, infectious hemolytic anemia.*

Ethiopatogenesis: swine eperythrozoonoosis is caused by Eperythrozoon suis, a cocoid bacteria with dimensions of 0.8 to 1.0 mm, which "parasitize " red cells, framed in 2002 in Haemoplasmosis group and renamed Mycoplasma suis (hemosuis). Icteroanemia in pigs has been described in the United States since 1943. Later were found many bearing animals, after drafting an indirect hemagglutination test in 1975. The disease affects all types of pigs.

Clinical view may vary, particularly where there are infections side concerned.In small piglets and in those weaned , the primarily form is acute anemia and secondary infections, while in subacute or chronic delays occur with growing pigs uneven ,growth immature, weak. Chronic infection in sows is associated with reproductive problems and whether there is stress during birth may occur fever and agalactia. The bacteria can cross the placenta and cause birth of frail piglets , pale and high mortality in the pre and post weening.If there are anemic piglets , pale or in the period immediately following weaning received injectable iron, should be considered the possibility of infection with M .Suis. (2) Although M. suis causes high mortality rarely in herds, is recognized as a occasional cause for acute hemolytic anemia or jaundice in young pigs (6). Currently it is believed that although most pigs are healthy carriers, in particular under stress or are immunosuppressed may trigger the disease, the trigger is still unidentiffied .There are hypothesizes that it would be associated with swine circovirosis PCV2 virus type 2. Mycoplasma suis has been described as adhessive to erythrocyte membrane, but a 2009 study shows that after accessing the erythrocyte produces invasion by deepintrosusception of the membrane (Figure 1); as red blood cells invasion progresses the cells become star shaped (2) (Figure 2) subsequently causing lysis of red blood cells. In electronic microscopy with SEM scanning they apear like a erythrocyte intracytoplasmic inclusions (2). (Fig 1).

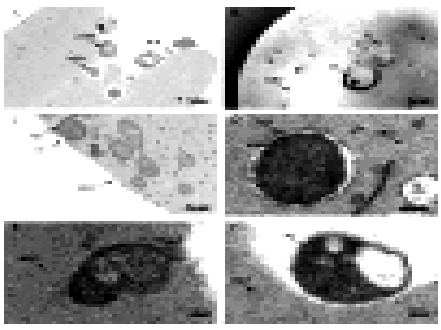

Fig.1. M.suis in SEM Images (2)

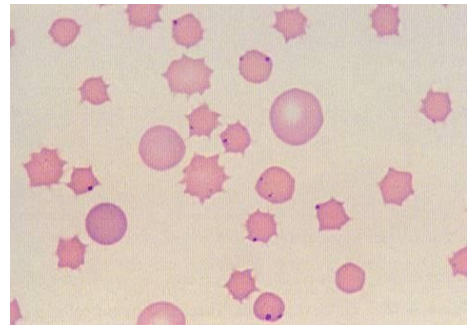

Fig.2.Bloodsmears, MGG. Stain (www.studyblue.com)

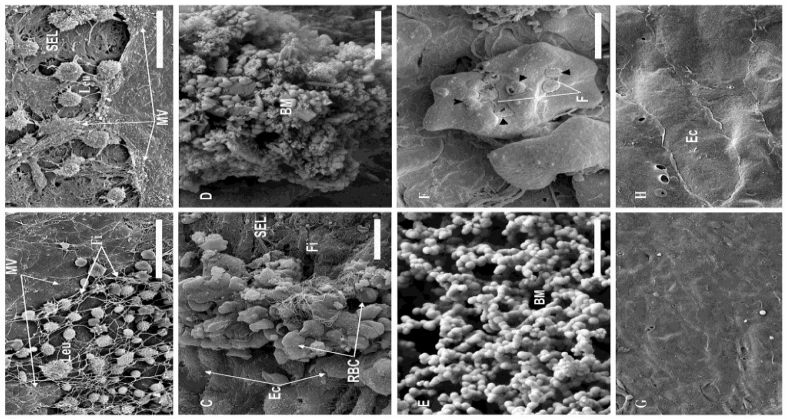

Fig. 3. Immune modulation and intravascular coagulation and biofilms

Recent studies have elucidated the mechanism by which M. suis as well as infected red blood cells adhere to the vascular endothelium both in large vessels such as the aorta and result in capillary endothelial cell activation, inflammatory chain initiation, that leads to alterations in the vessel ,explaining the presence of blood in cavities and organs. These endothelial changes were accompanied by hemorrhage, intravascular coagulation, vascular occlusion and massive morphological changes in organs and in the muscle parenchyma (3) and myocardial muscle .Fibres appear disorganized or destroyed. These effects could be explained by the aggregation of infected erythrocytes in blood vessels, occlusion of capillaries leading to cardiac ischemia. This would be consistent with the fact that some cases of acute M. suis infection lead to death within a few days. They highlighted that red cell aggregates adhering to vessel wall and liver sinusoidal capillaries bring on the obstruction (3). In addition, the ability to form biofilm of M. suis - microcolonies on the vascular endothelium, which protects from the antimicrobial and immune factors of the host, may contribute to the persistence of the infection. M. suis interfere with the protective function of the endothelium, causing bleeding diathesis accompanied by immune modulation and intravascular coagulation (Fig 3) (3).

In recent years, reports of zoonotic infections with hemo-micoplasmosis have increased significantly. Strains of Mycoplasma suis, M. haemofelis and M. ovis were isolated from sick people [1,4,5] and have been associated with clinical symptoms of fever and hemolytic anemia [4]. A study from China shows that nearly 50% of pig farm workers and veterinarians were carriers of an identical philogenetic strain with the strain of M suis detected in pigs, suggesting the possibility of interspecies transmission; however, research is ongoing.

Porpouse :

From our documentation on Swine Eperythrozoonosis we have not found any data in literature from our country. Therefore we believe that this is the first study which is trying to correlate the etiological diagnosis with the cases that present the specific clinical and pathological view that can accurately assign with eperythrozoonosis and also providing its incidence in North-east of Romania. In current conditions when the import of live pigs in EU

has grown, it is important to determine whether the disease has been or it will be endemic in Romania.

Objective: to establish the incidence Swine Eperythrozoonosis in farms in north-eastern Romania by correlating the clinical and pathological view with laboratory histopathological examinations, highlighting the Mycoplasma suis bacterium (Eperythrozoon suis) in the blood of sick animals and specify the diagnosis of hemolytic anemia infection through laboratory examinations: smears of whole blood, blood count for the constants of red blood cells; WBC counts and dispenses the general biochemical parameters.

Methods: The incidence was investigated swine eperythrozoonosis with Mycoplasma suis in a number of fattening pigs, in the region Moldova – Iasi, Suceava, Bacau, Vaslui. The investigated pigs were from Germany, Netherlands and Denmark, rarely from closed-circuit farms from Romania. Usually clinical signs appear 2-3 weeks after the introduction into fattening, at 120-150 days old piglets: sudden departure, loss of appetite, apathy- adinamya, accentuated pale skin, sometimes necrosis of the ear, diarrhea with blackish feces, no fever in this stage of disease. The clinical signs and the pathological view are very similar and typical in all the investigated farms, especially when there are no secondary infections that complicate the clinical view, for the specimens that the disease starts suddenly and death is fast (2-3 days) and which are usually in good wellfare. Morbidity is between 1% of the winter herd and 3-4% of the summer herd, mortality is 100% in individuals with clinical signs. Death occurs within days or 1-2 weeks after onset of clinical symptoms, even if it starts with the symptomatic treatment or with the treatment with antibiotics of general use. Necropsy examinations were performed in farms and data were collected during the last 5 years by Dr.Mihailescu Manole and laboratory tests were performed in the laboratory SC Laborvet Serv SRL Bacau by Dr. Sonia Caragea and Dr. Hojbota Georgian in September 2015. Blood samples were collected from farms where there were cases with suspected symptoms and injuries associated with known necropsy, from clinically healthy individuals and in from those with clinical signs both from the same herds. Pigs were totally free from Aujeszky, dysentery with Brachispira, usually they were vaccinated for Mycoplasma Hyopneumoniae, APP, Circoviroza, PRRS.

Results and Discussion: At necropsy we noticed: accentuated paleness of the corpse (skin white as paper, pale muscles with boiled muscles apperance), hemorragic exudate or into the thoracic and abdominal cavities, organs (liver, kidney, heart, lung) had paleness and appearance of parenchymal degeneration, spleen with hyperplasia and subcapsular bleeding, bloody small intestin content and dark colon content, stomach contents with blood and sometimes clotted or with appearance of coffee grounds without ulcers and erosions justifying the the presence of blood. The appearance of the opening corpse: bleeding diathesis is generalized with aqueous blood hemolysis (Fig 4).

Figure 4. Pathological lesions: hyperplasia with bleeding spleen; hemorrhagic stomach content and content aspect ,, coffee grounds'; Hemorrhagic exudate in large cavities; dystrophic pale kidneys; serohemoragic exudate into the chest cavity; dystrophic pale liver and stomach distended; stomach, small intestine and colon with hemorrhagic content.

In blood smears from pigs with clinical signs of the disease it has revealed the presence of small cocoid corpuscles dispozed on erythrocyte surface which were identified as Mycoplasma suis (Eperythrozoon suis), star shaped red blood cells and in various stages of cytolysis.(fig.5).

The intensity of the erythrocyte infection was 3 to 6 erythrocytes in a microscopic field. Blood smears performed from blood harvested on anticoagulant from dying pigs we noticed an intense hemolysis and numerous messed up cells and broken cells, this aspect is suggesting an intravascular hemolysis.

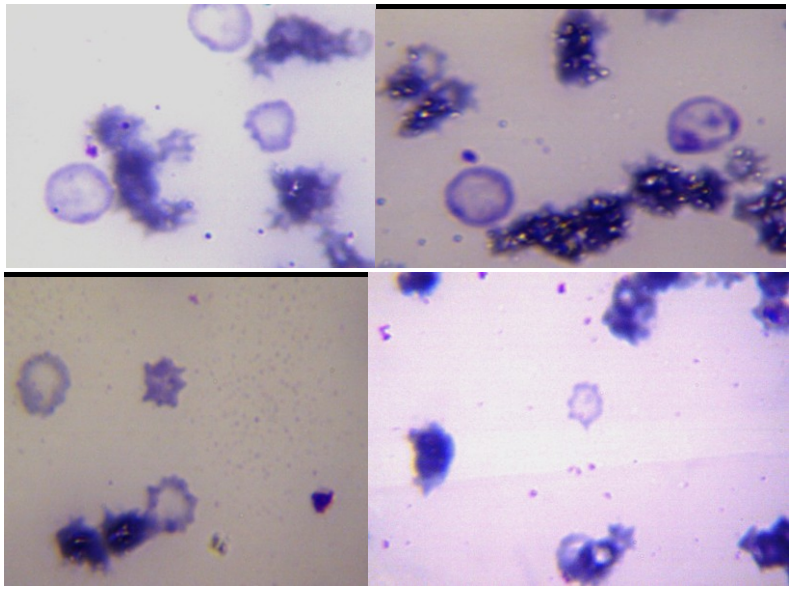

Fig. 5. M. suis erythrocyte membrane
and cell adherent stelate. MGG Stain x 1000

In test the tubes samples we observed low content of sedimented solid mass compared to the expressed serum (fig.6).

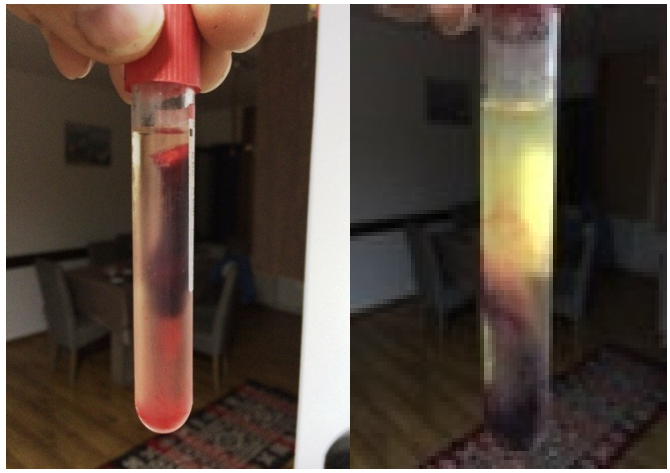

Fig 6. Appearance of fibrin clot in tests tubes

In samples from clinically healthy individuals Mycoplasma suis was not detected and there were no red cells with altered forms indentified, and complete blood count and biochemical examination were placed in the reference range of the species and categories.

The sick pigs did show increases in total leukocytes (up to 54.10 x 109 / L compared to 12.40 x 109 / L in healthy individuals) based on the increase of granulocytes (up to 96.9% from 68.5 % health); mononuclear leukocytes were below normal for growing pigs (L / M: 1.5 x 109 / L compared to 3.9 x 109 / L clinically healthy individuals) (table 1).

Table1. WBC counts :

Param. / Cathegory Profil:	WBC Limits: 17.60 X 10^9/L	NEU Limits: 3 X 10^9/L	GRANS Limits: 9.20 X 10^9/L	GRANS Limits: 49±7,5 %	L/M Limits: 8.4 X10^9/L	L/M Limits: 55-66 %	PLT Limits: 180-300/µL
Below values	12,40-13,35	-	8,50-8,68	-	1,5-7,6	3-48	124-167
In range	17,20-17,70	2,99-3,22	9,10-9,35	50,1-52,0	8,3-8,5	31	210
Above values	47,90-55,90	4,98-5,99	46,40-54,10	68,5-96,9	-	-	461

Hematocrit values were between 4% and 7% vs. 32, 2 to 34.4% in healthy individuals, and hemoglobin between 2.2 g / dl - 3.2 g / dl compared to 10.3 - 11.4 g / dL in healthy individuals; the average hemoglobin concentration (MCHC) showed no significant decrease (from 29.5 to 30.1 g / dL compared to 31.3 g / dL in healthy individuals); platelets were mostly below the reference range of the species, but were also elevated among individuals with up to 461 K / ml. (table 2)

Table 2.Blood count :

Parameters / Cathegory Profil:	HCT Limits: 37±3 %	HGB Limits: 11,5±0,8 g/dl	MCHC Limits: 31±3,0 g/dl
Below values	32,3-33,2	10.3	28,9-29.9
In range	34.5-35,5	10,8-11,2	30,0-32.2
Above values	3,3-7,1	1,1-2,2	-

- values below the reference range of the species and age
- values above the reference range of the species and age
- reference values in the range of species and age

The analysis of biochemical parameters showed growth in all infected individuals ALT, GGT variations with values below the reference range but above the limit; AST no significant changes compared to the reference period at any of the categories of individuals; increasing total serum protein due to albumins and globulins increase; normal values actually showed a decrease in albumins and globulins growth; low values had normal albumin value and decreased globulins (table 3). These variations shows various degrees of damage to organs involved in the proteic homeostasis of blood (liver, kidney), the amount of globulins variable is related to the immune system to respond to infection aggression and it's exhaustion is correlated with depletion of white blood count of mononuclears (monocytes and lymphocytes).

Table 3. Blood serum biochemical examination:

Parameters / Cathegory Profile:	ALT Limits: 9 - 43 U/L	AST Limits: 16 - 65 U/L	GGT Limits: 16 - 30 U/L	Total proteins Limits: 6,0 ± 0.5 g/dl	ALB Limits: 2,7 ± 0.3 g/dl	GLOB Limits: 3.3 ± 0.5 g/dL
Below value	-	-	10-15	4,3-5,4	2,0-2,6	2,4-2,7
In range	15-41	17-29	19-28	6,5	2,6-2,8	3,0-3,1
Above value	48-50	26-60	33-40	7,4-7,6	3,0-3,3	4,0-4,6

CONCLUSIONS:

1. Clinical and phatological views were almost identical in all investigated farms throughout the observation period reported. Pathological lesions justify characteristic symptoms for category of fattening piglets with acute and subacute forms of evolution described by various authors in international publications.
2. Characteristic clinical signs related to the presence of bacteria in red cells offer a positive diagnosis for Swine Eperythrozoonoosis.
3. In favor of diagnosis of infectious hemolytic anemia advocates very low hematocrit and hemoglobin; average concentration of hemoglobin did not show significant declines (which excludes Deficiency anemia) and leukocytosis (neutrophilic and lymphocytic depletion granulocytosis like.
4. Platelets were in most cases with lower values, which confirms the state of diathesis, vascular endothelial damage (3). This actually reveals their consumption and would be interesting to follow their dynamics during the disease state.
5. Clinically healthy individuals of the same herds were not confirmed healthy carriers (absence M. suis in red blood cells).
6. The nature latency and clinical symptoms of the disease without organ specificity enables the prevalence of E. suis in swine populations to be considerably higher than the number of clinical cases (5). So far, E. suis was not officially diagnosed in Romania, but should not exclude the possibility that there may be a eperythrozoonoosis occult disease in pig herds, the symptoms being attributed to Circovirosis or PRRS, especially farms with breeding sector in which there are problems.

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RESEARCH FINDINGS ON PATHOGENICITY AND ANTIBIORESISTANCE OF STAPHYLOCOCCUS SPP. STRAINS ISOLATED FROM BREEDING BREEDING FLOCKS, EMBRYOS AND BROILERS IN A CLOSED CIRCUIT POULTRY UNIT

SONIA CARAGEA, GABRIEL PREDOI
SC LABORVET SERV SRL, Bacau
U.S.A.M.V. Bucuresti,
gabriel.predoi@yahoo.com

Abstract: it was investigated the pathogenicity and antibiotic susceptibility of 73 strains of Staphylococcus spp. strains isolated from 74 sets of samples collected from breeding herds, embryos and 8-day resulted in a closed circuit poultry production unit. They were examined 74 sets of samples, locking in a total number of 73 strains of Staphyilococcus spp. which were tested for pathogenicity and antibioresistance.

Identification and confirmation was made by bacteriological exams and with automatic method bioMerieux Vitek 2 Compact. They were identified a total of 15 (20,54%) coagulase positive strains (CPS-S.aureus) and 58 (79,46%) coagulase negative strains (CNS- S.gallinarum, S.epidermidis si S. saprophyticus).

Antibiotic susceptibility testing was performed by the Vitek 2 Compact, C.L.S.I. standardized method with GP AST-P 592 cards based on MIC (Minimal Inhibitory Concentration). The 15 strains of S. aureus have been tested with diffusimetrical method Kirby-Bauer, for predictive tests of the mecA gene-mediated resistance and and to investigate susceptibility to other antibiotics for investigation of antibiotics susceptibility used commonly in birds therapy.

Antibiograms performed made by both methods have revealed a resistance to oxacillin and cefoxitin of 26,66% (MRSA strains) and sensibility in 73,33% percentage (MSSA Strains) and also the existence of phenotype BORSA (Borderline Staphylococcus aureus) with a rate of 13,33%. Vancomycin resistance was correlated with the constitutive resistance to oxacillin (26,66%). Pathogenicity of S. aureus was demonstrated by experimental infection of 2 batches of 1-day chicks, used for replication of the infection , and highlighting the enterotoxin production.

Keywords: Staphylococcus,pathogenicity, antibioresistance, resistance phenotypes.

Staphylococcus infection affect many species of birds, having either acute septicemic evolution (day old chicks to 3 week old chicks and laying hens in battery during warm season) or localized infection manifested by arthritis, bursitis and skin infections (youth 9 -16 weeks and adults). Coagulase positive Staphylococcus (SCP- S. aureus) that are pathogenic for birds, animals and human. Coagulazo-negative Staphylococcus (SCN) are considered non-pathogenic, commensals of the skin and mucous membranes in birds:S. epidermidis (skin infections), S.hycus (produces blepharitis in chicken and turkeys and osteoarthritis only in turkeys) and S.gallinarum (was isolated from poultry meat contaminated during technologycal processing [1].

Given the continuous evolution of the phenomenon of resistance to antibiotics, including methicillin-resistant of S. aureus (MRSA) it is important to determine accurately the antibiotic susceptibility of strains circulating. S. aureus resistance to methicillin) is mediated by the mecA gene that encodes the production of penicillin-binding protein 2a (PBP 2a). This is expressed homogeneous or inhomogeneous in microbial population (eg. 1 in 100 000 cells is a bacterial resistant phenotype)[4].

Methicillin is currently removed from the therapy and of susceptibility tests, being replaced by the MIC- method cefoxitin and oxacillin, and difuzimetric only with cefoxitina 30 μg. reporting of results is done as oxacilino-susceptible/resistant.

Oxacilino-resistant Staphylococcus are resistant to all current β-lactam antibiotics, except the last generation (III and IV) cephalosporin with anti-MRSA activity. Some strains, called BORSA (borderline Staphylococcus aureus) have no intrinsic resistance but have β-lactamase hyperproduction. They are susceptible to β-lactam antibiotics in combination with inhibitors of b-lactamase - clavulanic acid. [6,7]. Oxacillin resistance of S. aureus indicates a low resistance to Vancomycina [4]. This is highlighted only by turbidity antibiograms MIC(Minimal Inhibitory Concentration) and is characterized by values between 4-16μg/ml [4].

In recent years there have been reported SCN isolated from nosocomial infections, but also in veterinary practice, with multi-resistance to antibiotics including methicillin and vancomycin reduced susceptibility. Methicillin resistance was also detected in SCN strains (including S. epidermidis strains, S. saprophyticus)

that do not contain the mecA gene but who possess other resistance mechanisms (including S. epidermidis strains, S. saprophyticus).

Since 2003 there have been studies that have investigated the association of MRSA infections in animals raised in households, infections occurring mainly in pig farms [5]. In this context, the European Commission asked EFSA to support a program for monitoring MRSA in pig holdings in EU countries. The prevalence of MRSA in livestock shelters in the EU was 14% and in production farms was 26.9% [5]. Because the presence of MRSA in food is important, EFSA recommends expanding research upon such products, including those from cattle and poultry .

STUDY GOAL

The research was conducted from January to May 2015, using a unit in Bacau County with poultry breeding farm systematized in two separate areas and two hatcheries in different locations. It investigated the existence of an infectious circuit between the reproduction population - hatcheries - week old broiler chicks, by collecting samples from all segments of the technological flow.

OBJECTIVES

Making cultural examinations, strain identification, antibiotics susceptibility testing and pathogenicity research in S. aureus strains isolated from breeding herds by experimental inoculations chicks of different origin.

MATERIAL AND METHOD

Laboratory tests were conducted in a private veterinary laboratory, Laborvet Serv Hemeius, Bacau. We examened 74 sets of samples collected simultaneously from the breeding farms, the two hatcherys and the broiler farm (chicken series which were investigated in the incubation on the day of hatching)

Isolation was achieved by conducting bacteriological examination by seeding non-selective and selective media (nutrient broth Chapmann and Baird-Parker) and the identification was done on the basis of cultural, tinctorial and morphological characters (Gram-staining smears), biochemical tests (catalase test, oxidase test, mannitol fermentation) and investigation of ,,in vitro'' pathogenicity characters (hemolysis on Colombia Blood Agar 5% medium, highlighting of the nuclease enzyme (S. aureus- marker) on the DN-ase Agar medium).

Some isolates were cryopreserved at -20˚ C, using Criobile system (ceramic beads in glycerol), to be used in further laboratory tests.

Strains were confirmed and tested simultaneously for susceptibility to antibiotics using the Vitek 2 Compact automatic system (bioMérieux) by the standardized method M.I.C.(CLSI-based and Phenotipic) with the identification cards for Gram positive cocci AST-P592, and was completed by antibiograms performed through Kirby-Bauer disk diffusion method, to investigate prediction of mecA gene, by surrogate oxacillin test with cefoxitin 30µg, (VET01-S2,Vol.33.Table 9D), and also susceptibility to other antibiotics that are used in therapy birds.

The quality control in diffusimetric antibiograms was done with the strain of Staphylococcus aureus ATCC 25923 [4] (Liofilchem). The acceptance rate at QC was consistent with the prescribed values shown in CLSI -VET01-S2 No.8. Table 4.

In addition, we conducted the Screening test for resistance to oxacillin of Staphylococcus aureus (VET01-S2, Number 8. Table 9C). Resistant strains to oxacillin and cefoxitin were cultivated on the MHA agar with 4 % NaCl and oxacilina 6 mcg/ml. We used commercial formula MRSA Screen Agar (Biolab).

Investigation of S. aureus strains pathogenicity "in vivo" (highlighting the lethal toxins and enterotoxins[8]) was achieved by experimental infection with MRSA strain isolated from bone tissue of breeding chickens by inoculating 2 groups of 1-2 days old chicks of different origin, clinically healthy, as follows: group 1, consisting of 10 chicks, was inoculated within femoral muscles using 0.3 ml of strain selected, filtered, inactivated culture broth; group 2, consisting of 10 chicks was inoculated ,,per os'' with 1 ml of the same culture, inactivated 60 minutes at 65˚ C, to highlight a staphylococcal enterotoxin (which is thermally stable for 30 minutes at 100˚C[1]).Post-inoculation chickens were submitted to necropsy and the organs and legs with arthritis were used insemination to isolate staph.

RESULTS AND DISCUSSIONS:

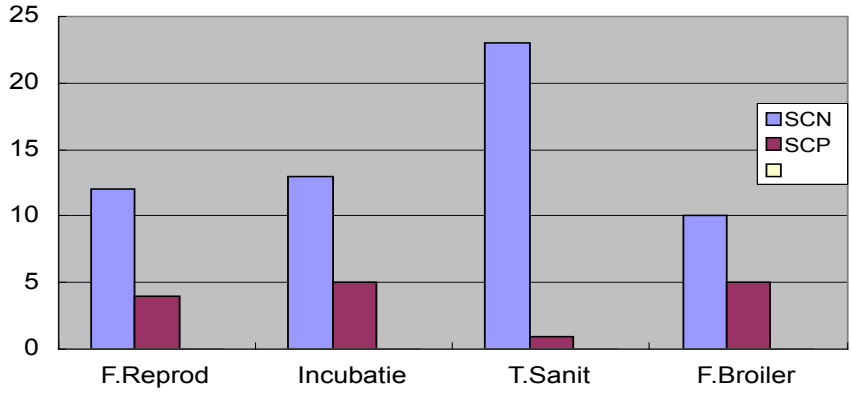
1. Of the 74 sets of samples analyzed, we have isolated a total of 73 strains of Staphylococcus spp., of which 58 SCN and SCP 15 strains were identified as S. aureus.

Distribution strains within stream segments was as follows (Table No.1): Breeding Farm for 16 strains of which 12 SCN (75%) and 4 SCP (25%); Hatchery: 18 strains of which 13 SCN (72.22%) and five SCP (27.77%); The broiler farm 15 strains of which 10 SCN (66.66%) and five SCP (33.33%); of the 36 samples sanitation 24 strains were isolated from the CNS 23 (95.83%) and one SCP (4.17%).

Table no. 1

Obiectiv//Tipul probei	**Nr. Total Tulp.**	**Nr. Tulp. SCN**	**Nr. Tulp. SCP**
Breeding Farm Sector I +II : eggs from in nest boxes nests and bedding, feces and long bone	16	12	4
Hatchery 1+ 2 : non-incubated eggs, embryos before and after Mirage, dead in shell, chicks hatched viable and sick	18	13	5
Broiler farm: clinically healthy chicks (the cecum, colon); chicken enteric syndrome (heart, liver, spleen, vez bil, cecum, colon)	15	10	5
Sanitation tests on surfaces spaces and egg shells from warehouse of breeding farm; sanitation tests on surfaces spaces and shells eggs from Hatchery	24	23	1

Diagram 1. Total isolated Staphylococcus spp. Strains on all technological circuit. The ratio between the SCN and SCP



2. Research of susceptibility to antibiotics using method Vitec 2 Compact showed: of the 15 S. aureus strains, 4 strains (26.66%) were oxacillin-resistant (MRSA) and 11 strains (73.34%) oxacillin -sensitive (MSSA).The 58 strains of CNS were 100% of oxacillin-sensitive. The phenotype analysis indicated that 2 strains (13,33 %) have a reduced resistance to oxacillin

and were categorized as Intermediate sensitive(with resistance phenotypes) which is reported as resistant. These strains were tested by diffusion method with cefoxitin (I) and and amoxiclav (S) were categorized as phenotype BORSA.

Vancomycin resistance was present in 6 strains (40%), being present in all 4 strains of MRSA. The 58 strains of SNC were 100% sensitive to vancomycin and oxacillin.

Induced resistance to the MLSB+A (reported on the basis of resistance related to erythromycin-clindamycin) was 100% in MRSA strains and 36,36% in MSSA; resistance to erythromycin was 100% at strains of S.aureus and 20,68% from SCN (Table 2).

Table no.2

Antimicrobial	**Types of strains**						
	Totally resistant strains	**SCN 58 strains**		**MSSA 11 strains**		**MRSA 4 strains**	
		S	**R**	**S**	**R**	**S**	**R**
Penicillin	73 tulpini	0	58	0	11	0	4
Oxacillin	6 tulpini	58	0	9	2	0	4
Gentamicin	47 tulpini	50	8	3	8	0	4
Teicoplanin	8 tulp	56	2	7	4	2	2
Vancomycin	6 tulp	58	0	9	2	0	4
Linezolid	0 tulp.	58	0	11	0	4	0
Eryithromycin	27 tulp	46	12	0	11	0	4
Clindamycin	11 tulp	58	0	7	4	0	4

S = sensitive; R = resistant; I = intermediate

3. The antibiograms carried out by the method of diffusion for the 15 strains of S. aureus have shown sensitivity of 100% to florfenicol. Cefoxitin susceptibility test and MRSA agar screening test results confirmed the results by the Vitec 2 method : 11 strains (73.34%) was MSSA and 4 strains (26.66%) MRSA; the 2 strains (13.33%) initially reported on Vitec with intermediate susceptibility to oxacilina have shown moderate sensitivity to Cefoxitina and Amoxiclav sensitivity, which we denote as phenotypes were BORSA.Resistance to erythromycin and lincomycin were 100%. Susceptibility to other antibiotics is shown in Table 3:

Table no. 3

Antibiotic	**Tipuri de tulpini S.Aureus (15 tulp)**		
	R	**I**	**S**
Florfenycol 30 µg	0 (0%)	0 (0%)	15 (100%)
Clortetracyclin 30 µg	4 (26,66%)	7 (46,66%)	4 (26,66%)
Gentamicin 10 µg	8 (53,33%)	5 (33,33%)	2 (13,33%)
Neomicyn 30 µg	6 (40%)	7 (46,66%)	2 (13,33%)

Amoxi & Clav acid 20/10 µg	4 (26,66%)	0 (0%)	11 (73,34%)
Enrofloxacin 5 µg	3 (20%)	2 (13,33%)	10 (66,66%)
Fosfomycin & Tylozin 50/15 µg	13 (86,66%)	0 (0%)	2 (13,33%)
Cefoxitin 30 µg	4 (26,66%)	0 (0%)	11 (73,34%)
Lincomycin 10 µg	15 (100%)	0 (0%)	0 (0%)
Erithromycin 15 µg	15 (100%)	0 (0%)	0 (0%)
MRSA Screen Agar	4 (26,66%)	0 (0%)	11 (73,34%)

S = sensitive; R = resistant; I = intermediate

4. ,,In vivo'' pathogenicity research has shown that: 30% of chickens inoculated intramuscularly developed infection septicaemic infection and and died 24 hours after inoculation, the rest died after 48 hours, showing anorexia, hyperthermia and arthritis tibio-tarso-metatarsal in member of injection inoculum. Chickens inoculated orally with inactivated culture survived 48 hours, showing deviation, loss of appetite and diarrhea with dark brown feces with chalky deposits.

At necropsy was evident chickens inoculated septicemic lesions: in cavities serous hemorrhage exudates, bleeding in the gallbladder and epicard,, spleen hyperplasia, digestive lesions (catarrhal and hemorrhagic enteritis and liver dystrophy) and arthritis. Chickens inoculated orally with inactivated strain experienced chataral and hemorrhagic enteritis, hemorrhagic abdominal cavity serosa, hepatic dystrophy [3].

From chickens which died with the septicaemic forms, Staphylococcus was isolated from parenchymal organs, and from those with arthritis was isolated from synovial fluid. In all inoculated chickens all have seen in organs and intestines a proliferation of Gram Negative flora: E. coli and Pseudomonas spp.

CONCLUSIONS :

Similar percentages of S. aureus strains isolated in all sectors of the technological flow (Breeding Farm 25%; Hatchery 27,77%; broiler farm 33,33%) shows that endemic infection in the poultry flocks is maintained by birds with chronic forms of the disease that eliminates the germ through feces and contaminated shell eggs , but can be counted and vertical transmission, assumption made in the literature already argued egg germ isolation[1]; in this study revealed the presence of non-incubated eggs and in ovarian mature follicles.

It also correlates percentage of embryonic mortality with the rate of morbidity to the chiks in the first week of life.

Sanitation standards indicate a poor cleaning but the presence of s. aureus was low (4.17%).

2. S.aureus strains isolated from adult birds with arthritis, from dead embryos and broiler as showed resistance to methicillin (26,66%), in a percentage similar to that found in studies of pig holdings [5]; CNS species were resistant to more than 4 antibiotics.

3. Tests for mecA gene prediction (diffusion method with cefoxitin 30 µg and oxacillin resistance test on MRSA Agar provides results comparable with VITEC 2 MIC- method.

4. We believe that the experimental model reproduced and explained the clinical and morphological lesion of the embryos and the broiler chiks.

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OBSERVATIONS REGARDING THE PRESENCE OF SOME GENES IN APEC STRAINS

NICOLAE CĂTANA[1], VIRGILIA POPA[2], VIOREL HERMAN[1], IULIA BUCUR[1], IONICA IANCU[1]

1-USAMVB- Faculty of VeterinaryMedicine, Timișoara, România

2 – S.N. Institutul Pasteur S.A., București

epirovet_tm@yahoo.com;

Abstract: *Colibacilosis produces significant economic losses, in broilers, either as primary disease or as a secondary infection.It is produced by E. coli strains, included in APEC pathotype (Avian pathogenic Escherichia coli), that have as site of penetration, the body's respiratory mucosa.APEC strains have more pathogenic factors, especially adezins with tropism for cell receptors of epithelium respiratory.In the organism produce septicemia and localized infections, while the disease is considered an extra intestinal infection). In 2003, EWERS CHRISTA et al., have developed an extensive study that outlined for the first time, the phenotypic and genotypic features of this pathotype.Further, this area of avian infectious pathology, was approached by numerous collective of researchers, including our country. The research followed the presence of some genes wich encodes the synthesis of some virulence factors characteristics for APEC pathotype strains.There were studied a total of 118 strains of E. coli isolated from colibacillosis outbreaks that have evolved in broiler chicken flocks.The strains were included in E. coli species, based on phenotypic characters and with multiplex PCR screening (Polymerase Chain Reaction) were detected the ompA, iss, fimH and lac Z genes, whose frequency was variable. OmpA gene that encodes the synthesis of an outer membrane protein,with the role of an adesin,was present in 83,10% of strains; the iss gene which also encodes the synthesis of an outer membrane protein, that has the role of an adesin, andinduces the resistance to the complement was present at 88.14% of the strains. FimH gene that encodesthe synthesis of type I fimbria, , was present in 83.9% of the strains and the lacZ gene encoding beta-D-galactosidase enzyme synthesis, was present in all of the tested strains.*

Keywords: avian colibacillosis, *E.coli,* virulence genes.

INTRODUCTION

In intensive poultry farming, colibacillosis is the main cause of morbidity and mortality in broilers, in chickens intended for breedingor for eggconsumption and in youth around the age onset of lay.Colibacillosis producesignificant economic losses either as primary disease or as a secondary disease, triggered by some viruses, some vaccines you and numerous extrinsic factors, technological factors and microclimate (2,4).

Colibacilosis is produced by strains of *E.coli* included in APEC pathotype (*Avian Pathogenic Escherichia coli*) as an extra intestinal infection with septicemic evolution (colisepticemia),followed by sequels (meningitis, panoftalmia, osteoarthritis, synovitis and coligranulomatoza turkey osteomyelitis) or as a localized infection with several forms (omphalitis and yolk sac inflammation, swollen head syndrome, cellulitis, turkey diarrheal disease, venereal colibacillosis, salpingitis and coliform orchitis) (4).

In 2003, EWERS CHRISTA et al., elaborated a ample research regarding the phenotypic and genotypic *E.coli* strains isolated from poultry and based on these characteristics delineated a new pathotype called *Avian Pathogenic Escherichia coli* (2).

Researches in this field were continued for more collective wich established that this pathotype strains were assigned to several genes, detaching as important *ompA, fimH* and *iss* gene.

The genes encoding the synthesis of virulence factors represented by the synthesis of outer membrane proteins, type I fimbriae, and resistance to complement.

These factors ensure cell attachment to bacterial respiratory mucosa, followed by penetrating the mucous and blood multiplication realizing septicemia (1,3,6).

In Romania, research in this area were initiated by Dr.VIRGILIA POPA in 2001 and continued by other collectives (5).

The research was conducted in order to determine the frequency of these three genes and the *lacZ* gene of *E. coli* strains from pathotype APEC isolated from outbreaks that have evolved colibacilosis flocks existing in broiler farms from the west of the country.

MATERIALS AND METHODS

The research was performed over a number of 118 strains of *E. coli* isolates from broiler carcasses with colisepticemia lesions or colibacilare localized infections located.Strains were typified cultural, tinctorial and biochemical species to fit into *E.coli* species, which was established after the behavior to antibiotics.

The presence of *ompA*, *fimH* and*iss*genes was determined by multiplex PCR screening (Polymerase Chain Reaction) using primers synthesized by classical trading methodology and for detection of *lacZ* gene detection were used two primers (5'ATGAAAGCTGGCTACAGGAAGGCC-3'and
5'GGTTTATGCAGCAACGAGACGTCA- 3').

Results of the frequency of these genes have been processed and graphically presented.

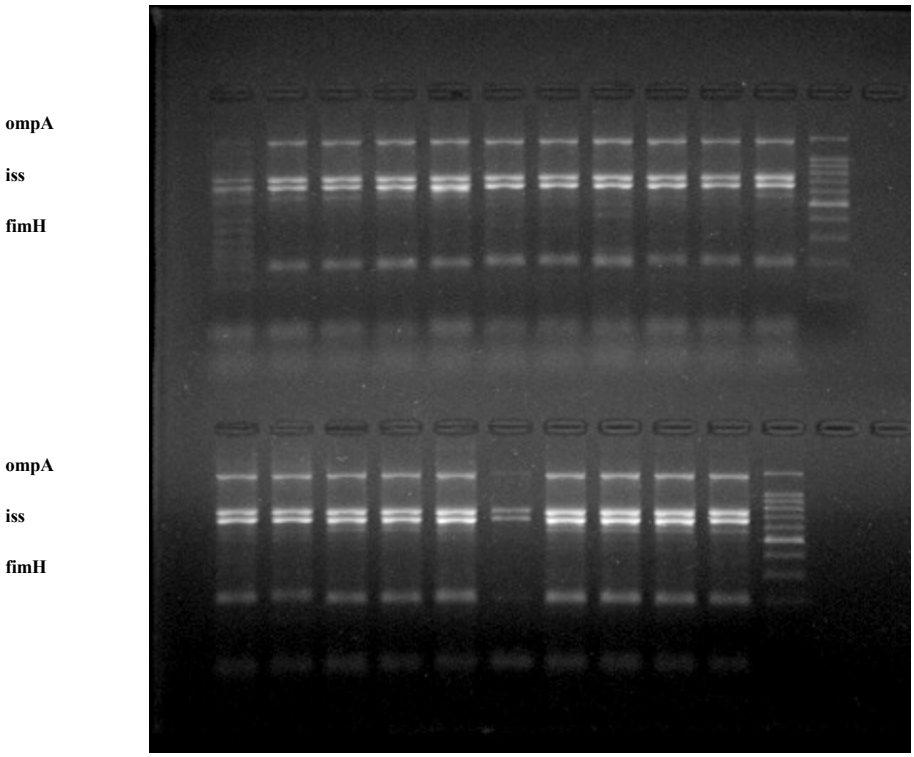
RESULTS AND DISCUSSIONS

Multiplex PCR technique allowed the detection of*ompA*, *iss*and *fimH* genes and, amplicons of which are at the level with the control amplicons finally being obtained following base pairs (bp): *ompA* 1421, *fimH* 770, *iss* 737 (Figure 1).

The *ompA* gene encoding the outer membrane protein responsible for bacterial attachment, was present in 98 (83.10%) of the strains tested, the gene iss which encodes a protein of external membrane,inducing the resistance to complement favoring the multiplication of colibacilli in blood was present at the 104 (88.14%) of the tested strains and *fimH* gene, component of the *fim*operon that encodes the synthesis of type I fimbre (adhesin), was present at 99 (83.90%) of the strains tested.

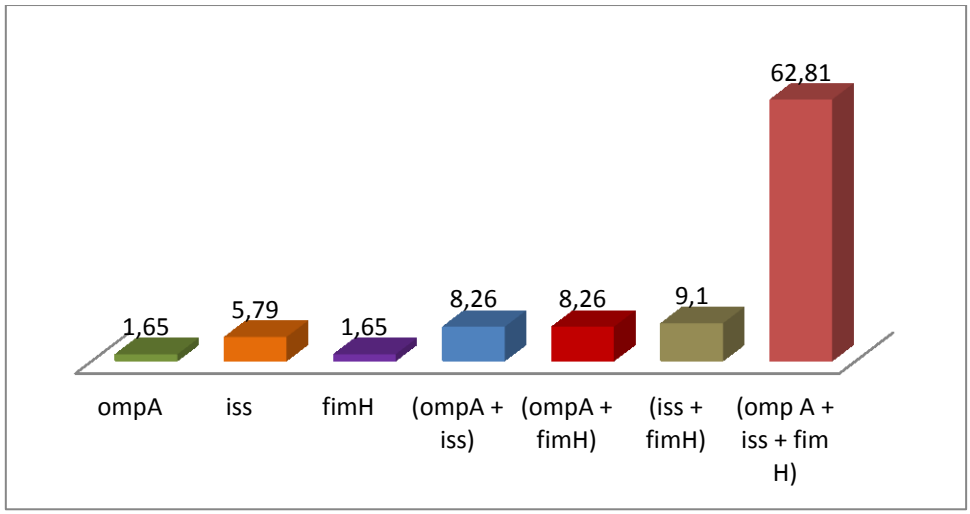
Analyzing the results obtained by screening carried out, results that only 11 strains (9.32%) was present only a single gene.The *ompA* gene was present in the two strains (1.70%), *iss* gene was present in 7 strains (5,93) and *fimH* gene was present in the two strains (1.70%).The association of two genes was more frequent, as follows:*ompA* + *iss* association gene was present in 10 strains (8.47%) *ompA* + *fimH*gene association was present in 10 strains (8.47%) and the association of genes *iss* + *fimH* was present in 11 strains (9.32%), but the association of the three genes form was present at the 76 strains (63.56%) (Figure 2).

Figure 1



Escherichia coli 3535-3553.Molecular typing by multiamorsa PCR (multiplex PCR), the APEC pathotype.Control by gel electrophoresis after PCR: 3535-3544 strains.B: strains 3545-3553.Lines 11 (A) and 10 (B) positive control E. coli 4293 GDP ((ompA + - ISS + - FimH +) Line 12 (a) and 11 (B): Standard DNA - 100 bp.

Figure 2

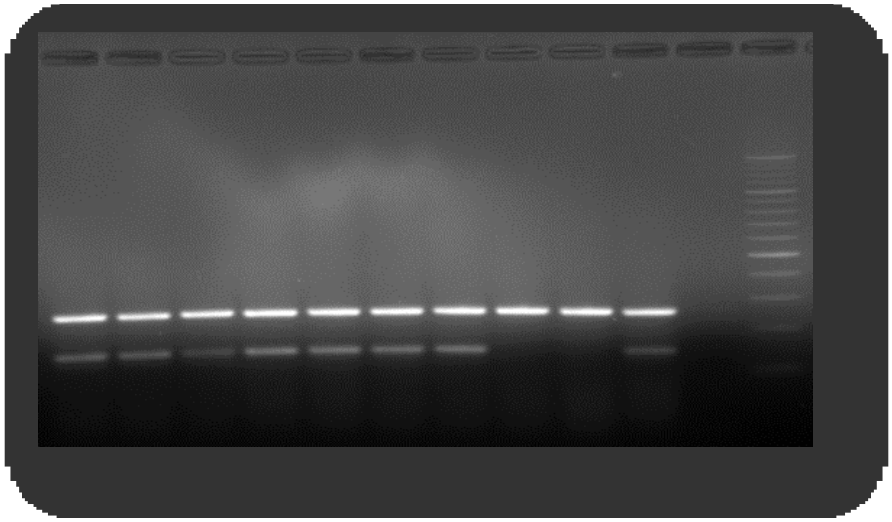


Gene association ompA iss and fimH in APEC strains

LacZ gene that encodes the synthesis of beta-D-galactosidaseenzyme, which hydrolyzes the lactose, was present in all of the tested strains (Figure 3).The frequency of this

gene was identical to results achieved on the lactose fermentation by all of the strainstested in culture media.The screening carried out by multiplex PCR technique has determined that APEC strains have in their genetic structure, of these genes in varying proportions, the results are similar to dates in literature (2,3,6).

Figure 3



The presence of the lacZ gene of E. coli strains

These genes are transmitted between strains of *E.coli* by conjugative and unconjugative type processes, and these strains are spread through primary and secondary sources both in poultry flocks farms and hatcheries in and through poultry material trade are worldwide spread.

CONCLUSIONS

Screening carried out multiplex PCR variant has established a high frequency of APEC strains isolated from outbreaks of avian colibacillosis.

The strains tested *ompA*, *fimH* and*iss*genes were associated,two or three at the most of the strains tested.

Multiplex PCR technique can be used to identify strains APEC and represents a useful method for monitoring avian colibacillosis.

The PCR in all strains tested were detected *lacZ* gene, which encodes the synthesis of the beta-D-galactosidase enzyme, which hydrolyzes the lactose, these results being identical to the results provided by biochemical tests.

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REVIEW REGARDING THE EVOLUTION OF AN OUTBREAK OF REOVIRUS IN BROILERS

NICOLAECĂTANA[1],OANA PETREC[1], VIRGILIA POPA[2], IONICA IANCU[1]
1-USAMVB- Faculty of VeterinaryMedicine, Timișoara, România,
2 – S.N. Institutul Pasteur S.A. București;
epirovet_tm@yahoo.com

Abstract: *Avian reovirosis are infectious and contagious diseases spread in intensive poultry, especially broilers, evolving with anatomoclinical multiple forms.*
Intensive trade with poultry material, contributed to the spread of avian reovirus infection and, after 1990, these infectious diseases often evolve also in our country.
The research was conducted in a flock of 10,000 broilers, from COBB 500 hybrid, raised to the ground, on a farm from the western country. The number was monitored by epidemiological, clinical and pathological exams, made biweekly throughout the growth.
To confirm the reovirosis, blood samples were taken at the age of 21 days (R1) and at the age of 42 days (R2), revealing the specific antibodies by ELISA technique, using the FlockChek Avian Reovirus antibody test Kit, provided by IDEXX Laboratories, Inc.
Cumulative mortality had weekly variable values and in the monitored period was 7.91%.
Clinical examination revealed the presence of characteristic symptoms: malabsorption and arthritis-tenosynovitis syndrome.
Serological examination confirmed the evolution of reovirosis at the age of 21 days, identifying 6 groups of titer (0-5), with a minimum titer of 12 D.O. and a maximum titre of 1022 D.O., and at the age of 42 days, were identified 8 groups of titers (0-7), with a minimum titer of 63 DO and D.O. and a maximum titre of 1453 D.O..
At the age of 21 days were positive 29 serums, respectively 60.42% and at the age of 42 days 35 serums were positive, respectively 77.77%. These data show that within 21 days, the proportion of positive serums increased 1.29 times, which suggests the horizontal extension of the infection in the flock of monitored chickens.

INTRODUCTION

Avian reovirosis are infectious and contagious diseases encountered in chickens and turkeys, manifested clinically by many evolutionary forms. There are widespread in intensive poultry, especially in broilers, to which produce significant economic losses (2,4,5).

In 1954, FAHEY and CRAWLEY isolated a virus from chickens with chronic respiratory syndrome, which in 1957, was classified by PETEK in Orthoreovirus genus. Later OLSON et al. Isolated orthoreoviruses from chickens with arthritis and tenosynovitis, while DESHMUTH et al. Isolated the same viruses from chickens and turkey hen with enteritis (2,4,5).

In 1978, KOUWENHOVEN describe in broilers a clinically form localized digestive as "runtingsyndrome", named by VELTMAN in 1985, "malabsorbtion syndrome" (2,4,5).

Intensive trade with poultry material contributed to the spread of infections with avian reovirus worldwide, which after 1990, frequently evolve in our country, too (4).

Researches were made in a farm specialized in breeding broilers, from western country, where evolved avian reovirosis in several series of chicken, in order to investigate the epidemiological, clinical and anatomopathological issues of this disease.

MATERIALS AND METHODS

In a farm from thewest of the country, specialized in breeding broilers, was studied a herd of 10,000 chickens, from COBB 500 hybrid, raised to the ground. The number was monitored by epidemiological, clinical and pathological exams, made biweekly through out the growth, from the first day stocking and until the day of slaughtering.

The results were processed and showed in the tables and charts.

To confirm the reovirosis, blood samples were taken at the age of 21 days (R1) and at the age of 42 days (R2), revealing the specific antibodies by ELISA technique (Enzyme - linked Immunosorbent Assay), using the FlockChek Avian Reovirus antibody test Kit, provided by IDEXX Laboratories, Inc.

RESULTS AND DISCUSSIONS

Epidemiological examination carried out as an ample investigation, followed the evolution of cumulative mortality, of weight gain and the existence of infection sources. In the farm,the following vaccination are made: against bursal infectious disease made *in ovo* hatchery; against infectious bronchitis made in hatchery (vaccination I) and in farm (vaccination II) and against Newcastle disease made in hatchery (vaccination I) and in farm (vaccination II).

Weight gain, determined by weekly weighing showed a lower feed conversion. Thus, the average weight was 30% lower than the standard weight of that hybrid and the expected increase was higher by about two weeks. The evolution of weight gain is the result of malabsorption syndrome, the specialized literature mentioning that weight gain can be reduced by 25% -66%.

Epidemiological investigation revealed that reovirosis was vertically transmitted in broilers from the monitored flock and within the farm through secondary sources of infection.

The mortality losses were recorded daily, as absolute value, and quantified as relative value by the **cumulative mortality** whose evolution is shown in Table 1.

Table 1

Weeklyevolution of cumulative mortality

Week	Cumulative mortality		Corpsesnecropsied	
	Nr.	%	Nr.	%
I	216	2.16	15	6.94
II	149	1.49	18	12.08
III	113	1.13	18	15.92
IV	101	1.01	16	15.84
V	73	0.73	22	30.13
VI	139	1.39	7	5.03
Total	**791**	**7.91**	**96**	**12.13**

Analyzing the data presented in the table, results that this indicator evolved differently in the monitored period, with a maximum value in the I week.

During the monitoring period, the cumulative mortality value was 7.91%, similar with existing data from literature. The values of this indicator are influenced by several favoring factors, biotic and abiotic, existing on farms, and thus influencing the progress of reovirosis and of cumulative mortality, most of the authors considering that in broilers the cumulative mortality 20% (1,2).

Clinic examination performed individually on ckchickens, showed typical symptoms of the two main syndromes that have evolved simultaneously in the monitored herd. Symptoms were more evident after the age of 12 days, along with the two syndromes are present and respiratory and digestive symptoms. Symptoms were more obvious after the age of 12 days, along with the two syndromes also being present respiratory and digestive symptoms.

The specific symptoms of **malabsorption syndrome** appeared after the age of 10 days being clearly observable after the age of 3 weeks, when it was noticed a marked nonuniformity of the offspring, suggesting the existence of chicks of different ages. Other symptoms reported were: diarrhea, capricious appetite, higher consumption of wáter, adinamia and maintaining the fluff.

After the age of 3 weeks, changes on wings feather appeared, which turned sideways or upwards, characteristic to"helicopterchickens".

The specific symptoms of **arthritis tenosynovitis syndrome** appeared in chickens after the age of 3 weeks. The sick chickens had difficulty on walking, lameness, unilateral or bilateral arthritis at tibio-tarsus-metatarsal joint. Joints were increased in volume, fluctuating and painful.The joints were increased in volume, fluctuating and painful.This is why the chickens refused to move in order to drink water and tofeed, thus gradually weakening.After the age of 4 weeks, in some chickens, the gastrocnemius tendon rupture occurred, followed by the impossibility of movement and keeping extension of the affected limb.

Pathological examination was made biweekly from the age of 6 days to 42 days,when the herd was slaughtered. In the mentioned period,were necropsied 87 corpses, which revealed some macroscopic lesions characteristic in avian reovirosis. The results of this examination are shown in Table 2.

Table 2

Macroscopic anatomopathological lesions observed at necropsy

Nr.crt.	Lesion	Number	Percentage
1.	Unabsorbed yolk, peritonitis, omphalitis	10	11.49%
2.	Rhinitis,tracheitis	18	20.68%
3.	Congestion and pulmonaryedema	42	48.27%
4.	Aerosaculitis	8	9.19%
5.	Unilateral necrosis of femural head	25	28.73%
6.	Bilateral necrosis of femural head	35	40.22%

7.	Hyperplastic spleen	8	9.19%
8.	Renal dystrophy, liver dystrophy, uric gout	49	56.32%
9.	Fibrinouspolyserositis	7	8.04%
10.	Catharral/ haemorrhagicbursitis	9	10.34%
11.	Ascites	7	8.04%
12.	Hidropericard	9	10.34%
13.	Proventriculus	28	32.18%
14.	Catharral/ haemorrhagicenteritis	23	26.43%
15.	Artritis/tenosynovitis	7	8.04%
16.	External lesions	19	21.83%

Necropsy results revealed lesions characteristic in reovirosis with the highest frequency on bilateral necrosis of the femoral head (40.22%) and proventriculus (32.18%).

Other lesions characteristic in reovirosis (ascites, hidropericard and arthritis-tenosynovitis) had a lower incidence similar to the frequency communicated by other authors (2,3,5).

There were also revealed lesions characteristic colisepticemia, avian mycoplasmosis and infectious bursal disease, which evolved as a secondary infection consecutive to immunosuppression induced by avian reovirus.

Serological examination, made with immunoassay test,confirmed the presence of reovirosis in the monitored flock of broilers and the results are shown in Table 3.

Table 3

R1 – 21 days		**R2 – 35 days**	
Titer group	**Number of samples**	**Titer group**	**Number of samples**
0	19	0	10
1	5	1	3
2	6	2	5
3	7	3	5
4	6	4	5
5	5	5	6
-	-	6	5
-	-	7	6
Maximum titer	1022 O.D.	Maximum titer	1453 O.D.
Minimum titer	12 O.D.	Minimum titer	63 O.D.
Geometric mean of titer	89	Geometric mean of titer	245

At the age of 21 days, there were identified 6 groups of titer (0-5), with a minimum titer of 12 D.O. and a maximum titre of 1022 D.O., and at the age of 42 days, were identified 8 groups of titers (0-7), with a minimum titer of 63 DO and D.O. and a maximum titre of 1453 D.O.

At the age of 21 days were positive 29 serums, respectively 60.42% and at the age of 42 days 35 serums were positive, respectively 77.77%. These data show that within 21 days,

the proportion of positive serums increased 1.29 times, which suggests the horizontal extension of the infection in the flock of monitored chickens. The extension of reovirosis was also confirmed by seroconversion and the geometric average of the titers at the age of 35 days was 2.75 times higher than the geometric average of the titers at the age of 21 days.

CONCLUSIONS

- In the monitored broiler flock reovirosis was suspected based on clinical and pathological examination.
- The epidemiological investigation made in the farm, showed the vertical transmission of reovirois and subsequent extension of the disease by horizontal transmission.
- In the monitored broiler flock reovirosis evolved with malabsorption and arthritis tenosynovitis syndrome, but were also present other clinical forms: hidropericard, ascites, respiratory symptoms and gastrointestinal symptoms.
- Serological examination conducted by immunoassay test confirmed the presence of reovirosis and showed the seroconversionphenomenon characteristic to postinfectious immune response.

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ANTIBIOTICS RESISTANCE DYNAMICS OF ISOLATED PSEUDOMONAS AERUGINOSA STRAINS IN A HOSPITAL FROM NORTHEASTERN ROMANIA IN THE PERIOD 2012 – 2014

OANA – ALEXANDRA CIOCAN[1], ANDREEA – PAULA COZMA[1], IRINA LIPOVAN (ABALASEI)[1], ELEONORA GUGUIANU[1], CARMEN - VALENTINA PANZARU[2], MIHAI MAREȘ[1], MIHAI CARP – CĂRARE[1], CĂTĂLIN CARP – CĂRARE[1]

[1]Faculty of Veterinary Medicine, Iasi

[2]Grigore T. Popa University of Medicine and Pharmacy Iasi

veterinarians_phd@yahoo.com

Abstract:

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. In fact, Pseudomonas aeruginosa is the epitome of an opportunistic pathogen of humans, is one of the leading Gram-negative organisms associated with nosocomial infections (3). The increasing frequency of multi-resistant Pseudomonas aeruginosa strains is concerning as efficacious antimicrobial options are severely limited.

Aims: *determining the dynamics of antibiotic-resistant strains of Pseudomonas aeruginosa isolated in a hospital in northeastern Romania.*

Materials and methods

Our study was based on the number of bacteriological examinations and antibiograms performed in the hospital under study.

Strains from pus, urine, blood cultures, catheter and aspirate/ sputum were analyzed.

The statistical analysis working method was performed using standard programs.

Results

During the 3-year study, we recorded a total of 12,317 strains out of which: in 2012, 317 strains proved to be resistant to first-line antibiotic treatment, in 2013, 387 resistant strains and in 2014, a total of 449 resistant strains were identified.

The highest number of recorded strains that do not respond to primary treatment were strains from urine, followed by those from sucked, and the last being the catheters.

Conclusion

Following the above data analysis, we conclude that the incidence of drug-resistant strains has an increasing trend. The resistance to antimicrobial agents is an increasing public health threat every year (1). It limits therapeutic options and leads to increased mortality and morbidity (2).

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Keywords: *hospital, Pseudomonas aeruginosa, resistance to antibiotics*

INTRODUCTION

Antimicrobial resistance is when microbes are less treatable with one or more medication used to treat or prevent infection. This makes these medications less effective in both treating and preventing infection. Resistant microbes may require other medications or higher doses – often with more side effects, some of which may be life threatening on their own. Some infections become completely untreatable due to resistance. All classes of microbes develop resistance. Antimicrobial resistance is a growing problem in the world, and causes millions of deaths every year. (6)

Pseudomonas aeruginosa is one of the leading gram-negative organisms associated with nosocomial infections. The increasing frequency of multi-drug-resistant Ps. aeruginosa strains is concerning as efficacious antimicrobial options are severely limited.

The reasons for the widespread use of antibiotics include the following: the increasing global availability over time since the 1950s, the uncontrolled sale in many low- or middle-income countries, where they can be obtained over the counter without a prescription, potentially resulting in antibiotics being used when not indicated. This may result in the emergence of resistance in any remaining bacteria. Prescription of or obtaining broad-spectrum antibiotics incorrectly: these are more likely to induce resistance than narrow-spectrum antibiotics.

These were the arguments for which, during 2012-2014, we analyzed the dynamics of Pseudomonas aeruginosa antibiotic resistance dynamics in a hospital in northeast Romania.

AIMS

The purpose of this study was to determine the incidence of antibiotic resistance phenomenon and analyze its dynamics.

MATERIALS AND METHODS

Our study was based on the number of bacteriological examinations and antibiograms performed in the hospital under study.

The strains of Pseudomonas aeruginosa were isolated, identified and analyzed from pus, urine, blood cultures, catheter and aspirate/ sputum. Strains were identified by classical methods in microbiology, using common culture media, the simple Agar and Cetrimide Agar medium, and they were biochemically confirmed using the RapID NF Plus test (9).

The samples analyzed were taken from patients admitted to the following units: intensive care unit for coronary artery disease patients, paid ambulatory care, day hospital admission to the cardiology unit, outpatient surgery, laboratory for examination of functions, intensive care unit – cardiovascular surgery centre, day hospital admission at the cardiovascular surgery centre.

This study was conducted over three years: 2012, 2013, 2014, the total number of samples amounted to 12, 317 and those of sensitivity tests amounted to 1,207.

The statistical analysis working method was performed using standard programs.

RESULTS

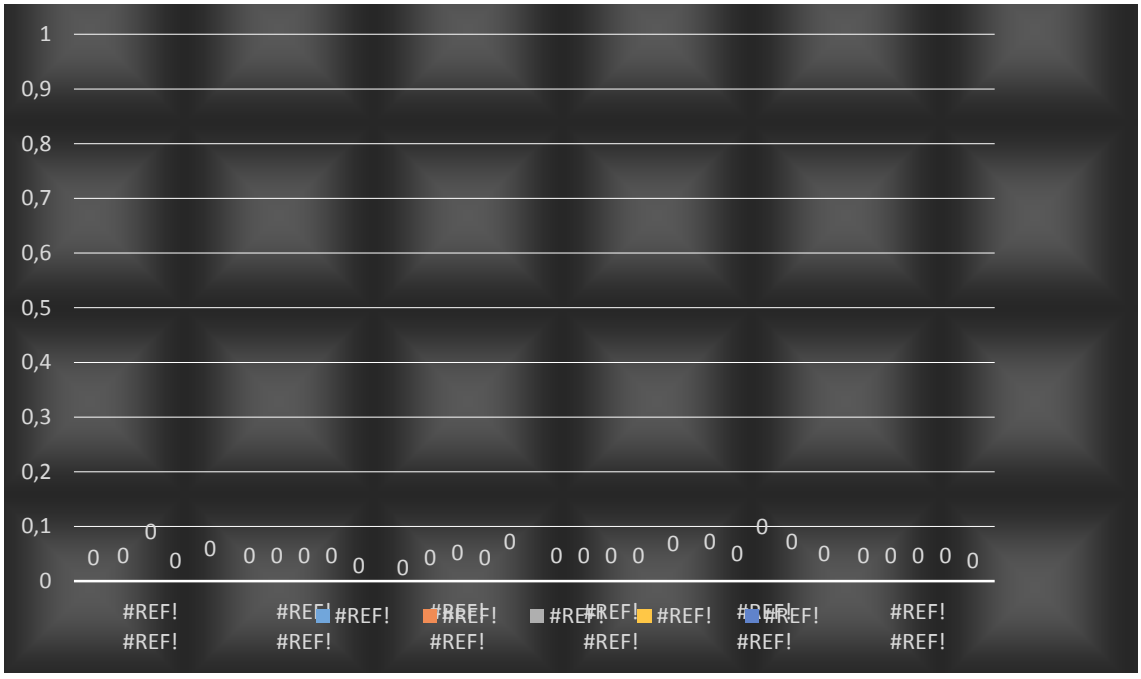
Table no. 1

SITUATION OF THE TOTAL OF PUS, URINE, BLOOD CULTURES, SPUTUM SAMPLES AND A NUMBER OF SENSITIVITY TESTS CONDUCTED ON THE STRAINS RESISTANT TO THE INTIAL TREATMENT

YEAR	2012		2013		2014	
PRODUCT	I	A	I	A	I	A
Pus	299	72	387	81	381	84
Urine	1677	163	1850	159	1865	172
Blood cultures	320	34	300	27	484	43
Sputum	1287	88	1493	105	1559	122
Total	3671	371	4147	387	4499	449

Figure no. 1

SITUATION OF THE TOTAL COLLECTED SAMPLES



I – INITIAL TREATMENT

A – ANTIBIOGRAM

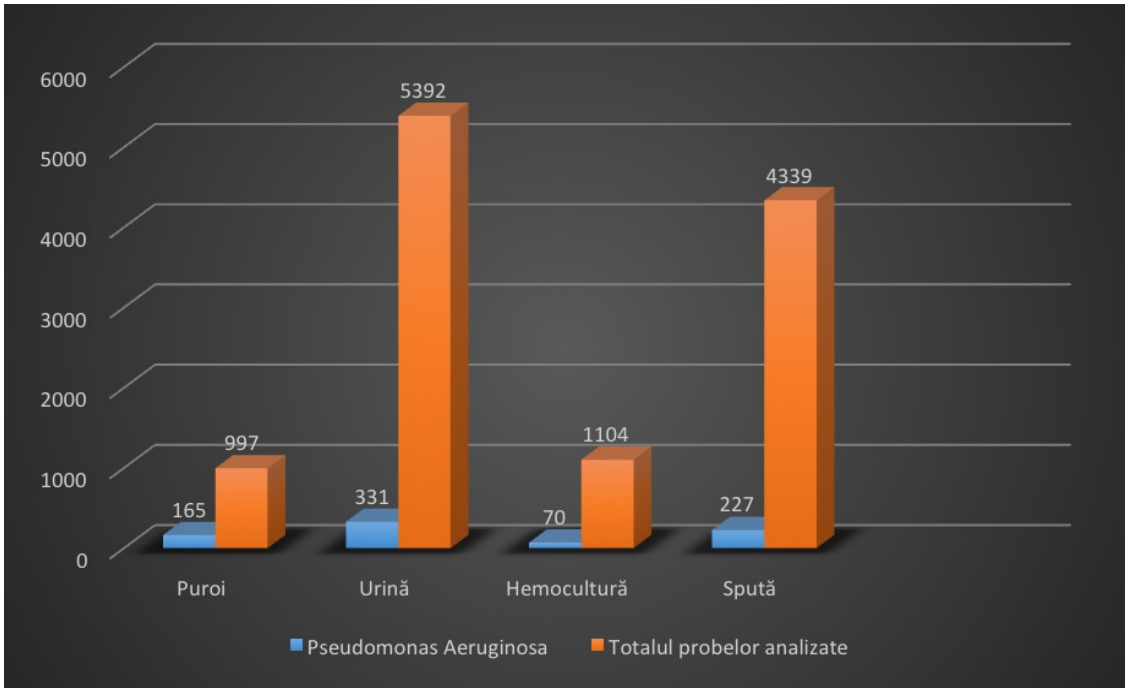
Table no. 2

SITUATION OF THE PSEUDOMONAS AERUGINOSA ISOLATED STRAINS

PATHOLOGICAL PRODUCT	PSEUDOMONAS AERUGINOSA	TOTAL OF ANALYZED SAMPLES
PUS	***165***	***997***
URINE	***331***	***5392***
BLOOD CULTURES	***70***	***1104***
SPUTUM	***227***	***4339***
TOTAL	**793**	**11832**

FIG. NO . 2

GRAPHICAL REPRESENTATION OF PSEUDOMONAS AERUGINOSA ISOLATED STRAINS



In the cases we studied, the share of the infection caused by Ps aeruginosa is 6.70% of the total number of samples analyzed. The highest percentage of ps aeruginosa strains, 16.54, was isolated and identified from the purulent secretions that were analyzed. As regards the percentage of ps aeruginosa strains isolated and identified from urine cultures, blood cultures and bacterial sputum cultures, the results were the following:

- 6.13% of Ps aeruginosa strains from urine cultures;
- 6.34% of Ps aeruginosa strains from blood cultures;
- 5.23% of Ps aeruginosa strains from sputum cultures.

Comparing the results we obtained with the data from literature, we can notice an increasing dynamic of the percentage of Ps aeruginosa strains resistant to the antibiotics action. (7)

CONCLUSION

- It is necessary to perform the antibiogram of samples from pus secretions since, in this study, the largest number of bacterial strains that did not respond to the primary treatment come from pus, considering the fact that there is the information transfer of the antibiotic between bacteria and the mechanism of resistance, we recommend conducting antibiotic-sensitivity testing for all the collected samples.
- Supervision under standardized conditions of the sensitivity to antibiotics of pseudomonas aeruginosa strains is useful for understanding the current spectrum of bacteria sensitivity to antibiotics, with direct implications in updating the therapy guidelines and limiting the spread of multiresistant strains.
- Antibiotic resistance is a serious and an increasing global problem: a World Health Organization (WHO) report released in April 2014 stated that "this serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country. (8). Antibiotic resistance—when bacteria change so antibiotics no longer work in people who need them to treat infections—is now a major threat to public health." (5)
- The recorded percentages indicating resistance issue a warning about the correct use of antibiotics.

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RESISTANCE PHENOTYPES OF *PSEUDOMONAS AERUGINOSA* STRAINS OF HUMAN ORIGIN IN AMINOGLYCOSIDES

OANA – ALEXANDRA CIOCAN[1*], CĂTĂLIN CARP – CĂRARE[1], ANDREEA – PAULA COZMA[1], ELEONORA GUGUIANU[1], CRISTINA RÎMBU[1], ELENA PETRARU[2], STRATULAT PETERCĂ EMMA CECILIA[3] CARMEN - VALENTINA PANZARU[4] , MIHAI CARP – CĂRARE[1]

[1]*Faculty of Veterinary Medicine, Iasi*

[2]*"St. Mary" Emergency Hospital for Children "St. Mary", Iasi*

[3] *SC Eris Vet SRL, Iasi*

[4]*Grigore T. Popa University of Medicine and Pharmacy Iasi*

veterinarians_phd@yahoo.com

Abstract:

Introduction

P. aeruginosa has become an important cause of infection, especially in patients with compromised host defense mechanisms. Aminoglycosides (1) are a vital component of antipseudomonal chemotherapy involved in the treatment of various infections (2), particularly pulmonary infections in cystic fibrosis patients (3).

Aims: *This study was intended to detect the aminoglycoside resistance phenotypes production in Pseudomonas aeruginosa and to evaluate the susceptibility pattern.*

Materials and methods

The batch studied included 793 multi-resistant strains of Ps. aeruginosa, which were isolated from the Institute of Cardiovascular Diseases in Iași during 2013 – 2014. 61 strains of Pseudomonas aeruginosa with resistance profile were included in this part of the study.

The susceptibility to antibiotics of the bacterial strains included in the study was tested using the diffusimetric method. The interpretation of results was based on the rules established by CLSI 2014.

Results

Following the tests carried out, according to the literature, the group identified 61 isolates with resistance profile and the results were the following:

- *15 strains present wild-type phenotype aminoglycosides, these strains are sensitive to all the aminoglycosides tested.*
- *G Phenotype: 8 strains*
- *GT Phenotype: 11 strains*
- *GTNtA Phenotype: 6 strains*

Conclusion

In this study, the GT phenotype was most frequently defined (Gentamicin and Tobramycin resistant, sensitive AK, I, Nt).

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Keywords *: Pseudomonas aeruginosa, aminoglycosides, nosocomial infection*

INTRODUCERE

Aminoglycoside is a medicinal and bacteriologic category of traditional Gram-negative antibacterial therapeutic agents that inhibit protein synthesis and contain as a portion of the molecule an amino-modified glycoside (sugar), the term can also refer more generally to any organic molecule that contains aminosugar substructures.

Aminoglycoside antibiotics display bactericidal activity against gram-negative aerobes and some anaerobic bacilli where resistance has not yet arisen, but generally not against Gram-positive and anaerobic Gram-negative bacteria.

Aminoglycosides display concentration-dependent bactericidal activity against "most gram-negative aerobic and facultative anaerobic bacilli" apart from some bacilli and methicillin-resistant staphylococci, but not against gram-negative anaerobes and most gram-positive bacteria.

They require only short contact time, and are most effective against susceptible bacterial populations that are rapidly multiplying. These activities are attributed to a primary mode of action as protein synthesis inhibitors, though additional mechanisms are implicated for some specific agents, and/or thorough mechanistic descriptions are as yet unavailable.

Bacterial resistance to aminoglycosides has several mechanisms:

- the main mechanism is represented by enzymes from the plasmids of Gram-negative bacilli (aminocglycoside acetyltransferase, aminocglycoside posphotransferase, aminoglycoside nucleotidyl-transferase);
- another mechanism resides in altering the action target: modifying an aminoacid in the structure of a ribosome protein, which will lead to reduced affinity of the 30S subunit with aminoglycosides;
- mutations that affect the passive diffusion at the level of porines, which leads to a reduction in accumulation of aminoglycosides in the bacterium.
- impermeability, efflux, enzymatic inactivation, respiratory mutants, a combination of these mechanisms.

The resistance to aminoglycosides through impermeability is common for this species. However, numerous possible combinations of enzymes have been described.

Considering the risk of selecting resistant strains for *Pseudomonas aeruginosa*, **it is recommended to use aminoglycosides in combination therapy rather than in monotherapy**; Aminoglycosides are synergistically combined with beta-lactam antibiotics, glycopeptides or with fluoroquinolones.

The wild phenotype is sensitive to all the aminoglycosides. The following table displays the most common phenotypes.

Table 1

PHENOTYPES OF RESISTANCE TO AMINOGLYCOSIDES IN *PSEUDOMONAS AERUGINOSA* STRAINS

Phenotype	Enzyme	G	T	Nt	A	I
G	AAC (3)-I	R	S	S	S	S
GNt	AAC (3)	R	S	R	S	S
GT	ANT (2") -I	R	R	S	S	S
GNtT	AAC (6')-II	R	R	R	S	S
	AAC (3)-II,IV,V					
TntA	AAC (6')-I	S	R	R	R	?
GTNtA	AAC (6')+ANT(2")-I	R	R	R	R	?
EFLUX (30-10%)		R	S	R	R	R

G – gentamicin
T – tobramycin
Nt – netilmicin
A – amikacin
I – isepamicin

MATERIAL AND METHOD

In the study were analyzed the same strains of *Pseudomonas aeruginosa*, isolated and identified during 2013 – 2014, strains that were tested in order to characterize phenotypes of resistance to β - lactams. The identification of bacteria was performed based on the microscopic, culture-related and biochemical properties.

The batch analyzed included **61 strains of *Pseudomonas aeruginosa* with a resistance profile**.

The susceptibility to antibiotics of the bacterial strains included in the study was tested by using the diffusimetric method. The results were interpreted based on the CLSI – 2014 rules.

Antibiotic susceptibility testing

The susceptibility to antibiotics of the bacterial strains included in the study was tested by using the diffusimetric method. The results were interpreted based on the CLSI (Clinical Laboratory and Standard Institute) rules.

RESULTS AND DISCUSSIONS

The wild-type strains of *Ps. aeruginosa* wild-type strains are sensitive to all the aminoglycosides. The acquired resistance is due to several mechanisms such as: **impermeability, efflux, enzymatic inactivation, respiratory mutants or a combination of these mechanisms**. The most common resistance is the one through impermeability.

As a result of the tests conducted, according to the literature, out of the batch of 61 strains that were isolated and identified as having a resistance profile, the results were the following:

- 15 strains from the batch taken from the Cardiology Hospital presents the wild-type phenotype in aminoglycosides, these strains are sensitive to all the aminoglycosides tested ***G – gentamicin, T – tobramycin, Nt – netilmicin, A – amikacin, I – isepamicin***.

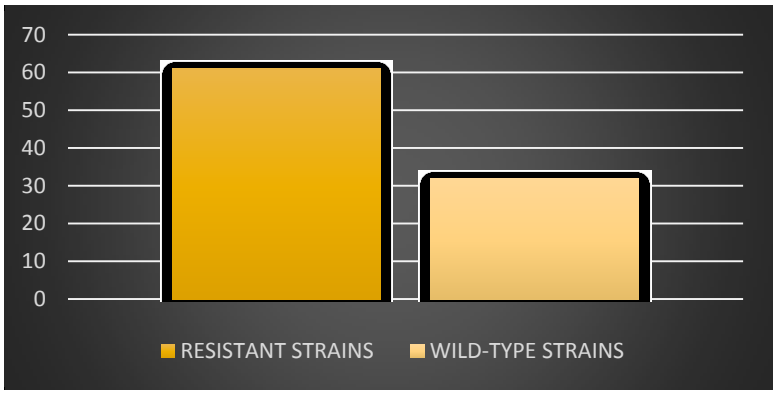

Fig. 1. Graphical representation of the strains with a wild-type resistance profile

As a result of tests, the following resistance profiles were determined:

- ***G phenotype: 8 strains (resistant to Gentamicin and the rest of the aminoglycosides tested)***
- ***GT phenotye: 11 strains (resistant to Gentamicin and Tobramycin, sensitive to AK,I,Nt)***
- ***GTNtA phenotype: 6 strains (resistant to all the aminoglycosides tested)***

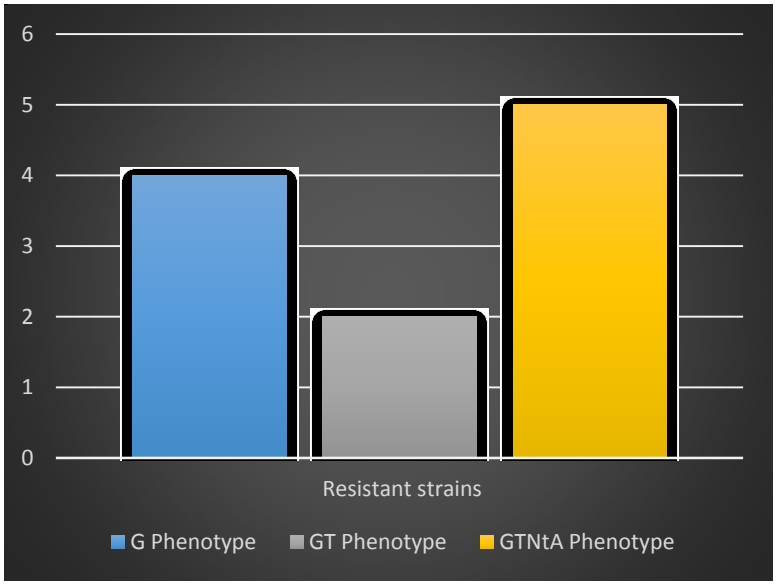

Fig.2. Graphical representation of strains with resistance profiles

CONCLUSIONS

- **In this study, the GT phenotype was most frequently determined (resistant to Gentamicin and Tobramycin, sensitive to AK, I, Nt)**
- this group of antibiotics is particularly useful in treating infections caused by *Pseudomonas aeruginosa*;
- their effectiveness is increased to the maximum, especially in combination with beta-lactam antibiotics, that is why they are never administered in monotherapy;

- The most frequent use of aminoglycosides is the empiric therapy for serious infections such as septicemia, complicated intra-abdominal infections, complicated urinary tract infections, and nosocomial respiratory tract infections. Usually, once the cultures of the causal organism are grown and their susceptibilities tested, aminoglycosides are discontinued in favor of less toxic antibiotics.

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RESEARCH ON THE NEED FOR USE DECONTAMINATION OF POULTRY CARCASSES

CARMEN CREȚU*, CRISTINA HORHOGEA*, CRISTINA RÎMBU* IONEL BONDOC*
*Facultatea de Medicină Veterinară Iași
carmenccretu@yahoo.es

Abstract: *Microbiological risk assessment can be considered as a tool that can be used in the management of the risks posed by foodborne pathogens and in the elaboration of standards for food in international trade. The researches found that there were no quantitative data available on the effects of specific interventions applied during live animal production on the prevalence and/or level of contamination with Salmonella spp., Campylobacter spp., Staphylococcus aureus, Yersinia enterocolitica, Listeria monocytogenes.*

Most processing procedures are similar in the different regions, but there are differences in processing practices, since the product wanted by the consumers can vary a lot between the different regions. The decontamination of poultry carcasses can help to reduce human foodborne infections.

The results obtained from the carcasses of poultry we reflect a contamination at the parameters examined : Escherichia coli a percentage of 34% for Staphylococcus aureus 14%, a rate of 12% for Campylobacter jejuni. The presence of Listeriei monocytogenes from carcasses examined showed a percentage of 4%, which indicates a major contaminatio during processing. Salmonella species was identified in 2% of the surface of carcases examined.

Keywords*: Poultry carcasses, decontamination, microflora patogens*

INTRODUCTION

The frequency of pathogenic bacterial species present on poultry carcasses varies from one country to another, from one farm to another. The comments gathered so far shows that poultry slaughter in slaughterhouses, where slaughter process runs flawless hygienic conditions, however contamination with pathogenic microflora can not be avoided, but only limited. The European Food Safety Authority (EFSA) gave a favorable opinion use solution in based on peroxyacetic acid (PAA) for the treatment of carcasses of poultry meat to reduce contamination with pathogens, considering that there is no risk of toxicity for consumers or environment.

MATERIAL AND METHODS

The research was conducted in a poultry slaughtering units, Iasi county. The samples were processed at the Food Microbiology Laboratory of the Faculty of Veterinary Medicine. May visits were carried out at intervals of one month in which were made poultry meat, on which occasion by 10 carcasses were removed at random from the technological line. From selected carcasses were removed under conditions which prevent contamination of carcasses additional one piece of skin from the neck about 25 grams. Skin fragments were collected using sterile scissors, sterile plastic bags (special bags for Stomacher - 305/175 mm) and kept on ice harvesting to the laboratory. Samples were processed for examination within 5 hours of collection.

To obtain the serial dilutions were respected SR EN ISO 6887-1.-1996. Bacteriological evaluation of the surface of poultry carcasses was done 78323/1998 and 74032/1999 according ANSVSA Program.

Identification of *Escherichia coli* bacteria - was made according to STAS ISO 4832/1992. Microbiological assessment of carcasses was done according to Agency programs National Sanitary Veterinary and Food Safety, as standard SREN ISO 6579 / AC / 2006, which states that identification of microorganisms of the genus *Salmonella spp*. On carcasses of birds will make samples of 25 grams the skin of the neck. According to this standard, shall *Salmonella spp.* is absent in samples of 25 grams. The evaluation was conducted in accordance carcasses ANSVSA programs: SR ISO 10272/2007, on the surface of carcasses. Confirmation of *Campylobacter* species was done by tests: examining the morphology and mobility, study growth at 25 ° C (microaerob) and 41.5 ° C (aerobic) oxidase detection.

Coagulase-positive staphylococci determination was made after SR ISO 6888/1/2/2002. Isolation was done on the environment Baird - Parker where colonies of *Staphylococcus aureus* are black with a halo. Confirmation testing was performed using Api Staph. To determine the presence and number of *Listeria spp*. Was used method SR ISO 11 290/2000, *Listeria monocytogenes* confirmation was made with tests: the test of hemolysis; use carbohydrates; CAMP test.

Yersinia enterocolitica determination was made according to ISO 10273/2003 SR. According to this standard enrichment broth made PSB (peptone, sorbitol and bile salts) and CIN agar isolation. Peroxyacetic acid has been used for treatment of poultry carcasses, it was originally a concentration of 5%, then did dilution to a concentration of 0.01%. Peroxyacetic acid degradation products are non-toxic and can easily dissolve in water. Peroxyacetic acid is a colorless liquid with a slightly pungent odor and a strong oxidant. No risk of human and environmental toxicity. Determination of pH 0.01% peroxyacetic acid solution indicated a value of 2.8. Sampling was carried out on 25g skin of the neck, then for each bacterial species proposed to apply for isolating and identifying the steps according to SR. ISO. Peroxyacetic acid treatment was carried out by immersion in a sterile bag with a solution of a concentration of 0.01% for 10 seconds. I applied a rinse with potable water in another sterile bag for 20 seconds, after which I made again the isolation and identification of pathogenic bacteria to the appropriate standard.

RESULTS AND DISCUSSIONS

Although meat is kept under natural conditions, temperature and humidity, with the biochemical processes that generate better organoleptic properties and development occurs which modifies microorganisms, both aerobic and anaerobic processes, properties of meat. Pathogenic bacteria capable of causing food poisoning in humans can be found on / in poultry. Of these the most important are *Salmonella spp., Campylobacter spp., Listeria monocytogenes* and strains of enterohaemorrhagic *Escherichia coli* or entericpathogens. Other bacterial species *Yersinia enterocolitica* such as *Staphylococcus aureus*, known for their pathogenicity.

Tests for *Escherichia coli* in poultry carcasses seeks to detect the presence of generic *E. coli* colonies (biotip1) on the surface of carcasses. During the 5 visit to the slaughterhouse species *Escherichia coli* was isolated from most of carcases inspected both housings with an incidence of 34% (Table 1).

Salmonella spp. inhabit the intestinal tract of a wide range of animals, birds including species of animals providing meat. The incidence varies depending on the technology of growing, processing hygiene in meat slaughter and subsequent handling.

The main source of *Salmonella* contamination of poultry meat is the animal itself, the incidence of bacteria on / in poultry meat closely linked to the incidence in poultry flocks destined for slaughter. The investigations carried out on the surface of poultry carcasses found a rate of 2%. The presence of bacteria of the genus *Staphylococcus* surfaces carcasses was evident, the most frequently isolated *S. aureus* in terms of food safety, important as it may produce enterotoxin. The origin of strains "endemic" *Staphylococcus* is not fully elucidated but it is thought that most of the strains coming from poultry skin brought to slaughter. Part of poultry carcasses contaminated with *Staphylococcus aureus*, which is an indicator of contamination on the hands of the technological and personnel. Poultry carcasses examined showed a 14% contamination with this species, which indicates a high percentage.

Campylobacter infection was the most common food-borne bacterial diagnosed in humans, which is why reducing the incidence and level of contamination of turkey meat with *Campylobacter* is a priority for the authorities involved in protecting the health of consumers worldwide. Since *Campylobacter spp*. is sensitive to high temperature heat treatment and prevention of contamination of the meat after the heat treatment, the main method to reduce the risk of poisoning with this bacterium. All high percentage found *Campylobacter jejuni* 12% and *Yersinia enterocolitica* 8%.

L. monocytogenes can grow at low temperatures and therefore has a tendency to colonize surfaces in the machining gap, not only during processing at the slaughterhouse by the housing and often during subsequent processing of the carcass (cutting, packaging , etc.). The poultry carcasses examined we identified 4%, contaminated carcasses.

Evaluation of the antimicrobial effect of 0.01% peroxyacetic acid (PAA) on poultry carcasses revealed the following results: significant reduction in the species *Escherichia coli* percentages found in only 6%, 4% *Stapylococcus aureus* and *Campylobacter jejuni* 2% (Table 2). Peroxyacetic acid has antimicrobial effect *Yersinia enterocolitica* cid total species, *Listeria monocytogenes* and *Salmonella spp* been assessed on poultry carcasses was not treated any bacteria identified in these genres.

CONCLUSION

1. In poultry slaughterhouses processed a large number of animals (thousand birds / hour) with the same facilities, carcases are very close, cross-contamination being more frequent.
2. From the results it is observed that the poultry carcasses aerobic conditioning predominant pathogenic bacterial flora represented by *Escherichia coli, Stapylococcus aureus, Campylobacter spp., Listeria monocytogenes, Salmonella spp., Yersinia enterocolitica*
3. European Food Safety Authority (EFSA) gave a favorable opinion-based solution in the use of peroxyacetic acid (PAA) for the treatment of poultry carcasses and meat to reduce contamination with pathogens, considering that there is no risk of toxicity on consumers or the environment
4. To reduce pathogenic microflora most efficient technological flow would be necessary after the introduction of a first stage of gutting peroxyacetic acid treatment and then step before cooling to carry out a second treatment by immersion in pools of cooling carcasses.

Table 1.

The incidence of pathogenic bacterial species isolated from poultry carcasses

Nr. visit	*Escherichia coli*	*Staphylococcus aureus*	*Campylobacter jejuni*	*Yersinia enterocolitica*	*Listeria monocytogenes*	*Salmonella spp.*
1.	6/10	2/10	1/10	1/10	1/5	0/5
2.	4/10	1/10	1/10	1/10	0/5	1/5
3.	2/10	1/10	2/10	0/10	0/5	0/5
4.	3/10	1/10	1/10	1/10	1/5	0/5
5.	2/10	2/10	1/10	1/10	0/5	0/5
TOTAL	17/50 (34%)	7/50 (14%)	6/50 (12%)	4/50 (8%)	2/50 (4%)	1/50 (2%)

Table2.

The incidence of pathogenic bacterial species isolated from poultry carcasses after treatment with 0.01% peroxyacetic acid

Nr. visit	*Escherichia coli*	*Staphylococcus aureus*	*Campylobacter jejuni*	*Yersinia enterocolitica*	*Listeria monocytogenes*	*Salmonella spp.*
1.	2/10	1/10	0/10	0/10	0/10	0/10
2.	1/10	0/10	0/10	0/10	0/10	0/10
3.	0/10	0/10	1/10	0/10	0/10	0/10
4.	0/10	1/10	0/10	0/10	0/10	0/10
5.	0/10	0/10	0/10	0/10	0/10	0/10
TOTAL	5/50 (6%)	2/50 (4%)	2/50 (2%)	0/50 (0%)	0/50 (0%)	0/50 (0%)

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VETERINARY MEDICINES: QUALITY, SAFETY AND EFFICACY

ALINA DRAGHICI, SIMONA STURZU, DANIELA TIRSINOAGA, MARIANA DUMITRACHE, IOANA TIHULCA, CRISTINA POPICA
Institute for control of veterinary biological products and medicines, Bucharest, Romania
draghici.alina@icbmv.ro

Abstract: *Just few know that behind the success of a doctor, there is a team of other doctors and specialists. There are people who have no direct contact with the patients or consumers and so they do not know about their existence or their work.*
For example medicines are components of a complex treatment scheme for various diseases. Behind every product there are thousands of hours of work and research for the development of the product, for assessing the product in order to grant the marketing authorisation and to maintain product quality over time.
This paper aim is to produce a short presentation of a few steps that must go through veterinary medicinal product after they have been produced in order to reach to the consumers and to be indicated as a good quality, efficacy and safety product.

Keywords: veterinary medicines, quality, safety, efficacy.

INTRODUCTION

Making treatment often involves the use of medicinal products (allopathic or homeopathic). Treating physician's success is actually the joint success of a team of doctors, pharmacists, chemists, biochemists, with whom the patient never makes contact with.

Few patients know that behind the product lying on the pharmacy shelves stands the work of a whole team of several specialists. Few are questioning how these products get in the pharmacies, how and who makes them, who allows them to reach to the consumer and whether these products are checked after being "manufactured".

Scope of the workpaper

This paper aim is to take us on a short journey into the medicines world, in the gate area between producer and consumer of veterinary medicinal products.

Marketing authorisation of a veterinary medicinal product

Production after thousands of hours of study, research and physical work of a veterinary medicinal product does no mean that the product is automatically "sold". The product should be assessed and examined from the point of view of quality, safety and efficacy. To this end the company producing the product is preparing the technical documentation which must be prepared in accordance with European standards and guidelines. Then this documentation is submitted with an application form at the Institute for control of veterinary biological products and medicines from Bucharest. The product is now entering into a complex verification process in which are involved veterinarians, chemists, biochemists.

The assessment can last from several months to several years. It is different depending on the product type (biological or medicine), depending on the type of

authorisation procedure (national or community), depending on the type of application, on the active substance etc. For all the above situations there are assessment guidelines developed by working groups of the European Medicines Agency (EMA).

What does comprise this technical documentation? As veterinarians we may be tempted to think that it is enough that a medicines has a good efficacy in order to be authorized. Efficacy is one of the conditions the product must fulfill, but it is not enough.

In fact the technical documentation for a veterinary pharmaceutical product includes 4 parts:

- Part I - here you can find summary information about the product, about the producer of active substances and finished products, who is the one who made the request and authorisation of the product, if any, who is the importer. Here we can find the resume of the product characteristics (RPC) and, also, the package leaflet. An important part of the file included in this section are the reports from the experts who, after analyzing the studies provided by the manufacturer, recommends the product for use.

- Part II - the data related to the analytical product; the documentation is usually checked by chemists or biochemists. Data supplied by the manufacturer in this part must prove the quality of that product (in terms of physico-chemical, biological, microbiological): check product composition (both active substances and excipients, the action of each component in part, constituents that cover the product), packaging, method of preparation and product validation, control of raw materials are checked, tests on intermediate products, finished product, stability tests, it is also checked whether the substances used in the product may be involved in the transmission of spongiform encephalopathies.

- Part III - is the folder designed to check the safety of the product. It is assess both by the veterinarians and by the chemists. Product safety issues are particularly important, as the European community is putting great emphasis on them. The documetation addresses aspects related to products' toxicity, embryotoxicity, fetotoxicity, teratogenicity, mutagenicity, carcinogenicity, immunotoxicity, metabolic disorders etc., for the safety of the medical staff but also for the the people who come into contact with the animal after the treatment is done. An important part of the evaluation of technical documentation is the checking of residues documentation. The issue of residues has gained great importance in recent years through the emergence of antimicrobial rezistance. Also in Part III of the technical documentation there is a section which must include data on the effects of the medicinal product on the environment when it is used in animals,

- Part IV is assessed by veterinarians and should include data about the efficacy. After assessment of this part of the technical documentation, the assessor should be fully cleared and convinced that the product is correct regarding the indications and dosage for the species indicated. For this, the documentation includes the results of the studies conducted by the producer over several years, both in vitro and in vivo. The types of studies are very varied, depending on the species for which the product is indicated, on the the active ingredient of the product, on the pharmaceutical form, on the directions etc.

Evaluation of the technical documentation leads to a report of evaluation at which all the involved persons contribute and ends with the conclusions of the assessors.

The report thus issued will be submitted to the Commission for authorisation of veterinary medicinal products comprised of members from the Institute for control of veterinary biological products and medicines and members of the National Sanitary

Veterinary and Food Safety Authority (ANSVSA), which may or may not agree to issue marketing authorisation.

Renewal of the marketing authorisation

At a certain time (usually five years) the authorized product is reviewed regarding the renewal of the marketing authorisation. At this stage the pharmacovigilance report has an important role.

Renewal of authorisations is done whether the benefit/risk ratio of the product, meaning if the ratio of the positive therapeutic effects of the product and the risks involved in using it, is positive. If these conditions are met medicinal product receives authorisation indefinitely. That does not mean that the product will not subsequently be monitored and controlled.

Post authorisation control of the veterinary medicinal products

Quality control of veterinary medicinal products is made based on the Annual plan sampling and testing of veterinary medicinal products marketed in Romania, prepared by the Institute for control of veterinary biological products and medicines and aims to improve animals' health and the quality of veterinary medicinal products. The Plan for sampling and testing of veterinary medicinal products is part of the actions of surveillance, prevention, control and eradication of disease in the animals, those transmitted from animals to humans, animal protection and environmental protection, identification and registration of cattle, pigs , sheep, goats and equine.

The selection of veterinary medicinal products to be tested and drawing of the Annual Plan for sampling and testing of veterinary medicinal products marketed in Romania is a complex process that is based on risk analysis so that all veterinary medicinal products to be tested several times during their lifecycle, at intervals not exceeding 5 years, depending on their score on risk analysis.

Institute for control of veterinary biological products and medicines is designated by the central authority - National Sanitary Veterinary and Food Safety Authority (ANSVSA)- as the only official laboratory for performing quality control of veterinary medicinal products before and after their approval.

In the process of quality control for medicinal products there are two laboratories involved: microbiology laboratory (where are performed: sterility of products, determination of the degree of contamination of the products, determination of the active substance content of products, LAL test, etc), and physico-chemically control laboratory (where they make determinations for active substance content and other physico-chemical analyzes).

In case the analysis performed on a veterinary medicinal product does not meet the conditions to be defined as a quality, safe and effective, then the competent Authority - National Sanitary Veterinary and Food Safety Authority (ANSVSA)- is notified in order to take the necessary actions, according to the legal provisions.

CONCLUSIONS

Marketing authorisation of a medicinal product is a complex process, which has to respect well-established rules. Even so, assessor meets freqvent unprecedent situations and has to handle them, to take some decision. So their job is very demanding.

This brief overview wishes to make known the activity of the specialists from the Institute for control of veterinary biological products and medicines and their effort to have on the Romanian veterinary medicines market good quality products, with high safety and good efficacy.

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A SEROLOGICAL SURVEY OF RESPIRATORY BACTERIAL PATOGENS IN TURKEYS FARMED IN BRASOV COUNTY

VLAD-MIHAI DUMA, OANA-IRINA TĂNASE, FLORENTINA DARABAN, ELENA VELESCU
Facultatea de Medicină Veterinară, Universitatea de Științe Agricole și Medicină Veterinară Ion Ionescu de la Brad, din Iași
elenavelescu@yahoo.com

Abstract: *Mycoplasma gallisepticum and Ornithobacterium rhinotracheale can cause respiratory diseases or result in a silent infection turkeys, that may lead to important economical losses. The breeding of turkeys in Romania is not well developed, therefore only a few studies have been conducted in order to show the importance of these two pathogens in turkey flocks. The aim of the present study was consisting in the de analysis of the prevalence of Mycoplasma gallisepticum (MG) and Ornithobacterium rhinotracheale (ORT) antibodies in commercial turkey flocks from Brasov County estimated by using of ELISA assay. For this study, blood samples were collected from 4 Farms at different ages: Farm nr.1 at day 139 respectively day 152, Farm nr. 5 at day 0 and day 100; Farm nr. 6, at day 100; from Romad Farm at day 125 and 131 respectively. The presence of the pathogens was previously confirmed by PCR. In Farm 5, at day 0 no specific antibodies for MG were identified, instead for ORT the average titer was 5551. At day 100, specific antibodies for both MG and OR were identified with an average titer of 4625 for MG and 7641 for ORT. In Farm 6, at day 100 no antibodies were identified for MG nor ORT. Further, in Farm 1, at day 139 the average titer was 5130 for MG and 7698 for ORT; at day 152 the average titer for MG was 5626, while for ORT was 7857. For Ferma Romad, at day 125 the average titer was 101,while at day 131 was 67 for MG; for ORT, the average at day 125 was 5511 and 3493 at day 131. The absence of the specific antibodies anti-MG at day 0 suggest that turkey are free of these specific pathogens, and lately, according to the antibody titration, the occurrence of pathogens is supported by the different change in the breeding technology. Moreover, in the light of these results, there is the need of the implementation of a vaccination schedule according to the epidemiological situation founded in the turkey flock.*

Keywords: serological assay, mycoplasmosis, respiratory disease.

INTRODUCTION

The breeding of turkeys in Romania is not well developed, therefore only a few studies have been conducted in order to show the importance of the bacterial pathogens in turkey flocks.

Mycoplasma gallisepticum and Ornithobacterium rhinotracheale can cause respiratory diseases or result in a silent infection turkeys, that may lead to important economical loses (Back et al, 1998).

The most important mycoplasmas isolated from domestic avian species include Mycoplasma gallisepticum (MG), M. synoviae (MS), M. meleagridis (MM) and M. iowae (MI). MG causes chronic respiratory disease of chickens and infectious sinusitis in turkeys, resulting in economic losses. MS causes infectious synovitis or mild upper respiratory disease. MM infects only turkeys, causing airsacculitis and sub-optimal production and hatchability. MI is associated with reduced hatchability in turkey flocks. Transmission is either direct, from bird to bird or through the egg, or indirect (Fritz et al., 1992, Kleven, 1998).

Mycoplasma gallisepticum is the most economically significant mycoplasma pathogen of turkeys and poultry, and has a world-wide distribution. In common with other

mycoplasmas, M. gallisepticum is minute in size with minimal genetic information and with a total lack of a bacterial cell wall. Infection with M. gallisepticum has a wide variety of clinical manifestations, but even in the absence of overt clinical signs, the economic impact may be significant. The most dramatic disease presentation of M. gallisepticum is chronic respiratory disease in meat-type birds, often as one of several aetiological agents in a multi-factorial disease complex (Levisohn and Kleven, 2000).

Ornithobacterium rhinotracheale (ORT) is a bacterium that has been associated with respiratory disease in both chickens and turkeys. This bacteria is able to cause potentially severe disease in turkeys characterized by respiratory distress, facial edema and swelling of infraorbital sinuses. The most striking lesion is unilateral or bilateral fibrinopurulent pneumonia (Glisson, 1998). Some researchers believe that the different clinical outcome of ORT infections may be attributed to a variation in virulence (Travers et al., 1996). However, very little is known about the pathogenesis of ORT infections in turkeys and the virulence determinants of this agent (Canal et al., 2005). ORT can be a primary or secondary etiological agent depending on strain virulence, adverse environmental factors, immune state of the flock, and presence of other infectious agents (Van Empel and Hafez, 1999).

Therefore, respiratory disease in turkeys is a multifactorial problem, with viral and bacterial respiratory pathogens often concurrently present and most probably influencing one to each other.

Diagnosis is based on isolation and identification of mycoplasmas and ornithobacterium, according to biochemical, serological or molecular biology tests, or serological examination of host sera by slide agglutination, haemagglutination inhibition or enzyme-linked immunosorbent assay (ELISA) tests.

The aim of the present study was consisting in the de analysis of the prevalence of Mycoplasma gallisepticum (MG) and Ornithobacterium rhinotracheale (ORT) antibodies in commercial turkey flocks from Brasov County estimated by using of ELISA assay.

MATERIAL AND METHODS

In turkeys reared in intensively farms located in Brasov County, the occurrence of respiratory manifestation followed by losses by mortality was registered. The farms were represented by Ferma nr.1, Ferma nr. 5, Ferma nr.6 and Ferma Romad. The farm were populated with 1 day old baby turkeys BUT 6 hybrids.For this study, blood samples were collected from 4 Farms at different ages: Farm nr.1 at day 139 n=10, respectively day 152 n=10, Farm nr. 5 at day 0 n=10 and day 100 n=20; Farm nr. 6, at day 100 n=20; from Romad Farm at day 125 n=10 and 131 n=10 serum samples. In order to determine the antibody titer against MG and ORT, the serum samples were delivered to the Pasteur Institute, Bucharest, where they were analyzed by indirect ELISA assay.

RESULTS AND DISCUSSIONS

The farms Ferma nr. 1, Ferma nr. 5, Ferma nr. 6 and Ferma Romad were located in Brasov County (figure 1). Each farm was composed of several halls.

Figure 1. The farms nr. 1 and nr. 5 (left); the farm nr. 6 (right)

Respiratory signs were observed in turkeys from all the tested farms. The clinical signs were consisting in nasal discharge, infraorbital sinusitis, head edemas, respiratory rales, lameness and prostration; often, the birds were found dead.

The indirect ELISA assay for MG antibodies revealed the presence of the specific antibodies in the tested serum samples. The results of ELISA assay are given in Table 1.

Table 1

The results of ELISA assay for MG antibodies

Titer	*Mycoplasma gallisepticum* - ELISA						
	Ferma nr.5		Ferma nr.6	Ferma nr.1		Ferma Romad	
	0 days	100 days	100 days	139 days	152 days	125 days	131days
0	10	0	20	0	0	10	10
1	0	0	0	0	0	0	0
+2	0	20	0	10	10	0	0
Maximum titer	109	6648	427	6097	6058	200	116
Minimum titer	6	1988	58	837	4510	15	30
Average titer	32	4625	155	5130	5629	101	67
Results	negative	positive	negative	positive	positive	negative	negative

In Farm 5, at day 0 no specific antibodies for MG were identified, while the farm was considered free of MG. However, specific antibodies for MG were identified at day 100 with an average titer of 4625 and the epidemiological status of the farm was changed as positive-contaminated. With respect to Farm nr. 6, no specific antibodies were revealed, since the average titer was 155, and the farm was considered free of MG. Instead, in Farm nr.1, at day 139 the average titer was 5130 and 5629 at day 152; the farm was considered positive for MG. In Ferma Romad, at day 125 the average titer was 101, while at day 131 was 67; since no specific antibodies were revealed, this farm was considered as free of MG.

The indirect ELISA assay for ORT antibodies revealed the presence of the specific antibodies in the tested serum samples. The results of ELISA assay are given in Table 2.

Table 2

The results of ELISA assay for ORT antibodies

Titer	*Ornithobacterium rhinotracheale*- ELISA						
	Ferma nr.5		Ferma nr.6	Ferma nr.1		Ferma Romad	
	0 days	100 days	100 days	139 days	152 days	125 days	131days
0	0	0	20	0	0	0	2
1	0	0	0	0	0	0	0
+2	10	20	0	10	10	10	8
Maximum titer	7593	8727	185	8499	8301	8087	8961
Minimum titer	3445	6681	14	7177	7405	3154	271
Average titer	5551	7641	61	7698	7857	5511	3493
Results	positive	positive	negative	positive	positive	positive	positive

In Farm 5, at day 0 specific antibodies for ORT were identified with an average titer of 5552, while the farm was considered positive for ORT. Specific antibodies for ORT were identified at day 100 with an average titer of 7641 and the epidemiological status of the farm was positive-contaminated. With respect to Farm nr. 6, no specific antibodies were revealed, since the average titer was 155, and the farm was considered as free for both MG and ORT. Instead, in Farm nr.1, at day 139 the average titer was 7698 and 7857 at day 152; the farm was considered positive for ORT. In Ferma Romad, at day 125 the average titer was 5511, while at day 131 was 3493; since no specific antibodies were revealed for MG, this farm was considered as free of MG and positive for ORT, therefore, in the occurrence of the respiratory disease the ORT pathogen may be incriminated.

Moreover, the presence of the specific antibodies against ORT in the first day of baby turkey suggests that the halls population was performed with contaminated birds, while the contamination with MG as revealed by serology at day 100, was probably caused by deficiencies during growing process.

The absence of the specific antibodies anti-MG at day 0 suggest that turkey were free of these specific pathogens, and lately, according to the antibody titration, the occurrence of pathogens is supported by the different change in the breeding technology. Moreover, in the light of these results, there is the need of the implementation of a vaccination schedule according to the epidemiological situation founded in the turkey flock.

The eradication of mycoplasma and ornithobacterium infection can be achieved through improvements in hygiene and management practices, therapeutic treatment of breeder layers and/or of hatching eggs and better monitoring procedures.

CONCLUSIONS

The presence of specific antibodies in the aforementioned farms may lead to some important changes in the prevention schemes previously used, while vaccination against ORT should be considered. Given the antigenic variability of ORF, it should be taken into account that in the future an auto vaccine should be produced with isolates from outbreaks of disease.

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RESEARCH REGARDING THE IDENTIFICATION OF PATHOGENS INVOLVED IN THE AETIOLOGY OF OF RESPIRATORY INFECTIONS IN INTENSIVELY REARED TURKEYS

VLAD-MIHAI DUMA, OANA-IRINA TĂNASE, FLORENTINA DARABAN, ELENA VELESCU
Facultatea de Medicină Veterinară,
Universitatea de Științe Agricole și Medicină Veterinară Ion Ionescu de la Brad, din Iași
elenavelescu@yahoo.com

Abstract: *Respiratory diseases cause significant economic losses in intensively reared turkeys mainly by the failure to gain weight, by the increasing of the mortality percentage, but also by the slaughter confiscations. Among bacterial and viral diseases with respiratory tropism, significant importance is attributed to infectious turkey rhinotracheitis (TRT), to respiratory mycoplasmosis and to ornithobacterium infection. During 2010-2013, in turkeys from farms placed in Brasov and Mures counties was recorded the occurrence of respiratory manifestations followed by losses by mortality. In order to identify the agents involved in the etiology of these infections, samples represented by tracheal swabs were collected from turkeys of different ages, as follows: at 23 days from the Farm Dealul Frumos; at 46, 86 and 118 days from the Farm No. 1; at 103 days and 117 days from Farm no. 2; at 96 days and 103 at Farm No. 5. The samples were analyzed by PCR assay in Anicon GmbH and LVL GmbH laboratories, Germany. The samples were constituted in pools and tested for TRT, MG (Mycoplasma gallisepticum) and ORT (Ornithobacterium rhinotracheale) and RA (Riemerella anatipestifer) antigens. In the case of 23 days turkeys from Farm Dealul Frumos, the occurrence of TRT was suspected and subsequently A and B TRT types were identified; for MG and ORT antigens the samples were negative. The samples from the 46 days turkeys from farm No. 1 were negative for all three antigens, instead, at 86 days were positive for MG and ORT; additionally, at 118 days the Riemerella anatipestifer antigen was identified. For Farm no. 2, at age of 103 days, the MG, ORT and Riemerella anatipestifer antigens were identified. At 117 days, the TRT type B was identified together with MG antigen. Regarding the Farm nr. 5, at 96 and 103 days are identified both MG and ORT antigens. Moreover, the ORT antigen typing revealed the presence of A, B and E serotypes. Following the laboratory findings, it might be suggested that the respiratory symptoms observed in turkeys are induced by a simultaneous intervention of viral agents and secondary bacterial agents. Under these conditions it is necessary the correction of the technological parameters, with a specific immunization schedule.*

Key words*: respiratory diseases, mycoplasmosis, ornithobacterium infection.*

INTRODUCTION

Viral and bacterial infections of the respiratory tract often result in disease in intensively reared turkeys of all ages and may lead to considerable financial losses due to reduced growth, an increased mortality rate, high medication costs and a higher number of condemnations at processing (Marien et al., 2005). Several pathogens play an important role in respiratory disease, alone, in synergy or triggered by noninfectious factors. Among bacterial and viral diseases with respiratory tropism, significant importance is attributed to infectious turkey rhinotracheitis (TRT, avian metapneumovirus), to Escherichia coli, Pasteurella spp., Bordetella avium, Ornithobacterium rhinotracheale (ORT), Mycoplasma spp., Chlamydophila psittaci and Riemerella anatipestifer (Marien et al., 2005). Environmental factors may augment these pathogens to produce the clinically observed signs and lesions (Glisson, 1998). Ornithobacterium rhinotracheale (ORT) is a bacterium that has

been associated with respiratory disease in both chickens and turkeys. This bacteria is able to cause potentially severe disease in turkeys characterized by respiratory distress, facial edema and swelling of infraorbital sinuses. The most striking lesion is unilateral or bilateral fibrinopurulent pneumonia (Glisson, 1998). Some researchers believe that the different clinical outcome of ORT infections may be attributed to a variation in virulence (Travers et al., 1996). However, very little is known about the pathogenesis of ORT infections in turkeys and the virulence determinants of this agent.

Avian pneumovirus (APV) is a respiratory disease agent of turkeys and chickens. A disease called turkey rhinotracheitis (TRT) was first described in South Africa in the late 1970s and is now present in many European countries (Buys and Du Preez, 1980, Naylor and Jones, 1993, Jones, 1996). TRT is caused by APV types A or B of the genus Metapneumovirus, which is distinguished from respiratory syncytial virus of humans, the type species for the genus Pneumovirus (Naylor et al., 1998). Clinical signs of the TRT disease consisted of coughing, nasal and ocular discharge, and swelling of the infraorbital sinuses. Infected flocks had high morbidity (50–100%) at all ages. Mortality rates up to 30% occurred in flocks with concurrent bacterial infections (Senne et al., 1998). Some flocks had high rates (up to 11%) of condemnation at slaughter because of airsacculitis (Jirjis et al., 2002). Mycoplasma gallisepticum is the most economically significant mycoplasma pathogen of turkeys and poultry, and has a world-wide distribution. In common with other mycoplasmas, M. gallisepticum is minute in size with minimal genetic information and with a total lack of a bacterial cell wall. Infection with M. gallisepticum has a wide variety of clinical manifestations, but even in the absence of overt clinical signs, the economic impact may be significant. The most dramatic disease presentation of M. gallisepticum is chronic respiratory disease in meat-type birds, often as one of several aetiological agents in a multi-factorial disease complex (Levisohn and Kleven, 2000).

Therefore, respiratory disease in turkeys is a multifactorial problem, with viral and bacterial respiratory pathogens often concurrently present and most probably influencing one to each other. The aim of this study was the identification of pathogens involved in the etiology of respiratory infections in intensively reared turkeys by molecular biology technique PCR.

MATERIAL AND METHODS

In turkeys reared in intensively farms located in Brasov and Mures Counties, the occurrence of respiratory manifestation followed by losses by mortality was registered. The farms were represented by Ferma Dealul Frumos, Ferma nr.1, Ferma nr. 2 and Ferma nr.5. The farm were populated with 1 day old baby turkeys BUT 6 hybrids and over their growth, tracheal swab samples were randomly collected. Around 23 days old, the occurrence of a high mortality was observed in Ferma Dealul Frumos, and subsequently n=30 tracheal swabs were collected; later, similar situation was observed in the others farms, since the baby turkeys came from the same source. Consecutively, the samples were collected as given: from Ferma nr. 1 n=10 at 46 days old turkeys, n=20 at 86 days old, and n=10 at 118 days old; from Ferma nr. 2 n=10 at 103 days old and n=10 at 117 days; from Ferma nr. 5 n=10 at 96 days old and n=10 at 103 days old were collected.

The collected samples were analyzed by PCR assay within the LVL GmbH and Anicon GmbH Laboratories, Germany. The samples were constituted in pools and tested for TRT, MG (Mycoplasma gallisepticum), ORT (Ornithobacterium rhinotracheale) and Reimerella anatipestifer antigens.

RESULTS AND DISCUSSIONS

The farms Dealul Frumos, Ferma nr. 1 and Ferma nr. 2 were located in Brasov County, while Ferma nr. 5 was located in Mures County (figure 1). Each farm was composed of several halls.

Figure 1. The farms Dealul Frumos, nr. 1 and nr.2 (left); the farm nr. 5 (right)

Respiratory signs were detected in birds from all the tested farms. The clinical signs were consisting in nasal discharge, infraorbital sinusitis, head edemas, respiratory rales, lameness and prostration (figure 2).

Figure 2. Infraorbital sinusitis (left); general prostration (right)

The clinical signs that were occurring were not possible to be linked to a specific disease, therefore, it was suspected a multi-factorial disease complex. At necropsy, the swelling of infraorbital sinuses with a fibrinous content was noted, with an obvious fibrinous polyserositis (figure 2).

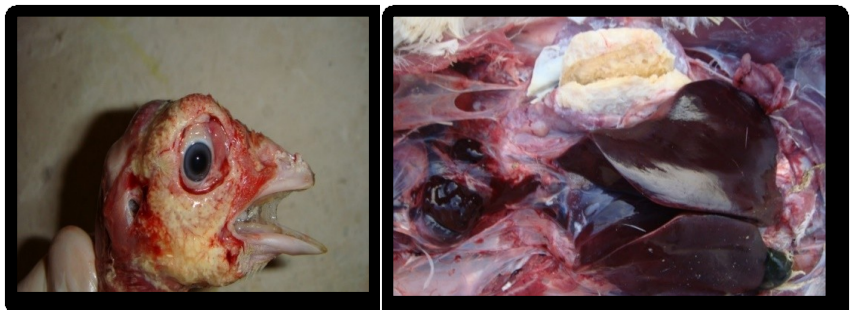

Figure 3. Necropsy showed head subcutaneous fibrinous deposits (left) and fibrinous polyserositis (right)

The PCR assay revealed the presence of the specific pathogens in the tested samples. The results of PCR assay are given in Table 1.

Table 1

The result of PCR assay for TRT, MG, ORT and RA antigens

Farm	Days	Pathogens				
		TRT		MG	ORT	RA
		A	B			
Ferma Dealul Frumos	23 days	+	+	-	-	-
Ferma nr.1	46 days	-	-	-	-	-
	86 days	-	-	+	+	-
	118 days	-	-	+	+	+
Ferma nr. 2	103days	-	-	+	+	+
	117 days	-	+	+	-	-
Ferma nr. 5	96 days	-	-	+	+	-
	103 days	-	-	+	+	-

In the case of 23 days turkeys from Farm Dealul Frumos, the occurrence of TRT was suspected, subsequently A and B TRT types were identified; for MG and ORT antigens the samples were negative. The samples from the 46 days turkeys from farm No. 1 were negative for all three antigens, instead, at 86 days were positive for MG and ORT; additionally, at 118 days the Riemerella anatipestifer antigen was identified. For Farm no. 2, at age of 103 days, the MG, ORT and Riemerella anatipestifer antigens were identified. At 117 days, the TRT type B was identified together with MG antigen. Regarding the Farm nr. 5, at 96 and 103 days are identified both MG and ORT antigens. Moreover, the ORT antigen typing revealed the presence of A, B and E serotypes. Following the laboratory findings, it might be suggested that the respiratory symptoms observed in turkeys are induced by a simultaneous intervention of viral agents and secondary bacterial agents. Under these conditions it is necessary the correction of the technological parameters, with a specific immunization schedule.

CONCLUSIONS

The respiratory diseases cause significant economic losses both by mortality rate and by technological and production failure. With respect to the clinical signs, turkey respiratory diseases are difficult to distinguish, requiring laboratory investigations. The occurrence of

TRT, MG and ORT may be a consequence of several technical errors, such as overcrowding, high temperature and humidity.

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RESEARCH ON THE FREQUENCY OF ONE OF THE INFECTIOUS DISEASES ASSOCIATED WITH RESPIRATORY SYNDROME AND REPRODUCTIVE PIGS

LARION FLUERAȘU, ADRIAN STANCU
Faculty of Veterinary Medicine, Timişoara

Abstract: *PRRS syndrome, is an infectious disease common in intensive rearing of pigs where is producing important economic losses. After 1990, the disease has spread all over the world and in Romania was diagnosed in 1998 by teams led by Dr. STĂNUICĂ and Dr. OLARU (4, 5).*
Laboratory research has shown that the etiological agent is a virus with two genotypes respectively type 1, European, and type 2, American, who have a degree of gene sequence similarity of 50-60% (1,3,6).
In our country the disease has an evolution characteristic for primary outbreaks affecting all categories of pigs, but also an endemic evolution, wich is associated with some bacterial infectious diseases (4,5).
The research was conducted in order to detect some bacterial infectious diseases that evolves in swine, as related diseases with this syndrome, in piglets up to the age of 8 weeks and in youth pigs after 8 weeks of age.

Key words: PRRS, associated disease, laboratory tests

MATERIALS AND METHODS

For this purpose were necropsied and examined anatomopathological and by laboratory tests, a number of 148 bodies from primary outbreaks of PRRS, grouped by age into two groups as follows: group 1, consisting of 119 corpses piglets up to the age of 8 weeks and group 2 consists of 29 youth bodies swine after 8 weeks of age.

The bodies were necropsied as swine necropsy technique and organs were examined for macroscopic lesions characteristic PRRS syndrome and other related diseases.

From organs with lesions were taken samples for the following laboratory tests: histologic, bacteriological and polymerase chain reaction.

Histological examination was carried on samples of lymph nodes and lungs by the classical methodology. The samples were fixed in formalin, embedded in paraffin and stained with hematoxylin-eosin-methylene blue.

Bacteriological examination was carried from lung samples, primary sowings being made in broth and agar with 5% defibrinated sheep blood, and isolated strains were identified on the basis of cultural, tinctorial and biochemical, characterics.

Polymerase chain reaction was performed in order to detect the virus PRRS, the Mycoplasma hyopneumoniae and Brachispira hyodisenteriae. This reaction was performed in the Laboratory of Molecular Biology from Pasteur Institute SN Bucharest.

RESULTS AND DISCUSSION

Necropsy performed on the bodies from the two age categories, has provided conclusive data on the presence of specific lesions for PRRS syndrome and other bacterial infectious diseases associated with the syndrome.

On anatomopathological examination bodies, were found external injuries, represented by weakening, deshydration, congestion of the extremities and enlarged ingvinale lymph nodes.

The results of the anatomopathological examination were processed and given in tables, according to age categories studied.

At the piglets **up to 8 weeks of age**, were found macroscopic lesions characteristic of the syndrome PRRS, in varying proportions (table 1). The catarrhal and haemorrhagic lymphoreticulitis was present in 35.2% of the bodies examined and pulmonary lesions was represented by congestion were at a rate of 28.5% and interstitial pulmonary edema at a rate of 42.85%.

Microscopic lesions were represented by lymphocytic depletion, outbreaks of necrosis, blastic type lymphocytes and small cysts, located in the cortex.

Microscopic lesions characteristic for interstitial pneumonia were represented by thickening of alveolar walls due to infiltration by macrophages, lymphocytes and plasma cells, hyperplasia of type II pneumocytes and by the presence of necrotic cells in pulmonary alveoli.

At autopsied bodies were discovered and macroscopic lesions with lung and pleural localization, characteristic for enzootic pneumonia and pasteurellosis. A relatively high frequency had fibrinous polyserositis, which was present in 26% of autopsied bodies.

At the digestive tract were present hemorrhagic gastritis (26%) and hemorrhagic enterocolitis (59.6%), anatomopathological lesion that is dominant in this category.

Through laboratory tests, who were effectuated, were confirmed next associated disease: enzootic pneumonia, pasteurellosis, Glasser disease and dysentery with *Brachispira hyodisenteriae.*

Table 1

The frequency of pathological lesions in the bodies of piglets up to 8 weeks

Nr. Crt.	Lesion	Nr. Corpses	%
1	Pulmonary congestion	34/119	28,5
2	Interstitial pulmonary edema	51/119	42,85
3	Catarrhal bronchopneumonia	29/119	24,36
4	Fibrinous bronchopneumonia	34/119	28,5
5	Fibrinous hemorrhagic bronchopneumonia	16/119	13,4
6	fibrinous pleuritis	27/119	22,6
7	Pericarditis	12/119	10
8	Lymphoreticulitis	42/119	35,2
9	Fibrinous polyserositis	31/119	26
10	Renal dystrophy	36/119	30,2
11	Haemorrhagic enterocolitis	71/119	59,6
12	Haemorrhagic gastritis	31/119	26
13	Myocardosis	16/119	13,4

In young swine **over 8 weeks of age** necropsy examination revealed gross pathological lesions characteristic of the syndrome PRRS and gross pathological lesions characteristic for other associated bacterial infectious diseases, in varying proportions (table 2).

Catarrhal and haemorrhagic lymphoreticulitis was present in 55.1% of corpses, the most affected being ingvinale lymph nodes. These were increased in volume, and on the section were bleeding or marbled. Histological examination revealed in lymph nodes and lungs, the same microscopic lesions.

Macroscopic lung lesions, characteristic of this syndrome was represented by pulmonary congestion (34.4%) and interstitial pulmonary edema (17.2%).

On lungs, pleura and pericardium were present inflammatory lesion like fibrinous in relatively large proportions. At this age category, being present and hemorrhagic pleuropneumonia, caused by A. pleuropneumoniae.

Fibrinous polyserositis were present in a smaller proportion (20.6%) compared with its frequency in age structure presented above.

The dominant anatomopathological lesion at this age structure, was still haemorrhagic enterocolitis (68.9%), accompanied by the hemorrhagic gastritis with a rate of 20.6%.

Laboratory tests have confirmed the following related diseases: enzootic pneumonia, pasteurellosis, hemorrhagic pleuropneumonia, Glasser disease and dysentery with *Brachispira hyodisenteriae.*

Macroscopic and microscopic lesions in the lungs and lymph nodes detected were similar with the lesions reported by other authors in herds where PRRS syndrome evolves both as primary and as evolving disease endemic (1, 2, 3).

Bacterial infectious diseases associated with this syndrome evolves both, in primary outbreaks and in endemic evolution, being produced by commensal bacteria from respiratory or digestive mucosa and are reported frequently and by other researchers as well (1, 3, 5, 6).

Table 2

The frequency of pathological lesions in the bodies of piglets over 8 weeks

Nr. Crt.	Lesion	Nr. Corpses	%
1	Pulmonary congestion	10/29	34,4
2	Interstitial pulmonary edema	5/29	17,2
3	Catarrhal bronchopneumonia	4/29	13,7
4	Fibrinous bronchopneumonia	9/29	31,03
5	Fibrinous hemorrhagic bronchopneumonia	6/29	20,6
6	fibrinous pleuritis	11/29	37,9
7	Pericarditis	4/29	13,7
8	Lymphoreticulitis	16/29	55,1
9	Fibrinous polyserositis	6/29	20,6
10	Renal dystrophy	8/29	27,5
11	Haemorrhagic enterocolitis	10/29	34,4
12	Haemorrhagic gastritis	20/29	68,9
13	Myocardosis	6/29	20,6

CONCLUSIONS

Anatomopathological examination revealed gross lesions characteristic of the syndrome PRRS in pigs in age categories monitored.

Gross pathological lesions were represented by: interstitial pneumonia, catarrhal and haemorrhagic lymphoreticulitis.

At piglets up to 8 weeks of age were diagnosed these bacterian associated infectious diseases: enzootic pneumonia, pasteurellosis, Glasser disease and dysentery with Brachispira hyodisenteriae.

At piglets over 8 weeks of age were diagnosed these associated bacterial infectious diseases: enzootic pneumonia, pasteurellosis, hemorrhagic pleuropneumonia, Glasser disease and dysentery with *Brachispira hyodisenteriae*.

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CODEX ALIMENTARIUS.
NEW CHALLENGES

VALENTI CANAL FONT
Faculty of Veterinary Medicine Bellaterra, Spain
canalfont2002@yahoo.es

Abstract: *Due to expanding globalization and international trade, in recent decades the relatedrisks have only increased continuously, on a rather exponential trend.*
On the other hand, even upon entry in EU, it is unclear who bears the responsibility to verify that all suppliers from abroad actually perform quality controls at source according to the same or similar rules? This is valid for producers in EU, who use imported ingredients in their production process, and also valid for supermarkets,where every day more and more exotic products can be found, many of them even marked as "ecological".
Though it is difficult to speak about 'international legislation' as such, a minimum legislative uniformity between countries has been achieved through the recommendations of the Codex Alimentarius. These are used by all countries as a reference to create national legislation, so that the highest possible matches result, in order to promote the world trade.Of course, in the process creation of their own national law, each country can choose the degree of application of the recommendations of the Codex, and choose to insert it in a more or less accurate manner. Codex Alimentarius has had a very bad press in Romania, however we must admit that standardization is indispensable in order to ensure a minimal level of food safety worldwide.
As a general comment, it should be noted that there is a lack of harmonisation of such legislation in many countries of the third world, and to be honest, even among EU countries there still does not exist a legislative uniformity, let alone that the real-life application is even less uniform.
There is a natural tendency to assume that everyone does/thinks/is like us, for instance we would tend to take for granted that the application of our European model of legislation and control is universal, but the reality is that often there is no control at all over the raw materials imported from abroad that enter the EU production food chain.

Key words:*Food safety Codex Alimentarius, Veterinary services,Worldwide and local legislation,.*

INTRODUCTION

The risks related to the food market and consumption by large numbers of population has increased continuously in the recent decades. We can asses this increase as being a dramatic one, for a variety of reasons, however in this material only those regarding with international trade and expanding globalization.

As we can notice in the following graphic, during the past 30 years, the worldwide trade has increased almost 10 times, from US 500.000 millions to 4.500.000 millions per annum. Furthermore, the figures for 2012 and 2013 have already shown a further increase, as compared to 2012.

Origin www.wto.org

World Trade Organization Statistics

In the context of such an increase of international transactions, we can look at the activity of **The World Trade Organization (WTO)** as both an upstream and downstream factor, we can see it both as a *cause*, a promoter, a generator and, on the other side, as a *consequence*, considering the need to further regulation of this field, as the international commerce is going every day into higher volumes.

To recap briefly on the WTO, it is an international organization built to supervise and liberalize international trade, and therefore involves the creation of laws of trade between nations, at a global level. It is responsable for the negotiation and implementation of new trade treaties and it is also responsable for the follow-up of these new treaties.

From its beginning the WTO TBT issued two specific agreements to restrict the barriers to free trade. The two agreements were based on technical and protectionist reglementations:

* *The agreement concerning the application of sanitary and phytosanitary measures* (WTO SPS) – which obliges the members to elaborate their food safety politics (contaminants, bacterial, pesticides, inspection and labeling.

* *The agreement regarding the Technical Barriers in Trading (WTO TBT)* – which obliges the members to reassure the fact that the technical reglementations, the voluntary standards and the evaluation procedures of compliance don't create useless obstacles in trading.

Although the latter agreement does not mention in an explicit way the Codex Alimentarius, in the context of the harmonization that it promotes, WTO makes extense references to *international standards*.

MATERIAL AND METHOD

For performing the analysis of this study, it has been proceeded, to identifying of the relevant materials/ pieces of legislation, to translating certain legal acts, where it was the case, and toevaluatingthe legal actsusing a documentary, comparative, critical and statistical approach.

RESULTS AND DISCUSSIONS

As already mentioned in the Introduction, it is obvious that the dynamics of the international trade on one side and of its standardization and regularization on the other side, inevitably go hand in hand. This is a typical "What came first? The chicken or the egg?" kind of dilemma. There is a clear correlation and dependence between the two, because the

entrance of less developed countries as suppliers on more sophisticated markets was only possible as a result of their submission to the *generally accepted international regulations*. And this is not only a question of legality at a governmental level, but also a matter of commercial risks attached to importing and distributing entities.

Furthermore, though it is difficult to speak about international legislation as such, a minimum legislative uniformity between countries has been achieved through the recommendations of the **Codex Alimentarius**. These are used by all countries as a reference to create national legislation, so that the highest possible matches result, in order to promote the world trade.

Of course, in the process creation of their own national law, each country can choose the degree of application of the recommendations of the Codex, and choose to insert it in a more or less accurate manner.

Codex Alimentarius has had a bad press in Romania, however we must admit that standardization is indispensable in order to ensure a minimal level of food safety worldwide.

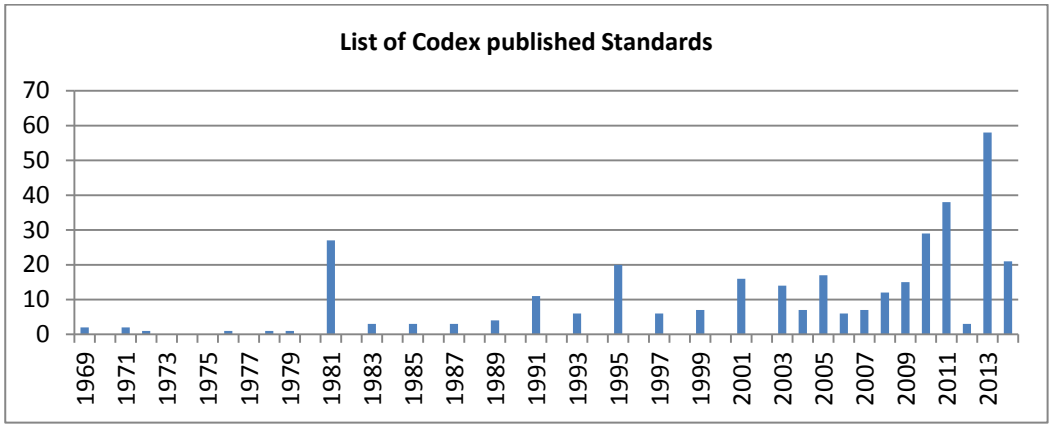
In this context, from its initiation, progressively the legal relevance of the Codes Alimentarius on the international trade scene grew.

Codex Alimentarius is a collection of international standards (quality standards, health and security standards, nutrition standards, ways of uniform standardization), codes of good food practice, guides and other recommandations regarding food products, their production and their safety. As regards its application domain, Codex Alimentarius incorporates standards for all the food products, cocked, semi-prepared or raw and for the distribution to the user, reglementations regarding food hygiene, food additives, pesticide residues, contamination factors, labeling and presentation, methods of analysis and sampling.

At an intent level, the documents issued by the Codes Alimentarius Commission are presumed to be elaborated on the principles of some analyzes and scientific proof well-fundamented that implies a thorough study of all relevant information for the standards to assure the quality and security of food products.

We can reasonably deduct the fact that the leading position is to establish a set of minimal rules to assure the fluency international trade, therefore a minimum level that has to be fulfilled by all the operators in the agricultural and nutrition fields so that international exchanges can take place in near-perfect conditions. Also, it has to be said that the Codex Alimentarius is recognized by WTO as a points of reference for the solving of consumer food security disputes.

In the last years there have been published **58 recommendations by the Codex Alimentarius**, which have already been adopted byworldwide countries within their national legislation, or will be adopted, sooner or later, if they wish to become or remain actively inscribed in the food international trade.



A crucial question arises: How much time will be needed by worldwide countries to introduce the new Codex Alimentarius proposals into their national legislation?

As already mentioned, when it comes to adopting the recommendations of Codex in the process creation of their own national law, each country can choose the degree of application, in a more or less accurate manner. As a general comment, it should be noted that there is a lack of harmonization of such legislation in many countries of the third world, and to be honest, even among EU countries there still does not exist a legislative uniformity, let alone that the real-life application is even less uniform.

As regards specifically the situation in the European Union, the measures taken by EU in connection with food safety frequently invoke Codex Alimentarius as a justification. The European Union regarding food safety frequently invokes Codex Alimentarius as a justification.

In an attempt to visualize the time lapse between the publication of any recommendations by Codex and their related transposition into the national legal systems, we could take a look to the example of a EU country such as Romania, that has a significant agricultural and food tradition, and has done in the last decades a major effort to fully harmonize its legislation with the EU one; we can see the rhythm of takeover of Codex recommendations:

1 CODEX: CODE OF PRACTICE FOR RADIATION PROCESSING OF FOOD (CAC/RCP 19-1979)

Order of 23.11.2001 for the approval of the Standard regarding food and food ingredients treated with ionized radiations.

2 CODEX: CODEX STANDARD FOR. FOOD GRADE SALT CODEX STAN 150-1985

Government Decision no 568/2002 regarding universal iodization of salt destined to human consumption, animal nutrition and the use in the food industry.

3 CODEX: GENERAL PRINCIPLES OF FOOD HYGIENE CAC/RCP 1-1969.Last modified 2003

Order of 24.10.2001 for the approval of Food product higiene Standard.

While we can reasonably conclude that the degree of assimilation of international standards into EU legislation, together with it own set of standards, is proportionate with the expected quality standards to ensure food safety, it is important also to check further how the situation stands in less developed countries that supply food and ingredients to EU producers and/or distributors.

As a general principle accepted by all countries, as well as laid down by Codex Alimentarius, the case is that the responsibility for the final product stays with the company responsible for the production of the product, while the health authorities only check the documents and examine the controls in place.

As regards the *practical application* by governments of underdeveloped or developing countries, sometimes the economical interests and needs dictate or put a lot of pressure on the equation. On one side, there are countries where a single product covers 80% of the country's exportations and 90-95% of the foreign exchange earnings. And on the other side, in Europe, there is much need of such raw materials / products as ingredients for the production, since in Europe such produce is zero, due to climate conditions etc, for example cocoa, pepper or coffee.

And speaking about economical interests placing pressures, we should probably admit that if health standards similar to the EU ones would be effectively applied in these developing countries, then obviously the general increase of costs for the respective industries would most probably make them relatively too expensive, leaving only the wage differential help they compete with their EU competitors.

CONCLUSIONS

Within the larger frame of the World Trade Organization (WTO) international regulations in order to promote worldwide trade, the number of recommendations published by the Codex Alimentariusin recent years has increased significantly, in direct connection with the higher volumes in global commerce.

The correlation is double, since the standardization and regulatory undertakings by WTO represent both an upstream and downstream factor. We can see it both as a *generator*of increased trade and, in the same time, as a *consequence*, considering the need to further regulate the growing volumes of international commerce.

In spite of voices complaining against excessive regulations manipulating the worldwide trade, we must admit that, in order to ensure a satisfactory degree of conformity in food safety for mass population, international regularization is a must.

Therefore, the numerous Codex recommendations issued in recent years have already been adopted by worldwide countries within their national legislation, or will be adopted, sooner or later, if they wish to become or remain actively inscribed in the food international trade.

Interesting issues arise in the rhythm and manner in which such international standards are legally adopted in less developed countries, and furthermore, the degree to which they are actually implemented in practice by their economic operators, as all these matters affect indirectly the food we get in our plates even in countries that have a strongly regulated system.

As we inevitably are going global, we cannot remain with a regional or national vision.

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OBSERVATIONS ON MAINTENANCE/GROWTH CONDITIONS OF THE MADAGASCAR COCKROACHES (*GROMPHADORHINA PORTENTOSA*)

CORNELIU GAȘPAR, IULIAN CĂTĂLIN GRĂMADĂ, LUMINIȚA IULIANA AILINCĂI
USAMV Iași - Faculty of Veterinary Medicine Iași, Romania,
cgaspar@uaiasi.ro

Abstract: *There are over 20 species of Madagascar cockroaches (Gromphadorhina portentosa) also called Madagascar hissing cockroach which although native live in retired places in Madagascar began to be reared as pets /pleasure because they are relatively harmless, have large dimensions (10-12 cm) and at handling the males make a sound (hiss) unique in the world of insects. This study concerns the possibilities of increasing their captivity.*

Keywords*:cockroaches, habitat, terrarium*

INTRODUCTION

Kingdom *Animalia*, Phylum *Arthropoda,* Class *Insecta*, Order *Blattodea*, Family *Blaberidae,* Genus *Gromphadorhina,* Species*G. portentosa*.This study presents the influence of environmental factors (growing space, bedding, temperature, humidity, etc.) which makes the holding of Madagascar cockroaches in captivity.

The Madagascar cockroach (*Gromphadorhinaportentosa*) called "hisser" or "hissing cockroach" has its origin on the island of Madagascar where they can be found on land in remote locations, usually under leaves or rotten logs. Although naturally grow in Madagascar (over 20 species), due to the size (10 cm female, 11 cm male), harmlessness, the rate increased of prolificacy and especially because when they are touched make a hissing sound characteristic and unique in the world of insects, it began to be bred as pets for pleasure or as a source of food for other living pets.

Pic. 1,2. Handling

MATERIAL AND METHODS

In the framework of this study were studied two generations of cockroaches Madagascar, respectively a total of 26 adults (21 females and 5 males) and 80 nymphs.

For this purpose were used hygrometers, thermometers and observation.

RESULTS AND DISSCUSION

1. Terrarium/Insectarium. Landscaping and needs.

The artificial microhabitat of growth for the species *Gromphadorhina portentosa* consist in the insurance of the main conditions regarding the natural development behavior and welfare.

In captivity, the Madagascar cockroaches is easy to grow, being a cockroach that needs very little space. The holding space, which can be diverse and improvised (glass bowls, boxes, plastic containers, etc.), needs to be fitted with aeration means but compulsory sealed and the diameter smaller than the nymphs to prevent escape into the environment.Therefore they can be kept in terrariums, aquariums or alimentary casseroles (Kristin Petrie, 2012).

Pic. 3. Example of box growth-side and top view.

Pic. 4. Lid with airing.

Their size can range from 20 cm lenght and 10 cm width for a pair of hissers, up to 40 cm lenght and 30 cm width in the case of colonies. Regarding height, it does not affect special the growth of cockroaches but still indicated to be about 20 cm in order to facilitate the introduction of hiding and for the good development of the territoriality behavior of males who prefer high spaces.

The Madagascar cockroaches are veritable climbers due to the soles nanostructures. For non-existence of a lid which is sealed, barrier can be achieved by drawing a line about 3 cm along the upper edge of the terrarium with olive oil or vaseline. Grease will prevent the advancing insects. However, it might see discontinuities in the layer of grease and beetles to escape.

It is also necessary and effective ventilation to prevent mold and mites multiplication. For this, it suffices to cut out a portion of the lid, then cover the opening with mesh in order to allow the air entering, but that does not allow the beetles out.

2. Substrate

The Madagascar cockroaches do not require a substrate. However different types of substrates is used to facilitate the hiding, such as: paper, wood shavings, leaves, sand / gravel, peat, coir, etc. If the substrate is chosen, should be considered the possibility of cleaning, so

the substrate should be easily changed (http://www.petbugs.com/caresheets/G-portentosa.html).

Tab.1. Types of substrates

Substrate	
Wood shavings, leaves, sand / gravel, peat, coir	+
Paper	++

To keep more acceptable the hygienic conditions is preferably a terrarium / insectarium without substrate.

3. Components of a space of growth

3.1. *With or without the substrate.*

3.2. *Hideouts.* The Madagascar cockroaches are nocturnal insects which are hidden during the day. The cockroaches are hiding and if someone approached them. These hideouts are essential in a terrarium with cockroaches. You can used formwork eggs witch are placed one above another. Also, you can opt for remaining cardboard tubes from rolls of paper or wooden sticks placed as high for the males to compete. So, if you are are several males in the colony, you will need more accessories.

3.3. *Bowl with water.* They are big water drinkers. The water tank is placed inside the container and does not have to be deep. To prevent drowning, especially the nymphs, it is advisable to put in the dish a sponge.

3.4. *Bowl for food*. The ideal would be to have a bowl for food and not scatter the food on the substrate. Food scraps unconsumed shall be removed from day to day. The Madagascar cockroaches are not picky eaters. In captivity they are fed with fresh fruits and vegetables and a protein source. Among vegetable it prefers apples, oranges, bananas, grapes, carrots and tomatoes. Leafy green vegetables are a delight: lettuce, cabbage and especially dandelion leaves. As a source of protein is selected dogs and cats dry food. It can opt for complete diets for the exotic birds, rodents, granules for the aquarium fish, etc.

Pic.5. The components of a space of growth:

a) Bowl with water;

b) Bowl with food (protein source);

c) Hideouts (egg crate);

4. Temperature

Being a tropical insect, the temperature is an important parameter. The ideal temperature is 26-28˚C. Beyond this limit the insects will become hyperactive (it will increase their metabolism), it will breed in excess and will die quickly. Under 18˚C their body functions slow down and become lethargic and will soon die. If the ambient temperature ranges, then should be introduced a system of heating proper for the container. Heat-pads are the most accessible solution. They will be placed under the vessel only in its half to create a temperature gradient.

5. Humidity

The humidity is an important parameter in the Madagascar cockroaches growth, which not respected easily lead to the animal's death, given that they come from rainforest which provides a high humidity of about 70%.Nymphs are most sensitive to low humidity. They molt several times to maturity and a humid environment will ease the removal of the exoskeleton. A bowl of water placed inside will satisfy this requirement. Another solution is regular spraying the vessel with water spray.

Temperature	Humidity	Length growing space	Width growing space	Height growing space	Substrate	
18-28 ˚C	60-80%	40 cm	30 cm	20 cm	-	Paper, wood shavings, leaves, sand / gravel, peat, coir

Tab.2. General microhabitual requirements for the Madagascar cockroaches

1. The Madagascar cockroaches can constitute an exotic alternative of holding only for the people „authorize".
2. Growing conditions must represent certain specific parameters similar to those of natural living environment.
3. The holding in captivity of these „creatures" requires adaptable facilities and hygienic conditions and even improvised.

WARNING / RECOMMENDATION

Due the prolificacy and high growth rate, their ownership should be done only by professionals, controlled, to prevent their escape into the environment.

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OBSERVATIONS ON GROWING AND HYGIENE CONDITIONS OF THE STICK INSECTS (*MEDAUROIDEA EXTRADENTATA)*

CORNELIU GAȘPAR, IULIAN CĂTĂLIN GRĂMADĂ, LUMINIȚA IULIANA AILINCĂI
USAMV Iași - Faculty of Veterinary Medicine Iași, Romania
cgaspar@uaiasi.ro

Abstract: *The stick insects along with the leaf insects comprise a large number of species, over 2500 which belong to the order Phasmida. They began to be bred in captivity, most recently in Romania due to certain characteristics (the diurnal apparent immobility) and their natural camouflage. The study presents the specific handling characteristics, their general characters and possibilities of holding which can be used for any possible desirous.*

Keywords*: stick insects, habitat, camouflage*

INTRODUCTION

Kingdom *Animalia,* Phylum *Arthropoda*, Class *Insecta*, Order *Phasmatodea,* Family *Phasmatidae,* Genus *Medauroidea,* Species *M. extradentata.*

The purpose of this paper is to present the handling, the growth possibilities and the living conditions for a group of exotic insects witch are becoming increasingly popular in the preferences of the breeders, respectively *Medauroidea extradentata*.

The stick insects are known as "crutch insect", "baston insect" or "stick insect Vietnamese", having their origin in the rainforests of South Asia, particularly in Vietnam. Their natural camouflage is their main characteristic, which makes them very difficult to find both in their natural environment, in the rainforests, and in the artificial microhabitat (insectarium). In addition they show apparent immobility daytime, offering an aspect of "still life".

Even though their life spawn is relatively short (6-7 months), their great length is another reason for which it are bred in captivity, which is 11-12 cm. Presenting the phenomenon of autotomy is recommended that their manipulation to be done carefully and never by the legs.

The insect need to be catch with the forefinger and the thumb from the metathorax with a moderate pressure. Also, as a method of defense (besides the camouflage) is the mimetism, so the insects will bend their abdomen and will remain motionless, "dead", when they feel threatened (http://animals.sandiegozoo.org/animals/stick -insect).

MATERIALS AND METHODS

This study fallow the hygienic and growth conditions of a total of 25 adults insects and 34 nymphs. For this purpose were used hygrometers, thermometers and observation.

Pic. 1,2. Manipulation

RESULTS AND DISSCUTION

1. Insectarium/ Insectivarium

Given that these insects have a big body length is required an insectarium which must have at least the height equal to three times the insect body length and a width equal to twice the body length.

Pic.3. Overview

Pic.4. Interior

In addition to providing sufficient space for growth, height is important in the process of molting, because insects will catch of the insectarium lid or of any decoration situated at height and through slow movements will leave the old exoskeleton. . It is therefore important to adopt a mesh cover for the insect in order to cling more easily.

Also, such a lid will also provide a good ventilation. Such an insectarium may can have the following dimensions: 40 cm height, 25 cm width and length. It can be made of glass, plexiglass or wood.

2. Substrate

The substrate may or may not be, depending on the choice of the space in which will be grown the stick insects. The interior space creates artificial conditions in their natural life, so it can be grown in substrate and other living animals as a source of food for other predators. In case of closed space intended for these insects, as the substrate is best paper / towels stacked. Most breeders appeal to the this type of substrate as it ensures an efficient cleaning of the insectarium and make it easier to collect the eggs. Also can waive all substrates, these insects spending the most time on walls of the insectarium or on the decorations (Valerie Bodden, 2014).

Tab.1. Types of substrate

Substrate	
Earth, moss, coconut fiber, gravel	++
Paper / towels	+++

++ Acceptable
+++ Good

Pic.5,6. Substrate of paper

3. The components of an insectarium

Their growth requirements and developments involving indoor environment that are presented in this paper (water dish sponge, support feeding / climbing). Hygienic conditions that must be respected consist primarily in the washing of food and water bowls and regular change of bedding / support of camouflage. The components of a insectarium are:

3.1. *With or without the substrate.*

3.2. *Decorations.* In case of these insects the decorations are important because most of the time the insects will spend sitting on it. As decorations it can opt for tree branches that will be ideal for camouflage or different types of bark. Feeding on leaves of rose, blackberry and raspberry branches, placing these plants in insectarium can be a genuine decor. Another way is to increase the plant directly into the insectarium, but this requires additional lighting.

3.3. *Water containers for plants*. It is necessary for the plant's location in order to provide food for a longer period. The ideal it would be to place a sponge in the bowl to prevent flooding of insects, especially of nymphs. In winter, the leaves are used successfully freezing and offering on a support or attached on decorations.

4.Temperature

This species prefer a temperature between 21-24˚C, generally are kept at room temperature.

5. Humidity

The humidity is recommended to be between 70-80%. It remains steadily due to water spray on the insectarium walls in order to ensure daily water requirement. So the insects will drink water from the confluence of drops on the sides of insectarium or directly on the leaves. It would be ideal that spraying the water to be made the evening when the insects are more active and seek food and water (Greg Rose, 2011).

Tab.2. General microhabitual requirements for stick insects

Temperature	Humidity	Length insectarium	Width insectarium	Height insectarium	Substrate	
21-24˚C	70-80%	25 cm	25 cm	40 cm	-	Earth, moss, coconut fiber, gravel, paper / towels

CONCLUSIONS

1. The stick insects of the species *Medauroidea extradentata* may be a ornamental variant for houses, providing a living corner, although apparently still life, the environment.
2. The increase in captivity require adherence to specific microhabitual parameters and certain environmental facilities tailored to specific dimensions.
3. Hygienic conditions consist of periodic cleaning of insectarium and the facilities (dish water, support feeding / camouflage, litter).

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LEGISLATIVE ASPECTS CONCERNING THE SAFETY, TRACEABILITY AND QUALITY OF TRADITIONAL PRODUCTS OF ANIMAL ORIGIN

OANA-MĂRGĂRITA GHIMPEȚEANU, MAGDA GONCIAROV
University of Agronomical Sciences and Veterinary Medicine of Bucharest,
Faculty of Veterinary Medicine, Romania
ghimpe_marga@yahoo.com

Abstract: *The concepts of safety, traceability and quality of traditional products of animal origin were introduced by the new food safety package that brings new regulations on implementation of the hazard analysis and critical control points in the food industry, general and specific hygiene requirements and new obligations for the food operators and competent authorities. The new regulations simplify the legislation, significantly reducing the number of used laws and strengthening the rules on European principles. Also, simplification of legislation brings the advantage of increasing transparency in food hygiene field.*

The novelty is represented by the primary responsibility of food operators to ensure that the requirements of hygiene in the unit are fulfilled.

Key words: traditional products, safety, traceability, quality

Today, the importance of product quality is uncontested and established itself as a determinant factor of the competitiveness of the company, paying attention and necessary funds. Manufacturers are trying to produce and put on the market diversified product assortments and continuously covering consumer demands. In this context, traditional meat products have become increasingly popular and more diversified. There is a specific legislation regarding the certification and registration of traditional products, namely Order No. 34 /2008. and Order no. 724/1082/360/2013 - certification of traditional products.

The need to implement appropriate programs in order to obtain safe food for all consumer groups, from children and young people up to elderly, convalescent or immune deficiencies people, requires the implementation of a system to ensure food safety-HACCP (Hazard Analysis Critical Control Points).

These goals are of utmost importance especially regarding the food. Therefore, the management of food establishments must ensure product quality, in order to fully satisfied the needs of use and not only guarantee the safety of their use or the absence of serious and immediate risks.

Principles regarding the production, circulation and marketing of food

The legislation in force establishes unified legal framework regarding the production, packaging, storage, transportation and marketing of food, the resposabilities of food manufacturers and retailers, food organization of official controls of food and sanctions in order to protect food quality. Application of the legislation protects consumers from malpractice in the manufacture, storage and marketing of food and ensure their full and correct information in accordance with legal regulations on consumer protection.

Legislative regulations regarding food safety does not apply to food produced in individual households for their own consumption and to food in transit or stored temporarily

as goods transiting the country, if they do not represent a threat to human health or for the environment.

Establishments in which are produced food ingredients, food additives and processing aids are placed only in those areas which meet the requirements of the regulations in force. For units producing traditional foods, they must meet the requirements for obtaining the hygienic products

Manufacturing, storage and transport of food are in compliance with technological standards, sanitary and veterinary health, to ensure that the requirements of public health, food hygiene and food quality.

Conditions regarding the composition and quality of food

The foodstuffs should have a composition that ensures both their quality and meet consumers' health and hygiene conditions laid down by the regulations.

At manufacture or handling of food intended for sale are prohibited:

a) the use of additives that have not been authorized, either alone or in combination with other substances;
b) using technological processes to obtain unauthorized additives in food;
c) use of additives above the admitted limit.

Conditions regarding pesticide residues and other products that are found in food:

It is prohibited the marketing of foods containing:

a) residues of substances for plant protection, fertilizers or other products to treat the plants and soil to protect stored products or their degradation products or reaction above the limits stipulated in the regulations in force;
b) residues of substances for plant protection, which have not been authorized and permitted to be used for food or for their raw materials.

General requirements for food establishments

The food establishments must be kept clean and in good working order and maintenance.

Planning, design, construction, location and size of the food establishments must:

a) permit appropriate maintenance, cleaning and / or disinfecting to avoid or to minimize the possibility of contamination by air, and provide adequate working space to allow the hygienic performance of all operations;

b) protect against the accumulation of dirt, contact with toxic materials and existence condensation or mold on surfaces;

c) permit good food hygiene practices, including protection against contamination and, in particular, pest control;

d) provide, where appropriate, adequate storage and handling of food at a controlled temperature, to have sufficient capacity for maintaining foodstuffs at appropriate temperatures and be designed to allow those temperatures to be monitored and after case be registered.

It must be provided suitable and sufficient natural or mechanical ventilation. It must be avoided mechanical air flow from a contaminated area to a clean area. Ventilation systems must be constructed so as to enable filters and other parts requiring cleaning or replacement to be readily accessible.

The food establishments must have natural and / or artificial lighting.

Drainage facilities must be adequate for the intended use. They must be designed and constructed so as to avoid the risk of contamination. When drainage channels are open in whole or in part, they must be designed to ensure that there is no leak of waste from a contaminated area towards or into a clean area, in particular in the area in which they are handled foods may present a high risk to the final consumer.

The cleaners and disinfectants must not be stored in areas where food is handled.

In rooms where are prepared, treated or processed food, except spaces for dining, layout and project should permit good hygiene practices of food, including protection against contamination between and during operations.

In particular:

a) pavement surfaces must be maintained in good condition and maintenance and be easy to clean and, where necessary, to disinfect.

b) the wall surfaces must be maintained in good conditions for maintenance and to be easy to clean and if necessary, disinfected. This requires the use of waterproof, nonabsorbante, washable and non-toxic, as well as a flat surface to a height adequate to perform the various operations,

c) ceilings or where there are no ceilings, the interior surface of the roof and hanging accessories must be constructed and finished so as to prevent the accumulation of dirt and reduce condensation, mold growth and spreading particles;

d) windows and other openings must be constructed to prevent the accumulation of dirt. Those which can be opened to the outside must be fitted with insect netting, which can be easily removed for cleaning. Where opening the windows will have the result the contamination, windows must remain closed and fixed during production;

e) doors must be easy to clean and if necessary, disinfected. This requires the use of smooth and nonabsorbante, unless that food business operators can satisfy the competent authority that other materials used are appropriate;

f) surfaces, including surfaces of equipment in areas which are handling food and in particular the surfaces in contact with food must be maintained in good conditions and be easy to clean and, when necessary, disinfect.

Suitable facilities must be provided, if necessary, for the cleaning, disinfection and storage of equipment. These facilities must be constructed of corrosion-resistant materials, be easy to clean and be supplied adequately with hot and cold water.

Must be developed, where appropriate, instructions for washing food. Each vat or any such facility of washing the food must have an adequate supply of hot water and / or cold potable water and be kept clean and,where necessary, disinfected.

Transport requirements

The vehicles and / or containers used for transporting of foodstuffs must be kept clean and in good repair and functioning in order to protect the food against contamination

and, if necessary, be designed and constructed to allow for cleaning and / or adequate disinfection.

Vessels and / or containers must not be used to transport other materials than foodstuffs where such use can result in contamination.

Foods in liquid form, granules or powder in bulk, must be transported in vessels and / or containers / tankers reserved solely for transporting food. Such containers must be marked visibly with letters that cannot be erased in one or more foreign languages, to emphasize that they are used solely for the transport of foodstuffs, or must be marked "for foodstuffs only".

Where necessary, conveyances and / or containers used to transport food must be adequate to preserve food at appropriate temperatures and allow those temperatures to be monitored.

To ensure the quality of animal products, including the traditional one, is compulsory the implementation of guides of good practice and HACCP system.

Food business operators must implement and maintain a permanent procedure or procedures based on HACCP principles. The system of self-control and the persons responsible for the implementation of self-control are established in unit.

The system of self control is endorsed by the competent authority supervising implementation. Units can use good practice manuals approved by the competent authority for the preparation of self-control system.

To ensure compliance with the conditions laid down by the legislation in force responsible veterinary units establish a sampling program allowing:

a) approval of self-control system to its implementation:

b) re-approval of the self system when products having other characteristics, or when the manufacturing process is changed;

c) ensuring that the provisions put in place permanent are valid and correctly applied.

The samples shall be analyzed according to the program's own unity - factory lab - or in another approved laboratory.

The responsable persons must have all the information relating to the implementation of self-monitoring and verification thereof and comprising:

a) a detailed document and completely covering data on product description and the manufacturing process, mentioning the critical points, risk assessment, planned measures to control critical points, and the procedures for the surveillance and control them, indicating the corrective actions provided the loss of control;

b) written reports and summaries of decisions regarding any corrective measures applied. It establishes a management system that allows documents to be easily retrievable corresponding to a batch of documents.

The documentation must be provided by the units to the competent authority.

The competent authority shall supervise the proper training of qualified staff inspection services for the official control, in order to assess the system of self-established unit responsible

The system of self-control has the following principles:

a) hazard identification, risk analysis and measures necessary to control;

b) identification of critical points;

c) establishing critical limits for each critical point;

d) establish procedures for the supervision and control;
e) establish of corrective actions to apply when necessary;
f) establishing procedures for verification and review;
g) establishing documents and records concerning all procedures.
Principles must be fit for every situation in the unit.

The advantages of implementing HACCP system:

1. satisfied customers
2. good reputation
3. business growth and profit
4. compliance with legislation
5. increasing the shelf life of preparations
6. proper working conditions
7. employees with high morale
8. increased productivity
9. a decline in the employment rate which will lead to stability.

Traceability of food, feed, food-producing animals and any other substance intended or expected to be intended for incorporation into food or feed must be established at all stages of production, processing and distribution.

Food and feed operators must be able to identify the origin and the source of the raw materials or any substance intended to be incorporated into the expected times a food or feed. For this purpose, food operators must have systems and procedures to allow, at the request of the competent authorities to provide this information. The food and feed operators must have systems and procedures to identify other activities which they are intended their products. This information will be made available to the competent authorities at their request. Food or feed which are placed on the market or are expected to be put on the market must be labeled or identified in an appropriate manner to facilitate traceability through documentation or information in accordance with the requirements .

"Quality system" involves all stages of the life cycle of a product or process, from market testing to identify customer needs and to use or consumption of the product. In this context ISO 9004 represented significant the "quality loop".The idea is based on the inseparable fact - the quality is built with the product from conception phase until it reaches the consumer.

In this context, all staff an undertaking to obtain quality products, should be actively and creatively involved in all steps indicated in the "quality loop", based on a good knowledge of the entire process.

CONCLUSION

1. The need to implement appropriate programs in order to obtain safe food for all consumer groups, from children and young people up to elderly, convalescent or immune deficiencies, requires implementing a security system, HACCP (Hazard Analysis Critical Control Points).
2. 2.The application of legal provisions protecting consumers from malpractice in the manufacture, storage and marketing of food and for ensuring their full and correct information in accordance with legal regulations on consumer protection.

3. To ensure the quality of animal products, including traditional, are mandatory implementation guidelines and HACCP good practices. Food operators mist implement and maintain a permanent procedure or procedures based on the principles HACCP.
4. Traceability of food, feed, food-producing animals and any other substance intended or expected to be intended for incorporation into food or feed must be established at all stages of production, processing and distribution.
5. Food operators and feed industry must be able to identify the origin and the source of food or a feed, animal intended for food or any substance intended or expected to be incorporated a food or feed. For this purpose, operators must have systems and procedures to allow, at the request of the competent authorities to provide this information.

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THE ACTIVITY OF THE IMMUNOCOMPETENT CELLS IN CORRELATION OF THE IMMUNE STATUS AT THE NEWBORN CALVES IN DEPENDENCE OF AGE

RITA GOLBAN
The State Agrarian University of Moldova, Chişinău,
golbanrita@gmail.com

Abstract: *The investigations reflected in this scientific research were performed with the main purpose to study the dynamics of the immunocompetent cells indices T and B at the newborn calves at the age of 5; 10; 20 and 30 days. There was determined in dynamics the number of the leukocyte, lymphocyte indices of the T and B cells. These data indicate the principal effector role in the activity of the immune system, as a efector of the immune reactions mediated cellular and humoral, capable of synthesizing some limfokine etc. Simultaneously increasing the concentration of the B lymphocytes justify early onset of humoral specific resistance of the immune status and receiving via colostrum antibodies in the neonatal period, as a result of the colostral immunity installation. The study results demonstrated that the newborn calves are born with an cell well formed lymphocytic immune system, represented by the organism's immune homeostasis correction.*

Key words: *Immunocompetent cells, Lymphocytes, Cellular immunity, Immune status, Reactivity.*

INTRODUCTION

The principal cellular immunity system is represented by the immunocompetent cells T and B lymphocytes, which determines the immune reactions of the animal organisms. Therefore the immunocompetence is attributed to all organs, tissues and cells, which contribute in some way at the achieving the immune response and is recognized due to the morphofunctional elements which produce by biosynthesis or from which results due to blast transformation immune effectors.

In the classical conception, it is considered that the activation of the immune function has a beneficial protector exclusive effect for the organism. The immune system ensures the preservation of the biochemical homeostasis of the organism, due to the immune function mediated by the molecules acting as receptors on the lymphocytes cells surface and by soluble molecules in humours of organisms.

The cells status of the immune system reflects both the resistance status of the newborn organism, as well as the cell damage, inflammation etc. By their reaction is appreciated the efficacy, norm and pathology of the inflamatory reaction both at the presence of the infectious agents as well as at the noninfectious agents [1], [5], [7].

The accelerated cells reactions induce the development of hypersensitivity, but those attenuated lead to insufficient resistance and immunity at pathogenes diversity of animals, with appearance of infections and of immunodeficiency status, because each infection doesn't appear and doesn't persist without an relative of absolute immunodeficit status

The performed scientific researches regarding the activity of the immunocompetent cells in correlation of the immune status of the new-born calves gave us the possibility to determine a set of particularities in development of the cellular and humoral immunity.

The elements of the immune system – macrofages, T and B lymphocytes are developing up till the birth of the animal, after which begin to function intensively. B

lymphocytes are the predecessors of the plasma cells, but T lymphocytes favors the possybility of developing the immune response in the first days of life of the new-born animal [3], [6].

At the present time an increasingly accute problem is connected with the immunodeficiency which is widespread in the practical medical clinic. This can be explained by the immunodeficiency results of the T and B lymphocytes of the immune system. Therefore the stimulation of the T and B lymphocytes which acts at the mucosal level increases the specific resistance of the entry gates and prevents the infectious processes from the very first days of life of animals [2], [4]

For this reason the main objective of this research is the study of the activity indices dynamics of the immunocompetent cells T an B in correlation of the immune status at the new-born calves in the neonatal period in dependence of age.

MATERIAL AND METHOD

The investigation were performed in the microbiology laboratory of the Faculty of Veterinary Medicine of the State Agrarian University of Moldova. For conducting investigations were used blood samples from the new-born calves aged up to 30 days which had 25-30 kg body weight with the purpose of determining the immunological blood indices.

Blood samples were collected from jugular vein with heparin on the basis of calculation of 0,3 ml heparin for 10 ml blood, for the purpose of anticoagulation. The samples were used for identification of the leukocites, lymphocytes and the immunocompetent T and B cells number.

The separation of T and B lymphocytes was performed by the method of spontaneous rosetting. The lymphocytes placed in contact with heterologous erytrocytes will form complexes with aspect of rosettes, resulting from coupling of major antigens of the erythrocyte surface and the lymphocyte receptors. Outside these „immune" rosettes, lymphocytes forms red blood cells, „non-immune cells", which results from the interaction of the antigenic determinants of erithrocytes with other receptor other than those involved in the immune recognition of effectors.

At the same from the heparinized blood were made smears, which were fixed with methanol and colored during 8-10 minutes after Romanovschii-Giemsa method. After calculating the number of leukocytes and their determination was appreciated the leukocyte formula and the lymphocyte percentage.

For the determination of the immunocompetent T and B cells was determined the lymphocyte layer using the solution of Ficol 17% with the purpose of cellular separation in density gradient having a density of 1,077 g/cm. The separation was performed in neutral glass tubes. In this scope in the centrifuge tubes was introduced 2,0 ml of physiological solution each, after which they were subjected to centrifugation at 1500 rotations/minute during 45 minutes. At the limit between plasma and the erythrocytes was observed a lymphocyte white ring. The lymphocyte ring was harvested with Pasteur pipette and resuspended by subsequent centrifugation, after which was enumerated the amount of cells in the Goriaev room.

The T lymphocytes were appreciated by the presence of the specific receptors compared to red cells of ram, using the reaction of spontaneous rosetting E-ROC, and B lymphocytes by the presence of receptors compared to three active component of the

complement in complementary reaction of rosetting with red cells of mice EAC-ROC. From the suspension of the fixed cellular components of the rosetting reaction fixed on blades carefully degreased were performed smears, which were colored using the Romanovschii-Giemsa method.

The results of the immunologic reaction of rosetting were determined by microscopy (40 X 10). To establish and define the absolute number of rosettes was determined the number of leukocytes in a ml of blood and lymphocyte percentage of a smear. The rosetted lymphocytes differed by placing around them of three markers arround the surface.

RESULTS AND DISCUSSIONS

The obtained results about the immunological investigations of the immune system regarding the activity of the immunocompetent cells at the new-born calves demonstrates that the leukocytes and lymphocytes level variates at diferent stages of life (table 1).

Significant results of the leukocites and lymphocytes indices were determined at the newborn calves at the age of 5 and 10 days, constituting the values of leukocytes 8,20± 0,81 and 7,90 ± 0,81 compared to obtained values at the newborn calves at the age of 20 and 30 days, where the indices constituted the level 6,95 ± 0,81 and 7,35 ± 0,81.

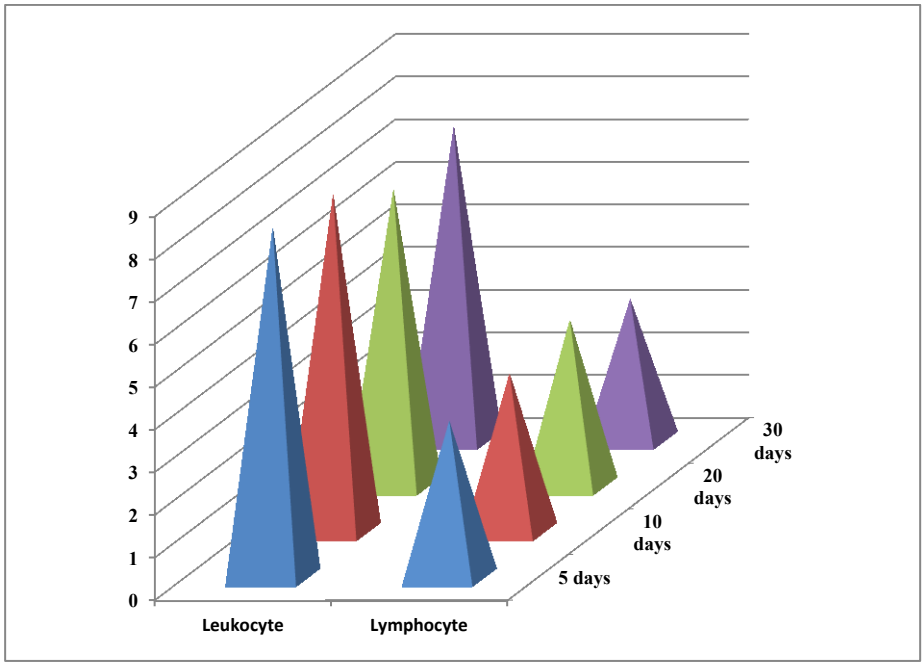

Fig. 1 The dynamics of the leukocytes and lymphocytes at the newborn calves in dependence of age, %

Simultaneously was determined and appreciated the number of lymphocytes at the newborn animals, which reveals appreciable values at the age of 5 and 10 days, constituting 3,69 ± 0,81 and 3,71 ± 0,81, compared with the calves at the age of 20 and 30 days constituting 3,90 ± 0,8 and 3,31 ± 0,81.

The organism is permanently affected by factor of the external medium which, coming in contact with its defense apparatus, triggers reactions against what is non proper (non-self)

for the cells of this apparatus, it can be a resulting reaction, or neutralization and elimination reaction, or a hypersensitivity immunological reaction, immunity reaction.

Thereby, the immune response is considered like a mechanism of defence, by which the organism recognize what is foreign for him. The self recognition difference of non-self, is very precise and proper to each organism. Among the mechanisms that generate illness or favors chronicization an important role is hold by the immune response deregulation.

The table 2 reveals the level of lymphocytes T-active and T-total at all the ages of investigation. Thereby at the age 5 and 10 days the lymphocytes concentration T-active constituted 23,4 ± 0,08 and 22,0 ± 0,08 compared to T-total lymphocytes, which constituted 14,4 ± 0,075 şi 13,4 ± 0,008. At the analyze of the registered indices at the 20 and 30 days the concentration of the T-active lymphocytes constituted 26,0 ± 0,08 and 23,8 ± 0,08, compared to T-total lymphocytes, which constituted 16,0 ± 0,08.

As a result of the cellular activation are initiated complex processes characterized by the initiation and realization of the cells functions involved in the immune response. The cells go through the cell cycle stages. The T cell activation is realized by signals carried out by antigen and a costimulatory molecule represented by the cytokine (IL-1). At the same time are triggered activation processes of the B lymphocytes as a result of antigen recognition by the BCR molecules. As a result of B cells activation is realized the proliferation and synthesis of antibodies.

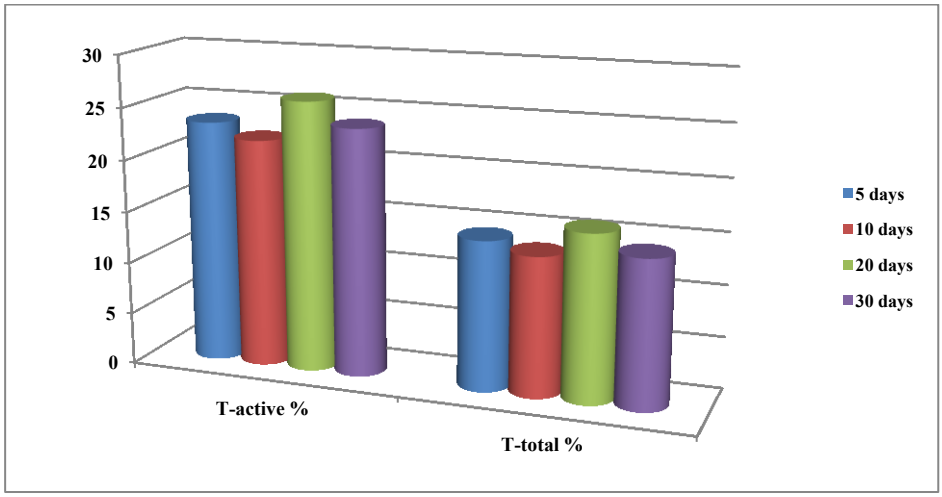

Fig. 2. The dynamics of the T lymphocytes at the newborn calves in dependence of the clinic status, %

This data reveales the efector principal role in the activity of the immune system as efector of the immune reactions cell and humoral mediated, capable to synthesize some limfokines etc.

As a result this values indicate that the level of concentration of the T-active lympphocytes at the newborn calves in different periods of age reveals significant increasing determined by the activity of these immunocompetent cells better expresses in terms of the immune tolerance to various bacterial and viral infections.

The separation of lymphocyte populations are based on the method of density gradient centrifugation with Ficoll separation medium (Fig. 3) As a result the lymphocytes which were

in contact with heterologous erythrocyte forms complexes with the rosette aspect, results of coupling between the major antigens of the erythrocytes surface and the lymphocytes receptors. Besides these immune rosettes, the lymphocytes form with the red blood cells non-immune rosettes, which result from the interaction of some antigenic determinants of erythrocytes with other receptors of lymphocyte membrane other than those implicated in the immune recognition of erythrocytes.

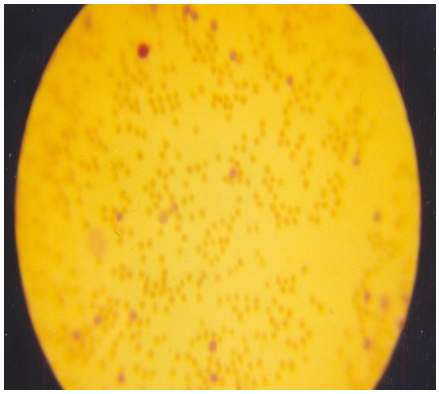

Fig.3. The separation of layer lymphocytes

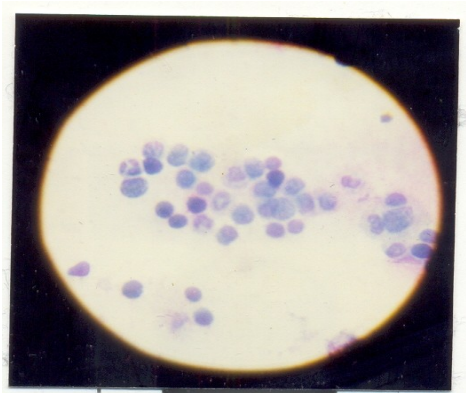

Fig. 4. Lymphocytes T and B

The T lymphocytes presents receptors for ram erythrocytes, B lymphocytes presents receptors for mice erythrocytes. The placement around the lymphocytes of 3 markers present a rosette (fig. 4). Both the rosette EA and the rosette EAC are non immune. They highlight the receptors of Fc fragment of the immunoglobulines and respectively the C3 component of the complement. The functional characteristic of the lymphocytes is the result of scientific researches made after 1960. The competence status is conditioned by the presence of receptors with whom the antigens from Moldova are well known.

The macrophages are esential cells for the protection function. They constitutes a line of defence, before the specific activation of T and B lymphocytes. They participate at the elaboration of the immune respones, in their capacity of cells which process and present the lymphocytes antigen. The macrophages are activated by lymphokines with stimulating functions of immunity.

The regulation mechanisms of the immune response are based on the immune reactions, which are controled by regulation systems by a complexity at least equal to those who stay at the base of their triggering and their expression. In the situation of blocking the regulation mechanisms, the clonal proliferation or the synthesis of immunoglobulines cannot be limited, leading to profound alteration of the immune response, accompanied by installation and evolution of diseases which usually have a lethal outcome.

At the same time the factors involved in the immunoregulation are very numerous. Some of these correlates with the antigen, type and quantity of antibodies.

The activity in dynamics of the Lymphocytes B at the newborn calves determined important results characterized in the table 3.

The level of the Lymphocytes B at the age of 5 and 10 days determined values of ,85 ± 0,008 and 10,0 ±0,81, compared to the age of the calves of 20 and 30 days, which constituted 12,0 ± 0,81 and 12,28 ± 0,008. The analyze of the registered indices reveals the level of the

concentration of lymphocytes B, which justifies the early instalation of the humoral resistance specific to the immune status and receiving the antibodies by colostrum in the neonatal period, as a result of instalation of the colostral immunity.

The cellular base of the immune cellular response is represented by the lymphocytes B. The immune cellular response protects the organisms against the aggression of fungi, parasites, viruses and bacteria with intracellular localization

The cellular base of the immune cellular response is represented by the lymphocytes B. The immune cellular response protects the organisms against the aggression of fungi, parasites, viruses and bacteria with intracellular localization

The lymphocytes T, responsible for the cellular imunity, express receptors which recognize only short peptide sequences from protein antigens.

Table 3 . The dynamics of the lymphocytes B at the newborn calves in dependence of age, %

Age (days)	Lymphocytes B, %
n=7	
5	5,85±0,008
10	10,0±0,81
20	12,0±0,81
30	12,28±0,008

The results of the scientific researches reveals important values at T and B lymphocytes at different ages. Therefore the level of T and B lymphocytes take values of 23,4 ±0,08 and 5,85±0,008 at age of 5 days, compared to the age of 10 days, when theses indices constituted 22,0±0,08 and 10,0±0,81. Simultaneously at the age of 20 and 30 days the values of lymphocytes T and B also determined important characteristic values constituting 26,0±0,08 and 12,0 ± 0,81compared to 23,8 ±0,08 and 12,28±0,008

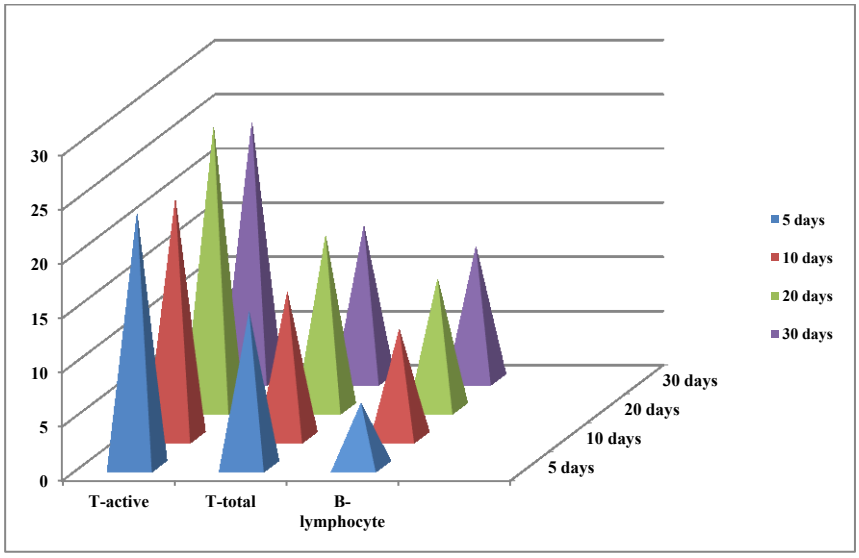

Fig. 5. The dynamics of the T and B lymphocytes at the newborn calves in dependence of the clinic status, %

Thus the researches performed for determination the immunobiological characters at the newborn calves from birth till the age of 30 days permited to reveal some peculiarities in the appearance and development of the cellular and humoral immunity. Thus the principal factor of cellular immunity is represented by the T and B lymphocytes with the subpopulations, which determined the immune reactions of the organism. Betweene these immunocompetent cells exist a specific interaction of the system T, which favors the immunocompetence of the lymphoid cells which regulated the system.The elements of the immune system, the macrophages, lymphocytes T and B are developing in the organism til the birth of the animal, after which begin intensively to function.

As a result at all periods of age, beginning with the 5 day of life till the 30 day of life, there were determined significant increases of the indices T and B lymphocytes, which give the possibility to conclude that is taking place the process of instalation of the immunological reactivity of the newborn organism and adaption at the changing conditions of the environment, especialy at the action of pathogenic microorganisms.

The cellular protection mechanism against the bacteria are performed by the efector cells with phagocytic function (neutrophyl, macrophages, etc.), cytotoxic cells etc.

By diverse mechanisms cell-mediated, the macrophages suffer a process of activation realized through secreted lymphokines by lymphocytes T. Therefore the macrophages have an important role in triggering and control of the cellular reactions, which will activate the lymphocytes B in order to synthesis the antibodies.

As a result the newborn calves are born with a lymphocyte system T and B well formed. From this point of view the young animal's capacity to develop immunologic responses depends on the link between the presence of the cellular system of immunity and the elaboration of the specific immunoglobulines.

Therefore it is important to know the methods of specific prophylaxis of some diseases of youth, no matter of species, which imposes to study the optimal age of doses, of ways of inoculation and of other parameters in function of the capacity of response of the animal.

CONCLUSIONS

1. The immunologic investigations demonstrated, that at the newborn calve in certain periods of growth and development gradually was registered the instalation of the resistance and reactivity of the immune system. Therefore the values of T and B lymphocytes reveal well determined functions of cellular reactions, determined by the processes of microorganisms phagocytosis

2.The study of the mechanisms of formation of the immune system of the animal organism offer the possibility to analyze the evolution of the cellular and humoral reactions, which mentain the immune homeostasis of the organism, of the cellular and hommoral protection factors considered the most important in the immune system regulation.

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THE SEROPREVALENCE OF *SALMONELLA SPP*. IN ASSOCIATION WITH SWINE INFLUENZA VIRUS H1N1 IN PIG FARMS LOCATED IN WESTERN ROMANIA

VIOREL HERMAN, CORINA PASCU., FINI CORINA GREMA., ADELA MARCU., ANCA SOFIANA SURPAT (HULEA)., DANIEL TĂTAR
Faculty of Veterinary Medicine Timişoara
viorel.herman@fmvt.ro

Abstract:*In the paper are presented the results obtained by detection of antibodies against Salmonella spp. and swine influenza virus H1N1 in Arad County, Bihor County, Caras-Severin County, and Timis County. The value of seroprevalence of Salmonella infection within farms ranged between 18.51% and 100%.*

Mixed infections (the presence of both swine influenza virus infection subtype H1N1 and infection with Salmonella spp.) were identified in four of the nine farms studied for both types of infection. The seroprevalence of Salmonella infections was increased in farms with mixed infections compared with the farms identified seronegative for swine influenza virus subtype H1N1. From a statistical point of view, a strong positive correlation between infections caused by swine influenza virus subtype H1N1 and Salmonella infections, in fattened pigs, was discovered. The correlation coefficient had a value of +0,919.

Key words*: pig salmonellosis, swine influenza virus H1N1.*

INTRODUCTION

Salmonella infection is a bacterial disease that is commonly found in pig herds. The disease has economic significance in the system of intensive growth of pigs. The economic losses are determined by mortality, expenses for prophylaxis, treatment, contamination of meat products, and meat product removal from public consumption (4).

Salmonellosis in pigs has become a major concern within the European Union (EU) because in the last few years, the contaminated pork meat has been considered the most significant source of human salmonellosis in country members. It is estimated that the consumption of contaminated pork meat may account for 10-23% of cases of human salmonellosis (4, 7).

The present researchers were aimed to investigate the seroprevalence of Salmonella infection in pigs in fattening units located in the Western region of Romania, in relation with the seroprevalence of infection caused by swine influenza virus subtype H1N1.

MATERIALS AND METHODS

The investigations regarding the infection with Salmonella spp. were performed in eleven swine fattening farms, located in four Western counties (two farms in Arad County; three farms in Bihor County; one farm in Caras-Severin County; and five farms in Timis County). Also, in nine swine fattening farms investigations were conducted to assess the seroprevalence of infection caused by swine influenza virus subtype H1N1. Farms were monitored during the production cycle. To perform serological screening, blood samples were collected from pigs between the ages of 45 days and 180 days from the eleven swine fattening farms located in the four counties mentioned above. The processing of serum samples was performed by ELISA using the Swine Salmonella Ab Test kit in the Laboratory

of the Deparment of Infectious Diseases and Preventive Medicine in the Faculty of Veterinary Medicine Timisoara. In order to read the results the Tecan Sunrise reader was utilized, while interpretation of the results was performed using the Xchek Software v. 3.3. program (IDEXX Laboratories Switzerland).

RESULTS AND DISCUSSION

Based on the results of the investigations conducted by ELISA , it was concluded that infections with Salmonella were detected in eleven monitored farms. In other words, Salmonella infections were identified in all studied fattening farms (figure 1). Seroconversion at the farm level, based on the number of samples examined, ranged between 18.51% and 100%, which highlights the significant differences among the investigated farms. Also, the results obtained confirmed that the Salmonella infection in pigs is widespread in swine fattening farms located in Western Romania.

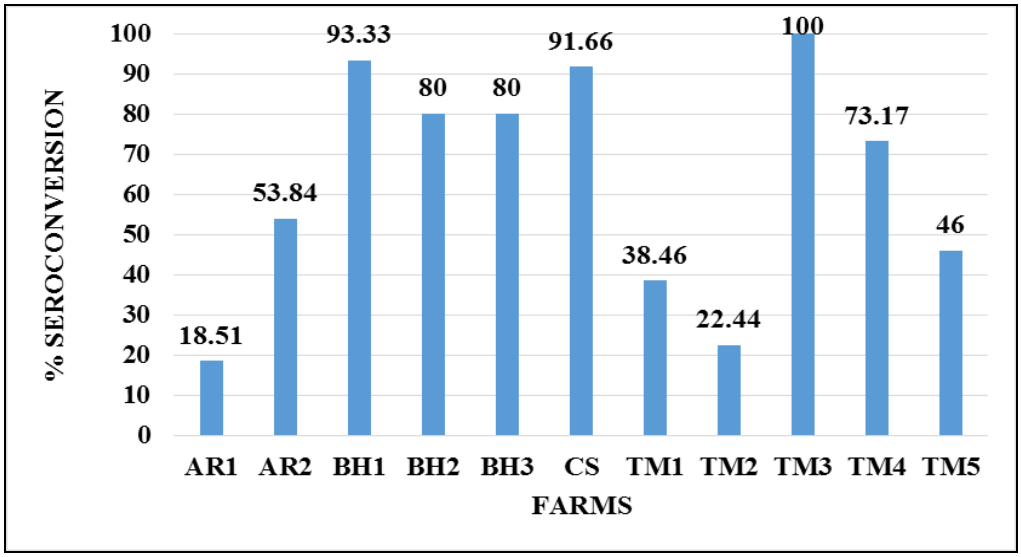

Figure 1. Seroconversion of *Salmonella spp.* in studied farms

Analysis of the results obtained from the serological investigations revealed that the highest rate of seroconversion (above 90%) was recorded in farms BH1, CS, and TM3, reported to the number of samples examined in these farms. In contrast, the lowest rate of seroconversion recorded was reported in farm AR1, which was 18.51%, based on the number of samples being investigated.

Figure 2 shows the distribution of infection with Salmonella spp., in the four Western counties studied. By analyzing the seroprevalence of Salmonella infections at the county level, it appears that there are significant differences among the counties analyzed. Therefore, the values of seroprevalence of Salmonella infections varied between 35.84%, in Arad County, and 91.66%, in Caras-Severin County.

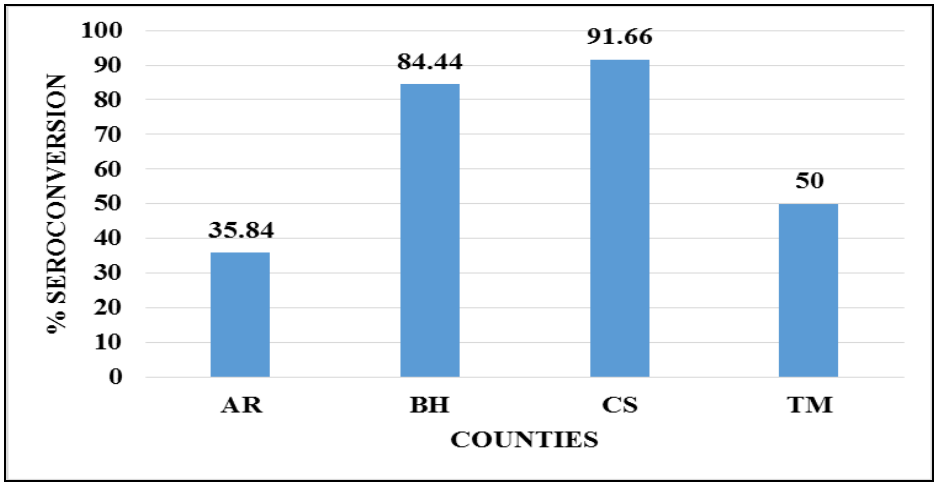

Figure 2. Distribution by counties of seroconversion of *Salmonella* infection

In contrast, the results obtained in our study, compared to similar studies, showed that the value of seroprevalence of Salmonella infections were different.

A study conducted, during a period of five months in Alberta-Canada by Rajic et al., (10), has revealed that from 90 farms studied, 60 (66.7%) of them had at least one positive sample for *Salmonella spp*. The results obtained in this study are somewhat similar with those obtained in our study.

In the present study, the values of seroprevalence of Salmonella infection, in the three age groups studied, within the swine fattening farms varied, ranging from 0% to 100% in the 45 day to 90 day category; 8.33% to 100% in the 91 day to 140 day category; and 14.28% to 100% in the 141 day to 180 day age category.

The research on the epidemiology of Salmonellosis in pigs has focused mainly on assessing the seroprevalence of infection in the finishing-slaughtering phase. However, the pigs, regardless of age, may play a major role in maintaining and disseminating Salmonella bacteria in pig farms as confirmed in this study. A longitudinal study, conducted by Keelara et al., (5), in swine fattening farms, has shown that there was an increase of seroprevalence of Salmonella infections in the final stage of production (finishing period), even though the principles of "all in-all out" were strictly respected. The results of this study highlight the potential role of farms as reservoirs of infection, taking into account that Salmonella bacteria could persist and survive in the external environment for a long period of time. The higher prevalence of Salmonellosis in pigs in the final stages of production represents a major public health concern from a food safety perspective (5).

In a cross-sectional study, the ELISA test was performed on four pig farms to evaluate when pigs became contaminated. Study results showed that seropositive pigs were only found for 20 week old finishing pigs. Also, in this study, the antibody response to Salmonella in piglets was investigated. It was found that maternal antibodies persisted until seven weeks of age and post-Salmonella contamination seroconversion was detected from eight weeks of age and up (9).

In France, a longitudinal survey was conducted by Beloeil et al., in a subclinically Salmonella-infected farrow-to-finish pig farm to describe the time-course of the serological response to Salmonella in growing pigs. It was demonstrated that seroconversion occurred

during the last third of the fattening phase, while shedding was observed during the first half of the fattening phase (1).

Experimental inoculations with Salmonella have shown that the onset of serological response and peak seroprevalence occurred approximately at seven and 30 days postinoculation, respectively. On the other hand, under natural conditions, the serological response had a different course because the pigs were infected at different points in time, with variability in exposure and host response (6).

A study done by Fedora-Cray has showed that about 90% of the examined pigs were seropositive at the age of one week. At nine weeks of age, this percentage fell to 15%, but increased to 52% in the finishing period. Study results suggest that there are fluctuations in the life cycle of Salmonella on farms. Therefore, it is widely accepted that seroconversion does not predict that Salmonella infections are present in slaughthouses (3).

In a study conducted in Catalonia-Spain by ELISA, serum samples obtained from 141 pig farms were tested. The seroprevalence of Salmonella infection had been estimated at 20% in pigs in the finishing phase (8).

A study conducted by Christensen et al., (2008), has revealed that the seroprevalence of Salmonella infection was 11.4% in the finishing period (Christensen et al., 2008).

A study conducted by Kranker et al., has showed that Salmonella occurrence varies between, and in, age groups within herds. Salmonella was predominant in growers and finishers (6).

By comparing the seroprevalence of swine influenza virus infection to that caused by *Salmonella spp.*, it was observed that swine influenza increases the susceptibility of infections caused by *Salmonella spp.* Analysis of the results obtained in this study showed that in farms AR2, TM2, TM3, and TM4, where the viral infection seropositivity values were between 5.88% and 25%, the dynamics of seroprevalence infection caused by *Salmonella spp.* had increased progressively from 53.84% in farm AR2 (Arad County) to 100% in farm TM3 (Timis County).

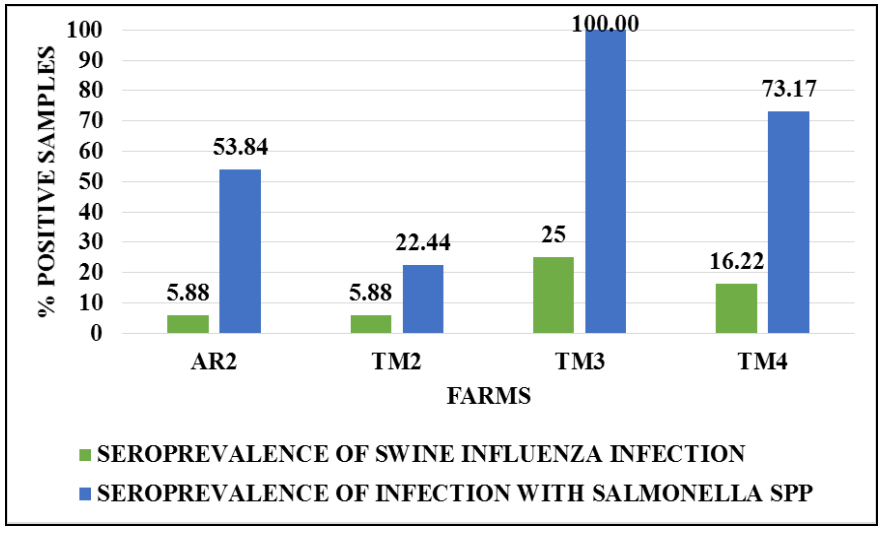

Figure 3. Comparative results of seroprevalence of swine influenza and *Salmonella spp.*

After analysis the data shown in figure 4, it was found that the seroprevalence of Salmonella infection was elevated in farms that were seronegative for infection with swine influenza virus subtype H1N1. The level of seroprevalence in these farms increased progressively from 18.51%, in AR1 farm, to 93.33% in BH1 farm.

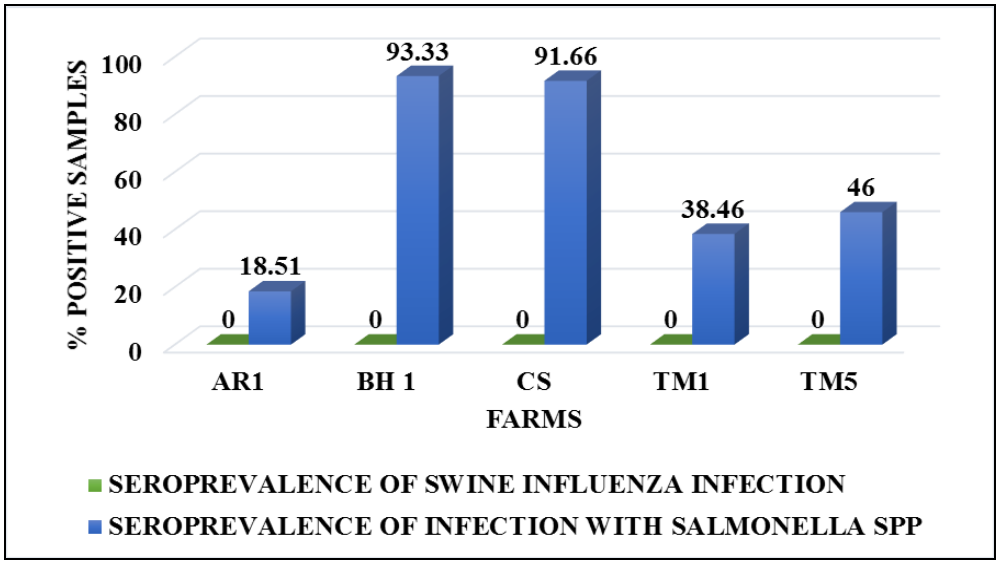

Figure 4. Seroprevalence values of Salmonella infections in farms identified seronegative for swine influenza virus H1N1

Until now, the association between the infection caused by Salmonella bacteria and the infection determined by swine influenza virus subtype H1N1 has not been studied, or at least the information regarding this association is not available in international literature. To the best of our knowledge, this is the first study designed to compare the seroprevalence of both infections. In the present study, the obtained results showed that mixed infections (the presence of both swine influenza virus infection subtype H1N1 and infection with *Salmonella spp.)* has been reported in four farms out of the eleven farms studied (figure 3). However, the interpretation of these results is difficult because the Salmonella bacteria caused infections by colonizing the digestive tract, while influenza virus affects the respiratory tract. The state of immunosuppression, caused by swine influenza virus subtype H1N1, is the only plausible explanation for the increased rate of seroprevalence of Salmonella infection in herds. In other words, it is possible that the virus acted first, taking into account the immunosuppressive effect which this type of virus manifests. As a consequence, a variety of viral and bacterial infections, including infections caused by Salmonella spp., can occur. It should also be emphasized that the existence of dual infections in studied farms depends on both the circulation of the virus and Salmonella bacteria in investigated areas and the possibilities of transmission and dissemination of these pathogens. From a serological point of view, the coinfection is characterized by the presence of antibodies against swine influenza virus and antibodies against *Salmonella spp.* which are present in serum of pigs studied. This aspect was confirmed by our study.

Analysis of the results obtained in this study from serological investigations revealed that, from a statistical point of view, a strong positive correlation between infections caused

by swine influenza virus subtype H1N1 and Salmonella infections, in fattened pigs, was discovered. The correlation coefficient had a value of +0,919.

In all four counties studied, Salmonella infections were widespread, and their expansion was correlated with the spread of swine influenza virus infection. Therefore, it is necessary to apply measures to reduce the economic impact and possible public health issues determined by both infections.

CONCLUSIONS

- By performing ELISA method for the detection of antibodies against *Salmonella spp.*, positive results were obtained in all 11 farms investigated, which belong to Arad County, Bihor County, Caras-Severin County, and Timis County. The value of seroprevalence of Salmonella infection within farms ranged between 18.51% and 100%.
- The values of seropositivity in farms from the four Counties studied varied between 35.84% and 91.66%.
- The values of seroprevalence of salmonella infections in the three age groups studied are variable, ranging from 0% to 100% in the 45 day to 90 days category; 8.33% to 100% in the of 91 day to 140 day category; and 14.28% to 100% in the 141 day to 180 day age category.
- Mixed infections (the presence of both swine influenza virus infection subtype H1N1 and infection with *Salmonella spp.)* were identified in four of the nine farms studied for both types of infection. The seroprevalence of Salmonella infections was increased in farms with mixed infections compared with the farms identified seronegative for swine influenza virus subtype H1N1. From a statistical point of view, a strong positive correlation between infections caused by swine influenza virus subtype H1N1 and Salmonella infections, in fattened pigs, was discovered. The correlation coefficient had a value of +0,919.
- Since Salmonella infections are spread in all four of the Counties investigated, it should be noted that the presence of Salmonella infections in pig farms are an important issue in pig farms located in Western Romania, and have public health, medical, and socioeconomic implications.

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EFFECTIVE TEMPERATURE ASSESSMENT IN LAYING HENS HOUSING

CRISTIAN FLORIN LĂZĂRESCU, IOAN ŢIBRU
Univeristatea de Ştiinţe Agricole şi Medicină Veterinară Regele Mihai I al României din Timişoara, Facultatea de Medicină Veterinară
cristi_lazarescu@yahoo.com

Abstract: *The present study monitored the effective temperature in the housing for laying hens. Temperature and air current speed were determined in 22 different points in a hall of 60,000 birds bred in vertical battery cages on 7 levels. Temperature was calculated in the 22 points and the following were concluded: in points 1, 6, 17 and 22 which were in front of the inlets, regardless their position horizontally-wise, there were differences of 4-5ºC between the air temperature and the effective temperature; in points 2, 5, 11, 12, 18 and 21, found at the middle of the shelter, differences were of 3ºC and in points 3, 4, 19 and 20, found in front of the fans, differences were again of 3ºC.*

Key words: *air temperature, poultry, effective temperature.*

INTRODUCTION

Air temperature, water vapor pressure, and air velocity are some of the most important factors in the physical environment of agricultural animals. In addition, factors related to animal health (i.e., infectious status) and genetics (i.e., transgenic modification) affect the thermal balance of animals and thus their behavior, metabolism, and performance. The range of environmental temperatures over which animals use the minimum amount of metabolizable dietary energy to control body temperature is termed the thermoneutral zone (2, 5). Homeothermic metabolic responses are not needed within this zone (6).

The preferred thermal conditions for agricultural animals lie within the range of nominal performance losses (5, 7). Actual effective environmental temperature may be temporarily cooler or warmer than the preferred temperature without compromising either the overall well-being or the productive efficiency of the animals. Evaluation of thermoregulation or of heat production, dissipation, and storage can serve as an indicator of well-being in relation to thermal environments (8, 3, 1).

MATERIALS AND METHODS

We have studied the housing of 60,000 hens in Timiş County. Birds were bred in ecological batteries on 5 rows with 7 levels each. In every battery there were 50 hens. Microclimate, feeding and watering are automatically done. Ventilation is of tunnel type, with inlets at the end side of the housing, provided with a device for air cooling [honeycomb type], and at the opposite end of the shelter, fans with variable speed. At the middle of each row and midway on the vertical, there is a sensor. Temperature and air current speed were determined for each level in 22 points so that the entire housing is monitored [154 measurements] (9).

Effective temperature was calculated based on the formula (4, 7):

$$13.12+ (0.6215 \times T)-(13.95 \times V \times 0.16)+(0.4863 \times T \times 0.16)$$

where T = air temperature, °C, and V = wind speed, m/s.

Points 1, 6, 17 and 22 were found at the end of the shelter where the inlets are. Points 3, 4, 19 and 20 were at the opposite side where fans were. Points 2, 5, 11, 12, 18 and 21 were

in the middle of the housing. And points 8, 9, 13, 16, 14 and 15 were midway between the middle of the shelter and fans, and inlets respectively.

Results and Discussions

After measurements were done and calculating the effective temperature, in table 1 is shown the air temperature and the effective temperature for each determination.

Table 1

Temperature values and there correspondents

Points		Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
1	**Temp.°C**	21.1	21.2	21.3	21.8	22.5	23.3	23.9
	Air speed m/s	0.34	0.11	0.04	0.05	0.1	0.09	0.09
	E. temp. °C	*26.03*	*26.23*	*26.33*	*26.64*	*27.05*	*27.56*	*27.94*
2	**Temp.°C**	24.6	24.6	25.3	25.9	27	27.5	28
	Air speed m/s	0.38	0.16	0.26	0.22	0.1	0.05	0.04
	E. temp. °C	*28.28*	*28.35*	*28.77*	*29.16*	*29.88*	*30.20*	*30.51*
	Temp.°C	27.6	27.4	27.4	27.5	29.4	29.4	29.8
3	**Air speed m/s**	0.12	0.04	0.07	0.19	0.33	0.32	0.35
	E. temp. °C	*30.26*	*30.14*	*30.14*	*30.19*	*31.41*	*31.40*	*31.67*
	Temp.°C	27.7	27.6	27.7	27.8	28.7	29	29.2
4	**Air speed m/s**	0.08	0.11	0.04	0.04	0.06	0.05	0.28
	E. temp. °C	*30.32*	*30.26*	*30.33*	*30.39*	*30.93*	*31.14*	*31.27*
	Temp.°C	28.6	28.1	28	28.2	28.7	28.9	29.2
5	**Air speed m/s**	0.3	0.11	0.13	0.09	0.22	0.09	0.10
	E. temp. °C	*30.89*	*30.57*	*30.51*	*30.64*	*30.95*	*31.08*	*31.27*
	Temp.°C	24.9	24.6	24.5	24.5	24.7	24.9	25.3
6	**Air speed m/s**	0.22	0.04	0.08	0.04	0.05	0.06	0.06
	E. temp. °C	*28.53*	*28.39*	*28.32*	*28.33*	*28.45*	*28.57*	*28.82*
	Temp.°C	21.7	22	22.4	22.9	23.8	24.4	24.8
7	**Air speed m/s**	0.43	0.11	0.22	0.29	0.28	0.22	0.26
	E. temp. °C	*26.37*	*26.73*	*26.93*	*27.22*	*27.80*	*28.21*	*28.45*
	Temp.°C	26.3	26.4	26.6	27	27.7	28.3	29.3
8	**Air speed m/s**	0.24	0.07	0.26	0.20	0.21	0.07	0.06
	E. temp. °C	*29.42*	*29.51*	*29.60*	*29.87*	*30.31*	*30.70*	*31.33*
	Temp.°C	27.2	27.4	27.5	27.8	28.3	28.7	29.1
9	**Air speed m/s**	0.21	0.22	0.27	0.28	0.35	0.31	0.31
	E. temp. °C	*30*	*30.12*	*30.18*	*30.37*	*30.69*	*30.95*	*31.21*
	Temp.°C	23.4	23.5	23.8	24.5	25.3	25.8	26.3
10	**Air speed m/s**	0.54	0.09	0.28	0.38	0.31	0.19	0.17
	E. temp. °C	*27.44*	*27.68*	*27.80*	*28.22*	*28.76*	*29.11*	*29.43*
	Temp.°C	26	26	26.2	26.8	27.6	28	28.4
11	**Air speed m/s**	0.32	0.12	0.09	0.07	0.09	0.04	0.22
	E. temp. °C	*29.21*	*29.25*	*29.38*	*29.76*	*30.26*	*30.51*	*30.76*
	Temp.°C	25.9	26	26.2	26.5	27.1	27.6	28
12	**Air speed m/s**	0.48	0.11	0.18	0.34	0.16	0.26	0.27
	E. temp. °C	*29.11*	*29.25*	*29.36*	*29.53*	*29.94*	*30.25*	*30.50*
	Temp.°C	24.9	24.8	24.9	25.2	25.5	26	26.4
13	**Air speed m/s**	0.17	0.14	0.11	0.05	0.11	0.08	0.16
	E. temp. °C	*28.54*	*28.49*	*28.56*	*28.76*	*28.94*	*29.26*	*29.49*
	Temp.°C	26.3	26.4	26.7	27.1	27.9	28.4	28.8
14	**Air speed m/s**	0.12	0.04	0.14	0.09	0.06	0.11	0.11
	E. temp. °C	29.44	29.52	29.69	29.95	30.45	30.76	31.02

	Temp.°C	28	28	28	28.2	25.5	28.7	29.1
15	**Air speed m/s**	0.15	0.08	0.11	0.09	0.21	0.16	0.03
	E. temp. °C	*30.51*	*30.51*	*30.51*	*30.64*	*30.82*	*30.95*	*31.20*
	Temp.°C	24.2	24.2	24.2	24.4	25.2	25.6	25.7
16	**Air speed m/s**	0.56	0.35	0.15	0.25	0.30	0.16	0.09
	E. temp. °C	*27.96*	*28.03*	*28.10*	*28.20*	*28.70*	*28.29*	*29.07*
	Temp.°C	19.8	19.8	20	20.3	20.9	21.3	21.8
17	**Air speed m/s**	0.12	0.11	0.09	0.06	0.05	0.07	0.05
	E. temp. °C	*25.34*	*25.34*	*25.48*	*25.69*	*26.07*	*26.31*	*26.64*
	Temp.°C	27.9	28.1	28.2	28.3	28.5	28.6	28.7
18	**Air speed m/s**	0.13	0.11	0.05	0.07	0.05	0.16	0.09
	E. temp. °C	*30.45*	*30.57*	*30.64*	*30.70*	*30.83*	*30.89*	*30.95*
	Temp.°C	26.9	26.9	26.9	27.2	27.6	27.9	28.4
19	**Air speed m/s**	0.06	0.07	0.11	0.13	0.28	0.36	0.44
	E. temp. °C	*29.83*	*29.82*	*29.82*	*30*	*30.24*	*30.43*	*30.76*
	Temp.°C	28	27.8	27.6	27.6	27.7	28	28.3
20	**Air speed m/s**	0.19	0.24	0.22	0.25	0.17	0.11	0.19
	E. temp. °C	*30.51*	*30.38*	*30.25*	*30.25*	*30.32*	*30.51*	*30.70*
	Temp.°C	26.6	26.3	26.2	26.2	26.4	26.7	27
21	**Air speed m/s**	0.17	0.22	0.19	0.24	0.17	0.11	0.09
	E. temp. °C	*29.62*	*29.42*	*29.36*	*29.35*	*29.49*	*29.69*	*29.88*
	Temp.°C	20.2	19.8	19.4	19.1	18.5	18.5	18.5
22	**Air speed m/s**	0.27	0.19	0.33	0.56	0.29	0.19	0.54
	E. temp. °C	*25.49*	*25.29*	*24.93*	*24.57*	*24.38*	*24.44*	*24.18*

Analyzing data from Table 1 the following were concluded: in points 1, 6, 17 and 22 which were in front of the inlets, regardless their position horizontally-wise, there were differences of 4-5ºC between the air temperature and the effective temperature; in points 2, 5, 11, 12, 18 and 21, found at the middle of the shelter, differences were of 3ºC and in points 3, 4, 19 and 20, found in front of the fans, differences were again of 3ºC.

CONCLUSIONS

1. Differences between air temperature and the effective one oscillates depending on the point (level) where hens are positioned at.
2. Differences are between 3 to 5ºC.
3. Mechanical ventilation and the means to direct it must reduce the temperature differences

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A NEW GLOBAL MEDICAL CONCEPT - "ONE HEALTH"

NICOLAE MANOLESCU[1], EMILIA BALINT[2]
[1]- Romanian Academy
[2]- Faculty of Veterinary Medicine, Bucureşti
manolescunicolae@yahoo.com

Abstract: *The authors present a new global medical concept "One Health" that was launched in Bucharest, Romania, in May 2014, by the Federation of European Academies of Medicine (FEAM).*
"ONE HEALTH" *concept demonstrates the imperative necessity of a new medical way of thinking through the reunion of the two medicines,* ***the human medicine*** *and* ***the veterinary medicine*** *into the unique framework of the comparative medicine, to which three more domains of activity for health should be added, in order to include them within the same notion of "medicine", namely,* ***the environmental medicine****, alongside with* ***the food and nutrition medicine****, as well as* ***the occupational medicine****.*
The drawing up of this genuine "medical hexagon" shall provide control over two huge problems of the planet health: ecotherapy –that is, the healing of the environment, and ecoprophylaxis, that is the primary prophylaxis for human and animal diseases.
The environmental medicine represents the "key" link of the whole global medical concept "One Health".

Key words: "one health", comparative medicine, environmental medicine, zoonoses, comparative oncology

INTRODUCTION

The concept, under various forms, extends back to antiquity. The acknowledgement of the fact that environmental factors may have an impact on health was recorded by the Greek physician Hippocrates (460 BC - 370 BC) who promoted the concept that public health depends on a clean environment.

Later on, the Italian physician Giovanni Maria Lancisi (1654-1720), a pioneer in epidemiology, and a veterinarian as well, laid emphasis on the role of the physical environment in the spread of diseases in humans and animals.

"One Health" concept has been recently used in its full form, since 2003, in connection with Ebola Hemorrhagic Fever – an infectious disease of animal origin which proved to be an extremely high-risk pathogen for humans. Under these circumstances, William Karesh has made the following statement: "Human or livestock or wildlife health cannot be discussed in isolation. There is only one health." Together with two colleagues, W. Karesh has subsequently given a series of lectures around the world on the theme of One World - One Health.

THE DEVELOPMENT OF THE CONCEPT

The United States of America

In 2008 the American Medical Association (AMA) and the American Veterinary Medical Association (AVMA) agreed upon the establishment of **"One Health Commission."**

On the other hand, **"One Health Initiative"** is the website that stores news and information on **"One Health".** The promoting agencies, American Medical Association, American Veterinary Medical Association, The American Society of Tropical Medicine and Hygiene, The American Association of Public Health Physicians, The Centres for Disease Control and Prevention (CDC), The United States Department of Agriculture (USDA), and The U.S. National Environmental Health Association (NEHA) as well as more than 800 prominent scientists, physicians and veterinarians around the world (from about 90 countries, including Romania) have all joined the initiative.

Worldwide

- In 2011 the first International **"One Health"** Congress was held in Australia
- In 2013 the second International **"One Health"** Congress took place in Thailand.
- In May 2015, the first Global **"One Health"** Congress was held in Madrid.

The European Union

Since 2008, the European Union has promoted the "One Health" approach and the concept is integrated in a number of EU strategic documents.

The translation into practice of "One Health" in the European countries is to be found as an item under discussion on the agenda of the annual session of the Federation of European Academies of Medicine.

Romania

Starting with 1968, in Romania there have been institutional concerns in the domain of comparative oncology, and, implicitly, of comparative medicine carried out by professor doctor Octav Costăchel and by the veterinary physician, doctor in medicine Nicolae Manolescu at the Bucharest Oncological Institute and the Bucharest Faculty of Veterinary Medicine, respectively.

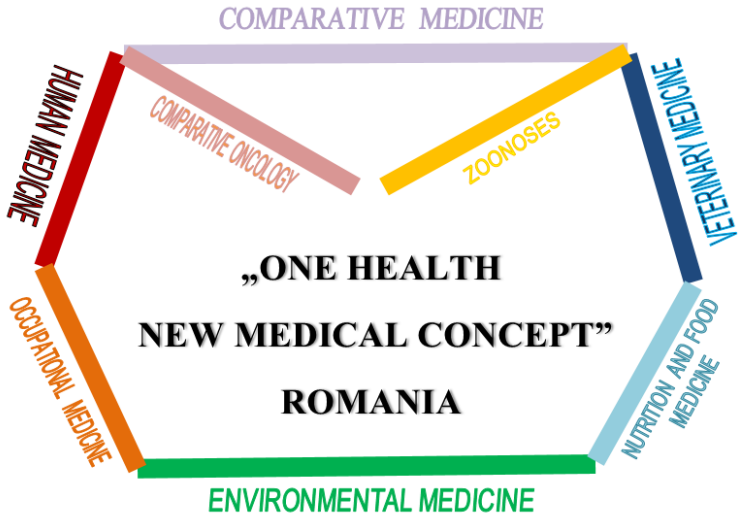
Mention should be made of other institutions and organizations with current preoccupations on this domain:

- The Institute of Comparative Medicine (1999) at The University of Agronomic Sciences and Veterinary Medicine from Bucharest, with subsidiaries in Cluj and Timișoara
- The Romanian Society of Comparative Oncology (1999) that became The National Forum of Comparative Oncology (2010), a subsidiary of The Mediterranean Forum of Comparative Oncology (2009)
- The Department of Comparative Medicine at the Romanian Academy (1995)
- The Department of Comparative Medicine at the Romanian Academy of Medical Sciences (2014)
- The National Forum of Comparative Medicine (2014)

The greatest achievement for Romania was the setting up of **"One Health – New Medical Concept"** Association, as an independent structure in May 2015, when it was granted legal personality.

"One Health"concept demonstrates the imperative necessity of a new medical way of thinking through the reunion of the two medicines, the human medicine and the veterinary medicine into the unique framework of the comparative medicine, to which three more domains of activity for health should be added, in order to include them within the same notion of"medicine", namely, the environmental medicine, alongside with the food and nutrition medicine, as well as the occupational medicine.

The drawing up of this genuine "medical hexagon" shall provide control over two huge problems of the planet health: ecotherapy –that is, the healing of the environment, and ecoprophylaxis, that is the primary prophylaxis for human and animal diseases.



The environmental medicine represents the "key" link of the whole global medical concept "One Health".

COMAPRATIVE MEDICINE: HUMAN MEDICINE AND VETERINARY MEDICINE

It develops and coordinates the issues of each and every medicine. It identifies their overlapping parts and provides human medicine both with elements of prophylaxis for common diseases and experimental medicine issues.

In Romania, comparative medicine has benefited from an institute specialized in comparative medicine (Institute of Comparative medicine – IMC) since 1999.

ZOONOSES

The activity of this element, part and parcel of the hexagon, will focus on four main directions:

1. Emerging zoonoses with reference to sylvatic and exotic animals

(with a special attention to cross-border migration).

2. Emerging zoonoses with reference to farm animals (livestock).
3. Emerging zoonoses with reference to pets.
4. Emerging zoonoses with reference to bio vectors.

COMPARATIVE ONCOLOGY

The Comparative Oncology in Romania, founded 47 years ago, has successfully carried out a prodigious clinical, didactic and scientific activity, materialized in the biannual issue of an English language journal benefiting from an international board of specialists, starting with 1999. Since 2000, the Romanian Comparative Oncology has become founding member, alongside Italy and Spain, of The Mediterranean Forum of Comparative Oncology, with its headquarters in Genoa – Italy.

From 2010 onwards , the scientific research in comparative oncology has been carried out together with our colleagues from Italy, who have all become titulary members of "One Health – New Medical Concept – Romania" Association.

ENVIRONMENTAL MEDICINE

It will lay the actual foundations of the "primary prophylaxis for human and animal diseases" by identifying, monitoring and eliminating the biotic and abiotic pathogen inducing factors in air, water, soil and plants.

All these will be organized on the basis of the results obtained from:

- microbiological analyses;
- clinical and radiological analyses;
- parasitological analyses, within the eco-prophylaxis and eco-therapy of the environment.

  The actual results will be turned to good account by the implementation of "biosentries"

OCCUPATIONAL MEDICINE

This department has in view the accomplishment of actions and activities aiming, among other things, at: the "healthy" and "unhealthy" processing of food, as well the respecting or unrespecting the compulsory relationship between the "health condition" of the members of a given family and their food content.

FOOD AND NUTRITION MEDICINE

This is an essential component of the new global medical concept "One Health", which aims at surveying the "food quality" in order not to contain chemicals (abiotic pathogen inducing factors) harmful to human health (including oncogenes) as well as biotic pathogen inducing factors that pollute food of both animal and non-animal origin, contributing to the improvement of food safety. All these factors will be studied in parallel with the nutrition typology specific to population groups.

The same will be done with animal feed, including the establishment of an adequate nutrition.

THE AIMS OF THE CONCEPT

- It creates and disseminates complex medical policies;
- It brings forth proposals to establish "specific laws" to be presented and voted in parliament;
- It is a body that gathers simultaneously information referring to the medical evolution of zoonoses and the pollution of the living environment, and then disseminates the processed data to The Ministry of Health, The Ministry of the Environment, The National Sanitary Veterinary and Food Safety Authority;
- It aims at realizing the "primary prophylaxis for human and animal diseases";
- It organizes activities for the smooth running of the "One Health" Conference from 2016 in Romania, under the auspices of The Federation of European Academies of Medicine (FEAM).

ACHIEVEMENTS

1. Granting international status to "One Health – New Medical Concept"- Romania Association, under the auspices of **The Federation of European Academies of Medicine** (FEAM).
2. The signing of a series of collaboration agreements. Among these, mention should be made of:
    - Ministry of Environment, Water and Forests
    - Faculty of Veterinary Medicine – Leon, Mexico
3. Following the participation to the ONE HEALTH global Congress in Madrid, we have been nominated to organize The First Interregional European Conference in Bucharest, in September 2016; therefore the first call for papers has been initiated.
4. The first issue of "One Health International Journal" in the English language has been edited (to be released in November 2015), whose board is made up of outstanding personalities.
5. We have laid the theoretical foundations of Environmental Medicine which consists of four elements:
    a. Eco-prophylaxis
    b. Eco-oncotherapy
    c. Onco-biotic inducing factors
    d. Onco-abiotic inducing factors

RESEARCH REGARDING THE CHANGES TO THE BIODIVERSITY OF THE BEGA RIVER WATER FAUNA

ANCA-ANDREEA MARIN[1], BENONI LIXANDRU[1], FLORICA MORARIU[1] , SORIN MORARIU[2], MILCA PETROVICI[3], ADRIAN SINITEAN[3], DUMITRU POPESCU[1]

[1]Banat University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" from Timisoara, Faculty of Animal Science and Biotechnology,

[2]Banat University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" from Timisoara, Faculty of Veterinary Medicine,

[3]West University of Timişoara, Faculty of Chemistry Biology and Geography, Department of Chemistry-Biology, Timişoara,

marin.aandreea@yahoo.com

Abstract: *The benthic macro invertebrates are good indicators of localized conditions, as many of the benthic macro invertebrates have limited migration patterns or a sessile mode of life. Most species have a complex life cycle of one year or more. Sensitive life stages will respond quickly to stress; the overall community will respond more slowly.*

In January 2015, were collected 20 quantitative samples of benthic zones, in the Bega River in order to highlight the links between pollution degree and the saprobionte organisms community.

After the identification of saprobionte organisms, have been performed the density, abundance and frequency of the sample. Based on these values, we can say that the upstream segment waters falls into the category of superior quality compared to the waters of the central segment, especially in the downstream segment.

The aim of this paper is to show the dynamics of the saprobionte organism in relation to sources of contamination of Bega River water.

Key words: *Bega river, saprobionte organism, water quality,.*

Studies of river macrobenthic invertebrates as biological monitoring techniques have been widely reported and described in the literature (Buikema *et al.,* Cairns, 1981; Cairns & Der Schalie, 1980; Cherry & Cairns, 1982; Herricks & Cairns, 1982; Mason & Parr, 2003; Matthews *et al.,* 1982; Ogbeibu & Oribhabor, 2002).

This is due to the fauna organisms being found along the river continuum. Most interestingly, freshwater macroinvertebrate species vary in sensitivity to organic pollution and, thus, their relative abundances have been used to make inferences about pollution loads. In natural pristine rivers, high diversity and richness of species could be found (Armitage *et al.,* 1983). However, high impact due to human activities caused many changes to the assemblages and biodiversity of the river fauna (Hellawell, 1986).

The benthic macro invertebrates are good indicators of localized conditions, as many of the benthic macro invertebrates have limited migration patterns or a sessile mode of life.

Most species have a complex life cycle of one year or more. Sensitive life stages will respond quickly to stress; the overall community will respond more slowly (Moldoveanu & Rîşnoveanu, 2010).

The aim of this paper is to show the effects of human impact (Ghiroda village, potable water treatment station, sewage water treatment station of Timisoara, Sânmihaiu Roman village) on dynamics of macrobenthic invertebrates in Bega River water and the changes to the assemblages and biodiversity of the river fauna.

MATERIAL AND METHODS

In January 2015 were collected 20 quantitative samples of benthic zones, in the Bega River in order to show show the dynamics of macrobenthic invertebrates in Bega River water water and the changes to the biodiversity of the river fauna.

Samples were collected from the upstream, middle and downstream of Timisoara city. The benthic samples were collected with Ekman-sampler with a surface of 225 cm^2 and were subsequently washed with benthic nets (meshes of 250 µm) and stored in 8% formaldehyde (Marin *et al.*, 2014; Lixandru, 2006; Péterfi & Sinitean, 2002; Petrovici, 2009).

The collecting stations (S) were located as follows (*figure 1*):

S1 is located upstream of Timisoara city, near Ghiroda village, upstream of potable water treatment station.

S2 is located upstream of sewage water treatment station of Timisoara.

S3 is located near Sânmihaiu Roman village from Timis County and downstream of sewage water treatment station of Timisoara.

S4 is located near Otelec village, before the border line with Serbia Country.

Figure 1: The location of the sample collecting stations on Bega River water

There have been calculated the density (Di = ni / Sp), the abundance (A= (ni / N)*100) and the frequency (F = Ni*100/Np), where ni represents the total number of individuals for the i series, Sp the total researched area, N the total number of individuals belonging to all species (from the sample or the studied samples), Ni the number of stations within which been identified the subjected species, Np the total number of stations (Sîrbu & Benedek, 2004; Stan, 1995).

RESULTS AND DISCUSSION

Once the laboratory work was carried out, it was identified ten groups of benthic macroinvertebrates (*table 1*): *Oligochaeta* subclass *Hirudin* class, *Lamelibranchiata* class, *Gastropoda* class, *Nematoda* phylum, Diptera order (larvae of the families *Chironomidae, Ceratopogonidae* and *Tipulidae*), *Isopoda* order, *Trichoptera* order, *Odonata* order, *Coleoptera* order and macro invertebrates density is presented in figure 2.

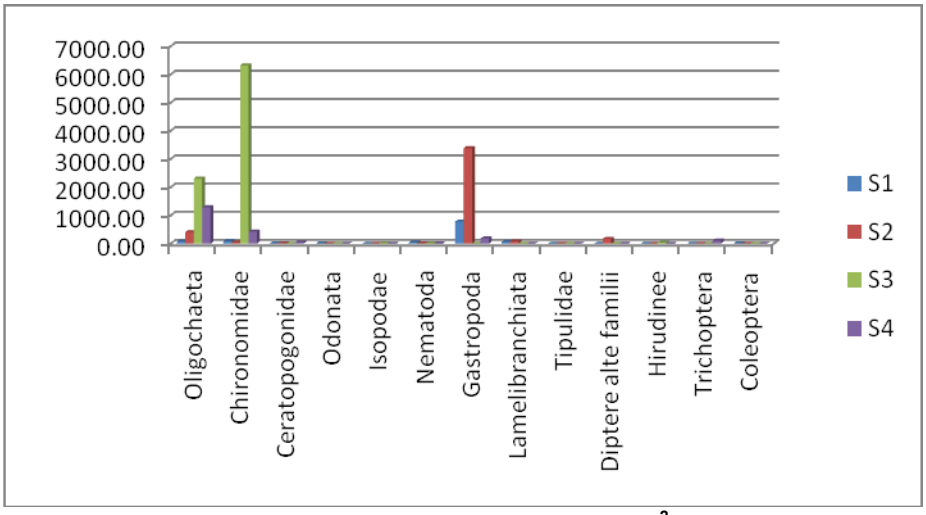


Figure 2: Macro invertebrate's density (individual's m^2) from Bega River

Table 1: Groups of saprobionţi in relation whith the collection stations

Groups	S1	S2	S3	S4
Oligochaeta	x	x	x	x
Hirudinea			x	
Lamelibranchiata	x	x		
Gastropoda	x	x		x
Nematoda	x	x		x
Chironomidae	x	x	x	x
Ceratopogonidae	x	x		x
Tipulidae			x	x
Isopodae			x	
Trichoptera				x
Odonata	x			
Coleoptera	x			

X= the presence

The high tolerance to the impurification of *Oligochaeta* subclass and *Chironomidae* order has been demonstrated in numerous studies (Benbow, 2009; Collier *et al.,* 2010; Courtney & Merritt, 2009; Marchese *et al.,* 2008). These two groups of invertebrates show significantly larger tolerance limits, adapting to various environmental conditions (Lucan-Bouché *et al.,* 1999; Verdonschet, 1999).

At the first station (S1) have been identified groups which Lorenz (2003) considered that they are indicators of unpolluted water (*Lamelibranchiata* class, *Gastropoda* class, *Odonata* order).

Analyzing the macro invertebrates density (fig. 2), we can say that the density of individual's that belong to the Gastropoda at the first stations is smaller than means that the degree of impurification of this station is very small.

At the second station (*figure 2*) we can say that it is notice a increase of the density values of groups which are classified as indicators of unpolluted water (*Lamelibranchiata* class, *Gastropoda* class). At this station we can notice the highest density values of sensitive groups to the pollution.

At the station three (S3) we can observe that the values of density for sensitive groups to the pollution (*Lamelibranchiata* class, *Gastropoda* class) start to decline and increase the density values of groups show significantly larger tolerance limits of pollution (*Oligochaeta* subclass and *Chironomidae* order).

At this station we can notice the highest density values to the groups who have a high tolerance to the pollution.

Also at this station are identified individual's that belong to the *Hirudin* class and *Isopoda* order, groups who show significantly larger tolerance limits at impurification and the disappear the individual's that belong to the *Lamelibranchiata* class, *Gastropoda* class, and *Odonata* order.

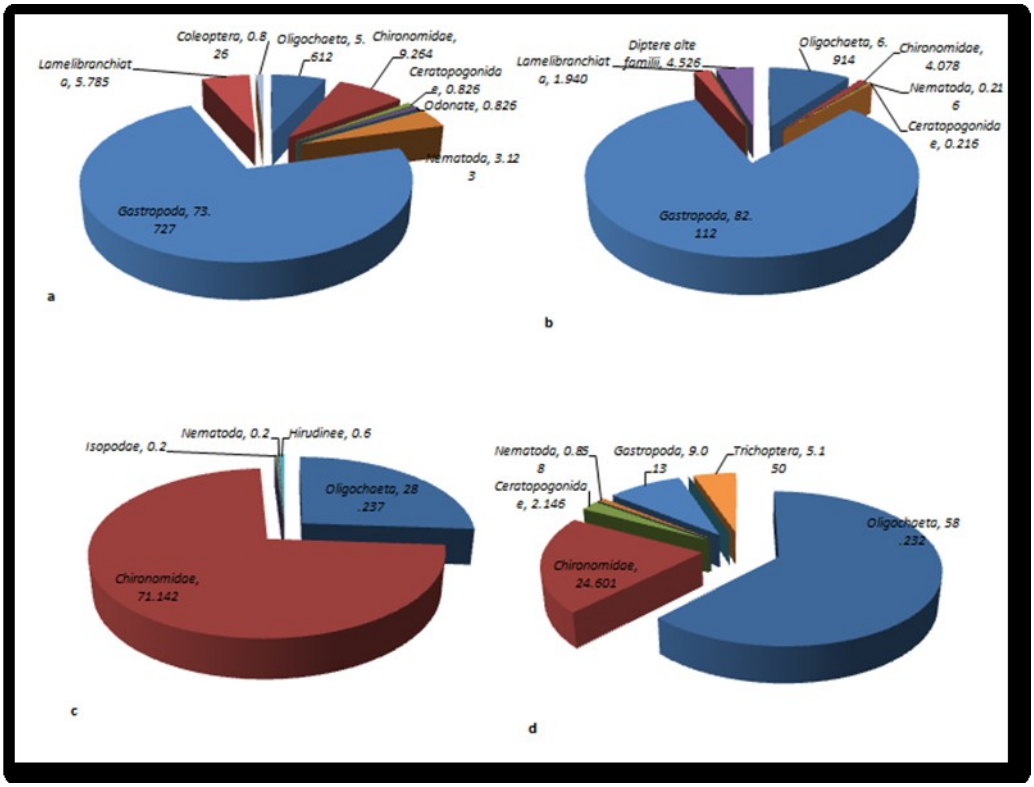


Figure 3: The numerical abundance of the invertebrates group at: a- first station, b- station 2, c- station 3, d- station 4.

The last station (S4) it is located near Otelec village, before the border line with Serbia Country. At this station there were lower densities of all macro invertebrates identified compared to other stations studied (*figure 2*).

Also at this station are identified individual's than belong to the Trichoptera order, macro invertebrates who are considered indicators of the indicators of unpolluted water (Lorenz, 2003).

Analyzing the numerical abundance we can notice that is proportional whit the density, if the density of one group of invertebrate is high then the numerical abundance of this group of invertebrate shows high values (*figure. 3*).

Analysing the frequency (*figure 4*), individual's belong to the *Oligochaeta* subclass show a 80 % frequency at the first station, a 60 % frequency at the second station and a 100% frequency at the three and the last station.

Individual's belong to the *Diptera* order (larvae of the *Chironomidae* families) show a 60 % frequency at the first station, a 80 % frequency at the second station and a 100% frequency at the three and the last station.

Individual's belong to the *Gastropoda* class show a 100 % frequency at the first station, a 80 % frequency at the second station and a 20 % frequency at the last station (*figure 4)*.

Macro invertebrates belong to the *Lamelibranchiata* class has show a 100 % frequency at the first station, a 80 % frequency at the second station and at station 3 and 4 the this invertebrates disappear.

Individual's belong to the *Trichoptera* order has a 40 % frequency at the last station.

Macro invertebrates belong to the *Nematoda* phylum show a 20 % frequency at the first and the second station, individual's belong to the *Hirudin* class has a 60 % frequency at the station three and individual's belong to the *Isopoda* order has a 20 % frequency at the same station (*figure 4*).

Individual's belong to the *Odonata* order show a 20 % frequency at station 1.

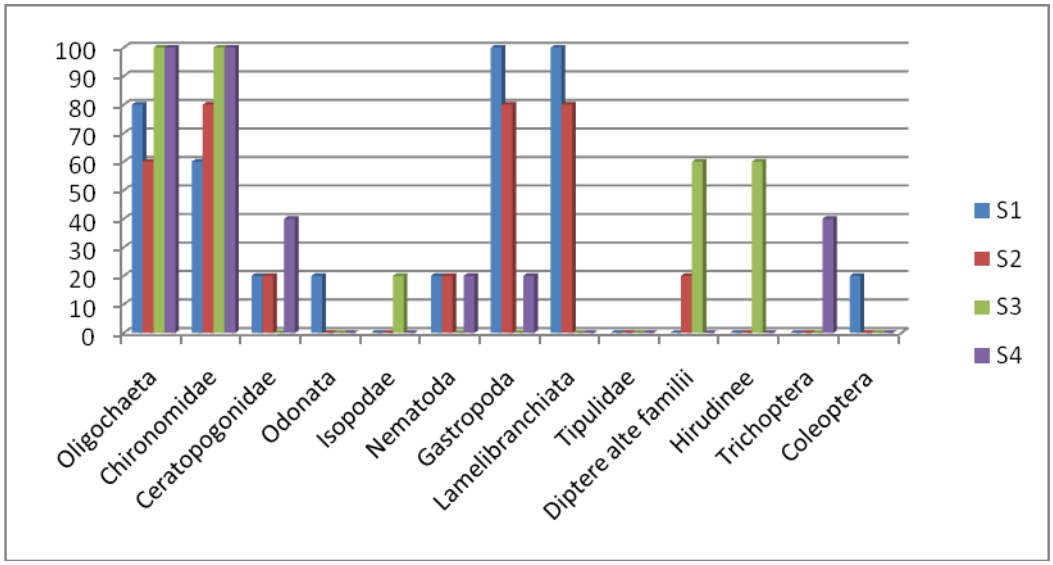


Figure 4: Macro invertebrate's frequency in the Bega River (%)

CONCLUSIONS

After the laboratory work was carried out, it was identified ten groups of benthic macroinvertebrates: *Oligochaeta* subclass *Hirudin* class, *Lamelibranchiata* class, *Gastropoda* class, *Nematoda* phylum, *Diptera* order (larvae of the families *Chironomidae, Ceratopogonidae* and *Tipulidae*), *Isopoda* order, *Trichoptera* order, *Odonata* order, *Coleoptera* order.

In terms of macro invertebrates density and frequency of these saprobionte groups in relation to the point of sampling, show the following conclusions:

- at the first and the second station has been identified groups who are considered indicators of unpolluted water (Lamelibranchiata class, Gastropoda class, Odonata order), and at the three station this groups disappear,
- at the second station was notice the highest density values to the groups who are indicators of unpolluted water,
- at the three station was notice the highest density values to the groups who have a high tolerance to the pollution (*Oligochaeta* subclass, *Diptera* order),
- at the last station decrease the density values of the groups who have a high tolerance to the pollution and appear macro invertebrates who are considered indicators of the indicators of unpolluted water (*Trichoptera* order and *Gastropoda* class).

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THE EVALUATION OF MILK PRODUCTION IN SOWS MAINTAINED IN INTENSIVE SYSTEM AND HOUSEHOLD BASED ON CONVERSION OF THE WEIGHT GAIN OF THE PIGLETS

OGNEAN L.[1]*, GR. ONACIU[2], RAMONA BLIDAR[1], S. MUNTEAN[1], CRISTINA TODORAN[1], S. OGNEAN[3], GH. BEREŞ[4]

[1]University of Agricultural Science and Veterinary Medicine Cluj-Napoca, România
[2]Faculty of Animal Science and Biotechnology Cluj-Napoca, România
[3]Technical University Cluj-Napoca, România
[4]EUROHYB – PIC breeding farm Coroi Sârmart-Mureş, România
lognean@yahoo.com

Abstract: *Background: The investigation and the surveillance of sow's lactation are already major concerns of research and practice in swine growth area. However, the available data regarding the evaluation of milk production in sows and its correlation with the growth performances of the infant piglets are still quite few. Based on these considerations, in this paper we intend to estimate the milk production for two samples of sows based on the conversion of realised gain by the infant piglets in the consumed milk.*

Materials and methods: Regarding the PIC sows (n=18) and the Large White (LW) (n=9), the milk production was estimated by adopting a protocol focused on the couple lactate sow – suckling piglets which consisted of calculating the weight gain realised by the lots of weaners and its processing into consumed milk by multiplying with the conversion factor of 4 L milk/kg live weight. (Hălmăgean, 1984). Finally, the milk/sow/lactation production was determined through equalization with the amount of milk consumed by the lot of weaned piglets. Following the statistical analysis of the resulted data, the productive performances of the couple lactate sow - suckling piglets were evaluated.

Results: The analysis of the resulted data revealed the high productive performances achieved by PIC sows, expressed by averages of 253 L/lactation, respectively 7.9 L/day, which ensured an average consumption of 20.63 L milk/piglet during those 32 days of lactation, in terms of an average number of 12 piglets/lot. The sows from the LW sample had the milk yields lower (244.38 L/lactation, respectively 6.6 L/day) and milk consumption/weaner higher (27.77 L). All this happens at a significant decrease in the average number of piglets/lot (8.8) and the extension of lactation period to 37 days.

Conclusions: The milk production of those two samples of sows exceeded the average yields of this species (5-6 L/day/8 weeks of lactation), reaching outstanding productive performances for PIC sows. All this ensured, during 32 days of lactation, an average consumption of 20.6 L milk/suckling piglet, for an average lot of 12 weaners, making an average yields of 7.9 L of milk/day.

Keywords: *Sows, milk production, suckling piglets, weight gain*

INTRODUCTION

According to consulted bibliographical sources, currently we have more and more data supplied by research into the area of sow lactation, which may open new directions in the rearing of pigs practice (Acie et al., 1999; Sărăndan et al., 2009; Williams, 1993). Although the documentary in this area abounds in data regarding the evolution of sow's milk production and especially of its influencing factors, research and available data on such assessments are still quite rare (Vlasiu et al., 2012).

In this paper we resorted, based on documentation and preliminary research, to estimating the milk production in sows by converting the realised gain of suckling piglets into

consumed milk. This being a sufficiently accessible and faithful method in relation to the provided results, it is more frequently used than procedures focusing on harvesting milk by milking, which are less accurate and more difficult to apply (Hălmăgean, 1984; Ognean et al., 2013). We also note that milking a sow is possible only in the first days after parturision, after which it becomes extremely difficult and dependent upon administration of oxytocin induction milk ejection. (Vlasiu et al., 2012; Ognean et al., 2013).

MATERIALS AND METHODS

For two samples of lactating sows (n=27) the milk production was evaluated by estimating the milk consumption corresponding to the achieved gain by the piglets. The research was conducted in an intensive type of farm on 18 PIC sows forming the sample I and in a microfarm of domestic type, on 9 Large White sows (LW) making up the sample II. It was adopted a procedure based on the use of lactate sow-piglets couple, as unite of study, including: the calculation of the weight gain made by the lots of weaners and its correction by subtracting the gain resulted from the additional consumption, extimated to 240 g concentrates/pig/foddered day; the transformation of the weight gain of weaners into consumed milk, by multiplying with the conversion factor of 4 L milk/kg body weight (Halmagean, 1984); the correction of the total consumption of milk/weaners lot by adding the quantity of milk consumed by the lost piglets, estimated depending on the number and depending on the duration of their breastfeeding; determining the production of milk/sow /lactation by equivalating with the amount of consumed milk by the lot of weaners; the calculation of average production of milk/sow /lactation/sample.

The resulted data were analised statistically with the help of some current tests (GraphPad InStat, OriginPro), finally establishing the individual and average values of daily total production of milk produced by the sows in sample I, during 32 days of lactation, respectively the ones in the sample II, whose lactation lasted for 37 days. At the same time were established correlations of Pearson type, which were interpreted depending on the correlation r coefficient, its field of action from -1 to +1. Based on these correlations the implementation of linear regression in estimating milk production in sows was proposed, which would allow the calculation of a variable depending on the values of another variable (y can be made out from x), meaning the achieving of a certain value of the total gain (x) with a certain milk quantity (y) in the case of our experiment.

RESULTS AND DISCUSSIONS

The summary of our results regarding the appreciation of milk production revealed that the sow lactation is influenced by many factors, intrinsic and extrinsic, the major impact belonging to the race and to the growth system. The analysis of the data regarding the milk production evolution for the researched sows revealed the existence of direct correlations between the influences exercised by this factors, the PIC sows performing individual (189.6 – 280.8 L) and average (252.9 L) values higher than those recorded in the case of LW sows (137.5 – 329.3; 244.3 L) (Tab.1 and 2). An exact confirmation of the higher values of milk production achieved in the case of PIC sows was also the superior level reached by the weight gain of the suckling piglets in this sample, compared to the sample of LW sows. The reaching of this performance is also backed by the fact that, in a short period of breastfeeding

(32 days) the PIC piglets reached an average gain/lot (67.36 kg) higher by 7 kg than the LW piglets (60.35 kg), LW having a longer breastfeeding period (37 days). We consider that this argument justifies entirely the comparison made between gain values achieved by the weaners in the two samples, although there were differences both in the breastfeeding period as also in the number of weaners (12 respectively 8,8). In this context we mention that the comparative evolution of the weight of the piglet lots during the lactation was a precise argument regarding the growth performances achieved by the PIC piglets, which reached gains (50.49 – 76.50 kg/lot) semnificatively higher than those recorded for the LW piglets, although the breastfeeding period for the latter was longer by 5 days (Tab 1 and 2).

The analysis of the data obtained from calculations also reflected the great relevance given to the individual and average values obtained from calculating the daily milk production reached by the sows in the two samples. Based on the values obtained from this parameter we consider that this calculation expressed best the productive performances achieved by the PIC sows, the average production level (7.9 L) reached by these being higher to the one recorded for the MR sows (6.6 L). This uptrend was also mantained after the calculation of average production of milk/sow/lactation, whose level was much higher for the PIC sows (252.9 L) than for the MR sows (244.3 L), athough the former had the lactation shorter. The productive performances of PIC sows from the analised sample are also very well revealed by the size of the weaned piglets (12), from which it can be concluded that these managed to ensure an average consumption of 20.6 L milk/suckling piglet.

From the conducted statistic analysis it was revealed that between the total gain/lot/lactation and the production of milk/lactation, respectively the production of milk/day there are important correlations and highly significant statistically, whose tendencies are expressively ilustrated in the grafic representation of correlations between the gains achieved by the lots of suckling piglets and the daily and total milk productions recorded in the case of the mother sows from the sample I (Fig.1 and 2).

Table 1

The values of weight gain of piglets and of milk production regarding the PIC sows

Sow (Lot)	Piglets/ lot (Nr)	Mean weight/ piglet/^{i} (Kg)	Mean weight/ piglet/^{f} (Kg)	Spore/ piglet/ lactation (Kg)	Spore total/ lot (Kg)	Milk prod./ lactation (L)	Milk prod./ day (L)
1	13	1.738	7.333	5.595	72.735	273.390	8.543
2	13	1.800	7.285	5.485	71.305	267.670	8.364
3	13	1.685	7.283	5.598	72.774	273.546	8.548
4	13	1.738	7.333	5.595	72.735	273.390	8.543
5	12	1.833	6.975	5.142	61.704	230.376	7.199
6	13	1.554	7.292	5.738	74.594	280.826	8.775
7	13	1.769	7.169	5.400	70.200	263.250	8.226
8	12	2.075	7.792	5.717	68.604	257.976	8.061
9	13	1.800	7.646	5.846	75.998	286.442	8.951
10	12	1.866	7.272	5.406	64.872	243.048	7.595
11	13	1.815	7.700	5.885	76.505	288.470	9.014

12	12	1.883	7.633	5.750	69.00	259.560	8.111
13	12	1.800	7.342	5.542	66.504	249.546	7.799
14	14	1.736	6.992	5.256	73.584	275.156	8.598
15	11	2.000	7.645	5.645	62.095	233.310	7.290
16	9	1.733	7.400	5.667	51.003	191.682	5.990
17	9	1.878	7.489	5.611	50.499	189.666	5.927
18	10	1.720	7.500	5.780	57.80	217.500	6.796
Mean	**12.055**	**1.801**	**7.393**	**5.592**	**67.361**	**252.934**	**7.904**
Dev. Std.	**1.433**	**0.116**	**0.231**	**0.196**	**7.948**	**30.024**	**0.938**

[i] – Initially = at parturition; [f] – Finally = at weaning

Table 2

The values of the weight gain for piglets and for milk production by the LW sows

Sow (Lot)	Piglets/ lot (Nr)	Mean weight/ piglet/[i] (Kg)	Mean weight/ piglet/[f] (Kg)	Spore/ piglet/ lactation (Kg)	Spore total/lot (Kg)	Milk prod./ lactation (L)	Milk prod./ day (L)
1	9	1.909	8.039	6.130	55.170	247.800	6.690
2	10	1.763	8.523	6.760	67.600	275.280	7.440
3	9	1.678	8.000	6.320	56.880	247.080	6.680
4	8	1.630	8.196	6.560	52.480	220.150	5.950
5	12	1.446	8.372	6.926	83.110	329.300	8.900
6	10	1.757	8.557	6.800	68.000	275.040	7.430
7	5	1.510	9.682	8.172	40.860	137.520	3.720
8	6	1.473	9.645	8.172	49.030	164.850	4.460
9	11	1.473	7.360	5.887	64.750	302.400	8.170
Media	**8.889**	**1.626**	**8.486**	**6.858**	**59.764**	**244.38**	**6.604**
Dev. Std.	**2.260**	**0.163**	**0.757**	**0.816**	**12.521**	**62.071**	**1.675**

[i] – Initially = at parturition; [f] – Finally = at weaning

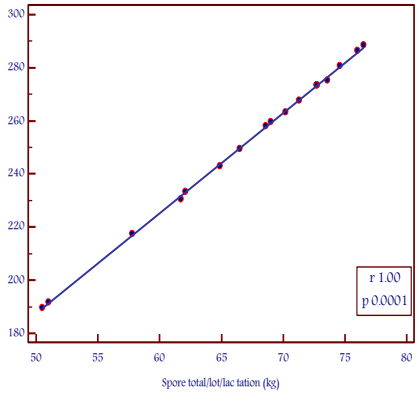

Fig. 1. *The correlational graphic between the gain of the piglets samples and the total milk production of PIC sows*

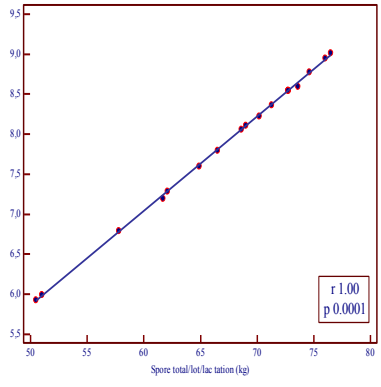

Fig. 2. *The correlational graphic between the gain of the piglets samples and the daily milk production of PIC sows*

After the use of linear regression in the calculus of milk production for the sows in sample I, we got the next equation:

$$y = -1,3385 + 3,7764\ x$$

In which: y is the production of milk/lactation/sow (L) and x the total gain/lot/lactation (Kg).

We consider that putting in practice such an equation will allow the farmer to quickly calculate the milk quantity needed for the achievement of a certain value for the gain of the weaners lot and to apply appropiate measures for the reaching of this desideratum.

We have also found out that the sows which in the previous lactation managed to keep their weight in the physiological limits reached higher milk production and implicitly higher values of weight for the lot of weaning piglets. At the same time we mention that, in the PIC farm, there were provided special conditions which led to the reaching of some high levels of calving rate (85%), of prolificacy (12.7), of the number of calving/year (2.11), of the number of weaners/year/sow (27) and high levels of the weight of the piglets at parturition (1.8 – 2.1 kg).

In practice, it is considered that the sows consume an average of one kg of combined fodder in order to produce one litre of milk, their capacity of ingestion significantly influencing the process of milk production and implicitly the milk quantity intended for the lot of suckling piglets (Sărăndan et al., 2009). On the one hand, although together with the rising of suckling piglets in the lot also rises the milk production of the mother sow, the available milk quantity/piglet decreases (Ladoşi, 2009). On the other hand, the individual milk consumption varies in very large limits, which explains why a good deal of piglets (10-30%) can become underfed (Petroman et al., 2008; Rada et al., 2010). No matter the provided conditions, the sow milk production presents quantitative variations during the lactation, which sign up in a true lactation curve. This includes a continuous growth in the first 3 weeks, with an almost constant plateau in the forth week, followed by a slight decrease in the sixth week and then a rapid decrease until the end of lactation. From the analysis of the sow's lactation curve it appears that, if we quantify the gain achieved by the piglets of at most three weeks old into consumed milk, the quantity of milk produced by the mother sow is best estimated, because in this period of time the weight gain is made almost exclusively through the consumed milk (Hălmăgean,1984; Sărăndan et al., 2008). Although the peak of the milk production is normally reached sometime around the 21th day of sow lactation, because of economical reasons in practice it is resorted to early weaning of the piglets, at three or even two weeks of breastfeeding.

However, it has to be mentioned that the level sow's mik production can also be influenced by a series of managerial factors together with the physiological ones. The fact that a sow with 10 piglets must produce around 10-12 kg milk/day determined some researchers to test the positive effects of porcine somatotropin on sow milk production (Olmos-Hernandez et al., 2010).

In quantitative terms, the milk productive sows can produce up to 350-400 L of milk during a 8 weeks lactation. Moreover, by the nutrients contained in the milk it was observed that, during those 8 weeks of lactation, the sows can eliminate up to 68-77 kg of dried substance, which includes: 23-26 kg protein; 24-27 kg fat; 17.5-20 kg lactose; 3.5-4 kg minerals (Klaver et al., 1981; Polen 2007, cit. de Vlasiu et al., 2014). This very intense rate of

mammary metabolic processes explains why the high milk production of sows can lead to significant weight loss, which have negative consequences when they reach a critical verge (King, 2000; Petroman et al., 2008). In the case of lactating sows it is essential to be provided a proper feeding in cantitative and qualitative terms throughout the entire lactation period. In conditions of nutritional requirements, it is thought that the milk production level reached by the sows from improved breeds can ensure the weight of the piglets to grow by 5 times in the first month, respectively by 10-12 times in the second month of life, at the age of 21 days being dependant on supplementary feeding (Vlasiu et al., 2012; Rada et al., 2010). According to other bibliographical sources, for a sow with 10 piglets the daily milk production is supposed to grow to 10-12 L of milk and the consumption of combined fodder is supposed to grow to 1 kg for each 100 kg of live weight to which it is added 0.4-0.5 kg for each breastfed piglet (Ognean et al., 2010).

After the analysis of the recorded data from our researches we can estimate the highly productive character of the PIC sows sample, average values of 253 L/lactation, respectively of 7.9 L/day. At the same time we notice that these values were consistent to an average consumption of 20.63 L of milk/piglet during those 32 days of breastfeeding and of an average number of 12 piglets/lot. Comparatively, regarding the sows in the LW sample we recorded lower milk production (244.38 respectively 6.6) and higher consumption of milk/weaner (27.77 L), this happening given the conditions of extended breastfeeding to 37 days and given a significantly decrease of the average number of piglets/lot (8.8).

CONCLUSIONS

1. The level of milk production reached higher quotas for the PIC sows, raised in an intensive system (252.9 L/lactation, respectively 7.9 L/day) than for the LW sows, raised in a domestic system (244.38 L/lactation, respectively 6.6 L/day);
2. The milk production of the sows in the two samples surpassed the average of the species, estimated to 5-6 L/day during a lactation of 8 weeks;
3. The productive performances of the PIC sows were most accurately expressed by the achieving of an average of 12 weaners/lot and of providing an average consumption of 20.6 L of milk/suckling piglet;
4. The sows which in the previous lactation maintained their weight between the normal linits reached higher levels of milk production, also reflected in the growth of the weight of the weaning piglets;
5. The preliminary evaluations on the samples constituted by lactating sows with suckling piglets are essential for the implementation of some proper models for the estimation of milk production regarding the source sample in the farms where the reproductive sows are raised;
6. The lactation curve, 32 days for the PIC sows, was characterised by an ascending phase in the first 15 days, a plateau of approximately 10 days and a shlight decrease around the weaning;
7. The losses due to death were prevailing at suckling piglets (45%), comparatively to the other swine categories from the two studied farms.

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ASSESMENT OF WEST-NILE VIRUS SEROCONVERSION IN HORSES FROM IASI AND SUCEAVA COUNTIES

LUANDA ELENA OŞLOBANU (LUDU), ANCA PÂSLARU, IOANA ANTON, DANA CORNEANU, GHEORGHE SAVUŢA
USAMV Iaşi, Faculty of Veterinary Medicine of Iasi,Romania
oslobanule@uaiasi.ro

Abstract: *West Nile virus (WNV) is an arboviral zoonotic agent transmitted mostly by mosquitoes. It is already known that WNV is well established in the south eastern Romania from 1996 but it was only in 2010, after the epidemics of WNV and reemergence in Europe and neighboring countries, that the infection in humans and high level of seroconversion in animals were signaled more often in the north and northeastern area of the country.The aim of the present study was to determine if, after five years from the last major WNV epidemics in Romania, the virus activity is still signaled in some areas from Iaşi (East) and Suceava (North) counties by investigating specific virus seroconversion in horses, known as susceptible to the infection.*

To determine specific anti IgG and IgM antibodies in horse sera we have used two commercial kits manufactured by ID VET Innovative diagnostic (Montpellier, France).

The results showed that in Iaşi County, where an epidemic was documented in 2010 and annually there are cases of WNV neurological infection signaled since then, the virus is still active. We have identified anti IgM specific antibodies in horse sera. In the northern Suceva county our results proved that even no human cases were signaled the WNV infection were evolving in the past years at least in horses and it is probable to be active at least in some regions where the enzootic cycle of transmission is possible.

Key words:*West Nile virus, anti WNV-IgG, anti WNV- IgM, horses, Romania*

INTRODUCTION

West Nile Virus (WNV) is the etiologic agent of the WN fever/encephalitis, a re-emerging zoonosis signaled frequently in the last decades in the whole world. The virus can cause severe neurological disease in horses and humans (some time with fatalities) and mortality in birds, mostly in USA (Zeller H. and al., 2010). It is maintained in nature in a sylvatic enzootic transmission cycle between birds and mosquitoes of *Culex spp*. (Nicolescu G. et al., 2009) but there were epidemics in which the transmission cycle was urban, as it was the one in Romania in 1996 when the first WNV encephalitis epidemics were registered (Savage HM and al., 2000).

Although statistical information indicates that WNV has been affecting humans since the 1950s in Romania the first major and well-documented epidemic dates from 1996 – when 393 cases with laboratory-diagnosed WNV infections, of whom 352 with acute central nervous system infections and 17 fatalities were signaled (Cernescu C. et al., 2000). After 1997, Romania has implemented a system for the surveillance of WNV meningitis/meningoencepalitis and between 1997 and 2009 a number of 78 cases were registered. The cases generally followed a descendent trendline (Neghina AM and Neghina R., 2011).

In 2010, another epidemic of WNV infection was registered in Europe and the pattern of the virus evolution has changed. If until 2010 the lineage 1 of the virus was

considered to be involved in the epidemics in humans and animals, starting with 2010 a distinct lineage (lineage 2) has been spreading in eastern Europe, causing outbreaks of higher virulence affecting a large number of birds, horses and even humans (ECDC, 2011).

Lineage 2 was isolated and totally or partially characterized from mostly of the outbreaks after 2010 in Europe and even in Romania (Sîrbu A. et al, 2011, Kolodziejek J et al., 2014).

In our country a total of 57 cases of West Nile virus infection were identified between July and October 2010 with a case fatality rate of 8.8%. Cases were distributed in 19 districts in the southern, western, central and eastern parts of the country. Molecular investigation in humans revealed that the lineage 2, related to the Volgograd 2007 strain was incriminated for the infections. Neither encephalomyelitis in horses nor mortality in birds has been reported, the majority of human cases were registered in the south of the country known as endemic (Sîrbu A. et al., 2011).

As for the rest of the country, starting with 2010 Romania has register a continuing apparition of human cases in the eastern and central area that stands for the spread of the infection apart from the southeastern territories (Dinu S. et al, 2015).

In horses, the elevated seroprevalence percent registered in localities from Iasi County in 2011 reflects the previous evolution of the infection in this area. In support of this statement is the fact that yearly after that cases of human infection were reported and diagnosed. We can assume that the spread of West Nile virus in the eastern area of Romania can be attributed to a reintroduction of the virus or to an endemisation of the infection in the country due to climate changes (Ludu L. and Savuţa G. 2012).

The diseases transmitted by vectors are in the spotlight of the public health specialists, due to environmental changes generated by climate (temperature and precipitation), geomorphology (altitude) and different land cover/land use. The above mentioned changes permitted the vectors to install on very large areas where pathogens entered the complex transmission cycles within the local ecosystems and there are still a lot of questions regarding WNV patterns of evolution in the eastern part of Romania. We consider that there is a need to determine the risk of WNV transmission in the eastern and northern area of Romania where the WNV is not considered to be endemic but yearly, cases of human encephalitis in humans are signaled (ECDC).

The aim of the present study was to determine if, after five years from the last major WNV epidemics in Romania, the virus activity is still signaled in some areas from Iaşi (East) and Suceava (North)- counties, by investigating specific virus seroconversion in horses, known as susceptible to the infection.

MATERIALS AND METHODS

The assessment of WNV circulation among horses in two counties from eastern Romania was made on 80 samples obtained from 12 localities, five located in Iaşi (47°9′44″N27°35′20″E) and seven in Suceava (24°57′ - 26°40′E 47°4′55′′ - 47°57′31′′ N) counties (Fig.1).

The horses were identified and their moving history inside/outside the county was verified. No horse travelled in the past years away from the home County.

Blood samples (n = 80) from Suceava (SV/n=36) and Iaşi (IS/n=44) were collected in the spring of 2015 by jugular venisection. Upon arrival at the laboratory, samples were centrifuged for at least 10 min at 2000 × g for serum separation and the serum was stored at −20 °C until testing.

To detect the presence of specific anti immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies in horse sera, we have used two commercial kits manufactured by ID VET Innovative diagnostic (Montpellier, France): Id screen® West Nile competition multi-species (for IgG detection in horse sera sampled from Suceava County) and ID Screen® West Nile IgM Capture (for IgM detection in horse sera sampled from Iaşi County).The tests were performed according to manufacturer's instructions.

Fig.1. Sampling areas and number of horses sampled from Suceava (left) and Iaşi (right) counties

RESULTS AND DISCUSSION

The aim of the present study was to determine if, after five years from the last major WNV epidemics in Romania, the virus activity is still signaled in some areas from IS (East) and Suceava (North) counties in Romania by investigating specific virus seroconversion in horses, known as susceptible to the infection.

In SV, where, to our knowledge, no case of human WNV infection was signaled till now, we have intended to determine the presence of specific IgG in horse sera.

Using the competitive ELISA (which is detecting antibodies directed against the PrM-E envelope protein common to flaviviruses) we have detected antibodies in 5 (13.88%) of the 36 sample tested (Tab. 1).

Table 1. Percent of seroprevalence in horses (IgG antibody detection) from localities situated in the northern area of Romania-SV

Localities	Total samples tested ELISA WNC	Positive samples	Seropositivity IgG(%)
Vultureşti	5	2	40.00
Voievodeasa	6	1	16.67
Dărmăneşti	5	0	0

Râşca	5	2	40.00
Preuteşti	5	0	0
Câmpulung Moldovenesc	5	0	0
Păltinoasa	5	0	0
Totals	**36**	**5**	**13.88**

In Iaşi County, where WNV seroconversion in horses (IgG) is well documented after the 2010 and 2011 epidemics and where at least one case of human neurological infection is signaled yearly we have tested for specific anti WNV IgM detection in order to see if in the spring of 2015 there are signals of WNV activity.

Using the West Nile IgM Capture ELISA we have detected antibodies in one (2,27%) of the 44 sample tested in Iaşi county (Tab. 2).

Table 2. Percent of positive horse samples (IgM antibody detection) in localities situated in the eastern area of Romania-SV

Localities	Total samples tested	IgM positive (%)
Scobinti	10	0
Popesti	10	10
Sinesti	10	0
Târgu Frumos	10	0
Sinesti Stornesti	4	0
Total	**44**	**2.27**

The IgM positive horse sample allows us to conclude that WNV is still active in IS given that it is well known that this class of antibodies in the case of WNV infection in horses can be detected until three months after infections.

The most interesting aspect is that in 2014 in IS county there was a case of WNV encephalitis in humans detected in Sineşti, locality placed near Popeşti where the IgM positive samaple was detected in 2015.

CONCLUSIONS

The results obtained in our study are well correlated with the evolution of West Nile virus infection in human population in Iaşi County, where an epidemic was documented in 2010. Since then, cases of WNV neurological infection are signaled annually. We have identified anti IgM specific antibodies in horse sera, meaning that in the spring of 2015 West Nile Virus is still active. Those data confirms that Iaşi County became a risk area for WNV encephalitis and the surveillance of the virus activity is an important issue of veterinarian and public health.

In the northern Suceava County our results proved that, even if, to our knowledge, no human cases were signaled until now, WNV infection were evolving in the past years at least in horses. It is probable that the virus is active, at least in some regions where the enzootic cycle of transmission is possible. A more specific laboratory test has to be used in order to confirm those results and to be sure that we didn't detect some other Flaviviruses specific antibodies that can cross react with WNV being closely related to it.

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EPIDEMIOLOGIC STUDY ON BLUETONGUE VIRUS CIRCULATION AMONG DOMESTIC RUMINANTS DURING 2014-2015 IN ROMANIA

EMILIA POPA[1], ADRIANA ANITA[2], DRAGOS ANITA[2], GHEORGHE SAVUTA[2]

[1]Sanitary-Veterinary and Food Safety Laboratory, Iași

[2] University of Agricultural Sciences and Veterinary Medicine Iași, Faculty of Veterinary Medicine

Abstract: *Bluetongue virus have recently emerged and spread in Eastern Europe. Bluetongue is an infectious, non-contagious disease of wild and domestic ruminants caused by the bluetongue virus (BTV) of the Orbivirus genus in the Reoviridae family. In Romania the first case of BTV infection was detected in Buzău County on August 2014. Since that moment passive and active surveillance (sampling for serological and virological testing for BTV) was established. Our epidemiologic study highlights the BTV spread in domestic ruminant during 2014-2015. The infected animals were detected by real-time rt-PCR, using specific primers for serotype BTV-4.*

Vector control and vaccination is necessary for successful BTV control. In order to control the virus spread, for commercial farms is possible to be applied the vaccination, voluntary at farmers requested. Elimination of the source of infection can be difficult because of the wide host range and the possible existence of latently infected carrier animals. Orbiviruses may be the "point of the spear" in terms of emergence of arboviral diseases driven by climate change and/or other factors.

Keywords: bluetongue, ruminants, PCR

INTRODUCTION

Bluetongue Disease (BTD) is internationally recognized as a notifiable disease with great economical relevance. *Culicoides* (Diptera: *Ceratopogonidae*) biting midges are cyclic vectors of the bluetongue disease virus (BTV). This infectious disease of wild and domestic ruminants is caused by bluetongue virus (BTV), a RNA virus belonging to the *Orbivirus* genus in the *Reoviridae* family. In the summer of 2014 this vector-borne disease was introduced into Eastern Europe. Multiple outbreaks of bluetongue virus serotype 4 in cattle and sheep have been reported across many regions in Albania, Bosnia, Bulgaria, Greece, Macedonia, Romania, Serbia and Montenegro. More recently infected animals have been reported in Croatia and Hungary. In this whole Balkan/Peloponnese/Central Europe region over 5,500 outbreaks have been reported.

Clinical manifestations of BTD are due to vascular damage, which leads to coagulopathy, tissue infarction, necrosis and excessive bleeding (Maclachlan, 2011). In goats and wild ruminants, BTV infection is typically asymptomatic despite prolonged viremia. These host species represent a potential reservoir for unnoticed dissemination of BTV in ruminant populations. Certain strains of BTV can cross the placental barrier, leading to infection of the developing fetus (Sagerman et al, 2011). Hence, abortions and malformations of offspring are frequently associated with infection of pregnant animals with certain strains of the virus. BTD outbreaks induce large economic losses, which are not only caused by morbidity, mortality and production losses in affected animals, but are mainly due to trade restrictions in affected countries.

In order to understand the BTV epidemiology in Romania, the aim of this study is to analyze the maintenance and dissemination of BTV serotype 4 in ruminants during the period 2014–2015.

MATERIAL AND METHOD

The study was made during autumn 2014-spring 2015 on EDTA blood collected from ruminants. The RNA was extracted using RNeasy MinElute Cleanup Kit (Qiagen) in order to detected BTV serotype 4. The quantitative real time RT-PCR protocol used primers targeting the non-structural protein NS1 of BTV (BTNS probe – 6 FAM – GCTTTTTGAGA AAATACAACATCAGTGGGGAT-TAMRA; BTNS – 1-F – TGG CAACCA CCAAACATGG; BTNS-1-R-CCAAAAAA GTC CTCGTGGCA). As reagents for amplification were used the OneStepRT-PCR kit (Qiagen), Trizol reagent (Life Technologies Inc.), Chloroform, Rnasin 40U/µl, MgCl2 25Mm. As special equipment was used: biological safety cabinet, class II, type B2, LightCycler Instrument (Roche Diagnostics) and LightCycler Software, version 2,0 (Roche Diagnostics).

RESULTS AND DISCUSSIONS

The study on BTV-4 consisted in virus detection during autumn 2014 in nine Counties, respectively in three counties in spring 2015. Beside domestic ruminants, in the time of investigations were tested three samples collected from wild ruminants (two Deers and one Llama) that were found negatives for BTV serotype 4.

Table 1

Ruminant samples tested for BTV-4 during autumn 2014

County	No. of samples tested	Bovine positive samples	Bovine negative samples	Sheep positive samples	Sheep negative samples
ALBA	10	-	10	-	-
VASLUI	2	2	-	-	-
IASI *	7	5	-	-	-
NEAMT	12	8	4	-	-
BACAU	3	3	-	-	-
COVASNA	41	35	4	1	1
HARGHITA	9	6	1	2	-
GORJ	331	222	6	103	-
SIBIU	39	34	3	2	-

*two deer samples - identified negative for BTV-4

All samples tested at LSVSA Iaşi during the surveillance program highlighted the presence and dissemination of BTV4 in Romania during August-December 2014. To detect effectively BT disease, nucleic acid based methods have been developed for group specific detection. By real-time RT-PCR the highly conserved genome segment of NS1 was targeted to detect BTV serotype 4 as well as geographic variants within the individual serotype. The real-time RT-PCR based assays are capable of rapid screening of field samples of BTV (Shaw et al, 2007). NS1 (MW: 64 kDa) forms tubules which are characteristic of orbivirus replication (Owens et al, 2004), plays a role in viral morphogenesis and release from infected

cells (Eaton et al, 1988), as well as participates in the upregulation of viral protein synthesis (Boyce et al, 2012).

Table 2

Ruminant samples tested for BTV-4 during spring 2015

County	No of samples tested	Bovine positive samples	Bovine negative samples	Sheep positive samples	Sheep negative samples
VASLUI	1 *	-	-	-	-
HARGHITA	1	-	1	2	-
GORJ	169	166	3	-	-
SIBIU	28	18	10	-	-

* one Llama sample that was identified negative for BTV-4

The results obtained during the surveillance program for BTV in 2015 highlight the remanence of the virus in ruminant livestock in Romania. More recently (September and October 2015) there has been an emersion of the virus in North - Eastern Romanian Counties: Botoşani, Iaşi and Suceava (www.oie.int/wahis_2/public/wahid.php/ Diseaseinformation/ WI). A number of different mechanisms may been involved in the process, including the movement of infected livestock and the passive movement of infected *Culicoides* on the wind. In addition to being spread between ruminants by *Culicoides*, evidence suggests that at least some strains of BTV can be transmitted directly from host to host by one or more secondary mechanisms, including transplacental, iatrogenic and oral transmission, as well as potentially being transmitted mechanically between hosts on the mouthparts of biting flies (Wilson et al, 2008).

CONCLUSIONS

Advances have been made in the knowledge of bluetongue disease and in the strategies for prevention and control. However, it is a complex disease which is expanding in the European Union. As a transboundary disease bluetongue needs to be tackled at international level also with regard to research.

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PREVALENCE OF *IXODID* TICKS ON ANIMALS IN EASTERN ROMANIA

CRISTIAN RAILEANU, DANIELA POREA, GHEORGHE SAVUTA
University of Agricultural Sciences and Veterinary Medicine Iasi,
Faculty of Veterinary Medicine, Romania
craileanu@uaiasi.ro

Abstract: *The prevalence of animal tick species in eastern Romania was studied from october 2013 to september 2014. Adult ticks were collected from 11 localities situated in six different counties. A total of 436 ticks were collected from four animal species (dog, cattle, goat, sheep) and identified to genera and species level. Six tick species of three genera were identified, in which one specie belong to genus Ixodes, two species to genus Dermacentor and three species belong to genus Rhipicephalus. Of all the total ticks collected, Ixodes, Dermacentor and Rhipicephalus represented 45.9%, 7.8% and 46.3% respectively. The tick species encountered were Ixodes ricinus (45.8%), Rhipicephalus sanguineus (22.4%), Rhipicephalus annulatus (19.7%), Rhipicephalus rossicus (4.1%), Dermacentor marginatus (7.3%) and Dermacentor reticulatus (0.4%). R. rossicus is a tick distributed in eastern Europe and western Asia and is considered to be rare. We found 18 ticks R. rossicus (4.1%) (4 females, 3 males and 11 nymphs) on six dogs from a public dog shelter in Tulcea county. According to previous reports it appears that regions from southeastern Romania are the western limit for Rhipicephalus rossicus distribution, a tick that is more common on humans and known to be involved in transmission of pathogens. The other tick species found in this study are more common for Romania, previous reports had shown their importance in carrying pathogens with medical and veterinary importance.*
To conclude, the tick species found in this study can be responsible for transmission of tick-borne diseases in addition to their physical damage during feeding. Therefore, further studies should be carried out on ticks and tick-borne diseases for prevention and control strategies.

Keywords*: Eastern Romania, prevalence, ticks, animals*

Ticks are divided in three families: *Argasidae* (soft ticks with 191 species), *Ixodidae* (hard ticks with 701 species) and *Nuttalliellidae* which has only one specie *Nuttalliella namaqua* (Guglielmone et al., 2010). Ixodid ticks currently consist in 13 genera, most important for humans being *Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Ixodes and Rhipicephalus* (Pfäffle et al., 2013). Ticks are vectors that usually do not use high energy levels in finding host compared to flying vectors; instead they use the energy for survival, which in many cases can happen for many years. Also, unlike other blood-feeding arthropods, every life stage of a tick feeds only once before moulting or, in case of adult females, oviposition (Pfäffle et al., 2013).

Ticks transmit a large group of pathogens that affect humans, companion animals and livestock. Ticks also cause harm to animals through blood loss, general stress and irritation, depression of immune function, damages to the skins (Ghosh et al., 2007). Economic losses due to ticks are mainly because of the diseases which they transmit (Garcia, 2003), financial losses associated with nagging irritation and depreciation of the value of skins and hides are also significant (Biswas, 2003).

Ixodes ricinus is the most widespread tick specie throughout Europe and is vector for many emerging pathogens with human and veterinary importance (viruses, bacteria and protozoa) (Lommano et al., 2012).

Dermacentor reticulatus is an important disease vector in Europe. *D. reticulatus* is a reservoir and the main vector of *Babesia canis* and other piroplasm species and it can be involved in transmitting *Rickettsia* spp., *Anaplasma phagocytophilum, Bartonella* spp., *Coxiella burnetii* and it may also be a potential vector of the tick-borne encephalitis virus (TBEv). Populations of *D. reticulatus* have been documented in Spain, Belgium, Germany, Switzerland, Austria, Poland, Slovakia, Hungary, Romania, Lithuania, Belarus, Ukraine, Moldova and in the European part of Russia (Paulauskas et al., 2015).

Ticks for *Rhipicephalus* genus are widely distributed, the brown dog tick *Rhipicephalus sanguineus* being the most widespread tick in the world (Dantas-Torres et al., 2013). *Rhipicephalus rossicus* is a tick with Eastern European and Western Asian distribution, considered to be a rare tick specie (Sándor et al., 2014).

In Romania, *Ixodes ricinus* is also the most common tick species representing 86.9% of ticks. *Dermacentor marginatus* is second most widespread tick in Romania (9.5 %) followed by *Haemaphysalis punctata* (2.6 %) and *D. reticulatus* (0.02 %) (Mihalca et al., 2012). *Rhipicephalus* spp are also found in Romania, more frequently in southeastern regions, two representative species of the genus, *R. sanguineus* and *R. rossicus* being reported to parasite dogs and less frequently humans (Sándor et al., 2014).

The objective of this study was to determine the prevalence of tick species collected from different animal species, to identify the favorable predilection sites for ticks and to establish the risk areas in eastern Romania.

MATERIAL AND METHODS

Study area is located in eastern Romania and includes 11 collection sites [Vatra Dornei (N 47.34476; E 25.35210); Borca (N 47.16110; E 25.77884); Brusturoasa (N 46.51721; E26.18707); Iasi (N 47.15845; E27.60144); Ibanesti (N 46.77040; E 24.93229); Tulcea (N 45.17162; E 28.79144); Slava Cercheza (N 44.90235; E 28.54727); Sulina (N 45.15674; E 29.65955); Slava Rusa (N 44.85081; E 28.60677); Enisala (N 44.87847; E 28.81922); Salcioara (N 44.78570; E 28.90301)] from six different counties (fig.1). Ticks were collected from october 2013 until september 2014 by using forceps from different regions of the body known to be specific sites of tick attachment.

In total, ticks were collected from 72 animals from four different species: dog – n=38 (52.77%); goat – n=10 (13.88%); cattle – n=14 (19.44%); sheep – n=10 (13.88%).

Collected ticks were preserved in pre-filled 70% ethanol tubes. Date of collections, place of collections, body sites from where ticks were taken and host specie were recorded. Counting and recording took place shortly after collection. Tick specie identification was performed under stereomicroscope using taxonomic key of Perez-Eid (2007). All ticks were conserved at -80^{o}C for further analysis regarding the capacity of ticks in transmitting pathogens.

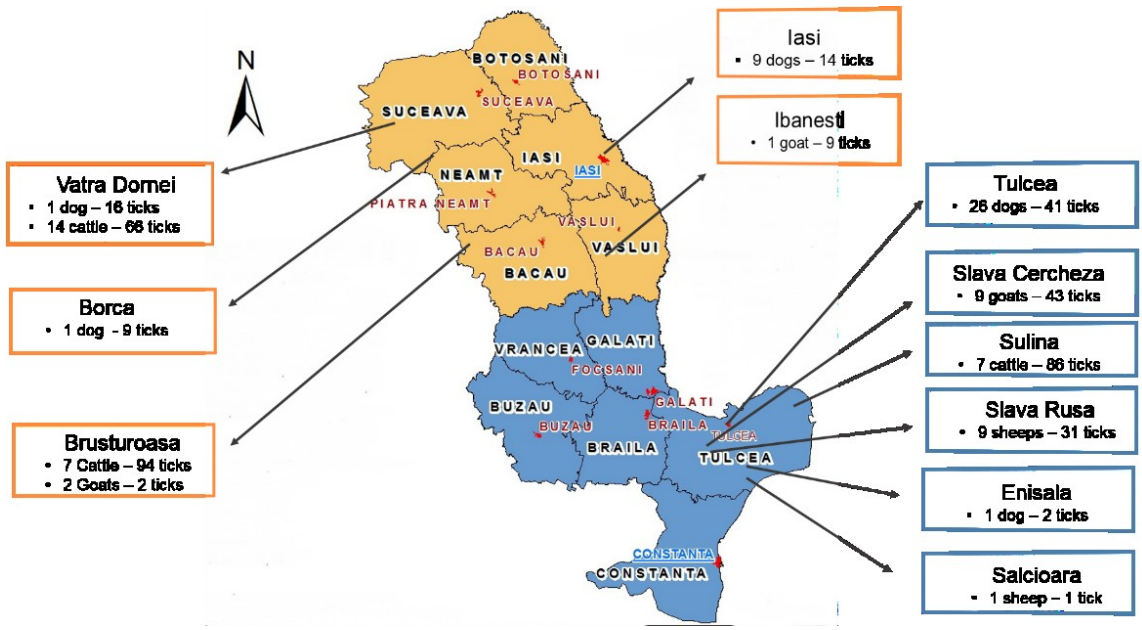

Fig 1 Ticks collection according to sampling sites and host species

RESULTS

Ticks collected from animals are shown by species and the species of the ticks were differentiated based on the morphological features using taxonomic key of Perez-Eid (2007). A total of 436 ticks were collected from which three genera and six species were identified. *Ixodes* (45.8%) was the most abundant and widely distributed genus in all study sites and *Dermacentor* (7.8%) was the least prevalent tick genus identified.

In this study *I. ricinus* was the most abundant tick species and it represented 45.8% (200/436) of the total ticks collected (table, fig. 2). This tick species was found in four study sites and was collected from three different species (cattle, dog, goat).

Second most abundant tick specie was *R. sanguineus* (98/436; 22.4%) (table, fig. 2) collected from southeastern Romania, in Tulcea county. This specie was collected also from three different animal species (goat, sheep, dog).

Rhipicephalus annulatus was collected from seven cattles in Sulina, Tulcea county. We collected 86 adult ticks (86/436; 19.7%) (table, fig. 2) from which 73 were females and 13 were males.

Rhipicephalus rossicus ticks were collected from six dogs in a shelter from Tulcea county (18/436; 4.1%) (table, fig. 2).

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We collected 32 ticks *Dermacentor marginatus* (32/436; 7.3%) from three animal species (dog, goat and sheep) in two counties (Iasi, Vaslui) (table, fig. 2).

Two *D. reticulatus* ticks (2/436; 0.4%) (table, fig. 2) were found on one dog in Enisala, Tulcea county.

Table

Tick species collected from animals according to developmental stage

Specie	F	M	N	Total ticks	%
I. ricinus	145	55		200	45.8
D.marginatus	16	16		32	7.3
D. reticulatus	1	1		2	0.4
R. sanguineus	51	47		98	22.4
R. annulatus	73	13		86	19.7
R. rossicus	4	3	11	18	4.1

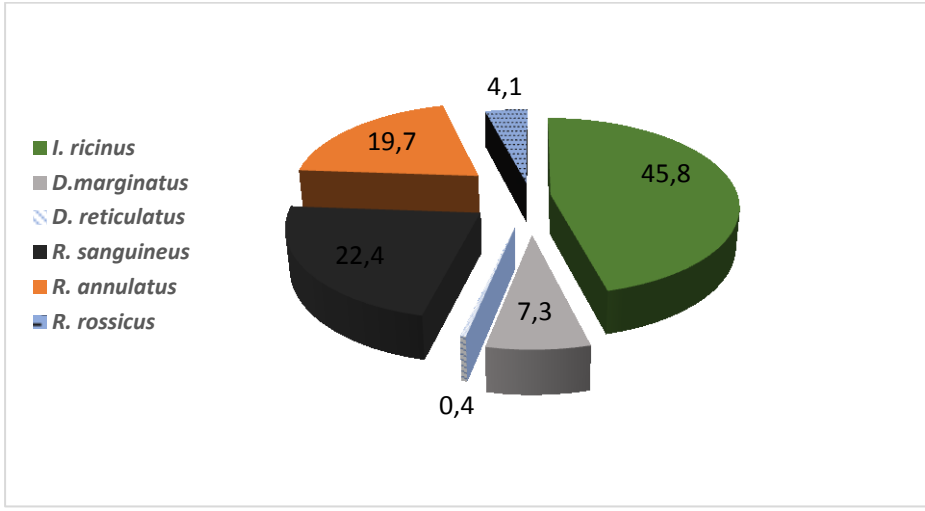

Fig 2 Prevalence (%) of tick species collected from animals

DISCUSSION

The distribution and abundance of the most common tick species infesting animals in eastern Romania vary from one area to another.

Ixodes ricinus is the most widely distributed tick in Romania (Mihalca et al., 2012) and also has a medical importance because it is an efficient vector of many tick-borne diseases (bacteria: *Borrelia* spp., *Anaplasma phagocytophilum, Rickettsia* spp., *Bartonella* spp., etc; viruses: tick-borne encephalitis virus; parasites: *Babesia* spp.) (Paduraru et al., 2012). In our study, we found 200 *I. ricinus* adult ticks (200/436; 45.8%) (table, fig. 2) representing the dominant specie collected in our study. Ticks were collected from three different host species (cattle, dog and goat) from Iasi, Borca, Brusturoasa and Vatra Dornei. The wide spectrum of host species for *I. ricinus* shows the role of this tick specie in transmitting pathogens and demonstrate the massive infestation of hosts with *I. ricinus*; the distribution of *I. ricinus* in eastern Romania still needs to be investigated.

Rhipicephalus sanguineus is a three-host tick that parasites dogs which are primarily hosts but also can be found occasionally on other hosts, including humans. *R. sanguineus*

ticks have a wide distribution around the world and are known vectors for pathogens such as *Babesia canis, Ehrlichia canis* and *Rickettsia conorii.* According to literature reports it seems that interaction between humans and *R. sanguineus* is more common than it is actually recognised (Smith et al., 2011). We found 98 (98/436; 22.4%) (table, fig. 2) *R. sanguineus* adult ticks on three host species (sheep, goat and dog) from three sites, all located in Tulcea county. Southeastern part of the country seems to be a suitable habitat for development of this specie.

A total of 86 *Rhipicephalus annulatus* adult ticks (86/436; 19.7%) (table, fig. 2) was collected from seven cattle in Sulina, an eastern locality of Tulcea county. *R. annulatus* together with *R. microplus* are known as main vectors for *Babesia bovis* (Bock et al., 2004). Unpublished data revealed high number of *Babesia* infected cattle in this area. Further research is needed to confirm the medical and economic importance of *R. annulatus* in infesting cattle and also their role in transmitting pathogens.

Rhipicephalus rossicus ticks were collected from six dogs in a public shelter in Tulcea. It appears that southeastern Romania is the western border of this specie distribution. Previous studies showed high infestation with *R. rossicus* in dogs from Danube Delta (Sándor et al., 2014). We confirm the presence of this tick specie on dogs from southeastern Romania, historically described as vector for Crimean-Congo haemorrhagic fever (CCHF) and call for further research to determine its distribution and role as a vector for pathogens.

Dermacentor marginatus ticks were collected from three different animal species (dog, goat, sheep) from two localities (Iasi, Ibanesti) (table, fig. 2). We collected 32 adult ticks (32/436; 7.3%) (table, fig. 2). We also found two *D. reticulatus* tick on a dog in Enisala, Tulcea county. *Dermacentor* spp ticks are known as vectors for pathogens but there are rare publications regarding their role as vectors in Romania.

We succeded by sampling a relatively small number of animals, to collect six different tick species [(*Ixodes ricinus* (45.8%), *Rhipicephalus sanguineus* (22.4%), *Rhipicephalus annulatus* (19.7%), *Rhipicephalus rossicus* (4.1%), *Dermacentor marginatus* (7.3%) and *Dermacentor reticulatus* (0.4%)] from three genera. This variety of tick species represents a high risk for infested hosts (animals, humans), being capable to transmit bacteria, viruses and parasites. They can also produce economic losses by harming animals through blood loss, general stress and irritation, depression of immune function and damages to the skins.

In order to limitate their impact, tick control strategies can be achieved by attacking one or more larval phases along the life cycle chain FAO, (1984). In addition to acaricide applications, appropriate livestock management, zero grazing and exploiting genetic resistance tick infestation are recommended.

Ticks distribution is determined by a complex interaction of factors such as host density, climate, host susceptibility, grazing habits and pasture-herd management (Tessema and Gashaw, 2010). Therefore, further studies in the distribution pattern of tick species and factors responsible for their distribution are necessary for understanding the issue and to improve control strategies.

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AN INFECTIOUS EPISODE OF EMBRYONIC DEATH TO PREGNANT VIPERS IN CAPTIVITY

CRISTINA RIMBU[1], ELEONORA GUGUIANU[1], CATALIN CARP CĂRARE[1], CRISTINA HORHOGEA[1], CARMEN CRETU[1], MARIANA GRECU[1], OANA CIOCAN[1], RITA GOLBAN[3], CATALINA SANDULEANU[2]

USAMV Iași, [1]Faculty of Veterinary Medicine, 8, Mihail Sadoveanu Alley,
USAMV Iași [2]Faculty of Animal Husbandry, 8, Mihail Sadoveanu Alley,
[3]The State Agrarian University of Moldova, Chişinău
crimbu@yahoo.com

Abstract: *Vipers bred in captivity for economic benefits or recreational request, primarily, strict conditions for microclimate. Its disruption in accommodation spaces can enable opportunistic pathogens, with major consequences.*

One such episode occurred in a farm of vipers in Iasi, with a reduced livestock of vipers that survived in conditions apparently own for their growth and development. All pregnant vipers and youth from livestock initially had breathing problems. The livestock held 20 adult vipers of which 10 (50%) were pregnant. Of these, five vipers had a general state of advanced disease. It disposed their slaughter in order to establish a diagnosis. On necropsy examination, all pregnant females had in the embryo sac and in different proportions, dead young, young stopped from embryonic development and rarely live young. Samples were taken from the living and dead embryos, and from organs.

Following microbiological examination was isolated in pure culture and identified on the basis of cultural, morphological, biochemical and pathogenicity, Enterobacter cloaceae species. Sensitivity to variations in microclimates and decreased immunity to pregnant vipers allowed multiplication and action of Enterobacter cloaceae species, an opportunistic pathogen responsible for various infections in reptiles, fish and numerous mammals.

Key words: *snake, pregnant viper, opportunist pathogen, infection*

INTRODUCTION

Breeding vipers in captivity has become a profitable economical alternative in Romania. Horned viper (*Vipera ammodytes*) is one of the preferred species for breeding.

Their ability to adapt to captivity is variable depending on the specific microclimate, which must imitate the natural microclimate as well. Even in these conditions, the vipers are forced to change their behavior. Variations in temperature, humidity, lighting, the substrate used in terrariums, push the limits of their physiological activities, influencing the growth and reproduction of vipers. The production of venom is determined and depends on these key factors. These conditions create the prerequisites for a nonspecific pathology of the natural environment, which negatively influences the economic purpose for which the vipers are grown in artificial conditions.

Captive snakes are sensitive to all kinds of infectious agents that cause different infections: dermatitis, stomatitis, pneumonia, enteritis, septicemia etc. (Ciudin E., 2006, Guguianu E., 2011).

Some authors believe that the most important factor is the quality of the terrarium substrate. A too wet substrate allows the accumulation and proliferation of different pathogens or parasites. The use of drying substances in the substrate to keep it dry can cause

respiratory and dermatological disorders (Rossi J.V., 2005; Zwart P., 2001,Gábor Árpád CZIRJÁK, 2015). Ingestion of parts of a rough substrate traumatizes the oral mucosa and skin, creating gateways for opportunistic microorganisms. In the absence of adequate treatment, infection leading to sepsis generalizes. In the absence of adequate treatment, infection generalizes leading to sepsis.

Other authors sustain that most bacterial infections are endogenous, etiologic agents being common germs conditioned pathogens or opportunistic, that express consecutive pathogenic potential intervention of favorable factors. In these conditions, the main factors incriminated in causing infections of portage are thermal discomfort, improper humidity, phonic stress and failure in exploiting technology (Page I.A., 1966, Guguianu E., 2011).

Bacterial infections can develop such as single entities or concomitantly with viroses determined by paramyxoviruses and specific retroviruses of reptiles, either as a complication of them either as synergistic infections (Johnathan K.L., 2008. Guguianu E., 2011).

MATERIAL AND METHOD

The biological material consisted of five pregnant vipers: 4 dead vipers and viper was sacrificed to establish a certain diagnosis (figure 1). The history showed that the vipers were withdrawn, did not consume food and water that would have endangered the life offspring of vipers, which depend in the womb of the female lipid reserves.

Figure 1. Pregnant vipers

Bacterial isolation and identification protocol consisted in following the classical steps specific to bacteriological examination. Samples were taken from the heart and embryos and inseminations have been performed on various culture mediums: nutrient agar, nutrient agar supplemented with horse blood agar TSA (Tryptone soy agar). The samples were incubated at 25 ° C for 48 hours.

The isolated strains were identified based on cultural, morphological and biochemical aspects. To test the metabolic activity were used oxidase strips and API's galleries for the detection of *Enterobacteriaceae.* Following these investigations, were isolated and identified strains of *Enterobacter cloacal*, in pure culture, the samples taken from sacrificed viper (heart and embryos) as well as from 2 of the 4 pregnant, found dead in terrariums.

Potential *Enterobacter cloacae* pathogenic strains was assessed by haemolysis test on agar with sheep blood and by experimental infection in mice (0.1-0.2 ml suspension culture in saline, sc). Antibiotic susceptibility profile was determined by antibiogram - diffusion

method, using Müller Hinton agar medium also oxytetracycline, amoxycillin, enrofloxacin, ciprofloxacin, amoxicillin-clavulanic acid, amikacin, gentamicin, chloramphenicol, flumequine, trimethoprim, ceftazidime also spectinomycin microcomprimate

RESULTS AND DISCUSSION

The vast majority of infections described in reptiles were by bacterial nature.

From history, it appears that the prostration state of vipers it is not the consequence of an obvious technological actions. Due to the absence of clinical signs exteriorization which can capture the animal in the early phase of the disease, changed behavior of vipers was notified late. From the viewpoint of the breeder, zoohygiene and environmental conditions were appropriate, food and water for consumption was from the same source. Cycle feeding pregnant females was done according to the physiological status and it was present in the vivarium the heat source. The substrate used consisted of chips and fir branches.

The five pregnant females bred in captivity maniested an acute form of the disease characterized by embryonic death and septicemia (figure 2 a and b; figure 3)

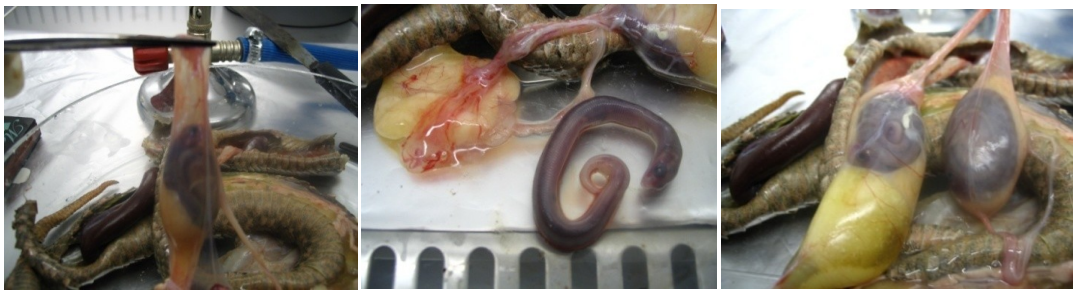

Figure 2 a and b. The aspect and position of the viper embryos in the second month of gestation and uterine wall congestion

Inseminations made from blood collected from the heart of pregnant viper and embryos, on TSA agar and blood agar, MacConkey medium, resulted in the isolation in pure cultures of alpha hemolytic strains of *Enterococcus cloacae* (Figure 4).

On microscopic examination of smears made from the obtained colony, were observed Gram negative bacilli and coccobacilli, medium sized, without grouping feature (figure 5a and figure 5b).

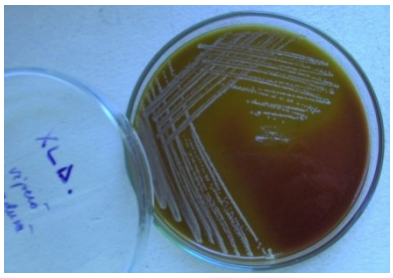

Fig.3. The congested aspect of the viper embryos

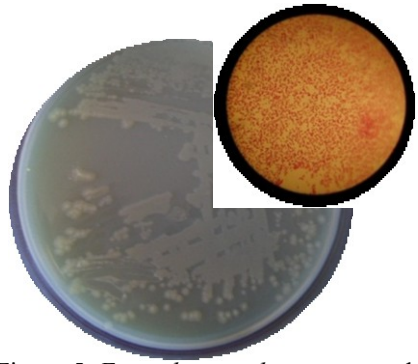

Figure 5. *Enterobacter cloacae* – a. colony morphology TSA agar; b- morphology Gram stain

Figure 4. *Enterobacter cloacae* –Mac Conkey medium

The isolated strains were negative for oxidase test and testing metabolic activity on API 20E test led to confirmation of the *Enterobacter cloacae* species (figure 6).

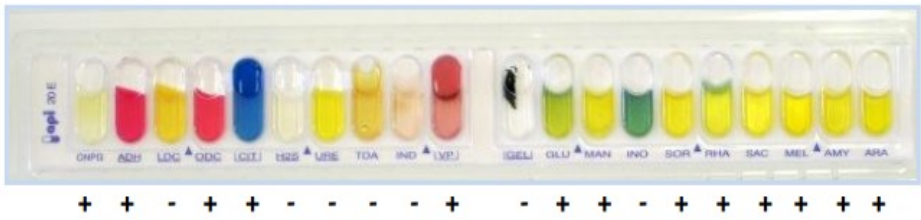

Figure 6. Biochemical characteristics of the *Enterobacter cloacae* isolates using API 20 E

The pathogenicity consisted in the ability of the isolated strains to produce alpha hemolysis and experimental infection in white mice, after their deaths that occurred within 72 hours.

The strain susceptibility testing for *Enterobacter cloacae* to antibiotics by antibiotic-diffusion method, indicated sensitivity to enrofloxacin, ciprofloxacin, gentamicin, amoxicillin, amikacin, ceftazidime and spectinomycin. Compared to other antibiotics tested (tetracycline, chloramphenicol, flumequine, trimethoprim), showed moderate sensitivity or resistance.

The reference literature is limited in studies on embryonic mortality of horned vipers reared in captivity.

Enterobacter cloacae is an opportunistic pathogen, acting on individuals with favoring physiological condition (gestation, age) or immunosuppressed (disease states, stress). Being a germ with an ecological intestinal niche contaminates and resists in the ambient environment. It is recognized as being responsible for respiratory infections, skin and soft tissue infections, urinary tract infections, intra-abdominal infections, eye infections, etc. (Stephen J. D., 2006).

A similar episode took place in the same batch of vipers, when infection with Enterobacter performed primarily with severe respiratory infections, mouth infection and death by septicemia (Guguianu E., 2011). Conducting on the same farm of vipers, of the two *Enterobacter cloacae* infection episodes, shows the persistence of favorable factors (unsuitable microclimate conditions, hygiene, nutrition, etc.) or technological malfunctions that are not recognized by the technologist.

Also, the location of the infection in the genital tract and correlated to the lack of other anatomopathological lesions, is guiding us to the possibility of the occurrence of cloacal microlesions that created gateways to this opportunistic germ.

Repetitive infections with these germs can be difficult to manage due to the selection of strains with multiple resistances, *E.cloacae* being known that has the ability to produce beta-lactamase (Conceicao T.,2004).

Therefore, appropriate treatment and the ability of these organisms to develop drug-resistant strains make the therapy to be complicated and with little chance of success.

Captive snakes are vulnerable to all kinds of infectious agents and the state of gestation increases the sensitivity to these pathological entities.

CONCLUSIONS

1. *Enterobacter cloaceae* it is an opportunist pathogen, with intestinal ecological niche that contaminates and resists in the ambient environment.
2. Captive vipers are sensitive to the inappropriate hygiene and microclimate conditions favoring the proliferation of the opportunistic pathogen germs.
3. Whereas the infection evolved mainly with embryonic death and septicemia, can be suspected a direct contamination with *Enterobacter cloacae* in the cloaca through microlesions created accidentally.

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THE INFLUENCE OF FEEDING SUGAR SYRUP WITH EGG ADDITION BASED SUPPLEMENTS UPON LATEN INFECTION IN BEES' MAJOR BACTERIAL DISEASES

AGRIPINA ŞAPCALIU[1], VASILICA SAVU[1], CRISTINA MATEESCU[1], MARIA MAGDICI[1], ION RADOI[2], CARMEN NEGOIŢĂ[2]

[1] Beekeeping Research and Development Institute of Bucharest, Romania
[2]University of Agronomical Sciences and Veterinary Medicine Bucharest
sapcaliuagripina@yahoo.com

Abstract: *During the inactive period (winter), when the reserve food is insufficient to raise the brood and feed the bee families, corn syrup based solid food supplements are fed with various added solid nutrients. The research aimed to establish the influence of sugar syrup with egg addition based supplement food upon the health of bees infected with major bacterial diseases etiologic agents. Samples were taken from adult bees and brood from two apiaries in the South and South-East of Romania that were monitored as part of a program monitoring the health of 202 initially clinically healthy bee colonies in the South and South-East of Romania, by comparison to 15 witness bee colonies in both apiaries that were not fed sugar with egg addition. Bacterioscopical and bacteriological tests prior to feeding sugar syrup with egg addition indicated sporadic presence of bacilli in bees' intestine in the samples. 48 hours after the syrup was fed, bacterioscopical and bacteriological tests were repeated. The following resulted from the clinical examination in both apiaries after feeding the sugar syrup with egg addition: large mortality, up to 50% in each colony, trembling bees, paralysed bees, bees crawling in front of the hive, cease of oviposition, strong colonies affected 92-100%. Bacterioascopic and bacteriologic results emphasized a boom of bacilli in bees' intestine as well as the presence of Paenibacillus larvae spores in the microscopic and bacteriologic examination for confirmation. Clinical testing after feeding the sugar syrup with egg addition indicated accute evolution of unspecific symptoms as compared to the witness colonies that had no clinical changes. Laboratory tests (bacterioscopical, bacteriological) confirmed a massive presence of bacilli and Paenibacillus larvae spores in the tested bees, and isolated bacilli in the witness sample. Symptoms started as result of the altered microbe balance in bees' intestine and of the isolated presence of bacilli noticed by direct microscopic testing.**Acknowledgements„This work was supported by a grant of the Romanian National Authority for Scientific Research, CNDI–UEFISCDI, project number PN 157/2014"*

Keywords: *Apis mellifera***,** *bacterial diseases, food supplements*

INTRODUCTION

In Romania, the supplementary feeding of bees is used by beekeepers both in case of insufficient honey reserves in the winter, and when it is necessary to obtain bee brood at the end of the inactive season (1, 2, 5). To stimulate brood growth, complex food supplements are needed that are quality sugars, proteins and lipids rich (6). The egg is a valuable source of these nutrients, an easy alternative to pollen and honey cake. Depending on the adapting capacity of bees' intestinal microflora, using egg-based supplements incorporated into sugar syrups may not always generate favorable effects. The purpose of this study was to establish the influence of egg-added sugar syrup supplements upon clinically healthy bees that however carry etiological agents of major bacterial diseases.

MATERIALS AND METHODS

By the end of the active season, adult bee and brood samples were collected from two apiaries in the South and South-East of Romania that were monitored as part of the bee health monitoring program. The study covered 217 clinically healthy bee colonies in the beginning of the inactive season. To stimulate brood growth, 202 colonies received egg-added sugar syrup, while the witness lot representing 15 bee colonies had only reserve honey (table 1). The bees were monitored before and after feeding the supplements by clinical and laboratory examination, respectively by direct microscopic examination (for ecto and endo-parasites, by bacterioscopy) and by bacteriological examination (3, 4).

Table 1

Distribution of monitored bee colonies

Apiary I		Apiary II	
Total number of monitored bee colonies 217			
Number of selected bee colonies		Number of selected bee colonies	
128		89	
Stimulation feeding (sugar + egg syrup)	Honey reserve feeding (WITNESS)	Stimulation feeding (sugar + egg syrup)	Honey reserve feeding (WITNESS)
120 (94%)	8 (6%)	82 (92%)	7 (8%)

RESULTS AND DISCUSSIONS

Previous clinical and parasitological examination, microbiological and toxicological examination showed no evolution of other bee diseases, and the presence of major disease etiological agents was noticed (*Figura 1*).

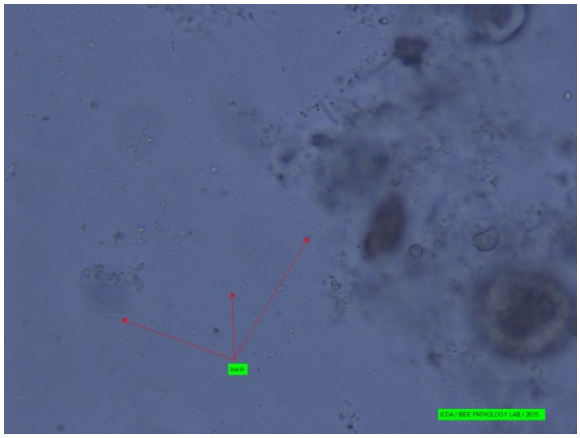

Figura 1. Vegetative forms of etiological agents in the loca before feeding sugar + egg syrup (direct microscopic examination of intestine content) x 400 (Original)

24-48 hours after feeding egg-based supplements, clinical manifestations appeared: trembling bees, paralyzed bees, bees crawling in front of the hive, oviposition ceased, strong colonies were affected. Moreover, clinical signs were accompanied by high mortality of up to 50% of each colony (table 2).

Table 2

Distribution of monitored bee colonies by depopulation degree

APIARY I				APIARY II			
Treated colonies		Witness		Treated colonies		Witness	
120		8		82		7	
No. of colonies with total depopulation after 48 h (100%)	No. of colonies with partial depopulation (50-90%)	No. of colonies with total depopulation (100%)	No. of colonies with partial depopulation (50-90%)	No. of colonies with total depopulation after 48 h (100%)	No. of colonies with partial depopulation (50-90%)	No. of colonies with total depopulation (100%)	No. of colonies with total depopulation (50-90%)
80 (67 %)	40 (33%)	0	0	0	82 (100 %)	0	0

48 hours after feeding the syrup, bacterioscopical and bacteriological tests were repeated. Test results showed a bacilli and cocci boom in bees' intestines as well as the presence of *Paenibacillus larvae* spores (*figura* 2). Clinical examination of egg-added syrup fed bees showed an acute evolution of non-specific manifestations as compared with the witness lot in which the clinical status remained unmodified. Laboratory ((bacterioscopical, bacteriological) tests confirmed the massive presence of bacilli, cocci and spores of *Paenibacillus larvae* (the agent of the american foulbrood) in the examined bees (f*igura* 2 and 3), while in the witness bees, rare presence of bacilli was noticed (*figura* 1).

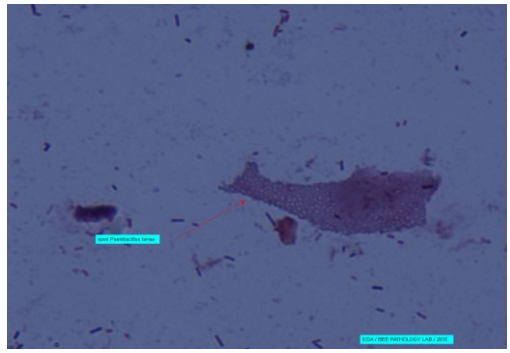

Figura 2. Marking the American loca etiological agent after feeding egg-added sugar syrup (Gram colored smear) x1000 (Original)

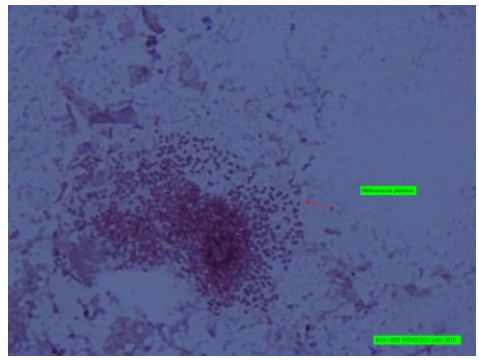

Figura 3. Numerous positive-Gram diplococci and association flora after feeding egg-added sugar syrup (smear of treated bees' intestinal content, Gram colored, x1000) (Original)

CONCLUSION

Acute symptoms started only in adult bees that had been fed egg-added sugar syrup supplements. This type of food supplement seriously affected the microflora balance of bees' intestine and *increased major bacterial diseases etiological agents* in the treated bees as compared to the witness lot. Intestinal microflora composition of the latter did not change. Therefore, feeding egg-added sugar syrup is not a favorable option for a food supplement for bees to stimulate brood growth, and neither for replacing their natural food, as it generates destabilizing effects on the intestinal microflora balance and affects the future brood generations, and it encourages subsequent evolution of major bacterial diseases in bee colonies. Consequently, we recommend periodical direct microscopic and microbiological examination of bee colonies, as well as avoiding egg-based food supplements. The egg addition sugar syrup supplement acted like a *climate favourable to the growth of etiologic agents of major bacterial diseases in bees*, by comparison with the witness in which no change occurred in the microflora composition in bees' intestine.

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DINAMICA EVOLUŢIEI RABIEI ÎN MOLDOVA ÎN PERIOADA ANILOR 2010 – 2015

T.SPĂTARU[1], N.STARCIUC[1], N.ANTOCI[2], NATALIA OSADCI
Universitatea Agrară de Stat din Moldova[1], CRDV[2]

Abstract: *Rabies is one of thoste spreading zoonoze in Republic of Moldova, which have meeting at different species of domestic and wild animals.*
The scientific was investigation implemented on the statistic dates about epizootological situation begin 2010 – 2014. Was estabilished the importance of fox in the epizootological procese of rabies the obtain dates let to make a conclusion that most important naturale source of rabies in Moldova are foxes and it depend of biological cycle of foxes.

Key words; *Rabies, endemic, domestic animals, wild animals, fox, season.*

Rabia este o boală infecţioasă zooantroponoză extrem de periculoasă . Boala este înregistrată pe tot teritoriul republicii, numai sub formă sporadică. Toate animalele domestice (de diferite specii) sunt receptive către virusul rabic. Animalele infectate şi purtătoare a virusului turbării reprezintă sursa principală de răspândire a acestei maladii la animalele domestice şi silvice de diferite specii şi prezintă un mare pericol pentru om.

Rabia este determinată ca o boală socială, care are o mare însemnătate economică. Studierea situaţiei epizootologice pe parcursul a mai mult de 50 ani ne demonstrează că Rabia în Republică rămâne destul de răspândită şi în present. Din cauza acestei maladii sectorul zootehnic şi particular au mari pierderi economice. Un rol important în răspândirea acestei maladii ân Republică îl joacă animalele sălbatice.
Oamenii muşcaţi de animalele turbate sunt stresaţi, îngrijoraţi de a nu se îmbolnăvi, necesită un tratament de urgenţă şi cheltueli material. În fiecare an pe glob mor peste 50 mii de oameni.

MATERIAL ŞI METODĂ.

Studierea situaţiei epidemiologice a rabiei în Republică pe parcursul anilor 2010 – 2015 a fost efectuată analizând datele din arhivă şi dările de seamă anuale a Centrului Republican de diagnostic în medicina veterinară (CRDV,secţia de virusologie şi epizootologie). Pentru stabilirea diagnosticului Rabiei, în calitate de material pathologic au fost creerul de la cadavrele animalelor mici agricole şi sălbatice, lafel capul de la animalele mari (bovine, cabaline).

REZULTATE ŞI DISCUŢII

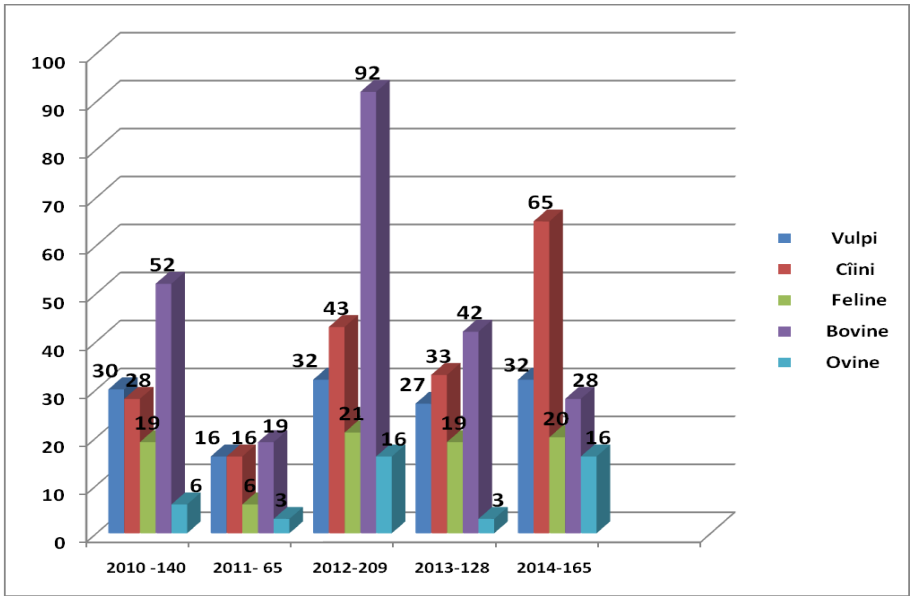
Starea epidemiologică şi dinamica evoluţiei turbării în Moldova.

Rabia in Republica Moldova începând cu anul 1951 şi până în present este una din cele mai răspândite boli iNfecţioase zooantroponoze, care se înregistrează la diferite specii de animale: domestice şi sălbatice. Această maladie provoacă mari pierderi economice sectorului zootehnic obştesc şi particular şi prezintă un mare pericol pentru om. Pierderile care au loc se explică prin aceea că animalele bolnave nu pot fi tratate şi au un sfârşit letal. Rezultatele cercetărilor situaţiei epidemiologice a Rabiei, care au fost efectuate în dinamică în anii precedenţi, începând cu anul 1951 şi până înprezent, ne relevă că turbarea este o boală persistentă pe parcursul acestei perioade de timp şi cu o evoluţie diferită în funcţie de ani.

Numărul cazurilor de rabie variază din an în an. În 1951-1952 numărul cazurilor au constituit – 66 şi 52, în 1978 – 1979 – 49 şi 72 cazuri, 1998 – 1999 corespunzător 38 şi 43, iar în 2000 - 2002 a fost înregistrată o scădere până la 15- 22 cazuri. În anii următori (2004-2008) a fost înregistrată o majorare de la 31 în 2004 până la 67 în 2007.

În ultimii 5 ani (2010-2014) (Diagrama 1) situaţia epizootică rămâne încordată.. În 2010 numărul cazurilor s-au dublat şi au constituit 140 cazuri. În 2011 s-a înregistrat o scădere de două ori a cazurilor de rabie până la 65 cazuri la diferite specii de animale. Dar cu regret în 2012 a fost înregistrat un record a cazurilor de Rabie în R. Moldova – 209 cazuri şi în anii următori : 2013 o scădere până la – 128, în 2014 au fost înregistrate 165 cazuri de rabie şi în 2015 pe parcursul a 9 luni 148 cazuri.

Fregvenţa cazurilor de Rabie în dependenţă de specia animalelor în R.Moldova în perioada anilor 2010 – 2014. **Diagrama 1**



La alte specii de animale pe parcursul acestor 5 ani au fost înregistrate căte 1-2 cazuri annual, în tota: la porcine – 5, cabaline -7, dihori – 7, jderi – 8.

Pe baza acestor date putem constata că în evoluţia procesului epizootic a turbării este caracteristic schimbul de faze (majorarea şi scăderea numărului de cazuri), prezente la ori şi ce altă boală infecţioasă, care depinde de mulţi factori: sursa de infecţie, virulenţa virusului, prezenţa animalelor receptive, nivelul imunităţii lor şi eficienţa măsurilor profilactice.

Această situaţie ne impune de-a îmbunătăţi măsurile de profilaxie şi combatere a rabiei care se efectuează în Republică. Este necesar de-a activa lucrul profilactic în zona silvică, în focarile natural staţionare Aşa dar teritoriul R.Moldova rămâne o potenţială zonă geografică rabigenă.

Către virusul rabic sunt receptive mai multe specii de animale domestice şi silvice. Dar tot odată s-a determinat că în diferite zone, procentul de contaminarea a diferitor specii de animale este variat.

Datele prezentate în diagrama 1 începând cu anul 2010 şi până în 2014 inclusiv, ne relevă că procentul cel mai mare de rabie la **animalele domestice** a fost înregistrat **la**

bovine, care au variat de **la 37,1% în 2010 până la 44,5% în 2012 (92 cazuri). În anul curent din 148 cazuri, 49 (33,1%)au fost la bovine.**

În anii **2010 – 2014** cele mai multe cazuri de rabie au fost înregistrate la **vulpi,** de la **19,4%** până la **36,8%,** şi corespunzător la **câinii –19,1% - 39,5%, la pisici – 13,6% în 2010 şi până** la **-14,8 % în 2012**, care şi sunt sursă principală de infecţie şi de răspândire a rabiei la animalele domestice şi servesc ca un mare pericol pentru om. În **2015** situaţia epidemiologică rămâne la acelaşi nivel : **20 (13,5%) la vulpi**
şi corespunzător 45 (30,6%) la câini şi 21 (14,2%) la pisici.

Conform datelor Institutului de Zoologie AŞM, populaţia de vulpi in republică s-a majorat şi au devenit cele mai răspândite dintre animalele sălbatice. La începutul anilor 1990 în mediu pe republică la 1000 hectare reveneau 2-3 vulpi, apoi începând cu anul 1998 numărul lor a crescut până la 8-10 exemplare ce a adus la răspândirea şi creşterea numărului cazurilor de rabie.

Analiza datelor despre răspândirea rabiei începând cu anul 2000 şi până în prezent ne demonstrează că cea mai mare densitate a focarelor sau a punctelor nefavorabile au loc în temei în raioanele din centrul şi nordul Republicii, care după datele Institutului de proiectare „Moldhiprozem" se află suprafeţe deluroase şi accidentale, fâşii de păduri în apropierea satelor, râpi, malurile stâncoase ale Nistrului şi Prutului, şi Codrii noştri, care sunt mândria noastră, pulmonii noştri naturali, dar tot odată ei „servesc" şi ca un focar natural al Rabiei în permanenţă, ca o sursă naturală de infecţie. Această sursă naturală o prezintă vulpile, căci anume în aceste zone şi este cea mai mare densitate a populaţiei de vulpi .

Studierea caracterului sezonier a procesului epizootic al rabiei în perioada anilor precedenţi şi pe parcursul anilor 2010 – 2014 ne relevă că pe parcursul fiecărui an se observă două majorări esenţiale a numărului de animale bolnave (Tabelul 1).

1 – în lunile **februarie – martie – aprilie** ce coincide cu perioada de împerechere a vulpilor. În această perioadă are loc un mare contact de animale şi lupte mari între masculi pentru femele.

2 – în lunile **octombrie până în ianuarie**, când se majorează densitatea populaţiei vulpilor datorită tineretului, care se stabileşte în noi teritorii, unde din nou au loc lupte pentru teritoriu, contacte directe şi tot odată răspândirea rabiei prin muşcături.

Datele din tabelul 1, ne permite să menţionăm că în aceste două perioade de timp, numărul cazurilor de rabie **în lunile reci ale anului** (I,II,III,IV,X,XI,XII) este mai mare decât în celelalte luni **de 2- 4 ori** şi constituie de la **62,0% până la 83,0%.**

Caracterul sezonier al Rabiei (2010- 2014)

Tabelul 1

N	Lunile	2010	2011	2012	2013	2014	Total pe lună
1.	Ianuarie	18	5	27	15	6	71
2.	Februarie	10	5	15	11	20	61
3.	Martie	19	4	15	7	8	53
4.	Aprilie	10	2	11	15	4	42

5.	**Octombrie**	**18**	**11**	**29**	**10**	**22**	**90**
6.	**Noembrie**	**5**	**15**	**27**	**7**	**34**	**88**
7.	**Decembrie**	**7**	**12**	**18**	**15**	**16**	**58**
	Total :	**87 (62%)**	**54 (83%)**	**142 (68%)**	**80 (62,5%)**	**110 (66,7%)**	**473 (66,9%)**
8.	**Mai**	**14**	**1**	**9**	**4**	**5**	**33**
9.	**Iunie**	**11**	**1**	**15**	**7**	**8**	**42**
10.	**Iulie**	**12**	**0**	**11**	**14**	**15**	**52**
11.	**August**	**8**	**3**	**13**	**12**	**10**	**46**
12.	**Septembrie**	**8**	**6**	**19**	**11**	**17**	**61**
	Total :	**53 (38%)**	**11 (17%)**	**67 (32%)**	**48 (37,5%)**	**55 (33,3%)**	**234 (33,1%)**
	TOTAL ANUAL CAZURI	**140**	**65**	**209**	**128**	**165**	**707 În 5 ani**

Din toate cele menţionate mai sus se poate de făcut concluzia, că sezoniera rabiei depinde în mare măsură de ciclul biologic a vulpilor, care la noi în Republică sunt rezervorul principal al Rabiei. Situaţia epidemiologică se agravează încă prin aceea, că în Republică insuficient se efectuează imunizarea cu scop profilactic a animalelor domestice şi sălbatice din zona silvică contra Rabiei.

Situaţia creată ne impune de-a analiza şi a îmbunătăţi măsurile de profilaxie şi combatere a rabiei în Republică. Este necesar de-a activa lucrul profilactic în zona silvică, în focarile naturale staţionare. Măsurile profilactice specifice în zonile focarelor naturale,vaccinarea animalelor silvice s-ă se efectueze regulat, conform unei scheme bine argumentată, fără întreruperi timp de 3-4 ani, pentru a râdica nivelul imunităţii faţă de rabie la animalele sălbatice şi câinii vagobonzi, ce ne va da posibilitatea de-a întrerupe lanţul epizootic natural.

CONCLUZII ŞI RECOMANDĂRI

1. În R. Moldova în ultimii 9 – 10 ani rabia este una din cele mai răspândite boli infecţioase zooantroponoze şi prezintă o problemă socială.
2. Epizootiile de rabie sunt strâns legate cu ciclul biologic a vulpilor, care servesc ca o sursă naturală în răspândirea turbării în Moldova
3. Conform rezultatelor din ultimii 10 ani, la vulpi si câini ca sursele principale a rabiei a fost înregistrat la vulpi de la 19,4% până la 36,8%, iar la câini corespunzător până la 39,5 în 2014.
4. Numărul cel mai mare de cazuri au fost înregistrate în raioanele din centrul şi nordul republicii unde sunt cele mai favorabile condiţii de trai a vulpilor.

5. Îmbunătăţirea situaţiei epidemiologice a rabiei poate fi ameliorată prin vaccinarea orală a populaţiei de vulpi şi a câinilor vagabonzi.

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OBSERVATIONS OF AN OUTBREAK OF MAREK'S DISEASE IN CHICKENS BROILLER

ADRIAN STANCU[1], VIRGILIA POPA[2], LARION FLUERAȘU[1], IONICA IANCU[1]
Faculty of Veterinary Medicine Timişoara[1]
S.N. Institutul Pasteur S.A. Bucuresti[2]

Abstract: *Marek's disease (WB) is the most common lymphoproliferative disease of chickens caused by a herpes virus, highly contagious disorder characterized clinically by neural and ocular general, acute or chronic evolving and pathologically by inflammatory and proliferative tumor appearance in peripheral nerves, endocrine glands, iris, visceral organs, muscle and skin, causing mortality, especially in the age of 8-22 weeks or serious economic losses through seizures.The research was conducted in farm B, in CarasSeverin country, in a flock of 10,500 broilers, Cobb hybrid, raised ground. The number was epidemiological, clinical monitored, pathological and laboratory examinations throughout the growth to the age and weight of slaughter.*

The research had as main objective epidemiological surveillance reovirusinfection that frequently evolve in flocks of broilers and bacterial or viral diseases that may occur consecutively this infection.

After 3 weeks of age in some corpses were found anatopathologic macroscopic lesions characteristic of the disease Marek reason that laboratory tests were performed to confirm these diseases.

Key words: Marek's disease, epidemiological, Broiller, Cobb hybrid.

MATERIALS AND METHODS

The research was conducted in farm B, in CarasSeverin, in a flock of 10,500 broilers, Cobb hybrid, raised ground. The number was monitored epidemiological, clinical, pathological and laboratory examinations throughout growth until slaughter age and weight.

The research had as main objective epidemiological survellancereovirus infection that frequently evolve in flocks of broilers and viral and bacterial diseases that can appear consecutively as secondary infectious diseases.

The bodies were necropsied twice weekly for detection of pathological lesions characteristic reovirus infection and bacterial and viral infectious diseases that have evolved.

The bodies were necropsied the bird necropsy specific technique. After 3 weeks of age in some corpses were found anatopatologice macroscopic lesions characteristic of the disease Marek. To confirm that disease samples were taken from the bodies of macroscopic lesions (liver, spleen) for histopathological examination and polymerase chain reaction (Polymerase chain reaction-PCR).

Samples intended for histopathological examination were processed by paraffin technique and stained by medoda HEA.

For detection of viral DNA was used acquis polymerase chain reaction carried out in the laboratory of molecular biology SN Pasteur S.A. Bucharest.

RESULTS AND DISCUSSIONS

At the age of 21 days, by pathological examinations carried out have been identified broiler carcasses that were highlighted macroscopic lesions characteristic of the disease to the liver and spleen Marek.

For the diagnosis of this disease took samples of spleen and liver for histopathological examination and polymerase chain reaction (PCR).Histopathologic examination was performed according to conventional methodology described above.

On gross examination of the liver was found an increase of approximately 3 times their volume and weight compared to its normal size, which is completely invaded by the tumor characterized by a whitish gray color with bacon aspect, aleucemice predominant characteristic shape. The friability of the liver is greatly increased. (Figure 1)

The macroscopic examination of the spleen was observed an increase in its volume and weight, surface slightly discolored and crumbly consistency. (Fig 2)

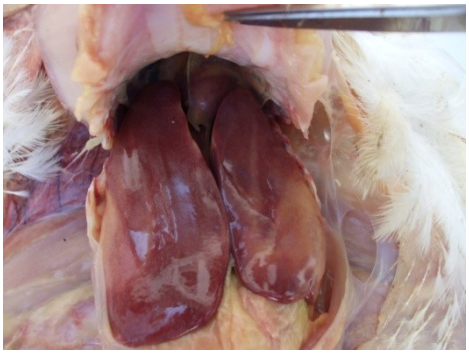


Fig1.Hepatomegaly
(broiler chickens 35 days)

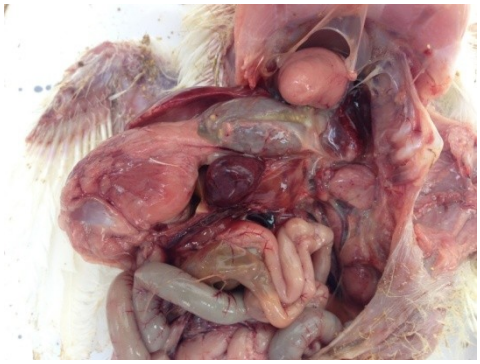


Fig 2. Splenomegaly
(broiler for 30 days)

Based onpathologicexamination resultsormacroscopiclesionspresent inthe liverand spleenwasdeterminedfrequency, shown inTable 1andChart 1.

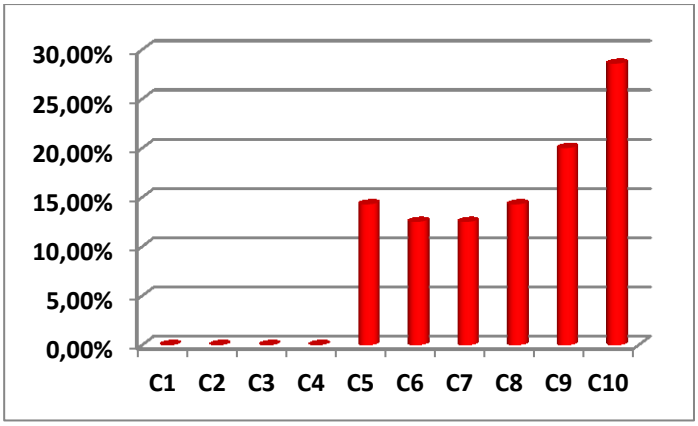
Table 1

Evolutionpathologicallesionscharacteristic of the diseaseby ageMarek

Control/age	Number of bodies	%
C1- 6 zile	0	0
C2-12 zile	0	0
C3-14 zile	0	0
C4-19 zile	0	0
C5-21 zile	1/7	14,28%
C6-26 zile	1/8	12,50%
C7-28 zile	1/8	12,50%
C8-33 zile	1/7	14,28%
C9-35 zile	3/15	20,00%
C10-41 zile	2/7	28,57%

Chart 1

The frequency of pathological lesions



Analyzing the results shown in the table and the graph shows that the frequency of gross lesions present in the liver and spleen increased progressively, the maximum age of 41 days.

The emergence of Marek's disease at the age of 21 days, is the result of infection with reovirus induced immunosuppression, which evolved from the herd of broilers, the examination confirmed by immunoassay and polymerase chain reaction. The emergence and evolution of Marek's disease in broilers at younger ages than ever, mentioned in the literature. The AK Schat, and Venugopal Nair, 2013, stated that the injuries induced by Marek's disease virus, broilers, appear in the thymus, and spleen stock Fabricius, after the age of 20 days in the natural infection, the disease is favored by states of immunosuppression induced by certain viruses present in flocks of broilers reared intensively. This phenomenon has been reported in experimental infections carried by conventional broiler chickens infected with certain viruses.

On microscopic examination of **liver** was highlighted mesenchymal hyperplasia polymorphic represented by massive infiltration of lymphocytes, plasmoblaşti, fibroblasts, diffuse and circumscribed. This infiltration compression produces atrophy, dystrophy and necrosis of liver cells. (Fig 3,4)

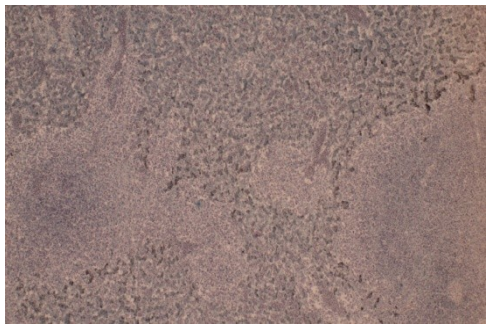


Fig3.Chicken Livers. Polymorphocelular Hepaticinfiltration. Col.HEAX10

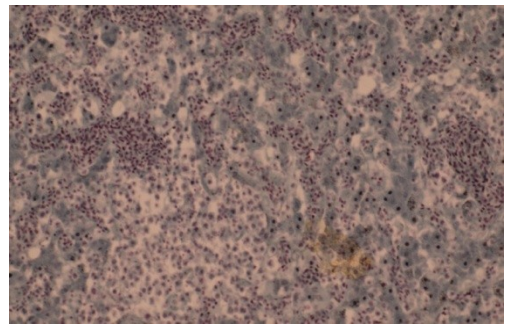


Fig. 4Chickenlivers. Polymorphocelular HepaticinfiltrationCol.HEAx 20

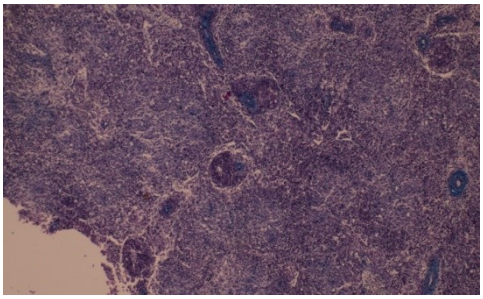


Fig. 5.Spleenchicken.
Mesenchymalhyperplasiapolymorphonuclear.
Microscopic-Col HEAx10

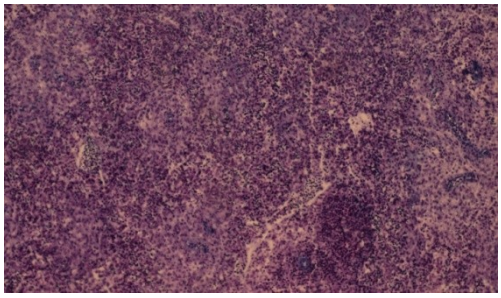
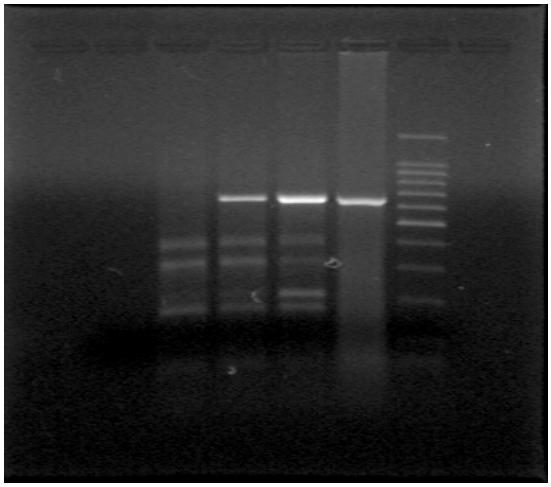


Fig 6. Spleenchicken.
Mesenchymalhyperplasiapolymorphonuclea.
Microscopic-ColHEAx 20

Marek's disease was confirmed by polymerase chain reaction which revealed the presence of viral DNA in liver and spleen. The results of this reaction are shown in Figure 7.



GelelectrophoresisPCRpostMDV-B virusdetectionMarek(GaHV2).
Line 1:negative control(NFW, negative);
Line 2: sample 1401, the spleen(negative v.Marek+non-specificamplicons);
Line 3: sample 1402spleen(positivev.Marek, 669bpampliconspecific, non-specificamplicons+);
Line 4: sample 1403spleen(positivev.Marek, 669bpampliconspecificamplicons+nonspecific);
Line 5: Marekpositiveinternal control-groundDNAsample2004 (669bp amplicon);
Line 6: Standard DNA-100bpDNAladder (Promega).

Fig. 7.Results of polymerase chain reaction

Note that the number of broiler reovirosis appeared after 10 days of age, being diagnosed clinically pathological and immunoassay test. This disease induce immunosuppression in chickens given that favor the emergence and development of several diseases, including Marek's disease.

CONCLUSSIONS

The number monitored broiler bird was confirmed reovirosis by immunoassay test;
After the age of 21 days, the carcasses broilers necropsy, gross lesions were identified, typical of Marek's disease in the spleen and liver;

Histopathology revealed both the spleen and liver mesenchymal very obvious hyperplasia represented by massive polymorfocelular infiltration feature Marek's disease;

By the polymerase chain reaction in liver and spleen tissues was detected viral DNA, thereby confirming the presence of Marek's disease in the flock monitored;
The emergence of the disease after the age of three weeks is the result of immunosuppression induced by the avian reovirus;

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OBSERVATIONS OF AN OUTBREAK OF PULMONARY ADENOMATOSIS IN SHEEP

ADRIAN STANCU, IOAN OLARIU-JURCA, ADRIAN OLARIU JURCA
Faculty of Veterinary Medicine TimişoaraFaculty of Veterinary Medicine Timişoara

Abstact: *Disease is often over-diagnosed adult sheep, the animal that presents a high rate of respiratory rate and breathing disorders, despite the fact that many infections that affect other organ systems can evolve to such signs, including mastitis, metritis, anaerobic enterotoxiemie painful conditions, severe lameness, acute fasciolosis respectively. The research was conducted in October 2011 - December 2011 to 11 bodies necropsy sheep aged 3-5 years, Turcana from the household, a herd of 250 dint ends. The autopsy was performed by specific technique mammals. Macroscopic lung examination, it was found that it has a whitish gray color to right lung apical lobe, cardiac lobe and diaphragm front third of them both on the surface and section, the consistent is fleshy, and the section aspect bacon. The affected areas were prominent, sharply demarcated from healthy areas.*

Key word: adenomatosis, pulmonary, sheep.

THE AIM

The research aimed to establish the diagnosis of pulmonary adenomatosis based on histological lesions (macro and microscopic).

MATERIALS AND METHODS

The research was conducted in October 2007 - December 2007 to 11 bodies necropsy sheep aged 3-5 years, Turcana bread from the household, a herd of 250 dint ends. The autopsy was performed by specific technique mammals.

Suspected pulmonary adenomatosis emerged from the necropsy of the first body when lung examination was a whitish gray color to right lung apical lobe, heart and diaphragm front third lobes, both at the surface and section, the consistent is fleshy and the section aspect bacon. The affected areas were prominent, sharply demarcated from healthy areas. In severing his windpipe observed a white foamy liquid with beaten egg aspect characteristic of pulmonary edema. The regional lymph nodes were swollen.

The samples were fixed in alcohol for 24 hours. The samples were transferred into the battery set dehydration of the alcohol in increasing concentrations of from 70 ° to absolute alcohol. in each bath, the five samples were stored for two hours, after which the alcohol was removed by placing in benzene for clarification section, and then made to incorporate wax in a thermostat bath at 56 ° C. The parafining blocks were obtained which contained samples (fragments) of organ lesions, which have been cut off at the microtome, six micrometers. Sections were fixed to slides obtained well degreased using albumin Meyer. Sections were stained slides glued by means of HEA. Histopathological sections staining was performed in the following steps: dissolving the wax with benzene; rehydrate using alcohols with decreasing concentrations and water; crossing blades with dye baths (hematoxylin, eosin methylene blue); removal of water using increasing concentrations of alcohols (70-90 ° amyl alcohol); colored sections between the blade assembly and slide.

RESULTS AND DISCUSSION

External examination of the bodies revealed a small amount of seromucos secretions around the nasal cavities.

Macroscopic lung examination, it was found that it has a whitish gray color to right lung apical lobe, cardiac lobe and diaphragm front third of them both on the surface and section, the consistent is fleshy, and the section aspect bacon. The affected areas were prominent, sharply demarcated from healthy areas. Retropharyngeal and mediastinal lymph nodes were swollen. (Figure. 1, 2)

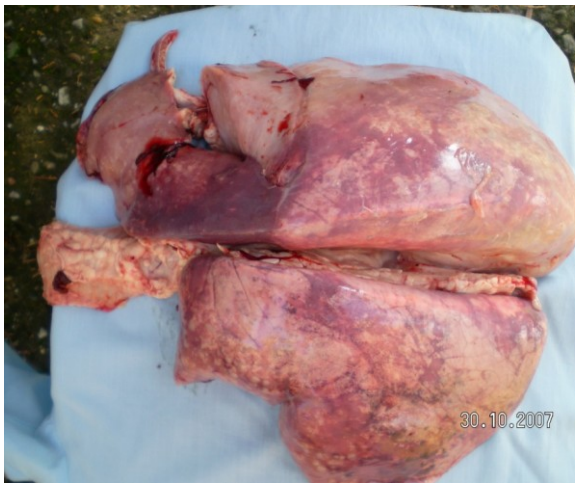

Figure 1.Lung sheep (Pulmonary adenomatosis.
Lung adenoma (exam at the entire lung surface).

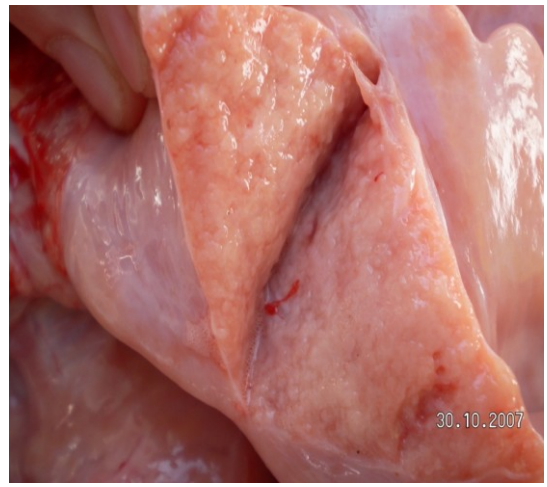

Figure 2. Lung sheep (Pulmonary adenomatosis)
Lung adenoma (exam section)

Microscopic found to contain both adenoma and adenoma papilifer simple.

Simple adenoma (tubular) consists initially of a moderate tumor alveolar epithelial hyperplasia, which is cuboid or prismatic tahicromatic-looking pseudoacinar.

Papilifer adenoma is characterized by a hyperplasia of alveolar epithelial tumor which protrude into the lumen of the papilla cellular form mono-, bi- or three-lobed. (Figure 3, 4)

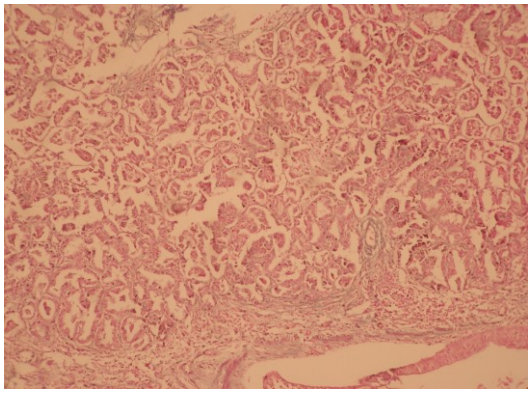

Figure3.Lung sheep. (Pulmonary adenomatosis)Simple adenoma (tubular) (Col. HEA x 10)

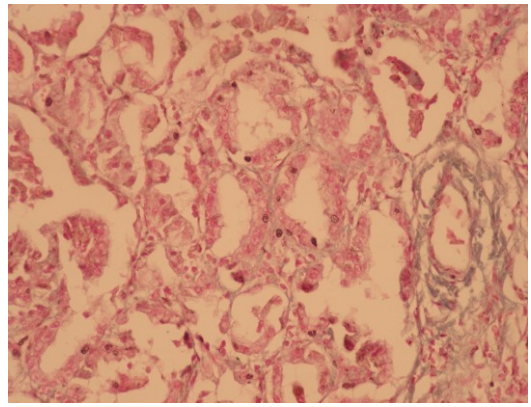

Figure 4 Lung sheep. (Pulmonary adenomatosis) Simple adenoma (tubular) (Col. HEA x 40)

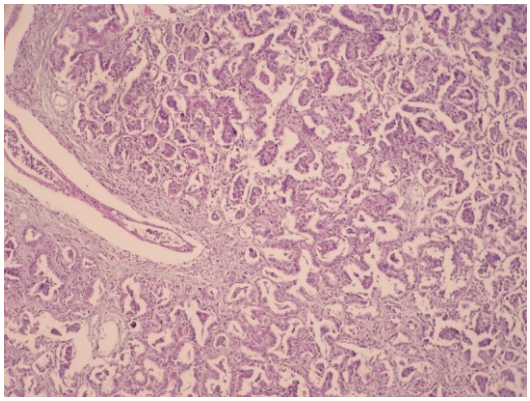

Figure 5.Lung sheep. (Pulmonary adenomatosis) Papilifer adenoma (tubular) (Col. HEA x 10)

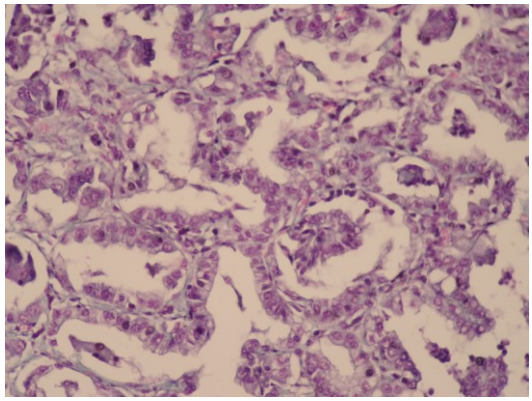

Figure 6..Lung sheep. (Pulmonary adenomatosis) Papilifer adenoma (tubular) (Col. HEA x 40)

CONCLUSSIONS

Pulmonary adenomatosis was diagnosed in all 11 necropsied corpses.

External examination of the bodies revealed a small amount of jetaj seromucos around the nasal cavities.

Macroscopic was a whitish gray color to right lung apical lobe, cardiac lobe and diaphragm front third of them both on the surface and section, the consistent is fleshy, and the section aspect bacon.

The affected areas were prominent, sharply demarcated from healthy areas.

Microscopic were found to contain both adenoma and adenoma papilifer simple.

Simple adenoma (tubular) initially formed a moderate tumor alveolar epithelial hyperplasia, which is cuboid or prismatic tahicromatic-looking pseudoacinar.

Papilifer adenoma is characterized by a hyperplasia of alveolar epithelial tumor which protrudes into the alveolar lumen in the form of mono- or multilobate papillae.

Specific injuries: the presence of solid tumors, whitish gray color with a translucent sheen glittering, separate areas adjacent normal lung and microscopic adenomas are found to contain both simple and papilifer adenoma.

Nonspecific Injury: seromucos jetaj around the nasal cavities, the presence of liquid sparkling white airways (pulmonary edema), alveolar macrophages in the lumen, swollen lymph nodes and mediastinal retropharyngeal.

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DETERMINATION OF THE BACTERIAN ENDOTOXINS IN VETERINARY PHARMACEUTICAL PRODUCTS

SIMONA STURZU, ALINA DRAGHICI, DANIELA TIRSINOAGA, IOANA TIHULCA, MARIANA DUMITRACHE, CRISTINA POPICA
Institute for Control of Veterinary Biological Products and Medicines
Bucharest, Romania
www.icbmv.ro

Abstract: *Determination of bacterian endotoxins in the parenteral pharmaceutical products for veterinary use involves using lysate (extract LAL) obtained from Limulus polyphemus blood cells by applying gel clot method. L.A.L. from Limulus polyphemus blood cells couples with endotoxins that inactivates them by forming a clot that will act as a protective barrier against bacterian infection.*
The aim of this testing for endotoxin contamination produced by Gram negative bacteria present in is the prevention of adverse effects after administration of the products and preventing reduction of efficiency of a product contaminated with endotoxins.

Keywords: *bacterian endotoxin, gel cloth, pharmaceutical products*

INTRODUCTIONS

The quality of the veterinary medicinal products is established by a set of parameters whose values must meet the requirements of the European Pharmacopoeia and give the user the confidence that the safety and efficacy of the medicinal products remains guarantor of their use. The level determination of the bacterian endotoxins in medicinal products is one of the quality parameters of an injectable veterinary medicinal product, and more.

The endotoxins constitute the basic structural components of Gram negative bacteria and are located intracellularly and intraparietal. The lipopolysaccharides and lipooligosaccharides belong to this category and both types of toxins are stored in the lipid wall of Gram negative bacteria (*Escherichia coli, Klebsiella, Shigella, Salmonella,* etc.). After destroying of bacteria, endotoxins can cause reactions of immune system such as the inflammatory type. The medicinal products can contain endotoxins when the bacterian cell debris are accidentally introduced into the product, during the manufacturing process.

The determination of the bacterian endotoxins in the medical products is carried out using an extract derived from the blood cells of Limulus polyphemus, using the gel-clot technique.

Gel-clot technique involves comparing of the serial dilutions of the appropriate sample dilutions of endotoxin standard for detecting or quantifying of the endotoxins on basis the coagulation of lysate in the presence of endotoxins.

A critical requirement for achieving this method is that all reagents, test tubes and pipettes to be pyrogen-free.

MATHERIAL AND METHOD

The working methodology refers to the determination of the endotoxin contamination caused by Gram negative bacteria present in the parenteral pharmaceutical products for

veterinary use have at base the technique gel - clot according to the European Pharmacopoeia. The samples under test are provided by sanitary veterinary inspectors, according to the Annual sampling and testing Plan, approved by ANSVSA.

The reagents and the solutions required to measure the contamination of bacterian endotoxins are:

- The Reagent L.A.L. (lysate) is an aqueous extract of the lysed blood cells of Limulus polyphemus as lyophilized form. The extract is obtained by collecting of the blood from the pericardium of "horseshoe" crab. The blood cells are separated from serum by centrifugation, and the component is then placed in distilled water that causes swelling and disintegration there of (lysis). This process releases the substances contained within the cells which are then purified and lyophilized.
- The standard of the endotoxin is necessary for the preparation of the positive controls and standard solutions. It has a predetermined endotoxin content, specified in the certificate of analysis. The reactivity lysate L.A.L. is correlated to the concentration of endotoxin.
- The dilution solutions: water LAL is used for reconstitution of reagent, performing of the sample dilutions and of endotoxin solutions.

The laboratory equipment required to measure of the bacterian endotoxins are simple and represented by incubator (set at the temperature of 37^{o} C), Accu-jet and stopwatch. The determination of the bacterian endotoxins involves the following steps:
1. Performing of the preliminary tests refers at the demonstration of the inhibiting properties and stimulation of the product (LAL test validation). The appropriate recovery of the endotoxin must be demonstrated before using the LAL test.
2. Sampling and preparation of working solutions
a. The sampling and processing is done aseptically using non-pyrogenic material and reagents. The sample solution is prepared by dissolving or diluting of the medicinal product using the LAL Water.
b. Preparation of standard endotoxin - After a proper homogenization of the stock endotoxin solution, serial dilutions can be prepared using water LAL.
c. The determination of the valid maximum dilution (VMD) - is the maximum acceptable dilution of a sample for which can be determined the endotoxin limit. VMD is determined using a standard calculation formula.
3. The determination of the bacterian endotoxin in the sample, implies the following steps:

- The confirmation of the lysate sensitivity – which is achieved when using a new set of lysate or when is identified any change in the experimental conditions that could affect the test result.
- The assay of the interference factors - is performed according to the European Pharmacopoeia by preparing of the working solution and the performing of the inhibition/enhancing test on the sample solution at a less dilution than VMD, which contains no detectable endotoxin.
- The testing of the bacterian endotoxins - involves comparing the serial dilutions of the sample with the appropriate dilutions of endotoxin standard

for detecting or quantifying of the endotoxins on coagulation of lysate in the presence of endotoxins.

After the allocation of appropriate dilutions in the test tubes, they are incubated for 1 h at 37 ° C ± 1 ° C.

RESULTS AND DISSCUTIONS

The test is valid if both replicates of the positive controls are positive and those of negative control are negative.

The result is considered to be positive if it is obtained a firm gel that remains inside the tube after a 180 ° rotation thereof. This result indicates the existence of a quantity of endotoxin in sample greater than the maximum allowed.

The sample under the examination passes the test when they get a negative result for both replicated of the test solutions at a dilution that not exceeding VMD test that was performed the interference factors test.

The result is considered to be negative if it is not not obtained a firm gel or if the substance obtained is viscous and not maintained inside the tube after turning it 180 °. This result indicates the absence or presence of endotoxins in sample in an amount which does not exceed the maximum limit allowed.

The sample examined falls the test when a positive result for both replicated of the test solution dilution at the dilution that not exceeding VMD test that was conducted interference factors.

The test is repeated if the different results from the two replicates of the test solution are that not exceeding VMD (a positive result in one of replicates and a negative result to the other). The sample complies with the requirements if a negative result for both replication of solutions that not exceed VMD at the retesting. The sample under examination is inappropriate if a positive result is from both or if one of replicate of the test solution not exceeding VMD in the repeat step of the test.

CONCLUSIONS

1. The test for determination of bacterian endotoxins from veterinary medicinal products is simple, easy to perform and does not require complicated laboratory equipment.
2. The determination of the bacterian endotoxins constitutes an important step in the assessment of the quality that offer guarantees on a safe and efficient use of veterinary medicinal products.

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EVALUATION THE PROTECTIVE EFFECT OF MODEFIED NANO PARTICALES ZNIC OXID ON TOXICITY OF NICOTINE ON THE LUNG OF MICE USING NANOTECHNOLOGY.

OMAYMA A.R. ABOUZAID;[1] ABDEL-MAKSOUD, H.A.[1] AHMED AWAD ABOUEL-ATTA[2], HANAN AHMED EL-KABANY[3] AND KHALED SAYED ABDEL-GAYED[1]

1- Faculty of Vet.Med. Moshtohor, Benha University Egypt.

2- Faculty of Vet.Med., Zagazeg University Egypt.

3- National Center for Radiation Research and Technology(NCRRT), Cairo –Eygpt

Omayma_ Ragb55@yahoo.com

Abstract:

The present study was aimed to investigate the toxicity of nicotine on this lung of mice using biochemical and histopathological study. Nicotine administration (2.5 mg/kg of body weight, intraperitonelly (ip) , 3 days a week for 4 weeks) enhanced Lipid peroxidation(LPx) and nitric oxid(NOx) accompanied by a significant increase in the marker enzymes alanine transaminase(ALT), aspartate transaminase(AST), alkaline phosphatase(ALP), lactate dehydrogenase(LDH), γ- glutamyl transferase (GGT) and elevated levels of cholesterol, triglycerides, createanine and urea in mice.meanwhile, suppressed glutathione peroxidase (GPx), catalase(CAT), p53gene experession, (p53)level and caspase-3(Cas3) activity.also, The histological examinations of this study revealed a damage and degeneration in the lung There was a significant protection by novel nano composite of modified Zinc oxide nps administration at dose (70mg/kg body weight) in nicotine-treated mice. The results suggest that novel nano composite exerts the protective effects by modulating the extent of lipid peroxidation, ameliorate and normalize most of the investigated parameters The results are supported by histopathological observations of lung. Hence, the intake of novel nano composite might suppress the toxicity and mutagenic activity of nicotine treated groups.

Key Words

Nicotine, novel nano composite, antioxidant stress, p53, caspase3, lipid peroxidation(LPx), nitric oxid(NOx), apoptosis.

1. INTRODUCTION

Metal oxide nanoparticles, including zinc oxide, are versatile platforms for biomedical application and therapeutic intervention. There is an urgent need to develop new classes of anticancer agents, and recent studies demonstrate that ZnO nanomaterials hold considerable promise. Focusing on ZnO NPs and their proposed mechanisms of cytotoxic action, as well as current approaches to improve their targeting and cytotoxicity against cancer cells may be improved further to make them attractive new anticancer agents. **Rasmussen, J. W., 2010.**

The major aim of medicine has long been the early and accurate diagnosis of clinical conditions , providing an efficient treatment without secondary effects. With the emergence of nanotechnology , the achievement of this goal seems closer than ever. To this end , the development of novel materials and devices operating at the nanoscale range , such as nanoparticles, provides new and powerful tools for imaging ,diagnosis ,and therapy. **Sanvicens, 2008**. Nanaoparticles are increasingly being recognized for their potential utility in biological application including nanomedicine **Hanley, C., et al., 2008**.

Curcumin has been shown to suppress the expression of epidermal growth receptor and estrogen receptors, which are cancer-associated growth factors . **Kunnumakkara, *et al*.,**

2008. Curcumin induces phase II enzymes (glutathione S-transferases and epoxide hydrolase), which play a protective role by eliminating toxic substances and oxidants and conferring benefit in the prevention of the early stages of carcinogenesis. **Liao, *et al*.,2008.**

Most studies have described that millimolar concentrations of ascorbate have a deep inhibitory effect on the growth of several cancer cell lines in vitro. **Chen, et al ., 2005.**

Nicotine is a naturally occurring alkaloid found in the nightshade family plants (solanaceae), predominantly in tobacco plant (nicotianatabacum). **Wu, et al., 2002. Nicotine** increased oxidative stress results from excess generation of reactive chemical species called free radicals from a number of sources and/or from decreased enzymic and nonenzymic antioxidant defences. **Nallella, et al., 2004.**

Free radicals and other reactive species have been implicated in the progression of not less than hundred different diseases. thus the interest in free radicals and oxidative stress has grown in recent times. Nicotine has been documented to alter the oxidant and antioxidant balance in rat lymphocyte in a dose and time-dependent manner. **Das. et al., (2012).**

Nicotine has many effects such as on heart rate, brain excitation, and blood pressure. **Shivij, et al., 2006.& Wu, et al., 2002**. reported that, nicotine induced a wide range of biological effects and is a major risk factor in the development of chronic obstructive lung diseases, cardio- vascular disorders and lung cancer. Morever nicotine through smoking, induced an inflammatory response in the lung and plays a role in pathogenesis of obstructive pulmonary diseases. **Carpagnano, et al., 2003.& Hackett, et al., 2003.**

The application of nanotechnology for cancer therapy has received considerable attention in recent years, cancer nanotechnology can interdisciplinary area of research in science , engineering and medicine is an upcoming field with extensive applications. It provides a unique approach and comprehensive technology against cancer through early diagnosis , prediction , prevention , personalized therapy and medicine. Target –specific drug therapy and method for early diagnosis of pathologies are the priority research areas in which nanotechnology would play a vital part. **Misra, et al., 2010.**

2- MATERIALS AND METHOD

2.1. Experimental animals:

Experimental animals were obtained from the breeding unit of National Cancer Institute (NCI), Cairo University; Cairo, Egypt . one handered and therity virgin male Swiss albino mice (8-10) weekes old weighing (20-25 gm body weight). mices were used in this study housed in separated wire mesh cages and kept at constant environmental and nutritional conditions throughout the period of experiment. The mices were fed on constant ration and fresh, clean drinking water was supplied ad- libitum.

2. 2. Induction of lung cancer:

To induce lung cancer, mices were injected nicotine at a dose level of 2.5 mg/kg b.wt intrapretoneally(i p) Hecht, 2003. three times weekly for 4 weeks Nicotine purchased from (Sigma, USA),99% pure nicotine dissolved in 0.9% saline,Molecular formula ($C_{10}H_{14}N_2$)

Mol.mass 162.23g/mol, Density 1.01 g/cm³, Melt. point -79 °C (-110 °F), Boiling point 247 °C (477 °F)

2.3.Preparations of novel nano composite (nanoparticeles zinc oxide with basic nan curcumin and ascorbate)

1- Curcumin was purchased from (Sigma, USA)**.** Curcumin has a melting point of 180°C; its molecular formula is ($C_{21}H_{20}O_6$) and molecular weight (368.39). **Aggarwal *et al*., 2003.**

2- Zinc oxide nanoparticeles purchased from (Sigma, USA), Molecular formula ZnO Molar mass 81.408 g/mol, Density 5.606 g/cm^3 Melting point 1975 °C Boiling point 1975 °C Solubility in water is Insoluble. Takahashi, et al., 2007.

3- Sodium bicarbonate purchased from(Indian combany)

Basic Nanocurcumin:

Basic nanocurcumin is prepared by mixing the pure curcumin and sodium bicarbonate by ratio (1:4) and grinding the mixture in the ball mail at 3500r.p.m for 8 hrs that allow the solid reaction between the curcumin and bicarbonate and the formation of the disodium salt of curcumin

Zinc Oxide nps Modified with Basic Nanocurcumin:

Modified ZnO nps was achieved by sooking ZnO nps for 24 h in basic nanocurcumin 0.05g in 50ml distilled water and stirred overnight to allow complete complexation. The resulting solids were dried in an evacuated desicator to give Zinc oxid***e*** nps modified with basic nanocurcumin.

Sodium Ascorbate Mixing to Zinc Oxide nps Modified with Basic Nanocurcumin:

About 0.5 gm powder of sodium ascorbate was add to nano compound and mixed well to produce noval nano compound which used dirctely in experimental study

Sodium Bicarbonate (Na HCO3) add to noval compound:

Approximatly 0.5 gm powder of sodium bicarbonate (Na HCO3) was added to adjuste pH till alkaline pH 8.5 The extracellular pH of malignant solid tumors is acidic, in the range of 6.5 to 6.9, whereas the pH of normal tissues is significantly more alkaline, (7.2 to 7.5). **Griffiths, 1991.** Acid pH was inhibited using oral NaHCO3, which has previously been shown to effectively reverse pH gradients in tumors and not affect the pH of normal tissues. **Raghunand. et al.,1999**.

Novel nano composite dissolved in 0.9% saline solution and administered to mice orally by stomach tube (70 mg/kg body weight for 28 days)

2. 4. Experimental design:

The present studied was carried out on 120 male swiss albino Mices were randomly divided into four main equal groups, each group placed in individual cages and classified as follow:-

Group 1: Control Normal group:

Received no drugs, served as control non-treated for all experimental groups

Group 2: Novel nano composite group:

Consisted of thirty mice treated only with novel nano compound administered orally by stomach tube at a dose of 70 mg/kg b.wt. three times per a week for 4 weeks

Group 3: Nicotine group:

Comprised thirty mice injected intraperitonelly (i.p) with nicotine at dose of 2.5 mg/kg body weight three times per a week for 4 weeks

Group 4: novel nano composite + Nicotine) group:

Included thirty mice treated with novel nano compounds administered orally by stomach tube at a dose of 70 mg/kg b.wt. three times per a week for 4 weeks as in group II, Two hours before nicotine injected as in the group III. until the end of experiment.

2. 5. Sampling:

Blood samples and tissue specimens (lung tissues) were collected at the end of experiment on 28th day for all groups (control and experimental groups).

2. 5. 1. Blood samples:

Blood samples for serum separation were collected from the heart at the end of experimental period in dry, clean, and screw capped tubes and serum were separated by centrifugation at 2500 r.p.m for 15 minutes .The clean, clear serum was separated by automatic pipette and received in dry sterile samples tube and kept in a deep freeze at -20^{0}c until used for subsequent biochemical analysis .All sera were analyzed for Liver function, kidney function and Lipid profil tests lactate dehydrogenase (LDH), γ- glutamyl transferase (γ-GT) and alkaline phosphatase (ALP) activities were also determined for all groups in serm.

2. 5. 2. Tissue specimen (lung tissue):

At the end of the experiment, mices of each group were sacrificed by cervical decapitation. The abdomen and chest were opened and the lung specimen was quickly removed and opened gently using a scrapper, cleaned by rinsing with ice-cold isotonic saline to remove any blood cells, clots, then blotted between 2 filter papers and quickly stored in a deep freezer at (-20°C) for subsequent biochemical analysis.Briefly, *lung* tissues were divided into appropriate portions, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH7.4) to make 10% homogenates. The homogenates were centrifuged at 6000 r.p.mfor 15 minutes at 4°C then the resultant supernatant were used for the determination of the following parameters: p53 gene, caspase 3 GPx, CAT, Lipid peroxidation(LPx)., and nitric oxid (NOx) content. Histopathological examination of lung tissues of each group was also performed.

2. 6. Biochemical analysis:

Molecular biology parameters: caspase-3 &p53 gene expression were determined according to the method discrebed by **Tribukait, B. 1984.**

Antioxidant activity &Lipid peroxide: glutathione peroxidase (GPx), and catalase (CAT) were determined according to the method using colorimetric detection kit. discrebed by **Necheles, et al., 1968**., **Sinha, 1972.** Respectevily, Lipid peroxidation(LPx)., and nitric

oxid (NOx) content. were determined according to the method discrebed by **Yoshioka, *et al*., 1979.**

Liver and Kidney function and Lipid profil parameters: Liver function (ALT and AST activities were determined according to the method discrebed by **Reitman and Frankel,1957**. γ- glutamyl transferase (γ-GT) was determined according to the method discrebed by **Szasz. 1974.** ALP was determined according to the method discrebed by **King. 1998**. kidney function Createnine was determined according to the method using colorimetric detection kit. discrebed by **Henry, et al.,1974.** Urea was determined according to the method using colorimetric detection kit. discrebed by **patton and Crouch. 1977.** and Lipid profil as Cholesterol was determined according to the method using colorimetric detection kit. discrebed by **Young. 1995.** Triglyceride was determined according to the method using colorimetric detection kit. discrebed by **Stein. 1987.** and lactate dehydrogenase (LDH) was determined according to the method using colorimetric detection kit. discrebed by **Dito. 1979.**

Histopathologic examination: Specimens of lung tissues were fixed in 10% formalin for 24 h, and standard dehydration and paraffin-wax embedding procedures were used. Sections (5 μ m) were cut in a microtome, adhered to glass slides with polylysine. Hematoxylin and eosin-stained slides were prepared by using standard methods and evaluated by light microscopy.

2. 7. Statistical analysis:

All mean values are reported as the mean ± SD. Data were analyzed using a one-way analysis of variance, followed by least significant difference (LSD) test. The level of significance was set at $P < 0.05$ in all cases. All statistical analyses were performed using SPSS software (Version 15.0) Data are means ± SD. Different letter on the top of each bar indicates $P < 0.05$ between them.

3. RESULTS:

Effects of treatment with noval nano composite on some lung tissues parmeters of nicotine-induced lung cancer (LC)

The obtained results in table (1) & figure (1) revealed that, a significant decrease in GPx, CAT, and, caspase 3 activities and p53 gene, were observed in nicotine- induced Lung Cancer in the mice. Meanwhile, a significant increase in Lipid peroxidation(LPx)., and nitric oxid (NOx) content in lung tissue

As well as, significant increase in Liver function (ALT,AST,ALP), kidney function (Createnine, Urea) and Lipid profil (Cholesterol, Triglyceride) tests and lactate dehydrogenase (LDH) activities in serm were observed in nicotine-induced lung cancer (LC) in mice when compared with control group.

Treatment groups with novel nano composite in nicotine-induced LC in mices resulted in improvement and significant increase in CAT, and GPx activities, p53 gene, and caspase 3 Furthermore,decreased in Lipid peroxidation(LPx)., and nitric oxid (NOx) content in lung tissue. at $p < 0.05$ as in table (1) & figure (1)

Table 1: **Effect of noval nano composite treatment on some lung tissue parameters of nicotine-induced lung cancer in mices.**

Experiment gps / Parameters	Control gp1	Nano gp2	Nicotine gp3	Nano+ Nicotine gp4
Caspase3(Unit/g.tissu	44.1±1.4	45.8±2.02[a]	34.08±2[abd]	40.8±2.8[abc]
P53 gene(Unit/g.tissue	13.6 ± 1.7	13.8±1.9	6.7 ± 1.2[abd]	11.6 ± 0.8[abc]
GPx (ng/ g.tissue)	26.1 ± 2.5	25±1.6[a]	17.3 ± 1.7[abd]	20.6 ± 1.6[abc]
CAT(mmol/g.tissue)	73.5 ± 2.8	75.5±3.1[a]	62.7 ± 2.2[abd]	73.4 ± 1.1[c]
LPx (µM/ gm)	82.5±4.(	73.5[acd]±3.1	103.2[abd]±2.3	82.2[bc]±2.5
NOx (µM/ gm)	52.1±1	50.3±3.2	90.2[abd]±2.4	48[abc]±8.8

a: significant from normal control group at $p < 0.05$

b: significant from nanocomposit supplement group at $p < 0.05$

c: significant from Nicotine induced-lung carcinoma group at $p < 0.05$

d: significant from (nanocomposit + Nicotine) group at $p < 0.05$

Data are presented as (Mean ± S.D). S.D = Standard Devation.Mean values with different superscript letters in the same row are significantly different at *(P<0.05)*.

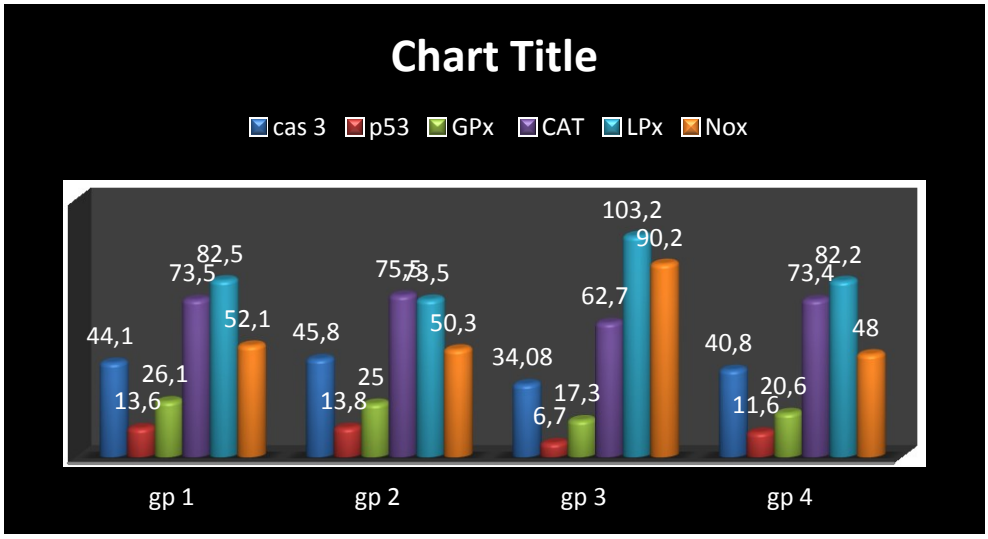


Figure (1) Effect of noval nano composite treatment on some lung tissue parameters of nicotine-induced lung cancer in mices.
gp(1) control gp, gp(2) nano composite gp, gp(3) nicotine gp,
gp(4) nano composite + nicotine gp.

And also, improvement occurred in biomarker enzymes as significant decrease in Liver function (ALT,AST,ALP and GGT), kidney function (Createnine, Urea) and Lipid profil (Cholesterol, Triglyceride) tests and lactate dehydrogenase (LDH) activities in serm were observed when compared with nicotine group. at $p < 0.05$ as in table (2)& Figure (2)

Table 2: Effect of noval nano composite treatment on some serm parameters of nicotine-induced lung cancer in mices.

Experimental gps / Parameters	Control gp	Nano Gp	Nicotine Gp	Nano+ Nicotine gp
ALT (U/L)	81.8±1.2	81.9±2.7	107.6^{abd}±1.8	84.4^{c}±6.1
AST (U/L)	131±2.7	133.4±1.8	141^{abd}±1.5	132^{c}± 3.3
ALP (U/L)	181.4 ± 2.7	181.6±1.1	214.6 ± 4.1^{abd}	184.5 ± 2.5^{c}
GGT (U/L)	8.7±0.4	9.1±0.3	18.4^{abd}±0.6	10.5^{abc}±1. 4
Cholesterol (mg/d	115.6±7..	122.6±6.8	210.8^{abd}±6.8	126^{ac}±8.4
Triglyceride (mg/dl)	135.4±4.'	132.8±9.9	153.8^{abd}±8.5	130.6^{c}± 6.6
Createnine (mg/dl	0.4±0.02	0.43±0.03	1.4^{abd}±0.2	0.46^{c}±0.07
Urea (mg/dl)	24.4±1.6	25.4±2.7	58.1^{abd}±1.8	27.2^{c}±3.4
LDH (U/L)	112.4 ± 2.5	112±3.8	175.6 ± 1.8^{abd}	119.8 ± 1.9^{abc}

a: significant from normal control group at $p < 0.05$
b: significant from nanocomposit supplement group at $p < 0.05$
c: significant from Nicotine induced-lung carcinoma group at $p < 0.05$
d: significant from (nanocomposit + Nicotine) group at $p < 0.05$
Data are presented as (Mean ± S.D).S.D = Standard Devation.

Mean values with different superscript letters in the same row are significantly different at *(P<0.05)*.

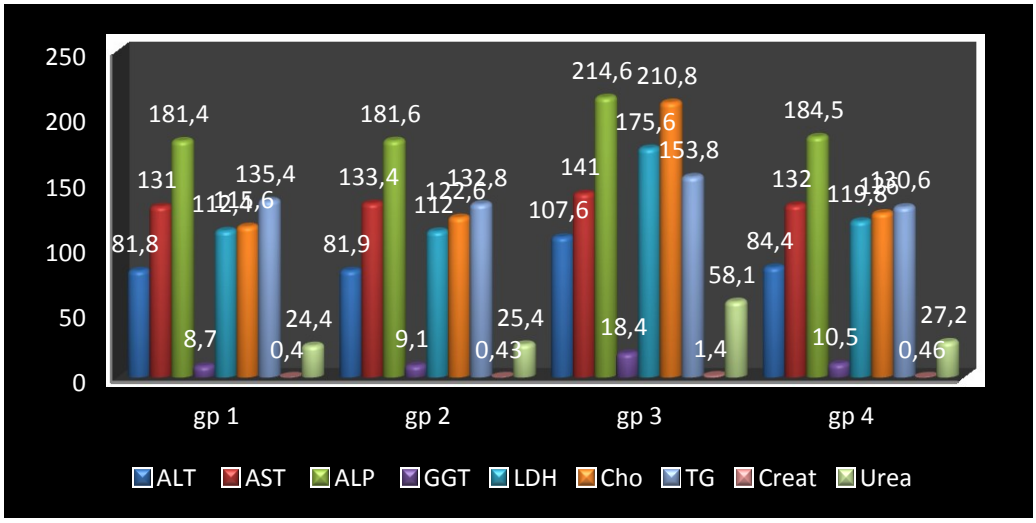
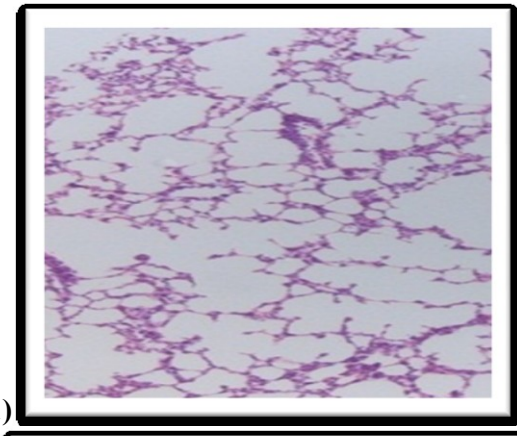


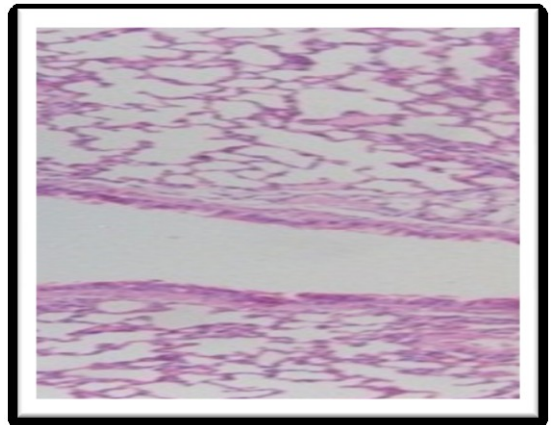
Figure (2) Effect of noval nano composite treatment on some serm parameters of nicotine-induced lung cancer in mices.
gp(1) control gp, gp(2) nano composite gp, gp(3) nicotine gp,
gp(4) nano composite + nicotine gp

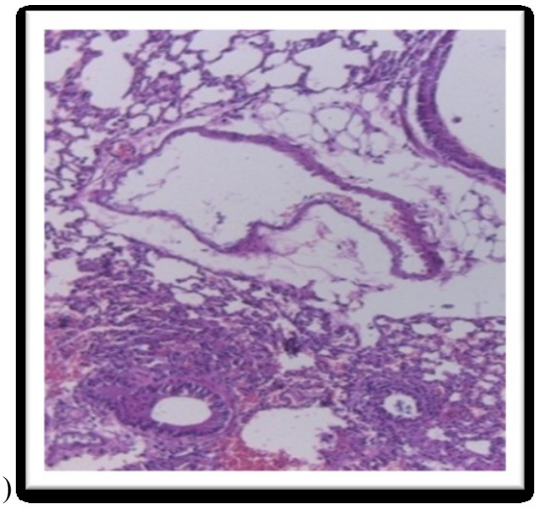
Histopathology examination of the lung tissues:

A(1)

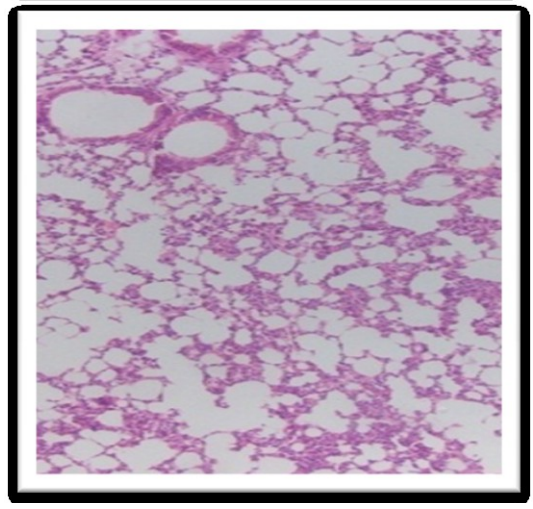


B(2)





C(3)



D(4)

Histopathological changes

Group(1) control gp Micrograph of lung section is normal The bronchoalveolar unit parenchyma, is normal (Fig. A(1)). Group(2) The (nanocomposit) group, showed parenchyma nearlly normal. (Fig.B(2)). Group(3) Nicotine treated animals showed cloudy swelling, medullary haemorrhage, emphysematous changes and showing thickened septa and marked dilated congested blood vessel in the alveolar septa. marked irregular air spaces (Fig. C(3)). Group(4) The (nanocomposit + Nicotine) group treated animals showed less normal appearance of interalveolar septa with mild infiltration of inflammatory cells, the congestion of lung was mild with no emphysematous changes and most of air space appeared normal and regular.(H & E) (Fig. D(4)).

4. DISCUSSION

Nicotine is considered a prototype polycyclic aromatic hydrocarbon (PAH), classic DNA damaging agent and carcinogen. Nicotine the major component of cigarette smoke plays an important role in the development of lung complications. Early-stage disease can be treated with curative intent although the risk for relapse is notoriously high. Unfortunately, the majority of

lung cancer patients present at an advanced stage. Despite an initial response to treatment, most of these late stage patients will eventually progress on standard therapy and die from their disease. Despite the complex nature of lung cancer biology, its molecular underpinnings are becoming increasingly clear. **Salgia, *et al*., 2011.**

Nicotine, the major component of cigarette smoke plays an important role in the development of lung complications Czernin & Waldherr. 2003. It causes oxidative damage to kidney, lung, liver and heart; it is a potential oxidant, which is capable of producing free radicals and reactive oxygen species Yildiz, et al., 1998. The nicotine-induced free radicals react with biomembranes causing oxidative destruction of polyunsaturated fatty acids and forming cytotoxic aldehydes by lipid peroxidation Yildiz, et al., 1999. Lipid peroxidation has been implicated in pathogenesis of a number of diseases. Morel, et al., 1998.

An increase in the activity of marker enzymes like AST, ALP, ALT and LDH in nicotine-treated rats indicates tissue damage. This increase in the LDH and ALP activities is the indicative of cellular damage due to loss of functional integrity of cell membranes. Wetscher, et al., 1995. Assay of ALP and LDH can be used for the prognosis of lung disorder. Rawson, & Peto, 1990. LDH, a cytoplasmic marker enzyme is a known indicator of cell and tissue damage by toxic compounds. Turna.2004. A high LDH level shows interstitial lung fibrosis following alveolar damage.Matusiewicz, et al., 1993. Oxidative damage to the cellular membranes produce marked changes in molecular organization of lipids resulting in increased membrane permeability. Lipid peroxidation can be used as an index for measuring the extent of damage that occurs in membranes of tissues as a result of free radical generation. **Jason et al., 1998**. Monitoring the extent of lipid peroxidation in the lung may aid in detection of lung disorders. **Janet et al., 1990**.Lipid peroxidation was enhanced in liver, lung and kidney of the nicotine treated rats. Nicotine is oxidized primarily into its metabolite cotinine in the liver, generates free radicals in tissues and induces oxidative tissue injury.**Wang, et al., 2000.** Thus the damage to the tissues in the nicotine treated animals may be due to the excessive generation of free radicals. In the present study the nicotine treated rats show increased levels of free fatty acids, and hydroperoxides. The increased free fatty acids in tissues of nicotine treated rats may serve as the substrate for lipid peroxidation. In the most study the cholesterol level was elevated in the nicotine-treated animals. The prevalence of hypercholesterolemia and triglyceridemia has been reported in heavy smokers. **Masora, 1977.** This increased level of cholesterol is attributed to the increased activity of 3-hydroxy-3-methyl-glutaryl CoA reductase (HMG-CoA reductase) and increased incorporation of labelled acetate into cholesterol. **Brunzell, et al., 1983.**

Nicotine decreases the activity of lipoprotein lipase resulting in elevated levels of triglycerides. This enzyme involved in the uptake of circulating triglyceride rich lipoprotein, chylomicrons or VLDL by the extra hepatic tissues. **Huttunen et al., 1976.** Chromaffin cells of adrenal medulla synthesize catecholamines by the stimulation of nicotine and adipose tissue lipolysis is carried out by catecholamines, which in turn elevates the levels of cholesterol, triglycerides and also increases fatty acids. **Cryer, 1981.**

Nanotechnology is the understanding and control of matter at dimensions between approximately 1 and 100 nanometers , where unique phenomena enable novel applications**. Boisseau. and Loubaton.2011.**

Antioxidants are the first source of protection of the body against free radicals and other oxidants, being the compounds that the attack and the formation of radical species within cells. The group of antioxidants inside the organism is known as the total antioxidantstate (TAS). **Teixeira, *et al*., 2013.**

The antioxidant protection of human cells includes enzyme mediated and non-enzymatic defense mechanisms. Superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (Gpx) are the most important antioxidant enzymes. SOD catalyses' the reaction of superoxide anion to hydrogen peroxide (H_2O_2); in turn, CAT converts H_2O_2 into water and oxygen. The affinity of CAT for H_2O_2 is relatively low, therefore, some H_2O_2 remains in the cell. GSH-px is capable of detoxifying the remaining H_2O_2. **Arrigoni, & De Tullio, 2002.**

Curcumin is a potent "scavenger" of the superoxide radical, a free radical that initiates potentially harmful oxidative processes such as lipid peroxidation **Sreejayan and Rao, 1996.** Through in Curcumin also increases survival of cells exposed in vitro to the enzyme hypoxanthine/xanthine oxidase, which stimulates superoxide and hydrogen peroxide production.Also curcumin demonstrates several other in vitro effects linked to free radical scavenging.Morever, curcumin has also been shown to quench reactive oxygen species and scavenge superoxide anion radicals and hydroxyl radicals and strongly inhibits nitric oxide (NO) production by down-regulating inducible nitric oxide synthase gene **expression Ghoneim 2009. and Wang, *et al*., 2008.** Decrease in tumorigenesis caused by turmeric is also associated with inhibition of DNA adduct formation. Curcumin inhibits of phase I enzymes systems consist of cytochrome P450 isoforms, the P450 reductase, the cytochrome b5 and the epoxide hydrolase and protect from the toxic effects of chemicals and carcinogens. **Jee, *et al*.,1998 .** On the other hand, curcumin induces phase II enzymes (glutathione S-transferases and epoxide hydrolase), which play a protective role by eliminating toxic substances and oxidants and conferring benefit in the prevention of the early stages of carcinogenesis. **Liao, *et al*.,2008**.

Furthermore, curcumin induces apoptosis in p53-null lung cancer cells. **Singh, 2009**. curcumin can block cell cycle progression or even apoptosis in a p53-independent manner as well, especially in the cells that lack functional p53. **Shankar and Srivastava, 2007.** Addetionally curcumin exhibits pleiotropic properties that involve the modulation of nuclear factor-kappaB (NF-κB), transcription factor activator protein-1 (AP-1), mitogen-activated protein kinase (MAPK), tumor protein 53 (p53), nuclear β-catenin signaling, and serine/threonine protein kinase (AKT) signaling pathways**. Hatcher, *et al.,* 2008.**

Zinc is one of the structural component of wide variety proteins and dependent enzymes like superoxide dismutase (SOD) that act as essential component of antioxidant defense system . **Bao, and choct, 2009.**

Nano ZnO is able to protect cell membrane integrity against oxidative stress damage, increase antioxidant enzyme levels and decrease MDA level**. Dawei, *et al.,* 2009** . Consequently, ZnONPs were shown to selectively induce apoptosis in cancer cells, ZnO NPs show much promise as new anticancer agents, given the specific apoptotic response of cancer cells.

Ascorbate has been examined in various epidemiologic studies as a potential chemopreventive agent for cancer. **Lee, *et al.,*2003.** Ascorbic acid is a good scavenger of free radicals and it protect cellular membranes their by preventing degenerative disease like cancer. **Gutteridge, *et al*., 2000 &Vijayavel, *et al*.,2006.** Caspase-3 was activated on sodium ascorbate

treatment, sodium ascorbate induced apoptosis via the mitochondria-dependent pathway in melanoma cells. **Shuw-Yuan. 2006.**

Histopathological findings revealed significant morphological alterations in the lung, of the nicotine-treated mice. This is due to the activation of circulating neutrophils, release of proinflammatory cytokines, free radicals, phospholipase A2 and a subsequent impairment of pulmonary vascular and alveolar structures leading to edema **Maritz, & Windvogel, 2003.** Microscopic changes observed in lungs of nicotine-treated mice showed congestion of the lung and emphysematous changes around collections of macrophages. Administration of novel nano composite at a dose of 70 mg/kg body weight effectively reduced these pathological changes induced by nicotine damage showing no emphysematous changes and normal parenchyma. Thus, novel nano composite showed its protective nature against nicotine induced lung toxicity.

5. CONCLUSION

From the obtained results it could be concluded that novel nano composite was an effective in protection against lung cancer induced by nicotine in mice Since, novel nano composite was able to ameliorate serum biochemical parameters, suppression of Lipid peroxidation(LPx)., and nitric oxid (NOx) content in lung tissue concentration, and increased activity of p53 gene, caspases -3 as well as enzymatic and non-enzymatic antioxidant defense system in lung tissue.

6.RECOMMENDATIONS

We recommend that these findings suggest that novel nano synthetic composite derivatives may potentially presents new hope for the development of lung cancer therapeutics, which should attract further scientific and pharmaceutical interest.

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BIOCHEMICAL EFFECT OF OLIVE OIL ON HYPERLIPIDEMIA INDUCED IN RATS

OMAYMA, A.RAGAB*; AFAF, D.ABDEL-MAJEED*; KAMEL, M.HASSANIN AND MOSTAFA, M.ABDELGHAFFAR*.**

*Biochemistry department, Faculty of Veterinary Medicine, Benha University.
**Biochemistry department, Faculty of Veterinary Medicine, Beni-Sweif University.

Abstract

This study was performed to investigate the effect of oral supplementation of olive oil on lipid profile, nitric oxide, adiponectin, endothelin-1, blood glucose and some inflammatory markers in normal, diabetic and hyperlipidemic rats supplementing high fat and cholesterol-enriched diet.

Forty female adult albino rats were divided into four equal groups of 10 rats each. Group (1): negative control received normal diet only, group (2): rats fed on normal diet and receive olive oil (0.5 ml/100g b.w.) orally, group (3): positive control received hyperlipidemic diet, group (4): rats fed on hyperlipidemic diet and received olive oil orally.

The obtained results revealed that, olive oil supplementations to hyperlipidemic rats showed a significant increase in serum HDL-cholesterol, nitric oxide, adiponectin and Endothelin-1 concentrations and significantly decrease in serum total cholesterol, triacylglycerols, LDL-cholesterol, Fasting blood glucose, Glycated Hemoglobin (HbA1C), high sensitive C-reactive protein and Interleukin-6 levels.

These results suggest that, olive oil supplementations may have some benefits in patients suffering from dyslipidemia and diabetes.

Key words: hyperlipidemia, diabetes, olive oil, lipid profile, inflammation, Interleukin-6.

1- INTRODUCTION:

Hyperlipidemia is a common disorder caused by lifestyle habits in developed countries and is the major cause of coronary heart disease. It results from abnormalities in lipid metabolism or plasma lipid transport or a disorder in the synthesis and degradation of plasma lipoproteins (Jang *et al*., 2008).

The consequence of hyperlipidemia can cause atherosclerosis, and thus the risk of coronary heart disease and stroke is increased.

Diabetes mellitus is associated with hyperlipidemia, which is a significant risk factor for cardiovascular diseases (El-Moselhy *et al*., 2011).

The incidence of type 2 diabetes mellitus is rapidly increasing worldwide. Type 2 (formerly called non-insulin dependent) diabetes mellitus accounts for over 90% of the diagnosed cases of diabetes (Pillarisetti and Saxena 2004).

Diabetes is a well-recognized risk factor for atherosclerotic and cardiovascular disease that confers a markedly increased risk of coronary heart disease (CHD). The altered lipid profile characterized by elevated levels of circulating free fatty acids (FFAs) and triacylglycerols, as well as a reduction in high-density lipoprotein cholesterol (HDL-C) along with excess fat deposition in various tissues including the liver (Banerjee *et al*., 2004). An abnormal accumulation of fat in the liver and muscle elicits insulin resistances that culminate in beta cell reduction in type 2 diabetes (Seo *et al*., 2008).

Olive oil improves the major risk factors for cardiovascular disease, such as the lipoprotein profile, blood pressure, glucose metabolism and antithrombotic profile (Abdel-Aal *et al*., 2008).

Some of these effects were attributed beside the monounsaturated fatty acids (MUFA) to the minor components of virgin olive oil (Al Jamal and Ibrahim 2011). Accordingly, the aim of this work was to investigate the antihyperlipidemic effects of oral supplementation of olive oil on some inflammatory markers, blood glucose, lipid profile, adiponectin and endothelin-1 in serum of female rats feeding high fat diet.

2- MATERIAL AND METHOD:

2-1- Experimental animals:

A total number of (40) adult female albino rats of (4 – 6) weeks weighting (140-160) gm were used in the experimental investigation of this study. Rats were obtained from the Research Institute of Ophthalmology, Giza, Egypt.

Animals were housed in separated metal cages and kept at constant environmental and nutritional conditions and allowed free access to standard pellet diet and water was supplied ad-libtum.

2-2- Diet:

Diets supplied to rats according to NRC (1995).

Table (1): Composition of the basal and fat-enriched diets for rats:

Feed Ingrdients	Level (%) in basal diet	Level (%) in fat-enriched diet
Oil	**15.00**	**13.00**
Yellow corn	**44.15**	**44.15**
Soya bean meal (44%)	**20.51**	**20.51**
Wheat Bran	**12.33**	**12.33**
Cholesterol	**0.00**	**1.00**
Coconut Oil	**0.00**	**2.00**
Molasses	**3.00**	**3.00**
Common Salts	**0.50**	**0.50**
Lysine	**0.18**	**0.18**
DL-Methionine	**0.74**	**0.74**
Min.-Vit. Premix	**2.00**	**1.50**
Ground Limestone	**1.59**	**1.09**
Total	**100.00**	**100.00**

2-3- Induction of Diabetes:

Streptozotocin powder manufactured by Sigma chemical Co. (USA) was used for induction of diabetes. According to (Mrudula *et al*., 2007).

Streptozotocin is an analogue of N-acetyl glucosamine which is readily transported into pancreatic beta cells by Glut2 and cause β-cell toxicity, resulting in insulin deficiency (Mrudula *et al*., 2007).

2-4- Dosage of olive oil:

Olive oil was given to female rats orally at a dose of (0.5 ml / 100g bw/ day) for 6 weeks (Nandakumaran *et al*., 2012).

3- EXPERIMENTAL DESIGN:

Rats were randomly divided into (4) main equal groups, 10 rats each, placed in individual cages and classified as follow: group (1): negative control, group (2): rats fed on normal diet and receive olive oil (0.5 ml/100g bw) orally, group (3): positive control, group (4): rats fed on hyperlipidemic diet and receive olive oil orally.

4- SAMPLE COLLECTION:

Blood samples were collected from all animal groups after 2, 4 and 6 weeks from the onset of curcumin, garlic and olive oil administration. The samples were collected in the morning after overnight fasting from the retro-orbital plexus of eyes.

Serum was separated by centrifugation at 3000 rpm for 10 minutes. The clear serum was aspirated and transferred into sterile labeled tubes and kept in a deep freeze at (- 70° C) until used for subsequent biochemical analysis. Total cholesterol according to (Schettler, 1975). Triacylglycerols (Schettler, 1975). HDL-cholesterol (Gordon *et al*, 1977). LDL-cholesterol (Friedewald, 1972). Blood glucose (Trinder, 1969). High sensitive C-reactive protein (Kimberly et al, 2003). Nitric oxide (Montgomery and Dymock, 1961). Endothelin-1 (Rolinski, 1994) Glycated Hemoglobin (Trivelli *et al*, 1971). Adiponectin (Yamauchi *et al*, 2002) and Interleukin-6 (Hirano, 1990).

5- STATISTICAL ANALYSIS:

The obtained data were statistically analyzed using one way analysis of variance (ANOVA) followed by the Duncan multiple test. All analysis were performed using SPSS (statistical package for social sciences, 1999; ver.10.0), values of $P \leq 0.05$ were considered to be significant.

6- RESULTS:

The obtained results presented in table (2) revealed that, Hyperlipidemia and diabetes caused significant increase in serum total cholesterol, Triacylglycerols, LDL-cholesterol, glucose, glycated hemoglobin, high sensitive C-reactive protein, interleukin-6 and endothelin-1 can confirm as compared with normal control group. Meanwhile, Oral administration of olive oil cause significant reduction in all of parameters as compared to that of positive control group.

Hyperlipidemia and diabetes induced significant decrease in serum HDL-cholesterol, adiponectin and nitric oxide can confirm as compared to that of normal control group.

7- DISCUSSION

The obtained results demonstrated that olive oil supplementation have potential effects in preventing hyperlipidemia, diabetes and on cardiovascular protection.

Interestingly, the results showed that, olive oil supplementation significantly improved serum lipid profile, as revealed by marked increase in HDL level and decrease serum total Cholesterol, triacylglycerols and LDL-C level.

It was found that olive oil improve lipid profiles and blood glucose in type-2 diabetic patients through the effect of monounsaturated fatty acids (MUFA), the minor components of virgin olive oil which prevents central fat redistribution and the postprandial decrease in peripheral adiponectin gene expression and insulin resistance induced by a carbohydrate-rich diet in insulin-resistant subjects (Al Jamal and Ibrahim 2011).

The obtained results also indicate that, olive oil supplementation were increased the serum NO level. This was matching with the study of (Song *et al*., 2013) showed that olive oil improve NO level in serum of patients with hypertriglyceridemia through the reaction of oleic acid with free radical $NO^{\bullet}$ - and nitrite (NO^{-}_{2})-derived species yields nitrated oleic acid. Although the mechanisms of biological fatty acid nitration remain incompletely characterized, recent studies reveal that during oleic acid nitration, vinyl nitro regioisomers represent a component that displays distinctive chemical reactivity and receptor-dependent signaling actions

Regarding serum adiponectin level similar results was reported by (Hafida *et al*., 2013) that olive oil increases serum adiponectin level in obese rats due to reduced fat mass could be explained mechanistically by increased β-oxidation and reduced lipogenesis in adipose tissue.

It was confirmed that olive oil to have anti-inflammatory effect and lower levels of hs-CRP and Interleukin-6 in obese rats and this suggestion was supported by the finding of (Sarda *et al*., 2012) investigate the effect of olive oil on serum hs-CRP and Interleukin-6 levels in subjects at high cardiovascular risk. They attributed the effect of olive oil to the components such as phenolic compounds, α-tocopherol, and carotenoids and to the high unsaturated/saturated fatty acid ratio with oleic acid (MUFA) as its main fatty acid.

The obtained results revealed that olive oil supplementations increased serum level of endothelin-1 level and our results were similar to the study of (Pontiroli *et al*., 2004), showed the effect of olive oil for improving serum Endothelin-1 level in morbidly obese subjects.

They explained that, olive oil might modulate the production of endothelin-1 (ET-1) and nitric oxide by arterial vessel, limiting the development of atheroma plaques. Moreover, they reported that eicosapentaenoic acid inhibited the ET-1 production stimulated by oxidized LDL.

8- CONCLUSION AND RECOMMENDATION

In conclusion, the present study demonstrated that olive oil supplementations showed positive effects on lipid profile, blood glucose level and serum inflammatory markers that may be developed by hyperlipidemia and diabetes. The antioxidant effects of olive oil have been shown to attenuate Streptozotocin-induced diabetes and may prevent diabetic cardiovascular complications.

Also, the present study demonstrated that olive oil is potent vasorelaxants as well as reduce the atherogenic properties of cholesterol.

So we recommended that, olive oil is useful in treatment of hyperlipidemia, cardiovascular disorders and insulin resistance. It must be used carefully and under medical supervision to get its therapeutic benefits and avoid their side effects.

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Table (2) : Effect of olive oil on serum total cholesterol, serum triglyceride, LDL-cholesterol, blood glucose , glycated hemoglobin, high sensitive C-reactive protein, interleukin-6, endothelin-1 HDL-cholesterol, adiponectin and nitric oxide level in the blood.

The table represents the mean value of the obtained results for each parameter of the samples collected after 2, 4 and 6 weeks from the onset of curcumin, garlic extraction and olive oil

Parameters	Groups			
	G1	G2	G3	G4
FBS	92.79a ±0.63	95.47b ±0.52	177.11e ±2.25	103.91c ±1.22
Adiponectin	5.93d ±0.05	5.68c ±0.08	2.77a ±0.08	8.15e ±0.05
hs-CRP (high sensitive C-reactive protein)	0.52b ±0.02	0.48ab ±0.01	1.22d ±0.04	0.93c ±0.04
IL-6 (interleukin-6)	67.26e ±0.69	62.28d ±0.45	83.35f ±0.54	57.45b ±0.56
NO (Nitric oxide)	76.24d ±0.47	71.18b ±0.78	66.63a ±0.47	110.96g ±0.7
Cholesterol	142.49c ±0.40	126.39b ±0.40	241.18g ±2.35	161.39f ±1.73
Triglyceride	99.27c ±0.38	89.33b ±0.39	199.48 ±1.55	141.18e ±1.29
HDL-C (high density lipoprotein cholesterol)	40.72d ±0.48	41.80de ±0.35	27.71a ±0.55	31.91b ±0.38
LDL (low density lipoprotein cholesterol)	130.31e ±0.71	117.85b ±0.76	195.81f ±0.80	121.71d ±1.28
HBA1C	5.02a ±0.11	5.04a ±0.07	7.02c ±0.11	5.70b ±0.09
Endothelin-1	0.96bc ±0.04	0.85ab ±0.03	1.67d ±0.05	0.94bc ±0.04

S.E. : Standard Error

a, b, c: Mean values with different superscript letters in the same row are significantly different at ($P \leq 0.05$).

MICROBIOLOGICAL RISK ASSESSMENT FROM THE INTEGRATED MILK CHAIN IN A MOUNTAINOUS AREA

Rodica SOMEŞAN, Laurenţ OGNEAN*, George NADĂŞ, and Nicodim FIŢ

University of Agricultural Science and Veterinary Medicine Cluj-Napoca, România
lognean@yahoo.com

According to current trends, worldwide cheese production is in constant growth and microorganisms is an essential component with important role in their maturation. The paper evaluates the microbiological risks of cheeses made from the milk collected from a mountainous area in central Transylvania. Microbiological risk assessment was done by tracking bacterial and fungal flora in milk samples from farms, collection points and brine in summer and winter as well as the cheese as finished products. Tests to determine the total number of mesophilic bacteria, total fungi, tests for identifying coagulase positive Staphylococcus, Salmonella identification, coliform bacteria and other pathogenic potential pathogens were performed. Results for raw milk, revealed a diverse bacterial, the bacterial profiles ranged between 3 and 5 bacterial genera (most common were Staphylococcus, Aerococcus, Micrococcus, Bacillus and Corynebacterium) mycotic profile highlighted 4 genera (Mucor, Aspergillus, Saccharomyces and Candida). The assortments of cheeses were abundant in lactic acid bacteria, small number of bacterial and mycotic species being found in milk and the brine used in the manufacturing process.The microbiological risk assessment of milk and cheese revealed that microbial flora collected in the mountains of central Transylvania is varied and includes potential pathogenic microorganisms. It was found that the process of cheese production inactivates most pathogenic bacteria and enriches the finished product in lactobacilli.

Keywords: microbiological risk, bacteria and fungi, milk, cheese, mountainous area.

INTRODUCTION

Microorganisms are an essential component of traditional cheeses with an important role in the production and maturation (Beresford et al., 2001). Biodiversity of mountain pastures can provide a higher value of raw milk by using milk in the production of traditional cheese products with superior flavor and texture (Martin et al., 2005).

According to current trends, raw milk cheese production is growing and consumers have increasingly high requirements regarding food with traditional character (Laurenčic et al., 2008). Today, consumers prefer less processed foods, no preservatives, low in salt, sugar or fat, but with an extended shelf life and quality (Zink, 1997). Also, the demand for convenient foods and ready to eat, and the food industry has developed new techniques for processing food products (minimum processing, prepared foods, refrigerated), in response to these requests (Hedberg et al., 1994).

This processing technique provides the proper environment for bacteria that produce toxins, such as *Staphylococcus aureus*, which is capable of growing and expressing the virulence in a variety of food (milk, mixed food, meat and meat products, eggs and egg products, cakes and ice cream). Thus, in 2009 the European Food Safety Authority (EFSA) reported that the cheeses followed by mixed meals or buffet were two main causes for food poisoning outbreaks caused by staphylococcal toxins (Schelin et al., 2011).

The flora fungal of milk and milk products may also be responsible for a series of negative effects, such as alterative processes, damaging the sensory properties of cheese, but can also produce relevant medical problems as in the case of mycotoxins produced by molds (Panelli et al., 2013).

Recent studies (Wallin Carlquist et al., 2010; Cretenet et al., 2011) focused on this indicated significant differences in the behavior of bacteria in plankton and real food matrices, thus the identification and quantification of microbiota throughout food chain is

essential in microbial risk assessment and prevention of food-borne diseases (Schelin et al., 2011).

As a result of these realities, food security remains an important issue for the food industry worldwide and is one of the major objectives aimed to improve public health (Thomas et al., 2013).

The arguments fully justify such a study designed to evaluate microbiological risks, the integrated chain of milk produced under a sub-Carpathian mountain areas of our country.

This paper focused on investigating milk and dairy products aimed the microbiological risk assessment by identifying and quantifying bacterial flora and fungi on the integrated chain of milk (raw milk, brine, cheese products), obtained in a mountainous area.

MATERIALS AND METHODS

Between December 2012 and December 2013 were conducted researches focussed on identification and quantification of bacterial flora and fungi samples of raw milk and processed cheese and brine (natural and conventional), used in the manufacturing process, in a private company. For this, milk produced in conditions of sub-Carpathian mountain areas from predominantly indigenous cattle breeds bred in traditional households and commercial farms was studied.

The research began by identifying raw milk suppliers, framed in product standards comply with legislation and with the processing unit legislation. Following assessments carried out the activities of the unit studied, we identified the following two sources for raw milk, including: collection centers (n = 6) and farms (n = 9). Milk from small producers was taken by the 6 milk collection points (MCP), which are organized and managed by processing units. Farms use their own cooling tanks and allowed receiving milk directly from the farm. The average quantity of milk supplied daily from the two sources was 35,000 l/day. The investigations were conducted over 12 months and included the following two milk samples: the sample A represented by raw milk originated from collection, sample B, raw milk from farms. From each sample of milk samples were collected in two seasons (summer and winter) and subjected to laboratory analysis, summing up a total of 30 analyzes.

The investigation was based on the surveys, identification and quantification of bacterial flora and fungi samples of cheese and brine (natural and conventional), used in the manufacturing process. Thus, based on assessments, was initially established that the two types of cheese, yellow cheese and feta, are obtained by a traditional method specific to the area of study. The specificity of this method is the use in the production of natural brine cheese that is abundant in the study area, being a natural reservoir.

The assortment of cheese investigated has a content of 54% DS (dry substance) and standardized raw milk is obtained from a ratio fat/protein of 0.96 and rennet chymosin. Its processing includes aging at a pH of 4.9-5.2, followed by slicing and blanching (74-78ºC) in natural brine bath with a concentration of 6-8%, and finally drying, packaging and storage at 2 8ºC.

Feta cheese, containing 40% DS is produced from standardized milk at a ratio of fat/protein of 0.87 pasteurized (85ºC for 3 minutes), connected using chymosin and addition of lactic acid and calcium chloride. Its processing includes slicing and keeping in natural brine with a concentration of 14-16%, for 7-14 days at a temperature of 2-8ºC; drying, packaging and storage at 2-8ºC.

Please note that, in order to quantify the impact of using natural brines, we have also studied conventional brine (salt water reconstituted) at the same NaCl concentration as the natural one. Initially, the concentration of NaCl in the brine (natural and conventional) was 24%, and then was diluted with water depending on the concentration used in the production

process (6-8% and 14-16% respectively). From each sample (cheese and brine) samples were collected (n = 2) and subjected to laboratory analysis, summing up a total of 16.

Samples collected from each specimen in the study were subjected to microbiological testing: for this purpose bacteriological and mycological examinations were conducted. Bacteriological examinations consisted in identifying mesophilic bacteria from milk, cheese and brine using phenotypic tests. Thus the samples were initially inoculated on nutrient agar with the addition of 5% sheep blood and incubated at 37ºC for 24 hours. Morphological tests, inoculations onto selective media, biochemical and API® bioMérieux tests were also performed. Protocol identification respected the international standards described by Markey et al. 2013. Coagulase-positive staphylococci were determined from the specimens (SR ISO 6888-1: 2002 / A1: 2005), the detection of *Salmonella spp* (SR ISO 6579: 2003 / A1: 2007) and the detection and enumeration of coliforms (SR ISO 4831: 2009). In order to determine the total number of bacteria in milk and cheese, the medium with glucose and yeast extract with incubation at 30ºC for 48 hours, and counting of yeast and molds was used in the medium of glucose, yeast extract and chloramphenicol, with incubation 25 ± 2ºC for 7 days.

Data were processed for identification and quantification of bacterial flora and fungi, and statistical analysis was performed using GraphPad InStat 3 (for statistical interpretation using a p significance threshold of 0.05), Fisher and Mann Whitney test.

RESULTS AND DISCSSIONS

Evolution of the bacterial and mycotic flora from raw milk. The results of bacteriological testing of raw milk, revealed a complex mesophilic bacterial flora for winter season (*Staphylococcus spp., Streptococcus spp., Corynebacterium spp., Bacillus spp., Neisseria spp., Aerococcus viridians, Lactobacillus lactis, E. coli , Bacillus subtilis, Micrococcus lutea, Staphylococcus epidermidis, Bacillus cereus*). During the summer season, although bacterial flora was similar for most milk samples, other species were also identified (*Proteus spp., Klebsiella spp., Prototheca zopfii*) (Tab. 1 and 2).

Comparative analysis of data obtained for two seasons (collections), revealed a microbial load of raw milk, higher in summer season (Fig. 1), but with statistically insignificant differences (p = 0.80).

Table 1. Mesophilic bacterial flora isolated from milk in farms

Sample nr.	Farms			
	Winter		Summer	
	Isolated species	TGC mesophilic bacteria (CFU/ml)	Isolated species	TGC mesophilic bacteria (CFU/ml)
1	*Lactobacillus lactis* *E. coli*	150	*Staphylococcus spp.,* *Lactobacillus lactis, Micrococcus,* *E. coli, Corynebacterium* spp.	750
2	*Bacillus subtilis*	70	*Staphylococcus* spp., *Aerococcus viridans, E. coli, Actinomices* spp.	80
3	*Staphylococcus epidermidis,* *Aerococcus viridans, E. coli*	520	*Staphylococcus* spp., *Streptococcus* spp., *E. coli,* *Prototheca zopfii, Micrococcus* spp.	350
4	*Staphylococcus* spp., *E. coli* *Streptococcus spp., Bacillus cereus*	430	*Staphylococcus* spp. *Streptococcus spp., E. coli*	210
5	*Staphylococcus* spp., *Lactobacillus lactis, Bacillus cereus, E. coli*	540	*Staphylococcus* spp. *Micrococcus spp., E. coli*	140

6	*Staphylococcus spp.* *Streptococcus* spp.	60	*Staphylococcus* spp., *Aerococcus viridans, Micrococcus* spp., *Bacillus spp., Corynebacterium* spp.	790
7	*Staphylococcus spp.* *Lactobacillus lactis*	210	*Staphylococcus* spp., *Aerococcus viridans, Micrococcus* spp., *E. coli*	750
8	*Aerococcus viridans* *Micrococcus lutea*	430	*Lactobacillus lactis* *Streptococcus* spp.	140
9	*Lactobacillus lactis, Aerococcus viridans, Corynebacterium* spp.	820	*Aerococcus viridans* *E. coli, Neisseria* spp.	320

Table 2. Mesophilic bacterial flora isolated from milk in collection centers

Sample nr.	Collection centers			
	Winter		Summer	
	Isolated species	TGC mesophilic bacteria (CFU/ml)	Isolated species	TGC mesophilic bacteria (CFU/ml)
1	*Aerococcus viridans* *Corynebacterium* spp.	120	*Staphylococcus spp., E. coli, Micrococcus, Lactobacillus lactis*	110
2	*Streptococcus* spp. *Staphylococcus* spp.	320	*Staphylococcus* spp. *Lactobacillus lactis, E. coli*	80
3	*Staphylococcus* spp. *Streptococcus* spp., *E. coli*	240	*Staphylococcus* spp. *Aerococcus viridans, E. coli*	320
4	*Staphylococcus* spp., *Aerococcus viridans, Bacillus* spp.	120	*Staphylococcus spp., Streptococcus spp., E. coli, Klebsiella* spp.	340
5	*Staphylococcus* spp. *Aerococcus viridans, Bacillus* spp.	350	*Lactobacillus lactis* *Streptococcus spp., E. coli*	230
6	*Streptococcus spp., Neisseria* spp., *E. coli*	240	*Staphylococcus* spp., *Streptococcus* spp., *E. coli, Proteus* spp.	120

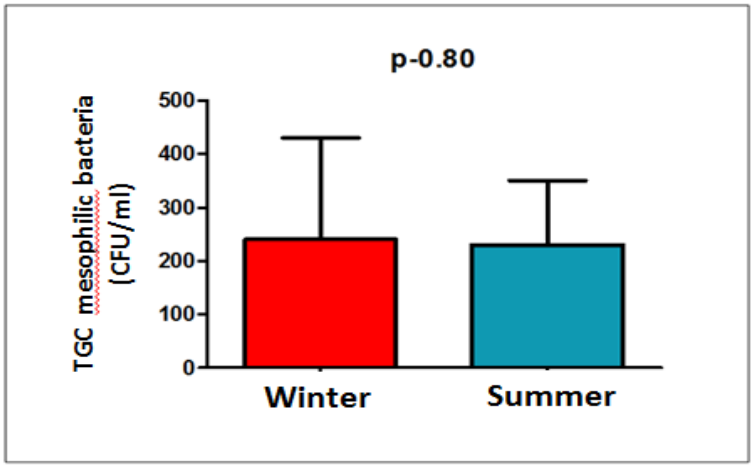

Fig. 1. Statistical difference in TGC average values (CFU/ml) between the two seasons.

Regarding mycological testing results, they revealed for the winter season, a complex fungal flora, represented by *Mucor spp*., *Aspergillus spp*., *Saccharomyces spp*., *Saccharomyces cerevisiae* and *Candida famata*. The microflora in the summer period was only represented by *Geothricum spp.* and *Saccharomyces cerevisiae* (Table 3, 4).

In comparison, the data for the two seasons, showed a fungal load of raw milk higher in winter (Fig. 2), but statistically insignificant differences (p = 0.60).

Table 3. Mycotic flora identified from milk in collection centers

Sample nr.	Collection centers			
	Winter		Summer	
	Isolated mycets	Mycets TGC (CFU/ml)	Isolated mycets	Mycets TGC (CFU/ml)
1	*Mucor* spp.	20	Negative	0
2	*Saccharomyces cerevisiae*	40	*Geothricum* spp.	10
3	*Saccharomyces cerevisiae*	10	Negativ	0
4	*Saccharomyces cerevisiae*	40	*Saccharomyces cerevisiae*	60
5	*Saccharomyces* spp.	60	*Saccharomyces cerevisiae*	20
6	*Saccharomyces cerevisiae*	10	Negative	0

Table 4. Mycotic flora identified from milk in farms

Sample nr.	Farms			
	Winter		Summer	
	Isolated mycets	Mycets TGC (CFU/ml)	Isolated mycets	Mycets TGC (CFU/ml)
1	*Saccharomyces cerevisiae*	30	Negative	0
2	*Aspergillus* spp.	10	Negative	0
3	*Saccharomyces cerevisiae* *Mucor* spp.	60	*Saccharomyces cerevisiae*	40
4	*Saccharomyces cerevisiae*	40	*Saccharomyces cerevisiae*	10
5	*Saccharomyces cerevisiae*	50	*Saccharomyces cerevisiae*	10
6	*Saccharomyces cerevisiae*	20	*Saccharomyces cerevisiae*	120
7	*Saccharomyces cerevisiae*	10	Negative	0
8	*Candida famata*	70	*Saccharomyces cerevisiae*	10
9	Negative	0	*Saccharomyces cerevisiae*	40

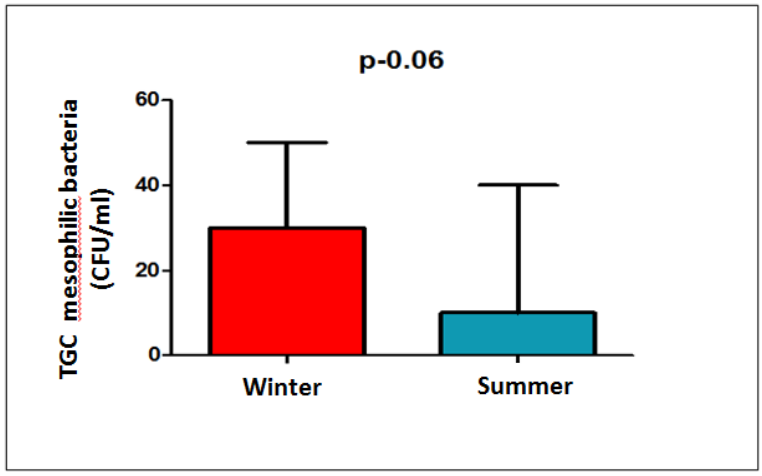

Fig. 1. Statistical difference in mycets average values (CFU/ml) between the two collections.

Evolution of the bacterial and mycotic flora from brine. The results from natural brine microbiological testing, showed that bacterial flora is represented by *Micrococcus lutea*, and the fungal by *Penicillium spp*. in both seasons (winter and summer). Regarding conventional brine, the results were negative in both bacterial and fungal flora (Tab. 5).

Table 5. Mycotic flora identified in brine (natural and conventional)

Sample nr.	Product name	Bacteria	bacteria TGC (CFU/ml)	Mycets	Fungi TGC (CFU/ml)
Winter					
1	Natural brine	*Micrococcus lutea*	50	*Penicillium* spp.	10
2	Conventional brine	Negative	0	Negative	0
Summer					
3	Natural brine	*Micrococcus lutea*	50	*Penicillium* spp.	10
4	Conventional brine	Negative	0	Negative	0

Evolution of the bacterial and mycotic flora from cheese.

The data obtained from bacteriological testing indicated for the winter season a complex bacterial flora both for yellow cheese (*Corynebacterium spp., Bacillus coagulans, Lactobacillus spp., Neisseria spp., Micrococcus lutea, Lactobacillus, Lactobacillus casei*), as well as for feta cheese (*Actinomyces spp., Lactobacillus lactis, Lactobacillus casei, Micrococcus lutea*). In the summer, the bacterial flora was similar in most samples, but two new species were also identified: *Lactobacillus lactis* (cheese) and *Bacillus spp.* (feta) (Tab. 6).

Regarding mycological test, the results indicated that fungal flora of cheeses studied, was represented by *Saccharomyces cerevisiae*, in both seasons.

Table 6. Bacterial and mycotic flora identified from cheese products in the winter season

Sample nr.	Cheese type	Identified bacteria	Bacteria TGC (CFU/ml)	Identified mycets	Fungi TGC (CFU/ml)
Winter					
1	Feta	*Lactobacillus casei* *Lactobacillus lactis*	20	*Saccharomyces cerevisiae*	110
2	Feta	*Micrococcus lutea* *Actinomices* spp.	30	*Saccharomyces cerevisiae*	430
3	Yellow cheese	*Lactobacillus* spp. *Bacillus coagulans, Neisseria* spp.	120	*Saccharomyces cerevisiae*	150
4	Yellow cheese	*Lactobacillus, Neisseria* spp. *Corynebacterium* spp	509	*Saccharomyces cerevisiae*	810
5	Yellow cheese	*Lactobacillus casei* *Micrococcus lutea*	450	*Saccharomyces cerevisiae*	620
Summer					
1	Feta	*Lactobacillus casei* *Micrococcus lutea, Bacillus spp.*	30	*Saccharomyces cerevisiae*	180
2	Feta	*Lactobacillus lactis* *Actinomices* spp.	40	*Saccharomyces cerevisiae*	90
3	Yellow	*Lactobacillus casei*	120	*Saccharomyces*	420

	cheese	*Neisseria* spp.		*cerevisiae*	
4	Yellow cheese	*Lactobacillus lactis, Neisseria spp., Corynebacterium* spp.	300	*Saccharomyces cerevisiae*	260
5	Yellow cheese	*Lactobacillus* spp. *Micrococcus lutea*	340	Saccharomyces cerevisiae	610

Overall analysis of the data obtained from microbiological testing showed that bacterial and fungal complexes in raw milk and cheese produced is complex and evolves differently during the manufacturing and storage process (Lafarge et al., 2004).

Mesophilic bacteria species detected in raw milk samples analyzed were 15, the most common being *Staphylococcus spp.* (N = 20), *E. coli* (n = 19), *Streptococcus spp.* (N = 11), *Aerococcus viridians* (n = 11) and *Lactobacillus lactis* (n = 9). The rest of identified species (*Corynebacterium spp., Bacillus spp., Micrococcus lutea, Bacillus cereus*), were less frequent isolated or identified in one sample (*Proteus spp., Klebsiella spp., Prototheca zopfii, Actinomyces spp ., Neisseria spp.*).

Results for raw milk revealed a bacterial biodiversity where the bacterial profiles varied. Some samples of raw milk only resulted in the isolation of two or three types of bacteria (*Staphylococcus spp., Streptococcus spp., E. coli*), others have shown more complex profiles, with up to five types of mesophilic bacteria (*Staphylococcus spp., Aerococcus viridans, Micrococcus spp., Bacillus spp., Corynebacterium spp*.).

Regarding the mycological profile of raw milk, the results indicated that fungal species identified were 5 in the winter season (*Mucor spp., Aspergillus spp., Saccharomyces spp., Saccharomyces cerevisiae* and *Candida famata*) and 2 in the summer (*Geothricum spp*., and *Saccharomyces cerevisiae*).

Regarding the microbiological profile of natural brine, the results indicated a bacterial flora represented by *Micrococcus lutea* and *Penicillium spp*. and negative results for the conventional brine.

Data for cheese showed a bacterial flora complex both for yellow cheese (*Corynebacterium spp., Bacillus coagulans, Lactobacillus spp., Neisseria spp., Micrococcus lutea, Lactobacillus, Lactobacillus casei, Lactobacillus lactis*), as well as feta cheese (*Actinomices spp. Lactobacillus lactis, Lactobacillus casei, Micrococcus lutea, Bacillus spp*). In both varieties of cheese, lactic bacteria predominated (*Lactobacillus, Lactobacillus casei, Lactobacillus lactis*) and the remaining species identified were found in raw milk (*Corynebacterium spp., Bacillus coagulans, Neisseria spp., Actinomyces spp*.) or natural brine (*Micrococcus lutea*) used in the manufacturing process and maturation.

In the case of cheese fungal flora investigated, it was confirmed that the species of fungi identified (*Saccharomyces cerevisiae*) found in yellow cheese and feta, was also found in both raw milk and natural brine used in the production and maturation process.

The results obtained in this study revealed that the microbiological composition of raw milk and natural brine used in the production process, determined the composition of bacterial and fungal community of the cheese tyopes, results outlined in other studies in this segment (Fuka et al., 2013). Microflora (especially the surface one) of ripened cheese is complex, composed of various species of yeasts and bacteria with strong effect on flavor, texture and appearance, reducing at the same time the risk of contamination with pathogens and alterations, such as *Listeria monocytogenes* (Monnet et al., 2012). In many cases, as in our study (*Corynebacterium spp., Micrococcus lutea*), a part of the species present on the surface of the cheese belong to the genera *Corynebacterium, Arthrobacter, Brevibacterium, Staphylococcus* and *Micrococcus* (Brennan et al., 2002, Feurer et al ., 2004, Mounier et al., 2005, Rea et al., 2007, Hunter et al., 2009).

It is known that many factors influence the composition of milk and therefore the nature and abundance of microbes, which then contribute to the sensory characteristics of the

cheese made from raw milk (Lafarge et al., 2004). However, the lack of pasteurization may favor the development of unwanted species and increase the probability of survival of pathogens in arrays of cheese, ready for consumption (Fuka et al., 2013).

The health status, the type of feed (silage) and production conditions (in particular hygiene practices) and storage of raw milk, are important factors that determine the composition of the microbial flora of the milk and finally the cheese quality (Fuka et al., 2013). Intensive washing of the milking equipments associated with low storage temperature of raw milk, offers a high level of contamination with coliform bacteria and *Pseudomonas spp.* (Wang et al., 2007; Culman et al., 2009). Instead, minimum udder hygiene keeps microorganisms, including halo-tolerant bacteria (*Micrococcus, Arthrobacter, Microbacteriu, Brevibacterium* and *Staphylococcus spp*.) and lactic bacteria (El-Baradei et al., 2008).

As a result of these realities, composition and microbiological characteristics of milk and milk products should be closely monitored and controled by biochemical processes and technology to develop final products with high food and safety values. Thus, food security remains an important issue for the food industry worldwide and is one of the major objectives aimed to improve public health (Thomas et al., 2013).

CONCLUSIONS

Mesophilic bacterial species detected in raw milk samples analyzed were 15, the most common being represented by *Staphylococcus spp.* (N = 20), *E. coli* (n = 19), *Streptococcus spp.* (N = 11), *Aerococcus viridians* (n = 11) and *Lactobacillus lactis* (n = 9).

Mycological profile of raw milk was represented by five species (*Mucor spp., Aspergillus spp., Saccharomyces spp., Saccharomyces cerevisiae* and *Candida famata*) in winter and two in summer respectively (*Geothricum spp.* and *Saccharomyces cerevisiae*).

In cheese varieties studied, lactic bacteria (Lactobacillus, Lactobacillus casei, Lactobacillus lactis) were predominant while the remaining species identified or were either found in raw milk (*Corynebacterium spp., Bacillus coagulans, Neisseria spp. , Actinomices spp.*) or natural brine (*Micrococcus lutea*) used in the manufacturing process and maturation.

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BIOMORPHOLOGY OF THE PAIRED FINS OF THE GILTHEAD SEA BREAM (*Sparus aurata*)

Oleg P. MELNYK[1], Kateryna GROM[1], Cristian DEZDROBITU[2], Melania CRIŞAN[2], Aurel DAMIAN[2]

[1]National University of Life and Environmental Sciences of Ukraine, Kiev, Ukraine.
[2]Faculty of Veterinary Medicine Cluj-Napoca. University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania.
museum@nubip.edu.ua, kateryna_grom@ukr.net

Abstract

The gilthead sea bream (Sparus aurata) is a specie of highly economic value. The present study was undertaken to establish the normal, healthy features of biomorphological structure of the paired fins in the gilthead sea bream, as well as to identify muscles that act on them with comparison of their relative masses. An anatomical dissection was carried out on the fresh specimens of fish. It was shown that the structure of the pectoral and pelvic girdles of Sparus aurata has its own peculiarities in comparison with other Percoidei fishes. The pectoral girdle consists of bones of both dermal (posttemporal, supracleithrum, cleithrum, postcleithrum) and cartilaginous origin (scapula, coracoid). The endoskeleton of the pectoral fin is composed of four mesopterygial elements and propterygium that are overlapped by the upper and lower rows of lepidotrichia. The fin rays are created by the fusion of lepidotrichia. The propterygium articulates with the first stiff fin ray that also attaches to the scapula. The pectoral girdle is linked with the axial skeleton by two prongs of posttemporal that attach to the supratemporal bones of the skull and by the ligament that, unlike other fish, originates on the supracleithrum and attaches to the basioccipital. The pelvic girdle of Sparus aurata is curved dorsally and has an incisure, where the apex of the heart enters. It consists of basal plates and basipterygium to which the fin rays attach. The basipterygium has a well developed crest that border the arrector dorsalis pelvicus and two needle-like processes which are directed anteriorly. The first fin ray of the pelvic fin is rigid and forms a spine.

The muscles that act on the paired fins were described along with the functions which they perform during fish locomotion. The group of adductors and arrectors of the paired fins are relatively equally developed. The abductor superficialis is more developed on the pectoral fin, while abductor profundus – on the pelvic fin.

Keywords: biomorphology, bones, gilthead sea bream, muscles, paired fins.

INTRODUCTION

The gilthead sea bream belong to the order Perciformes, family Sparidae that includes about 115 species of highly economic value. According to the data from 2006 the world aquaculture production of the gilthead sea bream, silver sea bream and porgies contributed 6,8 % in volume (244,153 mtn) and 14 % in value among other Percoidei fishes. Moreover, the gilthead sea bream is farmed not only for human consumption, but also for recreation purpose (Pavlidis and Mylonas, 2011).

To the paired fins of fish belong pelvic (ventral) and pectoral fins. It is known that the paired fins of fish are believed to be homologues of limbs of higher vertebrates (Yano and Tamura, 2013). The pectoral fins are places at the thoracic level behind the operculum, but the position of pelvic fins on the body of fish may vary in different families. In Percoidea (including the gilthead sea bream) the pectoral fins are places high up on the lateral side of the body, while the pelvic fins are placed at the thoracic level under the pectoral fins.

The function of the paired fins of percoidea type have been well documented in Harris's (1938) classical review, and the swimming behavior of the gilthead sea bream - in some recent papers (Montoya *et al.*, 2010; Svendsen *et al*., 2015). Numerous studies were dedicated to the osteological development of the paired fins in sparids (Matsuoka, 1985; Koumoundouros *et al*., 2001a; Koumoundouros *et al*., 2001b; Sfakianakis *et al*. 2004; Sfakianakis *et al*. 2005; Coban *et al*., 2009). There are also some investigations that were related to the embryological development of the skeletal elements of the paired fins of the gilthead sea bream (Faustino and Power, 1999; Faustino and Power, 2001) and to the development of lateral muscle (Patruno *et al*., 1998) that presumably rotate the pectoral girdle

backwards (Ostrander, 2000). However, much less attention was paid to the anatomical (biomorphological) peculiarities of the paired fins of this specie of fish and to the description of muscles that directly act on them.

It is no wonder that proper fish shape (Loy *et al.*, 1999; Russo *et al.*, 2007) and absence of morpho-anatomical abnormalities (Paperna, 1978; Georgakopouloua *et al*., 2010) are important components that affect the fish's health, as well as its market value. Besides, skeletal anomalies usually occur more often in hatchery-reared specimens than in wild-caught sea breams (Boglionea *et al.*, 2001). Thus, knowing biomorphological features, namely skeleto-muscular structure of the paired fins, could be helpful for veterinary specialists, ichthyologist and fish breeders.

MATERIALS AND METHODS

The study was performed at the Department of Animal Anatomy of National University of Life and Environmental Sciences of Ukraine (Kiev, Ukraine) on cadavers of fish (*Sparus aurata* L., 1758) that came from Turkey from Mediterranean sea. Since the gilthead sea bream is a protandrous hermaphrodite, who reverse to the female sex at the age of two years (Zohar *et al.*, 1978; Loukovitis *et al*., 2011), we did not take into consideration the sex of examined fish while performing our investigation. Totally 10 specimens of matured fish were used in this study. After skin removal, the muscles that act on the pectoral and pelvic fins were identified. Then the pectoral and pelvic girdles with corresponding muscles were removed for further macroscopic investigation of their elements. Farther the muscles were weighed in order to compare the development of muscle groups that act on the pectoral and pelvic fins.

RESULTS AND DISCUSSION

In the gilthead sea bream the pectoral fin and its supporting elements are the first to develop (Faustino and Power, 1999). Mainly because in early larval stages the pectoral fin plays a main role in propulsion. The development of the pelvic, caudal, dorsal and anal fins, that are responsible for more complex movements, occurs later (Faustino and Power, 1999) and is associated with a reduction of the locomotor role of the pectoral fins.

The pectoral fin is composed of endoskeleton (pectoral girdle, radials) and exoskeleton (fin rays), (Fig.1). The pectoral girdle consists of bones of both dermal and cartilaginous origin. The main body of the girdle is composed of bones of dermal origin (Faustino and Power, 2001). They are, from dorsal to ventral, the posttemporal, supracleithrum (hypercleithrum), cleithrum and the postcleithrum (metacleithrum). This part of the girdle is also called 'secondary girdle', because it is evolved after the 'primary' fin-bearing elements of cartilaginous origin (scapula and coracoid), (Ostrander, 2000). However, the dermal bones ossify before the cartilage replacement bones (Faustino and Power, 2001).

The cleithrum is the largest element of the pectoral girdle that is fused anteriorly with the same bone of the opposite side. A blade-like upper postcleithrum is attached to the posterior end of the cleithrum on the medial side. The lower postcleithrum associates with adistal margin of the upper postcleithrum and continue further beyond the conditional horizontal line that join the last fin ray with the coracoid. The supracleithrum is attached dorsally to the cleithrum on the lateral side. It is also a blade-like structure that together with the posttemporal bears a short section of lateralis canal. The medial surface of the supracleithrum has a strong cord-like ligament that inserts between the dorsal incisure of the cleithrum and connects the pectoral girdle with the basioccipital bone of the skull. The postemporal adjoints dorsally to the supracleithrum on the lateral side. Two prongs of this bone attach to the supratemporal bones of the skull. Thereby the pectoral girdle is linked with the axial skeleton. Moreover, the sternohyoideus muscle, which is involved in opening the mouth (Matsuoka, 1987; Ostrander, 2000), as well as pharyngoclavicularis externus and

internus muscles (Ostrander, 2000) are attached to the cleithrum. Thus, the cleithrum contributes to the essential feeding and breathing functions. Additionally, contraction of the anterior portion of lateralis superficialis will presumably rotate the pectoral girdle backwards (Ostrander, 2000).

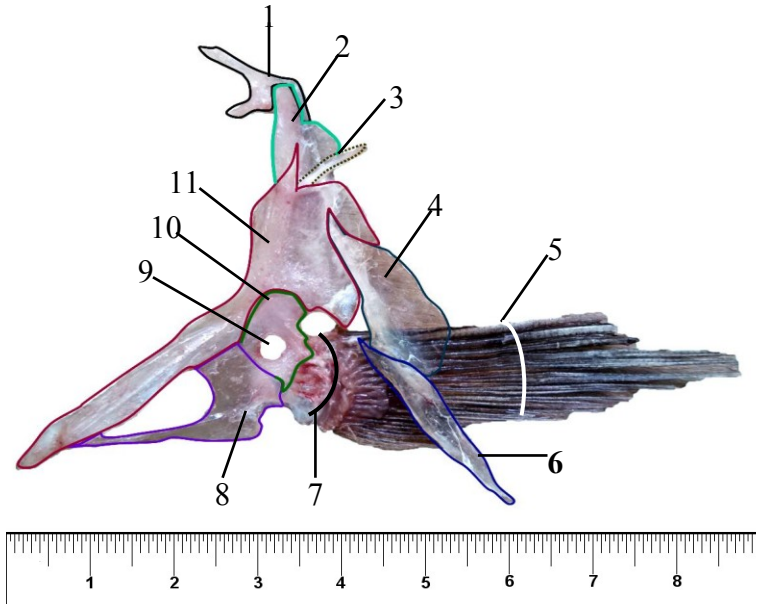

Figure 1. Skeleton of the pectoral fin of *Sparus aurata*, medial view.
1.posttemporal, 2.supracleithrum, 3.supracleithrum's ligament,
4.upperpostcleithrum, 5.fin rays, 6.lower postcleithrum, 7.proximal radials,
8.coracoid, 9.foramen of scapula, 10. scapula, 11.cleithrum.

The cartilage replacement bones of the girdle (scapula and coracoid) connects the pectoral fin with the cleithrum. The scapula is pierced with a round foramen for nerves and vessels of the pectoral fin. Ventrally the coracoid is placed. It has an anterior directed process. The anterior end of this process accrete ventrally to the anterior end of the cleithrum and form a large foramen that serves for passage of muscles that act on the pectoral fin.

The radials are divided into proximal and distal. However, the distal radials are very small and poorly visible on the non-stained preparations. The proximal radials are composed of four mesopterygial elements and propterygium. The mesopterygial elements are sometimes called pterygiophores (Sfakianakis *et al*., 2004; Sfakianakis *et al*., 2005; Koumoundouros *et al.*, 2001a). But we suppose that the term "pterygiophore" applies only to the supporting elements of the medial fins. The propterygium together with the first three mesopterygial elements articulates with the scapula. The forth and the largest proximal radial adjoin at the level of suture between the scapula and coracoid. The distal margins of the radials are overlapped by the upper and lower rows of lepidotrichia. Fusion of the two rows of lepidotrichia formes the fin rays. The propterygium articulates with the first stiff fin ray that also attaches to the scapula.

Muscles that act on the paired fins of the gilthead sea bream are divided into abductors, adductors and arrectors (Fig.2).

Abductor superficialis is the most laterally placed muscle that originates from the cleithrum and attaches to the bases of the pectoral fin rays. It covers abductor profundus and arrector ventralis muscles (Fig.3a). Contraction of the abductor muscles generates a forward rotation of the fin rays. In addition, contraction of the abductor profundus can lower the fin, because it has additional head that attaches to the basis of the first fin ray. Contraction of the

arrector ventralis lift the fin. This muscle also originates on the cleithrum and fixes on the basis of the first fin ray.

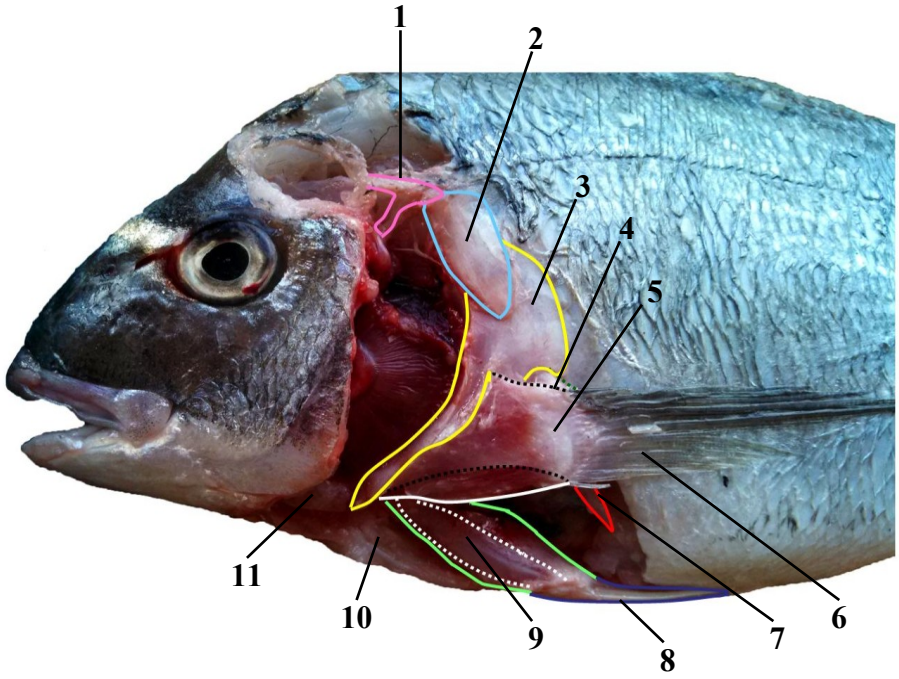

Figure 2. The paired fins of *Sparus aurata* with corresponding musculature, lateral view. 1.posttemporal, 2.supracleithrum, 3.cleithrum, 4.adductor superficialis, 5.abductor superficialis, 6.pectoral fin rays, 7.lower postcleithrum, 8.pelvic fin rays, 9.arrector dorsalis pelvicus, 10.ventral infracarinalis anterior, 11.sternohyoideus muscle.

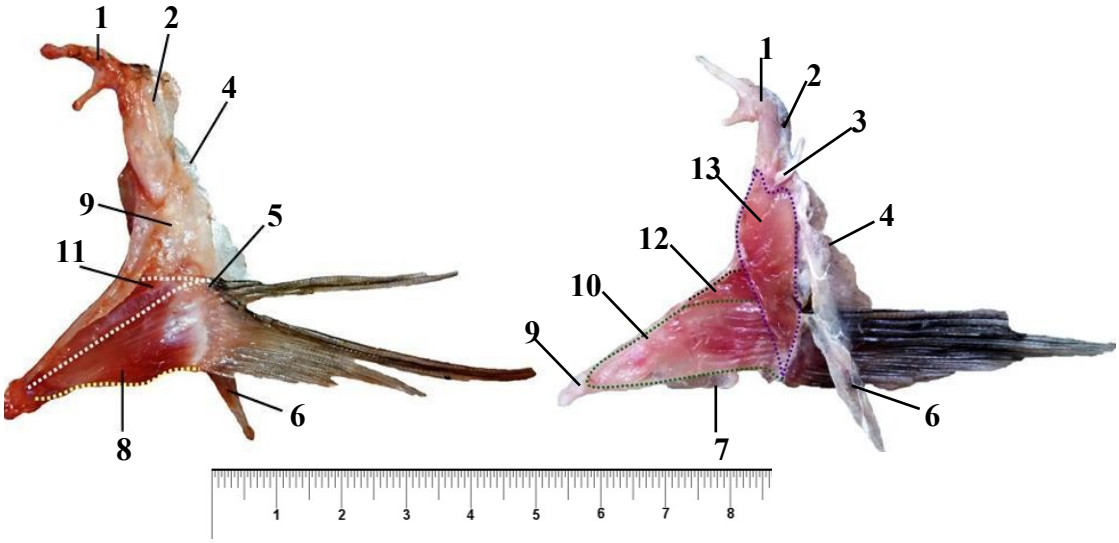

Figure 3. Skeleton and muscles of the pectoral fin of *Sparus aurata*.
(a) lateral view; (b) medial view. 1.posttemporal, 2.supracleithrum, 3.supracleithrum's ligament, 4.upper postcleithrum, 5.the first fin ray, 6.lower postcleithrum, 7.coracoid, 8.abductor profundus, 9.cleithrum, 10.adductor profundus, 11.arrector ventralis, 12.arrector dorsalis, 13.adductor superficialis.

Adductor muscles are placed on the medial side of the pectoral girdle. They are also divided into superficial and profundus (Fig.3b). Adductor superficialis muscle originates on the cleithrum and attaches to the bases of the fin rays. Unlike other muscles of the pectoral fin, his muscle fibers are directed ventro-posteriorly. It covers adductor profundus and arrector dorsalis muscles that additionally originate from the coracoid. Adductors rotates the fin backwards. Contraction of the arrector dorsalis, that is placed above the adductor profundus and attaches directly to the first fin ray, lift the fin.

The endoskeleton of the pelvic fin (pelvic girdle) is curved dorsally. It consists of the basipterygium and basal plates (Fig.4). The basal plates are fused into a single medial element, but forks anteriorly, thus creating a space where the apex of heart is placed. In addition, the anterior ends of basipterygium adjoin to the cleithrum of the pectoral girdle. Unlike other Percoidei fishes, ventro-medially the basipterygium has two needle-like processes that are directed anteriorly. Also the basipterygium of the gilthead sea bream has a well developed crest that border the arrector dorsalis pelvicus. Posteriorly the basipterygium associates with the fin rays. The first fin ray is very rigid and forms a strong pelvic spine. Ventrally the bases of lepidotrichia are connected by the pelvic ligament.

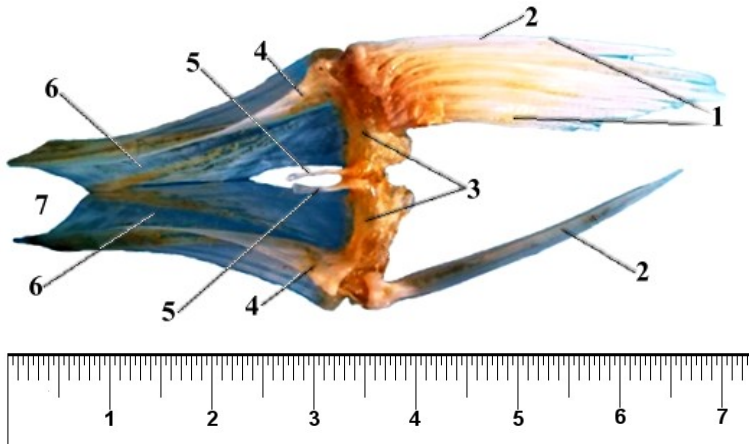

Figure 4. Skeleton of the pelvic fin of *Sparus aurata*, ventral view.
1.fin rays, 2.pelvic spine, 3.medial knobs of basipterygium, 4.crests of basipterygium, 5.processes of basipterygium, 6.basal plates, 7.heart's incisure.

The ventrally placed muscles that act on the pelvic fin are covered by the infracarinalis anterior muscle which run midline along the ventral surface of the body (Fig.5). Its tendons attach to the basipterygium and join with the infracarinalis medius muscle that posteriorly attaches to the medial knobs of the basipterygium. To the ventral muscles of the pelvic fins belong abductors (superficial and profundus). Their contraction enables the fish to stop quickly (Harris, 1938). The arrector dorsalis pelvicus is placed laterally in the groove which is formed by crest of basipterygium and corresponding basal plate. Its contraction additionally dilate the fin rays. On the dorsal surface of the pelvic girdle the adductor muscles are placed (superficial and profundus).

The relation of mass of each muscle that act on the pectoral and pelvic fin to the respectively total mass of the muscles that act on the pectoral and pelvic fins was defined (Fig.6). It was found that the abductor superficialis of the pectoral fin is more developed than the same muscle of the pelvic fin. At the same time the abductor profundus pelvicus is more developed than the abductor profundus of the pectoral fin. The degree of development of adductors was about the same. The determination of the degree of development of muscle groups (Tab.1) showed that, generally, they are equally developed in both pectoral and pelvic fins.

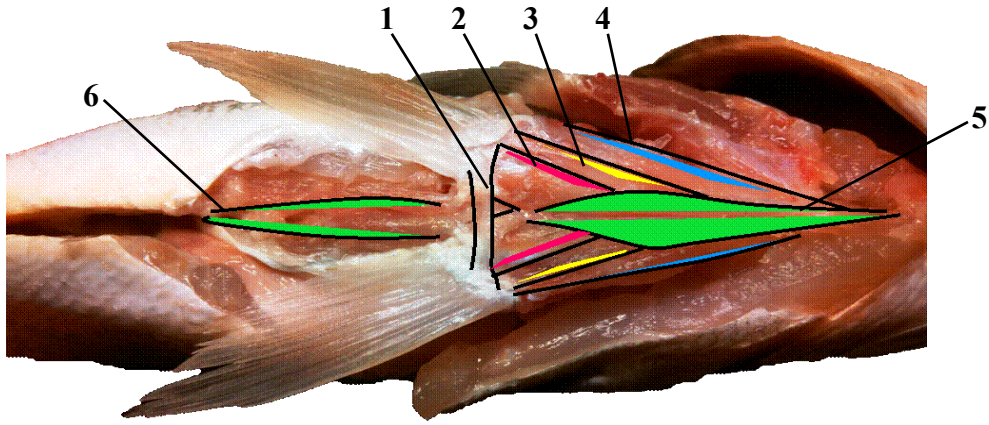

Figure 5. Muscles of the pelvic fin of *Sparus aurata*, ventral view.
1.pelvic ligament, 2.abductor superficialis pelvicus, 3.abductor profundus pelvicus, 4.arrector dorsalis pelvicus, 5.ventral infracarinalis anterior, 6.ventral infracarinalis medius.

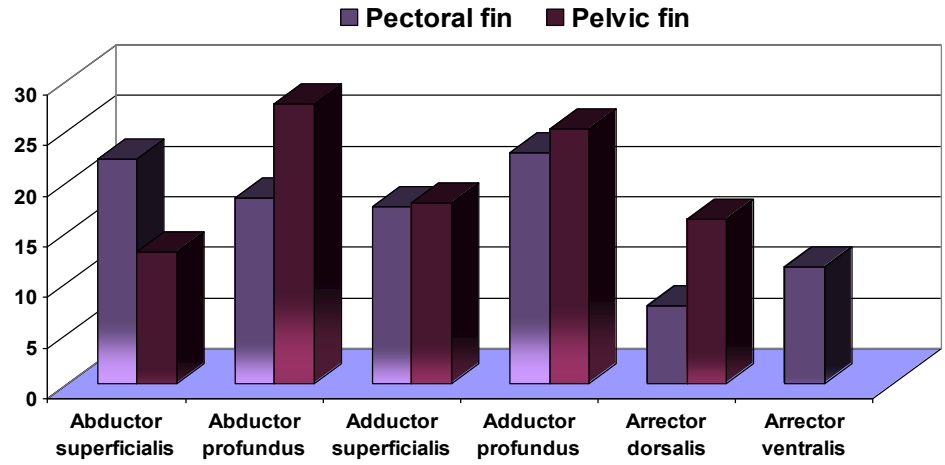

Figure 6. Comparison of the average relation of mass of each muscle that act on the pectoral and pelvic fin to the respectively total mass of the muscles that act on the pectoral and pelvic fins.

Table 1. Degree of development of muscle groups that act on the paired fins of *Sparus aurata.*

Muscle group	Mass relation (%)	
	Pectoral fin	Pelvic fin
Abductors	40,5	40,7
Adductors	40,3	43,1
Arrectors	19,2	16,2

CONCLUSIONS

The biomorphological peculiarities of the paired fins of the gilthead sea bream documented in this study provides an outline of characteristics that differ from other Percoidei fishes. It can be used for farther comparison with different sparids and for detection of musculoskeletal disorders that can occur with the paired fins of this specie.

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APPLICATION OF NANOTECHNOLOGY IN THE IMPROVEMENT OF SEMEN QUALITY – FUTURE TREND IN ASSISTED REPRODUCTION

B. DOROFTEI [1,2,3], Gabriela SIMIONESCU[1,3], Sabina NECULAI-VALEANU[1],
1. Origyn Fertility Center
2. University of Medicine and Pharmacy "Grigore T.Popa" – Iasi, Faculty of Medicine
3. Department of Obstetrics and Gynecology "Cuza Vodă" Hospital Iasi

Abstract: *Nanotechnology represents the 21st-century manufacturing technology, defined as the art of manipulating individual atoms and molecules in order to obtain structures with predefines properties.*
In the last years, the expansion of nanotechnology in different medical fields, including reproductive biology, has become more present, a series of tests based on nano-biotechnological tools being developed with the purpose of improving reproduction function and fertility of both human and animals. In this review, we present an overview on the emerging applications of nanomaterials in clinical assisted reproductive biology, focusing on nano purification technology that aims to select high-quality sperm cells for assisted reproduction.
Keywords: *Nanomaterials; Reproductive medicine; Reproductive Biology; Sperm Selection*

Nanotechnology refers to research, production and use of structures at the molecular level. The word "nano" comes from Greek and means "very small", for example, one nm (short for nanometer) represents 1/10000 of the thickness of a human hair. The nanoparticles represent the base of this science. The ability to use such nanomaterials and control the unique phenomena occurring at an atomic-scale makes possible the utilization of this technology in almost every field such as energy and conservation technology, IT business, the pharmaceutical industry and, of course, medicine. This technology may be better defined as the merging, at the atomic level, of biology, physics, mathematics, chemistry and material science into a single interdisciplinary science, thus erasing the fine demarcation line between different branches of life sciences.

Nanomedicine (integrant part of nanotechnology) evolved concomitantly with the growing need of finding new materials with high biocompatibility, thereby reducing the intolerance of living organisms.

Applications of nanotechnology in reproductive medicine

Reproductive biology is a constantly developing field that focuses on the structure and function of the reproductive system as well as on the complexity of processes that take place such as gametogenesis, fertilization, implantation and embryo development. In both humans and mammals, the success of fertilization depends on gamete fertility potential, sperm, and oocyte quality being equally important. Assisted reproductive technologies have been used extensively for humans and domestic animals in order to promote reproductive efficiency and preserve valuable genetics.

Due to their small size, nanoparticles may be easily integrated into the physiological processes of the cells and may migrate between different intracellular compartments, making

the selective targeting of certain tumoral cells possible ((Table 1). Another important characteristic is their high stability, which enables them to be used on a long distance, therefore improving drug delivery for example. According to Navath et al, 2011 polyamidoamine (PAMAM) dendrimer-based nanoparticles may be used for the delivery of drugs intravaginal. This application may be very useful in the case of pregnant women with ascending genital infections because by restricting the administration of the drugs to the vaginal area, the passage of the compounds towards the fetal membranes is limited, thus the fetotoxicity is minimized (Ensign et al, 2014). Nanoparticles have been successfully used for the detection and treatment of various genital infections such as Chlamydia trachomatis (Tang et al, 2010; Toti et al, 2011; Taha et al 2012). A nano-carrier may simultaneously carry and deliver a variety of substances (Table 1), the efficiency of targeting being adjustable depending on the physical and chemical proprieties of the carrier (Menjoge et al, 2010).

In the past years, the gold standard for several reproductive diseases was surgical treatment, but recent studies have shown that nanoparticles may be used for the detection and therapy of both reproductive cancers and non-cancerous diseases such as endometriosis (Weissleder et al., 1990; Zhao et al., 2012), uterine fibroids (Ali et al., 2013), ectopic pregnancy and trophoblastic diseases (Kaitu'u-Lino et al., 2013), offering a less invasive and safer alternative to standard diagnosis and treatment protocol.

Table 1. Application of nanomaterials in reproductive medicine

Applications	Principle
• *Treatment and imaging of reproductive system-related cancers*	Nanobiosensors for measuring cancer biomarkers Nanoparticles for biomedical imaging Nanoparticle Contrast Agents for Computed Tomography Imaging (Cormode, 2014)
• *Treatment of different gynecological affections*	Potentiation of the antitumor effect of chemotherapy by exposure to nanomaterials
• *Carrier particles*	Nanoparticles used for diagnosis, targeting and carriers for drug administration
• *Nanoparticle-assisted combination therapies*	Simultaneous delivery of different drugs
• *Gene therapy in reproductive diseases*	Therapeutic delivery of nucleic acid polymers using nanoparticles into a patient's cells as a drug
• *Sperm-mediated gene transfer*	application in obtaining transgenic animal
• *Magnetic-Activated Cell Sorting (MACS)*	Magnetic nanoparticle-based selection of live sperm with integral (not fragmented) DNA

Improvement of semen quality though nanotechnology

Semen quality measures the ability of semen to accomplish fertilization, thus estimating its fertility potential and implicitly, any decrease in sperm quality leads to reduced fertility. Although the advances in the cryobiology have led to the development of new methods that allow the conservation of gametes at low-temperature, a loss of fertility potential consecutive manipulation and preservation have been observed in both human and animals.

Routine semen assessment is usually done by microscopic evaluation of semen parameters. Such as total sperm count, sperm concentration, the percentage of motile sperm and percentage of normal sperm morphology. Some of these parameters may be correlated with fertility even though their capacity of predicting male fertility is limited (Sutovsky and Lovercamp, 2010).

The introduction of computerized technology in the process of semen evaluation such as computer-assisted semen analysis (CASA) increased the accuracy of the analysis by facilitating an objective and rapid count of the sperm cells, as well as offering essential data regarding the kinetic parameters of the cells.

Due to the fact that standard seminal parameters (concentration, motility and morphology) currently used for routine analysis in most species were insufficient for predicting fertility and detecting sub-fertility situations, a series of additional molecular marker-based test were elaborated. Consequently, flow cytometry analysis of semen passed the barrier from research to standard practice and fluorescent markers are now used to assess the acrosomal status, mitochondrial activity, the sperm chromatin integrity or oxidative stress of the sperm cells.

Now, a step forward is being made to the future by combining flow cytometry analysis and nanotechnology. A series of fluorescent biomarkers were developed with the purpose of assessing the structural and functional properties of sperm cells such as DNA condensation (sperm chromatin integrity), acrosome integrity, which permits the penetration of the egg during fertilization (acrosome status), mitochondrial membrane integrity which ensures the capacity of the sperm cells to produce the energy essential for sperm motility (mitochondrial membranary potential) as well as cell viability (expressed as live/dead ratio) (Payan-Carreira et al al, 2013).

An example of protein biomarker used for fertility assessment, which has been researched intensively is ubiquitin. This biomarker, which is located at the surface of abnormal sperm cells, possesses properties similar to lecithin ligands such as PNA (Peanut agglutinin) or PSA (Pisum sativum agglutinin) which are commonly used to assess acrosome status in different species (Mortimer, 1990; Cheng et al, 1996; Esteves et al., 2007). The abnormal sperm cells may be targeted by coating nano-particles with ubiquitin-binding antibodies, thus this technique may be useful in the process of nano-purification of semen.

Unlike agglutinin, ubiquitin has the ability to identify both the sperm cells showing abnormal acrosome, as well as those who have abnormalities of the tail and the head (Kuster et al, 2004), including those with altered sperm DNA. If an ejaculate contains a high

proportion of tagged sperm cells, this may indicate a poor quality of the sperm or infertility (Sutovsky et al, 2000), since ubiquitine-tagged sperm cells are an indicator of sperm quality. Due to this characteristics, a series of ubiquitin-based sperm assay were developed for the diagnosis of male factor infertility in different species such as men (Sutovsky et al, 2001), boars (Kuster et al., 2004), stallions (Sutovsky et al., 2004) or bulls.

Additionally, according to Ozanon et al., 2005, a negative correlation was observed, in humans, between sperm ubiquitin and embryo development in the case of assisted fertilization. The results obtained with ubiquitin were a cornerstone for the elaboration of other potential biomarker for male fertility, such as PAFR (platelet-activating factor receptor) or PAWP (postacrosomal WW domain-binding protein) which promotes pronuclear development during fertilization.

The new technology based on biomarkers facilitates the diagnosis infertility in men and enables the specialist to establish a custom and efficient treatment for each patient. In livestock reproduction, a high correlation has been observed between the routine semen parameters, conception rates after I.A and the parameters evaluated using flow-cytometry analysis based on biomarkers, therefore this technology may be a predictive tool for fertility potential of young males.

Another possible application of nanotechnology with the purpose of improving sperm quality is nano purification of semen using nanoparticles. Usually, semen used for assisted reproduction is desired to be of high quality, therefore, sperm cells with abnormalities are removed from the sample using different techniques such as swim-up, migration, filtration or density gradient centrifugation. These techniques are based on certain specific proprieties of the sperm cells (e.g. motility) or a set of proprieties (e.g. motility, membrane and chromatin integrity) such is the case of density gradient and aim to select superior quality sperm cells (membrane integrity, normal morphology, chromatin integrity) and even remove virus from the sample by combining the sperm selection techniques (Galuppoa et al., 2013). Over the years different commercial colloids have been developed for gradient centrifugation of both human semen (Percoll, PureSperm, Sage) and mammalian semen from different species (Bovipure, Capripure, Androcoll). Even if this method is quite efficient for selecting high-quality spermatozoa, it is time-consuming and requires expensive reagents and equipment, which makes it less suitable if larger amounts of semen must be processed for A.I. Therefore, new approaches are embraced by researches and andrologists, one of them being the magnetic separation method.

Having the antibody and lectins conjugation as example, Clemente Associates (Madison, CT) developed a sperm selection method using magnetite and iron oxides nanoparticles. The particles, coated previously with anti-ubiquitin antibodies or with lectin PNA, were projected to target the abnormal sperm cells by binding to the ubiquitin protein located on the surface of this cells. According to the field insemination trails conducted with nano purified semen, there were differences between males and also between heifers and cows which were inseminated but most importantly, no adverse effect was observed in the study conducted, fact which encourages further studies in this area and the development of new protocols for nano purification using magnetic separation (Sutovsky and Kennedy,

2013). Contrary to this finding are results obtained by Nasri et al, 2015, who concluded that iron oxide nanoparticles may have a negative effect on the Leydig cells at molecular level.

CONCLUSIONS

As any new technology emerges questions and controversy appear regarding the degree of safety and possibilities for implementation for human use, which is why the development of nano purifications protocols and biomarkers intended for human use must focus on the toxicity potential of the nanoparticles. The nano-reprotoxicity studies that were conducted on animal models revealed that the administration of some nanoparticles either orally or intravenously may have a harmful effect over the central nervous system and reproductive system by determining disturbance in the hormone synthesis and spermatogenesis process. Thus, special attention must be paid for the development of highly biocompatible nanomaterials destined for reproductive biology.

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FOWL TYPHOID (SALMONELLA ENTERICA) OUTBREAK IN INTENSIVELY REARED QUAILS

Oana TĂNASE, Alexandru MERLĂ, Constantin PAVLI, Florentina DARABAN, Elena VELESCU
USAMV Iassy, Faculty of Veterinary Medicine,
tanase_oana@yahoo.com

Abstract: *Fowl typhoid (Salmonella enterica) is an infectious and contagious disease in poultry, manifested mainly by digestive disorders and high mortality in embryos and chickens, while in adult birds is characterized by laying disorders and weight loss, (1).*
Given the lately explosive development, in order to ensure nutritional supply and demand for different adjuvant treatments, the global population resorts more and more to various homeopathic products. Following to this demand it began to emerge small businesses in order to ensure the offer for the required need, hence the growing interest for intensively reared quail.
With respect to quail flock, the disease had an insidious onset, with an oscillating evolution causing significant losses in the chicken batch; the incubation was negatively affected, while the egg production decreased by 10%; there were no evident clinical signs in the adult birds.
In order to diagnose the fowl typhoid, epidemiological, clinical, morphopathological and laboratory investigations were performed.
Following disease progression, the offspring that survived were raised until the age of 7 weeks, when they were sacrificed, as well as the adult quails that have responded positively and/or dubious to the rapid-plate hemagglutination assay. The adult quails with negative results were raised for consumer egg production for other 4 months, then they were slaughtered.
After stamping out the entire shelter, it was resorted to mechanical cleaning of the farming equipment and incubators. For the final disinfection, a potassium peroxydisulfate solution, Ecocide S, in 1% concentration was used; the solution was applied to all surfaces by spraying. The disinfection was repeated after 7 days and 14 days after the first disinfection the building was repopulated with quail chicks from a known source, free from fowl typhoid.
To prevent the new flock against fowl typhosis, the quails were immunized with Tiforomvac, live attenuated vaccine with Salmonella gallinarum strain 9R.

Key words: quails, Salmonella enterica, embryo, laying disorder.

INTRODUCTION

In quail exploitation is often resorting to an intensive growth system in batteries. Of these facts we owe a decreasing predisposition of the birds to contact various viral, bacterial and parasitic diseases if the growth rules are strictly respected and there is achieved a uniform and monitored flow, (4).

Fowl typhoid is important because of its evolution precourse and of its more frequent occurrence among breeders because of failure to comply with hygiene and environmental conditions. Another important factor is consisting in overcrowding, especially in the case of the beginner breeders with the desire to obtain a higher profit with a minimum investment in growth technology. Although the number of quail breeders has greatly increased in recent years, the pathology of these birds is still ignore, since the breeders are relying on so-called myths, according to which the quail would be one of the most resistant birds, in spite that the domestication was performed in its totality, and its genetic heritage regarding the resistance of the of wild quail preserved very little. It is important to know that not all breeders, unfortunately, are using appropriate feed and therefore not all the eggs and quail carcasses sold on the market are of the highest quality, an aspect important especially when the eggs are used raw for various cure and the quail meat, because the intensive growth may easily modify their physic, chemical and organoleptic properties after a deficient feeding.

The aim of this study was consisting in diagnosis of fowl typhoid by epidemiological, clinical, morphopathological and laboratory investigations.

MATERIALS AND METHODS

The observations were performed in a quail microfarm located in Iasi county, Pascani city, which had a total number of 1700 quails of different age, of which 1200 adult quails: 950 hens quail, 250 males and 500 chicken quails of different ages, where 300 under the age of a week, 100 between one week and 3 weeks, 100 aged between 3 and 5 weeks and 100 aged between 3 to 5 weeks. The first signs of the disease occurred in January 2013, when the birds were non-immunized against fowl typhoid but only against Newcastle disease.

The building intended for the quails rearing have dimensions of 5 m / 7m, the space was divided into two rooms, separated by a small access hallway and a cloakroom. One room was destined for hatching eggs and chickens growth, while in the second room were kept the adult laying quails. The space was provided with an air cooling system and a wood-fired heating system. The ventilation was achieved with 2 fans that pulls the air out, one in each room.

The ambient temperature had an average value of 24 ° C and a relative humidity of approximately 60%.

The adult quails were reared in an intensive system, in battery backup system with automatic watering system, connected to a central pool, with feeding trays manually filled, and with manure trays that were cleaned every two days. Egg harvesting was made 2 times per day, and the eggs were conditioned depending on current needs. When the eggs were used for treatments, they were packed in trays and conditioned immediately at 4 ° - 6 ° C, and if they were kept for hatching, they were subjected to sorting, then put into formwork and maintained at 15 ° - 18 ° C until the introduction in incubation. The chicken separation was performed as: up to age of one week they were kept in a separate room, in maternity room with glass walls, at an ambient temperature of 34 ° C, while the temperature was reduced 1 ° C every day, reaching the value of 26 ° C. At the age of one week they were moved in the same room with the adult quails, but in specially designed shelves, with filled walls, where they were held for up to 3 weeks of age. After this age the chickens were moved in battery cages similar to those of adult quails and separated were gender, (2).

With respect to quail flock, the disease had an insidious onset, with an oscillating evolution causing significant losses in the chicken batch; the incubation was negatively affected, while the egg production decreased by 10%. At dead birds a pathological examination were performed, paying attention to the segments of the digestive tract, to the cord and to the genitals, (3).

In order to diagnose the fowl typhoid, epidemiological, clinical, morfopathological and laboratory investigations were performed, as well as specific prevention and control measures.

In order to confirm the disease, from the blood samples collected from the adult quails and from the other birds that cohabited with them, respectively 10 hens and 30 chickens, serological examinations were performed (rapid slide haemagglutination test and slow agglutination in tubes)

RESULTS AND DISCUSSIONS

Of the 1700 birds, of which 1200 adults and 500 chickens of different ages taken under study in the first week of the disease progression, specific clinical and lesion aspects were identified in 10 adult quails and 334 chicken quails (table 1).

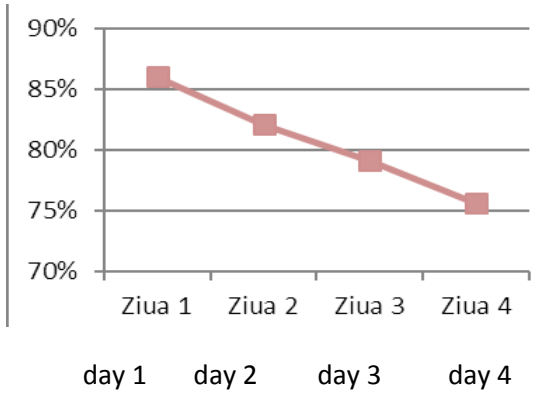
Table 1

The disease onset in the first week of: observations based on clinical signs

No. crt.	Quails category	Quails number	Affected quails		Death out of affected quails	
			No.	%	No.	%
1	Adult quails	1200	10	0.83	1	10
2	Quails< 1 week	300	**281**	**93.6**	**215**	**76.5**
3	Quails 1- 3 weeks	100	45	45	9	20
4	Quails 3-5 weeks	100	8	8	2	25

After analyzing the data from Table 1, it can noted that the percentage of morbidity and mortality parameters vary in a quite large frame, depending on the age of the category affected. In the case of the adult quails, the morbidity was 0.83%, while in the one week age chicken category, the morbidity reached a value of 93.6%, being the he highest affected category, in the same time having the highest value of mortality, probably as a consequence of the sensitivity given by hatching stress. The chicken quails aged between 1 to 3 weeks were affected in a proportion of 45%, while those aged between 3 to 5 weeks were affected in a proportion of 8%, these two groups having similar values of mortality, 20% respectively 25%.

Clinical symptoms prevailing in the adult quails were consisting mainly in prostration and ruffled feathers. Of the 10 the adult quails with clinical signs, two showed orange diarrhea and conjunctivitis. Although the clinical signs were observed in a low percentage of adult quails, the laying percentage decreased by about 10% from a value of 86% within 4 days (figure 1). At the same time it began to be affected the eggs quality, with soft and depigmented shells (figure 2).



The evolution of laying percentage during the first 4 days of disease

In the case of chicken quails under the age of one week, the main symptoms were consisting in white-cretaceous diarrhea and a typhous condition, some of them however showing respiratory symptoms and marked by rales. A total of 114 chickens, quail have pronounced shown symptoms since day one, those being the quails who 100% died within 2 days. In 167 chicken quails, the symptomatic onset was more subdued, manifested by poor appetite over a period of 2-3 days, with continually cheeping, transient diarrhea and death in 101 chickens that occurred after 6-7 days of the disease.

The symptoms seen the other groups of chickens, characterized in particular by their listlessness, loss of appetite, transient diarrhea, chickens weakening an their death.

After this period with an explosive start, the symptomatic evolution has ceased, the morbidity was reduced to zero, the only change being the maintenance of laying percentage around 75%, with the occurrence of soft-shelled eggs, 2 weeks after the first signs onset. During the last 5 months, the quails were kept for the production of consumption eggs, the monthly mortality ranged from 0.5 to 1% of, and the laying percentage began to rise gradually in the second month, after progression of the disease.

The farm had a closed circuit, the fecundity and the embryo viability were affected. From an initial hatching rate of 72%, with egg fecundity up to 85%, the hatching the reached the threshold of 56% and fecundity decreased to 71%.

In order to identify the morphopathological changes, it was performed the necropsy of several cadavers, observing the changes on the digestive system in the adult quails, such as represented by enterocolitis, biliary stasis, friability with liver congestion and spleen congestion (figure 2).

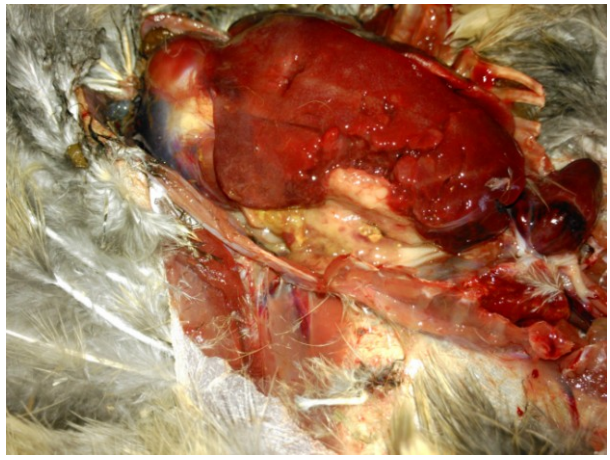


Figure 2 Congestion and liver friability an adult quail

The three days old quail chicken that were necropsied showed non-absorbable yolk (figure 3), hemorrhagic myocarditis, and infiltration at the base of the cord (figure 4) and generalized ischemia. At the same time, a lack of food could be observed throughout the digestive tract, while in cloaca, an obstructing white plug was observed, because of the abundant diarrhea that had agglutinated the fluff around cloaca.

Figure 3 Non-resorbable yolk in chicken quail

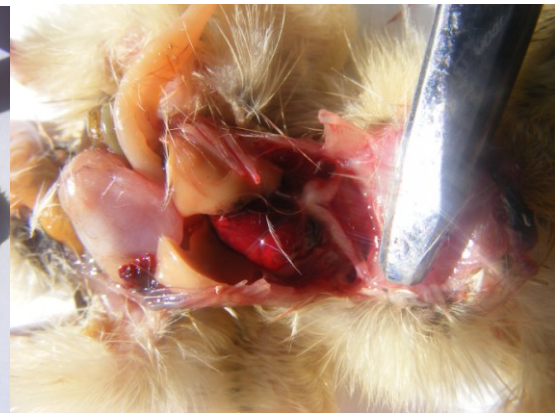
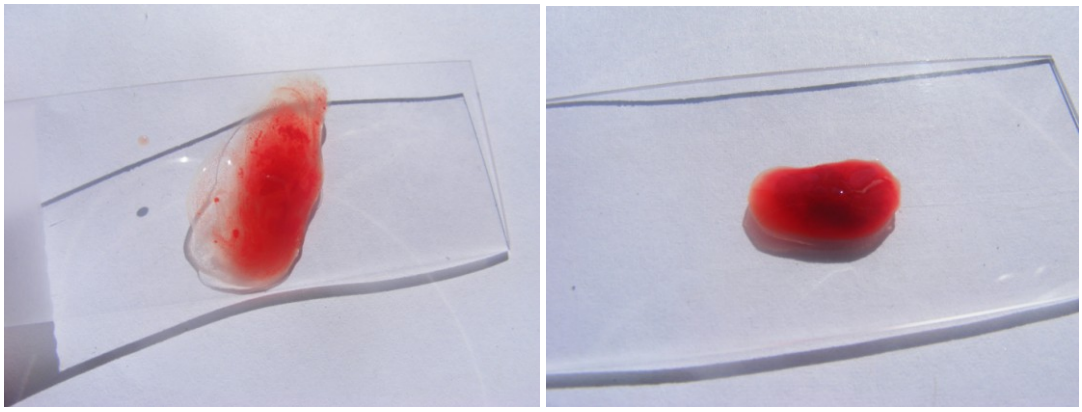


Figure 4 Myocarditis and generalized ischemia

Following analysis of the laboratory results, respectively rapid slide haemagglutination test (figure 5A and B) and slow agglutination in tubes, the presence of *Salmonella enterica* has been confirmed as responsible for the evolution of the outbreak.



A **B**

Figura 5
A. Positive rapid slide haemagglutination test
B. Negative rapid slide haemagglutination test

Following the hemagglutination reactions performed in adult quails with clinical symptoms, all samples tested positive, while after the testing of the samples from quails without clinical signs 85% were negative. 1011 of 1190 samples tested negative, while the presence of antibodies for fowl typhosis was highlighted in 143 samples, while 36 provided non-specific results.

The slow agglutination reaction in tubes was performed in 9 adult quails with clinical signs. Following this reaction, specific antibodies were detected from a dilution of 1/20 to 1/320 in blood serum, while 100% of the obtained result were considered positive (++++) (table 2).

Table 2

The slow agglutination reaction in tubes results

Quails	**Positive SART**	**Agglutinating titre of the serum**										**Total positive**	
		1/20		**1/40**		**1/80**		**1/160**		**1/320**			
		No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Female adults	6	-	-	**2**	**33.3**	1	16.7	**2**	**33.3**	1	16.7	6	100
Male adults	3	-	-	-	-	1	33.3	1	33.3	1	33.3	3	100
Total	**9**	**-**	**-**	**2**	**22.2**	**2**	**22.2**	**3**	**33.3**	**2**	**22.2**	**9**	**100**

The analysis of the data shown in table 2, it might be observed that 100% of the birds tested positive. In two female quails a total agglutination of the antigen was recorded at 1/320 dilution, which represent a great amount of antibodies present in the blood of the bird.

After the first two days of the disease progression, it was started a treatment with a polypeptide antibiotic, namely Colistin, and to potentiate the effect of its, this was associated with a sulfonamide, Sulfamidina respectively. This combination was administered in the drinking water, once per day for 7 days. Beside this treatment, in order to help the birds immune system, were administered some medicinal products based on vitamins, amino acids and oligoelements, such as Selevit Sol, over 14 days and repeated after a 7 days.

Following the disease progression, the surviving chickens were reared until 7 weeks of age when they were slaughtered as well as adult quails who have responded positively quails and dubious to the slide rapid hemagglutination test. The adult quails with negative results were kept for the production of egg consumption for other 4 months, after which they were slaughtered.

After stamping out the entire shelter, it was resorted to mechanical cleaning of the farming equipment and incubators. For the final disinfection, a potassium peroxydisulfate solution, Ecocide S, in 1% concentration was used; the solution was applied to all surfaces by spraying. The disinfection was repeated after 7 days and 14 days after the first disinfection the building was repopulated with quail chicks from a known source, free of fowl typhoid.

To prevent the new flock against fowl typhosis, the quails were immunized with Tiforomvac, live attenuated vaccine with *Salmonella gallinarum* strain 9R, using the following schedule:

- The first vaccination at the age of one week by subcutaneous inoculation of 0.2 ml of reconstituted vaccine in the dorsal region of the neck,
- The second vaccination at 5 weeks old quails, by subcutaneous inoculation of 0.5 ml of reconstituted vaccine in the dorsal region of the neck, with revaccination every 3 months.

CONCLUSIONS

Following the clinical, pathological and laboratory observations performed in an outbreak of fowl typhosis in a quail microfarm from Pascani, the next conclusions may be drawn:

1. The diseases occurred in adult quails during of laying peak, when 10 out 1200 adult quails were affected, 281 out of 300 chicken quails under the age of one week.
2. With respect to the clinical signs, the disease began with non-specific symptoms for fowl typhosis in adult quails, while in chickens under one week, evolved with specific signs.
3. The pathological examination revealed several changes but non-pathognomonic
4. The laboratory tests (serological) showed the presence of specific antibodies for Salmonella enterica, confirming the presence of the bacteria.
5. The treatment with Enrofloxacin applied on the second day of the disease progression reduced the mortality among the chicken quails with the aged between 1 to 5 weeks.
6. A final disinfection applied to the all equipments and buildings, followed by the acquisition of a batch of quail from a trusted source along with a specific immunization, prevented the appearance of a new outbreak.
7. There was designed a special room intended for decontaminating the hands and the objects of the persons that enter, together with a rug at the main entrance, for shoes decontamination.

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INFECTION WITH AEROMONAS HYDROPHILA IN SNAKE: CASE REPORT

Oana TANASE, Mădălina IRIMIA, Cristina RÎMBU, Constantin PAVLI, Florentina DARABAN, Elena VELESCU

USAMV Iassy, Faculty of Veterinary Medicine,
tanase_oana@yahoo.com

Abstract: *Reptiles are vertebrate tetrapods that emerged to the terrestrial fauna during Carboniferous period of the Paleozoic Era and their dominance peaked in the Mesozoic Era. Reptiles are exclusively terrestrial, with some exceptions. They are poikilothermic animals generally oviparous, with the pulmonary respiration. Reptile skin is provided with scales or cornified plates, however, the skin is poor in producing glands, therefore it is dry and waterproof, as a result of adaptation to terrestrial life. The heart is three-chambered (two atria and a ventricle partially closed), the exception are the crocodiles who have a tetra-chamber heart. The reptiles are the first class of vertebrates' completely adapted to the terrestrial life.*

Most species of the snakes raised as pets are feed with mice or rats that were previously stun / kill in order to avoid reptile injury, since a rodent alive unexpectedly introduced in a snake cage may attack.

The compliance with the environment cleaning, of the water, proper humidity and temperature are essential for the health of snakes. Microclimate conditions must also ensure snakes moult, which is a physiological periodically phenomenon, by which the tissues retain their freshness, permanent and attractive pigmentation.

Snakes shed their skin once all, except for the large snakes that can break the skin. Shedding moment is signaled by the partial disorder of the eyes and food refusal.

In this work it was analyzed an albino python snake, originating from a zoological garden that after several weeks of anorexia and dyspnea it was found death.There were performed: clinical examination, pathologic examination and laboratory tests. The bacteriological investigations and the oxidase test have identified Aeromonas hydrophila bacteria as the main etiology of the shake disorder.Changing the terrarium, the stress of adapting to new terrarium, the presence of air currents and temperature variations have led to loss of appetite, weakened the immune system, thereby the bacteria had the opportunity to multiply and create disorder to the internal organs, with a septicemic character leading in the end to the reptile.

Key words: snake, Aeromonas hydrophila, microclimate

INTRODUCTION

Aeromonas hydrophila is one of the bacterial species belonging to the genus *Aeromonas*. Is a heterotrophic organism that can exist in both aerobic and anaerobic medium. *Aeromonas hydrophila* has pili that allow it to attach to host organisms. It able to produce a cytotoxic enterotoxin that causes tissue damages, (1, 2).

The reptiles are the first class of vertebrates fully adapted to terrestrial environment. Only a few of them lead an aquatic or a semi aquatique life. By now, there are known over 8,000 species of reptiles. There are four orders of reptiles: Chelonian (turtles), Lacertilians (lizards), Ophidian (snakes) and Crocodylomorpha (crocodiles).

The Ophydians are of great variety and arouse a great interest of researchers during the time. The snakes like vipers and cobras are very venomous, the venom helping to slay the prey and defend themselves from predators. Not all snakes need venom to kill the preys, some rely on muscle strength, such as anaconda, boa and python. Despite are very feared,

most of snakes are harmless to humans. The snakes hunt invertebrates such as frogs, other snakes, birds to deer, while the preys are swallowed whole.

In this work it was analyzed an albino python snake (*Python bivittatus albino*), originating from a zoological garden from Barlad that after several weeks of anorexia and dyspnea it was found death. Consecutively to the bacterial examination, *Aeromonas hydrophila* was isolated and incriminated as the cause of the death.

MATERIALS AND METHODS

The observations were made in the zoo garden from Barlad, where the albino python snake (*Python bivittatus albino*) considered as an attraction, was ill for several weeks. There have undertaken investigations regarding the environmental conditions, the diet and was performed a clinical examination of the animal.

After the animal death, the investigations have continued with the necropsy and laboratory tests: bacteriology, bacterioscopically, completed by performing antibiogram since in the zoo from Barlad were other three pythons, with no clinical signs.

For the bacteriological test, the medium used was represented by Blood Agar. The oxidase test was performed on bacterial cultures, where 2-4 days old culture was covered by tetrametylparafenilendiaminhidroclorid 1% aqueous solution, freshly prepared.

For the bacterioscopically exam were collected samples represented by lung, kidney and cord. The sample collection was performed in a sterile environment, under the fume hood. Smears were prepared by crushing the sample between two blades, then dried at room temperature, fixed by passing through the gas flame three times in succession, followed by Gram staining, (3).

The antibiogram was performed by disc diffusion method on solid media.

RESULTS AND DISCUSSIONS

Following the clinical examination and the case history received from the reptile caregivers, it has been found that albino python did not consume food for about a week, was lethargic, was moved to a new terrarium, where were present airflows. The reptile was submitted to the Infectious Diseases Clinic from Faculty of Veterinary Medicine, Iasi, where it was found that the reptile was a 4-5 years old female. After anamnesis and clinical examination, it was observed that the python was listless, lethargic, and his eyes were turbid.

Shortly after the presentation to the clinic, the python died and there were carried out: necropsy, the bacteriological test, bacterioscopically examination and antibiogram.

At the necropsy, the external body examination revealed the presence of ecchymosis, signs of septicemia (figure 1). In the oral cavity it was present a seropurulent deposit, malodorous, mixed with remnants of litter (figure 2).

Figure 1. The presence of ecchymosis

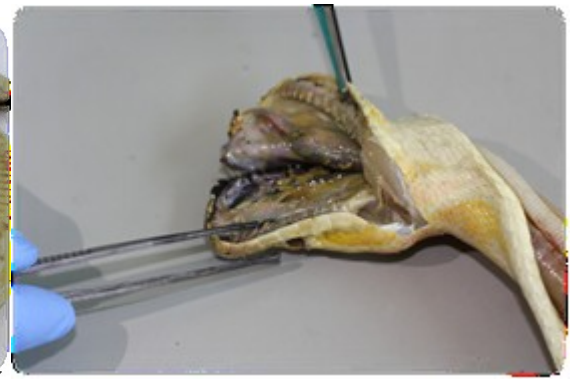

Figure 2. Seropurulent deposit in the oral cavity

During necropsy, on the internal organs were observed: in the lung (figure 3) and in the kidney the presence of two nodular processes (figure 4), on the liver the presence of two lesions on its surface (figure 5) with the size of a pinpoint, in the pericardial sac was present a citrine fluid (figure 6), that tends to clot in contact with the air.

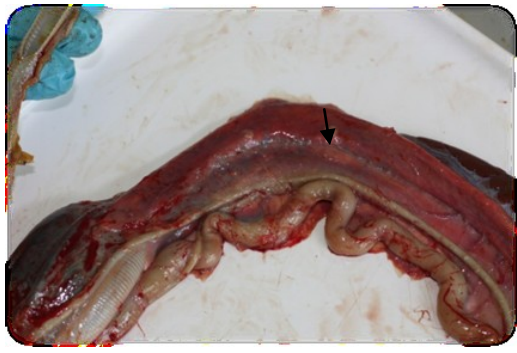

Figure 3. Pulmonary lesions (black arrow)

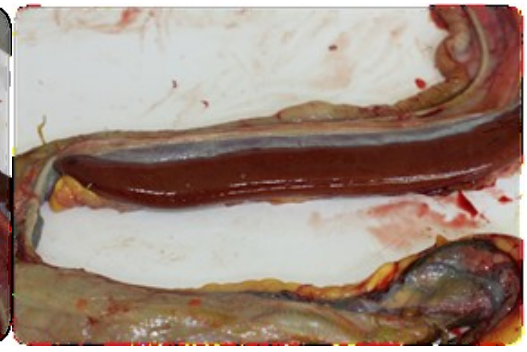

Figure 4. Liver lesions

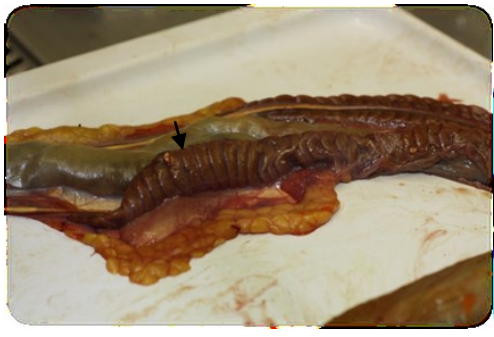

Figure 5. Kidney lesions (arrow)

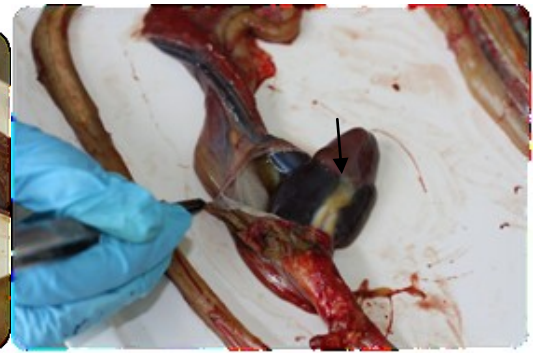

Figure 6. Fluid in the pericardial sac (arrow)

The bacteriologic examination was carried out on samples taken from the long, heart, kidney and liver. After storing the samples in a thermostat, on both media, round, no pigment or cream colonies of 2-3 mm diameter developed within 24 h (figure 7).

Following microscopically examination it has been identified a high microbial load, composed of coccobacillus and Gram negative bacilli similar to *Aeromonas hydrophila* (figure 8).

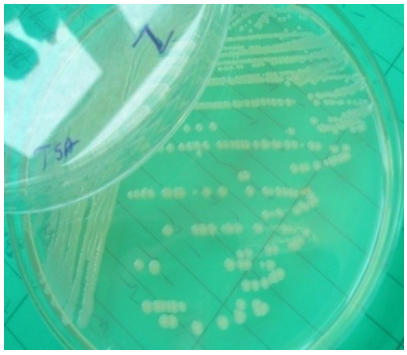

Figure 7 Cultural characters of Aerom*onas hydrophila s*train on TSA medium

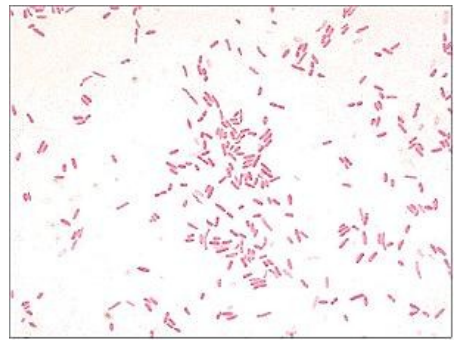

Figure 8. *Aeromonas hydrophila* – Gram negative bacilli

All the strains that were tested showed mobility and oxidase-positivity.

The results of the oxidase test performed on 24 h bacterial cultures indicated that the cord and kidney samples were oxidase „ + ", while the liver sample was „ oxidase -". In order to read the reaction, there were used strips of paper soaked in reagent, where it was observed that oxidase- positive bacteria were stained in purple brown (figure 9).

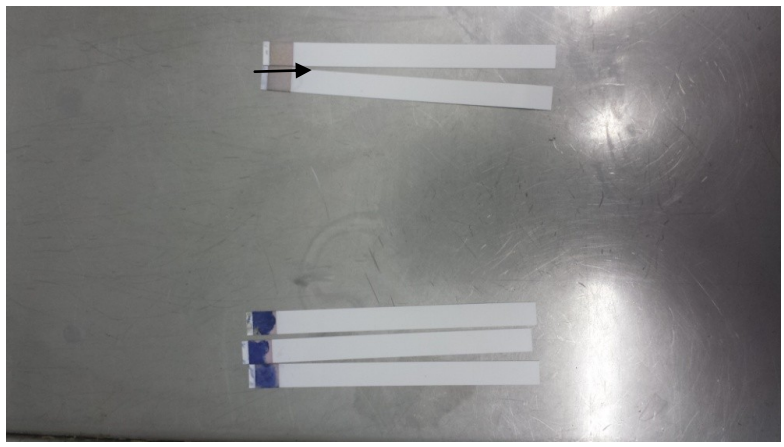

Figure 9. Oxidase positive and negative strips (black arrow)

For the exam to biochemical properties have been used API 20E. These were inoculated with bacterial strains to be tested according to the standard protocol specified by the manufacturer. The results have confirmed the species *Aeromonas hydrophila* (figure 10).

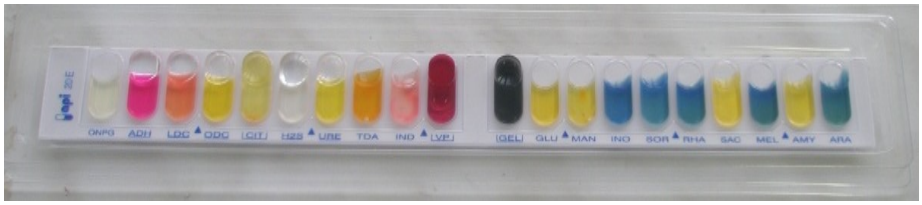

Figure 10. Biochemical characteristics of the *Aeromonas hydrophila* isolates using API 20 E

After performing the antibiogram it was found that the highest sensitivity was to Enrofloxacin, moderate sensitivity to Micacin, according to the 2015 CLSI and resistant to the other antibiotics: Oxytetracycline, Cephalexin, Ceftazidime, Lincospectin, Amoxicillin, Ampicillin, Chloramphenicol and Trimethoprim.

In the literature the most commonly incriminated in snake's diseases are encountered *Aeromonas* and *Pseudomonas* species, (4). *Pseudomonas* is small or medium bacillus found isolated or diplo, enveloped, non sporulated, Gram-negative and oxidase producing. *Aeromonas* is a bacillus or coccobacillus, gram negative and oxidase positive, (5).

In the case of the albino python the changing of the terrarium, the stress of adaptation to the new terrarium, the presence of airflow and the temperature variations have led to loss of appetite, to decreased immunity, so the bacteria had the opportunity to developed and to create the conditions in the internal organs, with a septicemic character leading eventually to death of the reptile.

For the other pythons from the zoo garden of Barlad, it was recommended a treatment with Enrofloxacin in a dose of 5 mg / kg for 12 days, preventively in order to prevent the clinical manifestations seen in the albino python.

CONCLUSIONS

The investigations regarding the microclimate, clinical, pathological and laboratory may conclude the following:

1. When the terrarium was changed, the albino python suffered and addaptation stress because of the airflow presence and temperature variations;
2. The decrease of immunity led to the development of bacteria *Aeromonas hydrophila*, with the occurrence of some conditions in the internal organs, with a septicemic character leading eventually to death of the reptile.
3. *Aeromonas hydrophila* bacteria was isolated from the cord and kidney, demonstrating that it is a positive oxidase strain.
4. The results of the antibiogram showed that the isolated strain was sensitive to Enrofloxacin and resistant to Oxytetracycline, Cephalexin, Ceftazidime, Lincospectin, Amoxicillin, Ampicillin, Chloramphenicol and Trimethoprim.

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ANTI- *MYCOPLASMA AGALACTIAE* THERAPY INFLUNCES THE SYSTEMIC IMMUNITY IN SHEEP

Aurel VASIU, Gh. F. BRUDAŞCĂ, Mihaela NICULAE, Diana OLAH, Carmen Dana ŞANDRU, Marina SPÎNU
University of Agricultural Sciences and Veterinary Medicine,
Faculty of Veterinary Medicine,Cluj-Napoca, Romania,
aurel_vasiu@yahoo.com

Abstract. *The experiment aimed to clarify the changes induced in the immunological profile of sheep with contagious agalactia by the antibiotic treatment applied to the animals to treat the disease. The investigations were conducted on 18 animals with clinical signs of contagious agalactia, with different locations (articular, ocular or combined), aged 2 to 8 years. On day 0 of the experiment, treatments were applied depending on the location of the lesions. Two samplings were done, collecting the blood on heparin for carbon particle inclusion test, on EDTA for total leukocyte counts and on clotting gel for serum, one before and one after the antibiotic treatment, 18 days apart. Total leukocyte numbers and their phagocytic capacity (carbon particle inclusion test) as well as total Ig (zinc sulfate precipitation test) and circulating immune complexes (CIC)(4.2% PEG precipitation test) levels were evaluated. In vitro treatments were also performed by using an Echinacea angustifolia alcoholic extract to investigate its effects on phagocytic activity. There was a highly significant ($p<0.001$) decrease in total leukocyte numbers (from 24520.00±6366.66/mm^3 to 11392.90±1999.80/mm^3) after the treatment, accompanied by a highly significant ($p<0.001$) decrease in spontaneous phagocytosis, which correlated statistically significantly ($r=0.492$, $p<0.01$) with the total number of white blood cells. Values, lower than physiological limits, indicate the activation of immune complexes' clearance and are concordant with the decrease of total Ig levels, suggesting the enhancement of the complexing and clearance of CIC following the treatment of Mycoplasma infection. Thus, the results would possibly contribute to a better understanding of immunopathological processes that occur during contagious agalactia in sheep.*

Key words: *therapy, contagious agalactia in sheep, immune status, phagocytosis, humoral changes*

INTRODUCTION

Contagious agalactia of sheep and goats mostly present in traditional husbandry, represents a disease of dairy animals, defined by different locations of the lesions such as mammary, joint and ocular ones, leading to interstitial mastitis, stiff joints and keratoconjunctivitis (Almeida et al., 1992, Cottew and Yeats,1982, DaMassa et al., 1992). The agent is usually *Mycoplasma agalactiae* , nevertheless *M. mycoides capri* and *M. capricolum capricolum* and *M. putrefaciens* were also isolated from goats with mastitis and arthritis (Fox et al., 2003). The use of specific cellular response investigation tests brings, apart from possibilities of standardization in what concerns the ovine species, considerable contributions to the characterization of some particular aspects linked to the outlining of the immunologic profile of mycoplasmosis in this species (Avramidis et al., 2002). Assessment of the functional level of different specific effectors during the evolution of the disease allows the elucidation of some aspects connected to the infection pathogenesis and, is probably, linked to the re-emergence of the disease in spite of the anti-agalactia constant vaccination in the areas of high epidemiological risk (Rodriguez et al.,2002, Szeredi et al., 2003).

The experiment aimed at a better understanding of the cellular reactivity, which may contribute to the elucidation of pathogenetic mechanisms and establishment of some enhanced measures toward the control of the disease. The study also sought to establish correlations between the functional capacity and numeric changes of circulating phagocytes,; similarly, the *in vitro* effect of the *Echinacea angustifolia* extract, as a potential, immunemodulating agent, was tested.

MATERIALS AND METHODS

Biological material and experimental protocol. The investigations were conducted on a number of 18 animals with clinical signs of contagious agalactia with different localizations (joints, eyes or mixed), 2 males and 16 females, aged from 2 to 8 years. The animals were subjected on day 0 to an antibiotic treatment with streptomycine, administered twice a day, for 5 days. A supportive therapy was also applied in conjunction with local therapy, in case it was necessary.

Blood samples were taken twice, 18 days apart, before initiating the antibiotic therapy and after its conclusion. The blood was collected on heparin 50 UI/ml for the carbon particle inclusion test, on EDTA for total leukocyte counts and on clotting gel for serum The blood samples were processed in maximum 4 hours from the sampling (Ghergariu et al., 2000).

Leukocyte counts were performed according to Campbell, 1994.

Carbon particle inclusion test. Heparinized blood samples (0.50 ml) were mixed with 2 microliters of supernatant of India ink, which were obtained by centrifugation at 1308g for 40min (Hettich Zentrifugen, Germany). One hundred and fifty μl of the mixture were transferred immediately to 2 ml of saline and the rest was incubated for 30 min at 37°C. Other aliquots of 0.15 ml sample were similarly processed at 30 and 45 min of incubation respectively. In parallel, the blood samples were treated *in vitro* with the alcoholic extract of *Echinacea angustifolia*, using 1.5 microliters of each extract and 70° alcohol per 0.5 ml of heparinized blood, to evaluate the *in vitro* influence of the extracts on the carbon particle inclusion process.

All tubes containing saline, blood and ink were centrifuged at 419g and the supernatants were read spectrophotometrically (l=535 nm, *d*=1 cm). The absorbance of carbon particles decreased with time, as the phagocytosis was in progress. Phagocytic activity index was calculated as the difference between the natural logarithms of the optical densities of the phagocytosis at 0–30 min and 30–45 min (Khokhlova et al., 2004).

Circulating immune complex measurements. Measurement of the level of circulating immune complexes (CIC) allows evaluation of the molecular clearance capacity at a particular moment. Part of the collected blood was allowed to clot for 30 min at 37°C and then centrifuged at 1308 g for 10 min. Sera were removed and kept at –20°C until tested. A 4.2% polyethylene glycol (PEG) solution in borate buffer was used as the precipitating agent, while buffer-treated samples served as controls for borate-induced precipitation. The reaction was performed in a 96-well-plate to enhance spectophotometrical readings. Volumes of 196.7 ml of borate buffer and PEG solution, respectively, were mixed with 3.3 ml samples of the serum, for each sample, in parallel wells. The samples were allowed to precipitate at room temperature (22–23°C) for 60 min, then read spectrophotometrically at a wavelength of 450 nm in the test plate (*d*=0.5 cm)

(multichannel spectrophotometer SUMAL PE2, Karl Zeiss, Jena, Germany). CIC concentrations, expressed in optical density units (ODU) were calculated by subtracting the value of the control (serum + buffer) from that of the PEG precipitate.

Immunoglobulin measurements. Total immunoglobulin, known as opsonins, play an important role in the 'first line of defense', that is innate immunity, against aggressors. At a pH 7.4, the electric charge and colloidal stability of gamma globulins are lower than those of serum albumins. Thus, concentrations as low as 24 mg l^{-1} of metal salts precipitate the immunoglobulin. A volume of 6.6 ml of serum was mixed with 193.4 ml of a 0.024% barbital buffer zinc sulphate solution and allowed to precipitate for 30 min at room temperature (22–23°C). Optical density (ODU) then was read spectrophotometrically (l=475 nm, *d*=0.5 cm).

RESULTS AND DISCUSSIONS

Leukocytes are essential not only for their protective attributes but also for their capacity of producing, in a variety of species, endocrine mediators which were believed to be produced solely under the direct control of the hypothalamus-hyphophysis axis (Melchers and Rolink, 1999). The total number of leukocytes offers a perspective into the modulation capacity of the immune system (innate cellular component), of adaptation to the continuous impact of the environment (Roitt and Delves, 2001).

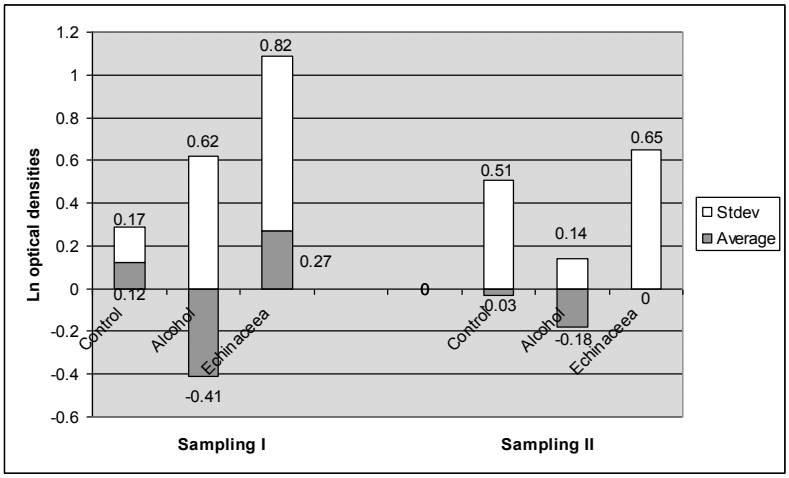
Total leukocyte numbers are presented in Table 1. The high standard deviation of the values during the first sampling indicated various gravity of the inflammatory process, depending on the individual and time elapsed from the onset of the infection.

Table 1

Dynamics of the total numbers of white blood cells before and after the antibiotic treatment (x ±s)(10^3 /mm^3)

Parameter	Sampling I	Sampling II
Average	24,520	11,390
Stdev	6.37	2.00

Initiating the anti-infectious and support therapy in affected animals lead, in 18 days from the first blood sampling, to a statistically highly significant decrease of these values. The individual values at the second sampling ranged between 8.4 and 14.8 thousand/mm^3. These values were closer to the physiological limits of the species (Ghergariu et al., 2000).
The effectiveness of the treatment was demonstrated not only by the clinical healing of the animals, but also by the depletion of the leukocytes subpopulations, with rebound of the total number to normal ranges, which indicated the gradual reduction in the inflammatory process. The observed decline was over 50% ($p<0.001$). Phagocytes are able to ingest not only bacteria, but also inert particles (carbon, of silicon etc).



The clearance of carbon particles from a mixture of whole blood and China ink, evaluated spectophotometrically, indicated the functional capacity of the circulating phagocytes. Testing of the *in vitro* functional potential of phagocytes offers information about their capacity to recognize and subsequently embed the "non-self" particles. The results obtained by carbon particles inclusion test were are presented in Fig.1. Spontaneous phagocytic activity found in the first sampling was reduced. Due to the decrease in phagocyte numbers, there was also a decrease in phagocytic capacity of the leukocytes, which indicated a diminishment of the inflammation. This result indicated a negative, time persistent influence of the mycoplasma infection on the spontaneous phagocytic process. The spontaneous phagocytic activity correlated statistically significantly ($r=0.492$, $p<0.01$) with the total leukocyte number before, but not after the antibiotherapy. When compared to spontaneous phagocytosis, both alcohol and Echinacea had negative effects during the initial sampling. The optical densities values were higher than those in the control. Nevertheless, a stimulation of the phagocytosis by the extract was recorded 30 minutes after the incubation. During the second sampling, both the alcohol and the vegetal extract inhibited phagocytosis, proving that in under the experimental conditions, neither the alcohol not the alcoholic extract of *Echinacea angustifolia* were stimulating.

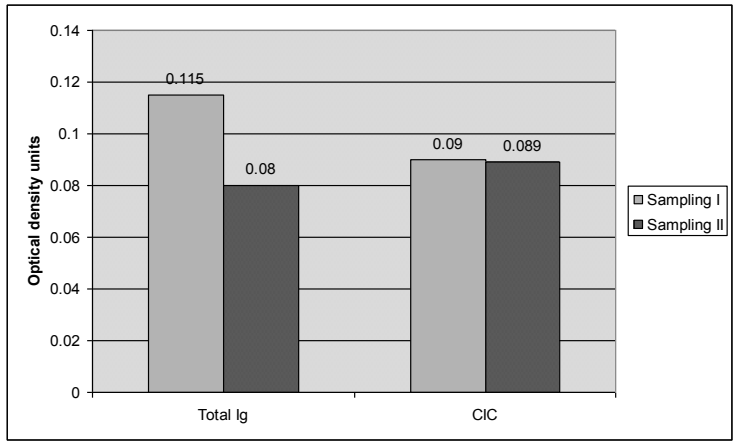


Fig. 2. Changes in total Ig and CIC values during the therapy (ODU)

Fig. 2 indicated the dynamics of the total Ig and CIC levels. The immune globulins highly significantly ($p<0.001$) decreased, while there were no changes in CIC levels from the first to the second sampling. This indicated a less pronounced inflammatory process and a clearance of the created immune complexes.

The obtained results showed that the involvement of the immune system decreases as the antigenic stimulation by *Mycoplasma agalactie* is diminished during the antibiotic treatment in diseased animals.

CONCLUSIONS

The antibiotic therapy during the clinical course of contagious agalactia, diminished antigenic pressure and the inflammatory response to *M. agalactiae* as well as the inflammatory response, thus reducing the total numbers of leukocytes, their phagocytic activity and the total Ig levels.

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