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CUPRINS

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INVESTIGATION ON HEPATITIS E INFECTION IN PIG FARMS

Adriana ANIȚĂ, Luanda LUDU, Dragoș ANIȚĂ, Gheorghe SAVUȚA
Faculty of Veterinary Medicine Iași
e-mail: aeanita@uaiasi.ro

Abstract
Swine hepatitis E virus (HEV) is identified as a zoonotic agent that is possibly transmitted to humans from pigs. Swine HEV is prevalent in pig populations and does not cause abnormal clinical symptoms in infected animals. In this study serum samples from 148 pigs were tested by using an enzyme immunoassay to detect the IgG antibodies against HEV. The blood samples were collected from eight farms in three counties, of which in only 4 farms, 12 pigs (8,1%), were detected as seropositive.

Keywords: swine hepatitis E, ELISA, seroprevalence.

Introduction

Hepatitis E was first named enterically transmitted non-A non-B hepatitis. The disease was first recognized in 1980 when a large waterborne hepatitis epidemic, in India. In 1997 a swine hepatitis E virus strain (swine HEV) was identified (Meng XJ, 1997) and HEV was suggested to be a zoonotic disease.

HEV the causative agent of hepatitis E, is a positive single-stranded RNA virus. Hepatitis E virus (HEV) is transmitted by the faecal-oral route, often causing water-born epidemics. An important issue raised from the discovery of swine HEV strains similar to human strains is the possibility of actual zoonotic transmission from swine to humans.

On the basis of comparative phylogenetic analysis, hepatitis E virus was reclassified as the sole member of the family Hepeviridae of the genus Hepevirus (Emmerson, 2004) Hepatitis E virus exists as a single serotype but there are at least five genotypes. A HEV genotype was defined by Worm *et al*. (2002) as these virus strains heaving nucleotide divergence of not more than 20% of the nucleotides in ORF2.

Domestic pigs and wild boars are the main animal reservoir for the genotypes 3 and 4 strains of HEV worldwide (Meng XJ, 2009). The natural HEV infection of pigs is produced between 10 and 12 weeks of age, so the majority of swine became infected at the age of 2 to 4 months, consistent with the high seroprevalence rate in pigs older than 4 months old. The IgG anti-HEV seroprevalence rate in sows is higher, ranging from 60 to 73%. (Pavio N, 2010)

The existence of anti-HEV antibodies in the swine population has been reported in many countries. Referring to the European swine population, during the last years, HEV has been detected in Spain (Peralta J., 2009), United Kingtom (Banks M, 2004), the Netherlands and Italy (Di Bartolo I, 2011).

The aim of this study was to analyse the prevalence of IgG anti-HEV antibodies in farm pigs from three counties in east of Romania.

Materials and methods

A total of 180 blood serum samples were collected from apparently healthy pigs from eight farrowing to finishing farms. The study was performed during january and may 2011. All blood samples included in this study were centrifuged and the serum was harvested and stored at –20^{o}C until testing.

The assessment of anti-HEV IgG in serum was performed with the use of a commercially available qualitative immunoassay method (HEV IgG ELISA, MP Biomedicals). The protocol was made according to the manufacturer's recommendations and modified using of anti-swine IgG horseradish peroxidase enzyme conjugate instead of anti-human conjugates. Each pig serum (10µl/well) was examined at a fixed dilution (1:21 in dilution buffer). The absorbance value was measured at 450 nm with reference wavelength of 620 nm. The results were expressed as optical density (OD). Pool serum from HEV positive and negative pigs (previously tested) were included as positive and negative controls, respectively.

Results and discussion

Serum samples were collected from healthy pigs from eight farms in three counties (Iași, Bacău and Tulcea). In this study we detected IgG anti HEV positive pigs in only four farms.

Table no.1

Distribution of the IgG anti-HEV positive pigs

County	Farm	Number of samples tested	Number of positive samples	Percent of positivity (%)
Iași				
	A	20	3	15
	B	26	4	15,38
	C	32	0	0
Bacău				
	D	8	4	50
	E	13	0	0
	F	19	0	0
	G	18	0	0
Tulcea				
	H	12	1	8,33
Total		**148**	**12**	

In the positive farms the prevalence of IgG anti-HEV antibodies was ranging from 8.33% to 50%. From the total of 148 serum samples collected and tested, 12 (8,1%) were found to be positive. The results suggest that swine could be an important reservoir for virus transmission in Romania as has been suggested for other non-endemic areas.

The highest prevalence of antibodies to HEV identified in the present study was in the farm D from Bacău county (4 positive serums out 8 tested). Out of three farm tested in Iaşi county two were found positive for IgG anti-HEV antibodies. There is an agreement among the present data and others (Aniţă A, 2010) on the concept that swine HEV causes almost universal infection in pigs at least in some regions. The different prevalence in the studied farms is probably due to the dynamics of the infection in swine, which is influenced by maternal immunity. Passive immunity protects piglets up to 2 months old and, after the infection, seroconversion occurs with IgG increase mainly at 15 weeks old.

Our results point out that HEV may be circulating among pigs in the east region of Romania. Similar results were obtained in Spain: 41.9% positive sera collected from 1998 to 2000 and 60.8% positive sera of gilts and sows collected from 1998 to 1999 (Seminati C, 2008). Other studies conducted on small-sized pig sera samplings showed seroprevalences in developed countries: United Kingdom (85%), Sweden (58%), Germany (23%), and the United States (34.5%).

The main concern is about the possibility of hepatitis E virus transmission to man, since pigs are very common domestic animals and whose meat is also largely consumed. Indirect evidences also support the hypothesis of zoonotic transmission of HEV to man, such as the high frequency of antibodies to HEV showed by animal handlers (Meng et al. 1999, 2002, Hsieh et al. 1999). Anyway, further studies will be made to establish the role of swine HEV in human disease.

In conclusion, our results suggest that HEV is circulating among several swine farms in the east of Romania. Further investigations are being conducted to identify the virus in swine as well as in sewage samples of pig origin for the genetic characterization of the virus.

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IDENTIFICATION OF NEW TICK-BORNE ENCEPHALITIS FOCI – A PILOT SEROPREVALENCE STUDY IN THE EAST OF ROMANIA

Oana COJOCARU, V. PĂDURARU, B. OLĂERIU, Gh. SAVU ȚA
Faculty of Veterinary Medicine Ia și
oana828282@yahoo.com

Tick borne encephalitis (TBE) is a viral zoonotic disease caused by TBE flavivirus (TBEV) and it is one of the most prevalent arboviruses in Europe and in many parts of Asia. Three subtypes of TBEV have been reported: the European (TBEV-Eu) subtype transmitted by Ixodes ricinus ticks and widely distributed in Europe, the Siberian (TBEV-Sib) and Far-Eastern (TBEV-FE) subtypes, carried by Ixodes persulcatus ticks and present from the Far-East to Baltic countries. Transmission of TBEV to humans usually occurs by bite of an infected tick or rarely by ingestion of unpasteurized milk products of infected livestock.
Risk of tick-borne encephalitis in Romania is reported only in Transylvania, at the base of the Carpathian Mountains.
The objective of this study is to identify new TBE foci in the east of Romania. A total of 180 serum samples taken from healthy animals were randomly selected from 6 counties and tested by immunoassay test EIA TBEV Ig (Test-Line Ltd., Czech Republic). The results suggest a potential risk of infection with TBEV in the east of Romania and indicate a need for more studies.
Key words: tick-borne encephalitis, ELISA, ticks

Tick borne encephalitis (TBE) is a viral zoonotic disease caused by TBE virus (TBEV) belonging to the genus *Flavivirus*, in the family *Flaviviridae*. Three subtypes of TBEV have been reported: the European (TBEV-Eu) subtype transmitted by *Ixodes ricinus* ticks and widely distributed in Europe, and the Siberian (TBEV-Sib) and Far-Eastern (TBEV-FE) subtypes, carried by *Ixodes persulcatus* ticks and present from the Far-East to Baltic countries *(Fauquet CM., 2005).* In Europe, TBE is regarded as the most important and potentially fatal human infection of the central nervous system, causing over 3000 cases annually *(WHO, 2011).*

Ixodes ricinus the principal vector of TBE is most abundant and widespread species of ticks in Romania *(Feider, 1965).* In *Ixodes ricinus*, TBEV prevalence in unfed ticks varies between 0.1 and 5% and increases during the tick development from stage to stage. Higher virus prevalences have been recorded in engorged ticks removed from humans *(Bormane et al., 2004; Süss et al., 2004).*

The main route of TBEV infection of humans is a tick bite; much more rarely, the infection was transmitted by the alimentary route involving dairy products from livestock. Large domestic animals such as goats, sheeps and cattles are potential hosts for *Ixodes ricinus* and may become infected by TBEV in endemic areas. These animals are viraemic over a very short period only and, as a rule, do not show any clinical symptoms. However, goats and sheeps, more rarely cattles, are of special importance for the so-called alimentary

acquired TBE. During the viraemic stage, the virus is excreted in milk and can be ingested oraly by consumption of non-pasteurized milk or cheese produced from raw milk. The clinical course of alimentary TBE is usually more often biphasic than that of TBE induced by tick bite, in which only 20–30% of the patients show a 2nd phase in the course of disease *(Jochen Süss, 2011).*

Romania has geo-climatic and ecological conditons allowing conservation and circulation of TBEV in natural foci. Serological tests performed in Romania on humans, in the '50s revealed data about the circulation of TBEV and offered the possibility to draw up its natural foci in relation with the presence of the vector and reservoir – the arthropods *(Lucia Ionescu et al., 2009)*.

During 1985-1993 was realized an extended sero-epidemiological study for humans and animals in 19 counties and Bucharest city in order to identify ares of circulation of arboviruses in Romania. Seroprevalence for human samples ranged from 1,1% (Constanța) to 19,4% (Maramureș) and for animal samples the seroprevalence ranged from 2% to 23,4% ,standing out the following counties with high seroprevalence: Suceava (23,4%), Hunedoara (18,5%), Maramureș (17,4%), Mureș (14,4%). Another investigation was conducted by the Local Public Health Authority in 1999, in the outbreak of TBE in Sibiu County, with 38 serologically confirmed acute neorological cases, established as etiology the TBEV. Epidemiological investigation established that raw goat milk and raw milk goat products were the source of contamination *(Lucia Ionescu et al., 2009)*.

Risk of tick-borne encephalitis in Romania is reported only for Transylvania at the base of the Carpathian Mountains, especially in the following counties: Alba, Bihor, Bistrița - Năsăud, Cluj, Covasna, Harghita, Mureș, Satu-Mare,Sălaj and Sibiu.

The aim of the present study is to perform a serologic survey among livestock assessing the prevalance of antibodies against TBEV in selected regions of Romania classified as non-endemic.

MATERIALS AND METHODS

The investigation was designed as a cross-sectional study. Serum samples collected in the years 2010-2011 from 180 animals were investigated for the presence of antibodies against tick-borne encephalitis virus. The study population consisted of clinically healthy sheeps, goats and cattles. Only animals over the age of 2 years were included, because animals of this age were more likely to have previously visited typical tick habitat such as fields or woodlands.

The animals blood samples were collected from six counties, placed in the eastern region of the country:
Suceava, Botoșani, Bacău, Iaşi, Galați and Vrancea. Figure 1 shows the places where samples were collected.

The number of the samples collected from each county was different, ranging from 7 to 30 blood samples.

Figura 1 Geographic distribution of the counties that provided blood samples

Serum samples were tested in commercially avaible EIA TBEV-Ig kit (Test-Line, Ltd., Czech Republic) according to the instructions of the manufacturer. Native serum samples were inactivated at 56^0 C for 30 min. The optical density was measured at 450 nm.

The test was regarded valid when the optical density (OD) value of the pozitive control was equal or lower than half the mean absorbance of negative control and when the mean absorbance of negative control was equal or greater than 0.200. Results were expressed as a ratio of averge OD value of the negative control / OD value of the sample.

Ratio of absorbances N/P	Interpretation
< 1.5	negative
1.5 – 1.99	borderline
≥ 2.0	pozitive

RESULTS AND DISCUSSION

Of the 180 samples tested, 6 were positive for TBEV serocomplex antibodies and 100 were categorised as boderline. TBE seroprevalence ranged widely over the counties from 3,33% to 14,28%. Analysis of all ELISA samples showed a seroprevalence of 3,33%. Here we report for the first time the presence of TBE infection in cattles and goats from the east of Romania.

Table 1

Results of the serologic exam for the detection of Ig anti -TBE

County	Species	No. of serum tested	ELISA results No. of positive serum	No. of borderline serum	No. of negative serum
Bacău	Goats	7	1	2	4
Galați	Cattles	1	-	1	-
	Goats	12	-	1	11
Vrancea	Goats	29	2	12	15
	Sheeps	13	-	4	9
Iași	Goats	21	1	12	8
	Sheeps	7	-	4	3
	Cattles	30	-	21	9
Suceava	Cattles	30	1	24	5
Botoșani	Cattles	30	1	19	10
Total		**180**	**6**	**100**	**74**

Seropozitivity to TBEV was found to be 5,79 % in goats, 2,19 % in cattles and 0,0% in sheeps. Results are comparable with another studies, for example in Poland from 358 goat samples, 17 (4,7%) were positive for anti-TBEV antibodies. In the investigation above seroprevalence in goats reached 14/151 (9,3%) in endemic and 3/207 (1,4%) in non-endemic provinces *(Pawel Stefanoff et al., 2008)*. Figure 2 shows the distribution of the seroreactivity or non-reactivity to TBE according to the kit interpretation in different counties when tested by EIA.

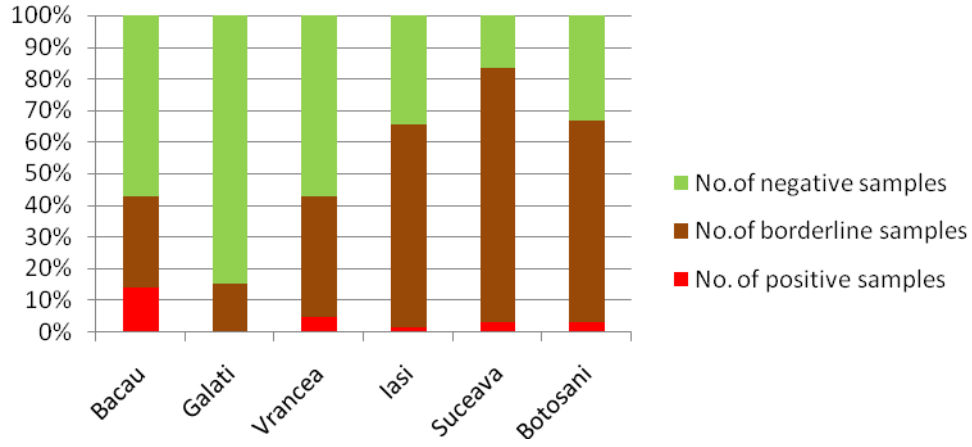

Figure 2 Distribution of tick-borne encephalitis virus ELISA positive, borderline and negative blood samples grouped according to counties

It could be observed that the serum samples from Galați county w ere non-reactive to TBE. The highest percentage of positive results was noted in goats from Vrancea county – 2/29 (14,28%). ELISA pozitive samples were found allmost in all regions of the east of Romania included in the study.

The present study explored the possible existence of TBE endemic foci in est of Romania, considered free of disease based on information provided by the routine communicable disease survillance system. Results obtained from goats and cattles support the hypotesis that undiscoverd endemic areas may exist in Romania. Animals included in the present study were living in farms which allowed them free grazing on local pastures and can be therefore considered as sensitive indicators of local presence of TBE virus in ticks. The counties where TBEV antibody-positive goats and cattles were found did not report a single human case.

The approach of using companion animals or livestock as indicators of TBEV local circulation was used by several authors in Germany and Russia (*Korenberg et al., 1984; Leutloff et al., 2006*).

This results are important because the consumption of raw milk from sheep, goats and cattles may be associated with risk of infection with TBEV. Experiments have demonstrated that infected domestic animals can excrete TBEV into milk for 3-7 days, beginning as early as the second or third day postinfection *(Holzmann et al., 2009).*

CONCLUSIONS

- The 6 positive serum for TBE, representing 3,33% in livestock were detected using an EIA kit for all species.
- Antibodies evidence show that TBE virus circulating among livestock in the eastern region of Romania.
- The results suggest a potential risk of infection with TBEV in the east of Romania and indicate a need for more studies.
- Romania has geo-climatic and ecological conditons allowing conservation and circulation of TBEV in natural foci.

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THE CONTRIBUTION OF ECOLOGICAL EDUCATION TO BIODIVERSITY CONSERVATION AND ENVIRONMENT PROTECTION

Victor Donea,
State Agrarian
University of Moldova

SUMMARY
The deterioration of the environment situation at national level in the last decades of the XXth century, as a result of irrational development of the agro - industrial complex and other branches of our economy, along with poor ecological consciousness of different social layers, represent the cause of extinction of many species of living beings.
The ecological education and its continuous character is the factor that directly ensures the conservation of biodiversity.
Key words: Conservation of biodiversity, Ecological consciousness and culture, Ecological education and training, Environment protection, Floristic and fauna resources, Laws of the nature, Protected areas.

INTRODUCTION

The interaction between human society and nature on different plans, not long ago, had a chaotic character, gradually replacing the living natural environment with anthropogenic ecosystems which constitute nearly 85% of the surface of the Republic of Moldova, inclusive 75, 6% - the agricultural ecosystems. The consequences of the intensive human agricultural activities are: permanent and increasing pollution of atmosphere, lithosphere, and as a result the extinction of many species of living beings or their inclusion in the category of living beings on the edge of extinction [16].

Within the international conference from Rio de Janeiro (1992), where more than 150 countries have participated, there have been debates on the present situation of the environmental biodiversity and on the global strategy of environment protection in present and future. In its turn, the Parliament of the Republic of Moldova has adopted in 2002 the National strategy and the Plan of actions concerning the conservation of biological diversity [7, 13].

It stands to reason that the future of all organisms living on Terra depends on the character of human economic activity. The conference from Rio de Janeiro (1992) had adopted the concept of sustainable development which stipulates the observance of the dynamic balance in the socioeconomic systems of different levels by the world countries, including the Republic of Moldova [12].

In the last decades, in the Republic of Moldova, a series of concrete measures concerning the problem of environmental protection and biodiversity conservation has been made such as:

- the legislative framework, concerning the environment, that is permanently completed with new laws and normative acts, has been created;
- they systematically accomplish researches in order to determine both flora/fauna resources and the situation of rare living beings and those on the edge of extinction registered in the Red Book of the Republic of Moldova;
- a big number of nongovernmental organizations organize concrete activities in order to solve the problems concerning biological diversity conservation [1, 5, 8].

It is necessary to mention that the ecological situation in Moldova determines us to undertake additional measures concerning environmental protection. As examples which prove the necessity of these kinds of actions can serve the following facts:

- the total surface of natural areas protected by state constitutes 1,96%, and by 2015 it will increase only till 2,4%, fact that situates the Republic of Moldova on the last places in Europe [11];
- the second edition of the Red Book of the Republic of Moldova [5], in comparison with the data from the first edition, constitutes a greater emergency signal which proves the necessity of efficient and urgent actions concerning the protection of species of plants, animals, mushrooms and other living beings that are on the edge of forced extinction: 126 species of plants in the second edition in comparison with 26 species in the first edition; 116 species of animals in comparison with 29. A series of other species are now in a deplorable ecological situation: 39 species of birds, 37 species of insects, 14 species of mammals, 12 species of fish, 8 species of reptiles, among plants - 91 species of vascular plants, 10 species of mosses, 16 species of lichens and others;
- most of Chişinău's population can see in early spring, in the markets and even in the center of the capital, the active marketing of decorative plants and of those on the edge of extinction from the spontaneous flora;
- unauthorized garbage sites appear in the periphery of forests, along the highways and water-pools and on the agricultural fields;
- the reduction of insects (with about 50-60%) and birds (with about 30-40%) number in vineyards and orchards because of vast amounts of pesticides used in the last century;
- vast surfaces of arable land became the victim of erosion because of their unreasonable use, and the protection bands have been almost cut. Nowadays, few protection bands are planted. An increasing source of environmental pollution is represented by sound pollution and republican auto park, the number of vehicles in which is growing extremely fast because of used and old motor cars imported from other countries [8].

Especially this fact confirms once again the necessity to apply concrete measures in order to improve the existing situation. Thus, the competent departments should initiate concrete activities that would ensure the observance of legislation regarding the problem of environmental protection and biodiversity conservation; the necessity to elaborate a unique state program concerning the continuous ecological education of the population is also of present day importance, beginning from early childhood and continuing during the whole period of professional activity.

MATERIAL AND METHODS

Aiming at stability, the ecological instruction and education, as a problem of education at all levels, gradually ensures the awareness of the obligations of every citizen (regardless of age and specific character of practical activity) concerning the environment. The stabilization and improvement of environmental factors will ensure biodiversity conservation and humankind existence as beings [2; 9; 14].

The researches have been accomplished using the forms, methods and technologies of classic and modern pedagogy [3, 10; 15].

ANALISYS AND DISCUSSIONS

The concept of ecological education of the population must aim at forming the consciousness regarding the protection and quality improvement of the environment, goal that can be achieved by:

√ Changing the consciousness of each person about the human being as a component of the living nature (but not as a hegemonic power as we used to consider it in the past), whose existence is possible only in harmony with the environment and other living beings surrounding us;

√ Being conscious about the fact that the environment has nothing in plus or dangerous, on the contrary, the living beings have mutual and stable relationships (such as: plant-plant, plant-animal, living nature-lifeless nature, human being-nature) and only in this way, due to the presence of living beings-producers (phototropic or chemotropic), living beings-consumers (herbivorous, carnivorous, parasitic heterotrophic organisms), living beings-reducers (organisms feeding on semi-decomposed organic substances from carcasses), the global biological circuit of substances in nature is ensured. Relationships character between living beings is varied (nutritional, antagonist, symbiotic, sexual relations, etc.), thus ensuring natural selection and life evolution. The result of ecological instruction and education will depend on child's and adult's capacity to become conscious about the phenomena previously described [2; 7; 8; 9; 15];

√ Basic directions of the ecological education of every citizen, and especially of children, depends both on theoretical training (storing corresponding ecological knowledge) and the formation of human responsibility for nature, i.e. the formation of ecological culture that implies the solving of socio-ecological problems without causing damages to the living environment and living beings' health.

Ecological education is focused on the present and future contributing to the renewal of didactic conception, to the reorganization of educational contents and means. It represents a moral duty of every employee working in the educational system of any level and practically, there is no speciality or school activity that doesn't touch upon aspects of the ecological education.

Ecological education begins in the family (from early childhood), continues in educational institutions of any level (preschool, primary, gymnasium, high school, university, post university degrees), and lasts the whole life. Thus, it is not a part of instruction; it represents the effect and purpose of the contemporary instructive process. Its object consists in the development of correct ecological thinking, creating an adequate ecological culture and ethics for each citizen.

At present there are two methods to accomplish the ecological instruction and education:

- The first one is called anthropocentric and it is based on the principle of considering the ecology as a sphere of interaction between society and nature, i. e. Only its practical aspect is taken into consideration. Attention is given to: human being (human society), scientific-practical progress and the problem concerning rational use of natural resources, pollution of hydrosphere, atmosphere and soil with technologic waste and the influence of these factors on the living nature components. It also focuses on the possibility to solve global and regional ecological problems using advanced technologies, concluding intergovernmental contracts (this will lead to rational use of planetary resources) and accomplishing various measures to protect vegetal and animal resources. The supporters of this method consider that present ecological situation requires the man to be the „nucleus" of solving the existing problems due to his universal relations with nature;
- The second method is called bio-centric and it is based on the fact that human society is a component part of nature and depends on it, obeying its laws. Thus, contemporary human beings realize the necessity to understand and not to neglect ecological laws that stand on the basis of existence formation and natural biological systems evolution of all levels. Knowledge and observance of nature laws create for the human beings objective conditions necessary to find their correct place within nature and to understand their responsibility for life keeping and evolution on Terra.

Thus, the general objective of ecological instruction and education is forming (to every member of society) modern ecological consciousness and ecological behavior, i.e. assimilating rich and correct scientific knowledge, about the living and lifeless nature, necessary to understand the problems of regional and global ecology; and finally - to save the life on Terra for the future generations protecting the environment and conserving biodiversity, at the same time ensuring the technical-scientific progress.

As a result of the concepts and objectives of national and international forums (Stockholm, 1972; Belgrade, 1975; Tbilisi, 1977; Rio de Janeiro, 1992; Chişinău, 1998, 2006 etc.), the ecological education in educational institutions must be accomplished on the basis of pluridisciplinary and interdisciplinary principles, both during class work (at every educational subject, especially at real disciplines) and within extracurricular activities [6; 9].

Since 01.09.2008, in the national secondary educational institutions, the project Curriculum for the optional discipline „Ecological and environmental protection education" has been implemented for preuniversity education (from Ist to XIIth forms), that was prepared with the help of author's participation [4].

CONCLUSIONS

Environmental protection and biodiversity conservation represent the basic problems the humankind is confronting with, the problems that will ensure the present and future of life on Terra in its natural diversity.

Ecological instruction and education is a continuous, pluridisciplinary and interdisciplinary process and its efficiency depends on the correct collaboration between family-human society and educational institutions-legislative and state bodies.

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FERMENTATIONS OF VARIOUS WHEY TYPES WITH USING *Kluyveromyces lactis* IN THE PRODUCTION OF BIOETHANOL AND ORGANIC LIQUID FERTILIZER

Gemilang Lara Utama[1], Tita Roostita Balia[1], Kurnani Tubagus Benito Achmad[1,2], Sunardi[2], Iwan Setiawan[1]

[1]Faculty of Animal Husbandry University of Padjadjaran
Jl.Raya Bandung Sumedang KM.21 Tanjungsari Sumedang, Jawa Barat Indonesia
[2]Institute of Ecology University of Padjadjaran
e-mail : thmfsc@yahoo.co.id

ABSTRACT

The research aimed to determine Kluyveromyces lactis concentrations and whey type that appropriate in producing the best bioethanol production with distillery waste as organic liquid fertilizer. This experimental research was done based on Completely Randomized Design with 3 x 3 factorial patterns. Treatments consisted of two factors, namely the addition of different Kluyveromyces lactis concentrations (5%, 10% and 15% v/v) and combinations of whey types (neufchatel whey, feta whey and mixed neufchatel and feta whey 1:1), with three replications. Whey was fermented by Kluyveromyces lactis with the concentration of 5%, 7.5%, and 10% (v/v) at the temperature of 33ºC for 24 hours and then distillated twice at the temperature range 78 ° -100 °C. The first distillation gives bioethanol purity until 86% and the second one gives purity until 95%. The result showed that 5% concentration of Kluyveromyces lactis on neufchatel whey (K1W2) produce best bioethanol content of 1.94% and liquid organic fertilizer with N content of 0.1%; P of 0.067%; K of 0.135%; and pH of 5.7.

Keywords : **Whey, *Kluyveromyces lactis*, Bioethanol, Liquid Organic Fertilizer**

Introduction

Whey is byproduct of cheese-making industry which potential to cause pollution. One of the pollution potential came from high BOD-COD and low pH. Whey also donates Nitrogen (N) and Phosphate (P) in enough concentrations to cause eutrophication if continuously discarded in large numbers to the waters. Beside the potential of pollution, the nutrients of whey allow to be processed into a commodity.

One of the important nutrients of whey is lactose. The content of lactose in whey reaches 4-5% (Ghaly, *et al.*, 2000). The ability to utilize lactose as a carbon source is owned by the yeasts such as *Kluyveromyces lactis*. As mentioned by Ghaly, *et al,*, (1993) and Maullu *et al.*, (1999), lactose from whey is widely used as a carbon source in bioprocess media for *Kluyveromyces lactis* growth.

Kluyveromyces lactis were used in many industrial activities because of lactase producing ability (Rech *et al.*, 1999; Moeini *et al.*, 2004). With the lactase producing ability, lactose can be hydrolyzed directly into glucose and galactose by *Kluyveromyces lactis*, then through the glycolysis and metabolic pathways it's hydrolyzed into pyruvic acid. Pyruvic acid

became the most important branching point in the process of *Kluyveromyces lactis* metabolism, in a state of limited oxygen the oxidation of NADH cannot be perfect, so ethanol fermentation occur through pyruvat decarboxylation into acetaldehyde which subsequently hydrolyzed into bioethanol (Breunig and Steensma, 2003).

Bioethanol with the expected purity can be obtained by distillation process. Besides bioethanol, the distillation processes were also generating distillery waste which can be used as an alternative of liquid organic fertilizer. Many nutrients left that can be used as a source of NPK for the soil and plants.

To produce best bioethanol production and liquid organic fertilizer necessary to observe several things such as the concentration of starter and raw materials used. The starter concentrations in the production of bioethanol are varies greatly depending on the type of raw materials and fermentation conditions. Therefore, research aimed to determine *Kluyveromyces lactis* concentrations and whey type that appropriate in producing the best bioethanol production with distillery waste as organic liquid fertilizer.

Materials and Methods

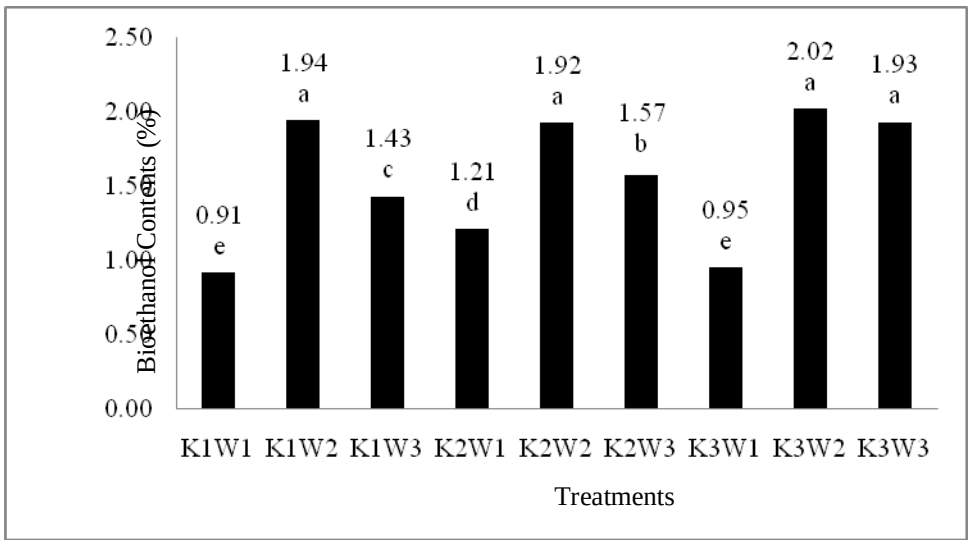
Neufchatel and feta whey was taken from PT. Yummy Food Utama East Jakarta and then analyzed at the Laboratory of Ruminant Nutrition and Feed Chemistry, Faculty of Animal Husbandry, University of Padjadjaran (2010) with results as follows: feta whey has a Lactose of 4.64%, Fat of 2.19%, Protein of 0.79%, Ash of 0.073% , Crude Fiber of 0.011%, Water of 92.29% and whey neufchatel have Lactose of 4.09%, Fat of 2.39%, Protein of 0.75%, Ash of 0.089%, Crude Fiber of 0.015%, Water of 92.67%. *Kluyveromyces lactis* was taken from The Laboratory of School of Life Science and Technology ITB.

All types of whey (neufchatel whey, feta whey and mixed neufchatel and feta whey 1:1) fermented by *Kluyveromyces lactis* with the concentration of 5%, 7.5%, and 10% (v/v) at the temperature of 33ºC (Steensma *et al.*, 1988) for 24 hours, it's because *Kluyveromyces lactis* through acceleration phase at 8-16 h at 35ºC (Barbosa *et al.*, 1985). After that, fermented whey was distillated twice at the temperature range 78 ° -100 °C (Erliza Hambali, *et al.*, 2009). The first distillation gives bioethanol purity until 86% and the second one gives purity until 95%. Bioethanol content were tested with using Gas Chromatography, N content with Kjedahl methods, P as P_2O_5 and K with *Atomic Absorbtion Spectrophotometer* (AAS), moreover pH with digital pH meter (Zenway).

Results and Discussions

Figure 1 showed that the highest average of bioethanol produced was 2.02%, which given by treatment combination of neufchatel whey with a 15% *K. lactis* concentration. While the lowest average of bioethanol produced was 0.91% that given by the treatment combination of feta whey with 5% concentration of *K. lactis*.

The production of bioethanol is not only due to the nutritional composition of the substrate but also influenced by pH, time, and also the amount of glucose and galactose which available on the substrate (Ramakrishnan and Hartley, 1993; Kargi, *et al.*, 2006; Dagbagli and Goksungur, 2008). All of the factors mentioned above was inter-related and affected the bioethanol contents. Kargi, *et al.*, (2006) states that pH 5-6 is the optimum pH in producing bioethanol with whey as raw material.



K_1 = 5 % *K.lactis* concentration, K_2 = 10% *K.lactis* concentration, K_3 = 15% *K.lactis* concentration
W_1 = *Feta* whey, W_2 = *Neufchatel* whey, W_3 = Mixed *feta* dan *neufchatel* whey 1 : 1

Figure 1. The average bioethanol contents that influenced by the treatment combination of *Kluyveromyces lactis* concentration in different types of whey

Similarly with Neri, *et al.* (2008) which showed at pH of 5.0 to 5.5, lactase enzyme produced by *K.lactis* through an adaptation phase and has a low activity and then lactase enzyme activity were increase and through the exponential phase along with the increasing pH of 5.5 to 6.0. Lactase enzyme activity by *K.lactis* reached peak at pH of 6.5, therefore the amount of glucose and galactose which can be hydrolyzed from feta and mix whey at a pH below 5.5 was not optimal, so the first 24 hours will be a shortage of glucose and production of bioethanol to be low. As mentioned Ramakrishnan and Hartley (1993) the first 24 hours *K.lactis* only utilize glucose and then break down galactose for bioethanol formation. Different with feta whey, neufchatel whey can produced higher bioethanol contents. Neufchatel whey has a pH of 5.7 which is the pH of *K.lactis* lactase activity experiencing exponential phase (Neri, *et al.*, 2008). Therefore, the amount of glucose and galactose which can be hydrolyzed would be better than feta whey and thus bioethanol produced becomes higher.

Beside the differences of whey types, the combinations of *K.lactis* concentration levels in different types of whey give significant effects on bioethanol contents. The high concentrations of *K.lactis* added resulted in higher competition in getting the available glucose so that glucose will be depleted and used only for respiration, it's resulting in a lack of glucose and results in lower amount of bioethanol produced (Mahmoud and Kosikowski, 1982).

Table 1. Average levels of NPK contents and pH of distillery waste affected by different types of whey and *K.lactis* concentration which compared with National Standard of Indonesia

Treatments	Results			
	N	P	K	pH
	%			
K1W1	0.12	0.060	0.127	5.4
K1W2	0.10	0.067	0.135	5.7
K1W3	0.12	0.058	0.112	5.4
K2W1	0.12	0.057	0.111	5.3
K2W2	0.11	0.062	0.130	5.6
K2W3	0.13	0.057	0.112	5.5
K3W1	0.13	0.049	0.116	5.4
K3W2	0.10	0.060	0.139	5.7
K3W3	0.11	0.055	0.171	5.5

K_1 = 5 % *K.lactis* concentration, K_2 = 10% *K.lactis* concentration, K_3 = 15% *K.lactis* concentration

W_1 = *Feta* whey, W_2 = *Neufchatel* whey, W_3 = Mixed *feta* dan *neufchatel* whey 1 : 1

Based on Table 1., note that the distillery waste at K2W3 and K3W1 treatment had a highest levels of N of 0.13%. *Kluyveromyces lactis* is one type of yeast that can utilize N for growth which derived from amino acids (Messenguy, *et al.*, 2006). The extracellular protease activities produced by *Kluyveromyces lactis* hydrolyze amino acids with peptides into N for metabolisms use (Walker, 1998). Nitrogen used by *Kluyveromyces lactis* and stored in their cells. Total Nitrogen in the yeast cells reached about 10% of the dry weight of yeast cells (Walker, 1998).

The results on Table 1 were also shown that K1W2 treatment gives highest P content of 0.067% on the distillery waste. Along with the increasing of *K.lactis* concentration the P content on the distillery waste was decreasing. It is caused by phosphorus in the form of nucleic acids and phospholipids contained in whey were an essential nutrient needed by *K.lactis* to grow (Parrondo, *et al*., 2009; Walker, 1998). Theobald, *et al.* (1996) states that *K.lactis* using phosphorus then it put in his cell in orthophosphate (H2PO4) form.

Distillery waste with highest K content was given by K3W3 treatments with the value of 0.171%. Walker (1998) mentions that K is macro-elements required as cofactor of various enzymes that involved in oxidative phosphorylation, biosynthesis of protein and carbohydrate metabolism. In the abnormal conditions, the role of Potassium in the metabolism of yeasts sometimes replaced by magnesium or sodium, but it will cause the slow rate of fermentation (Spencer, *et al.*, 1997).

Highest pH showed by K1W2 and K3W2 with pH of 5.7 and the lowest shown by K2W1 with the pH of 5.3. During the distillation, heating will trigger autolysis on yeast cells as mentioned by Stemwedel (2009), that heating at temperatures between 55-83ºC will cause autolysis of yeast cells which will lead the breakdown of cell wall and release the polysaccharides. Polysaccharides released can act as a source of carbon (C) and the hydroxyl group can trigger an increase of pH.

Conclusions

The results indicated that the 5% concentration level of *K.lactis* in whey neufchatel (K1W2) can be summed up as the best treatment to produce bioethanol. Even though the NPK content was not the highest; this treatment was taken as the best treatment on producing bioethanol because the treatment was not significant from other treatments that use a higher concentration of *K.lactis*. The addition of 5% concentration of *Kluyveromyces lactis* on neufchatel whey (K1W2) gives best bioethanol content of 1.94% and liquid organic fertilizer with N content of 0.1%; P of 0.067%; K of 0.135%; and pH of 5.7.

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INVESTIGATION OF FECX^{I} MUTATION IN THREE ROMANIAN SHEEP BREEDS

S.E. GEORGESCU[1], G. HRINCA[2], Marieta COSTACHE[1]
[1]University of Bucharest, Department of Biochemistry and Molecular Biology, Splaiul Independetei 91-95, Bucharest, Romania
[2]Popauti Research and Development Station for Sheeps and Goats Growth, Rachiti, Romania

Abstract
Sheep are a highly diverse species with more than 800 different breeds that vary substantially in their morpho-physiological characteristics as colour, milk production or fecundity. Recent studies have revealed that high prolificacy in some sheep breeds is due to a mutation (FecX^{I}) in the Bone Morphogenetic Protein-15 (BMP15) gene. The Inverdale mutation is characterized by a substitution (A/T) in the gene encoding bone morphogenetic protein-15. The effect of mutation is an increase in ovulation rate, in number of offspring per birth, and the number of follicles in the ovaries. Our objective was to develop an easy method to identify the FecX^{I} mutation in order to screen the sheep populations in terms of prolificacy. We designed primers to amplify a 154bp fragment from the BMP15 gene which may or may not contain the mutation. The PCR product was cut with XbaI endonuclease and the restriction products were analyzed by agarose gel electrophoresis. Using the PCR-RFLP technique, we established an easy and efficient method that can be used to screen the FecX^{I} mutation. Therefore, these new methods increase the panel of molecular tools available for sheep breeders to choose the most prolific genotypes for improving artificial selection.
Keywords: sheep, prolificacy, FecX^{I}, PCR-RFLP, screening.

Introduction

Recent discoveries have revealed that the ovulation rate was determined by a complex exchange of endocrine signals between the pituitary gland and the ovary. Three related oocyte-derived members of the transforming growth factor-b *(TGF-b)* superfamily, namely the growth differentiation factor 9 *(GDF-9)*, bone morphogenetic protein 15 *(BMP15)* and bone morphogenetic protein-1B have been shown to be essential for the ovulation rate and follicular growth (Kasiriyan *et al.,* 2011).

Galloway *et al.,* (2000) revealed that the high prolificacy in Inverdale Romney sheep is due to a mutation (FecX^{I}) in the Bone Morphogenetic Protein-15 *(BMP15)* gene. The effect of the mutation is an increase in the ovulation rate, respectively the increased number of offspring per birth and number of follicles in the ovaries (Davis *et al.*, 1992). In contrast, homozygous carriers (FecX^{I}/FecX^{I}) are sterile and have small ovaries, incompletely developed, containing only one layer of granulosa cells (Davis *et al.*, 1992). The hyperprolificacy phenotype of ewes is due to the presence of the FecX^{I} allele, recently identified as a single amino acid substitution (V299D) in the bone morphogenetic protein

15, caused by a single point mutation (T turn to A) in *BMP15* gene, located on sheep chromosome X (Galloway *et al.,* 2000). Animals which are homozygous for the FecX^{I} mutation are sterile due to arrested follicular development from the primary stage of growth.

The aim of this study was to develop a cost-effective and rapid method to identify the FecX^{I} mutation in order to screen ewes in terms of prolificacy.

Materials and methods

Sampling and DNA extraction

Blood samples were collected in EDTA-treated vacutainers from three breeds: Karakul de Botosani, Palas Milk Line, and Palas Merino. For each breed we analyzed 25 animals. The isolation of genomic DNA from fresh blood was performed with Wizard Genomic DNA Extraction Kit (Promega).

PCR-RFLP reaction

In order to amplify a 154bp fragment we have used a pair of primers (forward: 5'-gaaagtaaccagtgttccctccacccttttc-3'; reverse: 5'-catgattgggagaattgagacc-3') corresponding to the FecX^{I} mutation (Davis *et al.,* 2006). The PCR conditions were optimized in order to determine the best annealing temperature for the two primers, between 51-61˚C on a gradient thermocycler (BioRad).

After determining the optimum temperature of 58˚C, the amplification reactions were carried out in 25µL final volume and consisted of 1X PCR Buffer, $MgCl_2$ (1.5mM), 250µM of each dNTPs, DNA template, 0.5 units of AmpliTaq Gold DNA Polymerase, 10mM of each primer and nuclease free water. PCR amplifications were performed using a program with 45 cycles: denaturation for 30 seconds at 95˚C, annealing for 30 seconds at 58˚C and extension for 1 minute at 72˚C. The first denaturation step was of 10 minutes at 95˚C and the final extension was of 15 minutes at 72˚C.

The PCR products obtained were digested with *XbaI* restriction endonuclease (Promega) for 3 hours at 37˚C. The restriction fragments were directly analyzed by electrophoresis in 3% agarose gels, stained with ethidium bromide. The genotypes of the analyzed individuals were established directly using the restriction fragments observed in the gel.

Sequencing

The obtained PCR products were subjected to the sequencing reaction. In order to undergo this reaction, the amplicons were initially purified using the Wizard PCR Preps DNA Purification System Kit (Promega) according to the manufacturer's instructions. The next step was to mix them with ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit. The purification of the amplified products was done using the BigDye XTerminator® Purification kit. The products were analysed on a ABI Prism 3130 Genetic Analyzer and the nucleotide sequences were aligned with the BioEdit program.

Results and Discussions

In this study, we have developed a fast and accurate DNA-based screening method to identify the FecX^{I} carriers. This versatile method employs PCR amplification of DNA extracted from blood samples, followed by restriction fragment length polymorphism (RFLP) analysis to generate fragment patterns that can be resolved on agarose gel electrophoresis. Initially, to discriminate between individuals we used PCR amplification. The PCR products obtained were separated on 2% agarose gel and the results showed we have obtained a fragment of 154bp that was consistent with the target one and that had good specificity.

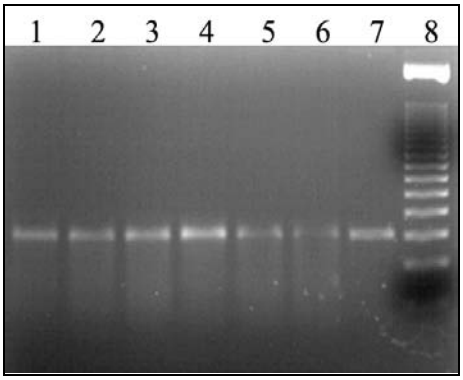

Figure 1. Results after *XbaI* digestion of a 154bp fragment of *BMP15* gene. 1-2: Karakul de Botosani; 3-4: Palas Merino; 5-6: Palas Milk Line; 7: uncut fragment; 8: 50bp Molecular Weight Marker (Promega).

In order to evaluate the presence or absence of FecX^{I} carriers, the amplified products were subjected to enzymatic digestion using the *XbaI* restriction enzyme. The potential polymorphism will modify the restriction site of this enzyme and thus we will be able to diferentiate between carriers and non-carriers. After enzymatic digestion with *XbaI* and agarose gel electrophoresis, the wild type (non-carrier) yields just one fragment of 154bp, the homozygous individuals for FecX^{I} yield two fragments of 124 and 30bp, while the heterozygotes (carriers) have all three fragments. The results after restriction on a 3% gel electrophoresis are represented in Figure 1. After the digestion with the *XbaI* restriction enzyme, we have obtained in all three sheep breeds only the wild allele which represents the non-carrier animals.

In order to confirm the results obtained using the PCR-RFLP technique we have sequenced the amplicons. After aligning the obtained sequences with the reference sequence from GenBank Database we have obtained a homology of 100% (Figure 2).

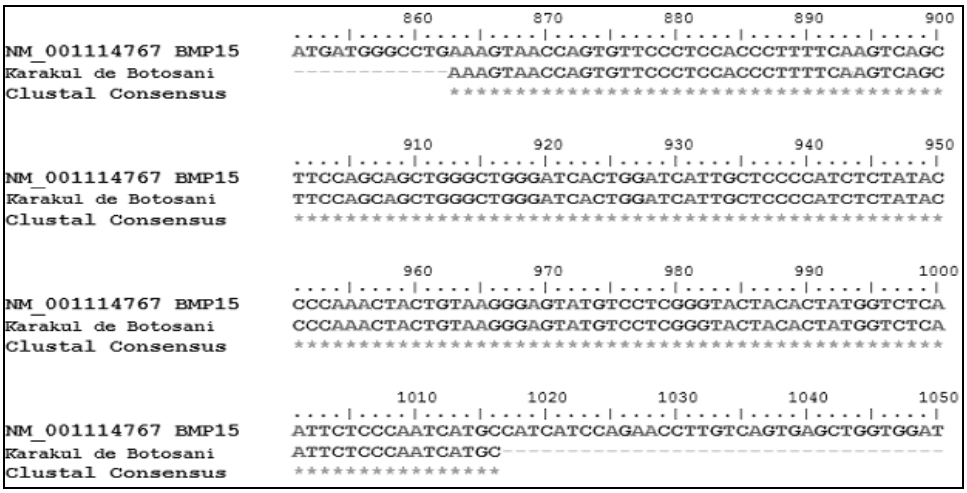

Figure 2. BioEdit alignment between a reference sequence (Genbank Database Accesion Number NM001114767) and the sequence obtained for Karakul de Botosani.

In the present study, PCR-RFLP tests were carried out only for the $FecX^I$ mutation and no carrier has been identified. However, there are three other known mutations of the sheep *BMP15* gene that produce the same phenotype of increased ovulation rate in heterozygous individuals and infertility in homozygous ewes: $FecX^B$ (Belclare), $FecX^G$ (Galway) and $FecX^H$ (Hanna) (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004).
Our results showed that, by using this technique, it is easy to identify the animals which have the favourable allele for reproduction. The method presented is reliable, fast and can be successfully applied in the wide-scale screening of different ewe populations.

Conclusions

The $FecX^I$ mutation can have a positive effect on ovine prolificacy and production. For this reason we wanted to use a modern, efficient and economic method in order to identify the animals carrying the T/A substitution in the ovine *BMP15* gene.
The results obtained highlighted the absence of $FecX^I$ carriers in the analysed Romanian sheep breeds.
This method increases the panel of molecular tools available for sheep breeders to choose the most prolific genotypes for improving the artificial selection.

Acknowledgements

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INFLUENCE OF GROWTH CONDITIONS ON B.U.T. BIG 6 TURKEY HYBRID AT SLAUGHTER

Roxana Lazăr, Alina Narcisa Postolache, Boişteanu P. C.
University of Agriculture Science and Veterinary Medicine „Ion Ionescu de la Brad"
Mihail Sadoveanu Alley no. 3, Iaşi, 700490, Romania
lazarrxn@yahoo.com

Summary
Creating of lighting programmes based on expression of physiological status it's a goal of poultry technologies through development of poultry breeding and maintenance technologies based on physiological needs.
For turkey hybrids, development of lighting programs is a scientific argument for physiological connections, represented by the hypothalamic - pituitary - epiphysis - gonad axis and the biological potential they have. As biological material we used BUT BIG 6 turkey hybrid variety, with a total number of 30816 females and 24552 males, high ground growth technology. Determinations had focused on live weight evolution, carcass weight and the weight of cutting anatomical regions (upper leg, lower leg, breast and wings). Other parameters were the quantification of water and feed consumption and the average daily gain. The females of the experimental group were slaughtered at 20 weeks old and males at 24 weeks.

Key words: *turkey meat, microclimate parameters, productive performance*

INTRODUCTION

This research is a part of a postdoctoral program, were the objective is to identify the relationships between morphology and physiological status of turkey pineal gland in relation to the degree of somatic development. The inquiries were made at S.C. Galli Gallo Codlea, who is the only firm with high capacity for growth and turkeys slaughter from Romania [4].

The conducted research supports the ability to identify technological solutions involved in maximizing the growth and development of somatic maintenance, implying the increase in meat production through experimental research in avian physiology [1, 2]. Duality of basic research with the assessment of turkey growth and maintenance management was done by establishing the technological parameters; through them was demonstrated that functional context of the body has the ability to express their genetic potential for meat production [3, 5].

MATERIAL AND METHODS

As biological material was used BUT BIG 6 turkey hybrid, with a total number of 30816 females and 24552 males.

The experimental protocol required the use of a biological material divided by gender and distributed in six blind poultry houses, each of them with an area of 2000 m^2. The technology of turkey growth implied permanent straw bedding with a thickness of 10 cm, the birds being exploited through technology of growth on land.

Determinations had focused on live weight evolution, carcass weight and the weight of cutting anatomical regions (upper leg, lower leg, breast and wings). Other parameters were the quantification of water and feed consumption and the average daily gain. Females of the group were slaughtered at 20 weeks old and males at 24 weeks.

RESULTS AND DISCUSSION

The poultry house was populating with turkey chicks of one day, after we ensured the optimal hygienic and microclimate conditions. Ventilation system operation is based on achieving a negative pressure inside the poultry house (forced evacuation and natural inlet) through the central fan with a capacity of 12000 m^3/h, 8 roof fans, 5 groups of tunnel fans and 23 inlets with valves located on the sidewalls, which adjusts its position according to outside temperature. The ventilation program according to turkey age is detailed in table 1.

Table 1

Ventilation program in growth poultry houses

VENTILATION PROGRAM							
AGE (days)	WEIGHT (g)		TEMP. (^{O} C)	HUMIDITY (%)	VENTILATION (m^3/h)		
	REF.	REAL.			Min.	Max.	Mean
HATCH	42	44	31	50-60	213	2181	1303
1	59	62	31	50-60	299	3063	1830
2	71	74	30	50-60	359	3686	2202
3	84	88	30	50-60	425	4361	2606
4	100	105	29	50-60	506	5192	3102
5	118	124	29	50-60	597	6126	3660
6	138	145	28	50-60	699	7165	4281
7	160	168	28	50-60	810	8307	4963
8	184	193	27	50-60	931	9553	5708
9	211	221	27	50-60	1068	10955	6545
10	241	252	26	50-60	1220	12512	7476
11	273	286	26	50-60	1382	14173	8469
12	308	323	25	50-60	1559	15991	9554
13	346	362	25	50-60	1751	17963	10733
14	386	404	25	50-60	1954	20040	11974
15	429	449	24	50-60	2172	22273	13308
16	476	499	24	50-60	2409	24713	14766
17	525	550	24	50-60	2658	27257	16286

18	577	604	23	50-60	2921	29956	17899
19	633	663	23	50-60	3204	32864	19636
20	691	724	23	50-60	3498	35875	21435
21	753	789	22	50-60	3812	39094	23359
22	817	856	21	50-60	4136	42417	25344
23	885	927	21	50-60	4480	45947	27453
24	956	1002	21	50-60	4839	49633	29656
25	1031	1080	21	50-60	5219	53527	31982
26	1108	1161	21	50-60	5609	57525	34371
27	1189	1246	21	50-60	6019	61730	36884
28	1273	1334	20	50-60	6444	66091	39489
29	1360	1425	20	50-60	6884	70608	42188
30	1450	1519	20	50-60	7340	75280	44980
31	1543	1616	20	50-60	7811	80109	47865
32	1640	1718	20	50-60	8302	85145	50874
33	1739	1822	20	50-60	8803	90285	53945
34	1842	1930	20	50-60	9324	95632	57140
35	1948	2041	20	50-60	9861	101135	60428
36	2057	2155	19	50-60	10412	106794	63810
37	2168	2271	19	50-60	10974	112557	67253
38	2283	2392	19	50-60	11556	118528	70820
39	2401	2515	19	50-60	12154	124654	74481
40	2522	2642	19	50-60	12766	130936	78234

For summer ventilation it is used so-called wind-chill effect, that partial use the tunnel ventilation with roof ventilation, causing an optimal ventilation of the poultry house. The ventilation system is designed to ensure draft-free ventilation above the allowed limit and the renewal of 100% of floor area with fresh air. In the growth poultry houses it is allowed the following amount of pollutants from microclimate: CO_2 max. 3000 ppm, the max. permissible value for CO is 30 ppm, max. 20 ppm of NH_3 and airflow of 1-2 m/s.

Another important factor is the ambient temperature. It must be uniform throughout the poultry house.

Table 2

Modulation scheme for temperature increase in poultry house

Specification	Temperature (ºC)
Preheating phase for 2-4 days	38 – 40
Concrete floor temperature	28
Litter temperature	34
The temperature of poultry house at:	36-37
• **0 – 7 days:**	34
• **8 – 14 days:**	28
• **15 – 21 days:**	24
• **22 – 28 days:**	22

The building cooling is secured by a system mounted on the building length, with special nozzles, the main propose being to obtain water as small particles for tunnel ventilation handling.

Table 3

Variation of poultry growth house temperature depending on age and location of radiators

AGE (days)	TEMPERATURE(ºC) (depending on the location of the radiator)			Humidity (%)
	Under radiator	Ambient space	Air temperature	
D 1 – D 3	39 - 40	30	33 – 34	50 - 60
D 4 – D 7	36 - 38	28 – 29	32 – 33	50 - 60
D 8 – D 14	32 – 34	26 – 29	30 – 31	50 -60
D 14 – D 21	30	24 – 27	28 - 29	50 - 60
D 21 – D 28	-	23 – 26	27 – 28	50 - 60
D 29 – D 35	-	22 – 25	26 – 27	60 - 65
D 36 – D 42	-	-	24 – 25	60 - 65
D 43 – D 49	-	-	23 – 24	60 - 65
D 50 – D 56	-	-	22 – 23	60 - 70
D 57 – D 63	-	-	21 – 22	60 -70
D 64 – D 70	-	-	20 – 21	60 - 70
D 70 – D 91	-	-	18 – 19	60 - 70
D 92 – D 105	-	-	18 – 19	60 - 70
> D 105	-	-	18 – 19	60 - 70

Environmental factors are closely correlated with population density. Females are kept in the growth rooms up to a maximum weight corresponding to 52 kg/m^2 and males up to 58 kg/m^2. In table 4 are given the values for the density of the birds from the shelter.

Table4

Density of turkeys

Specification	Density (turkeys/m^2)
Up to 5 weeks old (males and females)	9-10
Females up to 16 weeks	5,1
Males up to 21 weeks	2,8

The main rule in design of turkey breeding system is the easy access to feeding and watering places. Feeding and watering facilities are distributed so that birds can find them at a distance of up to 6 m respectively 4 m. The distribution of watering and feeding devices by gender and age are presented in tables 5 and 6.

Table 5

Feeder devices distribution by age and sex

Turkeys category	Feeding space /kg live weight
Youth during the growing	0,80 cm
Females for meat	0,18 cm
Males for meat	0,18 cm

Table 6

Watering devices distribution by age and sex

Turkeys category	Space for watering/kg live weight
Youth during the growing	0,40 cm
Females for meat	0,10 cm
Males for meat	0,10 cm

In the experimental growth protocol we followed the distribution of 80 - 100 turkeys/ watering device.

To assess the degree of body development was monitored daily feed consumption, determining daily body weight for 20 weeks and consumption index (CI). Parameters were followed for both, males and females. The obtained results are recorded in tables 5 and 6.

The obtained values for daily weight were always compared with reference values presented in the technological growth guide; these results are presented in table 7.

Table 7

The changes in body weight at male and female

AGE (weeks)	Reference values for females (g)	Mean values for females (g)	Reference values for males (g)	Mean values for males (g)
1	160	182	160	192
2	340	386	390	415
3	640	684	750	773
4	1050	1212	1270	1396
5	1590	1779	1950	2005
6	2230	2487	2770	2936
7	2960	3120	3730	4080
8	3760	3950	4810	5008
9	4620	4920	5980	6312
10	5510	5760	7230	7690
11	6420	6685	8540	8845
12	7320	7495	9880	10596
13	8220	8263	11240	11500
14	9090	9368	12610	12480
15	9940	10080	13960	14460
16	10740	11045	15300	15306

17	11500	11577	16610	16522
18	12220	12050	17900	17472
19	12880	12920	19160	19063
20	13490	13562	20390	20104
21	-	-	21600	21702
22	-	-	22800	22339
23	-	-	23980	23755
24	-	-	25150	24727

The quality of technology management applied of turkey chickens from the experimental protocol conditions reflect the percentage of mortality, recorded during the course of monitoring.

From track recordings, it result a mortality rate at four weeks old of 0.13% at females and 0.07% at males. At the age of 20 weeks, the mortality rate was 2.31% for females and 2.75% for males. At 24 weeks, when it started the male slaughter, the mortality rate was 3.08%.

Regarding the feeding scheme provided until the age of 5 weeks, the turkeys received a starter feed containing 26% BP. At this age category, female's body weight was with 189 g higher than provided weight from the hybrid growth guide while males have exceeded the weight with 126 g.

At the age of 10 weeks, the feed was changed with a second type of growth feed that had a concentration of 20.88% BP; this leaded to an excess weight at females with 250 g and 460 g at males compared to the growth guide of hybrid technology.

At 14 weeks was introduced a finisher feed (type I) with 17.5% BP, which was replaced at 18 weeks with a second finisher feed (type II) containing 16% BP. At 19 weeks old, when it started the females slaughter, the average weight of females was 12.92 kg; for the males, at 24 weeks, body weight reached an average of 24.73 kg.

The results obtained after slaughtering were assessed by determining carcass weight and the proportion of anatomical cutting parts from carcass. From the experimental groups, turkeys were slaughtered weekly in order to determine the changes of assessing indicators at slaughter.

The maximum value for turkey carcass weight was achieved at 20 weeks, being 15.7 kg for males and 10.3 kg for females.

Slaughter results assessment was made based on the anatomical parts of carcass cutting. The main part of turkey carcass is the chest, and the analysis of this indicator requires an assessment of skin weights, due to birds subcutaneous fat deposits and consumer preference.

At 12 weeks, the chest represented 27.8% from female carcasses compared with 25.3%, value at males of the same age. At 20 weeks the females had a maximum value of 33.7% compared to males who had a share of the chest in carcasses of 32.1%.

In order to assess the results of turkey carcasses at slaughter, measurements were made both on the breast with skin and the breast without skin. The skin of the males chest

showed a variation between 2.3% at 12 weeks and 4.9% at 20 weeks compared with females that had a variation of 3.0% at 12 weeks and 6.3% at the end of study.

At 12 weeks the upper thighs of females had recorded a maximum percent value of 14.3% and at 20 weeks the value decreased at a rate of 13.8%. At males was recorded a decrease that started from a value of 14.2% at 12 weeks and finished with the value of 13.7% the las value 20 weeks. Analyzing the proportion of lower legs, at 20 weeks this cutting parts had a percentage of 10.3% from male carcasses and 9.2% from female carcasses.

The highest value was recorded at the males of 12 weeks old and was 10.1%, the lowest weight resulting at 20 weeks at females being 7.5%.

CONCLUSIONS

The compliance of strict hygiene and microclimate parameters insure the obtaining of individuals which have a high yield at slaughter, increasing the economic efficiency.

If we insure the proper technological parameters and the correct feed recopies we are able to obtain superior results to those provided in user growth guide.

Values for actual losses are, in terms of growth management, a plurality of outputs by all livestock mortality study.

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EPIDEMIOLOGICAL INVESTIGATION REGARDING PRRSV INFECTION ON PIGLETS FROM NE MOLDAVIAN AREA

MIHAI (MILEA) LEONTINA-SMĂRĂNDIȚA[1] , Elena VELESCU [1], TOMASZ STADEJEK[2], TAHAR AIT-ALI[3]

[1]Universitatea de Ştiin țe Agricole si medicină Veterinară' Ion Ionescu de la Brad', Iasi

e-mail: marutza85@yahoo.com

Institutul Veterinar de Stat Piwet, Pulawy Polonia

[3]Institutul Roslin Edinburgh, Scoția UK.

REZUMAT

Sindromul tulburărilor respiratorii şi de reproducție suin (PRRS) este una dintre cele mai importante boli pentru industria porcină din toată lumea (Neumann şi colab. 2005; Pejsak şi colab 1997).

Lucrarea de față are la bază cercetări privind profilul serologic cu ajutorul testului ELISA în infecția cu PRRSV la purceii proveniți din NE Moldovei. Pentru probele recoltate am observat o incidență scazută a prezenței anticorpilor PRRSV în ser, având în vedere faptul că nu toți purceluşii prezentau semne clinice. Incidența scăzută se justifică prin imunizarea pasivă în urma trecerii prin boală sau printr-o infecție latentă. Totuşi în literatura de specialitate detectarea anticorpilor şi a virusului poate fi o surpiză de diagnostic deoarece prezența semnelor clinice nu este întotdeauna identificată.

Cuvinte cheie: purceluşi, PRRSV, testul ELISA.

ABSTRACT

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the most important diseases for swine industry worldwide (Neumann and colab. 2005; Pejsak and colab 1997).

This paper is based on research regarding serological profile in PRRSV infection using the ELISA test on piglets from NE Moldavian area. For the collected samples we observed a low incidence of PRRSV antibody level in serum, considering the fact that not all piglets had clinical signs. Low incidence is justified by passive immunization after going through the disease or by a latent infection.

However, in literature antibodies and virus detection may be a surprise because the clinical signs are not always present.

Key words: piglets, PRRSV, ELISA test.

Introduction

PRRSV is a Arterivirus, which has a genome consisting of a single molecule of linear single-stranded RNA. Porcine Reproductive and Respiratory Syndrome (PRRS) was for the first time recognized in 1987 in US and in Europe in 1990 and since then, the disease has spread worldwide.

The clinical manifestation may be acute or chronic, marked by two main components: the respiratory and /or reproductive. Clinical signs may be limited in a first step to

respiratory tract (influenza syndrome) or, equally may start with the reproductive component. Reproductive disorders are mainly expressed in late abortions, premature births or abnormal extension of the duration of pregnancy. Neonatal mortality is high. Sows shows agalactia, anestrus and loss of appetite.

Respiratory disorders are present in all animal categories and represented as flu syndrome (loss of appetite, moderate hyperthermia, cough and dyspnea). Respiratory disorders have a higher incidence on piglets, and usually have a multifactorial origin, association with other infectious agents by the action of the PRRS virus.

On piglets, the PRRSV infection is often subclinical. It is however indirect responsible for large economic losses in liverstock finishing, due to its important role in pigs Respiratory Disease Complex. If clinical signs appears, they are: fever, sneezing, dyspnea, cough, pneumonia, lethargy, periocular edema.

Materials and methods

Serum samples were collected from piglets belong to 4 farms and backyard of two county in NE Moldavian area aged between 1month and 6 months. In this farms there were no clinical manifestation for PRRS. Only farm 1 had one year before PRRV infection.

Farms	No pigs	Samples	Age
Farm 1	28	Serum	1-4 months
Farm 2	30	Serum	1-5 months
Farm 3	1	Serum	4.5 months
Farm 4	2	Serum	3 months
Backyard	9	Serum	3-4 months

For detection of PRRSV antibody level in serum we have used the ELISA kit home made by the OIE Laboratory in the National Veterinary Research Institute Piwet Pulawy Polonia (Fig.1). We performed ELISA test according to the manufacturer's instructions, as follows: All reagents must be allowed to reach room temperature before use.

First step is the preparation of the samples: dilute sample 1/100 in "Sample and conjugate diluent". Control sera has must be prepare (dilute) as follows: Positive control-dilution 1/200 or 1/400 and the negative control-dilution 1/100. Conjugate must be dilute RAS 1/5000 in "Sample and conjugate diluents".

Washing solution: dissolve PBS tablets in distilled water according to the instructions on the PBS tablets container. If the PBS is without Tween 20, add 0.5 ml Tween 20 per liter of PBS solution.

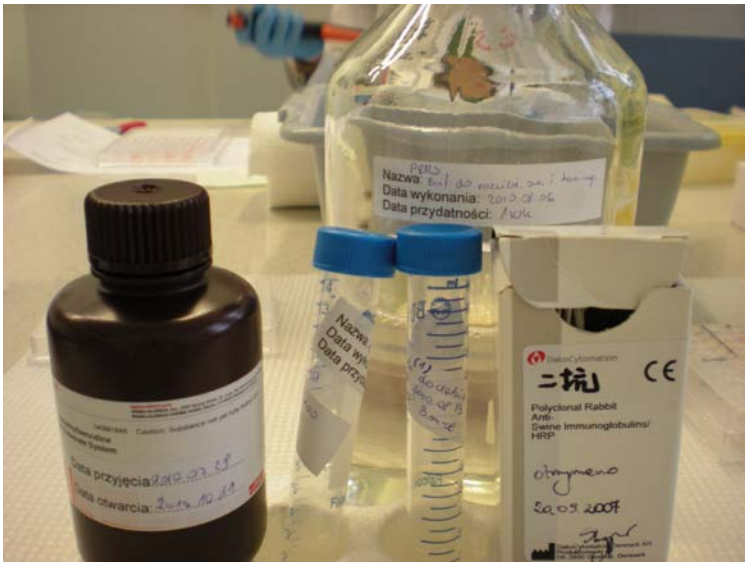

Fig.1 ELISA kit

After this start the test procedure. Add 50 µl of positive control to four wells: A1, A2, B1, B2 of the plate, 50 µl of the negative control to another four wells C1, C2, D1, D2 and 50 µl of serum samples to the remaining wells of the plates in duplicates (for example: serum no. 1 – E1, E2;, serum no. 2 – F1, F2; etc). The plates are coated in the following manner: Odd columns are coated with natural host antigen (NHC). Even colunmns are coated with PRRSV N protein (PRRS).

Plates must be incubated for 30 minutes with shaking (shaking platform) at room temperature (18-25°C), then 4 washing times .

The next step is the adding of 50 µl of diluted conjugate dilution to each well. After this, plates must be again incubate 30 minutes with shaking at room temperature (18-25°C), wash 4 times, and adding 50 µl of substrate to each well. Then plates must be incubated for about 15 minutes (in dark, for example in a drawer) at room temperature (18-25°C). Still add 50 µl of stop solution (2M H_2SO_4).

Read the OD of each well at 450 nm.Validation of the test: OD of positive control must be greater than 0,6 and . Interpretation: For each sample, calculate the S/P ratio:

$$S/P=\frac{ODsample:PRRS-ODsample:NHC}{ODpos.control:PRRS-ODpos.control:NHC}$$

Samples that have S/P lower than 0,5 are considered negative and with greater than or equal 0,5 are considered positive.

Results and discussions

From 70 serum samples that we have tested, 4 were positives. Two of them belong to backyard and other two positive samples belong to farm 1, wihch had PRRSV infection one year before. Considering that the farm 1 had one year before PRRSV infection, we was thinking that is possible to find antibody in piglets serum, which happened. In Fig.2 it can see the results from the plate.

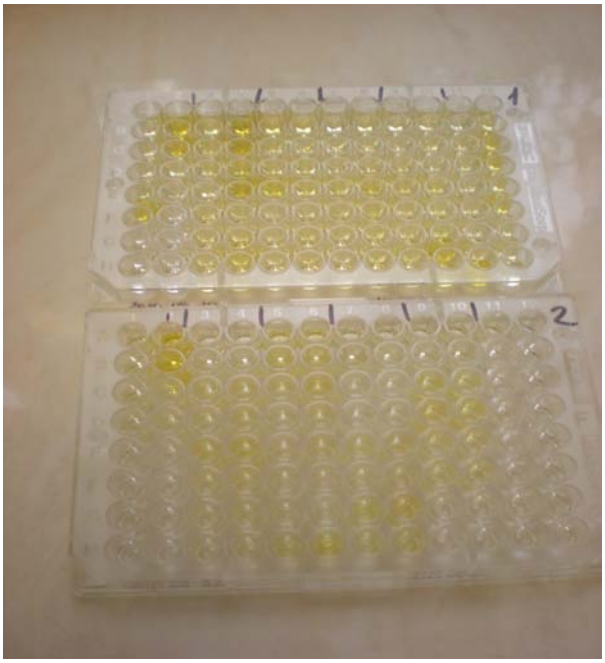

Fig.2 ELISA results

It is possible that the presence of the antibody in the first farm to be a new beginning of another outbreak. Low incidence is justified by passive immunization after going through the disease or by a latent infection.

Conclusions

The presence of the PRRSV infection in very important for all the farmers worldwide. It is good to have situation under control, considering the fact that PRRSV infection is very hard to manage.

ELISA test can detect antibody against PRRS virus and is very important to know which is the serum status . It is also important that the piglets whom we collected to be unvaccinated because this way we can have a clear idea of the level origin of the antibody in serum.

Our results shows that the PRRS virus circulates and and the presence of the antibody can be a start for a new outbreak.

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EFFECTS OF THE REMEDY BIOR ON THE HEALTH AND PRODUCTIVITY IN YOUNG RABBITS

V.MACARI, Natalia IACUB, Ana MACARI, D. MAȚENCU
Agrarian University of the Republic of Moldova

ABSTRACT

In the present paper are displayed the results of a scientific study performed in production conditions, on a rabbit farm in order to emphasize the results of the BioR products influence obtained from Spirulina plantesis on health and the young rabbits youth productivity recently weaned. The researches were realized on 4 allotments being seven young rabbits in each. To young rabbits youth from the allotments 1-3 have been administered intramuscularly the BioR remedy three times: in the weaning day, on the 14-th day and on the 26-th day after weaning the dose of 0,1-0,5 and respectively 1,0 ml/head, but to the rabbits form the allotment- 1,0 ml sol. 0,9 NaCl. The research has found that the BioR remedy obtained by modern technologies by Spirulina plantesis administered to the young rabbits from the weaning day have very good local and general tolerance contributing simultaneously to the improvement of the bioproductive indices.

Key words: BioR, Spirulina plantesis, young rabbits, bioproductive indices

INTRODUCTION

It is well-known the fact that bioactive substances are recommended for birds and animals being essential to growth, development of diverse organs and systems, also being involved in various physiological-metabolic processes, enzymatic and obviously for products of animal origin(2; 6; 8).

Both at national and international levels the researches directed towards the elaboration, study and testing of new bioactive products have intensified, one of the reasons being also the total ban of antibiotic forage in the European Union since January 1 2006 (3; 9). The study of literature in this field has enabled us to highlight the fact that out of the wide range of bioactive remedies, algae and especially the remedies of algal origin worth particular attention (1; 5; 8; 10).

The above mentioned facts and not least the physiological peculiarities of rabbits have determined us to initiate this research with the purpose to elucidate the influence of the BioR remedy on the health and productivity of these animals.

MATERIALS AND METHODS

The experiment was performed at the rabbit farm SRL ”Fieditehnologii”, v. Băcioi, mun Chişinău on recently weaned young rabbits. Scientific researches have been carried out on 4 groups of 7 rabbits each begging on the day of weaning, and the principle of organization and realization of this study is reflected in table 1.

BioR remedy proposed for testing, of algal origin, obtained form Spirulina platensis, with the use of modern technologies, is autochthonous and certified in Republic of Moldova(7). This product contains a series of bioactive substances: , amino acids, especially immunoactives, carbohydrates, oligopeptides, phycobiliproteins(C-phycocyanin), microelements. Veterinary care, weaning, maintenance, feeding of the young rabbits in all groups were identical and in accordance with the existing technology.

In order to carry out the laboratory examination blood was collected from the external auricular veins, at the beginning of the researches, before the administration of the BioR remedy from 5 rabbits and at the end of the study from 5 rabbits, from 5 animals from each group.

During the researches, the young rabbits were permanently clinically examined, periodically, at established intervals the rabbits were individually weighted, at the same time permanently keeping record of the number of rabbits, for 5 rabbits from each group the body temperature also being determined.

Table 1. Schedule of injection of BioR to young rabbits

Rabbit groups	No. of rabbits	Injection	Dose, ml	Injection schedule
Control	7	I/mus	1,0-0,9% sol. NaCl	3 times: on the day of weaning, on the 14th and on the 26th day after weaning.
E_1	7	I/mus	0,1	
E_2	7	I/mus	0,5	
E_3	7	I/mus	1,0	

OUTCOME AND DISCUSSIONS

During the researches , for a period of 70 days, it was observed that the tested bioremedy BioR, in field condition, did not cause any adverse reactions or other health and development deviations of the young rabbits. A fundamental role in the appreciation of the health of the animals and highlighting the adaptive and antistress properties is played by essential clinical parameters and especially the level of the body temperature (4, 5). The analyses of the results of the effectuated research attest maintaining of body temperature of all rabbits within normal limits. Nevertheless we mention that the BioR remedy administrated to rabbits manifested adaptive and antistress properties confirmed indirectly through the fact that body temperature of the rabbits treated with BioR in doses of 0,5 – 1ml/head, at the end of the study was lower with 0,06 – ^{o}C compared to the control group, a fact that might indicate a better metabolic and circumambient adaptation (in the warm period of the year) of the young rabbits treated with BioR 3 time consecutively.

It is worth mentioning the fact that in testing any product thought to manifest antistress and stimulative properties a fundamental role is played and by the bioproductive indices which are reflected in our study in table 2.

Analyzing the data from table 2 we can conclude that all rabbit groups that were studied have good bioproductive indices. In this context we point out that the growth of the rabbits in 73 days, in the control group was of 1,23 kg, meanwhile in the in the experimental groups it was bigger with 0,24-0,48 kg. More broadly the bioproductive indices in meat rabbits are reflected in the medium daily growth, that compared to the control group is 19,3 – 38,9% higher.

Another argument regarding the high feeding and hygiene standards is the viability of the young rabbits involved in the study, an index that is 100% in all groups included in the experiment.

The results obtained and discussed are confirmed also by the analysis of some hematological and biochemical indices in the animals involved in the research. Thereby, in the rabbits treated with BioR ,at the end of the study, before slaughtering was registered a rise of hemoglobin and erythrocytes of 2,86 – 17,9% and 4,9 – 15,2% respectively at the experimental groups 2 and 3 compared to the control group.

At the same time, in the sanguine serum of rabbits from the experimental groups before scarification, was registered an increase of the total proteins of 1,8 – 3,9%, of the urea of 3,3 – 4,5% and of the creatinine of 1,8 – 12,2% respectively, compared to the control group.

We point out the fact that BioR remedy of algal origin contributed essentially to maintaining of the young rabbits health, because it is determined also by the hematologic and biochemical indices, especially by the indices that reflect directly the protein metabolism.

Table 2.Bioproductve indices in broilers under the influence of the BioR remedy

	Units of measurement	Animal groups			
		Control	Experimental 1	Experimenalt2	Experimental 3
Number of animals	Cap	7	7	7	7
Remedy dose	ml	1,0-0,9% NaCl	0,1	0,5	1,0
Weight of a rabbit at debut (weaning)	kg	1,111±0,036	0,958±0,034	0,945±0,042	1,029±0,014
Weight of a rabbit on the 14^{th} day after weaning (74 days)	kg	1,310±0,015	1,230±0,036	1,359±0,031	1,364±0,052

Weight of a rabbit on the 26th day after weaning (86 days)	kg	1,406±0,010	1,481±0,036	1,501±0,019	1,658±0,029
Weight of a rabbit on the 60th day after weaning (120 days)	kg	2,141±0,045	2,223±0,03	2,295±0,05	2,481±0,051
Medium weight of a rabbit on the 74th day after weaning.	kg	2,340±0,097	2,424±0,054	2,509±0,068	2,736±0,072
Duration of the research	days	73	73	73	73
Weight growth of a rabbit	kg	1,229	1,466	1,564	1,707
Daily weight growth of a rabbit	g	16,83	20,08	21,42	23,38
Viability	%	100	100	100	100

CONCLUSIONS

1. Administration of the BioR remedy obtained by the means of modern technologies form Spirulina platensis, to the young rabbits, did not induce any deviations from the general state or phenomena of local. intolerance
2. It was experimentally established that in zoo-technical farm conditions the BioR remedy, administrated to young rabbits intra-muscularly 3 times consecutively since the weaning day, has benefic effects on the bioproductive indices.
3. The effectuated research confirms the necessity of undertaking more profound and detailed studies regarding the elaboration of doses and plans of administration of the BioR remedy in rabbit breeding.

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THE EVALUATION OF THE IMMUNE RESPONSE LEVEL INDUCED BY A NEW ANTI – CONTAGIOUS AGALACTIA VACCINE FORMULATION IN GOATS AND SHEEP

Laurenț OGNEAN,.[1], C. CHIURCIU [2], F. ZĂVOIU [2],
Cristina CERNEA[1], P. SEVCIUC[2], I.NICOLAE [2],
P. ŞTIUBE [2], C.TUDORAN [2], A.NICA [2], Meda MOLDOVAN[1]
[1]Universitatea de Ştiințe Agricole şi Medicină Veterinară Cluj
[2]S.C. Romvac Company S.A.
e-mail: lognean@yahoo.com

ABSTRACT

The immune response of a new vaccine formulation against contagious agalatia, *Agalaxin forte,* using aluminum hydroxide (as mineral compound) and inactivated *Staphylococcus aureus* cultures (as organic compound) has been evaluated on indigenous populations of sheep (n=129) and goats (n=25), through the use of ELISA serological tests, in order to determine the optical densities (OD) and thus the level of antibodies, respectively the ratio between the optical density of the serum sample and the positive control (S/P) in order to determine the level of effectiveness.

The analysis of immunological effectiveness was reported to the pre-vaccination individual and mean values of the optical densities, which showed a decreased frequency of positive cases, with variable antibody titers, these being statistically unimportant for the studied groups. The post-vaccination evolution of the effectiveness indicators highlighted an evident level of immunization in the *Agalaxin forte* vaccinated groups, regardless of the production series of the employed vaccine in testing. The increased level of the immune response was confirmed by the post-vaccination dynamics of the optical densities, which showed a relevant increase in the anti agalactia antibody titers starting from the 14-th day when the rappel was performed in most of the groups. More relevant is the presentation of this effectiveness analysis on the test groups and experimental variables, correlating general aspects with some particularities, mostly regarding species, area, and vaccine formulation.

In the goat groups was predominant an evolution of the OD characterized predominantly by post-vaccination oscillations, compared to those found pre-vaccination. In all goat groups, more relevant was the comparison of the evolution of post-vaccination OD means, situated between 0.11±0.165 and 0.35±0.153, and the mean values from the control group, which fluctuated between 0.08±0.037 and 0.21±0.158.

The evolution of the immunological tests in the sheep groups was characterized by important oscillations of the mean OD pre-vaccination, being situated between 0.14 ±0.135 and 0.22±0.099. Comparative to these values post-vaccination showed important increases in the titers of antibodies. Thus the increase of the OD reached a maximum value at 17 days after the rappel, situated at 0.24±0.257 in case of the 01 vaccine series and 0.47±0.132 in case of the 03 vaccine series. On the other hand in case of the vaccination with the known formulation (*Agalaxin,* series 50) the most prominent immune response (0.34±0.244 OD) was situated at the rappel. The increasing trend of the immune response was more evident in case

of the last two groups of sheep, in which immunological controls were done on an interval of 7 months post-vaccination.
The relevance of these tendencies was highlighted by the evolution of the OD values and even more so by the S/P ratio in the individuals from group 3 (Floreşti, Cluj county) and 4 (Zimbru, Călăraşi county) investigated during the 7 months of the experiment. Thus, in case of group 3 the S/P ratio registered values of 18.09%, for the series 04, 57.46% for the series 06 and 68.66% for the series 50. Likewise relevant increases were noted in the levels of antibodies in the sheep of group 4, the values being elevated even at 7 months after the rappel. Regarding this aspect an important significance was attributed also to the trend of maintaining elevated values of the OD and S/P in the individuals from this group which showed values of 81,56% - 126,11% (at 4 months post-vaccination) and 85,71% - 104,41% (at 7 months post-rappel).
Based on the obtained results we attribute a high level of effectiveness to the new vaccine formulation, *Agalaxin forte*, expressed by significant increases in post-vaccination values of OD and S/P ratio in the groups of goats (0.35 ± 0.153, respectively 50.61%) and sheep (1.19 ± 0.859, respectively 104.41%), the differences were statistically ensured ($p < 0.05$) for most vaccinated groups.

Key words: organic and mineral adjuvant, *M. agalactiae*, inactivated vaccine, optical densities.

INTRODUCTION

Contagious agalactia (CA) of sheep and goats is a pathological entity known for approximately two centuries, having as the main etiological agent *Mycoplasma agalactiae* and as clinical manifestations, mammitis, arthritis and keratoconjunctivitis (*Cottew,* 1979; *Da Massa*, 1983; *Thiaucourt* et al, 1996). Similar clinical infections can be caused, in small ruminants by other *Mycoplasma* (*M. Mycoides*, *M. capricolum M. Putrifaciens*) (*Da Massa* et al. 1992; *Kusiluka* et al., 2001; *Bergonier* et al., 1997). CA is a frequent pathological state in Mediterranean countries, North Africa and in the Middle East, areas in which has caused notable losses (*Erdag*, 1989; *Kusikula* et al., 2000). After a decade from the first isolation of *M. Agalactiae* from milk produced an inactivated vaccine which has then been used in limited areas. Gradually the strategyes for vaccination against CA was diversified, focusing on live vaccine as well as inactivated ones (*Leon Vizcaino* et al, 1995; *Tola et al.*, 1999).

The evaluation of anti agalactia vaccine effectiveness usually relies on evaluating the titers of specific antibodies, based on determining the optical density. In Europe, live vaccines against *M. agalactiae* not being accepted, attention was focused on utilizing inactivated microorganisms, formaldehyde being used as the inactivation agent and aluminum hydroxide or mineral oil as adjuvant (*Leon Vizcaino* et al. 1995, *Sarris* et al., 1989). Anti agalactia vaccines inactivated with phenols or saponins ensured a superior level of protection in experimental infections in comparison to those inactivated by formaldehyde, sodium hypochlorite or heat (*Tola* et al, 1999). In Turkey attention is focused on live, attenuated vaccines, which offer a higher level of protection in comparison to the inactivated ones but present at the same time the risk of producing transient infections with release of *Mycoplasmas* (*Erdag*, 1989).

The majority of anti agalactia vaccines posed many problems regarding effectiveness, especially in field conditions (*Bergonier* et al.,1997). It is considered that the inefficiency of some vaccines may be caused by the high level of antigenic variability of the *M. Agalactiae* strains (*De la Fe* et al., 2006) or the diversity of mycoplasma strains that produce infections in the populations of goats and sheep (OIE, 2006).

Aiding the effectiveness of specific CA immuno-prophylaxis in goats and sheep is

the focus of our study regarding the elaboration and implementation of *Agalaxin forte* vaccine, with aluminum hydroxide as a mineral adjuvant and inactivated cultures of *Staphylococcus aureus* as organic adjuvant.

MATERIALS AND METHODS

This effectiveness study focuses on the evaluation of level of the immune response induced by the *Agalaxin forte* vaccine formulation, produced by ROMVAC Company on groups of goats and sheep from different areas of the country. For this purpose we resorted to determination of optical densities respectively the concentration of antibodies.

Agalaxin forte vaccine formula has as main active compound a suspension of inactivated *M. Agalactiae* (strain S/94; Cod ATC: QI 04 AB). This strain isolated in 1994 from a sheep with mammitis was utilized from 1998 in the preparation of the commercially available *Agalaxin* vaccine (an inactivated vaccine with aluminum hydroxide as adjuvant), summing a number of 55 vaccine series with over 12.000.000 doses. The novelty element of the *Agalaxin forte* vaccine formulation is the complexity of the adjuvant, consisting of a mineral compound (aluminum hydroxide) and an organic compound (inactivated cultures of *Staphylococcus aureus*). Using the *Staphylococcus aureus* strain as adjuvant is based on its potential imunomodulatory effect and an eventual protection against *Staphylococcus aureus* induced mammitis, protection which is hard to prove because of the difficulty of demonstrating immune response to anti staphylococcus vaccines. The results of initial investigations undertaken on groups of goats and sheep experimentally vaccinated with single doses and overdoses of *Agalaxin forte*, have proven that this new immunologic product has a good level of safety and innocuity (*Ognean et al., 2010*).

The tested animals were composed of groups of milking sheep and goats (between 2 and 5 years) from different areas of Romania (Cluj, Bistriţa-Năsăud, Călăraşi). In the test were introduced exclusively clinically healthy animals selected on the bases of situation between physiological limits for the main hematological and biochemical parameters.

Vaccination and testing of the animals – the goats and sheep from the experimental groups were vaccinated subcutaneously with an initial dose of 1 ml, repeated at rappel (at 14 days in most groups). Before vaccination, at rappel and at 2 or 3 intervals after the rappel (1, 4 and 7 months after) blood samples were collected and the serum was submitted for immunological tests using POURQUIER ELISA kits as standard hyperimune sera for sheep or goats (positive controls) and negative (negative controls). The tests were the basis of determining the evolution of the OD in each group, in comparison with positive control sera for *M. agalactiae* (0.300 - 0.350). Thus we determined the values of the OD as primary parameter, with a sensibility of 99% and a specificity of 78% for *M. Agalactiae*.

Evaluation of the immune response and presentation of the results followed a protocol focused on the experimental immunization of 4 groups of animals from different areas summing 4 groups of goats and sheep. Group I summed all of the goats (Ic, IIc, IIIc, şi IVc), and groups 1-3 all of the sheep. Thus group II included 3 subgroups (Io, IIo and IIIo), group III 4 subgroups (VIo, Vo, VIo and VIIo) and group IV 4 subgroups (VIIIo, IXo, Xo and XIo). The immune response was evaluated on the basis of individual and mean values of the OD and of the S/P ratio, respectively the titers of antibodies, the obtained results being differentially analyzed in view of the experimental variable (group, species, area, and vaccine formulation-series)

Data processing included statistical calculation performed using GraphPad In Stat using ANOVA categorical analysis, completed with the calculation of the "p" probability index after the comparison of OD values. For the comparative statistical analysis of the OD values at different time intervals we resorted to the Dunnett Multiple Comparisons Test, the level of comparison being the pre-vaccination OD values (*Motulsky*, 2004).

Finally the antibody concentration was calculated through comparison with standard positive and negative samples (S/P ratio) utilizing the following formula:

S/P = [(OD serum sample – OD negative control)/(OD positive control – OD negative control)] x 100

S/P = ratio between optical density and serum samples positive control;
OD Serum sample = optical density of serum samples subjected to analysis;
OD Negative control = optical density of the standard negative control;
OD Positive control = optical density of standard positive control.

The S/P ratio was determined for each group, this parameter representing also the prediction curve respectively its equation.

RESULTS

Results of the immunological investigations have been influenced to a greater or lesser degree by the experimental variables, advocating for their individual presentation – for each group.

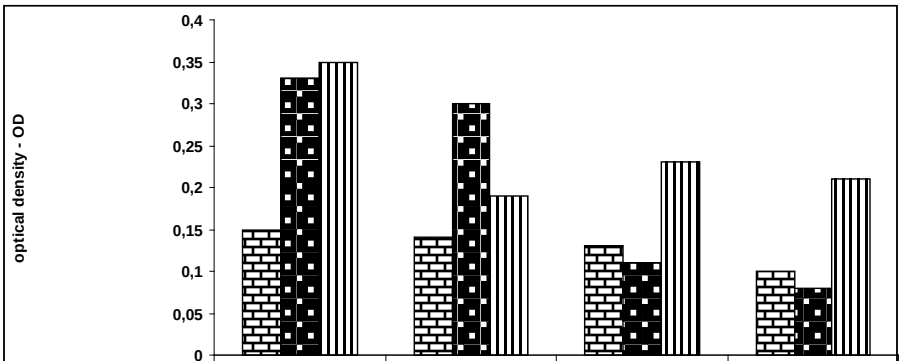
Group I summed the developments registered in goats (Bistrița-Nasaud area), which were characterized by large individual fluctuations of OD and significant increases in mean values at 14 days after the rappel in the animals from subgroup Ic (control-effectiveness *Agalaxin forte*), whose post-vaccination OD levels (0.35 ± 0.153) were significantly higher than the pre-vaccination (0.15 ± 0.149). A similar trend and dynamics was observed in the goats from of subgroup IIc (control effectiveness at expiration date *Agalaxin forte*), but with post-vaccination increases of lesser importance, the maximum OD (0.30 ± 0.208) occurring post-vaccination, before the rappel. Less evident, however, were increases in post-vaccination OD of goats from subgroup IIIc (control vaccinated with *Agalaxin*), ranging between 0.11 ± 1.165 (before the rappel) and 0.23 ± 0.24 (at 14 days after the rappel).

Relevance was ensured, however, by the increased post-vaccination OD levels (0.30 ± 0.208 to 0.35 ± 0.153) in vaccinated animals compared to the means values obtained from unvaccinated goats in the control subgroup (IVc), which fluctuated between 0, 08 ± 0.037 and 0.21 ± 0.158 (figure 1).

The mentioned evolutionary trends were highlighted also by the dynamics of the S/P ratio, which was characterized by pre-vaccination differences of lesser importance (7.09 to 15.10%). In contrast mean S/P value, post-vaccination showed particularly important increases in the *Agalaxin forte* vaccinated goats (48.01 to 50.61%) than those vaccinated with *Agalaxin* (9.61 to 26.52%) and the unvaccinated controls (1.55 to 25.41%). This data reveals high levels of immunization with *Agalaxin forte* respectively the antibody titer. High levels of this parameter (22.15 to 50.61%) at 14 days after the rappel with the new formula, indicates the persistence in time of the high level of antibodies.

Of important significance was the evolution of the immune response indicators in goats from subgroup Ic, in which, were recorded the highest mean values of OD and S/P

ratio. These indicators also showed an increasing trend, from the initial mean value of 15.1% to a significantly higher post-vaccination level of 48.01 % before the rappel and 50.61% at 14 days after the rappel. This development actually indicated the post-vaccination increase of antibody level to about 50%, compared with positive control (figure 1).



	group Ic (01)	group IIc (02)	group IIIc (50)	group IVc (negative control)
Pre-vaccination	0,15	0,14	0,13	0,1
Rappel (14th day)	0,33	0,3	0,11	0,08
Post-rappel (at 14 days)	0,35	0,19	0,23	0,21

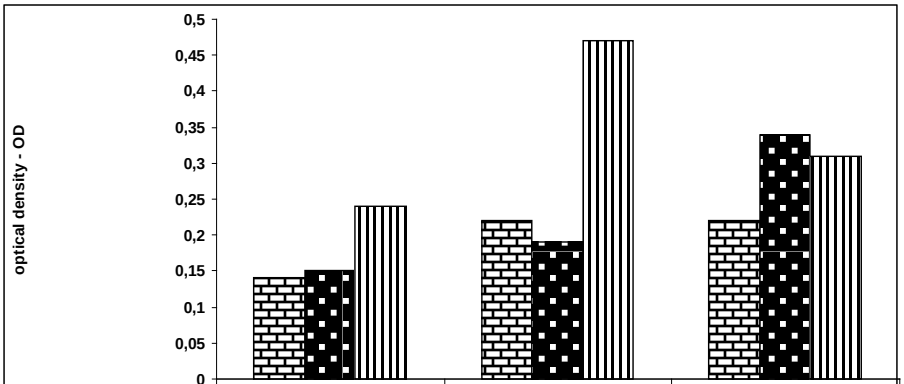
Figure 1. *The mean values of the immunological parameters for the goats in group I*

Group II included 3 subgroups of sheep (Io from Bistriţa –Năsăud area, IIo and IIIo from Călăraşi area) in which individual and mean optical densities variations were influenced to some extent by the lot, respectively by location. Thus, for sheep in subgroup Io, the mean OD, situated pre-vaccination at a value of 0.24 ± 0.257, increased progressively so that at the 17-th day after the rappel, reached a value of 0.14 ± 0.135. The same could be said for the values of S/P ratio which increased from 14.55% before the rappel to 30.91% after the rappel (figure 2).

Significant oscillations were noted in the OD dynamics of subgroup IIo, initially recording slightly negative variations; the mean pre-rappel value (0.19 ± 0.168) being lower than pre-vaccination (0.22 ± 0.099). In contrast, post-rappel values in this subgroup reported very high elevations in the mean values, which reached 0.47 ± 0.132, being statistically significant ($p < 0.05$). This level of immune response was confirmed by the positive developments of the S/P ratio, which increased from 27.27% (pre-vaccine) to 72.73% (17 days after the rappel); the levels reached post-vaccination indicated a significant increase in antibody titer for this subgroup.

The dynamics of the OD values in animals from subgroup IIIo was characterized by insignificant fluctuations, the post-vaccination increases reaching levels of 0.31 ± 0.216 at the last measurement. Likewise, for this subgroup a particular relevance given to the S/P ratio, which reached its highest level at the rappel (49.09%), followed by a slight decrease (43.64%) (figure 2). According to the presented data, for this group, pre-vaccination levels of OD in the sheep vaccinated with *Agalaxin forte* were relatively homogeneous (0.14 ± 0.135 to 0.22 ± 0.099), the most significant antibody titers were recorded at 17 days after the rappel in case of vaccination with the series 01 (0.24 ± 0.257 OD) and 03 (0.47 ± 0.132).

The situation was slightly different for the control group vaccinated (with *Agalaxin* 50 series), the most pronounced immune response was recorded at the rappel (0.34 ± 0.244).



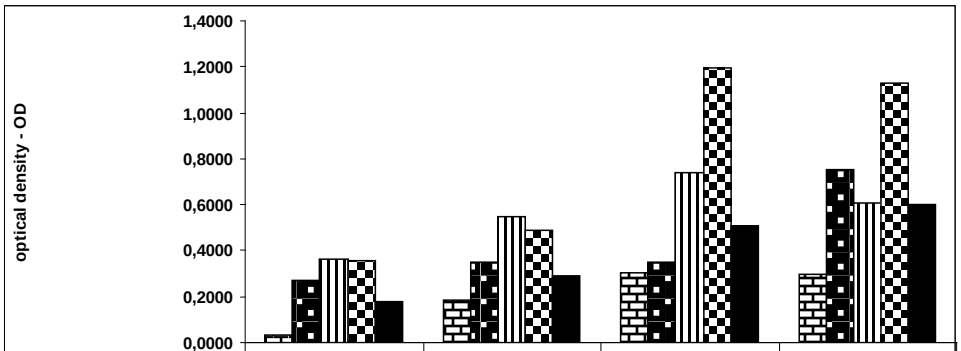
	group Io (01)	group IIo (03)	group IIIo (50)
Pre-vaccination	0,14	0,22	0,22
Rappel (14th day)	0,15	0,19	0,34
Post-rappel (at 14 days)	0,24	0,47	0,31

Figure 2. *The mean values of the immunological parameters for the sheep in group II*

Group III included results reported for the four subgroups of sheep (IVo, Vo, VIo and VIIo from Cluj area) vaccinated with *Agalaxin forte* series 04, 05 and 06, respectively *Agalaxin* series 50 (figure 3). The evolution of the immunization process in this group showed OD values ranging from 0.03 ± 0.027 (pre-vaccination) to 0.30 ± 0.43 (post-vaccination). The post-vaccination dynamics of OD showed differences referring to animals and vaccination series. Thus, in case of subgroup IVo (series 04), the post-vaccination values of antibody titers showed a moderate increase, which was ensured before the rappel ($p < 0.05$) and 30 days post-rappel respectively at 4 months ($p < 0.01$), followed by a decrease in the OD (0.18 ± 0.219 OD) at 7 months after the rappel. The same could be said about the levels of S/P ratio associated with the analysis of the regression curve which had a constant evolution between ante-and post-rappel (30 days) when it showed the highest mean values (20.83% and 28.75 %). However, less relevant were the developments observed in case of subgroup Vo (series 05), characterized by insignificant differences in post-vaccination antibody titers, the mean values being situated between 0.18 ± 0.421 (pre-vaccination) and 0.54 ± 0.548 OD (30 days post-rappel) (figure 3). The dynamics of the S/P ratio in this group was relatively constant, with the maximum positive level at 30 days post-rappel (43.63%).

The highest immunization level was recorded in the animals from subgroup VIo, in which the use of *Agalaxin forte* vaccine (series 06) caused a distinct statistically significant ($p < 0.01$) increase in antibody titer at 4 months post-rappel, the slightly increasing dynamics being maintained throughout all of the observation period. The same could be said about the regression curve for the evolution of the S/P ratio which showed an evident increasing trend, with maximum values of 59.03% and 57.46%, recorded at 30 days and 7 months post-rappel (figure 3). The immunization indicators evolved similarly in case of the subgroup VIIo, the highest antibody titers being reported at 4 months post-rappel (1.13 ± 0.909 OD), when the differences from pre-vaccination titers were statistically ensured ($p < 0.01$). The S/P ratio dynamic also showed an increasing trend, with a primary peak pre-rappel (60.07%) and a secondary one at 7 months post-rappel (68.66%).

Comparative analysis of these results shows that in case of the series 04, 05 and 06 of *Agalaxin forte* the maximum titers of antibodies were reached at 30 days and at 4 months post-rappel. In comparison for *Agalaxin* (series 50) the maximum levels were reported at the rappel and 4 months post-rappel (figure 3). Slightly increasing trends were also maintained in the S/P ratio and the regression curve for all subgroups of this group, indicating the maintenance of important values at 7 months post-rappel (figure 3).

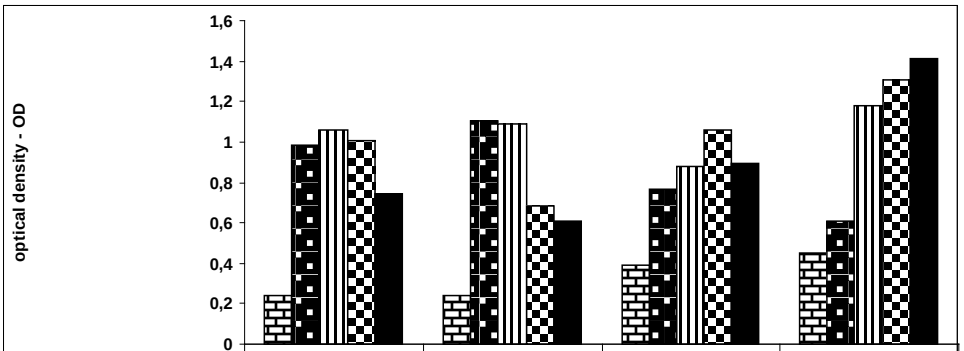


	group IVo (04)	group Vo (05)	group VIo (05)	group VIIo (50)
Pre-vaccination	0,0322	0,1847	0,3006	0,2995
Rappel(14th day)	0,2687	0,3501	0,3472	0,7513
Post-rappel (at 30 days)	0,3661	0,5491	0,7386	0,6095
Post-rappel (at 4 months)	0,3537	0,4910	1,1937	1,1325
Post-rappel (at 7 months)	0,1815	0,2924	0,5069	0,5995

Figure 3. *The mean values of the immunological parameters for the sheep in group III*

Group IV summed the results reported for the last four subgroups of sheep (VIIIo, IXo, Xo and XIo, Călăraş area), vaccinated with *Agalaxin forte* (series 04, 05 and 06) respectively with *Agalaxin* (series 50), which revealed high titers of antibodies pre-vaccination, the mean OD values being situated between 0.23 ± 0.294 (group IXo) and 0.44 ± 0.585 (group XIo) (figure 4). A special significance was attributed to the dynamics of the S/P ratio, which fluctuated between 27.99% and 53.09%, the post-vaccination recorded differences being statistically significant for enhancing the immune response in all immunized subgroups (figure 4). In case of the IXo subgroup, statistical analysis showed similar aspects to the previous subgroup, the maximum antibody titers being recorded pre-rappel (1.10 ± 0.580 OD) and 21 days post-rappel (1.09 0.543 OD). In the same context evolved also the S/P ratio, showing elevated values even 7 months after the rappel and a relatively constant trend of the regression curve. In the assessment of subgroup Xo also was emphasized increased post-vaccination levels of antibodies, with a primary peak at 4 months (1.06 ± 0.712 OD) and a secondary one at 7 months (0.89 ± 0.569) post-rappel, this increasing trend was revealed by the dynamics of the S/P ratio, which reached at the end of investigations a value of 104.41%.The dynamics of immunological parameters was characterized also, in case of the XIo subgroup, by a progressive evolution of the antibody titer, the most important levels of OD being recorded after 7 months from the rappel (1.41 ± 0.190). Statistically, all these increases in the post-rappel antibody titers were distinctly significant ($p < 0.01$). The marked increasing trend, registered in the case of antibody titers was confirmed by the dynamics of the S/P ratio correlated with the appearance of the regression curve. Overall evolution of the immune response of group IV showed the

prevalence of very high antibody titers and their clear tendency to maintain or even increase during a period of 7 months post-rappel (figure 4).



	group VIIIo (04)	group IXo (05)	group Xo (05)	group XIo (52)
Pre-vaccination	0,2383	0,2378	0,3911	0,4484
Rappel(14th day)	0,9844	1,103	0,7663	0,6082
Post-rappel (at 30 days)	1,0597	1,0917	0,8814	1,179
Post-rappel (at 4 months)	1,007	0,6873	1,0611	1,3084
Post-rappel (at 7 months)	0,74	0,6067	0,8944	1,4141

Figure 4. *The mean values of the immunological parameters for the sheep in group IV*

Global analysis of the development of the tested effectiveness indicators for the tested *Agalaxin forte* vaccine series showed strong relatively homogeneous levels of immunization, expressing obvious increase in antibody titers for groups III and IV. The relevance of immune response stimulation was more obvious in the developments of the S/P ratio, between 18.09% and 57.46% for group III, and between 81.56 and 126.11% at 4 months post-rappel, respectively 85.71 and 104.41% at 7 months post-rappel for group IV.

DISCUSSIONS

Finding anti-mycoplasma antibodies in the unvaccinated control groups of goats and sheep may be due to a possible natural oral immunization. We support this possible route of immunization by highlighting the common presence in milk of macrophages, containing mycoplasmoid formations that underwent phagocitosis. We also consider that the natural immunization is significantly influenced by mycoplasma porting. In this context, we assume that natural immunization can be achieved mainly through suckling, a process that facilitates contact of the kids and lambs with mycoplasma antigens that are eliminated mainly through milk.

The induced immune response of different types of anti-agalactia vaccines has been investigated by various researchers in the field. Thus, *Tola* et al. (1999) showed that saponin and phenol inactivated vaccines maintain high levels of antibodies, reaching a primary peak at 3 months, followed by a secondary one at 8 months after vaccination. In Turkey, *Erdag* (1989) tested an agalactia inactivated vaccine, to which he begins to detect the antibody titers (complement fixers and inhibitors of growth) at two weeks after vaccination, peaking 4 weeks later, at 10 weeks after vaccination he recorded a significant decrease in baseline titers. After *Vior* et al. (1990), inactivated vaccines provide inadequate clinical immune protection against epidemics of CA. The low level of immunization has led

some researchers (*Leon Vizcaino* et al., 1995;) to recommend agalactia immunization three times a year, although the effectiveness of vaccination is controversial in infected herds with very high infection pressure.

Regarding the influence of species on the anti-agalactia immune response, it is considered that the immune response against *M. agalactiae* is better in sheep than goats, which advocates the use of vaccine strains isolated from the species in question, namely creating their specific vaccine. All these studies outline a new strategy based on the combined production of vaccines against CA, which is more efficient because it is prepared from inactivated *Mycoplasma* strains isolated from the field and selected according to their protein and antigenic profile (*Scherm* et al., 2002).

CONCLUSIONS

1. Evaluation of immune response in the groups of goats and sheep vaccinated with the tested *Agalaxin forte* formula was based on the determination of mean OD values and the S/P ratio compared with those obtained from non-vaccinated control groups (0.10±0.037 to 0.19±1, 39 OD, 1.55% - 25.41%) and control groups vaccinated with reference *Agalaxin* formula (0.13 ± 0.143 to 0.23% ± 0.240 OD).
2. Pre-vaccination recorded antibody titers were predominantly low, except for two groups of sheep from flocks previously vaccinated anti-agalactia some years before (Călăraşi area), where OD levels were elevated (0.39 ± 0.607 to 0, 44 ± 0.585%).
3. The groups of goats immunized with *Agalaxin forte* showed significant increases in antibody titers, with fluctuating mean values of OD, reaching maximum levels at 14 days post-vaccination in sample IIc (0.30 ± 0.208) and 14 days post –rappel in case of the Ic group (0.35 ± 0.153).
4. Significant fluctuations were observed in the immunological indicators of the sheep of group II, OD values indicating large post-vaccination increases, with a peak (0,24±0,257-0,47±0,132) at 17 days after rappel for series 01 and 03 of *Agalaxin forte*, respectively at the rappel in case of *Agalaxin* series 50 (0.34 ±0.244 OD).
5. The increasing trend of the immune response was particularly evident in the last two groups of sheep in which serological tests extended to 7 months post-vaccination, showed increased levels of the S/P ratio (from 18.09 to 57.46%) for group III, respectively extremely high rates at 4 months (81.56% to 126.11%) and even at 7 months (85.71% to 104.41%) post-rappel for group IV.
6. Analysis of the influence of vaccine series on the evolution of the immune response showed the reach of maximum levels of antibodies in case the series 04, 05 and 06 of *Agalaxin forte* in the interval of 1-4 months post-rappel, the evolution being similar to the reference formulation *Agalaxin* series 50.
7. Based on the obtained results we attribute a high level of effectiveness to the new vaccine formulation, *Agalaxin forte*, expressed by significant increases in post-vaccination values of OD and S/P ratio in the groups of goats (0.35 ± 0.153, respectively 50.61%) and sheep (1.19 ± 0.859, respectively 104.41%), the differences were statistically ensured ($p < 0.05$) for most vaccinated groups.

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PRION PROTEIN DETECTION IN SMALL RUMINANTS FROM SOUTHEAST ROMANIAN FARMS

MARIA RODICA OTELEA[(1)], STELIAN BARAITAREANU[(1)], MIHAELA ZAULET[(2)], FANEL OTELEA[(4)], DOINA DANES[(1,3)]

[(1)]*University of Agronomical Science and Veterinary Medicine Bucharest, Faculty of Veterinary Medicine,*
E-mail: otelea_maria@yahoo.com,
[(2)]*University of Bucharest, Molecular Biology Center,*
[(3)]*Institute of Comparative Medicine, University of Agronomical Science and Veterinary Medicine Bucharest,*
[(4)] *Braila County Veterinary Laboratory,*

Abstract

The paper describes the PrP^{Sc} detection in four traditional Romanian sheep/goats farm, by immunoenzymatic (sandwich format) technique and histopathological examination. The studied outbreaks evolved between 2007 and 2009, in Braila County, in surroundings areas within a 30 km radius. Epidemiological study revealed that the movements of animals between farms were present, new animals were bought from EU countries, and in animals feed has not been used animal flour.

The incidence of scrapie positive animals was under 0.5% which demonstrates that the disease was diagnosed in early stages. The histopathological exams matched with the immunoenzymatic technique results and confirmed the diagnosis of scrapie. Also, all immunoenzymatic and histopathological positive results were confirmed by IHC and immunoblotting performed by National Reference Laboratory for Transmissible Spongiform Encephalopathies. The farms were officially confirmed with scrapie and the animals have been disposed by slaughter or euthanasia.

These results support the reliability of screening methods used in active surveillance of scrapie in Romania.

Keywords: TSEs, scrapie surveillance, EIA, brainstem neuronal disorders

Introduction

Scrapie is the transmissible spongiform encephalopathy (TSE) of small ruminants, including sheep (*Ovis aries*), domestic goat (*Capra aegagrus hircus*) and European mouflon (*Ovis ammon musimon*), produced by a protein infectious particles named prions [4, 6, 7, 12, 13]. The posttranslational modification of the normal cellular host-derived PrP^{C} into an abnormal protease-resistant PrP^{Sc} isoform (prion) represents the main feature of these fatal neurodegenerative diseases [5, 9, 10, 12].

TSEs refer to a group of fatal neurodegenerative disorders affecting mammals and human beings [8, 12, 14]. TSEs affecting human beings include Creutzfeldt-Jakob-disease, Gerstmann-Sträussler-Scheinker-syndrome (GSS), Kuru, fatal familial insomnia (FFI) and sporadic fatal insomnia (SFI). TSEs affecting mammals are scrapie in sheep and goats,

transmissible mink encephalopathy (TME), chronic wasting disease in elk, mule deer and white-tailed deer (CWD), bovine spongiform encephalopathy in cattle (BSE, mad cow disease), exotic ungulate encephalopathy in exotic ungulates (EUE) and feline spongiform encephalopathy in cats (FSE) [14]. The first European scrapie reports were in England (1732) and Germany (1759). Romanian official report of scrapie was in 2002 [1].

Disease often occurs in animals aged 3-4 years and rarely under 18 months age. Scrapie was the first spongiform encephalopathy whose transmissibility was proved [3].

The main purpose of the study was to present the PrP^{SC} detection by immunoenzymatic technique and histopathological examination in four traditional sheep/goats farm in southeast Romanian area and to establish what was the incidence of scrapie positive animals.

Materials and Methods

Biologic material

Brainstems samples collected from 5345 sheep and 828 goats (Table 1) coming from four surrounding farms within a 30 km radius.

Table 1. Sheep and goats breeds in studied farms

	Farm A	Farm B	Farm C	Farm D
SHEEP BREED				
Merinos	242	305	-	-
Lacaune	339	-	-	-
Meat Line of Merinos	417	-	-	-
Milk Line of Merinos	705	-	-	-
Tigae with Black Head	728	-	-	-
Suffolk	213	25	-	-
Texel	4	-	-	-
Turcana	3	-	-	-
Țigae	-	265	-	-
Half breed of Merinos	-	514	1312	273
Total	**2651**	**1109**	**1312**	**273**
GOATS BREED				
Friza	269	-	-	-
White Banat	106	-	-	-
Saanen	6	-	-	-
Alpine	4	-	-	-
Carpatine	12	-	-	-
Half breed	-	197	89	145
Total	**397**	**197**	**89**	**145**

The official diagnostic of scrapie was performed in National Reference Laboratory for Transmissible Spongiform Encephalopathies (NRL-TSE, IDAH Bucharest, Romania) by immunohistochemistry and immunoblotting, according with Regulation (EC) 999/2001 [15], and the outbreak was reported in 2007-2009 to World Animal Health Information Database (WAHID) [21].

Histological examination used only sections of medulla oblongata at the level of obex, in accordance with sampling recommendation of OIE Terrestrial Manual 2009 [19]. The rapid immunodiagnostic test used fresh medulla taken at the obex or just caudal to the obex, in accordance with the manufacturer's instructions.

In vitro purification and detection of PrP^{Sc} by immunoenzymatic (sandwich format) technique

The immunoenzymatic technique (EIA) used the TeSeE Purification and Detection Kit (Bio-Rad, Marnes-la-Coquette, France). The protocol of vitro purification and detection of PrP^{Sc} was performed according with the manufacturer's instructions.

Histopathological examination

The medulla oblongata fragments were formalin-fixed, embedded in paraffin, cut at 4µm and haematoxylin-eosin stained. The histological exam was performed in accordance with the method used at the European TSE Community Reference Laboratory, VLA Weybridge [20].

Note: NRL-TSE performs immunohistochemistry and immunoblotting in accordance with the method used at the European TSE Community Reference Laboratory, VLA Weybridge [20].

Results and Discussions

In vitro purification and detection of PrP^{Sc} by immunoenzymatic (sandwich format) technique is a rapid test used for scrapie screening and the results must be confirmed by other diagnosis methods like histopathological examination, immunohistochemistry and immunoblotting, in accordance with the method used at the European TSE Community Reference Laboratory, VLA Weybridge [20, 18].

The studied farms were in surroundings areas within a 30 km radius. The animals had 5-6 years aged and were from different breeds (table 1). In animals feed has not been used animal flour and there were movements of the animals through buying and selling between the four farms. In the farm B were brought animals from the farm A and in farm D were brought animals from the farm C. In the farm A the animals were imported from France or bought from different Romanian areas and in the farm C, the animals were from own breeding. The movements of the animals have direct correlation with the scrapie positive cases in all the four farms [19, 18].

The EIA results revealed from 3048 animals, 12 positive sheep and 1 goat in the farm A, in farm B, 1 positive sheep from 1306 animals, in farm C, 3 positive sheep from 1401 animals and in farm D, 1 positive sheep from 418 animals. In the four farms there are 18 cases of scrapie (Figure 1). The incidence of scrapie is under 0,5% which demonstrates that the disease was diagnosed in early stages. All the animals in the outbreaks have been disposed by slaughter or euthanasia. In the farm A were more positive cases in comparison with the other three: 12 cases in farm A, 1 case in farms B and D and 3 cases in farm C). That means an increased susceptibility to scrapie in farm A. Further genotyping investigations are recommended to demonstrate the scrapie susceptibility in all farms [19, 17].

Clinical picture seen in scrapie positive animals, showed the following symptoms:

- behavioral changes: aggressiveness, anxiety, retiring;
- locomotors changes: tremors, balance;

- sensitive changes: pruritus, lips reflex movements, abnormal head movements, paresis and paralysis or in some cases missing clinical signs (Figure 2).

Lack of immune response and intravitam diagnosis test, make the scrapie clinical picture an important diagnosis element but not determinant [11, 12].

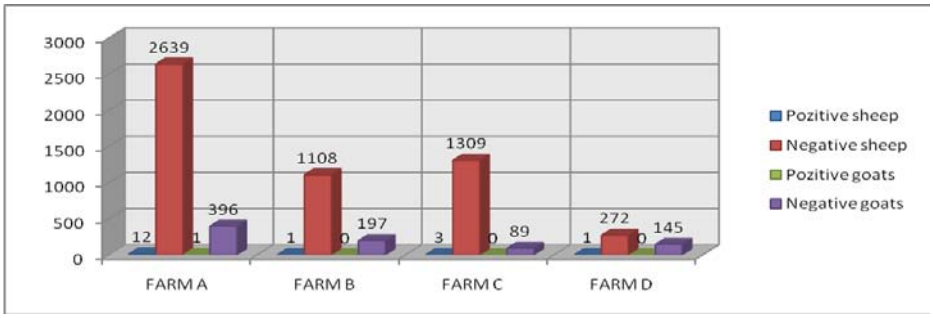

Figure 1. The incidence of scrapie in four small ruminants farms from southeast Romanian area

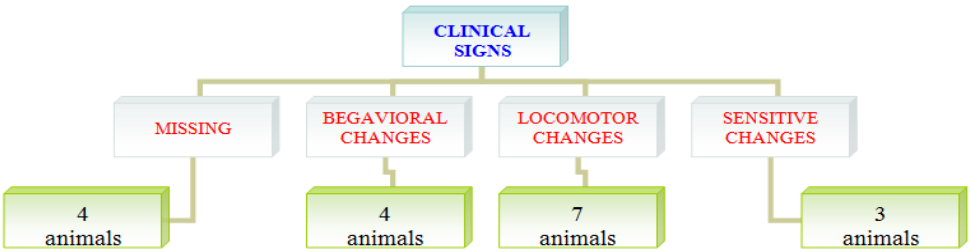

Figure 2. Clinical signs of the positive animals

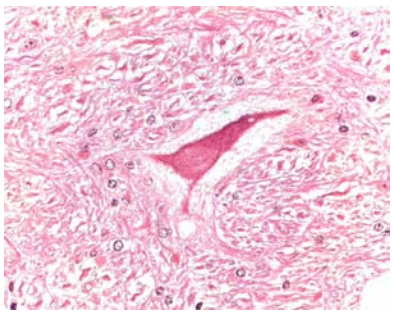

Figure 3. Medulla oblongata at the level of obex: the degenerescence of perykaria and microvacuola (x400, HE).

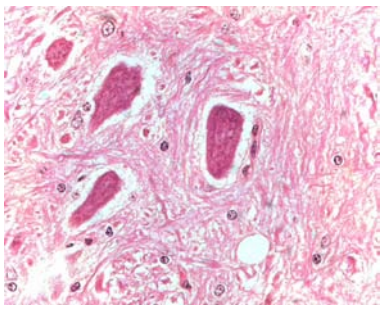

Figure 4. Medulla oblongata at the level of obex: the degenerescence and atrophy of perykaria (x630, HE).

The histopathological exams (hematoxylin and eosin stain) matched with the EIA results, and NRL-TSE confirmed scrapie diagnosis by immunohistochemistry and immunoblotting. NRL-TSE is using the same methods for scrapie diagnostic as the European TSE Community Reference Laboratory, VLA Weybridge [20]. Those results make confident the Romanian scrapie survey program.

In the medulla oblongata fragments were identified typically lesions for scrapie: neuronal vacuolation, neuronal degeneration, loss of neurons, astrocytosis and spongious aspect of the grey matter neuropil. The vacuoles have been different sizes are empty and

clearly separated. The vacuoles have been intraneural, paraneural, perineural or non-associated with the perykaria (fig. 3). However, the absence of lesions is not evidence for the absence of scrapie infection, as this can exist without either clinical signs or detectable morphological changes [5, 16]. Also, it is no direct correlation between clinical signs severity and pathological changes [2, 4].

Conclusions

The incidence of scrapie positive animals in the studied outbreaks was under 0.5% which demonstrates that the disease was diagnosed in early stages.

The movements of the animals have direct correlation with the scrapie positive cases in all studied farms.

Lack of immune response and intravitam diagnosis test, make the scrapie clinical picture an important diagnosis element but not determinant. Currently, only postmortem diagnosis by various laboratory methods can certainly establish the scrapie.

The EIA positive results it must be confirmed by others laboratory methods (immunohistochemistry and immunoblotting) by.

NRL-TSE confirmed the diagnosis of scrapie so it supports the reliability of screening methods used in active surveillance of scrapie in Romania.

The results of screening methods used in active surveillance of scrapie in Romania fit to those of the NRL-TSE, making reliable their use.

Genotyping investigations are recommended to establish the scrapie susceptibility in the farms and to create scrapie resistant population by sheep high susceptibility removal.

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THE INFLUENCE OF THE REMEDY BIOR ON THE HEALTH AND PRODUCTIVITY IN BROILER CHICKS

V. MACARI, V. PUTIN, Natalia PAVLICENCO, Gh. CAZACU
State Agrarian University of the Republic of Moldova

ABSTRACT
This work presents the results of a scientific research carried out in poultry factory conditions with the purpose of delineating the impact of BioR and improves the adaptive properties and the properties of stimulation of the bioproductive indices of young broilers.
The study involved 150 broilers divided into 5 groups with 30 broilers each. The young broilers from the experimental groups 1-4 received intramuscular BioR injections twice as follows: on the 9th day - 0,3-0,6ml/head and on the 21st day – 0,4-1,0ml/head. The broilers in the control group were injected physiological serum respectively 0,4 and 0,6 ml/head. The study showed that the BioR product obtained from Spirulina platensis through modern technologies and injected into broiler chicks starting with the 9th day of life is very well tolerated both generally and locally and improves the adaptive properties and the properties of stimulation of the bioproductive indices of young broilers.

Key words: BioR, Spirulina platensis ,young broilers, bioproductive indices

INTRODUCTION

Aviculture being a basic branch of the agro-alimentary sector in the Republic of Moldova has the task to ensure the food security of the state, which is difficult to achieve without the modernization of the poultry business, improvement of the genetic potential, providing forage base and modern technologies. It is important to note that the advanced development of this branch in particular broiler production is unthinkable without the use of growth bio-stimulators (5; 6; 7; 8).
It is already known that the usage of antibiotics for the stimulation of the productivity in birds and animals has been banned in the European Union since 2006, on this basis the research on the development, study and testing of the new pure ecologic remedies with adaptive and obviously biostimulating properties intensified (1; 2; 5; 8).
The study of literature in this field has enabled us to perform this research to determine the influence of the BioR remedy on the health and bioproductive indices in broilers.

MATERIALS AND METHODS

The study involved 5 groups of 9 days broilers each, in poultry farm conditions at Avicola Saver, Bucovat, Republic of Moldova. The principle of organization and carrying out this study with the use of the BioR remedy is shown in table 1.
The remedy submitted for testing is autochthonous and is obtained by biotechnological means from Spirulina platensis (3). This remedy contains a wide range of bioactive substances such as: carbohydrates, microelements, phycobiliproteins(C-phycocyanin), oligopeptides amino acids, especially immunoactives (4).
Veterinary care, maintenance, watering and feeding of the chicks in all groups were identical and in accordance with the existing technology. The chicks involved in the study

have been permanently examined, and were weighted once in 7 to 10 days intervals. In order to carry out the laboratory examination (hematologic, biochemical) blood was collected from 5 chicks, in the 9th day, prior to the administration of the BioR remedy. For the same purpose blood was collected prior the slaughter, from 5 chicks from each group.

Table 1.Schedule of usage of BioR remedy in broiler chicks

Group	No. of chicks	Type of injection	Schedule	Dose, ml	
				1st time	2nd time
Control	30	IM	Twice: on the 9th and on the 21st day of the life of the chicks.	0,4 ml 0,9% sol. NaCl	0,6 ml 0,9% sol. NaCl
Experimental 1	30	IM		0,3	0,4
Experimental 2	30	IM		0,4	0,6
Experimental 3	30	IM		0,5	0,8
Experimental 4	30	IM		0,6	1,0

OUTCOME AND DISCUSSIONS

The administration of the BioR remedy of algal origin to boiler chicks, two times consecutively in poultry factory conditions, for a period of 30 days did not cause any adverse reactions or other health and development deviations of the broilers.

The undertaken researches allow us to conclude that the chicks that were administrated 2 times consecutively the BioR remedy during catching, examination, administration of the tested product and weighting were more accessible, did not react so tough at the contact with the humans and did not provoke so many cutaneous injuries to those who manipulated them when carrying out these actions.

It is well known that multilateral study of remedies thought to manifest antistress and stimulative properties is successful only through the means of basic zoo technical indices: viability, growth rate reflected in body weight, weight growth and not the least – the daily addition. Table 2 contains the values of the bioproductive indices in dynamic in chicks from the experimental variants, for the entire experimental period.

From the data presented in table 2 the following aspects regarding bioproductive indices can be inferred:

- All broiler groups during the experiment have shown good bioproductive indices, due to the high housing standards ,food, hygiene, and probably due to the conduct of the research.
- For the whole period of growth of broilers (1-41 days), the experimental 1 group registered the highest body weight (2439,69 g), this being with 313,4 g more than the control group, or respectively with 14,7% than the control group.
- Throughout the whole experimental period, after the administration of the BioR remedy (9-41 day), the total growth increase at the experimental groups was of 103,9 – 310,7 g compared to the one if the control group.
- Tracking the data from table 2, it could be concluded that throughout the whole experimental period (9-41 day), in the experimental groups the medium daily growth was 63,47 g-69,93 g, this being higher than in the control group with 5,4-16,1%.

- Throughout the whole growing period the highest medium daily growth was registered in the experimental 1 group – 59,98 g, followed closely by experimental 2 group – 59,53 g, followed then by experimental 3 and experimental 4 groups with 58,54 g and respectively with 54,7 g, this being higher with 4,9 – 15,0% compared to the control group.

Table 2. Bioproductve indices in broilers under the influence of the BioR remedy

Indices		Chicks groups				
		Control	Experi-mental-1	Experi-mental-2	Experi-mental-3	Experi-mental-4
Weight in the 1 day, g		40,0	40,0	40,0	40,0	40,0
The dose of the remedy	In the 9 day	0,4 ml 0,9%NaCl	0,3	0,4	0,5	0,6
	In the 21 day	0,6 ml 0,9%NaCl	0,4	0,6	0,8	1,0
No. chicks, head debut		30	30	30	30	30
No. chicks, head at the end of the study		30	30	30	29	30
Weight on the 9th day (debut of research)		199,0± 2,99	201,66± 4,36	208,36± 3,22	211,30± 2,88	197,06± 2,53
Weight on the17th day		552,0± 2,99	584,50± 17,05	602,0± 16,03	606,0± 15,9	576,0± 19,63
Weight on the28th day		1188,13± 32,81	1347,46± 34,88	1380,63± 26,05	1348,93± 26,96	1244,76± 35,79
Weight on the35th day		1783,06± 41,06	2005,56± 40,10	1995,30± 34,48	1960,13± 31,56	1859.86± 35,80
Weight on the41 day (end of the study)		2126,16± 41,09	2439,56± 48,16	2421,50± 45,21	2381,82± 41,30	2228,16± 48,96
Period of the study, days		32	32	32	32	32
Total growth/period/ chicks, g		1927,16	2237,9	2213,14	2170,52	2031,10
Medium daily growth / period, g		60,22	69,93	69,16	67,83	63,47
Viability ,%		100,0	100,0	100,0	96,6	100,0
Period of growth, days		40	40	40	40	40

Total growth/ growth period , g		2086,16	2399,56	2381,5	2341,82	2188,16
Medium daily growth / growth period , g		52,15	59,98	59,53	58,54	54,70

For the confirmation of the results reflected in table 2, arguments regarding registered hematological and biochemical indices could be brought. At the end of the study, the level of hemoglobin and the number of erythrocytes is higher in the experimental groups with 7,4-38,8% and respectively with 56,7-65,2% compared to the control group ($p< 0,001$). Hereby, the tested remedy on broilers influences positively erythropoiesis, which itself determines basically all the physiological-metabolic processes in the organism. Therewith in the sanguine serum of the chicks treated with BioR the level of pseudocholinesterase is higher with 12,7 – 41,5% than in the control group ($p<0,01$), the level of urea in the sanguine serum exceeds 1,4 – 2,3 times the values of the control group ($p<0,001$), indices that influenced also the level of serum creatinine that exceeds 1,9 – 2,9 times this index in the reference group ($p<0,001$), parameters that influence and reflect directly the protein metabolism especially in growing animal.

CONCLUSIONS

1. The BioR product obtained from Spirulina platensis through modern technologies, injected to broiler chicks on the 9th day of life is very well tolerated both generally and locally.
2. It was experimentally established that in poultry farm conditions that BioR remedy, administrated intra-muscularly 2 times consecutively, has benefic effects on productive indices in broiler chicks.
3. The productive indices observed in broiler chicks under treated with BioR remedy can be confirmed and by the means of hematologic and biochemical indices in these birds.

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DETECTION OF CIPROFLOXACIN-RESISTANT *E.COLI* ISOLATES FROM POULTRY FAECES USING THE EUCAST AGAR DILUTION METHOD

Ramona MORARU[1], Anne-Marie Pourcher[2], P. Dabert[2], V. Nastasa[1], Mariana Grecu [1], M. Mares[1]

[1]University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad", Iasi, Laboratory of Antimicrobial Chemotherapy

[2]CEMAGREF, Research Unit "Environmental management and biological treatment of waste" (GERE)

rmoraru82@gmail.com

Abstract

The livestock production becomes more and more important worldwide and together with this phenomenon, it increases as well the consumption of antimicrobial agents that are used to prevent diseases and to improve animal production (Yang et al, 2004). The antibiotics are used in veterinary medicine field as growth promoters, for prophylactic and therapeutic purposes. The use of antimicrobials seems to be the key factor that determines the appearance of resistance, moreover, it seems that the selection pressure of resistance take place mainly in calf, swine and poultry farming (Teuber, 2001). The minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (EUCAST Def. 3.1, 2000). There was carried out an experimental study on two groups of Ross chickens (control and treated group), samples were collected from chicken fresh faeces and E. coli strains were isolated after identification. The changes that took place in susceptibility values towards ciprofloxacin were assessed in a two weeks period of time, consecutively to the therapy with enrofloxacin. It may be noticed that the MICs (MIC_{50} and MIC_{90}) of ciprofloxacin in the case of treated group, is placed in resistance area, having a value of 128 µg/mL, fact that shows a rapid induction of resistance to ciprofloxacin after its administration in water/feed. The obtained results when there were tested the susceptibility of E. coli strains isolated from the treated group, showed that only 27.77% from strains were susceptible to ciprofloxacin, the rest being resistant even to high concentrations of 128 µg/mL. Consequently, this may be correlated with a high level of clinical resistance and therapy failure together with the presence of resistant bacteria that may contaminate the meat at slaughter, reaching in human consumption.

The obtained results indicate the fact that antimicrobial resistance represents an emergence phenomenon related to the clinical (or other nature) use of the antimicrobial agents, towards which resistance is against

Keyword*: E. coli, resistance, ciprofloxacin, poultry,*

1. Introduction

Intensive farming systems determined the need of antimicrobial use in order to prevent and treat diseases (Yang et al, 2004). Attention was draw lately to the antimicrobial resistance phenomenon that is the result of antibiotic use and that seem to lead to medical hazards such as untreatable bacterial disease and foods contaminated with such resistant bacteria. In poultry farms, Fluoroquinolones are used for both prophylactic and therapeutic purpose and it seems that the selection pressure of resistance takes place mainly in poultry, together with calf and swine (Teuber, 2001). Since their approval for use in animals, Fluoroquinolones determined resistance to almost all bacteria they were used for, especially in *E. coli* (Yue at al., 2008). To determine the minimum inhibitory concentration (MICs) of antimicrobial agents are used dilution methods and reference methods for antimicrobial susceptibility testing. MIC methods are mainly used to

establish the susceptibility of organisms that give equivocal results in disk diffusion tests, where disk tests may be unreliable and the results are important to be accurate for clinical management. The method tests the ability of microorganisms to produce visible growth on a series of agar plates (agar dilution) containing dilutions of the antimicrobial agent. Thus, the MIC represents the lowest concentration of an antimicrobial agent that will inhibit the growth of a microorganism (EUCAST Def. 3.1, 2000; Andrews, 2001).

The agar dilution method described further is the same method described in the report of an international collaborative study of antimicrobial susceptibility testing and is very similar to those described in countries such as France, Germany, Norway, Sweden, the UK and the USA (EUCAST Def. 3.1, 2000).

2. Material and Method

The susceptibility to ciprofloxacin was assessed by standard method for MIC determinations on *E. coli* strains. There was an experimental study carried out on two groups of Ross chickens, samples were collected from chicken faeces and *E. coli* strains were isolated. There were assessed the changes in susceptibility values towards ciprofloxacin in a two weeks period of time, consecutively to the therapy with enrofloxacin (ciprofloxacin is its active metabolite). The chickens were separated in two groups, one treated group (n = 20) and a control non-treated group (n = 20). The chickens used in the experiment are from the same hatchery and they were housed in similar microclimate conditions (temperature, relative humidity, light/ darkness), water and feed. The enrofloxacin (Baytril 10% solution) was administrated in drinking water for 5 days time period. The samples were collected at different times for the better understanding of the susceptibility dynamics towards ciprofloxacin. The first faeces sample was harvested at 4 days before beginning of the treatment (T_0) from both groups, separately. There was a collection of faeces samples at second day of treatment (T_1) and then in the last day of treatment, the 5th day (T_2) from both groups. There were also faeces samples harvested at two days (T_3) and at one week after treatment (T_4). The bacterial strains were cultivated on TSYE agar (Oxoid, LTD, Hampshire, England), were isolated, purified and then identified by using TBX (Oxoid, LTD, Hampshire, England) selective agar and for confirmation Api 20 E kit (bioMerieux, Marcy l'Etoile, France).

After the identification of the bacterial strains, there were selected only the *E. coli* strains (n = 45), 27 strains from the control group (60%) and 18 strains from the treated group (40%). There were prepared fresh cultures for the ciprofloxacin MIC determinations. The results of this method present reliable data and helps in establishing a good clinical management.

2.1. Medium

The used medium for susceptibility testing is Mueller-Hinton (MH, Fluka, Sigma Aldrich, India) agar, which shows no performance advantages in comparison to other media but is probably the most widely used medium internationally and the MH agar meets the requirements of the CLSI standard being considered the reference medium (EUCAST Def. 3.1, 2000; Andrews, 2001).

2.2. Antimicrobial agents

The antimicrobial powder, Ciprofloxacin, was obtained directly from the manufacturer (Fluka, Sigma Aldrich, China). The antimicrobial powder has specified a stated potency (mg or International Units per g powder, or as percentage potency), Ciprofloxacin 98.0% (HPLC), the expiry date and the details of recommended storage conditions. The Ciprofloxacin powder was stored in a sealed container at 4°C as recommended by the manufacturer. The agent was dispensed into aliquots used on each test occasion. Before using an aliquot, the containers were allowed to warm to room temperature before opening them in order to avoid condensation of water on the powder (EUCAST Def. 3.1, 2000; Andrews, 2001).

2.3. Preparation of stock solutions

The Ciprofloxacin was weighted using an analytical balance (Kern, Germany). Allowance for the potency of the Ciprofloxacin powder was made by use of the following formula:

Weight of powder (mg) =

$$\frac{\text{Volume of solvent (mL) x Concentration (mg/L)}}{\text{potency of powder (mg/g)}}$$

this meaning (for an **initial antimicrobial concentration of stock solution of 2560 mg/L ciprofloxacin with purity ≥ 98.0%):**

$$\frac{\text{1000 mL x 2560 mg/mL}}{\text{980 mg/g}} = \text{2612 mg (weight of ciprofloxacin powder)}$$

The volume of diluents needed may be calculated with a given weight amount of antimicrobial powder from the formula:

Weight of solvent (mL) =

$$\frac{\text{Weight of ciprofloxacin powder (mg) x Potency of powder (mg/g)}}{\text{Concentration (mg/L)}}$$

This meaning (for **a 2612 mg weight ciprofloxacin powder of 98.0% purity):**

$$\frac{\text{2612 mg x 980 mg/g}}{\text{2560 mg/L}} = \text{999.9 mL (approximately 1 L)}$$

There were followed the recommendations of the manufacturers for solvents and diluents and the antimicrobial agent, ciprofloxacin in this case, was dissolved and diluted in half volume sterile distilled water and as alternative solvent a minimum volume of 0.1 M KOH to dissolve, then make up to total volume with water. The stock solution was stored frozen in aliquots at - 60° C for at least 6 months. Stock solutions were frozen as soon as possible after preparation, used immediately after preparation and not refrozen (EUCAST Def. 3.1, 2000; Andrews, 2001).

2.4. Preparation of working solutions

A volume of twenty-milliliter agar was used in 9 cm Petri dishes for agar. The EUCAST document schemes involve adding 19 mL volumes of molten agar to 1 mL volumes of ciprofloxacin solution. The conventional method described in the document is based on diluting the stock solution (in this case 2560 mg/L), always measuring 1 mL volumes of antimicrobial solution as follows:

Table 1. Preparation of dilutions for the agents to be used in agar dilution susceptibility tests

Antimicrobial concentration (mg/L) in stock solution	Volume stock solution (mL)	Volume distilled water (mL)	Antimicrobial concentration obtained (mg/L)	Final concentration in medium after addition of 19 mL of agar (mg/L)
2560	1	0	2560	**128**
2560	1	1	1280	**64**
2560	1	3	640	**32**
2560	1	7	320	**16**
320	1	1	160	**8**
320	1	3	80	**4**
320	1	7	40	**2**
40	1	1	20	**1**
40	1	3	10	**0.5**
40	1	7	5	**0.25**

5	1	1	2.5	**0.125**
5	1	3	1.25	**0.06**
5	1	7	0.625	**0.03**
0.625	1	1	0.3125	**0.015**
0.625	1	3	0.1562	**0.008**
0.625	1	7	0.0781	**0.004**

2.5. Preparation of plates

The Muller Hinton (MH) agar was prepared as recommended by the manufacturer. The sterilized agar was cooled at 50° C in water-bath (Memmert, Germany). In this period of time was prepared a dilution series of antimicrobial agent, as described in table 1, in 25-30 mL containers. It was included a drug free control as positive sample. 19 mL of molten agar was poured into prelabeled sterile Petri dishes inside the sterile niche and mixed thoroughly (ABS Class II Cabinet BioQUELL, UK). The plates were allowed to set at room temperature and dried so that no drops of moisture remained on the surface of agar, but not over dried. The plates were used immediately after the proper dry of the agar and not stored as we do not know if the agents are stable on storage (EUCAST Def. 3.1, 2000; Andrews, 2001).

2.6. Preparation of inoculum

The standard document requests to standardize the density of the inoculum to give 10^4 colony-forming units (CFU) per spot on the agar. The inoculum was prepared by suspending four or five overnight colonies of a pure culture from Plate Count (Oxoid, Hampshire LTD, England) agar medium. A 0.5 McFarland (BioMerieux, France) standard was used for visual comparison to adjust the suspension to a density equivalent to approximately 10^8 CFU/mL. This bacterial suspension was serially diluted in 0.85% saline solution (0.85% NaCl) to give 10^4 CFU per spot. Plates were inoculated within 30 minutes since the suspension of organisms was prepared in order to avoid changes in inoculum density (EUCAST Def. 3.1, 2000; Andrews, 2001).

2.7. Inoculation of plates

All Petri dishes were marked both with the concentration of ciprofloxacin and with a sign so that the orientation was obvious. Under each Petri dish was placed a paper with numbers that was identifying with precision each tested microorganism. Using the micropipette was transferred 5µl inoculum prepared for each bacterial strain of 10^4 CFU/spot to the series of agar plates on the surface of MH agar. The method allowed testing 31 bacterial strains plus a control organism (*Escherichia coli* ATCC 25922) at once on each ciprofloxacin concentration. There was also included a control plate without antimicrobial agent in order to prove the viability of the inoculum (fig.1). The inoculum spots were allowed to dry inside the sterile niche at room temperature before incubating the plates. The plates were incubated at 35-37° C in thermostat for 18 h. There were made duplicate tests and even triplicates where was necessary for each microorganism.

3. Results and discussions

The EUCAST document define the MIC as being the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot (fig.2) (EUCAST Def. 3.1, 2000; Andrews, 2001). A small number of colonies growing on concentrations several dilutions above that which inhibits most organisms were investigated by retesting. Such results may indicate contamination, resistant variants or if incubation was prolonged regrowth of susceptible organisms following deterioration of the antibacterial agent.

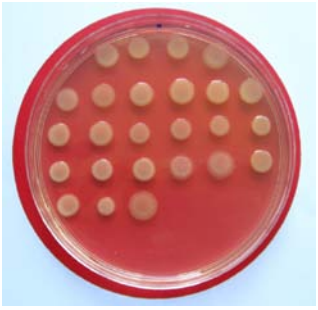


Fig.1. Positive control (*E. coli* strains were spoted on Mueller Hinton agar containing no antibiotic).

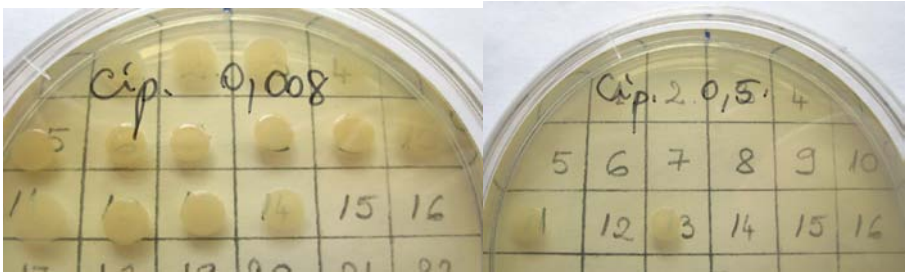


Fig.2. Interpretation of the MICs by reading the overnight growth (18 h) of *E. coli* strains, on different concentrations of ciprofloxacin.

Table 2. Ciprofloxacin breakpoints values in conformity to different standards (EUCAST, 2010; CA-SFM, 2010; CLSI, 2008 ;)

References standard	MIC breakpoints values (µg/mL)	
	Sensible	resistant
EUCAST	**≤ 0,5**	**> 1**
CA-SFM	**≤ 0,5**	**> 1**
CLSI	**≤ 1**	**> 2**

CLSI – Clinical Laboratory Standard Institute, USA
EUCAST – European Committee on Antimicrobian Susceptibility Testing
CA-SFM – Comité de l'Antibiogramme de la Société Française de Microbiologie

After the ciprofloxacin MIC breakpoints were obtained, the following statistical parameters were calculates (Mareș et al, 2007): MIC_{50}, MIC_{90}, and GM (geometric mean) (table 3). The interpretation of the results was performed in conformity with the EUCAST Standard (table 2).

Tabel.3. *In vitro* susceptibility of *E. coli* strains to ciprofloxacin

The tested microorganism *E. coli*	The ciprofloxacin concentration ranges	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	GM (geometric mean)
T^a strains (n = 18)	**0,25 – 128**	**128**	**128**	**19,253**
NT^b strains (n = 27)	**0,03 – 16**	**0,06**	**1**	**0,1839**

T^a – *E. coli* strains isolated from the treated group of chicken with ciprofloxacin for 5 days
NT^b – *E. coli* strains isolated from the control group, untreated chickens
MIC_{50} – the minimal concentration that inhibits 50% from *E. coli* strains
MIC_{90} –– the minimal concentration that inhibits 90% from *E. coli* strains

From the presented data, we may conclude that MIC_{90} of ciprofloxacin, determined for the control group, untreated chickens (NT), with a value of 1µg/mL, underlines the fact that a minimal concentration which inhibits 90% of the *E. coli* strains is placed into the susceptibility area.

On the other hand, it should be noticed that both MIC_{50} and MIC_{90} of ciprofloxacin determined for the treated group of chickens (T), have a value of 128 µg/mL, this being the highest tested concentration.

The distribution and the cumulative frequency of MIC of ciprofloxacin in untreated group of chickens (NT) are presented in table 4.

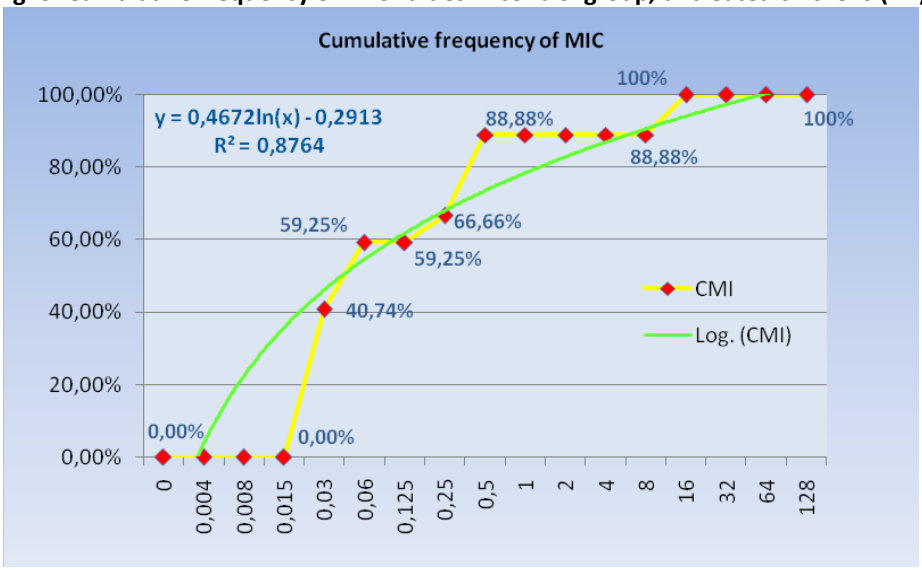
Tabel.4. The cumulative frequency of MIC values for ciprofloxacin, in untreated group of chickens (NT)

CIP mg/l	0,004	0,008	0,015	0,03	0,06	0,125	0,25	0,5	1	2	4	8	16	32	64	128
AF (*n*)	0	0	0	11	16	16	18	24	24	24	24	24	27	27	27	27
RF (%)	0	0	0	40,74	59,25	59,25	66,66	88,88	88,88	88,88	88,88	88,88	100	100	100	100

AF – absolute frequency (the number of strains)

RF – the relative frequency (percentage of the total number)

Fig. 3. Cumulative frequency of MIC values in control group, untreated chickens (NT)



The frequency of the inactivated strains depends in percentage of 87.64% by the ciprofloxacin concentrations, the multiple correlation coefficient having a value of R^2 = 0.8764 (fig.3) for the control group, untreated chickens. Although out of the total number of strains, 88.88% are susceptible to ciprofloxacin, this fact does not recommend the use of ciprofloxacin for

prophylaxis reasons, as obtained data, after analyzing the samples from treated group of chickens, indicate rapid acquisition of resistance.

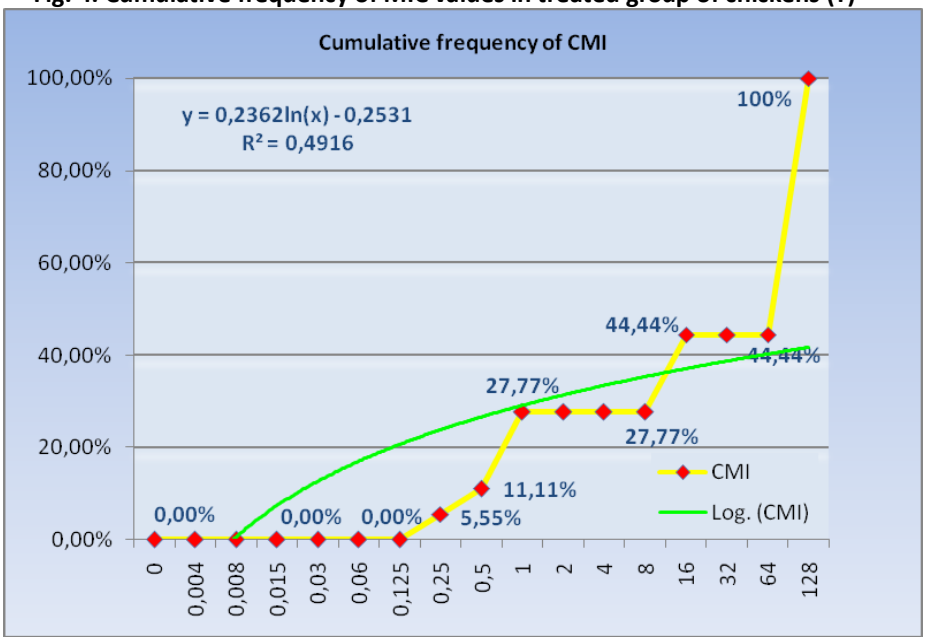
The distribution and the cumulative frequency of MIC of ciprofloxacin in treated group of chickens (T) are presented in table 5.

Tabel.5. The cumulative frequency of MIC values for ciprofloxacin, in treated group of chickens (T)

CIP mg/L	0,004	0,008	0,015	0,03	0,06	0,125	0,25	0,5	1	2	4	8	16	32	64	128
AF (*n*)	0	0	0	0	0	0	1	2	5	5	5	5	8	8	8	18
RF (%)	0	0	0	0	0	0	5,55	11,11	27,77	27,77	27,77	27,77	44,44	44,44	44,44	100

RF – the relative frequency (percentage of the total number)

Fig. 4. Cumulative frequency of MIC values in treated group of chickens (T)



The frequency of the inactivated strains depends in percentage of 49.16% by the ciprofloxacin concentrations, the multiple correlation coefficient having a value of R^2 = 0.4916 (fig 4) for treated group of chickens. It is important to be noticed that only 27.77% from the *E. coli* isolated strains are susceptible to ciprofloxacin, the rest being resistant even to high concentration such as 128 µg/mL. This fact may correlate with a high clinical resistance rate and therapeutically

failure together also with the presence of resistant bacteria that may contaminate the meat at slaughter.

Within a similar study that took place in China, there were harvested faeces samples from poultry from different regions. The obtained results highlighted that antibiotic resistance (88.1% for nalidixic acid and for fluoroquinolones with a range of 57.1% to 66.7%) was reaching to highest values within the chicken farms were antibiotics were used repeatedly and without limits. In farms with intensive animal growth, the antibiotics will be administered to the whole flock and not to the individuals, thus it will lead to a high selective pressure for the antimicrobial resistant bacteria in chickens (Song Li et al., 2008).

The antimicrobial drugs represent important weapons in the fight against diseases, to keep the healthy status of the animals and productivity (Yang et al., 2004). Hence, their misuse was blamed to be the cause for the appearance of resistance, selection and dissemination of the antimicrobial resistant microorganisms, both in human and veterinary medicine (Song Li et al., 2008).

4. Conclusions

1. From the data presented at MIC determinations for ciprofloxacin on *E. coli* strains, it may be said that MIC_{90} in the case of control group, is situated within the susceptibility area, having a value of 1µg/mL.
2. It may be noticed that the MICs (MIC_{50} and MIC_{90}) of ciprofloxacin in the case of treated group, is placed in resistance area, having a value of 128 µg/mL, fact that shows a rapid induction of resistance to ciprofloxacin after its administration in water/feed.
3. Out from the total number of *E. coli* strains isolated from the treated group, only 27.77% are susceptible to ciprofloxacin, the rest being resistant even to high concentrations of 128 µg/mL. This fact may be correlated with a high level of clinical resistance and therapy failure, consequently, together with the presence of resistant bacteria that may contaminate the meat at slaughter, reaching in human consumption.
4. The obtained results indicate the fact that antimicrobial resistance represents an emergence related to the clinical (or other nature) use of the antimicrobial agents, towards which resistance is against.

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STUDY ON SOME CHARACTERISTICS OF KOSTA MALE GOAT

Jan Alex SIWI, Enang Wasja ADIKARTA, Primiani EDIANINGSIH
Faculty of Animal Husbandry, Padjadjaran University, Bandung, INDONESIA
siwijanalex@gmail.com

Study on some characteristics of Kosta male goat, was conducted in Bandung, Indonesia. This research is using 30 post weaning 12 week male kids and six of 2-3 year Kosta bucks, to investigate the characteristic aspects of Kosta male goat. This research is a descriptive approach with an experimental method, and the data were analyzed by using the regression and correlation analysis with a repeated measurement of the subject. Results indicated that Kosta kids reach the sexual maturity at 19.47 ± 1.30 weeks with 7.56 ± 0 .71 kg body weight.

Key words : Kosta male goat, characteristic aspects, sexual maturity and body maturity

INTRODUCTION

Kosta goat is a cross bred between Kacang goat dan Kashmir goat. The colour of Kosta goat are between dark brown to black; and mostly 61% are black, 20% dark brown, light brown 10.2%; red brown 5.8% and 3.4% grey hair. Usually, the colour consists of two colours and the spotted colour usually is white. Kosta goat, usually are kept in Jakarta area and Banten province. Kosta goat has medium body size, flat nose, even sometimes has concave nose, short horn and short hair (Setiadi, et al, 1997 and Pamungkas et al, 2009). Goat breeding plays an important role in the agricultural production of developing countries, where 95% of the world's goats are kept. As goats are especially associated with the poorer part of the population, development efforts to better lives of the poor have to consider goat keeping as an important part of food production for both home consumption and sale (Stemmer, et al, 1998). In Table 1, the production and reproduction abilities of Kacang goat and Kosta goat.

Tabel 1. Production and reproduction abilities of Kacang Goat and Kosta Goat

Criteria	Kacang goat	Kosta goat
Litter size (heads)	1.57 (1.29 – 1.76)	1.76 (1.69 – 1.81)
Birth weight (kg)	1.90 (1.45 – 2.40)	1.95 (1.60 – 2.40)
Birth interval (months)	7.40 (7.00-7.70)	8.00 (7.70-9.00)
Body weight (kg)	21.69 – 28.45	26.20 – 32.24

Source : Setiadi et.al (1995)

In Table 1, showed that the production and reproduction of Kosta goat are better than Kacang goat, in traditional management in rural area.

MATERIALS AND METHODS

Materials :

This research using 30 post weaning 12 week male kids and six of 2-3 years Kosta bucks.

Methods :

This study used a descriptive approach with an experimental method, and the data were analyzed by using the regression and correlation analysis with a repeated measurement of the subject.

RESULTS AND DISCUSSION

1. Body Characteristics as Sex Maturity

Body condition is meant, the body weight and condition of the testes when the Kosta male goat reached sexual maturity. In Table 2, included the condition of adult male sex of Kosta Goat.

Table 2. Kosta Adult Male Goat Sex Condition

Parameters	Average	Standard Deviation
Age (weeks)	19.47	1.30
Body weight (kg)	7.56	0.71
Testis size (cm)	11.13	1.10
Testis length (cm)	4.95	1.05

From Table 2, it appears that adult male sex maturity of Kosta Goat begins at age 19.47 $\pm$ 1.30 weeks with a range of ages between 18-20 weeks. At sexual maturity condition, the Kosta male goat, earlier than Kacang goat males; is 20.23 $\pm$ 0.83 weeks (Soeparna, 1984).

In addition, adult male sex of Kosta Goat reached at body weight of 7.56 $\pm$ 0.71 kg. The body weight of male Kosta goat reached when the growth was slowly , according to the opinion of Sutherland (1971) which states that tropical goats grow slowly, to reach 15 kg body weight, and it takes one year.

For more details, about the average weaning weight, body weight and age sex maturity of adult male goats Kosta, can be seen in Table 3.

Table 3. Weaning weight, Body Weight and Age in Sex Maturity Male Kosta Goat.

Weaning Weight (kg)	Sex Maturity		
	Body Weight (kg)	Age (week)	Sample (heads)
5.70	6.95	22	3
6.00	7.60	20	25
6.54	8.30	18	2

From Table 3, it appears that Costa male goat weaning weight ranged from 5.70 to 6.54 kg with an average age of sexual maturity reached at 18-22 weeks, and the body weight ranged between 6.95 - 8.30 kg. The high weaning weight at sexual maturity has a higher body weight. This is in accordance to the opinion of Abdul Gani (1981), that the goat with high weaning weight will grow faster than lighter weaning weight goat.

2. Characteristics of Male Kosta Goat Body Weight

The body weight gain of adult male Kosta goat, occurred at the time when it show no significant growth. In Table 4, included Adult Body Characteristics, Body Weight and Age of sexual maturity.

Table 4. Body Weight and Age of Adult Male Kosta Goat.

Parameter	Average	Standard Deviation
Age (weeks)	52	1.17
Body weight (kg)	18.63	1.51

Increased body weight Kosta male goats will rise since the age of 12 weeks to 52 weeks of age. After 52 weeks of age, the rate of weight gain, has no significant increase. This is caused by the Kosta male goat have reached the body maturity with average body weight 18.63 ± 0.76 kg.

CONCLUSIONS

Results indicated that Costa male goat as a local goat, reach the sexual maturity at 19.47 ± 1.30 weeks with 7.56 ± 0.71 kg of body weight. Sex maturity of adult male Costa goat, reached at the age of 52.00 ± 1.17 weeks, with body weight 18.63 ± 1.51 kg.

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THE MONITORING OF WATER TEMPERATURE, PH, AND DISSOLVED OXYGEN VARIATION, IN IZVORU-MUNTELUI BICAZ MAN-MADE LAKE, BETWEEN 2009-2010.

Ramona SORIC, Liviu MIRON, Dumitru ACATRINEI

Department of Clinics, Faculty of Veterinary Medicine, "Ion Ionescu de la Brad" Univesity of Agricultural Sciences and Veterinary Medicine.
ramonas@uaiasi.ro

Izvoru-Muntelui man-made lake, have like all mountain lakes a great fish farming potential. Measuring water chemistry and its effect on fish is essential for fish breeding, because it helps to prevent parasitic, bacterial and viral diseases. Our goal was to register monthly variation of water pH, temperature, and dissolved oxygen, in order to identify the period of the year with the most pathological effects on fish. We used two work-station on the lake for monthly data samples, between oct.2009-sept.2010, from the surface to the bottom of the lake. To monitor this parameters we used an Environmental monitoring system 6600 V2, Multiparameter Water Quality Sonde YSI 2. We found that the most critical period of the year is in summer, when the highest water temperature touched 24°C, and pH average value was 8,50. The homothermy period was established for the dimictic reservoir analysed, in January and April.

Keywords: water quality, diseases, homothermy

Izvoru - Muntelui Bicaz lake is a mountain reservoir with bioproductive potential in aquaculture. Situated at 350 m altitude, the lake has a volume of water of 1,3 billion cubic meters, with a stretch of 32 Km in length and a water surface of 3000 ha. These characteristics allow the development of over 30 salmonid farms with a production capacity of approximately 50 tonnes of rainbow trout a year [4]. For lakes deeper than 1.5 m it can be observed thermal stratification of the water column, with the existence of 3 layers: one at the surface called epilimnion; one at the bottom called hypolimnion; and one, called metalimnion, that is in the middle and and with a temperature that decreases with more than 1 °C per meter [6].

The quality of the water in which fish are contained is very important in their livelihood. Adverse environmental parameters can have direct or indirect effect on fish like reducing resistance to parasitic, bacterial and fungal diseases and reduced tolerance to other stress factors [1, 5, 8].

MATERIAL AND METHOD

Between oct.2009-sept.2010 it was monitored water temperature, pH, and dissolved oxygen variation, from the surface to the bottom of the lake with an Environmental monitoring system 6600 V2, multiparameter Water Quality Sonde YSI 2.

We established two work – station on the lake, one situated next to the dam (Bicaz-dam station) and one situated between two trout's farms in Potoci bay (Potoci – Farm station) for monthly data samples evaluations.

RESULTS AND DISCUSSIONS

Water temperature variation was analyzed monthly and the most important alteration was observed in the epilimnion in the period of the spring to summer.

After completing the evaluation of the water characteristics, it has been confirmed that the lake is a dimictic reservoir with two homothermic periods. The winter homothermy it was established in January and that is due to the size and form of the lake[4]. The spring homotermy was registered in April. Between January and April was register an inverse thermal stratification of the water column, when the temperature from the hipolimnion layer (~ 3,5 °C)was greater than the water from the epilimnion (0 °C). This period is very important in fish husbandry because, under this conditions, the feeding activities and the metabolic rate are very low [2, 7]. Daily average of weight stoped in the same period and also the trout growth.

In May the temperature registered at the surface of the lake was of 11,2°C and is decreasing from the surface to the bottom of the lake. In this month the metalimnion layer, was registered from the depth of 14 m. to 22 m (fig.1). In the summer the water temperature in the epilimion reaches 24 ° and the metalimnion layer was registered from the depth of 34m to 36 m, much deeper but thinner than the one registered in May. In the period of spring to summer, water temperature increases from 10°C to 23,8°C (fig.1) in two months, and this has a major impact to the pathogens life cycle.

From September to December the water temperature, from the epilimnion layer, decreases proximally 4°C/ month and it can be observed that, in September and November, the metalimion layer, is found at the same height. In November and December height of the metalinion layer, greatly decreases.

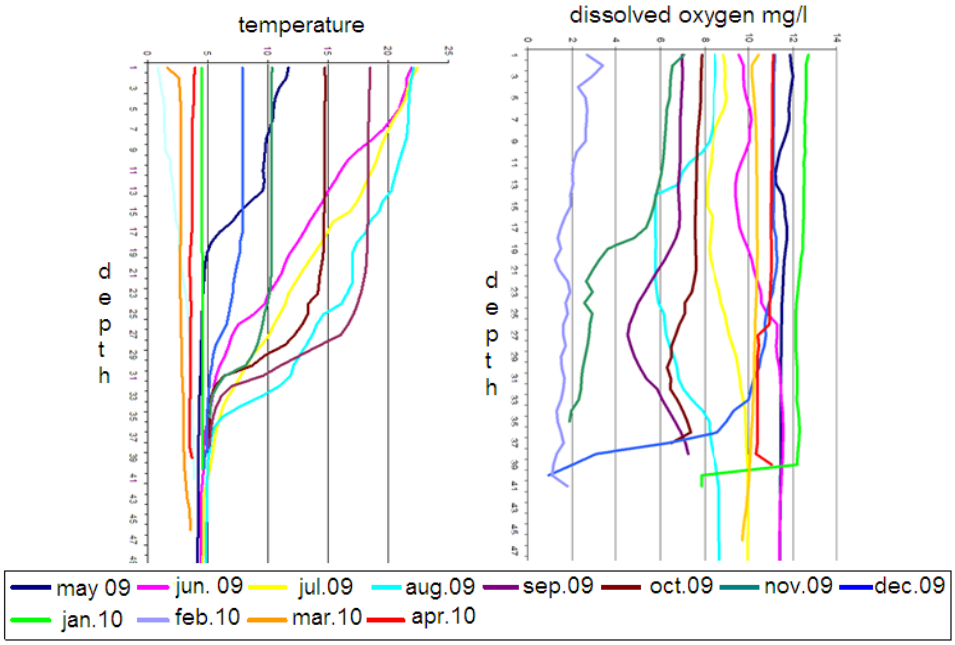

Fig. 1. Water temperature, dissolved oxygen variation from the surface to the bottom of the lake.

In autumn dissolved oxygen quantity presents a slight decrease, because of the absence of plant photosynthesis and, small oxygen exchange rate with the atmosphere.

The maximum quantity of dissolved oxygen vas observed in January (winter homotermy), 12 mg/ l (fig.1) in the entire water column. This is due to the wind that leads to the increase diffusion of oxygen from atmosphere, and also because of disappearance of some oxygen consumers like plants. Also in the period of spring homotermy, it was registered, a high value of dissolved oxygen ~ 11 mg/l (fig.2). The most important alteration was in winter when the surface is frozen (23 cm thickness), dissolved oxygen quantity is 3 mg/l under the trout necessary (~6/8 mg/l) but because of this, parasite diseases are extremely rare [3].

The importance of the homotermy period is also, observed, by analyzing water pH that reach a minimum value, of 7,4 (fig.2) in Ianuary due to the vertical water currents that mixes the entire water volume. In the study period it was not observed an important variation of water pH, that might influence fish activities.

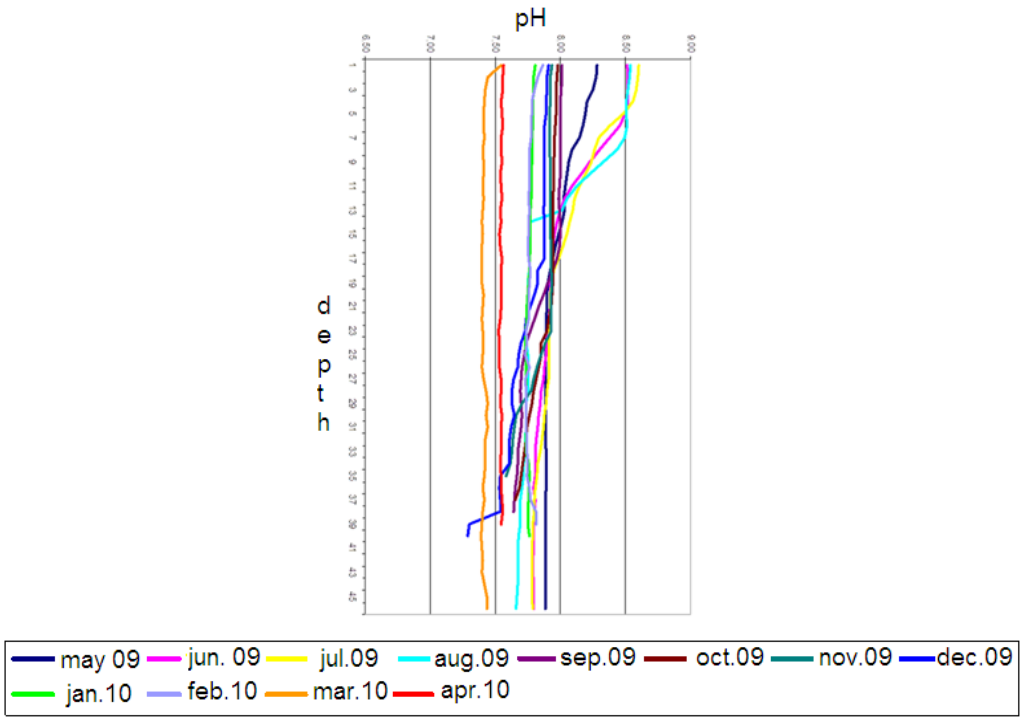

Fig. 2 Water pH variations

Bicaz lake is a dimictic reservoir and the homothermy period was established in January and in April when the vertical currents mixes the entire water volume, from the bottom to the surface and leads to the uniformity of the water characteristics.

Critical period in trout growth is in August when the high water temperature represents a stress for the trout and in January – March when the dissolved oxygen quantity is much under trout necessary [3] .

Regarding this fact, in august 2011 it was registered an algal bloom with colonies of *Aphanothece* spp. When the densities increased from 73 to 5529 times, and the dominant diatom

remains *Cyclotella distinguenda* var. *unipunctata*, reaching the highest peack comparative with anterior years of analysis int the same period, according Aoncioaie & col, 2011.

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IMMUNOMODULATING PROPERTIES OF *CALENDULA OFFICINALIS* AND *ECHINACEA ANGUSTIFOLIA* EXTRACTS IN ANTIGEN PRIMED HENS

Marina SPINU, Carmen SANDRU, Mihaela NICULAE, D. CADAR, Timea KISS, Armela BORDEANU, Florina-Alexandra KRUPACI, Silvana POPESCU, R. ŞTEFAN

University of Agricultural Sciences and Veterinary Medicine, Str. Manastur No.3-5, Cluj-Napoca, marina.spinu@gmail.com

Abstract. *Vegetal extracts from various sources are more and more often used, showing favorable influence in diminishing the negative impact of numerous agents or in increasing the non-specific or specific resistance of the body to infections. The aim of the study was that of establishing the role played by Calendula officinalis and Echinacea angustifolia extracts, containing mainly carotenoids, in the augmentation of the immune response to Newcastle disease virus in primed hens.*

The experiment was carried out on 28 Leghorn hens, with an average weight of 1.5 kg. The birds were divided into four equal groups (n=7), subjected each, for seven days, to a differentiated treatment, starting on day 0 and using the same pattern (0.5 ml/bird/day), as follows: group I was injected with an alcoholic Calendula officinalis extract*; group II – with an alcoholic Echinacea angustifolia extract; group III, a control for the extracts – with 70º alcohol and group IV, control for environmental and injection stresses – with saline. On days 0 and 7 the birds were subcutaneously primed with Newcastle disease vaccine. Clinical examination of the birds, to monitor the tolerance to the injected vegetal extracts, was carried out.*

The C. officinalis extract induced a gradually increasing specific humoral activity, persistent after the stimulation, during both primary and booster vaccination while the Echinacea extract induces an increase during the first phase, but the stimulating effect was not persistent. The active principles in the Calendula extract positively influenced the anti-Newcastle antibody titers, without attaining the values in the control groups. Injecting the birds with the Echinaceea extract seems to negatively influence the antibody synthesis, especially during the primary response.

Key words: chickens, age, vegetal extracts, phagocytosis, *in vivo* treatment

A number of immunomodulatory effects have been attributed to the medicinal plants *Calendula officinalis* and *Echinacea angustifolia*; however, little is known about whether treatment with these plants can enhance antigen-specific immunity. Investigations conducted on rats over a period of 6 weeks showed that the Echinacea treatment significantly augmented of the primary and secondary IgG response to the antigen. These results suggested that medicinal plants like *Echinacea* may enhance immune function by increasing antigen-specific immunoglobulin production (El-Gengaihi et al., 1994; Kaufman et al., 1999). In this context the stimulation of the oxidative burst as well as the modulation on monokine secretion were reviewed (Attele et al., 1999). Meanwhile, from the water or alcaline-water extracts of *Calendula officinalis L.,* polysaccharide fractions with molecular weights in the range of 25000 to 500000 and higher have been isolated, which, according to the granulocytes- and carbon clearance tests, showed significant immune stimulating activities. The isolated compounds belong to the group of water soluble, acidic branched-chain heteroglycans (Mashaly et al., 2000).

Still, the results concerning biological activities of various plant extracts could be contradictory. Their influences on the non-specific cellular immunity of the mouse after intra-

peritoneal, intravenous or per oral application were investigated. Under various conditions no effects on the immune system could be found using the carbon clearance test (Larsson et al., 1993).

Resistance to diseases is a multigenic trait governed mainly by the immune system and its interactions with many physiologic and environmental factors. In the adaptive immunity, T cell and B cell responses, the specific recognition of antigens and interactions between antigen presenting cells, T cells and B cells are essential. It occurs through a network of mediator proteins such as the molecules of the major histocompatibility complex (MHC), T cell receptors, immunoglobulins and secreted proteins such as the cytokines and antibodies. The interaction of disease resistance with production traits and the environment is crucial therefore thorough studies aim to find ways to clarify its mechanisms and means of control (Masteller and Thompson, 1994). Vegetal extracts could be an easy-to-obtain, biologically available help in this direction.

The aim of the study was that of establishing the role played by *Calendula officinalis* and *Echinacea angustifolia* extracts, containing mainly carotenoids, in the augmentation of the immune response to Newcastle disease virus in primed hens.

MATERIALS AND METHODS

The experiment was carried out on 28 Leghorn hens, with an average weight of 1.5 kg. The birds were divided into four equal groups (n=7), subjected each, for seven days, to a differentiated treatment, starting on day 0 and using the same pattern (0.5 ml/bird/day), as follows: group I was injected with an alcoholic *Calendula officinalis* extract; group II – with an alcoholic *Echinacea angustifolia* extract; group III, a control for the extracts – with 70º alcohol and group IV, control for environmental and injection stresses – with saline. On days 0 and 7 the birds were subcutaneously primed with Newcastle disease vaccine. Clinical examination of the birds, to monitor the tolerance to the injected vegetal extracts, were carried out.

Blood samples were taken from the wing vein, on days 0, 7 and 14. After clotting, sera were separated by centrifugation of the samples at 3000 rpm for 10 min. All the sera were kept at -20ºC, till the tests were performed.

Hemagglutination inhibition test was used to quantify the antibody titers against Newcastle disease vaccine, in the injected hens, by use of a in a U-bottom 96 well plate, by the two fold (1/2, 1/4, 1/8...1/2048) dilution technique of the whole sera (25 microliters) in phosphate (pH=7.6) buffer (25 microliters), adding 50 microliters of antigen and the same ammount of a 0.15% SRBC suspension. After one hour of incubation at room temperature, the last dilution entirely inhibiting the agglutination of the erythrocytes was red for each sample. Natural logarithms (ln) of the antibody titers were calculated and statistically interpreted.

Mean values, standard deviations and the statistical significance of the differences between the vegetal extract treatments and against controls were calculated.

RESULTS AND DISCUSSION

The immune system of birds offers an attractive model for studying adjuvant activities of conventional or modern compound (Davison, 2003). The involvement of ontogenesis in eliciting an immune response is recognized in birds. Based on experimental data, a relationship that exists between the age of the chick, the functional activity of the heterophil, and the susceptibility to organ invasion by *Salmonella* was proved (Wells et al., 1998).but there is no exact data on the age differences on the simultaneous administration of a thymus dependent antigen and potentially immune stimulating/ modulating (adjuvant) vegetal extracts in these species. Various ways of antigen presentation to the cells of the immune system, depending on the administration route were already mentioned.

A less investigated field of the vegetal extracts' use, but one with exquisite practical perspectives, is that of identification of novel adjuvants for vaccines, a stage considered to be essential in the development of modern vaccines (Vogel, 2000; Kaufman et al., 1999). Total vegetal extracts or extractive components from various plants could show such qualities.

The *Calendula officinalis* and *Echinacea angustifolia* extracts, accompanied by thymus dependent antigens, sheep red blood cells (SRBC) or Newcastle disease vaccine, to elicit adaptive immune responses, were monitored for their adjuvant capabilities, on the injection route, with the recording of the innate cell mediated activity expressed by phagocytosis.

The ln of the hemagglutination inhibition antibodies are presented in table 1. The lowest mean was recorded for the birds injected simultaneously with vaccine and the Calendula extract (6,124±0,78). The differences calculated for the previous group before the first and the last samplings are statistically significant ($p<0.05$). The maximal value (8,172±0,30) characterized the antibody synthesis in group III, after the booster vaccination. For this group the differences are statistically significant between the first and the intermediate samplings, and the difference becomes statistically very significant between the first and the last samplings ($p<0,001$).

Table 1

Anti-Newcastle antibodies in the experimental groups

	Group I			Group II			Group III			Group IV		
	I samp.	II samp.	III samp	I samp.	II samp.	III samp	I samp.	II samp.	III samp	I samp.	II samp.	III samp
Mean	5,12	6,51	7,06	7,06	6,93	7,48	6,37	7,45	8,17	6,93	7,27	7,76
Stdev	0,79	1,05	0,76	1,14	0,98	0,90	0,59	0,35	0,31	1,27	1,20	0,76

A central role within the immune system of mammals is being represented by a large repertoire of antibodies capable to ensure anti aggression potential. In chickens, the diversity of this repertoire is being created by the intra cromosomial genetic conversion of singular genetic fragments of heavy and light chains of the Ig. The diversification needs the bursa of Fabricius (Masteller and Thompson, 1994). Numerous compounds intervene the antibody synthesis dynamics, influencing one or more of the sequences of the process. Thus, dimetil dioctadecil ammonium bromide (DDA) amplifies the cell-mediated response, increasing simultaneously the antibody titers against Newcastle disease. The titers induced were slightly lower than those compared by injecting simultaneously the vaccine and Freund complete adjuvant (Katz et al., 1993).

The vegetal extracts whose adjuvant value was studied in the present experiment were alcoholic extracts with un-typed chemical composition, but with immune stimulating potential, according to the literature. Haemagglutination inhibition revealed antibody titers with a minimal value at the first sampling in the birds that were injected with *C. officinalis* extract, this fact being probably correlated with a lower reactivity in that group, compared to the others. The antibody titers calculated for the final sampling, indicated a statistically significant difference between the two main samplings (II and III).

The obtained data indicated a slight adjuvant effect exerted by the vegetal extract in hens multiply primed with the antigen, considering the previous Newcastle disease vaccinations included in the technology. The *E. purpureea* extract apparently exerted a suppressing effect in the first phase, since the intermediate sampling revealed lower titers compared to the previous one. Although the birds were stimulated multiple times with the antigen during the exploitation technology, before the experiment, there was no positive influence correlated with that of the extracts, in both the treatments the titers being under the values of the control and alcohol treated groups. In the experimental protocol we used, the cell-to-cell cooperation in antibody

synthesis was negatively influenced by both extracts, the effect exerted by *Calendula* being more pronounced, but statistically not significantly different from that exerted by *Echinacea.*

CONCLUSIONS

1. The *C. officinalis* extract induced a gradually increasing specific humoral activity, persistent after the stimulation, during both primary and booster vaccination while the *Echinacea* extract induces an increase during the first phase, but the stimulating effect was not persistent.

2. The active principles in the *Calendula* extract positively influenced the anti-Newcastle antibody titers, without attaining the values in the control groups. Injecting the birds with the *Echinaceea* extract seems to negatively influence the antibody synthesis, especially during the primary response.

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THE ACTION OF STREPTOMYCETES BIOMASSES AT IMMUNOLOGICAL EFFICACY AND SOME BIOCHEMICAL BLOOD INDEXES OF VACCINATED CHICKENS

Nicolae STARCIUC, OLGA POSTOLACHI, Ion SCUTARU,
Tudor SPATARU, NATALIA OSADCI, Ruslan
ANTOCI
State Agrarian University of Moldova

Abstract: *Today in many countries is the large development and fruit production of biologically active substances, growth stimulators, premixes, preparations of microorganisms that are used to increase the percentage of viability in animals and birds, body mass, immune status and organism physiological resistance. As biologically active status in many groups of actinomycetes are streptomycetele.*

Use of actinomycetes as a source of biologically active substances is one of the main prposes of studing. So was studded the biological blood properties and immune post vaccinated status of chickens which was feeding with supplement of biomass of streptomycetes.

Data are presented confirming the low post-vaccination immune efficiency in chickens vaccinated at one day old and immune stimulating effect in case the vaccine was administered in combination with biomass streptomycetes by recommended schedule.

Key words: *streptomycetes, antibody titre, immunostimulating, body weight, albumins, globulins, total proteins, biomass.*

MATERIAL AND METHODS

Investigations on obtaining and examining biological and biochemical properties of streptomycetes and biomass streptomycetes composition was performed CNMN (National Collection of Nonpathogenic Microbiology) Institute of Microbiology and Biotechnology, Academy of Sciences of Moldova.

Basely strains studied material was obtained Dulone mineral medium for 3 days at 27° C temperature with agitation. Subsequently (biomass) of streptomycetes BM-11 was separated by centrifugation of liquid culture. Quantity BM-11 was determined by weighing method.

The strain of Streptomyces canosus CNMN-Ac-03 was used after γ-irradiation where and kept in laboratory conditions for more than 5 years and after numerous passages. Absolute value of protein mass was 38.5 to 40.0% , the composition of the essential amino acids was 52.0 to 53.0% and 50.0 to 52.0% immunoactive amino acids. Total lipid ratio was at 14.0 to 14.8%. The phospholipids composition was 21.5 to 22.0% and14.0 to 14.3% starriness.

This study aimed at assessing the immunological efficiency vaccine strains of "PA" and "Winterfield 2512" which was administrated at the one day age in combination with biomass ratio and streptomycetes and its action on some blood biochemical indices.

Investigations were conducted on six groups of one day chickens, cross "Hi Land", separated 20 chickens in each group who were kept in analog conditions as follows:

First group – was a control group, the second group - the chickens who have received only biomass ratio streptomycetes, the III-rd group - chickens vaccinated with vaccine strain "PA" with the addition of biomass of streptomycetes, the IV-th group – the chickens vaccinated with strain "Winterfield 2512" with the addition of biomass of streptomycetes, the V-th group - only vaccinated chickens with vaccine strain "PA", and the VI-th group - chickens vaccinated with vaccine strain "Winterfield 2512.

Vaccination of chickens was carried out at the age of one day, the vaccine was administered with drinking water. Streptomycetes biomass was given in a report 1g/1kg combined feed up to 21 days age and 2 g / 1 kg combined feed up to 21-45 days age.

After 15, 30 and 45 days post vaccination in each group were sacrificed five chickens and examined the stock weight, was collected blood samples for biochemical examination and serum samples to determine post vaccination antibody titers.

REZULTS AND DISCUSSIONS

The results of serological investigations are presented in table 1, where we see that the chickens from groups post vaccine antibody control group were not detected in anyone of the examinations group, because chickens have been obtained from vaccinated hen's eggs.

At 15-th day after vaccination antibody levels were established in the vaccinated chickens groups with strain "PA" and "Winterfield 2512"in combination with biomass of streptomycetes in serum dilution 1:50 that formed two symbols "+" of the four possible.

Antibody levels equivalent to one symbol "+"were established in the group's vaccinated chickens only with vaccine strains "PA" and "Winterfield 2512" without the addition of streptomycetes biomass.

At the 30-th day after the vaccine, antibody levels that constituted one symbol "+" have been detected in chickens vaccinated with strains "PA" and "Winterfield 2512" in combination with biomass streptomycetes. In anther groups, post vaccine antibody levels were not detected.

Also at the 45-th day after vaccination in now one group of vaccinated chickens was detected post vaccine antibody.

Table 1. The level of antibody titers in chickens vaccinated against infectious bursal disease with substitution in combined food biomass of streptomycetes

Nr. gr.	Nr. of chickens	Age vac-cinated chickens (days)	Vaccine straun / biomass streptomy-cetes	Time management streptomycetes biomass (days)	Serum dilution 50/100	Examination period (days)		
						15	30	45
I	20	1	C	-	50 / 100	- / -	- / -	- / -
II	20	1	C/ BM	45	50 / 100	- / -	- / -	- / -
III	20	1	PA / BM	45	50 / 100	+ + / -	+ / -	- / -
IV	20	1	Winterfield / BM 2512	45	50 / 100	+ + / -	+ / -	- / -
V	20	1	PA / -	45	50 / 100	+ / -	- / -	- / -
VI	20	1	Winterfield / -2512	45	50 / 100	+ / -	- / -	- / -

In the table 2 are presented some of the blood biochemical indices in chickens vaccinated with strains of "PA" and "Winterfield 2512" which were administered separately and fill in combined food streptomycetes biomass.

Analyzing data from the table 2 may be noted that at 15 days after vaccination, on chickens from control group total protein was 14.6 g / l, albumins 11.4 g / l and 3.2 g / l globulins. The highest protein level group was established at chickens in the control group which received with feeding biomass of streptomycetes. The value of these indices was respectively, total protein -22.6 g / l, albumins and globulins 13.4 g / l and 9.2 g / l properly.

A higher protein level was established in the chickens group which was vaccinated with strain "PA" complemented with the biomass of streptomycetes, contained 20.9 g / l. In the other groups this index ranged from11g / l up to 16.5 g / l.

Albumins and globulins ranged between 13.2 and 9.49 g / l, the lowest being 6.8 g / l in group chickens were vaccinated with strain "Winterfield 2512" without the addition of the ratio of biomass of streptomycetes.

On the 30-th day after vaccination the total protein at the chickens from the control group were 15.1 g/ l. In the offspring of experimental groups, this index was higher in groups vaccinated chickens with vaccines "PA" and "Winterfield 2512" which contain 18.6 and 21.6 g / l, but in the group of chickens vaccinated only with vaccines without streptomycetes biomass this index was respectively 17 and 16 g / l. The level of albumins and globulins in control group was 10.9 g / l. Highest level of albumins was established in group of chickens were vaccinated with strain "Winterfield 2512" in combination with biomasses of streptomycetes, representing respectively 15.8 g / l and globulins level in the group of chickens that received vaccine 'AP' without streptomycetes biomass was 39.3%. The lowest albumins level was established in group of chickens vaccinated with strain "Winterfield 2512" without biomass of straeptomycetes being 9.7 g / l and the lowest level of globulins - 4.1 g / l was established in the control group.

For examinations performed at the 45-th day after vaccination in chickens of control group, protein level was 16.3 g / l, but in the groups that were vaccinated with strains of "PA" and "Winterfield 2512" with added biomass of streptomycetes this index ranged from 18.2 up to 18.6 g / l.

Slightly fewer indexes were established in the group of chickens that received only the vaccine without streptomycetes biomass ratio, representing a change of values from 17.6 and 15.1 g / l.

So as albumins and globulins level was higher in experimental groups of chickens, where vaccines were administered in combination with the biomasses of streptomycetes

At the same time the highest level of globulins was recorded in chickens group vaccinated with strain "Winterfield 2512" ration which was supplemented with biomass streptomycetes, being 8.1 g / l, while the smallest index - 5,2 g / l was established in chickens group vaccinated with strain "PA".

Table 2. Blood biochemical indices in chickens vaccinated against infectious bursitis with feeding of streptomycetes biomass

Gr. nr.	Nr. of chickens	Vaccines strains / Biomass of streptomy-cetes	Biochemical indices of the blood (days after vaccination)														
			15					30					45				
			Total proteins g/l	Albumins		Globulins		Total proteins g/l	Albumins		Globulins		Total proteins g/l	Albumins		Globulins	
				g/l	%	g/l	%		g/l	%	g/l	%		g/l	%	g/l	%
I	20	C	14,6±0,36	11,4±0,26	78,0	3,2±0,45	21,9	15,1±0,17	10,2±0,17	67,5	4,9±0,17	32,4	16,3±0,23	10,2±0,15	62,5	6,1±0,11	37,4
II	20	C / BM	22,6±0,26***	13,4±0,25**	59,2	9,2±0,17***	40,7	16,3±0,26*	12,2±0,2**	74,8	4,1±0,25	25,1	18,4±0,25**	12,0±0,34**	65,2	6,4±0,15	34,7
III	20	PA / BM	20,9±0,17***	13,2±0,1**	63,1	7,7±0,2***	36,8	18,6±0,25***	12,5±0,26**	67,2	6,1±0,15**	32,7	18,2±0,32**	10,7±0,32	58,7	7,5±0,20**	41,2
IV	20	Winter/BM	12,5±1,05	9,4±0,52*	75,2	3,1±0,17	24,8	21,6±0,26***	15,8±0,25***	73,14	5,8±0,2*	36,8	18,6±0,15**	10,5±0,14	56,4	8,1±0,15***	43,5
V	20	PA / -	16,2±0,81	12,1±0,55	74,6	4,1±0,1	25,3	17,0±0,43*	10,5±0,2	61,7	6,5±0,34*	38,2	17,6±0,25*	12,4±0,15***	70,4	5,2±0,25*	29,5
VI	20	Winter / -	11,0±0,36**	6,8±0,1***	61,8	4,2±0,2	38,1	16,0±0,1*	9,7±0,2	60,6	6,3±0,17**	39,3	15,1±0,32*	8,7±0,23**	57,6	6,4±0,25	42,3

*** p>0,001; ** p>0,01; * p>0,05

CONCLUSIONS

1. Vaccination against infectious bursitis of one day chickens dasen, t stimulate a antibody level that could protect chickens from disease contamination during critical age period (2-6 weeks).

2. The ratio suplining with biomass of streptomycetes have the positive affect at some biochemical indexes which increase the level of blood total protein, albumins and globulins.

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ASSESSMENT OF VIRAL ARTERITIS IMPACT BY SEROLOGICAL EXAM IN HORSES FROM IASSY COUNTY

Oana Tanase, Constantin Pavli, Remus Plesca,
Luanda Ludu, Gheorghe Savuta, Elena Velescu
Facultatea de Medicină Veterinară Ia și,
tanase_oana@yahoo.com

Equine viral arteritis (EVA) is a contagious disease of horses caused by equine arteritis virus (EAV). The virus is present in horse populations throughout the world, aided by international movement of horses and infective cryopreserved semen.

EVA outbreaks don't occur often, but when they do, they are frequently associated with the movement of horses or shipment of semen.

The primary pathologic feature of acute EVA infection is inflammation, edema and necrosis of blood vessels in multiple organ systems. Horses with clinical EVA usually make a full recovery and mortality associated with the virus is rare in adult horses. Foals infected with EVA in utero may die within 2-4 days after birth due to severe progressive interstitial pneumonia.

The economic importance of the disease is due firstly by the material losses produced consequently imposing severe restrictions on the international movement of equines, the ban of import and export of very valuable horses, semen, ova or embryos. On the other hand, in case of an outbreak of equine viral arteritis, many mares will abort and some of the foals will die shortly after birth. As it happened in any countries around the world, equine viral arteritis become an important veterinary issue in Romania too.

The prevalence of EAV infection differs considerably both between countries and between particular horse breeds in the same country and region.

These are the reasons that initiated the researches described in this article.

Keywords: equine arteritis virus (EAV), ID screen equine viral arteritis, the prevalence

Equine viral arteritis is a contagious and infectious disease, affecting horses, regardless of breed, age, sex or physiological condition. It is clinical characterized by febrile syndrome, accompanied by profound fatigue, lacrimation, ocular and nasal catarrh, inflammatory edema of the subcutaneous connective tissue, icteric staining of conjunctival mucosa and abortion in pregnant females. The most important lesions are those of vasculitis of the the blood vessels, with 5 mm maximum diameter and media degeneration and necrosisd of these vessels.

In Romania, the disease was reported for the first time by Aurelia Ionescu et al. Subsequently, there were reported episodes of equine viral arteritis in almost all the studs from the country and the clinical evolution had different forms, from very severe forms with high mortality rate and massive abortion, to discrete evolution, but with high percentage of seropositive animals.

The economic importance of the disease is due firstly by the material losses produced consequently imposing severe restrictions on the international movement of equines, on the import and export at worldwide level, particularly of the valuable horse and due to the number of abortions and the loss of foals shortly after birth.

MATERIALS AND METHODS

Investigations were undertaken on a number of 39 equine spread in households from Iasi county. It has to be mentioned that investigations were initiated because in the past two years in this equine flocks has unjustified increased the percentage of abortions and infant mortality.

The research was conducted on the equine that are equine infectious anemia free (EIA), the infected animals being removed from the flock gradually as required by ANSVSA plan.

It was used indirect-ELISA immunoassay test for detection and titration of anti-virus specific antibodies of equine viral arteritis serum samples, as a confirmatory test produced by ID-VET.

Immunoassay test is based on specific reaction between antigen fixed on polystyrene plate and antibodies for equine viral arteritis from equine test sera.

The presence of specific antibodies for equine viral arteritis in the test sera will block the antigen from the plate which in the second phase will no longer bind specific monoclonal known antibodies from the kit.

The presence or absence of monoclonal antibodies specific bindings is distinguished by a color reaction obtained in the presence of the substrate and by peroxidase adding.

RESULTS AND DISCUSSION

The studied sample consists of 39 horses of different ages, in the study being presented animals of both sexes.

All the 39 serum samples were deposited on a plate with 96 wells in duplicate, including controls (positive and negative). After deposition of sera samples in the wells and performing EVA ELISA reaction, following results were obtained (table 1):

Table 1

Results of EVA ELISA, placed on the working plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.057	0.057	0.179	0.213	0.083	0.183	**1.035**	**2.841**	0.126			0.343
B	0.15	0.078	**0.289**	**3.428**	**0.198**	**3.776**	**0.171**	**3.709**	0.31	0.572	0.588	0.542
C	0.085	1.638	**0.142**	**3.677**	0.294	0.871	0.085	0.179	0.125	0.135	0.683	0.468
D	0.121	1.382	**0.17**	**3.602**	0.115	0.268	0.102	0.092	0.255	0.228	0.075	0.08
E	0.195	0.308	0.13	0.345	**0.073**	**3.55**	0.079	0.088	0.07	0.209	0.064	0.061
F	0.14	0.311	0.198	0.233	0.1	0.359	0.098	0.198	0.086	0.071	0.167	0.586
G	0.114	0.292	**0.152**	**2.889**	**0.129**	**3.647**	0.093	0.083	**0.147**	**3.533**	0.149	0.672
H	0.106	0.166	**0.124**	**3.231**	**0.108**	**2.303**	0.169	0.095	0.158	0.356	0.058	0.06

Using indirect ELISA assay were examined a total of 39 sera from horses, of which 12 positively reacted, with a seroprevalence average of 30.76%.

The equine flock that was the subject of our research has the age ranging between 48 and 120 months, in this experiment participating both female and male. Thus, 23 subjects were males (stallions) which represent 58.97%, and 16 of the test participants were females (mares) which represent 41.02%.

Out of 39 examined samples, a total of 12 samples gave a positive result at EVA ELISA assay. Of these, a total of 7 samples are coming from the stallions, which represents 30.43% of all tested stallions and 5 samples are coming from mares, which represents 31.25% of the total tested mares (Table 2, Fig. 1).

Table 2

The results of tested animals by gender

Results								Total of tested animals
Males (stallions)				Females (mares)				
Pozitives		Negatives		Pozitives		Negatives		
No.	%	No.	%	No.	%	No.	%	
7	30.43%	16	69.56%	5	31.25%	11	68.75%	39

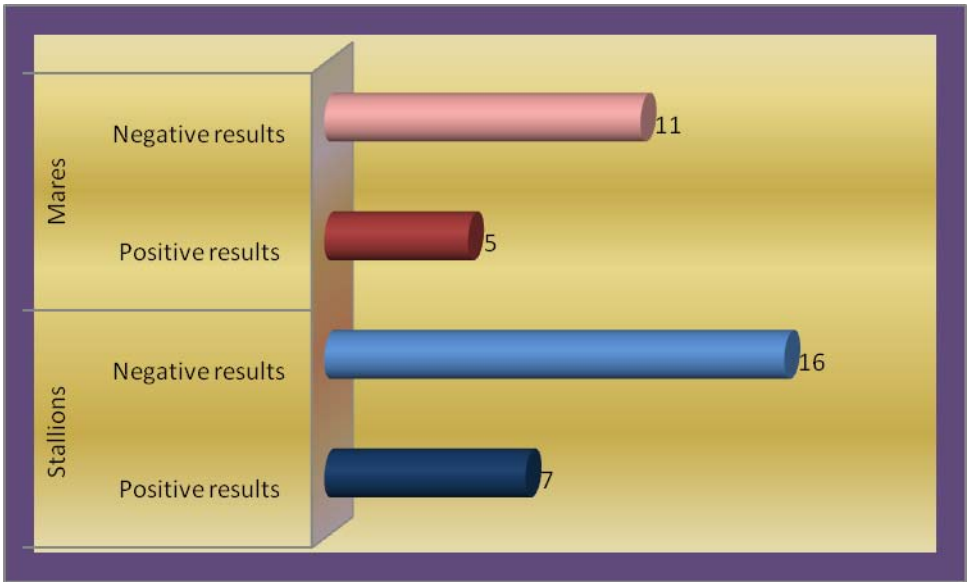

Fig. 1
The results gouped on sex groups

Those seven stallions serologically positive at EVA ELISA assay have the age ranging between 64 months and 120 months and those five tested mares detected positive by EVA ELISA assay have the age ranging between 52 months and 140 months.

To limit the effects of these disease and to prevent its extension to the equine flocks from other neighboring CSV, the movement of the horses will be restricted.

International Animal Health Code prohibits the movement of horses if in the last three months, the horses have showed clinical signs of disease or illness or if the disease was reported in the flock that they belong.

It has to be mentioned that the equine group (39 horses) is not infected with infectious anemia virus (EIA) according to the results from LSVSA-Iasi, as a result of Coggins test (agar gel immunodiffusion test).

Horses that were the subject of research in the present experiment are coming from households and there were no swine at the moment when the samples were collected, to avoid in this way any possible cross-reactions with PRRS virus.

To limit losses caused by abortions, the positive stallions detected by EVA ELISA assay will be castrated, thus interrupting their role of the disease dissemination in the flocks. Also it has been collected semen from which will be performed a virological examination to reveal the presence of equine viral arteritis virus (EVA).

CONCLUSIONS

1. Given that in Romania the horses are not vaccinated against equine viral arteritis, the conclusion is that specific antibodies has appeared consecutively natural infection of the animals.
2. Out of 39 sera samples coming from a group of equine serological tested using EVA ELISA diagnostic test from ID^{VET}, there were identified as positive a number of 12 samples, representing 30.76% of analyzed samples;
3. Out of 23 samples coming from tested stallions with age ranging between 64 months and 120 months, 7 samples were detected as positive, which is indicating a seroprevalence of 30,43%.
4. Out of 16 samples coming from mares with age ranging between 52 months and 140 months, 5 samples were detected as positive, which is indicating a seroprevalence of 31,25%
5. Because there were no swine in the households when the samples were collected, it can be concluded that there are not present possible cross-reactions with PRRS virus.

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EVALUATION OF THE ANTIFUNGAL EFFICIENCY OF ORGANIC ACIDS IN OILSEED CONSERVATION IN PRODUCTION CIRCUMSTANCES

Alina TEODOR, Ioan COMAN, Daniel TEFELE Ş
Faculty of Veterinary Medicine,
University of Agricultural Sciences and Veterinary Medicine, Iasi,
dralisarbu@yahoo.com; profcoman@yahoo.com

ABSTRACT
The purpose of this study was to limit the growth and proliferation of filamentous fungi, potentially pathogenic, on oilseeds, using two lower fatty acids, lactic and acetic, which will not alter the vegetal substrata in terms of quality. The main objectives set to achieve the intended purpose were represented by the preservation of chemically treated oilseeds in bulk storage, in a warehouse, for a period of ten months, from November 2009 to August 2010, and the assessment of fungal contamination for evaluating the effectiveness of conservation agents. Although used in low concentrations (10% for treatment of soybeans and 50% of lactic acid or 25% of acetic acid and their mixture for sunflower seeds), the chemical substances have shown a strong destructive effect on storage fungi, regardless of seed storage conditions, of their level of moisture and pH values. The efficiency of organic acids in preservation of soybeans and sunflower seeds was different during the experimental period, but for the first eight months of storage, these substances have caused a reduction in fungal contamination in excess of 98%, regardless of seed type or chemical agent.
Regarding the specific micoflora for these categories of seed, in general, the same types of fungi were developed on both varieties, especially Penicillium, Cladosporium and Alternaria. The inferior fungi also showed resistance to organic acids, competing for substrate with those of genera mentioned above.

KEYWORDS: oilseeds, organic acids, preservation, antifungal

INTRODUCTION

During their evolution, plants have developed defence mechanisms to prevent the growth of pathogenic microorganisms, which attack them both mechanically and chemically. The protective envelope, previously considered only a physical barrier, is important for regulating tissue water transport and maintaining their integrity, blocking the pathogens, due to the molecules in its structure (Santos et al., 2008). The plants induce a complex defensive response, typically called hypersensitive response, usually associated with host cell necrosis at the site of contact with the pathogen (Hyun et al., 2004). In soybean external layer were discovered many proteins with antimicrobial properties, such as peroxidase 41 kDa (kilodalton or kDa is the unit used in the case of molecules with atomic mass greater than 1,000 units), 32 kDa class I chitinase, trypsin inhibitor 21 kDa and 8 kDa hydrophobic protein. Peroxydases from soy beans shell are very stable at high temperatures, extreme pH values and organic solvents. They are also protective during germination, having values 100 times higher than the antifungal effect of latency seeds (Varga et al., 2000). Also, soybeans are protected against insect attacks and micromycetes invasion because of the isoflavones, which have antifungal activity (Mebrahtu et al., 2004). However, if significant quantities of oil seeds are stored for long periods of time, plant substrates ability to defend itself against pathogens becomes ineffective.

The main types of filamentous fungal that are normally developing on these categories of seeds are: Aspergillus, Penicillium, Fusarium and Alternaria (Ramos and Hernandez, 1997). For example, to grow on the soybeans and to produce mycotoxins, strains of *A. flavus* requires a moisture content of 17 to 17.5%. This species grows relatively quickly at 24°C, this temperature being the lowest for aflatoxin production. Maximum threshold temperature above which are not produced mycotoxins is at 42°C (Bhattacharya et al., 2002). *A. flavus* is the dominant species inside the store throughout the year. *A. niger* dominate the species of Rhizopus, Penicillium, Fusarium and Alternaria genera between July and October, for the period of storage, with a maximum of 48%. This species is able to produce losses of up to 25% caloric to lentils in just 15 days and 49% in 30 days. *A. candidus* grows after several months of storage, when the nutrient substrate and moisture conditions are favourable, as a result of the activity of other fungi. These species affect seed germination and cause a slight calefaction of the substrate (Moss, 2002). On sunflower seeds, selected from several isolated localities in Egypt*, A.ochraceus* was reported as the predominant species, and in Tunisia, *A. flavus* and *A. parasiticus* (Abdel-Mallek et al., 1994). In Spain, commonly found on oil seeds, are the filamentous fungal species belonging to the genus Aspergillus: *A. niger*, *A. fumigatus*, *A. flavus*, *A. parasiticus*, *A. versicolor* and *A. candidus* (Jimenez et al., 1989).

From genus Penicillium, on oilseeds, are commonly isolated the following species: *P. crustosum*, *P. nordicum* and *P. verrucosum*. The optimal conditions that *P. verrucosum* requires to develop are: about 17% substrate moisture and heat range of 5°C- 40°C. This kind of fungal is able to produce mycotoxins (Kokkonen et al., 2005).

When seeds are stored for long periods of time, under inappropriate conditions of the microclimate, development of filamentous fungi can be controlled only by using synthetic substances, often selected being the organic acids, particularly lactic and acetic acid (Cabo et al., 2002, Chitarra, 2003).

But there are hiperacidophilic microorganisms that maintain a relatively neutral pH, pumping protons outside the cell, setting a low membrane permeability for them. Among fungal species, some are resistant to acid chemical formulations, such as: *Mucor racemosus* that withstand a pH of 2.0, *A. stolonifera*, *A. flavus*, *A. fumigatus*, *F. culmorum* and *Penicillium sp.* uninhibited in development at a pH value of 3.0, *A. niger*, which normally grows at a pH of 2.3, and *Cladosporium herbarum* at pH 3.5 (Gross and Robbins, 2000).

MATERIALS AND METHODS

To perform the monthly mycological examinations, there were used standardized equipment and working techniques. All, however, had as its starting point sample collection and processing of biological material. Samples of biological material were represented by: soybeans and sunflower seeds in a quantity of 200 kg for each variety, selected so as to be relevant and representative samples, distributing 50kg for each sample.

For the chemical treatment of the biological samples, were used two organic acids, lactic acid and acetic acid, individually or in combination. Also, depending on the assortment of seeds, there were used different concentrations of each acid and also the equal mixture of these substances, for dilution using saline. Thus, for the treatment of soybeans were used: 10% lactic acid, 10% acetic acid , 10% AA mixture and for the sunflower seeds: 50% lactic acid, 25% acetic acid and 25% AA mixture.

As necessary equipment for the first phase of research were needed a thermohygrometer, a container to spray the preservatives, and special tools used for obtaining samples and for their homogeneity. Dispersion was achieved with chemical spray, the seed being sprayed in thin layers and then homogenized by hand using rubber gloves. With the thermohygrometers were determined throughout the storage period of seeds, the main parameters of microclimate in warehouse areas, namely air temperature and relative humidity.

RESULTS AND DISCUSSION

Seed samples were divided into four batches of 50 kg each, being stored, after treatment with organic acids, for a period of ten months, from November 2009 to August 2010. In terms of temperature and humidity values in the warehouse, where the samples were stored, those were slightly different, except for the summer months, when they exceeded the limit allowed for seed storage conditions that do not cause losses nutrients, in the temperature case (chart 1).

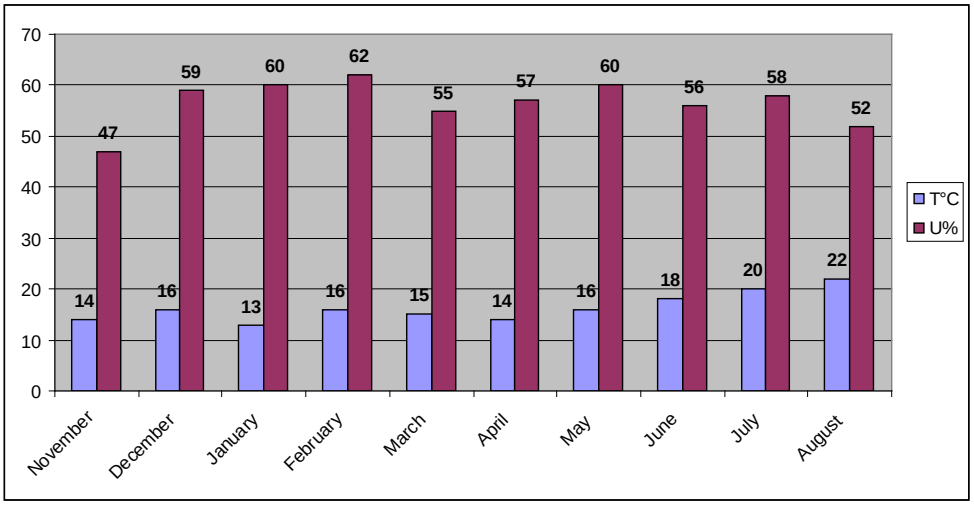


Chart 1- Values of the main microclimate parameters in the warehouse during November 2009 - August 2010

● **Efficiency of organic acids in soybean conservation**

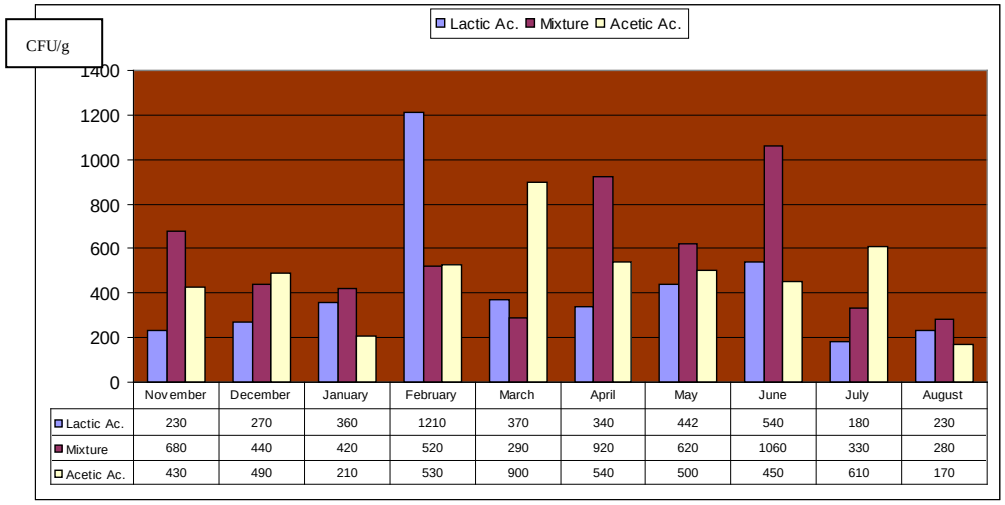
The main purpose of the monthly quantitative mycological tests was to establish the degree of contamination with filamentous fungal of soy beans, recorded the dynamic incidence throughout the experimental period.

In terms of quantitative mycological examinations of the blank sample, in the first month of storage, the total number of colony forming units (CFU)/g was 96000, increasing gradually during the winter months, then falling to 51800 CFU/g in the last month of the experiment. The greatest value of the number of CFU/g was observed in February, being 194000, and in March recording a total of 178 000 CFU/g.

From December to February, the degree of fungal contamination progressively increased from 120000 CFU/g, in the first month of winter, to 176000 CFU/g, in January, reaching its highest level in February, when the total amount was 194000 CFU/g. In March, the filamentous micromycetes decreased, in comparison to the previous month, remaining, however, at a higher value than those observed in December and January, more precisely, to 178 000 CFU/g. This phenomenon can be explained by climatic variations, which influenced by temperature and humidity levels, much higher than those in the winter months, the micromycetes development. Since April, the degree of fungal contamination was significantly reduced until the tenth month of storage, from 86200 CFU/g to 51800 CFU/g, amount recorded in August. In May and June, the results of quantitative mycological examinations were close in value, registering 71067 CFU/g, in the first month, and 72733 CFU/g, in the second month. July and August were those who achieved the lowest values of the total number of CFU/g: 59100 CFU/g and 51800 CFU/g. These values, reduced by nearly half over the first month of experiment, may justify, on the one hand, the microclimate conditions less favourable for the micromycetes development, such as, for example,

high temperatures and low atmospheric humidity, and on the other hand, losses of nutrients in soybean meal, normal for a relatively long period of preservation.

Regarding the soybean samples treated with the selected organic acids, the results obtained, using quantitative mycological examination, revealed a very low degree of contamination by filamentous fungi compared with the reference sample (Chart 2).

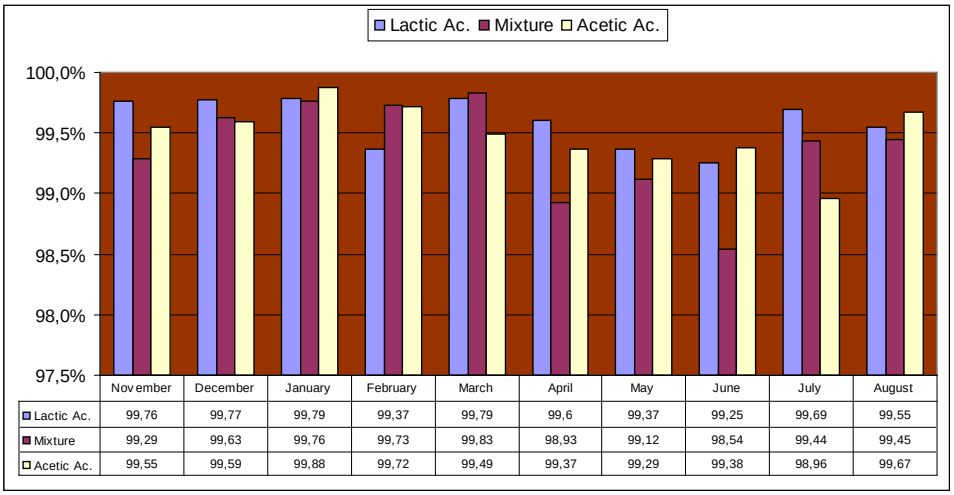


	November	December	January	February	March	April	May	June	July	August
Lactic Ac.	230	270	360	1210	370	340	442	540	180	230
Mixture	680	440	420	520	290	920	620	1060	330	280
Acetic Ac.	430	490	210	530	900	540	500	450	610	170

Chart 2- The degree of fungal contamination (CFU / g) of soybean samples treated with lactic acid and acetic acid, used individually or in combination, during November 2009 – August 2010

Comparing the antifungal effect of the acids used in the conservation of soybeans for a period of ten months, it was observed that, whatever was the used substance and, given its very low concentration, of only 10%, all chemical formulations drastically reduced the filamentous fungi within the seed mass (chart 3).

Taking into account the main parameters of microclimate variations, depending on the season, and the long storage period, which influenced greatly the loss of nutrients and, consequently, the power plant's own defence against filamentous fungi, the fact that the chemicals were very active throughout the period of this experiment show high efficiency of these substances, both in terms of hygiene and health, and economic, given the low quantities in which they were used.



	November	December	January	February	March	April	May	June	July	August
Lactic Ac.	99,76	99,77	99,79	99,37	99,79	99,6	99,37	99,25	99,69	99,55
Mixture	99,29	99,63	99,76	99,73	99,83	98,93	99,12	98,54	99,44	99,45
Acetic Ac.	99,55	99,59	99,88	99,72	99,49	99,37	99,29	99,38	98,96	99,67

Chart 3- Comparative analysis of the effectiveness of antifungal lactic and acetic acids, used individually or in combination, in soybean conservation during November 2009 – August 2010

Regarding the micoflora observed on the soybean samples, it was composed of species of filamentous fungi belonging to the main genres that normally colonize the vegetal substrata during the storage period. Filamentous fungi of the genera Penicillium and Cladosporium were found consistently in all samples of seeds, followed in incidence by *Alternaria sp*.. It seems that these fungal categories are resistant to the chemicals compounds that were tested. Also, the inferior fungi developed mainly in the plates corresponding to the first dilutions, allowing colony count of filamentous fungi only at the following ones. These results correspond with those made by Ramos and Hernandez (1997), and Kokkonen et al. (2005).

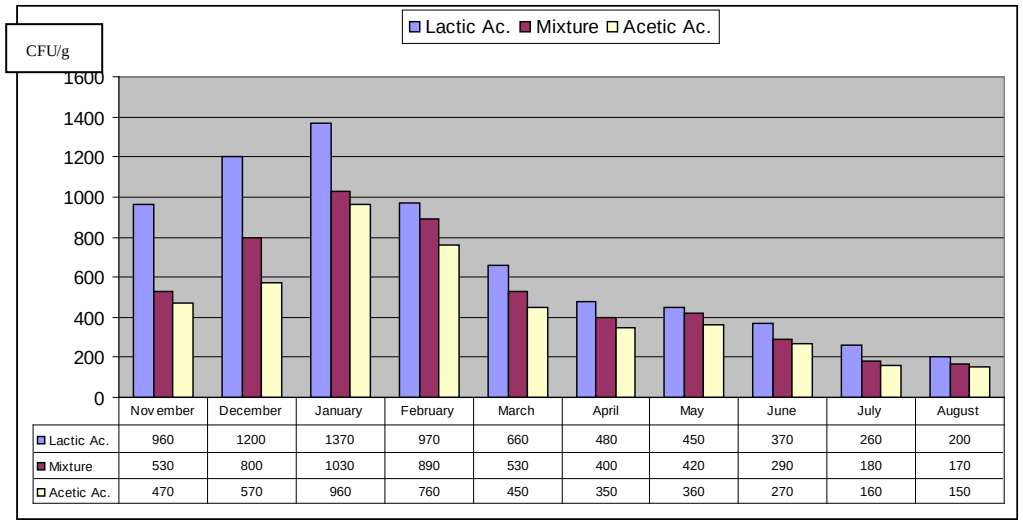
● **The efficiency of organic acids in sunflower seed conservation**

Samples of sunflower seeds, stored in a warehouse for the entire period of the experiment, were represented by four groups, of which one was kept as reference/control sample or blank, another was previously treated with lactic acid 50%, the following with 25% acetic acid, and the last with a mixture of the two organic acids, having 25% active ingredients.

Analyzing the results obtained on the blank, in the first month of storage, the total number of CFU/g was 84200, varying between 104000 during the winter season (January) and 115000 (in February), in December, the value recorded being also higher than in November, registering a total value of 110067 CFU/g.

Starting with March, the incidence of filamentous micromycetes pretty much began to fall, compared with the three winter months, the recorded values being 44000 in the first month of spring, and, in April and May, the results having close values, 37300 CFU/g and 36400 CFU/g. In June, the degree of fungal contamination on the reference sample of this type of seed was 23700 CFU/g, a value that was drastically reduced in the next two months at 7773, in July, 3400 respectively in August.

Regarding the results of quantitative mycological tests carried out on samples treated with organic acids, these can be interpreted using chart 3.



	November	December	January	February	March	April	May	June	July	August
Lactic Ac.	960	1200	1370	970	660	480	450	370	260	200
Mixture	530	800	1030	890	530	400	420	290	180	170
Acetic Ac.	470	570	960	760	450	350	360	270	160	150

Chart 3- Organic acids efficiency in the conservation of sunflower seeds from November 2009 - August 2010

Comparing the results of the blank with those of chemically treated samples, it was noted that all three selected chemical formulations have shown a strong destructive effect of storage fungal flora, characteristic to these types of oilseeds. Also, making a comparison between the results reported for the chemically treated samples it can be observed that the acetic acid was most effective in reducing fungal contamination during the whole period of experiment. Similar effect showed the equally mixture between the two organic acids, thus, the sample treated with lactic acid, although in concentrations two times higher than the other two, recorded the high degree of contamination with filamentous fungi.

Regarding the micoflora observed on the samples of sunflower seeds, it was composed of species of filamentous fungi belonging to the main genres that normally colonize plant substrates during storage. Fungi of the genus Penicillium occurred steadily on the growing medium, especially on the control sample, and, from the chemically treated groups, on that with lactic acid. Towards the end of conservation, its tendency was to develop as the unique species of filamentous fungi on all seed samples.

With low incidence, due to the sensitivity at the preservatives destructive action, or because of the micromycetes competition for the substrate, colonies of filamentous fungi belonging to the genera Cladosporium and Alternaria were observed during the period of storage.

CONCLUSIONS

Antifungal efficiency of organic acids on soybean sample was maintained above 99% throughout the entire period of storage.

Organic acids effectiveness in combating filamentous fungi decreased, especially in the last three months of the experiment, in case of sunflower seeds.

The samples of both seed types have been stored under the same conditions, and under the same influence of microclimate parameters. Thus, the differences between the reported results are due to the different seed structure, especially the coat, or to their internal defence factors.

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PLANT-ANIMAL MODELS FOR HEAVY METALS ACCUMULATION IN AGRICULTURAL SYSTEMS

Liliana TUDOREANU, Mario CODREANU, Gheorghe V. GORAN, Victor CRIVINEANU
Faculty of Veterinary Medicine,
University of Agricultural Sciences and Veterinary Medicine Bucharest,
Interdisciplinary Laboratory for Heavy Metals Accumulation in the Food Chain and Modelling (HEVMETFOOD)
105 Splaiul Independentei, 050097, 5th district
liliana_tudoreanu223@yahoo.com

ABSTRACT
There are only very few attempts to model heavy metals accumulation in the plant- animal system. Many traditional farms such as the ones in the Copsa Mica area are using local feed and local pastures for dairy farm animals. We propose a modelling approach for Cd and Pb accumulation in the plant-animal system which is considering Cd and Pb accumulation in pasture plants as well as Cd and Pb accumulation in some other local produced fodder plants.
The soil/root and root/shoot transfer coefficient for local pasture plants was found to be dependent on plant growth and of the distance from the pollution source (concentrations of heavy metals in soil), and the complexity of the pollution. Accumulation of Cd and Pb in animal organs and blood is mainly dependent on the duration of exposure and the complex exposure to heavy metal and mineral nutrients in fodder/pasture plants.

Key words: heavy metals, plant-animal models

A large variety of modeling techniques is in current use each of the models being highly personalized, serving a very specific purpose. It seams that mechanistic and regression models are the majors modeling techniques in use.

The main purpose of all these models is to predict the value of a certain parameter (yield, nutrients uptake, heavy metals accumulation) at a certain time with or without predicting how the system unfolds with the passage of time.

Many traditional farms such as the ones in the Copsa Mica area are using local feed and local pastures for farm animals.

The proposed model approach is considering Cd and Pb accumulation in pasture plants as well as Cd and Pb accumulation in some other local produced fodder plants.

The proposed models were constructed to simulate the uptake and accumulation of cadmium and lead by plants from the soil solution and aerial depositions as well as from more traditional soil parameters such as pH and organic matter and clay. Plant nontoxic accumulation was found to be the most common situation for field grown plants under normal agronomic practice and thus the plant models take into account accumulation below the upper critical concentrations of Cd and Pb in plants tissues which would affect plant growth. Cadmium uptake is described by Michaelis-Menten parameters and its accumulation in plant tissues are governed by the rate of Cd sequestration by class III metallothionines. The proposed cadmium foliar accumulation onto and in the plant leaves is compartmentalised into wet deposition, dry deposition and deposition from sewage sludge. The model is flexible and may be likened to growth

models which calculate dry matter production in relation to intercepted radiation, air temperature, soil water status and tissue N-concentration.

The animal models presented are published linear models or endogenous models derived from data collected in Copsa Mica from the period 2000-2010. As the Copsa Mică agronomical system is very complex the plant-animal model is limited for the moment therefore future developments are needed.

Materials and methods

The plant – animal model

The proposed plan-animal model with several modules is aplicable to agronomical systems containing farm animals such as sheep cattle and equines as well as pastures or maize and wheat cultures.

The overall model consists of several soil-plant modules including foliar deposition and several animal modules.

The soil –plant module

At this stage of the soil-plant module several approaches are proposed:

Empirical model M1

Calculation of Cd concentrations in plants based on the soil Cd total concentration, soil pH, organic matter and clay content.

log[Cdplant] = Constant+ a·log[organic matter] + b·log[clay] + c·log[Cdsoil] + d·[pH]

Constant, a, b, c, d, are statistically derived parameters for the linear model

Calculation of Cd concentrations in plants based on soil solution Cd concentration (Tudoreanu and Phillips, 2007)

Module M2- Cadmium accumulation in maize/ wheat/ ryegrass/ pasture plants

The M2 module for maize is bazed on the following assumptions:

-Cd Concentration in soil solution does not exceed 0.1□mol/l

-Cadmium accumulates in plant tissues in organic form.

-Upper critical limits for Cd concentration in Plant tissues are not reached for Cd concentration in the soil solution less then 0.1 □mol/l

-Plant parameters describing cadmium influx kinetics in the roots are the Michaelis-Menten parameters: I_{max}; K_m; E. Influx rate (I_n) is known for maize plants

-Cadmium adsorption to roots is considered to be about 70 □g /100g root fresh weight for shoot Cd excluders of maize inbred lines and 5-25 □g/100 g rootfresh weight for non shoot Cd excluders maize inbred lines the root desorbable Cd fraction from the total Cd concentration in the root is 13% - (15.7±2.3) % for maize shoot Cd excluders and (8.7±2.4.7) %- (10.3±3.2) % for non-shoot Cd excludes

- Cd uptake by roots will depend on root distribution in the soil profile and by the fraction of the total root length found in the contaminated layer

-Accumulation of cadmium at root level is due to sequestration by phytochelatins and adsorption on root cel walls.

-Average daily rates of phytochelatin concentration per unit of dry root mass is denoted by PHYd and will depend on soil solution concentration (Keltjeans, 1998)

-Root ionic Cd will be denoted by the term IrootCd

-Ionic Cd in Shoots IShootCd and IleafCd may be used for shoot and leaves respectively) [At this stage of the model they will be considered '0'].

-Daily Cd accumulation in grains for the period of grain filing is not bigger then 0.1 from the daily shoot concentration.

-Each plant variety will be characterized by a specific quantity of cadmium sequestered per unit mass of phytochelatins, denoted by the term Seq

-It is assumed that the plants will not reach phytotoxic levels of cadmium accumulation that might affect growth rate values.

-Rates of aerial depositions should follow the equation proposed by Struck et al (1989) when applicable. Ccadmium foliar accumulation in and onto the plant leaves from aerial depositions will depand on deposition velocity v_g on a specific surface (for ryegrass v_g=0.15m/s) and the LAI.

-When applicable, cadmium foliar accumulation in and onto the plant leaves from sewage sludge may follow the rationale presented Struck model and the foliar deposition is considered to be dry and wet as well.

-Phytochelatins are present in the plant tissue even when plants are grown in the absence of Cadmium.

-Shoot / root ratio for maize plants are 4.0 to 4.5 for shoot Cd excluders and 5.2 to 5.5 for non-shoot Cd excluders

- Phytochelatins are present in the plant tissue even when plants are grown in the absence of Cadmium.

- Phytochelatin concentrations are linearly related to internal Cd concentration Cd_{max} is 100□g of Cd /gDM for both maize and wheat.

- Shoot / root ratio for maize plants are 4.0 to 4.5 for shoot Cd excluders and 5.2 to 5.5 for non-shoot Cd excluders

- Cd accumulation in maize under toxic stress is considered to be governed by the relationship between shoot phytochelatins (PC-SH) concentration and shoot dry matter(DM) :

Maize: Y= -4.90x + 5.00 (r = 0.61)

Wheat: Y= - 22.71x +5.48 (r = 0.68)

Y= Shoot PC-SH in □mol/g DM

x = shoot DM in g

Several root / shoot partitioning coefficients for Pb concentrations were calculated for pasture plants from the Copsa Mica area. It was found that the Pb partition coefficient varies with time.

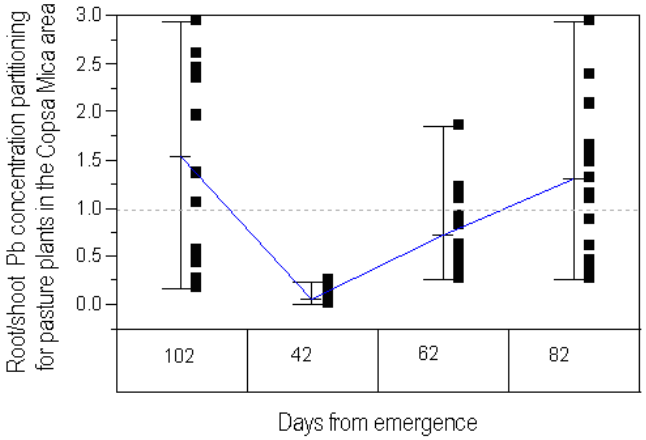

Figure 1. Root/shoot Pb concentration partitioning index (It) for pasture plants in the Copsa Mica area.

The variation with time of the partitioning index for Pb concentration in the pasture plants in the Copsa Mica area is following the polynomial equation (figure 2)

$I_t = -0.75 + 0.025\ Z - 0.0002697\ (Z-72)^2$

where Z is the number of days from emergence.

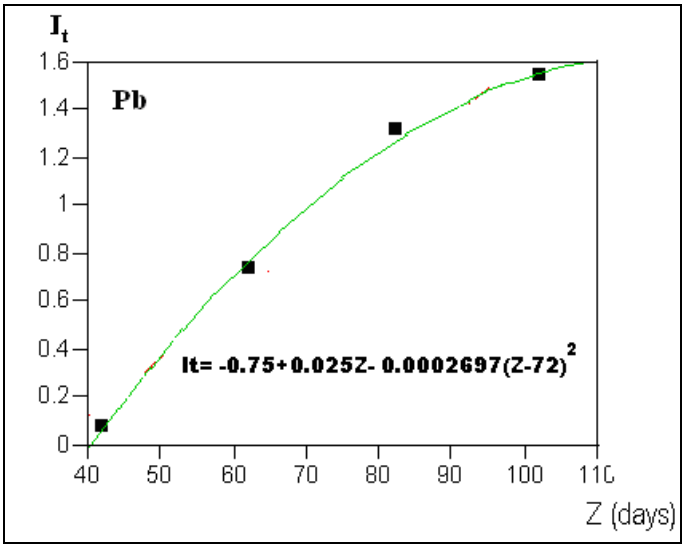

Figure 2. The temporal variation of the partitioning index for Pb concentration in pasture plants in the Copsa Mică area.

The animal module

Module M3 Cattle - for Cd accumulation in cattle.

The M1 animal module (Fels-Klerx et al, 2011) can be used for the calculation of the total daily and annual Cd intake by cattle considering the intake of roughage (grass and maize), compound feed, water and soil.

Differences in the consumption patterns of younger and older cows are also considered.

The daily Cd intake (DI) for cattle (Fels-Klerx et al, 2011) in mg Cd per day was calculated as the sum of the intake of soil, roughage (including grass and maize in a fraction and compound feed, each of the three multiplied by their respective Cd contamination levels

$DI = \Sigma\ (Cd_{soil} \times Co_{soil}) + (Cd_{com} \times Co_{com}) + (Cd_{rou} \times Co_{rou})$ (Fels-Klerx et al, 2011)

Co_{soil}, C_{com} and Co_{rou} represent the animal daily consumption of respectively, soil, compound feed and roughage, expressed in kg dry matter per day.

The biotransfer rate ($\mathbf{B_{Cd}}$) of Cd BTR (Franz et al., 2008) is defined as the increase of the Cd_concentration in the organ tissue per day divided by the additional Cd intake per day,and expressed per 1 kg of tissue.

The $\mathbf{B_{Cd}}$ of Cd into kidneys is 9.0×10^{-5} for kidneys and 1.7×10^{-5} for liver (Franz et al., 2008).

The cadmium concentration in organs (Ct) in mg /kg is a linear function of $\mathbf{B_{Cd}}$, daily intake (DI) and time (t):

$Ct = \mathbf{B_{Cd}} \times DI \times t$

The M1 cattle model calculates the levels of Cd in liver and kidney based on

1) a linear bioconcentration model assuming accumulation only for up to 5 year old animals

2) a non-linear accumulation-excretion model

Module M3- sheep

The M2 module consist of the sheep animal model for Cd accumulation in liver and kidney for sheep (Phillips and Tudoreanu, 2010)

Module M3- equines

The M1- equines module estimates Cd and Pb accumulation in hair, kidney and liver tissues for horses and is still under current development. The M3 model will attempt to estimate also the rate of excretion for Cd (Crivineanu et al, 2009)

Conclusions

1. The proposed plant-animal model is only partially covering only a part of the agricultural systems from Copsa Mica.
2. The data from Copsa Mica suggests that the heavy metals partitioning between root and shoots for pasture plants is time dependent
3. The monthly accumulation of heavy metals in the animals organs grazing in the Copsa Mică area is dependent of the portioning index between root and shoot for the pasture plants. This fact suggests that grazing after 45 days from plant emergence will substantially increase Pb accumulation in animal organs.

Acknowledgment

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