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THE RESEARCH OF CIRCULATION OF PATHOGENIC LEPTOSPIRA AMONG POPULATIONS URBAN BROWN RATS IN KYIV, UKRAINE

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Summary. Urban brown rats (*Rattus norvegicus*) are frequently implicated in the spread of *Leptospira* spp. Urban brown rats (n = 116) were trapped in different places near the lakes and ponds in Holoseevsky and Obolon districts of Kyiv and tested for *Leptospira* spp. using an agglutination test (MAT), which was conducted with 21 *Leptospira*'s serological groups.

It is identified the circulation of pathogenic *Leptospira* among urban brown rats population of Kyiv, Ukraine. In total, 65 (56%) rats were positive by MAT. The antibodies against serogroups *Grippotyphosa, Mini* and *Ballum* were dominant in positive reactions.

Keywords: Leptospirosis, urban brown rats (*Rattus norvegicus*), antibodies, microscopic agglutination test (MAT), Kyiv, Ukraine

Introduction. Leptospirosis is a major emerging infection with a worldwide distribution (Levett, 2001). It is a systemic disease of humans and domestic animals (Adler and la Peña Moctezuma, 2010). Regarded globally as a zoonosis because it is acquired by humans from contact with animals or from water contaminated with the urine of infected animals, it is presumed to be the most widespread zoonotic disease in the world (Adler and la Peña Moctezuma, 2010; Levett, 2001).

Leptospirosis is caused by leptospira species, which has over 20 serogroups and more than 250 serovars and distributed worldwide (Bharti et al., 2003).

The natural reservoir of *Leptospira* is wild vertebrate animals, mostly mammals (Turner, 1967). Today we know that a wide variety of animals sheds *Leptospira* into the environment and thus puts others, including human, at risk of infection. An incomplete list of wild mammals known to be infected includes such orders as Rodentia (rats, mice, voles, gerbils, coypus), Insectivora (hedgehogs, shrews), Carnivora (dogs, foxes, mongooses, jackals, civets, skunks, raccoons), Marsupialia (bandicoots, opossums), Chiroptera (bats), Artiodactyla (deer), and Lagomorpha (hares, rabbits). Among the domestic animals, dogs, cattle, swine, and goats are frequently affected by this organism (Stoenner, 1976; Stoenner and MacLean, 1958). In addition, birds (Frezza, Sindoni, and Tredici, 1968), snakes, turtles, lizards (Lindtner-Knific et al., 2013), frogs (Gravekamp et al., 1991), and fish (Maestrone and Benjamenson, 1962) may be rare sources of infection.

Wild mammals, and in particular rodents, are the primary reservoirs of the infection; whereas domestic animals such as cattle, dogs and pigs may act as carriers for several months (temporary carrier) while rodents usually remain carriers throughout their life (permanent carrier). Rodents are therefore considered to be the major reservoir of infection (Bharti et al., 2003).

Two commensal (*L. cum mensa*) species are common inhabitants on farms worldwide: the house mouse (*Mus musculus*) and the brown rat (*Rattus norvegicus*). Both species originated from Asia, from where they spread over the world along with the development of agriculture, which provided shelter and supplies of food. They are underground dwellers, omnivorous and can breed year-round when conditions are optimal (Hanney, 1975; Nowak, 1999).

Generally, the urban rodent problem is caused by three species: the Norway rat (*Rattus norvegicus*), the roof rat (*Rattus rattus*), and the house mouse (*Mus musculus*). Although the Norway rat is known also as the brown rat and the roof rat as the black rat, these names are misleading because the colors of both species range from light brown to black. These three rodents, of all rodent pests (nutria, cotton rat, and the like),

are most often identified with rodent-borne diseases and economic loss through destruction and contamination (Clinton, 1969).

The rat was the first incriminated as a source of human infection and was long thought responsible for most human illness. Thus, the *Leptospira Icterohaemorrhagiae*, was first reported from a human with Weil's disease in 1916 and then from wild brown rats (*Rattus norvegicus*) in 1917 (Inada et al., 1916; Noguchi, 1917).

In 1990–1991 were reported, that most, if not all, rat populations are infected with *Leptospira Icterohaemorrhagiae* at a prevalence of 50–70% (Golding, 1990; Waitkins, 1991).

Nowadays rats are the primary carriers of *Leptospira* serovars *Icterohaemorrhagiae* and *Copenhageni* (i.e., the *Icterohaemorrhagiae* serogroup) and serogroup *Ballum* (Aviat et al., 2009; Ko, Goarant, and Picardeau, 2009; Levett, 2001). These serovars are the major in leptospirosis of human (Ko, Goarant, and Picardeau, 2009), dogs (Cerri et al., 2003) and pigs (Cisneros Puebla et al., 2005).

The brown rats are regarded as the carriers of other serovars which are dangerous for human and animals (Babudieri, 1958; Keenan et al., 2009.

Rats are colonial and territorial animals that promote intra-colony interactions (Barnett, 1963). In the urban environment, limited available space leads to the formation of colonies with a small home range, and inter-colony contacts may occur. Therefore, the urban rat population more likely functions as a meta-population, as described by Viana et al. (2014), in which the sub-unit is the rat colony. Determining rat meta-population behavior with regard to *Leptospira* strain distribution would provide evidence of rat interactions resulting in rat infections.

Over the last twelve years, studies of rodent infection in the vicinity of index cases have associated rats with human infection in both rural (Ganoza et al., 2006; Johnson et al., 2004) and urban settings (Jansen et al., 2005; Pezzella et al., 2005; Vinetz et al., 1996).

For example, five studies published during the period 2003–2014 the report on the prevalence of *Leptospira* infection among urban rats to range between 11.1% (N = 592) in Vancouver, Canada (Himsworth et al., 2013), 16% (N = 127) in Tokyo, Japan (Koizumi et al., 2009), 48% (N = 23) in Santa Fe, Argentina (Vanasco et al., 2003), 65.3% (N = 201) in Baltimore, USA (Easterbrook et al., 2007), and 63% to 83% (N = 226) in Salvador, Brazil (Costa et al., 2014).

The aim of this study is to investigate a large number of blood sera samples from wild brown rats living in urban areas to determine the current leptospira status, both in terms of its prevalence and the range of serogroups and serovars that were carried out.

Materials and methods. *Strains:* Twenty-one pathogenic *Leptospira* spp. strains were genotyped. These strains were part of the bacterial collection of the Leptospirosis Laboratory of Farm Animals with the Museum of Microorganisms of the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences of Ukraine.

Rodents trapping: The investigation was carried out in two districts of Kyiv during the period from May 2014 to September 2015. Overall 116 rodents were collected, with various types of traps: sticky traps 'Catch Expert' and trap metal mesh. Traps were set on the banks of reedy lakes and ponds in parks and recreation areas consistently with the observations of rodent signs, droppings, nests, and burrows, and were controlled every day. On arrival at the laboratory, trapped Rodents were immediately anesthetized with ethyl ether and numbered, and the record of species and places of trapping was prepared.

Study sites: Sera from 116 urban brown rats, which were caught in the parks and public recreation areas in Holoseevsky and Obolon districts of Kyiv during 2014–2015, had been studied.

In Holoseevsky district, urban brown rats were restored in the following locations: Holoseevsky Park (cascade Horihuvatsky Pond and Holosiivsky Pond, Didorivka Lake), park 'Feofania' (Palladinski Lakes), ponds near the residential complex 'Towers-2', Mysholovski Lakes, and Kytayevski artificial ponds.

In Obolon district, rodents were caught near Verbne, Jordan, and Kirilivske Lakes, and the cascade of Opechensky Lakes.

Serological test (MAT): The research was carried out by microagglutination test (MAT) using antigens of 21 *Leptospira* serogroups which are listed in Table 1.

Table 1 – List of strains used for research

№	Serogroup	Serovar	Strain
1	*Javanica*	javanica	*Veldrat Bataviae 46*
2	*Bataviae*	*djatzi*	*HS 26*
3	*Mini*	*szwajizak*	*Szwajizak*
4	*Sejroe*	*polonica*	*493 Poland*
5	*Hebdomadis*	*kabura*	*Kabura*
6	*Tarassovi*	tarassovi	*Perepelicyni*
7	*Pomona*	*pomona*	*Pomona*
8	*Grippotyphosa*	*grippotyphosa*	*Moskva V*
9	*Canicola*	*canicola*	*Hond Utrecht IV*
10	*Icterohaemorrhagiae*	*copenhageni*	*M 20*
11	*Louisiana*	louisiana	*LSU*
12	*Shermani*	*shermani*	*LT 821*
13	*Panama*	*panama*	*CZ 214 K*
14	*Semaranga*	*patoc*	*Patoc 1*
15	*Celledoni*	*whitcombi*	*Whitcomb*

16	*Australis*	erinaceieuropaei	*Jez 1*
17	*Autumnalis*	*autumnalis*	*Akiyami A*
18	*Cynopteri*	*cynopteri*	*Vleermuis 3868*
19	*Pyrogenes*	*pyrogenes*	*Saline*
20	*Ballum*	*ballum*	*Mus 127*
21	*Australis*	bratislava	Jez-bratislava

MAT was used in dilutions: 1:50, 1:100, 1:500, 1:2,500. Antibody titers 1:50 and above were considered as positive.

The study of blood sera of wild rodents was conducted in the Laboratory of Leptospirosis with the Museum of Microorganisms of the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences of Ukraine and in the Laboratory of Special Danger Infections of the Kyiv Regional Laboratory Center of the State Sanitary-and-Epidemiologic Service of Ukraine.

Results. During 2014–2015, 116 samples of blood sera from urban brown rats were investigated, 65 samples of them (56.0%) were positive.

In Holoseevsky district over the period, 79 samples of blood sera from urban brown rats (68.1% of the total) have been analyzed (Table 2).

As shown in Table 2, the urban brown rats contamination by pathogenic leptospira in the analyzed period was lower in 2014 (45.0%). In 2015, the rate was 62.7%. We see that in two years there is a significant percentage of mixed reactions, representing respectively 66.7% and 83.8%. This fact contributes to significant increase in the total number of positive reactions (mono + mixed).

In 2014, 20 samples of blood sera were investigated and 9 received reactions (45.0%) were positive in MAT. Antibodies to serogroup *Grippotyphosa* (31.6%) and *Icterohaemorrhagiae* (26.3%) of the total number of positive reactions were often diagnosed in urban brown rats this year. In significantly lesser amount of cases the serological groups *Pomona* (15.8%), *Cynopteri* (10.5%) and *Ballum* (10.5%) were registered. The smallest number of animals reacted positively to leptospirosis serogroup *Mini* (5.3%) (Table 2).

During 2015, 59 samples were analyzed and 37 positive reactions (62.7%) were received. The dominant serogroup, as in 2014, was *Grippotyphosa* — 17.3% of positive reactions. In 2015, antibodies to Leptospira of serological group *Mini* was diagnosed in 17.3% of samples while in 2014 — in 5.3% of samples. Other serological groups — *Ballum* (12.7%) and *Pomona* (11.0%) — were found in smaller quantities.

In 2015, the percentage of positive reactions to serogroup *Icterohaemorrhagiae* decreased by 18.6% compared to previous year; the following serological groups was recorded for the first time: *Celledoni* — 5.5% and *Australis* — 5.5% (serovar *bratislava*), *Javanica* — 5.0%, *Shermani* — 4.0% and *Australis* — 3.1% (serovar *erinaceieuropaei*). The lowest etiological role was played by *Sejroe* (0.9%), *Hebdomadis* (0.9%), and *Pyrogenes* (0.9%) serogroups.

Table 2 – The results of serological studies of blood sera samples of urban brown rats caught in Holoseevsky district, Kyiv

Years			**2014**	**2015**	**Total**
Studied samples of blood sera			20	59	**79**
Positive results		total	9	37	**46**
		%	45.0	62.7	**58.2**
Monoreactions, %			33.3	16.2	**19.6**
Mixed reactions, %			66.7	83.8	**80.4**
Positive results (mono + mixed)			19	220	239
Antibodies to leptospira serogroups	*Javanica*	total	—	11	**11**
		%	—	5.0	**4.6**
	Mini	total	1	38	**39**
		%	5.3	17.3	**16.3**
	Sejroe	total	—	2	**2**
		%	—	0.9	**0.8**
	Hebdomadis	total	—	2	**2**
		%	—	0.9	**0.8**
	Pomona	total	3	24	**27**
		%	15.8	11.0	11.3
	Grippotyphosa	total	6	38	**44**
		%	31.6	17.3	**18.4**
	Icterohaemorrhagiae	total	5	17	**22**
		%	26.3	7.7	**9.2**
	Shermani	total	—	9	**9**
		%	—	4.0	**3.8**
	Celledoni	total	—	12	**12**
		%	—	5.5	**5.0**
	Australis	total	—	7	**7**
		%	—	3.1	3.0
	Cynopteri	total	2	18	**20**
		%	10.5	8.2	**8.4**
	Pyrogenes	total	—	2	**2**
		%	—	0.9	**0.8**
	Ballum	total	2	28	**30**
		%	10.5	12.7	**12.6**
	Australis (bratislava)	total	—	12	**12**
		%	—	5.5	**5.0**

Over the period, in parks and places of public entertainment in Obolon district 37 wild rodents (31.9% of the total) were trapped and their blood sera samples were analyzed (Table 3).

Table 3 – The results of serological studies of blood sera samples of urban brown rats caught in Obolon district of Kyiv

Years			2014	2015	Total
Studied samples of blood sera			17	20	**37**
Positive results		total	9	11	20
		%	53.0	55.0	**54.0**
Monoreactions, %			55.6	9.1	**30.0**
Mixed reactions, %			44.4	90.9	**70.0**
Positive results (mono + mixed)			9	44	**53**
	Mini	total	2	10	**12**
		%	22.2	23.3	**22.6**
	Pomona	total	1	6	7
		%	11.1	13.6	**13.2**
	Grippotyphosa	total	3	10	**13**
		%	33.4	23.3	**24.6**
	Celledoni	total	—	1	**1**
		%	—	2.3	**1.9**
	Cynopteri	total	1	7	**8**
		%	11.1	16.2	**15.1**
	Ballum	total	2	10	**12**
		%	22.2	23.3	**22.6**

As shown in Table 3, during the whole period of research, the leptospirosis infection in urban brown rats was relatively at the same level (53.0% in 2014 vs. 55.0% in 2015). The percentage of monoreactions sharply reduced by 46.5% (from 55.6% in 2014 to 9.1% in 2015).

During 2014, we have studied 17 samples of blood sera and received nine positive reactions in MAT (53.0%). Leptospira serogroup *Grippotyphosa* was dominant (33.4%). At the second place — *Mini* (22.2%) and *Ballum* (22.2%). The lowest percentage of positive reactions was recorded for serological groups *Pomona* (11.1%) and *Cynopteri* (11.1%).

During 2015, 20 samples were analyzed and 11 positive reactions (55.0%) were received. Among them, prevalent serogroups were the following ones: *Mini* (23.3%), *Grippotyphosa* (23.3%), and *Ballum* (23.3%). Serogroups *Cynopteri* (12.5%) and *Pomona* (13.6%) were registered in significantly lesser amount of cases. This year, antibodies to serogroup *Celledoni* (2.3%) were diagnosed for the first time.

In general, 15 monoreactions were registered in urban brown rats from both areas for two years (Obolon district — 6, Holoseevsky district — 9). The etiological structure for their serogroups is shown in Fig. 1.

As shown in Fig. 1, the monoreactions were detected only to four leptospira serological groups.

In the samples of blood sera of wild rodents, which were trapped in Holoseevsky district of Kyiv, antibodies to serogroups *Grippotyphosa* (44.5%) and *Ballum* (33.3%) prevailed; *Mini* (11.1%) and *Pomona* (11.1%) serogroups were recorded less frequently.

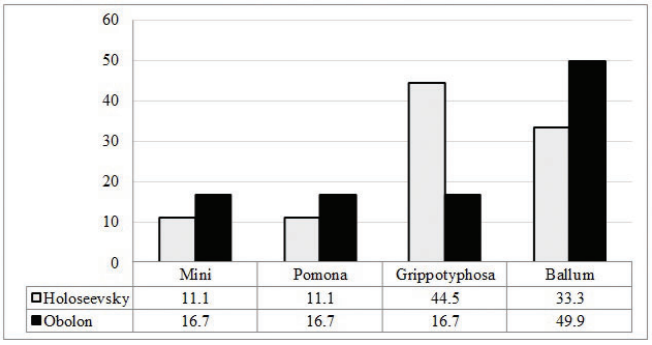

Figure 1. The etiological structure of monoreactions

Among the samples from Obolon district, the serogroup *Ballum* was dominant — it was registered almost half of positive cases (49.9%) of monoreactions. The other half is made up of serological groups *Mini* (16.7%), *Pomona* (16.7%), and *Grippotyphosa* (16.7%).

Thus, based on the analysis of the results, we see both mixed and monoreactions in the samples of blood sera from urban brown rats trapped in Holoseevsky and Obolon districts of Kyiv during 2014–2015. Amount them, serogroups *Mini*, *Grippotyphosa*, and *Ballum* were prevalent. The percentage of positive reactions to each serogroup in the total number of animals reacting shows in Fig. 2.

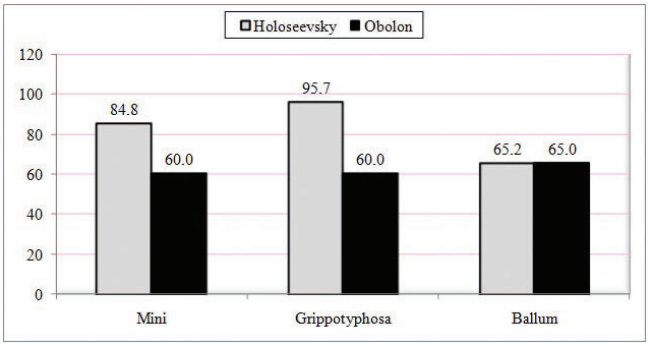

Figure 2. The percentage of each serogroup in the total number of positive reactions

After analyzing Fig. 2, it can be argued that all three serological groups play an important role in the etiology of leptospirosis of urban brown rats in Kyiv. In Obolon district, these groups play almost the same etiological role (12–13 cases of 20 positively reacting animals). In Holoseevsky district *Grippotyphosa* and *Mini* were dominating (44 and 39 cases of 46 positively reacting animals). At the same time, serogroup *Ballum* was diagnosed in 65.2%.

Discussion. Leptospirosis is the most wide spread zoonosis worldwide, which is present in all continents

except Antarctica (Adler and la Peña Moctezuma, 2010) and evidence for the carriage of *Leptospira* has been found in over 150 mammalian species (Ko, Goarant, and Picardeau, 2009).

Animals, including humans, can be divided into maintenance hosts and accidental (incidental) hosts. The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts (Babudieri, 1958). A maintenance host is defined as a species in which infection is endemic and is usually transferred from animal to animal by direct contact. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans. The extent to which infection is transmitted depends on many factors, including climate, population density, and the degree of contact between maintenance and accidental hosts. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroups *Icterohaemorrhagiae* and *Ballum*, and mice are the maintenance hosts for serogroup *Ballum* (Levett, 2001).

On the whole, we have studied 116 samples of the blood sera from urban brown rats: Holoseevsky district — 79, Obolon district — 37. In both areas, the percentage of positively reacting rodents is rather high (58.2% and 54%) that correlates with studies of urban brown rats in other countries (Costa et al., 2014; Easterbrook et al., 2007; Himsworth et al., 2013; Koizumi et al., 2009; Vanasco et al., 2003).

Our research on the samples of blood sera from urban brown rats in Kyiv showed the presence of diagnostic titers of *Leptospira* antibodies to the 14 serogroups in Holoseevsky district and to 6 — in Obolon district of 21 serological groups used for the study. Serogroups *Grippotyphosa*, *Mini*, and *Ballum* were dominating in the structure of positive reactions in both districts as well as in most countries (Levett, 2001).

In Holoseevsky district, antibodies to serogroups *Pomona* (11.3%), *Icterohaemorrhagiae* (9.2%), *Cynopteri* (8.4%), *Celledoni* (5%) and *Australis* (5%) (serovar *bratislava*) were less common. At the same time, in Obolon district serogroups *Cynopteri* (15.4%) and *Pomona* (11.5%) were recorded.

As we see, *Icterohaemorrhagiae* serological group was found only in the samples of blood sera from Holoseevsky district and played a minor etiologic role, despite the fact that urban brown rats are considered as its main carriers (Aviat et al., 2009; Golding, 1990; Inada et al., 1916; Ko, Goarant, and Picardeau, 2009; Levett, 2001; Noguchi, 1917; Waitkins, 1991).

Antibodies to other serological groups, such as *Javanica, Sejroe, Hebdomadis, Shermani, Australis* (serovar *erinaceieuropaei*), and *Pyrogenes*, had been registered less often.

The percentage of mixed reactions is significantly higher than monoreactions in both areas (80.4% vs. 19.6% in Holoseevsky district; 70% vs. 30% in Obolon district).

Antibodies to serological groups were found in MAT in various diagnostic titers: from 1/50 to 1/2,500. Their structure in positive reactions was the fallowing: 1/50 — 14.7%, 1/100 — 30.1%, 1/500 — 40.8%, and 1/2,500 — 14.4%.

The circulation of the pathogenic leptospira among the population of urban brown rats in Kyiv was indicated by the analysis of the research data and by systematization of the data.

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THE VALIDATION OF ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DIAGNOSIS LEPTOSPIROSIS AMONG DOGS, PIGS AND CATTLE

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Summary. This study was aimed to develop a modification of ELISA for the diagnosis of leptospirosis in animals that would correspond to the following requirements:

1) ability to detect antibodies to all serogroups of *Leptospira* that are recommended for the diagnosis of this zoonoses in Ukraine (high sensitivity and specificity);

2) minimization of the risk of laboratory-acquired infection at the preparation of reagents for ELISA and its conducting.

The article presents the results of validation of ELISA for the diagnosis of leptospirosis among dogs, pigs, and cattle. During the validation, several technological stages were conducted. The first stage was carried out by selection and determination of the optimal concentration and titers of the main components of ELISA (antigen, enzyme conjugate, enzyme substrate, and blocking substance), and the second stage — by statistical analysis of the results after testing ELISA on the reference panel of sera blood samples.

Established that the sensitivity of the developed ELISA test is less than its specificity (89.8% against 96.7%), but the general efficiency of this method is high and equals 93.6%.

Keywords: leptospirosis, microscopic agglutination test, enzyme-linked immunosorbent assay, validation, titer

Introduction. Leptospirosis is the most wide spread zoonosis worldwide, which is present in all continents except Antarctica and evidence for the carriage of Leptospira has been found in virtually all mammalian species examined (Adler and la Peña Moctezuma, 2010).

The wide spectrum of symptoms confuses the clinical diagnosis and makes it undependable. The laboratory diagnosis of leptospirosis, a prerequisite for treatment, is usually achieved either by isolation of the causative organisms or by serological evidence indicating recent infection (Sharma et al., 2007). The microscopic agglutination test (MAT) is the reference test for diagnosis and detects antibodies at serovar levels (Levett, 2001). MAT has many advantages, but there are significant deficiencies. The maintenance of stock cultures and use of live organisms creates a risk of laboratory-acquired infection (Wautkins and Zochowski, 1990). Therefore, several methods have been developed for use in diagnosis of leptospirosis as an alternative to MAT, of which IgM ELISA is the most promising and detects genus-specific antibodies (Kurstak, 1985; Sharma et al., 2007; Bolin, 2008).

ELISA was also developed in the Laboratory of Leptospirosis of Farm Animals of the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences (IVM NAAS) for identifying the immunoglobulins G in the blood sera samples of cattle. Inactivated serovar wijnberg (serogroup Icterohaemorrhagiae) was used as the antigen. However, it has not gained widespread among specialists of veterinary medicine in Ukraine and needed, like MAT, working with pathogenic Leptospira culture (Ivanska et al., 2003).

The aim of this work is to develop a modification of ELISA for the diagnosis of leptospirosis in animals that would correspond to the following requirements:

1) ability to detect antibodies to all serogroups of Leptospira that are recommended for the diagnosis of this zoonoses in Ukraine (high sensitivity and specificity);

2) minimization of the risk of laboratory-acquired infection at the preparation of reagents for ELISA and its conducting.

Materials and methods. During the validation, we have conducted several technological stages. The first stage was carried out by selection and determination of the optimal concentration and titers of the main components of ELISA (antigen, enzyme conjugate, enzyme substrate, and blocking substance), and the second stage — by statistical analysis of the results after testing ELISA on the reference panel of sera blood samples.

To perform this research, ELISA was carried out with field samples of blood sera from healthy and sick with leptospirosis dogs, pigs and cattle, and seven samples of reference sera (OIE), that were obtained from the Royal Tropical Institute, Amsterdam.

The research had been carried out during the 2013–2015 in the Laboratory of leptospirosis of farm animals

of the IVM NAAS and in the Ukrainian Laboratory of Quality and Safety of Agricultural Products (ULQSP APC).

Results and discussion. Recombinant protein LipL 32 was used as the antigen for ELISA. It is a modified analog one of the major outer membrane lipoprotein of Leptospira (Tokuda, 2009). It is experimentally proved, that this protein is a part of the outer membrane only in pathogenic Leptospira species and is a secure to use for laboratory staff (Tokuda, 2009; Murray et al., 2009). All these qualities contribute to its widespread in the formulation of leptospirosis diagnosticums in different countries (Bomfim, Ko, and Koury, 2005; Sharma et al., 2007).

To determine the optimal dose of sensitizing antigen, we conducted its sorption within 0.5–4 µl volume in carbonate-bicarbonate buffer (CBB), pH 9.6. At the same time we have tested substances that eliminate the nonspecific interaction of antibodies with antigen-edible gelatin and skimmed milk powder (respectively, 0.5% and 5% solutions). This combination allows prevention false reactions and reduces the time for titration of the components.

The results of antigen titration with different blocking substances are shown on Fig. 1 and Fig. 2.

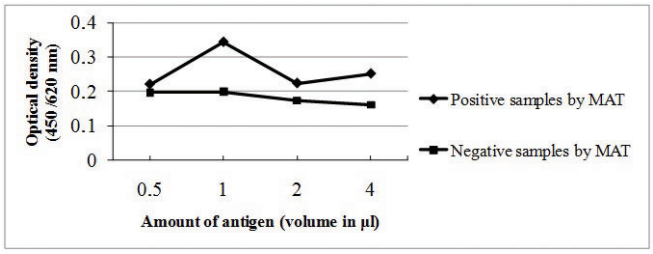

Figure 1. The sorption curve of antigen on polystyrene microplates with the use of 5% solution of skimmed milk powder

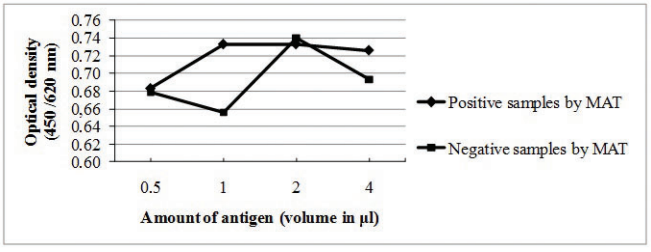

Figure 2. The sorption curve of antigen on polystyrene microplates with the use of 0.5% solution of gelatin

As the analysis of titration had shown, the optimum amount of LipL 32 in both cases was 1 µl volume. With such amount of antigen the high correlation coefficients were observed between the indicators of optical density in positive and negative blood sera samples by MAT, representing, respectively, 1.73 and 1.12.

However, in all investigated wells of microplate the high background reactions were registered when dilution of blood sera samples by phosphate buffer with 0.5% gelatin solution. Specifically, when the amount of antigen was 2 µl, the value of optical density of the negative control was higher than of the positive (respectively, 0.74 o. d. and 0.733 o. d.). At the same time, when a 5% skim milk solution was used, the indicators of the optical density of negative controls were much smaller, and the correlation coefficients of positive and negative blood sera samples were higher (Figs 1–2).

As a result of the previous research on the comparison of enzyme conjugates directed to different classes of immunoglobulins, it was found that the conjugate based on recombinant protein LipL 32 with horseradish peroxidase as the enzyme label (directed to Ig M and Ig G) is much more effective in the diagnosis of leptospirosis than the conjugate based on recombinant protein G of Streptococcus spp. and protein A of Staphylococcus aureus (directed on Ig G) (Pyskun, 2015). The titration of enzyme conjugate, based on recombinant protein LipL 32, and determination of the optimal dilution of blood sera samples for statement of ELISA with it, were conducted (Tabs 1–2).

The range of the titration for determination of the optimal blood sera dilution in ELISA was between 1/2.5–1/40 (2.5–40 µl volumes of blood sera) (Tab. 2). It was selected to provide the convenience for the further conducting an ELISA, for the reason that the process of the blood sera collecting (by doser) in volume less than 2 µl, and contributing to its homogenous dissolution in 98 µl of solution for the sample dilution is a very laborious process. At the same time, there is a high probability of background reactions in negative controls, *in case if* it is necessary to add blood sera in volume more than 40 µl in wells of microplate.

The results of titration are shown on Fig. 3 and Fig. 4.

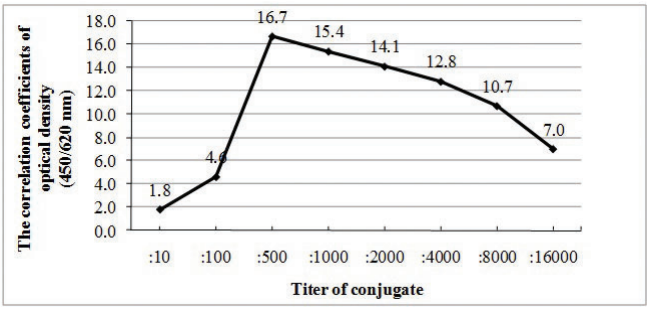

Figure 3. Dependence of the correlation coefficients of optical density between positive and negative samples on the titers of conjugate

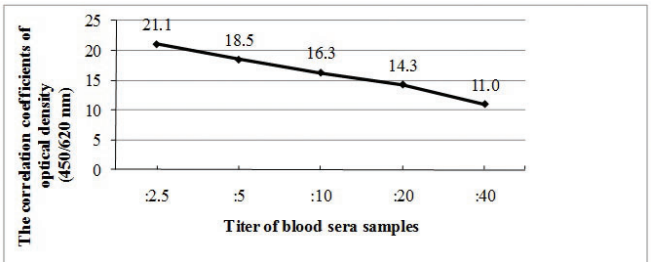

Figure 4. Dependence of the correlation coefficients of optical density between positive and negative samples on the titers of blood sera

The results of conjugate titration had shown, the highest correlation coefficients of optical density between positive and negative samples of blood sera were registered in its titer 1/500 and dilution of sera 1/2.5 (40 µl volume). Coefficients were, respectively, 16.7 and 21.1 (Figs 3–4).

Beyond that, the specific reagent in the development of ELISA is an enzyme substrate (substrate-chromogen). Due to the enzymatic reaction of conjugate with the substrate by using chromogen, the reaction products become colored that enables visually or automatically evaluate the presence of antibodies in the test material.

Table 1 – The results of the titration enzyme conjugate based on recombinant protein LipL 32

The titer of blood sera (OIE) by MAT	Indicators of optical density at 450/620 nm in a different dilutions							
	1:10	1:100	1:500	1:1000	1:2000	1:4000	1:8000	1:16000
Grippotyphosa ++ 1:4000	3.981	3.429	2.580	2.430	1.952	0.833	0.561	0.211
Icterohaemorrhagiae ++ 1:8000	3.320	3.111	2.133	1.721	1.514	1.128	0.623	0.114
Sejroe ++ 1:32000	2.247	1.914	1.052	0.613	0.543	0.327	0.221	0.094
Tarassovi ++ 1:16000	2.671	2.113	1.370	0.945	0.613	0.333	0.228	0.107
negative sample	1.706	0.573	0.107	0.092	0.082	0.051	0.038	0.019

Table 2 – The results of titration blood sera samples for statement ELISA with conjugate based on recombinant protein LipL 32

The titer of blood sera (OIE) by MAT	Indicators of optical density at 450/620 nm in a different dilutions				
	1:2.5	1:5	1:10	1:20	1:40
Grippotyphosa ++ 1:4000	2.412	2.121	1.921	1.66	1.23
Icterohaemorrhagiae ++ 1:8000	2.745	2.192	1.85	1.441	0.733
Sejroe ++ 1:32000	1.721	1.268	0.922	0.712	0.512
Tarassovi ++ 1:16000	1.32	0.947	0.647	0.422	0.22
negative sample	0.097	0.088	0.082	0.074	0.061

The o-phenylenediamine (OFD) and tetrametilbenzidin (TMB) are the most sensitive solutions of substrate-chromogens that are used for conducting ELISA nowadays. So we compared the obtained results of ELISA with these compounds (Tab. 3).

As the comparison of the enzyme substrates showed, the indicators of the optical density in the positive samples of blood sera by MAT from all species and in reference to blood sera were significantly higher by using TMB solution than indicators by using OFD chromogen, respectively, 0.836 ± 0.089 o. d. against 0.601 ± 0.066 o. d. and 1.490 ± 0.14 o. d. against 1.069 ± 0.13 o. d. The difference in optical values in both cases was significant ($p < 0.05$). At the same time, the optical indicators of the negative blood sera samples by MAT were not significantly different in both cases (0.098 ± 0.002 o. d. against 0.085 ± 0.0017 o. d.).

Thus, at the end of the first stage in the development of ELISA, we made the selection of antigen, enzyme conjugate, blocking reagent and chromogenic substrate, and established their optimum concentration.

The last technological stage was carried out by a statistical analysis of the results after testing of ELISA on the panel of blood sera samples.

Table 3 – The results of comparison the sensitivity of enzyme substrates OFD and TMB

The positive and negative by MAT blood sera samples	The number of samples	The mean values of optical density, o. d.	
		TMB	OFD
The positive blood sera samples from dogs	5	0.330	0.195
The negative blood sera samples from dogs	3	0.101	0.094
The positive blood sera samples from pigs	19	0.591	0.491
The negative blood sera samples from pigs	6	0.096	0.083
The positive blood sera samples from cattle	12	1.436	0.943
The negative blood sera samples from cattle	2	0.099	0.082
Reference blood sera (OIE)	7	1.490	1.069

To perform this phase of research, we used the blood sera panel that consisted of 128 positive samples (including 7 reference sera OIE) and 152 negative samples by MAT. The results of research are shown in Table 4.

As shown in Table 4, the indicators of the optical density in blood sera samples from dogs, pigs and cattle were increased respectively to antibodies titers by MAT. At the same time, the difference between them was highly significant in most cases ($p < 0.001$).

The limits and the average values of the optical density within each group of the blood sera by MAT were similar to each other in all of the listed species. Based on the obtained results, we have decided to calculate the statistical indicators of ELISA simultaneously by values of optical density of blood sera samples from all three species and the reference sera (OIE).

For further research, it was necessary to find with accuracy the determination of the limits of optical density indicators that interpreted as true or false. For this purpose, we used the method «cut-off», on the basis of which Table 5 was formed.

Table 4 – The results of testing ELISA on the panel of blood sera samples

The species of animal	The titers of antibodies by MAT	The number of samples	The values of optical density, o. d.	
			Lim	M ± m
Dogs	negative samples	48	0.079–0.125	0.101 ± 0.00076
	Monoreaction in titer ++1:50 – ++1:100	17	0.116–0.401	0.194 ± 0.012***
	Mixed reactions in titer ++1:50 – ++1:100	12	0.124–0.428	0.266 ± 0.015***
	Monoreaction in titer ++1:500 – ++1:2500	4	0.361–1.116	0.672 ± 0.08***
	Mixed reactions in titer ++1:100 – ++1:2500	4	0.763–3.175	1.762 ± 0.325*
Pigs	negative samples	53	0.075–0.129	0.102 ± 0.0008
	Monoreaction in titer ++1:50 – ++1:100	16	0.122–0.326	0.182 ± 0.0094***
	Mixed reactions in titer ++1:50 – ++1:100	9	0.136–0.425	0.246 ± 0.018**
	Monoreaction in titer ++1:500 – ++1:2500	11	0.117–1.1	0.508 ± 0.035***
	Mixed reactions in titer ++1:100 – ++1:2500	8	0.681–3.138	1.831 ± 0.192***
Cattle	negative samples	51	0.071–0.124	0.102 ± 0.0023
	Monoreaction in titer ++1:50 – ++1:100	14	0.119–0.392	0.212 ± 0.013***
	Mixed reactions in titer ++1:50 – ++1:100	13	0.122–0.505	0.321 ± 0.017***
	Monoreaction in titer ++1:500 – ++1:2500	6	0.119–0.945	0.483 ± 0.05**
	Mixed reactions in titer ++1:100 – ++1:2500	7	0.823–3.345	1.647 ± 0.239***
blood sera (OIE)	Monoreaction in titer ++1:4000 – ++1:32000	7	0.698–2.583	1.52 ± 0.139

Footnote: * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$

Table 5 – The results of comparison developed ELISA with MAT

The positive and negative blood sera samples by ELISA	The positive and negative blood sera samples by MAT	
	MAT+	MAT–
ELISA+	115	5
ELISA–	13	147

Analyzing the results, the indicators of diagnostic sensitivity (D-SN), specificity (D-SP) and overall efficiency (Ef) were calculated by the following formulas.

Diagnostic sensitivity:

$$D-SN=\frac{115}{(115+3)}\times 100=89.8\%$$

Diagnostic specificity:

$$D-SP=\frac{147}{(147+5)}\times 100=96.7\%$$

Overall efficiency:

$$Ef=\frac{(115+147)}{(115+5+147+13)}\times 100=93.6\%$$

Also, the additional efficiency indicators were calculated: the predictive values of positive (95.8%) and negative (91.9%) tests, the Youden's index (0.865), and the likelihood coefficients of positive (27.2) and negative (0.105) results.

Conclusions. 1. The selection of the main components for conducting ELISA for leptospirosis and determination of the optimal concentration were done.

2. Established that the sensitivity of the developed ELISA is less than its specificity (89.8% against 96.7%), but overall efficiency of this method is high and equal 93.6%.

3. The predictive values of positive and negative tests are high and equal, respectively, 95.8% and 91.9%.

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Part 2. Biotechnology

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REPRODUCTION CAPABILITY OF BOVINE LEUCOSIS FIELD ISOLATES IN SOME CELL CULTURES

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Summary. The article observes capability of cow embryo lung (CEL), calf coronary vessel (CVC), sheep kidney (SK), and calf kidney (CK) passaged cells cultures to reproduce bovine leukemia virus after integration of pathogen field isolates. The presence of pathogen proviral DNA was detected by molecular-genetic studies at different cultivation stages. Increasing of hemagglutinin titer in CVC, SK, CK, and CEL cell cultures was established in between 5–6 passages.

Keywords: leucosis, cattle, cells cultures, CVC, SK, CK, field isolates, reproduction capability

Kidney of a sheep embryo, chronically infected by bovine leukemia virus (FLK-BLV), is used in developing of specific disease prophylactic methods, as well as in manufacturing derivation of leucosis antigen that is a part of diagnostic kit for leukemia infected animals detection (Busol et al., 2000; Burba et al., 1996; Stegniy et al., 2011; Tsymbal et al., 1990). Solving the problem of practical veterinary medicine support with facilities for leukemia diagnosis involves searching of more effective ways of viral biomass accumulating in the technological scheme of drugs production. The quantitative detection of virus accumulating in culture medium is provided by its activity analysis after concentration (Stegniy et al., 2011; Tsymbal et al., 1990). The results of some authors (Syurin et al., 1998) obtain the possibility to draw a conclusion about presence of bovine leukemia virus pathogen in culture media by its capability to agglutinate the mice erythrocytes.

The purpose of our research is to integrate the BLV field isolates in separate cell cultures that are homology for correspondent animal species with the aim of pathogen adaptation, and accumulation of viral biomass for further practical use.

Materials and methods. CEL, CVC, SK, and SK-2 passaged cultures that are kept in cryobank of NSC 'IECVM' biotechnology laboratory has been taken for study. The cell cultures, derived from cryobank, were adapted to the nutritional medium in three passages. At that time, the morphofunctional characteristics of cultures had been studied. Three flasks of each culture were used for infection contamination. The preparation of infected material was begun from blood sampling, stabilized with 3.8% sodium citrate, in sterile-assembled blood collection systems. The cows infected with bovine leukemia virus (and positive by the results of immunodiffusion test) with clinical features of lymphoid malignancy (with haematological illness based on the results of the conducted pilot research) have been used as blood donors. The amount of leucocytes in blood of animals-donors at the time of sampling were 18.4–19.4 g/sm^3, the ratio of lymphocytes in leucocytic fraction was 87–92%. The leucoconcentrate was collected by providing of erythrocyte lysis using 0.83% ammonium chloride solution during 10 min at room temperature 20–22 °C.

The obtained material has been washed off ammonium and erythrocytes vestiges three times by physiological saline and with addition of bacteriostatic medications. The separate samples of leucoconcentrate were mixed. Working suspension was obtained by leucoconcentrate and physiological saline mixing. The method of supravital staining by 0.1% solution of trypan blue was used for accounting the saved cells. Working suspension contained 72,000 of live cells in 1 sm^3.

Leucoconcentrate was controlled for sterility according to DSSU 4483:2005.

The suspension was divided into two parts after cell account. The first part of native leucocytes was passaged into two flasks of each cell culture in a dose of 72,000 of live cells in 1 sm^3, one flask of each culture was left as a positive control. Nutritional culture media included 50% of Eagle medium and 50% of 199 medium without addition of blood serum. Plating was incubated at 37 °C for 48 h in the germinating apparatus.

 [1] Scientific mentor — Prof. A. P. Gerilovych

The second part of leucoconcentrate was divided into three parts.

The first part was subjected to osmotic lysis using distilled water at 3–4 °C for 30 min.

The second part was defrosted at (–20 … +20) °C.

The third part was mixed with Versene solution for preventing leucocytes adhesion. Then it was subjected to disintegration with intensity at 4.5 kHz, synchronization at 4 for 1 min (duration of each step was 30 s) in ice cooling regimen with the use of disintegrating machine UZDU-A.

All three parts were mixed in total capacity, where the general protein content was detected. The suspension was used for infection of one flask of each used cell culture with leucoconcentrate in a dose of 0.22% by general protein content in 1 sm^3 of culture medium. The infected cell cultures were incubated in culture medium with equal content of Eagle and 199 media without addition of blood serum for 48 h at 37 °C. The cattle blood serum was added to the culture medium in next passages, its ratio was slowly increased from 1–2% to 10%. The culture medium contained maximal concentration of cattle aglobuline blood serum at the sixth passage — 10%.

The control of antigen-production activity of cell cultures was provided using concentration of virus fluid after 3^{rd}, 5^{th}, 10^{th}, 12^{th}, 15^{th} passages for the purpose of antigen activity detection. At the same time, the part of virus fluid from separate passages was investigated for proviral DNA detection using molecular-genetic method (PCR).

The culture swabs has been investigated for leukemia virus presence using hemagglutination reaction with 1.5% suspension of mice erythrocytes. The suspension of erythrocytes opposite to physiological saline was used as negative control to exclude the spontaneous erythrocytes autoagglitination phenomena.

Isolation of total DNA was provided using commercial DNA kit 'Sorb-A' by firm 'Amplisens' (Moscow, Russian Federation), amplification was provided using commercial kit 'GenePak DNA PCR test' by firm 'Isogene Lab. Ltd' (Moscow, Russian Federation).

Electrophoresis assay was provided using electrophoresis kit by scientific-production organization 'Narvac' (Moscow, Russian Federation). The agarose concentration in gel was 1.5%, current electric intensity was 40 mA, voltage was 75 V.

Results. The visual estimation of cell cultures CVC, SK-2, CK, and CEL was provided before integration of native or lysed leucocytes. In all cases it showed that cell monolayer at 4–5 days of cultivating was uniform, separate peripheral cells were oblong and some of them were spherical, the regions without monolayer were particularly absent. Integration of lysed and native leucocytes from blood of animal, in which the clinical form of lympholeucosis was registered, was provided in mentioned cultures without cell monolayer violation. The visual estimation of experimental and control cell cultures was provided upon completing the second and following passages using inverted microscope at ×7.

The monolayer integrated by native leucocytes after the second passage in SK-2 cell culture was insufficient, a goodly proportion of cells was self-standing, separate parts of monolayer exfoliated and were present in culture fluid. The same cell cultures after integration of lysed leucocytes were marked by fulfill monolayer with good-visible cell borders via cell density. The last cells with normal morphology, separate from monolayer and with frank destruction have been rarely found. The control SK-2 cell culture showed density monolayer, the cells saved their normal morphology.

The CK cell culture, integrated with native leucocytes, was marked by well-visible monolayer with oblong cells. However, it was violated in some areas. Herewith, the separate cells with destructive changes were seen per visual field.

In turn, the CK cell culture, integrated with lysed leucocytes, was marked by uniform monolayer with good-visible cell borders. Cells with destructive changes have been rarely seen per visual field.

The monolayer of control CK cell cultures was scarcely no different from the monolayer of matrix initial passaged culture. It was uniform, mosaic, its cells had normal morphology, and cells that separate from monolayer were rarely seen per visual field.

The monolayer of CVC culture, integrated with both lysed and native leucocytes was marked by uniform fullness. The cells had normal morphology; destructive changes were rarely fixed among separate cells. The distinct destructive changes of control cell cultures monolayer were not found during visual estimating.

The destructive changes of CEL cell culture morphology after integration of native leucocytes were not found.

The monolayer cells were situated closely, with distinct borders. Vacuoles and oblong nucleuses were rarely occurred.

Similar passages estimating (monolayer structure, cells characteristics) was provided during 5–6 passages. It was fixed, that monolayer of all used cultures was marked by more clear areas with entirety violations with each subsequent passage. It fixed to the glass weaker and moved off easier. The number of cells with destructive violations increased. When estimating further passages, it was established the appearance of 'recovery' characteristics of cell cultures: the monolayer became more uniform, complete, the number of cells with destructive changes decreased.

In all cases when working with CVC, SK, CK, and CEL cell cultures, the syncytium formation was not observed. It only was noticed the appearance of separate cells with two or three nucleuses.

The culture fluid with cell monolayer was concentrated after the fifth passage according to the technology of leucosis antigen manufacturing. It was examined for activity in a standard immunodiffusion test with positive leukemic serum. Precipitin line on 48–72 h exposure was not observed.

The part of virus fluid of CVC and SK cell cultures was PCR-tested for proviral DNA detection after 4–14 passages in the NSC 'IECVM' laboratory of molecular biology. The results are shown in Table 1.

The results of the research conduct that samples number 2, 4, 5, 8, 9, 10 contain proviral DNA of bovine leukemia virus. The samples number 1, 3, 6, 7 has shown negative result.

Obtained results indicate the persistence of bovine leukemia virus to the 14th passage in CK cell culture, infected with native leucocytes; to the 12th passage in CK cell culture, infected with lysed leucocytes; and to the 4th passage in CVC cell culture, infected with lysed leucocytes.

Table 1 – Indication of proviral DNA in separate passages of cell cultures

№	Cell culture	Integrated leucocytes	Passage	Presence of BLV proviral DNA
1.	CVC	Control	4	–
2.	CVC	Lysed	4	+
3.	CVC	Lysed	6	+
4.	SK	Lysed	6	+
5.	SK	Lysed	13	+
6.	SK	Lysed	12	+
7.	SK	Lysed	14	–
8.	SK	Native	14	+
9.	SK	Lysed	13	–
10.	FLK-BLV	Control	14	+

Table 2 – Indication of BLV in cell cultures using haemagglutination test

№ of the passage	Antigen titer dilutions in haemagglutination test							
	SK		CK		CVC		CEL	
	Lysate	Native leucocytes	Lysate	Native leucocytes	Lysate	Native leucocytes	Lysate	Native leucocytes
0.	1:128	1:256	1:128	1:256	1:128	1:256	1:128	1:256
1.	1:8	1:16	–	1:8	–	–	_	1:16
2.	–	–	–	–	–	–	-	-
3.	–	–	–	–	–	–	-	-
4.	–	–	–	–	–	–	-	-
5.	–	1:8	1:8	1:8	–	–	1:8	-
6.	1:8	1:16	1:32	1:64	–	1:32	1:8	1:16
7.	1:16	1:64	1:16	1:64	1:8	1:8	1:4	1:8
8.	1:64	1:128	1:64	1:128	1:16	1:32	-	1:4
9.	1:64	1:128	1:128	1:64	Not cultivated	Not cultivated	Not cultivated	Not cultivated
10.	1:64	1:32	1:128	1:128	Not cultivated	Not cultivated	Not cultivated	Not cultivated
11.	1:256	1:128	1:256	Not cultivated	Not cultivated	Not cultivated	Not cultivated	Not cultivated
12.	1:128	1:256	Not cultivated	Not cultivated	Not cultivated	Not cultivated	Not cultivated	Not cultivated

Unfortunately, setting the proportion of virus in culture fluid in separate passages using the results of leukemia antigen activity in immunodiffusion test failed. We provided investigation of culture virus fluid using haemagglutination reaction with mice erythrocytes in order to determine this question. The results of this study are shown in Table 2.

The table shows, that culture fluid, obtained from 5–6 passages, contains antigen, agglutinating mice erythrocytes in 1:8–1:64 titer. Following passages increase titers. Titers of SK and CK cultural fluids were 1:128–1:256 at 11th passage. The SK and CK cultures have been more sensitive to infection and more addiction to virus adaptation. The CVC and CEL cultures have been not so sensitive to infection; haemagglutination test had shown the antigen presence only in 1:16–1:32 titer.

The negative result has been obtained in all control samples. It gives the evidence of the autoagglutination absence.

Conclusions.

1. The results of molecular-genetic analyses for BLV proviral DNA presence has shown that DNA fixes in CVC passaged culture to 4th passage, in SK culture with native lymphocytes to 14th passage, and with lysed lymphocytes to 12th passage.

2. Increasing of antigen concentration in culture fluids of SK, CK, and CVC passage series has been shown in haemagglutination test with mice erythrocytes.

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SNPS OF CALPAIN/CALPASTATIN SYSTEM GENES IN COMMERCIAL POPULATION OF ABERDEEN ANGUS IN KHARKIV REGION, EASTERN UKRAINE

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Summary. The calpain proteolytic system — the micromolar calcium-activated neutral protease μ-calpain encoded by the CAPN1 gene, and its inhibitor — calpastatin — encoded by the CAST gene, is involved in biological processes regulation by partial degradation of substrates — cytoskeletal proteins, signaling molecules and enzymes. Calpain system dysfunction caused by mutations or Ca^{2+} elevation is associated primarily with diseases affecting central nervous and cardiovascular systems, as well as skeletal muscles. For cattle breeding purposes the calpain hyperactivity in the presence of excess calcium ions (calcium passes out due to cell death) is considered to be desirable offering the possibility to obtain more tender meat. This study is aimed to analyze the polymorphic variants *CAPN316* and *CAST282* of calpain and calpastatin genes in Aberdeen-Angus bred within Kharkiv region and to compare obtained result with commercial herds of other countries.

For the SNP genotyping, PCR-RFLP methods were set up. Testing deviation from the Hardy-Weinberg equilibrium was performed using Pearson's chi-squared test. Spearman's correlation coefficient was used to measure the strength of association between two characteristics. Cluster analysis was used to classify the allele frequencies obtained within similar data obtained for cattle in other countries.

The allele and genotype frequencies of SNP *CAPN316* (AF252504.2:g.5709C>G) in calpain gene are: *C* — 0.398 and *G* — 0.602; *CC* — 13.6%, *CG* — 52.3% and *GG* — 34.1%. The allele and genotype frequencies of SNP *CAST282* (AY_008267.1:g.282C>G) in calpastatin gene are: *C* — 0.807 and *G* — 0.193; *CC* — 63.6%, *CG* — 34.1% and *GG* — 2.3%. Group-to-group variability in allele *C* frequency for SNP *CAPN316* (0.37%) is higher than *CAST282* (0.06%), due to the modulating effect of the calpastatin. The Aberdeen-Angus herd of Kharkiv region are comparable to the European commercial beef cattle herds by allele frequencies. Association between latitudinal zonation and frequency of allele C in cattle herds was demonstrated ($R = 0.53$, $p < 0.05$).

Keywords: Aberdeen-Angus breed, calpain gene, calpastatin gene, SNP, meat tenderness

Introduction. Mammalian calpain protease family includes more than 15 types of enzymes, being universal calcium-dependent, non-lysosomal cysteine proteases, acting as modulators of cell molecules function by selective degradation of proteins (Smith and Schnellmann, 2012). Calpains are ubiquitously present in cells and tissues of vertebrates as different isoforms (Goll et al., 2003). Calpain substrates are enzymes, signaling molecules and cytoskeletal proteins. Calpain activation occurs at neutral pH and depends on free calcium level. Different calpains require micromolar (μ-calpain) or millimolar (m-calpain) Ca^{2+} concentration (Grebinyk, 2012). Calpastatin is a specific inhibitor of calpain, activated by Ca^{2+} concentration required to achieve the half of μ-calpain maximal activity. Skeletal muscle calpain/ calpastatin system includes three proteases — calpain I (μ), calpain II (m), and calpain 3 (p94), and their inhibitor calpastatin. The large catalytic subunit of calpain is encoded by three genes *CAPN1*, *CAPN2*, *CAPN3* and small regulatory subunit is encoded by *CAPN4* gene (Nowak, 2011).

Calpains are involved in biological processes regulation such as cellular differentiation, apoptosis, synaptic transmission, muscle protein metabolism etc.

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(Sato and Kawashima, 2001), therefore calpain activity dysfunction mediated by calcium metabolism disorders or by mutations in calpain or calpastatin genes will be accompanied by pathological condition (Sakamoto et al., 2013). Increased calpains levels have been detected in various pathophysiological processes such as ischemia-induced tissue damage of heart (e.g., myocardial infarction), kidney, lung, liver or central nervous system (e.g., stroke), inflammations, muscular dystrophies, cataracts, diabetes, Alzheimer's disease, Huntington's disease, Parkinson's disease, multiple sclerosis (Goll et al., 2003), and tumors of the urinary system and bladder in cattle (Roperto et al., 2010), infectious diseases such as malaria and diseases caused by protozoa (Samanta et al., 2012; Li et al., 2007).

Investigating calpain system in animals as a model objects allows to simulate the physiological processes at the cellular and organismal level providing the high proteolytic activity of calpain in humans, additionally, function of calpain system has an economic value in cattle breeding. An important aspect of the calpain and calpastatin localized in the cattle muscle tissue is impact on meat quality expressed as tenderness appearing during postmortem proteolysis. After cell death Ca^{2+} concentration gradually increases and reaches the sufficient level for calpain activation, initiating the destruction of the muscle fibers in the lateral and transverse sections. Animals selection based on meat tenderness without molecular genetic studies is difficult, because this feature is not possible to estimate during animal's life. In this paper we analyzed *CAPN1* gene as the enzyme encoded by this gene is activated by less Ca^{2+} concentration than needed for calpain II activation. Calpain II is predominantly localized in the cytosol, but 70% of calpain I is bound with myofibrils — that ensures more effective scaffold proteins destruction during postmortem proteolysis. Since the calpain I is suppressed by calpastatin *CAST* gene was included in analysis too.

There are several SNP of *CAPN1* and *CAST* studied in cattle worldwide up to date: *CAPN316* (AF252504.2:g.5709C>G), *CAPN530* (AF_288054.2:g.4558G>A), *CAPN4751* (AF_288054.2: g.6545C>T), *CAST2959* (AF_159246.1:g.2959A>G), *CAST2870* (AF_159246.1:g.2870A>G), *CAST282* (AY_008267.1:g.282C>G) (Gill et al., 2009; Ribeca et al., 2009). However, the studies of these genes in cattle within neighboring countries are of inadequate attention. In Ukraine, SNP *CAPN530* was studied for six beef breeds (Dobrianska et al., 2013), in Russia - SNP *CAPN316* for Kalmyk cattle (Kosyan et.al., 2012). In Czech Republic, SNP CAPN1 c.947G> C and CAST c.155C>T were studied for seven beef breeds (Kaplanová et.al., 2013). The search and selection of candidate loci for *CAPN1* and *CAST* genes is carried out in Poland (Juszczuk-Kubiak et al., 2004) and in other countries (Barendse et al., 2007). Papers concerning the calpastatin gene in Ukrainian cattle or cattle of CIS countries were not found.

The aim of this study was to analyze the allele frequencies of genotypes for a polymorphic variant gene CAPN316 CAPN1 and CAST282 CAST gene in a group of animals Aberdeen-Angus.

This study is aimed to analyze the polymorphic variants *CAPN316* and *CAST282* of calpain and calpastatin genes in Aberdeen-Angus bred within Kharkiv region and to compare obtained result with commercial herds of other countries.

Material and methods. The study object were Aberdeen-Angus cattle (n = 88; cows: n = 82, bulls: n = 6) bred at PE 'Agrofirma Svitanok', Kharkiv region, Ukraine. DNA was extracted from blood samples using DNA extraction kits 'Diatom DNA Prep 100' ('Isogene', Russia). For the SNP genotyping, PCR-RFLP methods were set up, using primer pairs (Miquel et al., 2009; Schenkel et al., 2006). Restriction enzymes used were endonuclease RsaI and BtgI ('Fermentas', Lithuania). The digested fragments were electrophoresed on 2.0% agarose gel.

The deviation of allele frequencies from Hardy-Weinberg equilibrium was tested using Pearson's chi-squared test. For data distribution it was used normality test assessment. Spearman's correlation coefficient was used to measure the strength of association between two characteristics. The distance between two clusters in dendrogram was defined as an average distance between all pairs of objects therein, using sizes of the respective clusters as a weigh coefficients and Euclidean metric as a distance function (Khalafyan, 2007).

Results. Allele frequencies of SNPs *CAPN316* and *CAST282* for Aberdeen-Angus herd studied is given in Table 1.

Table 1 - Allele frequencies at two SNPs in the Aberdeen-Angus herd of Kharkiv region

Aberdeen-Angus	Allele frequencies			
	CAPN316		*CAST282*	
	C	*G*	*C*	*G*
Cows (n = 82)	0.390	0.610	0.793	0.207
Bulls (n = 6)	0.500	0.500	1.000	0.000
Total (n = 88)	0.398	0.602	0.807	0.193

C to *G* alleles ratio of SNP *CAPN316* for bulls and for whole sampling population were not significantly differ from the 1:1 ratio. *C* allele frequency of SNP *CAST282*

was quadruple to that of *G* allele for the whole sample and for cows. There were no bulls with allele *G*. *C* alleles of both SNP are associated with a loose muscle tissue structure following incubation at 5 °C, and respectively, with higher meat tenderness (Schenkel et al., 2006, Leveau, 2008).

The cytidine/guanosine (*C/G*) polymorphism in exon 9 of the gene *CAPN1*, which results in the amino acid substitution of glycine with alanine in large catalytic subunit of μ-calpain. Consequently, the enzyme activity in muscle tissue increases, resulting to the decrease of myofiber stiffness due to postmortem proteolysis. Expression of *C* allele in *CAST* gene, intron 5, position 282 results to non-functional calpastatin molecule synthesis. Therefore, when the Ca^{2+} concentration becomes sufficient to activate calpain inhibition of calpain does not occur and myofiber proteolysis prolongs. *C* alleles of both SNPs are associated with beef tenderness (Schenkel et al., 2006). Thus, the SNPs alleles distribution in population studied suggests moderate catalytic activity of calpain I and weak calpastatin inhibitory activity. The meat obtained from these animals will become more tender during prolonged storage period, because calpain activity is not inhibited by calpastatin non-functional molecules.

When tested deviation from the Hardy-Weinberg equilibrium using Pearson's chi-squared test there were no statistically significant differences between actual and expected genotype frequencies for both genes (see Table 2). The genotype frequencies in group studied was found to be at equilibrium.

Table 2 – Genotype frequencies at two SNPs in the Aberdeen-Angus herd of Kharkiv region

Object	Parameter	CAPN1			CAST		
		CC	CG	GG	CC	CG	GG
Cows (n = 82)	$n_{act.}$	10	44	28	50	30	2
	%	12.2	53.7	34.1	61.0	36.6	2.4
	$n_{exp.}$	12.5	39.0	30.5	57.3	27.4	3.3
	%	15.2	47.6	37.2	62.9	32.8	4.3
	Statistics	$X^2_{act.} = 1.346;\ p > 0.05$			$\chi^2_{act.} = 1.049;\ p > 0.05$		
Bulls (n = 6)	$n_{act.}$	2	2	2	6	0	0
	%	33.3	33.3	33.3	100.0	0	0
	$n_{exp.}$	1.5	3.0	1.5	6.0	0	0
	%	25.0	50.0	25.0	100.0	0	0
	Statistics	$\chi^2_{act.} = 0.667;\ p > 0.05$			-		
Total (n = 88)	$n_{act.}$	12	46	30	56	30	2
	%	13.6	52.3	34.1	63.6	34.1	2.3
	$n_{exp.}$	14.0	42.0	32.0	57.3	27.4	3.3
	%	15.9	47.9	36.2	65.1	31.2	3.7
	Statistics	$\chi^2_{act.} = 0.715;\ p > 0.05$			$\chi^2_{act.} = 0.788;\ p > 0.05$		

Note: df=2, $\Sigma\chi^2_{st.} = 5.99$.

The analysis of SNPs *CAPN316* and *CAST282* within Aberdeen-Angus group studied and literature data for Aberdeen-Angus and other breeds was performed (Tables 3–4).

According to the literature sources (Table 3), the maximal allele frequency *C* for *CAPN1* gene was observed in the Canadian Aberdeen-Angus population (0.71), and the minimal — in the Argentinian population (0.09). The highest allele frequency for SNP *CAST282* is observed in Ukrainian Aberdeen-Angus group (Table 4). Generally, the mentioned groups are characterized by relatively high frequency of *C* allele. Low frequency of *C* allele for *CAPN1* gene (less than 0.10) in Argentinian and Brazilian groups can be explained by several reasons.

Table 3 – Allele frequencies at *CAPN316* in the Aberdeen-Angus worldwide and other breeds by literature sources

Country	Breed	n	CAPN316		Reference
			C	G	
Canada	Aberdeen-Angus	158	0.71	0.29	Carruthers, 2009
International population [1]	Aberdeen-Angus	26	0.63	0.37	Carruthers, 2009
USA	Aberdeen-Angus	213	0.41	0.59	Van Eenennaam et al., 2007
Ukraine	Aberdeen-Angus	88	0.40	0.60	Present study
Sweden	Aberdeen-Angus	13	0.31	0.69	Leveau, 2008
Scotland	Aberdeen-Angus (50%) [2]	430	0.22	0.78	Gill et al., 2009
Argentina	Aberdeen-Angus	11	0.09	0.91	Soria et al., 2010
Spain	Retinta	89	0.64	0.36	Avilés et al., 2013
	Charolais	98	0.36	0.64	
	Limousin	99	0.32	0.68	
USA	Beef cattle [3]	1209	0.17	0.83	Quaas et al., 2006
Brazil	Nellore	638	0.08	0.92	Pinto et al., 2006

Notes: [1] International population represents an elite selection of international Angus cattle, the embryos donated by top purebred producers from Australia, Brazil, Denmark, Ireland, Scotland, Uruguay and the USA were implanted to Canadian cows. [2] Aberdeen Angus-sired beef cattle. [3] Hereford-sired, Charolais-sired Angus cattle

Table 4 – Allele frequencies at *CAST282* in the Aberdeen-Angus worldwide and other breeds by literature sources

Country	Breed	n	CAST282		Reference
			C	G	
Ukraine	Aberdeen-Angus	46	0.81	0.13	Present study
Sweden	Aberdeen-Angus	8	0.75	0.25	Leveau, 2008
Scotland	Aberdeen-Angus (50%) [1]	442	0.64	0.36	Gill et al., 2009
Canada	Aberdeen-Angus	12	0.63	0.37	Schenkel et al., 2006
USA	Beef cattle [2]	1209	0.72	0.28	Quaas et al., 2006
Spain	Charolais	98	0.76	0.24	Avilés et al., 2013
	Retinta	89	0.67	0.33	
	Limousin	99	0.65	0.35	
Spain	Piemontese	109	0.61	0.39	Ribeca et al., 2009
Brazil	Nellore	638	0.63	0.37	Pinto et al., 2006
Brazil	Nellore	290	0.55	0.45	Gomes et al., 2013
Turkey	Turkish Grey Steppe	132	0.51	0.49	Kök et al., 2013

Notes: [1] Aberdeen Angus-sired beef cattle. [2] Hereford-sired, Charolais-sired Angus cattle

The climate conditions of South America suggest that according Bergman's rule mammals inhabiting warmer climate seem to be smaller-bodied, than animals inhabiting cold climates. *C* allele for *CAPN1* gene is associated with hyperactive calpain synthesis involved in the formation of gaps in the microfibers during the growth of muscle tissue, thus animals with *CC* and *CG* genotypes will have advanced muscle mass. Calpastatin is associated with pleiotropic effect on carcass composition (Bolormaa et al., 2016).

Moreover, high calpain activity is associated with protozoa diseases, such as malaria and Visceral Leishmaniasis. In turn, the calpain inhibitors slow down the development of protozoa diseases. These diseases are prevalent in tropical and sub-tropical zones, therefore cattle would rather have alleles of normally active calpain and calpastatin in these region. For both SNPs studied these are *G* alleles. Additionally, calpain inhibitor has negative effect on IL-1α release, depressing immune function (Goll et al., 2003). This would justify smaller dispersion observed for *C* allele frequency of SNP *CAST282*, than *CAPN316*. Smaller dispersion can be explained by calpastatin modulating function, therefore *C* allele frequency distribution of SNP *CAST282* will have a more conservative pattern.

The traditional 'breeder's approach' can also explain resulting allele frequencies, because selection of phenotypic traits did not increase of *C* allele frequency in the South American cattle sample populations.

Aberdeen Angus, originated from Scotland are adapted to sufficiently cold conditions, therefore, this breed is likely to have high *C* allele frequency of SNP *CAPN316* which did not fix in a population inhabiting a warmer climate zone. Ukrainian Aberdeen-Angus by *C* allele frequency is similar to the North American population. Table 5 shows the *C* allele frequencies for SNPs *CAPN316* and *CAST282* considered as advantageous in terms of meat tenderness.

Table 5 – *C* allele frequencies at *CAPN316* and *CAST282* in the Aberdeen-Angus worildwide and other breeds by climate zones (Longitudinal length)

Country	Longitudinal length	C allele, SNP CAPN316	C allele, SNP CAST 282
Sweden	63°N	0.31	0.75
Scotland	57°N	0.22	0.64
Canada	56°N	0.71	0.63
Ukraine	49°N	0.40	0.81
USA	40°N	0.17; 0.41	0.72
Spain	40°N	0.32; 0.64; 0.22	0.76; 0.65; 0.67; 0.61
Turkey	39°N	—	0.51
Argentina	37°S	0.09	—
Brazil	11°S	0.08	0.63; 0.55
Spearman's correlation coefficient			
	R t	0.39 1.34	0.53 * 1.97 *

Note: * - $p < 0.05$; df=10, $t_{st.} = 1.82$.

C allele frequency of SNP *CAST282* is associated with latitudinal zonation, i. e. allele frequency is rather determined by a combination of climatic factors than animal breed. This trend is supported by previously described role of calpain inhibitor for immune response and susceptibility to the protozoan diseases.

Cluster analysis included only data on cattle groups having been studied for both *CAPN316* and *CAST282*. Since most papers describe different calpain and calpastatin genes SNPs composition for wide variety of cattle breeds there were found three papers for Aberdeen-Angus herds simultaneously studied for SNPs *CAPN316* and *CAST282*. Dendrogram (Fig. 1) showed Eastern Ukrainian herd to be separated from other groups. Dendrogram shape indicates that given groups do not form distinct classes; the closest groups are of hybrid cattle having 50% genes of Aberdeen-Angus breed or less. Purebred Swedish and Ukrainian Aberdeen-Angus herds are less similar to each other than crossbred animals.

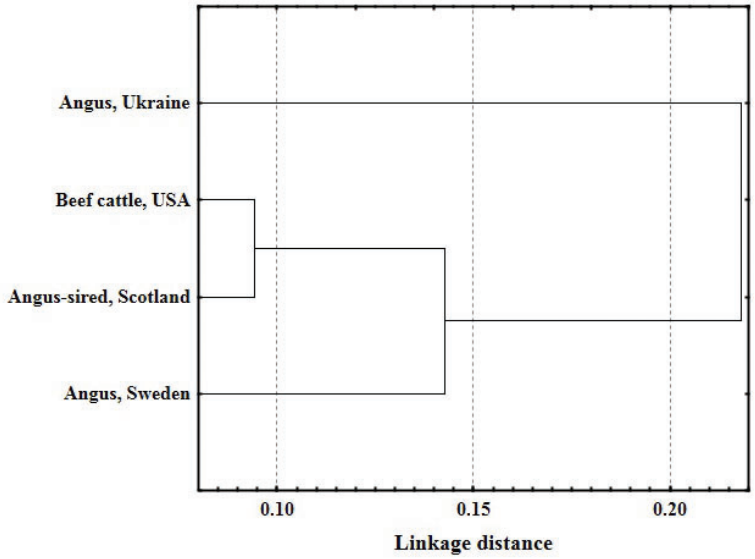

Figure 1. Dendrogram of Aberdeen-Angus based on *CAPN316* and *CAST282* (based on literature data and present study)

Extended analysis was based on papers describing SNPs *CAPN316 и CAST282* for other beef breeds (Fig. 2). There are two distinct groups on elongated diagram 'continental' group (European countries) and 'island' (non-European countries). Aberdeen-Angus herds of different countries were not allocated in one class, groups formation is rather based on geographic region then on breed. Eastern Ukrainian Aberdeen-Angus herd is close to the 'European' class, but there is a significantly greater distance between Ukrainian group than intra-group distance within class. The minor distance between groups within 'European' class refers to a greater similarity between them. Crossbred Aberdeen-Angus groups (50% or less) are more likely to be similar to Brazilian Nellore. The results obtained suggest that microevolutionary processes are tend to select the advantageous allele profile for specified climate and geographical conditions rather than ancestry history. Thus SNPs allele distribution of European Anguses is closer to that ones of other domestic European breeds than to Scottish sample population. Ukrainian sample population seems to be isolated from European herds due to later introduction and more continental conditions of Eastern Ukraine.

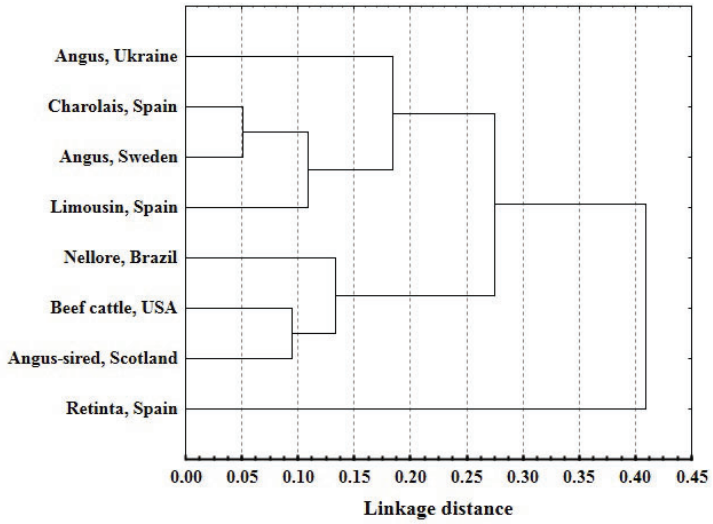

Figure 2. Dendrogram of beef cattle based on *CAPN316* and *CAST282* (based on literature data and present study)

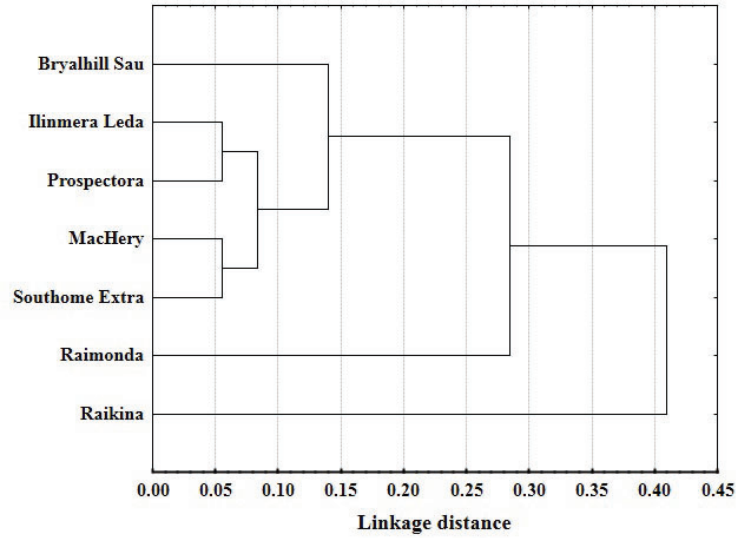

Figure 3. Dendrogram of Aberdeen-Angus lines of Eastern Ukrainian herd on *CAPN316* and *CAST282*

The Aberdeen-Angus group studied is produced under Canadian selection and predominately includes animals belonging to lines: Bryalhill Sau, Ilinmera Leda, MacHery, Prospectora, Raikina, Raimonda, Southome Extra (Kolisnyk et al., 2014). The highest *C* allele frequency of SNP *CAPN316* was reported for Raikina line - 0.67 compared with other lines — from 0.35 to 0.45 (see Table 6). The highest *C* allele frequencies of SNP *CAST282* were reported for Raikina and Southome Extra lines — 0.83. The lines do not form distinct classes (Fig. 3).

Conclusion. The preferred *C* allele frequencies of SNPs *CAPN316* and *CAST282* in the study population were 0.398 and 0.807, the population is in state of equilibrium. The genotype frequencies of *CAPN316* are *CC* — 13.6%, *CG* — 52.3% and *GG* — 34.1%, of *CAST282 CC* — 63.6%, *CG* — 34.1% and *GG* — 2.3%. The popuialion studied is close to European commercial beef herds. Group-to-group variability in allele *C* frequency for SNP *CAPN316* is higher than *CAST282*, due to the modulating effect of the calpastatin. Calpain/calpastatin system genes are likely to be associated with meat tenderness as well as some quantitative traits selected during beef cattle breeding. It was demonstrated the role of climate and geographic region on C/G distribution of SNPs rather than breed belonging, and, moreover, association between latitudinal zonation and frequency of allele C in cattle herds ($R = 0.53$, $p < 0.05$).

Table 5 – Allele frequencies at two SNPs in the Aberdeen-Angus herd of Kharkiv region, by lines

Aberdeen-Angus line	CAPN316		CAST282	
	C	G	C	G
Bryalhill Sau (n = 10)	0.35	0.65	0.90	0.10
Ilinmera Leda (n = 13)	0.38	0.62	0.73	0.27
MacHery (n = 9)	0.44	0.56	0.78	0.22
Prospectora (n = 21)	0.38	0.62	0.81	0.19
Raikina (n = 3)	0.67	0.33	0.83	0.17
Raimonda (n = 3)	0.17	0.83	0.67	0.33
Southome Extra (n = 9)	0.44	0.56	0.83	0.17

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Part 3. Biosafety and emergent diseases

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FUNDAMENTAL OBLIGATIONS AND RIGHTS OF BIOSAFETY COMMISSIONS IN THE ORGANIZATIONS WHICH WORK WITH PATHOGENIC AGENTS

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Summary. Biosafety is the system of preventing large-scale losses for the living systems directed on the maintenance of ecological balance and human health.

The pathogenic microorganisms and products of their vital functions which are agents of infectious diseases regardless of their origin and the methods of their receipt are the basic sources of biological danger for the population, animals, plants and the environment.

Each institution should develop its own biosafety programs for the following purposes:

- minimization of the negative influence of biohazards;
- prevention of the dangerous biological materials releasing;
- protecting of the experimental samples.

In each institution, the main points of the control over the compliance with the biosafety and biosecurity systems should be necessarily assigned to the Regime Commission or Biosafety Commission.

Based on this, each institution that works with the microorganisms of II–IV pathogenicity groups should have its regulations on the Regime Commission about the monitoring compliance with biosafety and biosecurity regimes when working with pathogenic microorganisms. It is noted that the provisions should include 3 sections: general provisions, obligations, and the rights of Regime Commission.

This article determines the basic provisions, rights, and responsibilities of the regime commissions from different departments. The main principles and approaches of 'Regulations on the Policy Commission on monitoring compliance with biosafety and biosecurity regime when working with pathogenic microorganisms' are also shown in this article.

Keywords: biosafety, veterinary medicine, laboratory, regime commission, pathogenic agents

Introduction. Biosafety is the ensuring of safety measures to reduce the risk of losses, thefts or applications of microbiological agents or toxins which could lead to the criminal or improper use of one or another agent as a biological weapon.

To prevent the release of biological hazard material that can harm the health of people, animals, plants, and the environment; as well as to defense the integrity of experimental materials, every institution that conducts scientific research using the pathogens of infectious diseases of livestock and poultry should develop its own biosafety programs. In the United States, Canada, and the European Union there are special committees on biosafety, genetic safety, biological ethics, and environmental safety aimed to maintain the biological security in a single facility or unit.

Most institutions in the CIS countries have only regime commissions that are directly responsible for biosafety and biosecurity.

The aim of this work is to investigate, analyze and define the basic obligations and rights of biosafety commissions among the organizations that work with II–IV pathogenicity groups of microorganisms.

Results and discussion. According to the order № 183 of the Ministry of Healthcare of Ukraine 'About the regime of working with pathogenic microorganisms' from 12.14.1992, each institution or laboratory that works with microorganisms of II–IV pathogenicity groups should have its regulations about monitoring of compliance to biosafety and biosecurity regimes when working with pathogenic microorganisms by the regime commission. This document should include three sections: general provisions, obligations, and rights of regime commission.

General provisions must include the following ones:

1. Regime commissions must be created in all institutions of the Ministry of Healthcare of Ukraine and other departments *that* work with the hazardous

material or with material that is suspected to be infected by pathogenic microorganisms.

2. Regime commissions in all institutions and departments must be created *on the basis of* the order of the head of institution, organization or department.

3. Regime commissions must work according to the relevant orders, instructions or applications.

4. The composition of the regime commission (5–6 members) should be confirmed by the order of the institution head. Commission must include specialists of the highest degree that have great skills in working with pathogen agents and in providing of antiepidemic measures. The head of the institution or his deputy in science, labor service or in the planning of the institution epidemiological readiness may hold a post of the Commission Head.

5. The head of the Regime Commission is responsible for executing of the regime inside the institution. In cases of his absence, Head's Deputy becomes a responsible person.

6. The head of the institution is responsible for executing of regime obligations.

Obligations of Regime Commissions:

1. To provide control measures for regime executing when working with pathogen microorganisms in different institutions regardless of their department participation.

2. To control obedience to orders in regime from the higher-level authority and the Head of the institution.

3. To render methodical assistance and to control activities of Regime Commissions in institutions regardless of their department participation and territory of activity.

4. To arrange necessary materials and documents for special echelons to gain the rights of working with pathogen materials or probably pathogen ones.

5. To evaluate methodical recommendations, instructions, projects of the laboratories to be built or reconstructed, and other documents for checking them by criteria of antiepidemic regime.

6. To register each occurrence of emergency when working with microorganisms of II–IV pathogenicity groups and to make accident reports to the higher-level authority regime commissions.

7. To determine and represent measures of accident liquidation to the Head of the institution.

Regime Commissions have the following rights:

1. To demand accomplishment of work standards with microorganisms of II–IV pathogenicity groups.

2. To involve other employees in the performance of Regime Commission work in accordance with manager's order.

3. To make an application for Heads of institutions and higher-level authority Regime Commissions about following questions:

- hiatus of certain employees to work with microorganisms of II–IV pathogenicity groups after systematic accepting of emergencies, or after the emergency with drastic consequence;
- prohibition of application of the methods that may be unsafe for medical stuff;
- hiatus of certain laboratories to work with pathogen agents.

4. If the Head of Regime Commission forbids working with pathogen organisms in the laboratory, he sends the direction to the Head of Institution that commits him to issue an order to review the activity of the laboratory. The copy of this order must be sent to the Regime Commission. In extreme case, related to the emergency or other situation, Regime Commission closes down the laboratory. The copy of this order must be sent to the state administration.

NSC 'IECVM' has its own 'Statute of the regime commission' related to the control of biosafety and biohazard regimes when working with pathogenic microorganisms in the laboratories of NSC 'IECVM'. It was ratified by the order of the Head, from 28.02.2014 with the signatures of members of regime commission and given under the Common Stamp of the Institution. It clearly determines the composition of Regime Commission, and regulations that are responsible for biohazard and biosafety modes accomplishment, as well as rights and obligations of NSC 'IECVM' Regime Commission.

Conclusions. Personnel reliability, its build-up oriented at the biosafety questions and strict observance of all procedures for defense from pathogens are wise and defendant using of scare resources. Most likely, these measures have greater influence at defending of important materials from using with the aim of sabotage than expensive measures of physical safety. Routine examination and renewal of set rules for pathogens and toxins defending, examination of safety measures for preventing their incorrect and sabotage use, documenting of storage, moving, using and disposal, all that is the passport to success of Regime Commissions for control of biohazard and biosafety in each institution that works with II–IV pathogenicity groups of microorganisms.

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Q FEVER IN POLAND – THE CURRENT EPIDEMIOLOGICAL SITUATION AND CONTROL

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Summary. The aim of the study was to assess the prevalence of Coxiella burnetii in small ruminants and cattle herds in different regions of Poland. Complement fixation test was performed on 1,200 serum samples collected from 449 cattle herds from 158 counties. Moreover, 1,287 samples of biological material from 180 cattle herds and 79 small ruminants herds were tested using real-time PCR. Molecular analysis by revealed that 320 from 1,287 tested samples (24.87%) were positive. The average rate of seropositive herds was 43.66%.

Keywords: Q fever, Coxiella burnetii, abortion, ruminants

Introduction. Q fever is a zoonosis caused by *Coxiella burnetii*. The etiological agent is a gram-negative, intracellular bacterium with a complex life-cycle. Sequencing of 16S rRNA has shown that the *Coxiella* genus belongs to the gamma subdivision of Proteobacteria, order Legionellales (Drancourt and Raoult, 2005). The pathogen can infect a wide range of mammals and non-mammalian species, including birds and arthropods (Babudieri and Moscovici, 1952).

In ruminants, which are considered as a main source of infection in humans, the bacteria caused non-specific reproductive disorders such as subfertility, abortion, stillbirth, delivery of weak offspring. Although, some infected animals remain asymptomatic and they constitute potential bacterial reservoirs capable of transmitting the disease. Ruminants shed a huge amount of the pathogen in birth products and a smaller number of bacteria in milk, urine, faeces, and semen.

Coxiella burnetii is transmitted to humans mainly by inhalation of contaminated dust or by direct contact with infected animals or contaminated wool, bedding, manure and birth products (ECDC, 2010). Therefore, cases of Q fever are usually notified among people occupationally exposed to the pathogen but the number of infected persons living in urban areas has been observed. A possibility of infection by alimentary route remains contradictory, but cannot be excluded (Masala et al., 2004; Angelakis and Raoult 2010, Signs et al., 2012).

The studies conducted by research from Central Europe have demonstrated that the prevalence of *Coxiella burnetii* infection in ruminants has been increasing in recent years (Astobiza et al., 2012, Czaplicki et al., 2009, Ryan et al., 2011). Detailed data about Q fever prevalence in Polish ruminants are limited and are mainly related to the endemic region in South-Eastern Poland (Recent monitoring studies showed a lot of seropositive results in milk cattle herd in Poland). Therefore, the aim of this study was to assess the prevalence of *Coxiella burnetii* in variety biological samples obtained from cattle and small ruminants herds from different regions of Poland.

Control of Q fever in Poland. In accordance with the regulation of Minister of Agriculture and Rural Development, Q fever is a notifiable disease in Poland. Moreover, since 2010, it has been included in the serological monitoring program performed by the Veterinary Inspection. Its results indicate that *Coxiella burnetii* has been consistently presented in a population of ruminants in Poland. At the beginning of this year, Veterinary Chief Officer has published the guidelines for veterinarians, which contains a surveillance and workflow schemes.

In the case of suspicion of the Q fever outbreak on the basis of clinical symptoms, it is recommended to perform serological tests. Samples should be collected from animals with clinical symptoms and from randomly selected. The age structure of the herd must be also taken into account and it is advisable to take samples from animals in each age category. When the result of a serological test performed by an official laboratory is doubtful or positive, information about the test result must be submitted to the appropriate District Veterinary Officer and the sample must be sent to National Reference Laboratory (NRL) for confirmation. If the sample, which was originally taken cannot be delivered to the NRL, the appropriate District Veterinary Officer should collect material from suspected animal. Along with the samples, information about the localization of the herd, its ID number, a number of ear tag and contact information to the District Veterinarian Officer should be given to NRL. If the serologic test performed in NRL is negative, withdraw from further proceedings. If, despite the negative serological test result, Q fever infection is still suspected, it is advisable to continue the diagnostic

procedure, as in the case of a positive result in a serological test.

In the case of positive result of a confirmatory serological test, it is recommended to collect the material from a seropositive animal for molecular testing using real-time PCR (qPCR). Depending on the availability it could be: bulk tank milk or individual milk sample and swab of the genital tract (if possible, collected in the perinatal period — up to 8 days after birth), placenta (fragment comprising of minimum three cotyledons) or sections from the internal organs of aborted fetuses (spleen, lungs, heart, liver). It is crucial to take a section from all of the mentioned organs because a lack of *Coxiella burnetii* in one of them does not exclude the presence of the bacteria in other tissues. If abortions occur in the herd, placenta, vaginal swabs and/or sections from the internal organs of aborted fetuses are the most suitable samples for molecular analysis.

A positive result in the qPCR test is the ultimate result confirming an outbreak of Q fever in the herd. The District Veterinary Officer is obliged to inform the Province Veterinary Officer and Sanitary Inspection about the outbreak. In this case, District Veterinary Officer should recommend the owner to isolate and treat/vaccinate or eliminate positive individuals. After isolation/culling of animals in the dairy cattle herds, it is recommended to test BTM and/or vaginal swabs using real-time PCR technique. If the herd was subjected to the treatment and/or vaccination, in order to evaluate the effectiveness of therapy, molecular tests should be performed but not earlier than 14 days after ending of therapy or vaccination. Type of material to study should be depended on its availability: bulk tank milk (from dried animals vaginal swabs in perinatal period should be collected), individual milk samples and/or vaginal swabs, placenta. According to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2015 published by OIE, if the abortions were noted in the herd, the real-time PCR tests (BTM and/or vaginal swabs — allowed the pooling of 9 swabs) should be performed every two months for a year.

If there is no possibility to test milk samples (beef cattle), a further diagnosis should be performed in the case of abortion. Then, to exclude *Coxiella burnetii* infection, placenta, and internal organs from an aborted fetus or vaginal swabs collected up to 8 days after birth should be tested by real-time PCR. If the bulk tank milk sample is positive in real-time PCR, it is recommended to test individual milk samples in order to separate the shedders. Non-shedding animals should be vaccinated. Infected cows should be treated and/or vaccinated or culled. Real-time PCR tests on BTM samples should be performed every two months for one year after treatment or vaccination.

Herd, which obtained positive serological test results in cases other than surveillance of animals' infections, could be typed for screening by District Veterinary Officer after analysis of an epizootic situation.

When the District Veterinary Officer receives information from the State Sanitary Inspector of the suspicion or diagnosis of Q fever in humans, then he undertakes actions ex officio to determine or exclude the disease.

Ethic statement. The samples were collected from animals by authorized veterinarians during clinical studies following standard procedures. The samples were collected specifically for this study with the agreement of the farmers. According to the Local Ethical Committee on animal Testing at University of Life Sciences in Lublin (Poland) from ethical approval is not required for this kind of study. We were using guidelines published by this ethic committee (Resolution No. 22/2006, November 7, 2006), which confirm that this work is acceptable without specific ethical approval.

Materials and methods. The serum samples were tested during the first part of the 3rd edition Multiannual Research Programme, the study was performed at the turn of 2014 and 2015 on 1,200 serum samples collected from 449 cattle herds from 158 counties. Most tested animals were milk cattle. Materials for diagnostic assay were collected from non-vaccinated animals. The complement fixation test using Siemens Healthcare Diagnostic Products (Germany), detecting both phase I and II antibodies, was done in agreement with the Manual of Diagnostic Tests and Vaccines according to the manufacturer's instructions. The temperature of inactivation of sera was 57±1 °C and 62±1 °C, respectively for cattle and small ruminants.

Moreover, a total of 1,287 samples of biological material from 79 small ruminants and 180 cattle herds were obtained for molecular analysis by real-time PCR. The material was sent to National Reference Laboratory for the confirmatory, research and service tests. 385 milk, 335 vaginal swabs, 539 blood, 2 tissues, 5 semen and 21 placenta samples were tested. DNA isolation was performed with commercially available DNA Mini Kit (Qiagen). The qualitative real-time PCR assay, detecting the IS*1111* element, was performed on blood and semen samples and for the other types of samples Adiavet COX RealTime PCR (Adiagene, Biomerieux Company) kit was used. PCR was performed according to the manufacturer's instructions. Only the samples presenting a typical amplification curve with a thresh hold value (Ct) values below 36 were considered positive.

Results and discussion. In Poland both human and animal cases of Q fever are notifiable. Cases of Q fever in animals are confirmed by the National Reference

Laboratories for Q fever. The information on animal cases or outbreaks is sent by the regional state veterinary officer to the National Sanitary Inspectorate. Elimination of the source of infection is achieved through established cooperation between veterinary and health services. Moreover, there is a monitoring program for Q fever in Poland for cattle and small ruminants. Outbreaks of Q fever in both humans and animals have been noted in Poland since 1956 (Lutyński et al., 1956). The largest epidemic of Q fever among humans and animals was recognized near Zamość (in the Lublin voivodeship in eastern Poland) in 1983 (Cygan et al., 1983, Mikołajczyk et al., 1986). More than 1,300 people fell ill in this epidemic centered around the area of Hrubieszów and Tomaszów Lubelski. Until 2007 when the large Q fever epidemic in the Netherlands broke out it had been considered the biggest Q fever epidemic in humans in the world. According to literature data from this time, anti-*C. burnetii* antibodies were found in cattle from this area (Cisak et al., 2003, Galińska et al., 2011, Niemczuk et al., 2011). Moreover, the serological studies performed by Cisak et al. (2003) among the farmers living in villages located in Lublin voivodeship showed the presence of specific antibodies to *C. burnetii* in 17.8% of 90 tested subjects for comparison in our studies the percentage of seroprevalence in tested farming population was higher in IFA (31.12%) and ELISA 39.07%.

The results of serological tests show a high percentage of seropositive cattle herds in Poland: a total of 524 serum samples were positive and the average rate of seropositive herds was 43.66%. Generally, the level of seroprevalence in Poland is similar to other European countries. The highest seroprevalence was noted in Mazowieckie and Lubelskie provinces. The lowest seropositive herds, below 20%, were in Warmińsko-Mazurskie and Łódzkie provinces.

Molecular analysis by real-time PCR revealed that 320 from 1,287 tested samples (24.87%) were positive. The percentage of positive cattle herds was 24.44%, for flocks of sheep it stood at 2.86% and for goat herds 22.22%. Goats seem to be more sensitive to acquire the pathogen because in many types of research prevalence in this species are higher than in sheep (Van den Brom et al., 2015). In this survey, a high percentage of positive goat herds cannot be representative of the whole goat population due to a small number of tested samples. The percentage of herds excreting *Coxiella. burnetii* in milk was 33.77% whereas presence in blood was noted only in 11.61% of cattle herds. *Coxiella burnetii* DNA in vaginal swabs was detected in 3 out of 15 tested herds. Molecular analysis showed a lower percentage of positive herds than serological test but it could be caused by high amount of blood samples, where bacteria are detectable in a short period during the infection.

Diagnosis of Q fever in animals is difficult because both the ELISA and the CFT test have some limitations. First antibodies appear about 14–21 days after infection and serological test performed in this period can give negative results. The literature data indicate that ELISA test loses positive results when the serum samples contained antibodies specific for phase II, and particularly when sera had low titer 1:10 (+ and ++) (Emery et al., 2012, Szymańska-Czerwińska et al., in press). It is due to a fact that ELISA is able to detect the IgG antibodies while CFT detects both IgG and IgM antibodies. On the other hand, some researches show that CFT has lower sensitivity compared with ELISA. Moreover, animals which shed *Coxiella burnetii* intermittently may remain seronegative but serological tests could be a reliable tool to identify heavy shedder, which are usually persistently highly-seropositive.

Real-time PCR is thought to be a fast and sensitive tool for detection of *Coxiella burnetii* shedders. Guatteo et al. (2006) observed that cattle shed the bacteria mainly by one route, but some animals excreted pathogen simultaneously in milk, faeces, and vaginal discharge. The data about predominant shedding route remain ambiguous, although it is thought that ewes shed more and longer in vaginal mucus than goats. The latter and cattle excrete bacteria more frequent in milk. (Arricau-Bouvery and Rodolakis 2005). What's interesting, most of the exanimated cows in this survey shed a huge amount of bacteria without any clinical signs, what is in agreement with results obtained by scientists in Europe. (Rodolakis et al., 2007)

Since 2013, the vaccine contains inactivated *Coxiella burnetti* bacteria, has been available for cattle and goats in Poland. It can reduce shedding of bacteria in these animals via milk and vaginal mucus but a drop in milk production is common in goats after the administration of a vaccine. Furthermore, the manufacturer informs that the immunization reduces the level of shedding only in non-infected animals. This fact was confirmed by many surveys (Hogerwerf et al., 2011; Van den Brom et al., 2013, Guatteo et al., 2008). Vaccinated ruminants cannot be distinguished from seropositive individuals by serological examinations, necessitating molecular techniques to confirm the infection.

Conclusion. The results of the survey performed in Poland show that *Coxiella burnetii* is widespread in ruminants population, mainly in cattle. Due to very low infectious dose and zoonotic character of this pathogen, further surveillance is crucial. Data about genotypes variations of *Coxiella burnetii* are limited in Poland, so the detailed genetic characterization of field strains will be valuable knowledge for epidemiological investigation in Q fever outbreaks.

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Contents

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