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PRELIMINARY STUDY IN RANGE OF PREVALENCE *CHLAMYDIA* SPP. IN THE POLISH CATTLE POPULATION

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Summary. *Chlamydia* spp. is an obligate intracellular agent that causes chlamydiosis in animals and humans. The aim of the presented study was to investigate the prevalence of *Chlamydia* infection in the Polish cattle population, both asymptomatic and having reproductive disorders. The study was performed on 900 serum samples collected from 14 Polish provinces at the turn of 2014–2015. The samples were tested by complement fixation test (CFT) and ELISA. Moreover, 40 samples of biological materials from cattle were tested by real time PCR. The study indicates that infections with *Chlamydia* spp. are present in Polish cattle. The study demonstrated that average percentage of seropositives herds is 26.40%. The real-time PCR gave the positive results for Chlamydiaceae into vaginal swabs in 10 tested herds. Moreover, the molecular studies showed that infection *Chlamydia pecorum* and *Chlamydia abortus* is more spread than with *Chlamydia pisttaci*.

Keywords: cattle, *Chlamydia* spp., chlamydiosis, complement fixation test, ELISA, PCR

Introduction. *Chlamydia* are obligate, intracellular, gram-negative bacteria that infect a wide variety of host species including vertebrates, arthropods, and amoeba (Wheelhouse and Longbottom, 2012). Chlamydiaceae only has a single genus *Chlamydia* that comprises eleven species according to the new taxonomy. Firstly, the division of Chlamydia was divided into genera *Chlamydia* and *Chlamydophila*. As their general importance is based on two aspects: economic losses to the animal owners and potential zoonotic transmission to humans. The most prominent *Chlamydia* agent in cattle is *Chlamydia abortus* (Markey, 2011). Moreover, recent evidence suggested that infection of cattle with *Chlamydia suis* and *Chlamydia psittaci* is also possible (Jee et al., 2004; Pantchev et al. 2010). Chlamydial infection in cattle has been associated with reproductive disorders including abortion, endometritis, repeat breeding, vaginitis, seminal vesiculitis, weak calves, and perinatal mortality (Reinhold, Sachse and Kaltenboeck, 2011). Moreover, cause bovine infertility and epizootic bovine abortion. In cattle *Chlamydia* such as *Chlamydia pecorum, Chlamydia abortus*, and *Chlamydia psittaci* are found in connection with infection of the respiratory tract as well (Sachse et al., 2009). *Chlamydia abortus* is a recognised cause of epizootic bovine abortion and a cause of bovine infertility worldwide. In bulls, the infection can cause epididymitis, seminal vesiculitis, and testicular atrophy, and affects semen quality. The organism has also been shown to be shed in semen, and multiplication of *Chlamydia abortus* via contaminated semen can result in local infections and inflammatory reactions in the uterus, which can subsequently lead to infertility in heifers (Livingstone and Longbottom, 2006). In dairy cows, the presence of *Chlamydia* was significantly associated with subclinical inflammation of the mammary gland, and while vaccination significantly lowered somatic cell counts in milk and increased antibody levels against *Chlamydia*, it failed to reduce shedding levels of these bacteria in milk. It is conceivable that chlamydial infections of the respiratory and/or intestinal tracts in calves originating from cows with subclinical chlamydia-associated mastitis may have an impact on developing calves, even in the absence of clinical illness. However, there are no data in the literature to verify this hypothesis (Uhe et al., 2005). Chlamydial infection in cattle has been reported in many countries including Poland but the data about prevalence are limited.

The aim of the study was to investigate the seroprevalence of the chlamydial infection in Polish cattle population in the individual region of the country. Moreover, the aim of the studies was identification of *Chlamydia* species and evaluation which one was the most common. The results may provide base-line data for the implementation of integrated strategies to prevent and control of chlamydial infection in dairy cattle in the future.

Materials nad methods. ***Blood samples and vaginal swabs.*** The study was performed at the turn of 2014–2015 on 900 serum samples collected from different region (voivodeships) of Poland. The samples were collected from randomly selected cattle herds. Totally, samples were taken from 317 herds. The number of samples from individual herd depended on availability for sampling. The blood samples was taken from coccygeal vein into a 10 ml vacuum tube, stored in a refrigerated bag, brought to the laboratory and centrifuged at 2500 g for 10 min within 24 h. Serum was then removed and stored at – 20°C. Moreover, from 40 seropositive herds the vaginal swabs were taken for confirmation the presence of active *Chlamydia* infection by real-time PCR. The samples were not taken from all herds because the farmers did not accept further research. Totally, we tested 400 swabs. The swabs were stored in conservation buffer SPG at – 80°C until subjected to DNA extraction.

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) formal ethical approval is not required for this kind of study. We were using guidelines published by this ethics committee (Anon, 2006), which confirm that this work is acceptable without specific ethical approval.

Serological examinations. The serological examinations were performed using CFT, a diagnostic technique recommended by the world Organisation for Animal Health (OIE). For CFT, Institut Virion/ Serion GmbH (Germany) and Sera and Vaccines Manufacturing (Biomed-Krakow, Poland) reagents were used. Before each examination, an intralaboratory evaluation including antigen titration against a positive control serum and checking the activity of other reagents used in the reaction, were carried out to find the actual titre versus activity ratio in relation to that declared by the manufacturers. The specific reaction of CFT, its consecutive steps, and result interpretation, were performed according to the 'Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees)' (OIE, 2012). The starting dilution of the examined samples was 1:4, and the ending dilution was 1:64. Serum was considered as positive when a partial inhibition of haemolysis was observed at the dilution of 1:32. Moreover, all seropositive samples were tested by ELISA (*Chlamydophila abortus* Antibody test Kit, IDEXX). This test detects antibodies against *C. abortus* in serum and plasma samples from ruminants. The sera, positive, and negative controls were tested in a dilution of 1:400 in duplicate. According to the manufacturer's instructions, the percentage of optical density (OD) was calculated as (sample OD-negative OD)/(positive OD-negative OD)×100 after averaging the duplicate values. Sera were considered to be negative when % OD < 30, dubious when % OD ≥ 30 and % OD ≤ 40, and positive when % OD > 40.

PCR. DNA extracts from swabs, were performed using the commercial QIAamp DNA Mini Kit (Qiagen), following the manufacturer's instructions. A Chlamydiacae-specyfic real-time PCR targeting the 23S rRNA gen (described previously by Pantchev et al., 2010) was used in this study. All samples with a cycle threshold (Ct) > 38 were considered negative. Normalization of target genes was performed using glycerinaldehyde-3-phosphate (GAPDH) as an endogenous standard. The positive control was DNA extracted from reference strain of *Chlamydia abortus* B-577 (ATCC-VR-656). The samples positives for Chlamydiaceae were tested by real-time test for species-specific detection of *Chlamydia psittaci* and *Chlamydia abortus* previously described by Panchev et al. (2009). The samples negative for *Chlamydia abortus* and *Chlamydia psittaci* were tested by nested PCR for *Chlamydia pecorum* according the protocol described previously by Kaltenböeck, Schmeer and Schneider (1997). The DNA form reference strain of *Chlamydia pecorum* obtained from Reference Laboratory for chlamydiosis in FLI, Jena, Germany.

Results. Serological results are presented in Table 1. Taking into account all CFT and ELISA results as positive, the seroprevalence of *Chlamydia* spp. infections in the Polish cattle population is presented. The average percentage of seropositives herds is 26.40% and ranges from 91.67% in the Mazovia Province to 0.00% in the Lubuskie Province. Whereas average the percentage the seropositive animals was 30.11%.

Based on the study, it was possible to observe that the highest level of the *Chlamydia* spp. seropositivity was in cattle herds localized in more industrialized regions of Poland, which have more intensive levels of agricultural and cattle breeding production. The highest percentage of positive samples was noted in the Mazovia Province (96.67%), the Pomerania Province (85.71%), the Silesia Province (50%), the Podlasie Province (43.48%), the Wielkopolska Province (30.77%), Świętokrzyskie Province (33.30%). The lower percentage seropositive herd was noted in Warmia-Mazuria Province (26.32%) and West Pomerania Province (21.43%). The lower percentage cattle herds with presence of antibodies against *Chlamydia* spp. was in Łódź Province (2.5%). While any seropositive herd was detected in Lubuskie Province. From three region Lower Silesia, Opole and Małopolska Province samples were not tested.

The results of molecular survey are presented in Table 2. The results showed that *Chlamydia* spp. organisms were present in the cattle. The real-time PCR gave the positive results for Chlamydiaceae to vaginal mucous in 10 tested herds. Both species of *Chlamydia abortus* and *Chlamydia pecorum* were detected in the tested samples but the most prominent was *Chlamydia pecorum*. However, the presence of other Chlamydia species (e.g. *Chlamydia psittaci*) was not confirmed. The positive herds for *Chlamydiacae* were in Mazovia Provine (two herds), Wielkopolska Province (one herds), Podlasie Province (three herds), Pomerania Province (three herds), Świętokrzyskie Province (one herds). *Chlamydia pecorum* was detected in 7 herds.

Table 1 – Seroprevalence of *Chlamydia* spp. in the Polish cattle in the individual region

Province	Number of tested herds	Number of seropositive herds (percentage)	Number of tested animals	Number of seropositive animals
Lower Silesia *	-	-	-	-
Kujawy-Pomerania	29	4 (13.79%)	61	25 (40.98%)
Lubelskie	12	3 (25%)	40	3 (7.50%)
Lubuskie	1	-	1	-
Łódź	40	1 (2.5%)	40	1 (27.50%)
Mazovia	12	11 (91.67%)	60	25 (41.67%)
Małopolska *	-	-	-	-
Opole *	-	-	-	-
Podkarpacie	106	21 (19.81%)	106	21 (19.81%)
Podlasie	23	10 (43.48%)	253	123 (48.62%)
Pomerania	7	6 (5.71%)	40	15 (37.50%)
Silesia	16	8 (50%)	102	32 (31.37%)
Świętokrzyskie	12	4 (33.33%)	40	4 (10%)
Warmia-Mazuria	19	5 (26.32%)	68	7 (10.29%)
Wielkopolska	26	8 (30.77%)	38	9 (23.68%)
West Pomerania	14	3 (21.43%)	51	6 (11.76%)
Total	317	84 (26.50%)	900	271 (30.11)

* from this region sampling was impossible

Discussion. The serological results showed that cattle revealed positive results of serological survey in all tested provinces of Poland excluding Lubuskie Province, and the presence of *Chlamydia* antibodies was noted. There is preliminary study and it is difficult to evaluate current epidemiological situation because the real-time PCR test was not performed in all provinces. These data show that the problem of *Chlamydia* spp. infection in cattle in Poland is presented and the pathogen is country-wide, and the control investigations have to be performed. these results compared with published data from Poland from several past years, indicate that the percentage of seropositive samples in cattle is now higher (Niemczuk 2006, 2005) although, the previous studies were performed on the selected population of cattle including cattle herds with reproductive disorders and the seroprevalence was not evaluated for herds but for all tested animals in individual provinces. To our knowledge, the present survey is the first epidemiological evaluation of the prevalence of *Chlamydia* in randomly selected cattle herds in a country.

Table 2 – Results of molecular studies (real-time PCR and nested PCR)

Province	Number of tested herds	Number of seropositive herds
Lower Silesia	-	-
Kujawy-Pomerania	-	-
Lubelskie	2	-
Lubuskie	1	-
Łódź	2	-
Mazovia	2 *	2
Małopolska	-	-
Opole	-	-
Podkarpacie	-	-
Podlasie	3^	3
Pomerania	3^	3
Silesia	5	-
Świętokrzyskie	1^	1
Warmia-Mazuria	2	-
Wielkopolska	1*	1
West Pomerania	-	-
total	40	10

* herds with presence of *Chlamydia abortus* infection confirmed by PCR and real-time PCR

^ herds with presence of *Chlamydia pecorum* infection confirmed by PCR and real-time PCR

Antibodies against *Chlamydia* were found in sera of cattle in several European countries (Borel et al., 2006; Kauffold et al., 2007; Wang et al., 2001; Wilson et al., 2012). Most of the surveys were performed by ELISA, so it is very difficult to compare them with our results because CFT is less sensitive but is still recommended by the OIE to detect antibodies against *Chlamydia*. The complement fixation test detects only IgG1 immunoglobulins persisting for 3–4 weeks after

infection, ELISA also detects other antibody subclasses. Moreover, most of the serological tests cannot detect the infection with these pathogens in the first phase because, generally, the titres are very low (Niemczuk and Truszczyński, 2012).

Pantchev et al. (2010) performed the examinations of cattle in Germany, and observed cases of mixed chlamydial infection as well. Combinations of *Chlamydia abortus* and *Chlamydia psittaci* or *Chlamydia suis* and less *Chlamydia pecorum* and *Chlamydia suis* in cattle were described. Recent data have demonstrated that subclinical chlamydial infections by both species, *Chlamydia abortus* and *Chlamydia pecorum*, are ubiquitous in cattle and often not detected due to low sensitivity of diagnostic techniques (Pantchev et al., 2010; Reinhold et al., 2008). However, frequently there is no correlation between the results of PCR or real-time PCR detecting the presence of DNA and serological tests determining the level of specific antibodies. The recent studies of bulls where *Chlamydia* antibodies were detected in 50.8% of bulls, while the PCR confirmed the presence of *Chlamydia* in semen in 9.2%, preputial washing in 10.7%, and faecal samples in 18.0% (Kauffold et al., 2007). PCR-positive but serologically negative bulls might have not exhibited a systemic immune response, possibly due to the obligate intracellular lifestyle of *Chlamydia* that might hide them from the systemic immune response. In contrast, other data showed that naturally infected calves were in 60% seropositive, while all of them were the shedders of *Chlamydia* (Reinhold et al., 2008).

Generally, based on the literature data it is known that chlamydial infections occur in cattle breeding. However, the epizootic situation varies in different European countries. For example in Sweden, there are 0.4% of seropositive cows, and performed studies suggested that *Chlamydia abortus* infection is absent or rare, whereas *Chlamydia pecorum* is probably more spread. Our results showed that both *Chlamydia pecorum* and *Chlamydia abortus* are present. The same researcher suggests that *Chlamydia* spp. are not related to reproductive disorders in the Swedish cattle (Godin et al., 2008). In Italy, serological investigation of cattle in different areas detected seroprevalence for *Chlamydia* ranging from 2 to 28% but association between seropositivity and abortion has not been examined (Cavirani et al., 2001). In Ireland, the percentage of seropositive cattle in ELISA was 4.44, in Taiwan — 51.3% for asymptomatic animals and 71.4% for aborted cows (Wang et al., 2001; Wilson et al., 2012).

The occurrence of *Chlamydia abortus* and *Chlamydia pecorum* in the Polish cattle can suggest that these pathogens can be one of the many factors responsible for these disorders in bovine, and can have impact on economic losses. Economic losses caused by late-term *Chlamydia abortus* infection, and the subsequent epizootic bovine abortion are readily apparent. However, infection may result in unrecognised economic losses as the consequence of subclinical infertility (Kaltenböeck, Hehnen and Vaglenov, 2005).

Conclusion. The study indicates that infections with *Chlamydia* spp. are present in Polish cattle. The study demonstrated that average percentage of seropositives herds is 26.40%. Moreover, the molecular studies show that infection *Chlamydia pecorum* and *Chlamydia abortus* is more spread than with *Chlamydia pisttaci*. It should be noted that if the *Chlamydia* agent is present in the Polish cattle population, there is a real risk to transmission of the infection to humans (Pospischil et al., 2002) but there is no information in the available literature about epidemiological situation in humans exposed to chlamydiosis in Poland.

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THE IMPACT OF POTENTIAL FEED ADDITIVE NANOCOMPOSITE (AG, CU, FE AND MN DIOXIDE) ON EGGS' QUALITY PARAMETERS OF LAYING HENS COMPARED WITH METAL SALTS

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Summary. The purpose is to study veterinary-sanitary characteristics of laying hens' egg quality under the conditions of influence of nanocomposite (Ag, Cu, Fe and Mn dioxide) (NcMe) and metal salts in the conditions of chronic toxicological experiment. Methods. The research was conducted on 72 laying hens cross Hayseks Brown (4 groups were formed: one control and three experimental n=18). Chickens from control group in addition were administered saline with feed; experimental chickens were given feed additives every day for 30 days: group I — a mixture solution of metal salts at a dose of 0.3 mg/kg, group II — nanocomposite metals in its biotic dose (0.3 mg/kg body weight) and group III — NcMe in toxic dose (4.0 mg/kg body weight), after cessation of additives administration the poultry was observed for 15 days. During the experiment there was conducted eggs' record from experimental poultry, and their veterinary-sanitary examination was carried out. Results. The data on the impact NcMe and metal salts on productivity of laying hens and veterinary-sanitary characteristics of eggs' quality was obtained. Conclusions. Introduction of NcMe with feed affect quantity and quality characteristics of laying hens productivity, predominating the effect of salts of the metals, that is characterized by increased levels of egg laying during the experiment in poultry from the third group in average by 36.1% and egg weight in the second experimental group — 24.7% ($P \leq 0.05$) and changes in pH level of egg white and yolk, but within the normative values (in accordance with DSTU 5028:2008 'Hen's eggs for human consumption. Specifications').

Keywords: laying hens, nanocomposite metals, metal salts, dose, veterinary and sanitary examination, eggs, productivity

Introduction. Industrial poultry farming is the most dynamic and knowledge-based industry that makes a significant contribution to the country's Food Program as a major manufacturer of high quality animal protein (eggs and meat). Productivity of poultry and usefulness of products of this industry depend largely on the balance of the diet, the presence of minerals, enzymes and other biologically active compounds in it (Egorov et al., 2011; Laptev et al., 2012). Currently, nanotechnology is recognized as the major driving force of science in the twenty-first century. They begin to be used in livestock, particularly in poultry farming. The literature data shows the superiority of metals in the form of nanoparticles before their salts, metal nanoparticles can easily penetrate all organs and tissues and in biotic doses stimulate metabolism (Nesterov et al., 2014). The purpose of our work was to study veterinary-sanitary characteristics of laying hens' egg quality under the conditions of the influence of nanocomposite (Ag, Cu, Fe and Mn dioxide) and metal salts in the conditions of chronic toxicological experiment.

Materials and methods. The experiment was performed at the Department of Toxicology, Safety and Quality of Agricultural Products, NSC 'IECVM' on 72 laying hens cross Hayseks Brown weighing 1.5–1.8 kg, aged 365 days (4 groups were formed: one control and three experimental, 18 chickens in each).

Experimental composite mixture — nanocomposite metals (NcMe) — was composed of silver nanoparticles (31.5±0.9 nm), iron nanoparticles (100.0±10.0 nm), copper nanoparticles (70.0±5.0 nm) and dioxide manganese nanoparticles (50.0±3.0 nm) in aliquots relation with the final concentration 100.0 μg/cm^3 for each metal, similar to the concentration of the metals in ionic (macro disperse) form in a solution of mixture of salts — $AgNO_3$, $CuSO_4 \times 5H_2O$, $MnSO_4 \times 5H_2O$ and $FeSO_4 \times 7H_2O$ respectively.

After holding experimental chickens from all groups on a standard diet for 15 days (equalization period), chickens of control group in addition were given additives to feed daily for 30 days, group I — a mixture solution of metal salts in doses of 0.3 mg/kg, group II — NcMe in biotic dose (0.3 mg/kg body weight) and group III — NcMe in toxic dose (4.0 mg/kg body weight), after cessation of supplementation the birds have been observed for 15 days. Biotic and toxic doses were established in accordance with previous studies (Kutsan, Roman'ko and Orobchenko, 2012; Orobchenko, Roman'ko and Kutsan, 2014) when studying acute and chronic toxicity NcMe on laboratory animals. During the experiment there was conducted clinical observation and collection of eggs from experimental birds to determine the level of productivity and quality of product by physical-chemical parameters.

Veterinary-sanitary examination of eggs was conducted under the rules (Ukraine. Chief Inspector of Veterinary Medicine of Ukraine, 2001), we were guided by the requirements of DSTU 5028:2008 'Hen's eggs for human consumption. Specifications' (DSSU, 2009). Statistical analysis of the results of research was carried out with the help of applied package Microsoft Excel 2003 (for Windows XP).

Results and discussion. According to the results of veterinary-sanitary examination of eggs from laying hens of control and research groups there was found that their quality meets the requirements of the current DSTU 5028:2008 'Hen's eggs for human consumption. Specifications' (DSSU, 2009) for the duration of the study: egg shell was intact, strong, without damage, smooth; the yolk was bright yellow, evenly colored, elastic texture, shape maintained; the white was pure and transparent, viscous, with no signs of damage; peculiar smell for fresh eggs.

Birds from experimental group III (NcMe 4.0 mg/kg body weight) were the most productive — from these birds during the experiment there were obtained 200 eggs, which exceeded the number of eggs received from poultry of control group in average of 36.1% ($P<0.05$). Slightly lower productivity was recorded in chickens of experimental group II (biotic NcMe dose, 0.3 mg/kg body weight) — 159 eggs, that meaningfully exceeded the number of eggs (n=147) received from the control birds, in average of 8.2% ($P<0.05$). The lowest productivity (n=138) was recorded in chickens from the group I which received a solution of metal salts at a dose of 0.3 mg/kg body weight, that was close to the level of the control group.

In accordance with the requirements of DSTU 5028:2008 'Hen's eggs for human consumption. Specifications' (DSSU, 2009), eggs are divided into categories according to weight (Table 1).

Table 1 – Classification characteristic of chicken eggs for food in compliance with DSTU 5028:2008 'Hen's eggs for human consumption. Specifications' (DSSU, 2009)

Category	The weight of one egg, g
Selected or XL	73.0 and more
Luxury or L	From 63.0 to 72.9
First or M	From 53.0 to 62.9
Second or S	From 45 to 52.9
Small	From 35 to 44.9

During 15 days of administration of the preparations the highest percentage of small eggs was found in the experimental group, where chickens received solution of metal salts 0.3 mg/kg, and the smallest — in the research group II (biotic dose of NcMe). The percentage of the second category of eggs was higher in the third experimental group; and in control, I and II groups it was almost on the same level.

The percentage of eggs weight from 53.0 to 62.9 g (first category) was the highest in the second experimental group, while for chickens from the third experimental group (NcMe 4.0 mg/kg body weight) there was recorded the minimum rate. Luxury eggs were found only in the second experimental group, where the chickens received biotic dose of NcMe.

After 30 days there was observed a similar picture, with the exception of second category eggs percent increase in the experimental group I. Also it was interesting from a practical point of view that the percentage of eggs of the first category was higher than the second category in the second experimental group (Fig. 1).

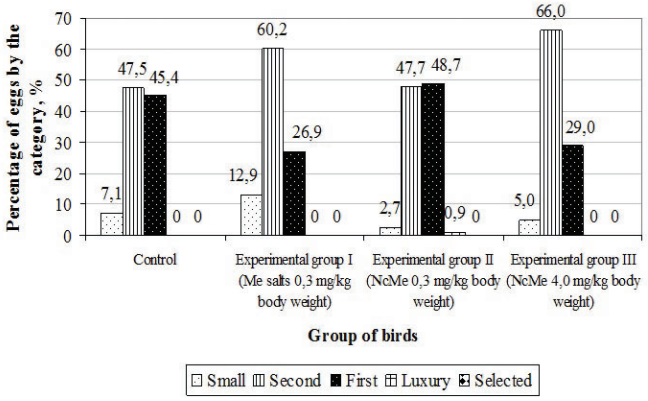

Figure 1. Percentage of eggs from experimental hens by the category on the 30th day of experiment

On the 7th day after the cessation of additives introduction there was determined unchangeable dynamic by the percentage of small eggs, increase the percentage of eggs of the second category in group II, and increase of eggs of the first category in control group.

And on the 15th day after the cessation of additives introduction there was recorded stabilization of eggs by the categories, except the third experimental group, where a higher percentage of eggs the second category maintained (Fig. 2).

Another indicator by which egg quality is regulated is yolk and white pH level (data is presented in Fig. 3–4). From the first to the 5th day of the experiment the pH level of egg yolk from hens of the first and second research groups was significantly lower than the control, 3.0% (Fig. 3), while in the third experimental group it had only a downward trend. In this term of study for pH of egg white from hens of experimental groups there were not served probable deviations from control (Fig. 4).

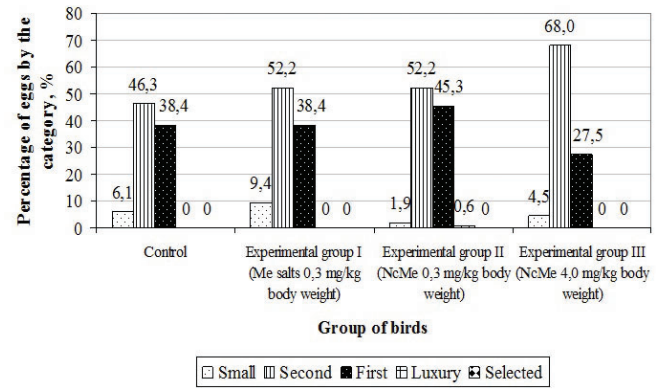

Figure 2. Percentage of eggs from experimental hens by the category in 15th days after cessation of additives introduction

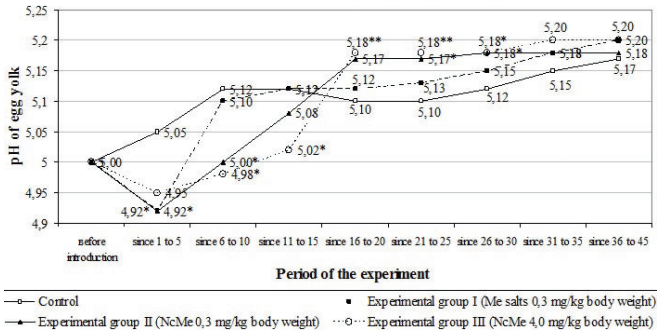

Figure 3. Dynamics of pH level of egg yolks from experimental laying hens (M±m, n=4), * — P<0.05; ** — P<0.01 — relative to control

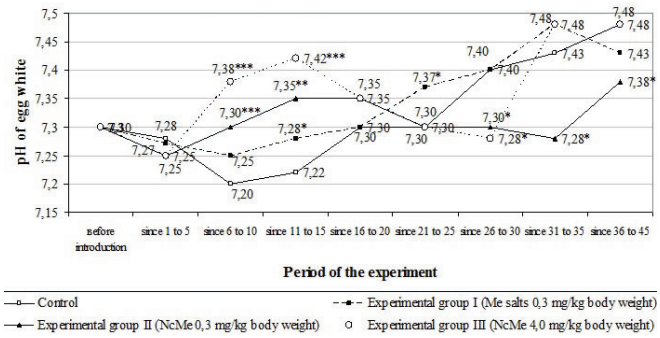

Figure 4. Dynamics of pH level of egg white from experimental laying hens (M±m, n=4), * — P<0.05; ** — P<0.01; *** — P<0.001 — relative to control

Since the 6th to the 10th day of the experiment likely decrease in pH level of egg yolks of hens, which received NcMe was recorded: in group II — by 2.3%, and in group III — by 2.7%, while in the experimental group I this figure did not differ from control.

pH level of egg white tended to increase in the first experimental group, and in the second and third groups was higher than the control (P<0.001) by 1.4 and 2.5% respectively. Since the 11th to the 15th day of the experiment the pH level of egg yolks from hens of the first experimental group did not differ from control, in the second experimental group there was observed a tendency to decrease, and in the third group there was probable decrease in pH level of 2.0%. In this term of the study pH of egg white in all experimental groups significantly exceeded control: in the group I — by 0.8%, in the group II — by 1.8% and in the group III — by 2.8%.

Since the 16th to the 20th day there was observed excess of control pH level of egg yolk in all research groups, and in groups I and II there was a tendency, in group III — significant increase by 1.6%, while the pH of the white had not probable deviations from control.

Since the 21st to the 25th day of the experiment there was noted the excess of control pH level of yolk in all research groups, and in group I there was a tendency. In groups II and III there was significant increase by 1.4 and 1.6% respectively. The pH of the white did not differ from control in the second and third experimental groups, and in the first group it was higher than the control by 1.0% (P<0.05).

A similar dynamics in the pH level of egg yolks has been observed since the 26th to the 30th day of the experiment, while the pH level of egg white in this period of the study did not differ from control in experimental group I, and in the second and third groups was significantly lower than the control by 1.4 and 1.6% respectively.

Since the 31st day and to the end of the experiment there was observed a tendency to increase the pH level of eggs yolks in all experimental groups. While the pH level of egg white since the 31st to the 35th day tended to increase in the first and third groups, and in the second group it was significantly lower than the control by 2.0%, and in the last period of the study pH level of white from hens in the experimental group III did not differ from control, in the second group there was a decrease by 1.3% (P<0.05) , and in the experimental group I there was a tendency to decrease.

Thus, the data indicates that metal nanoparticles affect the quantity and quality characteristics of laying hens' productivity, and they have an advantage over macro size forms of such metals (metal salts), as evidenced by the increase of egg-laying qualities(III experimental group) and egg weight (II experimental group) during the period of introduction of additions, and also it causes changes in pH level of egg yolk and white, but within the norms regulated by the requirements of DSTU 5028:2008 'Hen's eggs for human consumption. Specifications' (DSSU, 2009).

Conclusions. Introduction with feed of metal nanocomposite (Ag, Cu, Fe and Mn dioxide) affects

the quantity and quality of productivity of laying hens, predominating the effect of salts of the metals, that is characterized by increased levels of egg laying during the experiment in poultry from the third group in average of 36.1% and egg weight in the second experimental group — 24.7% (P≤0.05).

Introduction of metals as additives in macro and nanoscale form causes changes in the pH level of egg white and yolk, but within the norms regulated by the requirements of DSTU 5028:2008 'Hen's eggs for human consumption. Specifications' (DSSU, 2009)).

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BIOLOGICAL PROPERTIES OF *CAMPYLOBACTER* MUSEUM STRAINS AFTER LONG STORAGE IN LYOPHILIZED FORM

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Summary. Campylobacteriosis is dangerous disease of agricultural animals, which is characterized by lesions of the reproductive system and the gastrointestinal tract. Contaminated livestock products can be a source of infection for humans and cause toxicoinfection with severe disease. Domestic preparations for the in vivo diagnosis of the disease is missing.

The main condition for the creation of effective diagnostics and vaccines is the presence of stable production strains. An important focus in the work of veterinary microbiologists is the studying biological properties of *Campylobacter* museum strains and definition of the capacity for preservation of these properties during long storage.

We conducted experiments for determine of museum *Campylobacter* cultures viability, that have been isolated from animals and birds and were stored in lyophilized form for different terms. A total of 24 strains were studied.

It has been established that *Campylobacter* lose about 1.0% of their life potential for every year of storage in lyophilized form. After 10–12 years of storage only 44.4% strains retain their viability, therefore it is not feasibly to keep them for a longer time under such conditions.

It has been shown the ability to save typical properties of Campylobacter strains within the specified retention period.

Keywords: *Campylobacter* strains, biological properties, viability

Introduction. Campylobacteriosis is zoonotic infectious disease of animals and people, characterized by various manifestations (genital impression, temporary infertility, abortion, diarrhea). This disease is caused by bacteria of *Campylobacter* genus (Mshelia et al., 2010; Swai, Hulsebosch and Van der Heijden, 2005).

According to WHO, campylobacteriosis is widespread in the world and causes 15% of all acute intestinal infections of animals and people. The most significant natural reservoirs of pathogens are livestock and poultry. The disease results in high significant economic losses in livestock farms by losing of adult animals their reproductive qualities (van Bergen et al., 2005; Mai et al.; Molina et al., 2013).

There are no means for animal campylobacteriosis diagnosis and prevention in Ukraine. The main condition for the creation of effective diagnostics and vaccines is the presence of stable production strains. Therefore, an important focus in the work of veterinary microbiologists is studying the biological properties of *Campylobacter* museum strains and definition of the capacity for preservation of these properties during long storage (Babkin, Galishchev and Novakovskiy, 2002, 2003).

Materials and methods. *Campylobacter* museum strains were stored in lyophilized form in vials under vacuum at minus 2–8 °C. The contents of the vials were plated on nutrient media — MPB, semisolid MPA with antibiotics (cephalothin, fuzydin), Campilobacahar, blood MPA. Strains were cultured under microaerophilic conditions (5% oxygen, 10% CO_2, 85% nitrogen) at 42 and 37 °C, within 72 hours. Accounting growth was performed visually every day, after 72 hours — by smear microscopy.

When typical growth was found (small transparent colonies with a grayish tinge to MPA, and as gray-blue rings on the surface of the semisolid MPA) there done smear (Gram and Stamp staining). At revealing the typical morphology (Gram positive spiral S-shaped sticks) further studies were carried out on the biochemical properties (formation of H_2S and indole, growth in semisolid MPA with 1% glycine, growth in semisolid MPA with 1% bile, growth in semisolid MPA with 0.02% cysteine, growth in semisolid MPA with 3.5 % NaCl, catalase production, sensitivity to nalidixic acid and cephalothin, hydrolysis of sodium hippurate, growth in semisolid MPA with 15, 25, 37 and 42 °C).

Results. We conducted viability determination of museum *Campylobacter* cultures, that have been isolated from animals and birds and were stored in lyophilized form for different periods. Totally, 24 strains were studied. We spent 25 consecutive passages on nutrient media and did not determined the presence and intensity of cultures growth in any case after opening the vials. The research results are presented in Table 1.

We were able to revive only 7 of the 24 strains. We found that, 3 of the 15 cultures only, were kept during 33–34 years, were viable for 25 passages.

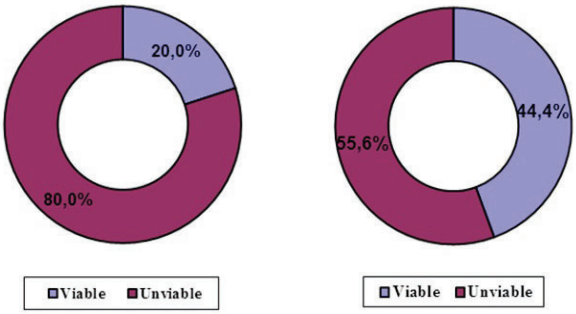
All of them belonged to the species *Campylobacter fetus* subsp. *venerealis*. 12 other cultures are kept their properties during 1–3 passages, but growth on nutrient media was absent.

Table 1 – Determination of the viability of *Campylobacter* cultures after long storage in lyophilized form

№	Name of strains	Term of the storage	Number of productive passages
1	*Campylobacter fetus* subsp. *venerialis* 4390	35	1
2	*Campylobacter fetus* subsp. *venerialis* 3121	33	3
3	*Campylobacter fetus* subsp. *venerealis* 1943 NaCl	35	1
4	*Campylobacter fetus* subsp. *venerealis* 60-312	34	1
5	*Campylobacter fetus* subsp.*venerealis* 2095	34	1
6	*Campylobacter fetus* subsp. *venerealis* 2095/2	33	2
7	*Campylobacter fetus* subsp. *venerealis* 2088	33	25
8	*Campylobacter fetus* subsp. *venerealis* 1707	33	1
9	*Campylobacter fetus* subsp. *venerealis* Rus	33	25
10	*Campylobacter fetus* subsp. *venerealis* 3816	34	1
11	*Campylobacter fetus* subsp. *fetus* 17	33	1
12	*Campylobacter fetus* subsp. *fetus* 372	11	3
13	*Campylobacter fetus* subsp. *fetus* 9SV	12	25
14	*Campylobacter fetus* subsp. *fetus* 2BGV	11	3
15	*Campylobacter fetus* subsp. *venerialis* 6913	33	25
16	*Campylobacter jejuni* 5779	10	25
17	*Campylobacter fetus* subsp. *venerialis* 6913/2	11	25
18	*Campylobacter fetus* subsp. *fetus* 15OV	10	25
19	*Campylobacter fetus* subsp. *fetus* 5OV	11	10
20	*Campylobacter jejuni* 5779	33	2
21	*Campylobacter jejuni* 5779/2	12	3
22	*Campylobacter jejuni* 5779/3	11	2
23	*Campylobacter fetus* subsp. *intestinalis* 240	33	1
24	*Campylobacter fetus* subsp. *intestinalis* 1767	34	1

Of 9 cultures, that were stored during 10–12 years, the were viable only 4. They belonged to the 3 species — *Campylobacter fetus* subsp. *fetus*, *Campylobacter fetus* subsp. *venerealis* and *Campylobacter jejuni*. None of the cultures of *Campylobacter fetus* subsp. *intestinalis* restore failed.

Thus, we have been a direct correlation between the degree of viability of the strains of their age (Figure 1).



33–35 years of storage 10–12 years of storage

Figure 1. Viability of *Campylobacter* strains

After 33–35 years storage, 20.0% of the strains only were viable. Whereas among the strains, that were stored during 10–12 years, this index was almost twice as much and was 44.4%. We calculated that the culture lose about 1.0% of their life potential for every year of storage.

For future work, we have selected three strains that were stored during 10–12 years. We examined their biochemical and cultural properties in a series of experiments (Table 2).

Table 2 – Biochemical and cultural properties of *Campylobacter* strains

№	Test	*C. fetus* subsp. *fetus* 15OV	*C. fetus* subsp. *venerialis* 6913/2	*C. jejuni* 5779
1.	Catalase production	+	+	+
2.	Growth with temperature 15 °C 25 °C 37 °C 42 °C	 – + + +/–	 – + + –	 – – – +
3.	Growth in semisolid MPA with 1% glycine	–	+	+
4.	Growth in semisolid MPA with 1% bile	+	+	+
5.	Growth in semisolid MPA with 3.2% cysteine	+	+	+
6.	Growth in semisolid MPA with 1.5% NaCl	–	–	–
7.	Sensitivity to nalidixic acid Sensitivity to cephalothin	– +	– +	+ –
8.	Formation of H_2S	+	+	+
9.	Formation of indole	–	–	–
10.	Hydrolysis of sodium hippurate	–	–	+

«+» — the presence of growth;
«–» — no growth; «+/–» — insignificant growth

We found that *Campylobacter* strains have retained typical properties. All 3 strains produced catalase; grew up with temperature 15 °C; grew up on in semisolid MPA with 1% bile and 3.2% cysteine; did not grow in semisolid MPA with 1.5% NaCl; form H_2S; did not form of indole.

C. fetus subsp. *fetus* 15OV and *C. fetus* subsp. *venerialis* 6913/2 grew up at 25 and 37 °C; were not sensitive to nalidixic acid and were sensitive to cephalothin; hydrolysed of sodium hippurate. *C. jejuni* 5779 did not grew up at 25 and 37 °C; grew up on in semisolid MPA with 1% glycine; were sensitive to nalidixic acid and was not sensitive to cephalothin; not hydrolysed sodium hippurate.

All strains had various sensitivity to temperature of 42 °C — *C. fetus* subsp. *fetus* 15OV showed insignificant growth, *C. fetus* subsp. *venerialis* 6913/2 did not grow and *C. jejuni* 5779 grew up at this temperature.

Conclusions. It was established that *Campylobacter* lose about 1.0% of their life potential for every year of storage in lyophilized form. After 10–12 years of storage only 44.4% strains retain their viability, therefore it is necessary to consider these storage conditions.

It has been shown the ability to save typical properties of *Campylobacter* strains within the specified retention period.

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ISOLATION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* FROM ZOO ANIMALS

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Summary. The article presents the results of cultural study of faeces samples from zoo animals and water samples. Five cultures were isolated from zoo animals (*Lama glama, Camelus bactrianus, Ammotragus lervia, Elaphurus davidianus, Bos frontalis frontalis*) and one culture from water sample. Culture-morphological, tinctorial, biochemical and biological properties of epizootic cultures of mycobacteria isolated from zoo animals were studied. It was the reason to attribute isolates to *M. avium* subsp. *paratuberculosis*. The results of conducted research indicate circulation of *M. paratuberculosis* among zoo animals.

Keywords: paratuberculosis, zoo ungulates, *Mycobacterium avium* subsp. *paratuberculosis*, insulation, cultivation, biological test.

Introduction. Paratuberculosis (Enteritis paratuberculosa, Johne's disease) — a chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*), characterized by granulomatous enteritis, temporary in the beginning and permanent afterwards diarrhea, progressive depletion, reduce of productivity and animal's death. Domestic ruminants, mainly cattle and sheep, are most susceptible to this disease. Buffaloes, camels, goats, deer, yaks rarely get sick. The range of susceptible to paratuberculosis animals is not limited by ruminant species. *MAP* has also been isolated from primates, rabbits, cats, foxes, badgers, bears, raccoons, rats, wood mice (Vansnick, 2004). Moreover, *MAP* is the cause of disease and mortality of many species of wild hoofed animals kept in zoos or endangered species. According to some researchers, species and breed of animals play not-significant role at risk of infecting. The differences in the overall infecting between species can be determined by the level and time of *MAP* exposure, resistance and susceptibility to the pathogen or other factors. According to the American Zoo and Aquarium Association one third of US zoos have at least one case with clinical paratuberculosis form since 1995. In a study by Weber and Gürke (1992) stated that *MAP* culture were isolated from fecal samples from 16.1% of the zoo ruminants. Infection was submitted in dwarf goats, moufflon, alpine ibex and Cameroonian sheep (Manning, 2001).

In 1999, problem of *MAP* spread among camelids in the Antwerp Zoo appeared after *MAP* culture isolation from faeces of okapi (Vansnick, 2004). The presence of *MAP* in feces and ileocecal lymph nodes in alpaca and camel were confirmed by PCR and by the cultural method in Germany (Burton et al., 2001). According to the large-scale study conducted in 1991–2007 at the San Diego Zoo (United States), *MAP* has been direct or indirect cause of death of many ungulates.

Paratuberculous infection was confirmed in 74 animals born before 1991 of 30 different species and 71 bovine animals born or imported from other zoos until 1991 in the research conducted on 3 298 animals representing 131 species. The main risk factors are the specific content of animals in captivity (limited space and high concentration), the exchange of animals with other zoos, stress during transportation. The true infections may remain unnoticed for years because of the long incubation period and the discontinuous *MAP* isolation even when prevention programs are carried out.

Thus in 2007 paratuberculosis was diagnosed in two David deer born in 1994 and 1997 from negative female reacting negative in cultural study of feces for more than 10 years. The infection source was unknown. These data demonstrate the importance of a long-term observation and testing of zoo animals on paratuberculosis (Münster et al., 2013).

As for the Ukrainian zoos, animal's research on paratuberculosis is not carried out and epizootic situation of the disease remains unknown.

The aim of the work was to study the fecal samples of zoo's ungulates and water samples on paratuberculosis.

Materials and methods. Fecal samples were collected for bacteriological examination from cloven-hoofed (*Camelus bactrianus, Lama glama, Vicugna pacos, Ammotragus lervia, Elaphurus davidianus, Bos frontalis frontalis*) and hoofed (*Equus ferus przewalskii, Equus asinus, Equus asinus asinus, Hippotigris*) animals. Water samples from the reservoir located at the zoo were also collected.

Fecal and water samples was treated with 0.9% sodium N-cetylpyridinium chloride. Suspensions were seeded on the developed in NSC 'IECVM' culture

media with and without growth factor, alcoholic extract of *M. scrofulaceum*, and on the culture media for *MAP* cultivation containing low-moor peat extract and 0.5% citric-ammonium ferric. Seeded cultures were cultivated in an incubator at 37.5±0.5 °C for 6 months. The cultural-morphological, biochemical and biological properties of isolated and adapted culture of mycobacteria with sufficient amount of bacterial mass was studied.

The biochemical properties of adapted subcultures were studied using following tests: hydrolysis of Tween-80, the recovery of potassium tellurite, determining of catalase and amidase activity.

The biological properties of isolated cultures were studied using double (with 7 days interval) intravenous infection of one-month old rabbits with culture suspension at a concentration of 2 mg/cm^3 on a sterile saline. The allergic test of infected animals was conducted every 30 days after infection using avian tuberculin (PPD). The experiments with animals was conducted based on the principles of bioethics.

Results. Four-six 'blind' passages have been made on a media with growth factor before visible growth of colonies from *Lama glama, Camelus bactrianus, Ammotragus lervia, Elaphurus davidianus, Bos frontalis frontalis* fecal samples and water samples was obtained. While smear microscopy of isolated cultures (n=6) stained by Ziehl-Neelsen method, characteristic clusters of very small acid-resistant sticks, cocci forms and less sticks arranged singly was observed. Primary colonies had the appearance of transparent beads of diameter less than 0.5 mm, but over time, the colony becomes more intense white color.

During further number of passages needed amount of bacterial mass for further study could only be obtained from cultures isolated from the *Elaphurus davidianus, Lama glama* and water samples. The remaining three isolates (*by Camelus bactrianus, Ammotragus lervia and Bos frontalis frontalis*) regardless of the number of performed passages, grew very slowly (4 month) in the form of tiny colonies. Thus, slow growth, dependence on growth factor, specific morphology and microorganism's location in smears was the reason to attribute isolated culture to *Mycobacterium avium* subsp. *paratuberculosis*. Culture obtained from *Elaphurus davidianus, Lama glama* and from water samples were adapted to the Pavlovskiy medium and egg medium with 0.5% citric-ammonium ferric. Colonies were white to gray or light cream color depending on the nutrient medium on which they were cultured. Colonies grew at a temperature 37–40 °C, had a smooth, moist, eventually folded surface and an oily consistency (S-form), at temperatures of 20 and 45 °C growth was not observed. The cultures did not grow on media containing 5% sodium chloride and sodium salicylate (1 mg/cm^3).

Basing on the study of the biochemical properties it was established that isolates have had a negative reaction with carbamide, catalase, pyrazinamide and weak reaction with nicotinamide, did not hydrolyzed Tween-80, restored potassium tellurite for 21 hours. Only culture from *Elaphurus davidianus* hydrolyzed Tween-80 after 10 hours.

Biological properties of three isolated cultures were studied. The following symptoms was conducted on experimentally infected rabbits while biotest (n=6, 2 bodies for each culture): cachexia, growth retardation, diarrhea, atrophy of hind limbs muscles. Rabbits died after 2–3 month. There were positive reactions to the avian tuberculin (PPD). Allergic reaction to mammal tuberculin (PPD) was absent. A big amount of aggregations of very small acid-fast rods were observed during microscopy of fecal samples. Specific for paratuberculosis changes were found in the small intestine, especially in the ileum, jejunum, and ileocecal valve during pathological examination of all animals. Intestinal contents represented light yellow transparent mucus with bubbles of gas. The walls of the intestine were 3–4 times thickened, sometimes with points of hemorrhages. There were areas with transverse and longitudinal furrows mucosa with fine gray-white nodules in the intestine. Serous nodules were also observed on mesenteric lymph nodes and liver. Original cultures were isolated from the lungs, spleen, liver and intestines as a result of seeding of biomaterial on nutrient medium with growth factor. Thus, specific for paratuberculosis lesions reproduced in experimentally infected rabbits confirmed that isolated from *Elaphurus davidianus* and *Lama glama* faeces and water samples cultures are *Mycobacterium avium* subsp. *paratuberculosis*.

Conclusions. Six mycobacterial culture isolates were attributed to *M. avium* subsp. *paratuberculosis* on the basis of study of culture-morphological, tinctorial, biochemical and biological properties. Isolation of *MAP* from fecal and water samples testifies circulation and risk of this infection spread among zoo animals. This fact justifies the need for further research on paratuberculosis.

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Part 2. Emergent diseases and biosafety

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BRUCELLOSIS — THE CURRENT SITUATION IN POLAND

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Summary. The surveys of animals for brucellosis in Poland are primarily based on serological tests. The examinations are performed by regional laboratories using RBT. In the case of positive results obtained in this test the samples are examined in SAT and CFT. The definitive confirmatory investigations are conducted by the National Reference Laboratory for Brucellosis in the Department of Microbiology of NVRI in Pulawy, which additionally uses Coombs' test, 2-Me test and ELISA. In the paper results of the examination of cattle in Poland in the years 2005–2014 are shown. Each year during examination period 130–420 cows were involved in confirmatory investigations and 6–34 animals were classified as positive for brucellosis. In bacteriological examinations of samples from seropositive cows, *B. abortus* has never been isolated. Only in 2006 *B. suis* biovar 2 has been recovered from the bovine internal organs. *B. melitensis* has never been reported and according to the Commission Decision from 2006, Poland has been regarded as 'Brucellosis officially free country'. At the moment, the aim of the ongoing testing is to maintain the *B. melitensis* free country status. Sheep and goats are tested using RBT. Samples reacting positive in the RBT are retested in the National Reference Laboratory for Brucellosis (NRL) using again RBT and CFT. Up to now all samples tested in NRL were finally regarded as negative. Ovine epididymitis (*B. ovis*) has been also reported in Poland. Diagnosis of the disease is based on the serological examination by complement fixation test (CFT) using the antigen obtained from the rough strain of *B. ovis* REO198. Similarly, examinations based on the antigen obtained from *B. canis* are conducted in dogs (rapid slide agglutination test – RSAT and slow agglutination test – SAT), but brucellosis in this species of animals has never been confirmed in Poland. Regarding the situation in porcine brucellosis, there were some outbreaks several years ago and active monitoring of pigs is performed. In general, positive results are mainly connected with false positive serological reactions (FPSR) due to *Y. enterocolitica* O:9. There is no active monitoring of brucellosis in the wildlife animals in Poland but samples are taken during the hunting season and tested for scientific purposes. When testing 4407 samples of the wild boar sera, 1077 sera reacted positive in ELISA. Also research done on hare sera several years ago indicated very similar percentage of the positives. These data show that the wildlife is a huge reservoir of the *Brucella* and it could play a role in an epidemiology of brucellosis also in domestic animals.

Keywords: *Brucella*, bovine brucellosis, porcine brucellosis, wildlife, *B. suis* biovar 2

Introduction. Brucellosis is an infectious disease, affecting many species of animals and man, caused by bacteria of the genus *Brucella*. The genus encompasses ten species: *B. abortus, B. melitensis, B. suis, B. ovis, B. canis, B. neotomae, B. cetaceae, B. pinnipediae, B. microti* and *B. inopinata* (Godfroid et al., 2005; Scholz et al., 2009; Jiménez de Bagüés et al. 2011). Brucellae are gram-negative facultative intracellular bacteria causing disease which remains a zoonosis of worldwide public health and economic importance (Godfroid et al., 2005; Franco et al., 2007). The main role play: *B. abortus*, responsible for bovine brucellosis; *B. melitensis*, the main agent of ovine and caprine brucellosis; and *B. suis*, which causes brucellosis in pigs. The testing is based almost entirely on serological assays. The most often used and important tests are Rose Bengal test (RBT), complement fixation test (CFT), ELISA, fluorescence polarization assay (FPA) and serum agglutination test (SAT). But unequivocal diagnosis of *Brucella* infection can be made only by the isolation and identification of the agent.

In Poland, bovine brucellosis has been eradicated in 1980 and since that time isolations of *B. abortus* have not been reported. In 2009 the country obtained official 'Brucellosis-free' status according to EU regulations (EC, 2009). On the other hand, *B. suis* is isolated from cattle from time to time (Szulowski et al., 2012). A huge reservoir of these bacteria constitutes a wildlife,

in particular wild boars and hares (EFSA, 2009). *B. melitensis* has never been reported and Poland has been 'Brucellosis officially free country' since 2006 (EC, 2006). What concerns brucellosis in pigs it was reported sporadically in the past but in recent years the only case concerned boars imported from one of the EU countries (Szulowski et al., 2011). In Polish conditions symptoms of *B. ovis* infections causing epididymitis, orchitis and impaired fertility in rams have been also reported. In turn, no evidence (isolation of *B. canis*) of brucellosis in dogs has been provided.

Bovine brucellosis. In Poland, bovine brucellosis (*B. abortus*) was eradicated in 1980, but due to EU regulations, the country obtained its official 'Brucellosis-free' status as late as 2009. On the other hand, wildlife constitutes a huge reservoir of *B. suis*. Surveys revealed that 12.3% of the sera from wild boars (Szulowski, Pilaszek and Iwaniak 2000) and 0.9% of the hare sera (Pilaszek, Szulowski and Iwaniak, 2000), collected from various part of the country, reacted positively in ELISA. The serology was confirmed by culture, which showed the occurrence of *B. suis* biovar 2, both in hares and wild boars (Szulowski, Pilaszek and Iwaniak 2000; Szulowski et al., 2008). The diagnosis of bovine brucellosis in Poland is primarily based on serological tests. All animals monitored for brucellosis and positive in the Rose Bengal test (RBT) undergo a further examination with the use of a serum agglutination test (SAT) and a complement fixation test (CFT) in accordance with the 'Manual of Diagnostic Tests and Vaccines for Terrestrial Animals' (OIE, 2012), and Annex C to Council Directive 64/432/EEC (EC, 2002). The National Reference Laboratory for Brucellosis (NRL) as a panel of confirmatory tests for further analysis uses also 2-mercaptoethanol test (2-ME), Coombs antiglobulin test (Coombs) and an indirect-ELISA commercial test (IDEXX). 2-Me and Coombs test are carried out according to official protocols (Królak and Stryszak, 1979; Wiśniowski, Królak and Drożdżyńska, 1978). All serum samples from those positive in RBT and SAT, which are positive in CFT and/or in one or more of additional tests (2-ME, Coombs, ELISA), in accordance with the Polish regulations, are classified as finally positive. In such cases, the animals are obligatorily slaughtered and subjected to bacteriological examination. For culture of the specimens (supramammary lymph nodes, liver, spleen, uterus) taken from the euthanized cattle the serum dextrose agar (SDA — home-made medium) is used. The plates are then incubated for 10 days at 37°C in an atmosphere with 5–10% CO_2 added. In parallel, the specimens are cultured in similar conditions in an enrichment liquid medium (serum dextrose broth — SDB, supplemented with antibiotic mixture) for up to 6 weeks with weekly subcultures on to a solid selective medium (Farrell's home-made medium). Colonies typical for *Brucella* are checked with anti-*Brucella* standard serum, examined in catalase and oxidase tests and stained by Gram's method. Further characteristics is performed by using monospecific anti-A and anti-M sera (ANSES, France) and further tests for: CO_2 requirement, production of H_2S (Hydrogen Sulfide Test Strip, Fluka) and urease, growth in the presence of thionin and basic fuchsin, and lysis by phages (Tbilisi at its routine test dilution — RTD and $10^4 \times$ RTD) (Alton et al., 1988). Additionally, molecular methods — a multiplex PCR assay (Bruce-ladder) and a multi-locus analysis of variable number tandem repeats (MLVA) are applied to confirm the identification of isolated *Brucella* strains (Lopez-Goni et al., 2008; Le Fleche et al., 2006).

From 2009, every year 1/5 of the bovine population (≥ 2 years old animals) in Poland is tested for brucellosis. Some of them react positive in a first screening test — RBT. Finally, several animals are recognised as a reacting positive for brucellosis. The next step is bacteriological examination of seroreagents. The data presented in the Table 1 show the results of serological examination of cattle in Poland from 2005 to 2014.

Table 1 – Results of serological examination of cattle in years 2005–2014

Year of testing	Number of seropositives
2005	12
2006	13*
2007	11
2008	25
2009	13
2010	34
2011	17
2012	13
2013	6
2014	19

* — isolation of *B. suis* biovar 2 from one cow

Brucellosis in sheep and goats (*Brucella melitensis* infection**).** *B. melitensis* has never been reported and according to the Commission Decision from 2006, Poland has been regarded as 'Brucellosis officially free country'. At the moment, the aim of the ongoing testing is to maintain the *B. melitensis* free country status. Sheep and goats are tested using RBT. Samples reacting positive in the RBT are retested in the National Reference Laboratory for Brucellosis (NRL) using again RBT and CFT. Up to now all samples tested in NRL were regarded as negative, it means that negative results have been obtained in CFT. If positive results in CFT are obtained, the District Veterinary Officer is asked to take the samples from the whole sheep or goat herd.

The samples are then tested again after the 30 days period from the last sampling.

Ovine epididymitis (*Brucella ovis* infection). *Brucella ovis* microorganisms cause brucellosis in sheep defined as ovine epididymitis. Disorders of reproductive tract is the main clinical sign of the disease. The disease produces inflammation of epididymis and testes in rams, infrequent abortions and poor neonatal lamb viability in ewes. Disease has been reported in many European countries, in both Americas, Africa, Australia and New Zealand. Probably disease occurs in most sheep raising countries. Ovine epididymitis has been also reported in Poland. Diagnosis of the disease is based on bacteriological or serological examination. Because of poor sensitivity, time-consuming and high costs of bacteriological methods, similarly like in diagnosis of 'smooth' *Brucella* infections serological methods are more often used. Following tests are used: complement fixation test (CFT), agar gel immunodiffusion test (AGID) and enzyme linked immunosorbent assay (ELISA). In Poland serological examination is carried out by CFT using the antigen elaborated in the National Veterinary Research Institute in Pulawy, in Department of Microbiology. The antigen has been titrated against Polish Working Standard of anti-*Brucella* ovis serum, which corresponds to International Standard of anti *Brucella ovis* serum. The antigen is in a permanent offer of the NVRI in Pulawy. At the moment, in Poland there is no ongoing national program of monitoring of *B. ovis* infection in a sheep population. Only animals devoted to reproduction, especially rams, are included in an examination scheme.

The last large scale examination has been performed in 1999. Results are shown on the Fig. 1.

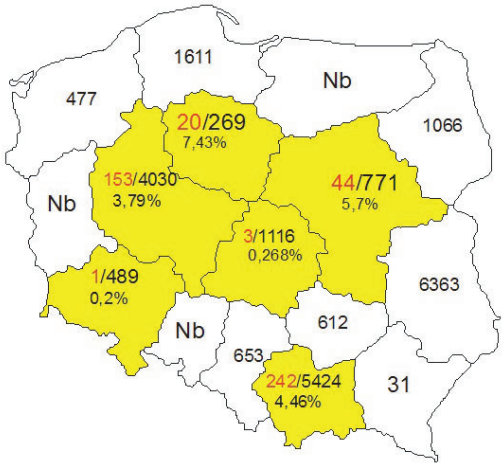

Figure 1. Distribution of the *B. ovis* seropositive sheep in voivodships in 1999

The obtained results indicate that *Brucella ovis* infections may be an important agent which affects health status and productivity of sheep flocks in Poland.

As far as *B. ovis* is not a zoonotic agent, the importance of the sheep testing has a great value, because infection in the flock leads to reduced productivity and significant economic loses in animal production.

Brucellosis in pigs. Porcine brucellosis is a zoonotic disease of widespread occurrence and global significance. However, the prevalence is low with the exception of South America and South-East Asia, where it is higher. Within the European Union (EU), the epidemiological situation is varied, with some countries free of the disease, others reporting sporadic outbreaks, and yet others reporting this disease as an emergent problem. Available epidemiological evidence shows that *B. suis* biovar 2 is the most common agent in Europe, and wildlife (wild boars and hares) constitutes a source of infection for pigs (Gyuranecz et al., 2011; Szulowski et al., 1999; Szulowski, Pilaszek and Iwaniak, 2000). There is a lack of systematic epidemiological data on porcine brucellosis in the member states of the EU, as there is currently no requirement for monitoring and surveillance of *B. suis* in domestic pigs and wild animals. However, in many disease-free countries statutory diagnostic testing is required, for example concerning boars in insemination stations, and is often a prerequisite for the movement of live animals. Testing is based almost entirely on serological assays, though the unequivocal diagnosis of *B. suis* infection can be made only by the isolation and identification of *Brucella*. Methods and tests used for the diagnosis of porcine brucellosis are very similar or even identical to those applied for the diagnosis of brucellosis in cattle. To date none of the serological tests has been shown to be reliable in routine diagnosis in individual pigs. The Rose Bengal test (RBT), the complement fixation test (CFT), indirect and competitive enzyme-linked immunosorbent assays (I-ELISA and C-ELISA), and the fluorescence polarization assay (FPA) are the prescribed tests for international trade purposes (OIE, 2012). In Poland the methods employed for diagnosing porcine brucellosis are RBT, I-ELISA, and additionally, to explain doubtful results, CFT, the serum agglutination test (SAT) and the 2-mercaptoethanol test (2-Me).

False positive serological reactions to brucellosis (FPSR). FPSR for brucellosis become a growing problem in international trade. The similarity of the O-antigenic side chain of *Brucella* with other microbes limits the specificity of serological diagnosis (Weiner et al., 2014). Most of the FPSR are caused by infections with *Yersinia enterocolitica* O:9, as the bacterium has identical O-antigen to that present in *Brucella* sp. (Cvetnic et al., 2003). In the last decade, *Y. enterocolitica* O:9 immensely increased the rate of FPSR (EFSA, 2007; EFSA and ECDC, 2013). The experts know the problem in their countries but there is no procedure on EU level

what to do in such case. In our opinion pigs originated from herds with the problem of FPSR for brucellosis should generally be avoided from international movement. But it is not always possible and pigs can be taken for trade from herds where the problem is not well recognized. If FPSR are found two different paths should be considered, to avoid slaughtering of animals. The first one — ignore it and introduce pigs into breeding herds. Such situation, connected with the presence of *Y. enterocolitica* O:9 infection may produce long lasting diagnostic, administrative and epidemiological problems in the future. The second path — to exclude animals from the breeding and allocate them for fattening. A clear guidelines for dealing in such cases should be created.

Brucellosis in the wildlife. The systematic brucellosis monitoring in wildlife does not exist as surveillance of the animal health status is strictly regulated for domestic animals only. But there are several publications showing the occurrence of brucellosis in wild boars in Europe. In Croatia Cvetnic et al. (2004) reported the presence of anti-*Brucella* antibodies by ELISA in 13.6% serum samples from wild boars. Garin-Bastuji et al. (2000) reported that in different regions of France positive serological reactions to brucellosis were found in wild boars in the range from 20% to 35%. In the Czech Republic the frequency of positive reactions to brucellosis was 15% (Hubálek et al., 2002) and in North-Eastern Germany 22% (Al Dahouk et al., 2005).

Last time in Poland a total of 4 407 sera of wild boars hunted in 2012, originated from territory of 11 out of 15 voivodeships from Poland were examined. Blood samples were taken from each animal from thoracic cavity, heart or pericardium into plastic tubes and allowed to clot. The sera were then separated by centrifugation and stored at – 20°C until tested. The sera have been tested by ELISA. Due to OIE Manual (2012), ELISA is one of the prescribed tests for international trade purposes in pigs. Besides, in contrast to other serological methods used in diagnosis of brucellosis, the test allows to detect anti-*Brucella* antibodies even when the quality of serum is poor, what is very common when we get material from wild animals.

Out of 4 407 examined samples, 1 077 (24.44%) reacted positively in ELISA for brucellosis. Figure 2 presents the distribution of OD values obtained in the ELISA in testing serum samples. Among sera classified as negative, the highest number of samples had OD values in the range 20–30% (N = 2 085) and 30–40% (N = 470) in relation to OD of positive control serum S+. On the other hand among sera classified as positive the largest number of samples had OD values in the range 100–150% (N = 274) and 150–200% (N = 215). The highest OD value of positive sample exceeded 800%.

Figure 3 presents the distribution of positive results of ELISA in respective voivodeships. The highest observed percentage of positive samples was observed in opolskie (39.9%), wielkopolskie (37.29%) and śląskie (34.48%) voivodeships. On the other hand the lowest ratio of positive results was observed in kujawsko-pomorskie (13.74%), łódzkie (15.47%) and warmińsko-mazurskie (18,66%).

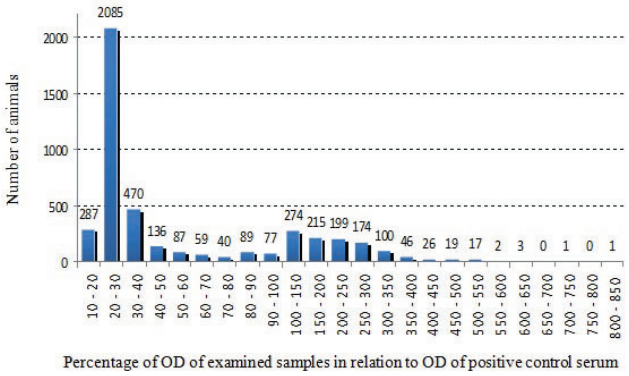

Figure 2. Distribution of OD values obtained in the ELISA in testing serum samples from wild boars

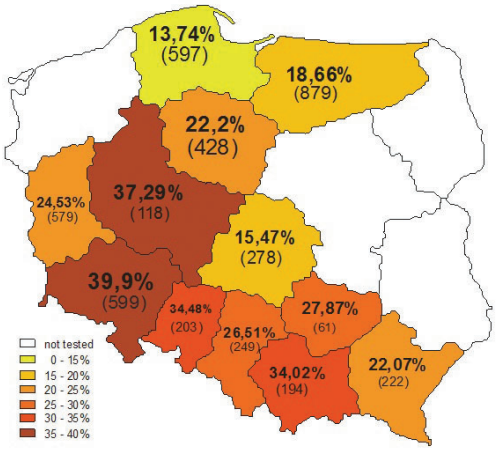

Figure 3. The results of examination of wild boars for anti-*Brucella* antibodies — voivodeships

The current results of investigations performed on much larger number of samples (N = 4 407) confirm that wild boars in Poland, similarly as in other countries of Europe, constitute a very important reservoir of *Brucella* microorganisms, undoubtedly major than hares. The prevalence, clearly higher than found several years ago, is particularly high in southwestern and lower in north and central part of the country. Bacteriological examinations performed in Poland on material from wild boars (usually lymph nodes) indicate that always the causative agent of brucellosis in wild boars and the presence of anti-*Brucella* antibodies is *B. suis* biovar 2 (Szulowski, Pilaszek and Iwaniak, 2000)

and it is typical for Europe (Kautzsch et al., 1995; EFSA, 2009). Fortunately this biovar is considered as rarely pathogenic or non-pathogenic for humans, and has only exceptionally been described as the causative agent of human brucellosis (Paton et al., 2001; Teyssou et al., 1989). But its importance stems from the fact, that *B. suis* biovar 2 can infect domestic pigs and even cattle. What concerns pigs, wild boars are potentially important source of infection especially in countries where porcine outdoor rearing systems are practiced (EFSA, 2009). It appears that only because this system is not popularized in Poland, despite so high level of *Brucella* infections in wild boars, outbreaks of brucellosis in domestic pigs are very sporadic — the last one was recorded in 1999. On the other hand, our previous investigations concerning cattle revealed that in Poland *B. suis* biovar 2 infuences the epidemiology and control of bovine brucellosis.

European brown hares along with wild boars are the natural reservoir of *B. suis* biovar 2. Small rodents and domestic animals (pigs, cattle, dogs) may be also involved in the infection chain. People can be infected while handling, skinning and eviscerating the carcases of infected hares or even by eating undercooked meat. The organism is rarely pathogenic for humans. A few years ago, in the wintertime, many hares was exported to France and to Italy. Before the shipment, the animals have been examined by palpation method. During that issue some hares showed clinical manifestation of brucellosis. The following pathological changed have been observed: nodes with purulent content, with greasy to dense consistency (more often mustard like) with yellow-green to yellow or white-creamy coloration. The nodes occurred occur in various parts of the animal body: hypodermic connective tissue, intramuscular tissue, spleen, liver, lungs and reproductive organs. Some of these changes are shown on Fig. 4–6.

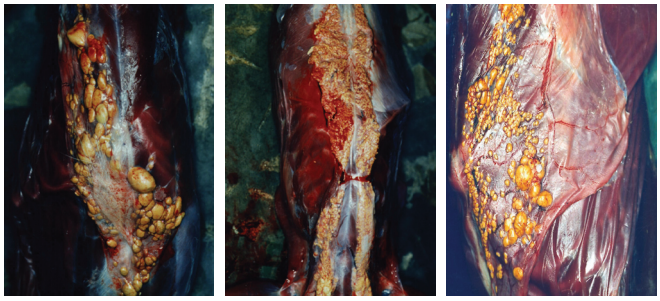

Figure 4. Purulent nodes in the subcutaneous tissue

Brucellosis in dogs. The etiological agent of brucellosis in dogs, named also contagious abortion in dogs, was first isolated by Carmichael in 1966. The causative agent is *B. canis*, similarly as *B. ovis* existing in nature in 'rough' form. The clinical signs of the disease in dogs are abortion and infertility in the female and epididymitis, orchitis, abnormal semen and testicular degeneration in the male with generalised lymph node enlargement and occasional discospondylitis and uveitis (Alton et al., 1988). In Poland the diagnosis of canine brucellosis is based on serological examinations. The antigen used in examinations has been prepared on the basis of less mucoid variant of *B. canis* strain. The methods used are the rapid slide agglutination test — RSAT, and the slow agglutination test — SAT. The examinations are not conducted in a large scale — concern primarily dogs travelling with their owners to other countries and animals from kennels. Till now *B. canis* infection has never been confirmed in Poland.

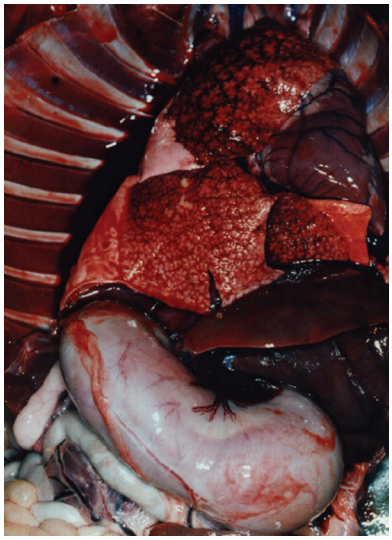

Figure 5. Caseous changes in lungs

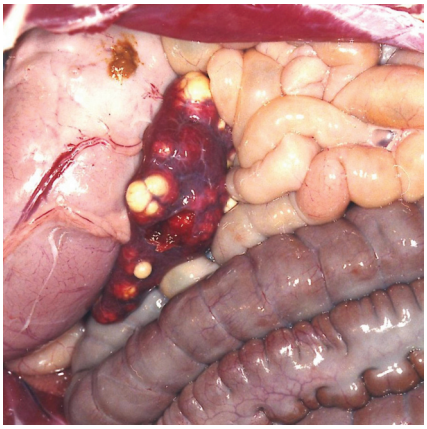

Figure 6. Enlarged spleen with purulent nodes

Conclusions. The diagnosis of brucellosis in Poland is conducted by the net of regional laboratories and the National Reference Laboratory for Brucellosis (NRL) in Department of Microbiology of the National Veterinary Research Institute in Pulawy.

The methods used are compliant with the requirements of European Community, and both serological and bacteriological methods are involved in examinations. The epidemiological situation of brucellosis in domestic animals in Poland is very good. The country has the 'brucellosis-free' status for bovine brucellosis and ovine and caprine brucellosis (*B. melitensis*). *B. melitensis* has never been confirmed in Poland while B. abortus has not been isolated from cattle since 1980. In pigs, brucellosis is recorded very rarely and, similarly as in other European countries, is caused by *B. suis* biovar 2. The reservoir of this biovar constitute wild boars (where the prevalance is relatively high) and hares. What concerns brucellosis caused by 'rough' *Brucella* species — *B. ovis* infections are recorded in sheep, whereas *B. canis* has never been confirmed in Poland.

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STUDY OF DRUG-RESISTANT TUBERCULOSIS IN KHARKIV REGION, UKRAINE

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Summary. A total of 93 isolates from pulmonary tuberculosis (TB) patients in Kharkiv region were characterized by drug susceptibility testing and genotypic analyses (VNTR using five exact tandem repeat loci). Obtained data demonstrated that the Beijing genotype was the most common (in 64 cases) among studied strains. Twelve isolates had individual VNTR profile. Among all patients 41 (44%) were diagnosed as MDR TB and 52 (56%) — TB with extending resistance. The most common resistance of *Mycobacterium tuberculosis* was observed to streptomycin. The frequency of resistance to kanamycin, 4-aminosalicylic acid and ethionamide was revealed significantly higher for LAM strain in compare to Beijing strain ($p<0.05$).

Keywords: tuberculosis, *Mycobacterium tuberculosis*, strains, Kharkiv region, Ukraine, resistance, PCR

Introduction. Today tuberculosis (TB) is a major public health and social problem not only in Ukraine but also all over the world (WHO, 2014). Drug resistance of *Mycobacterium tuberculosis* is one of the main factors limiting the effectiveness of TB treatment.

On the background of gradual decrease of epidemic indicators at the present stage there is the risk of the multidrug-resistant tuberculosis (MDR TB) spreading in Ukraine (ed. Tolstanov, 2015).

MDR TB is the form of tuberculosis when isolated mycobacteria have resistance to at least isoniazid and rifampin or often to more anti-TB drugs from I and II groups, which could be confirmed by laboratory method to the drug sensitivity test (MHU, 2014).

According to the WHO data in Ukraine MDR TB was found in 16 % of initially diagnosed patients and 44% of previously treated TB patients. About 8 % of newly diagnosed patients interrupt treatment and the death rate is up to 12 % (ed. Tolstanov, 2015).

The number of MDR TB cases has increased almost in three times last 10 years due to the implement of modern methods of molecular diagnostics. From the total number of patients with MDR TB 13% of them were registered with extending resistance to anti-TB drugs (Feshchenko and Melnyk, 2013).

Development of molecular genetics gave opportunities to carry out genetic typing of *M. tuberculosis*, which allows distinguishing between strains of the pathogen and determining their role in the further clinical course. The genetic families or clades of *M. tuberculosis* complex have been identified in different geographic regions (Beijing, East African Indian (EAI), Central Asian (CAS), T, Haarlem I, X and Latin American Mediterranean (LAM) families) as well as unidentified strains and other widely distributed and maintained epidemiologic TB at a high level. In a number of observations it found that the in difficult cases and non-effective treatment often associated with strains from Beijing family (Liashenko, 2015). This family genotype is also common in the other countries of the former Soviet Union (Lillebaek et al., 2003).

In this article, we report the analysis of 93 isolates of *M. tuberculosis* from MDR-TB patients collected during 2014–2015.

The aim of the study was to estimate resistant of different *M. tuberculosis* strains that were isolated from MDR TB patients in Kharkiv region.

Materials and methods. In the period between September 2014 and September 2015 the 93 cases of TB were studied in patients who were treated in the hospitals in Kharkiv region in Ukraine. Patients were selected by blind method.

Mycobacterium identification and testing of the drug susceptibility of these strains to four first-line anti-TB drugs (isoniazid, rifampin, ethambutol, pyrazinamide and streptomycin) and two second-line anti-TB drugs (kanamycin, amikacin, capreomycin, ofloxacin, levofloxacin, moxifloxacin, prothionamide, 4-aminosalicylic acid, cycloserine and ethionamide) were performed as recommended by WHO (WHO, 2014). The samples of expectoration were used for the strain isolation on Lowenstein-Jensen medium. A strain was referred resistant to the specific drug when the growth rate exceeded 1% compared to the control.

All obtained strains were inactivated by heating and used for DNA extraction by commercial kit 'Diatom DNA Prep 200' (Ukraine) according to the manufacture instruction. PCR was performed using 'GenPak PCR Core' (Russian Federation). VNTR genotyping was done by using sets of primers for amplification of five exact tandem repeat ETR loci (A, B, C, D, E) as previously described (Frothingham and Meeker-O'Connell, 1998;

Liashenko, 2015). PCR was carried out in a total volume of 25 μL using 5 μL of template DNA. PCR protocol included an initial denaturation of 5 min at 95 °C that was followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 62 °C for 1 min and extension at 72 °C for 25 s. A final extension was at 72 °C for 5 min. Obtained amplicons were analyzed in 1.8% agarose gel after electrophoresis followed by staining with ethidium bromide.

Statistical analysis was done using Excel 2007 package soft (Microsoft, license № RW2FR-7DFDD-TCF8J-9K9BJ-MJ678).

Results and discussion. Among 93 patients 80% were men (n=74) and 20% women (n=19). Age of the patients was between 23 and 84 years. Average age was 49±1.2 years.

All patients were observed by clinical, radiological and laboratory examination in accordance with Decree № 620 of Ministry of Health of Ukraine (MHU, 2014). There was widespread pulmonary tuberculosis in all patients with massive recovery of mycobacteria, which was confirmed by culture and smear.

As the first stage of our work we provided VNTR analysis by 5 ETR loci. It was determined the variability of each locus by PCR from all 93 strains. The results of VNTR-genotyping were shown that the most common profile was 42 435 that belong to the Beijing genotype (69%). It was demonstrated genotype LAM (13%), Africanum (3%) and Harlem (2%) (Fig. 1). This data correlated with previously studies in Kharkiv region by Dymova et al. (2011). Twelve strains were not identified by reason of unique VNTR profiles.

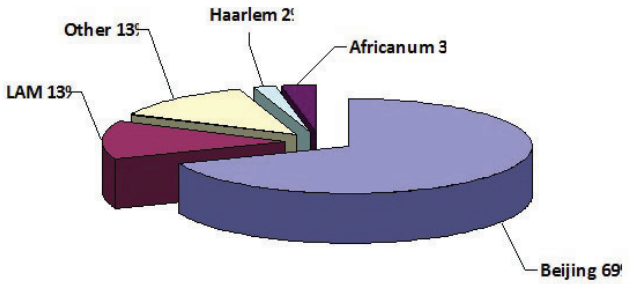

Figure 1. Prevalence of different genotypes among 93 *M. tuberculosis* isolates in Kharkiv region TB surveillance.

All isolates were tested for drug resistance using liquid and solid media. The results of resistant to the first-line anti-TB were shown in Table 1.

We observed the most common resistance to streptomycin (100%), followed by resistance to isoniazid (99%), rifampin (96%) and ethambutol (89%). There were no significant statistical differences in resistance frequency to the first-line anti-TB drugs among *M. tuberculosis* strains (p>0.05). Our findings showed that 26 patients with active TB could transmit MDR mycobacterium from Beijing clade that is stable to all first-line anti-TB drugs. All isolates were tested as well to the second-line anti-TB drugs (Table 2).

Table 1 – Resistant of *M. tuberculosis* isolates to the first-line anti-TB drugs

Resistance to	*M. tuberculosis* strains					
	Beijing (n=64)	LAM (n=12)	Haarlem (n=2)	Africanum (n=3)	Other (n=12)	Total (n=93)
Isoniazid (H)	63 (98%)	12 (100%)	2 (100%)	3 (100%)	12 (100%)	92 (99%)
Rifampicin (R)	63 (98%)	11 (91.5%)	1 (50%)	3 (100%)	11 (91.5%)	89 (96%)
Ethambutol (E)	58 (90.5%)	11 (91.5%)	0	3 (100%)	11 (91.5%)	83 (89%)
Pyrazinamide (Z)	26 (40.5%)	2 (16.5%)	1 (50%)	1 (33.3%)	6 (50%)	36 (39%)
Streptomycin (S)	64 (100%)	12 (100%)	2 (100%)	3 (100%)	12 (100%)	93 (100%)

Table 2 – Resistance of *M. tuberculosis* isolates to the second-line anti-TB drugs

Resistance to	*M. tuberculosis* strains					
	Beijing (n=64)	LAM (n=12)	Africanum (n=3)	Haarlem (n=2)	Other (n=12)	Total (n=93)
Kanamycin (Km)	42 (65.5%)	12 (100%)*	3 (100%)	0	7 (58%)	64 (69%)
Amikacin (Am)	8 (12.5%)	5 (41.5%)	1 (33.3%)	0	4 (33.3%)	18 (19%)
Capreomycin (Cm)	20 (31.2%)	5 (41.5%)	3 (100%)	0	4 (33.3%)	32 (34%)
Ofloxacin (Ofx)	37 (57.8%)	9 (75%)	3 (100%)	0	7 (58%)	56 (60%)
Levofloxacin (Lfx)	11 (17%)	3 (25%)	1 (33.3%)	0	1 (8.3%)	16 (17%)

Moxifloxacin (Mfx)	10 (15.5%)	4 (33.3%)	2 (66.6%)	0	2 (16.5)	18 (19%)
Prothionamide (Pt)	8 (12.5%)	3 (25%)	1 (33.3%)	0	3 (25%)	15 (16%)
4 Aminosalicylic acid (PAS)	13 (20.3%)	11 (91.5%)*	0	0	2 (16.5)	26 (28%)
Cycloserine (Cs)	2 (3%)	2 (16.5%)	2 (66.6%)	0	3 (25%)	9 (10%)
Ethionamide (Et)	21 (33%)	9 (75%)*	1 (33.3%)	0	4 (33.3%)	35 (38%)

* — $p<0.05$ vs. Beijing

Km and Ofx did not inhibit growth of *M. tuberculosis* isolates in 69% and 60% cases respectively. The most effective anti-TB drug was Cs (10%).

The frequency of resistance to Km, PAS and Et was revealed significantly higher for LAM strain in compare to Beijing strain ($p<0.05$). Among all 93 patients 41 (44%) were diagnosed as MDR TB and 52 (56%) — TB with extending resistance. It needs to prove preventive therapy with second-line anti-TB drugs for a long duration.

Conclusions. There is difficult situation for MDR TB in Kharkiv region. It was confirmed in 52 cases (56%) of TB from totally 93 cases. Using VNTR-genotyping it was found that 68.8% of MDR strains belong to Bejing family. Differences in the chemoresistance to the second-line anti-TB drugs depending on the *M. tuberculosis* strains have been not identified. Resistance test to the second-line anti-TB drugs showed tolerance to Km, PAS and Et. In the structure of chemoresistance to the second-line anti-TB drugs depending on the strains it was determined that the stability revealed significantly higher for LAM strain in compare to Beijing strain.

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Part 2. Emergent diseases and biosafety