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STUDYING OF PHYLOGENETIC RELATIONSHIPS OF LEUKEMIA VIRUS WITH OTHER RETROVIRUSES IN CATTLE

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Summary. Bovine leukemia virus (BLV) is one of the retroviruses, which genetically, structurally and functionally related with viruses of human T-cell leukemia. BLV is very convenient model for studying the pathogenesis of humans leukemia. Genomes of retroviruses is high variability due to lack of a mechanism of correct errors that occur when copying matrix during replication, and possible genetic recombination. In this regard, the study of the genetic variability of the virus is one of the major objective for biological monitoring. At this time molecular genetic analysis (polymerase chain reaction (PCR) is a necessary part of phylogenetic research. The aim of this work was to study the variability of the bovine leukemia virus, establishing phylogenetic relationships between isolates sequenced bovine leukemia virus, which circulates in farms of different regions in Ukraine, with other animals retroviruses. Was conducted the sampling of clinical material from cattle farms from different geographical regions in Ukraine and extracted proviral BLV DNA. Total received 831 samples of peripheral blood from cattle farms in Kharkiv region, 10 samples — Kirovohrad region; 10 samples — Donetsk region; 41 sample — Crimea, Simferopol region; 10 samples — Poltava region. Sequenced gene fragments of *env* sequences of bovine leukemia virus proviral DNA, circulating in different geographical regions in Ukraine. Established isolates of bovine leukemia virus, circulating in Ukraine, belonging to the Euro-Asian subtype. Proved genetic affinity of leukemia virus and bovine syncytial virus, Jembrana disease virus and bovine immunodeficiency virus.

Keywords: DNA, phylogeographic relationships, polymerase chain reaction, sequencing, retroviruses, virus bovine leukemia.

Introduction. Bovine leukemia virus (BLV) is one of the retroviruses representative. Humans T-cell leukemia viruses, HTLV-1 and HTLV-2, genetically, structurally and functionally related to bovine leukemia virus, and the development of diseases caused by these viruses is similar (Willems et al., 1993; Dube et al., 1997). That is why BLV is a very suitable model for studying the pathogenesis of human leukemia. An important aspect of these studies is the problem of bovine leukemia virus genetic variability.

It is known that viruses, which genome is represented by RNA, characterized by high speed variation of nucleotide sequence and associated with it significant lability structure of genetic material (Steinhauer and Holland, 1987; Parvin et al., 1986; Steinhauer et al., 1989; Darlix and Spahr, 1983; Katz and Skalka, 1990; Meyerhans et al., 1989; Manini, De Palma and Mutti, 2007). Genomes of retroviruses, like other RNA-containing viruses, have high level of variability due to lack of mechanism for correcting errors arising until copying the matrix during replication and potential genetic recombination. Nucleotide modifications can lead to changes an amino acid consist of synthesized proteins (Katz and Skalka, 1990). In this regard, the study of genetic variability of infectious agents is one of the main objectives of biological monitoring, that goal is the explanation of the phenomenon.

At this time, the necessary part of phylogenetic study is molecular genetic analysis (such as polymerase chain reaction (PCR) (Licursi et al., 2003; Giammarioli et al., 2008). The method of sequencing allows performing establish the existence of point and tandem mutations (Milos, 2009). Effectiveness and objectivity of molecular phylogenetic studies depends on many factors, such as: insufficient set of experimental data, errors in sequencing or sequence alignment, convergent evolution (i.e. formation of a complex of similar features in representatives from unrelated groups), horizontal gene transfer, etc. (Wendel and Doyle, 1998). In addition, different fragments of the genome provide unequal information during the molecular phylogenetic studies, because the result is more determined by the correct choice of gene or combination of genes in the sequence. Sometimes complementary DNA (cDNA), that includes coding sequences of structural genes, used for sequencing (Caraguel et al., 2009; Chang et al., 2009).

The aim of this work was to study the variability of the bovine leukemia virus, to establish phylogenetic relationships of sequenced isolates bovine leukemia virus that circulates in farms of different regions in Ukraine with other animals retroviruses.

Materials and methods. The total DNA was extracted from peripheral blood using a commercial kit 'DNA Sorbo-B' (Russia). Detection of proviral DNA was performed by PCR using basic kits Gene Pak™ (Russia) and a pair of primers III-BLV F/R developed in 2008. The length of the amplicon is 440 bp. Sequencing of proviral DNA of *env* gene fragments was performed on an automatic sequencer ABI PRISM 311D using the technology of ABI ('Applied Biosystems', USA).

Computer analysis of the primary structure of proviral DNA fragments of bovine leukemia virus isolates, multiple alignment of proviral DNA sequenced of major genes of retroviruses was carried out using programs Bioedit v. 7.0.0 (ClustalW modules and Neighbor) and Oligo Explorer v. 1.1.0. To construct phylogenetic trees used the program MEGA v. 4.1, and to view them — TreeView v. 1.6.6.

Results. There were sampling of clinical material of cattle from farms of different geographical regions in Ukraine and proviral DNA of BLV was extracted. There were obtained 831 samples of peripheral blood of cattle from Kharkiv region farms, 10 samples — Kirovohrad region; 10 samples — Donetsk region; 41 samples — Crimea, Simferopol region; 10 samples — Poltava region.

To set the primary structure of fragments of BLV genomic material by sequencing, the samples of proviral DNA were transferred to the National Veterinary Research Institute (Pulawy, Poland). To determine the possible divergence and the genetic variability of bovine leukemia virus was conducted a multiple alignment and comparison of BLV *env* gene sequences circulating in the Kharkiv region and other geographical regions. For this purpose from the international database GenBank we have selected three fully sequenced BLV *env* gene sequences from Belgium (AF503581), USA (AY078387) and Brazil (AF399704) with length 1,548 bp, and partially sequenced fragment with length 960 bp from Poland (AF111171). Fragment of the multiple alignment of selected sequences shown in Fig. 1 (nucleotides that are not the same in this position with other nucleotides of selected sequences are underlined).

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
AF111171        GGCCGATCAA GGGTCCTTTT ATGTCAATCA TCAGATTTTA TTCCTGCATC TCAAACAATG
AF399704        GGCCGATCAA GGATCCTTTT ATGTCGATCA TCAGATTTTA TTCCTGCATC TCAAACAATG
AF503581        GGCCGATCAA GGGTCCTTTT ATGTCAATCA TCAGATTTTA TTCCTGCATC TCAAACAATG
AY078387        GGCCGATCAA GGATCCTTTT ATGTCAATCA TCAGATTTTA TTCCTGCATC TTAAACAATG
Kharkiv         GGCCGATCAG GGATCCTTTT ATGTCAATCA TCAGATTTTA TTCCTGCATC TCAAACAATG
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
AF111171        TCATGGAATT TTCACTCTAA CCTGGGAAAT ATGGGGATAT GATCCCCTGA TCACCTTTTC
AF399704        TCATGGAATT TTCACTTTAA CCTGGGAAAT ATGGGGATAT GACCCCCTGA TCACCTTTTC
AF503581        TCATGGAATT TTCACTCTAA CCTGGGAAAT ATGGGGATAT GATCCCCTGA TCACCTTTTC
AY078387        TCATGGAATT TTCACTCTAA CCTGGGAGAT ATGGGGATAT GATCCCCTGA TCACCTTTTC
Kharkiv         TCATGGAATT TTCACTCTAA CCTGGGAGAT AAAGGGATAT GATCCCCTGA TCACCTTTTC
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
AF111171        TTTACATAAA ATCCCTGATC CCCCTCAACC CGACTTCCCT CAGCTGAACA GTGACTGGGT
AF399704        TTTACATAAG ATCCCTGATC CCCCTCAACC CGACTTCCCT CAGCTGAACA GTGACTGGGT
AF503581        TTTACATAAA ATCCCTGATC CCCCTCAACC CGACTTCCCT CAGCTGAACA GTGACTGGGT
AY078387        TTTACATAAG ATCCCTGATC CCCCTCAACC CGACTTTCCC CAGTTGAACA GTGACTGGGT
Kharkiv         TTTACATAAG ATCCCTGATC CCCTTCAACC TGACTTACCC CAGTGGAACA GTGACTGGGT
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
AF111171        TCCCTCTGTC AGGTCATGGG CCCTGCTTTT AAATCAAACG GCACGGGCCT TCCCAGACTG
AF399704        TCCCTCTGTC AGATCATGGG CCCTGCTTTT AAATCAGACG GCACGGGCCT TCCCAGACTG
AF503581        TCCCTCTGTC AGGTCATGGG CCCTGCTTTT AAATCAAACG GCACGGGCCT TCCCAGACTG
AY078387        TCCCTCTGTC AGATCATGGG CCCTGCTTTT AAACCAAACA GCACGGGCCT TCCCAGACTG
Kharkiv         TCCCTCTGTC AGATCATGGG CCCTGCTTTT AAATTCAAACA GCACGGGCCT TCCCAGACTG
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
AF111171        TGCTATATGT TGGGAACCTT CCCCTCCCTG GGCTCCCGAA ATATTAGTAT ATAACAAAAC
AF399704        TGCTATATGT TGGGAACCTT CCCCTCCCTG GGCTCCCGAA ATATTAGTAT ATAACAAAAC
AF503581        TGCTATATGT TGGGAACCTT CCCCTCCCTG GGCTCCCGAA ATATTAGTAT ATAACAAAAC
AY078387        TGCTATATGT TGGGAACCTT CCCCTCCCTG GGCTCCCGAA ATATTAGTAT ATAACAAAAC
Kharkiv         TGCTATATGT TGGGAACCTT CCCCTCCCTG GGCTCCCGAA ATATTAGTAT ATAACAAAAC
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
AF111171        CATCTCCAAC TCTGGACCCG GTCTCGCCCT CCCGGACGCC CAAATCTTCT GGGTCAACAC
AF399704        CATCTCCAGC TCTGCACCCG GCCTCGCCCT CCCGGACGCC CAGATCTTCT GGGTCAACAC
AF503581        CATCTCCAAC TCTGGACCCG GTCTCGCCCT CCCGGACGCC CAAATCTTCT GGGTCAACAC
AY078387        CATCTCCAGC TCTGGACCCG GCCTCGCCCT CCCGGACGCC CAAATCTTCT GGGTCAACAC
Kharkiv         CATCTCCAGC TCTGGACCCG GCCTCGCCCT CCCGGACGCC CAAATCTTCT GGGTCAACAC
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Figure 1. Fragment of multiple sequence alignment of *env* gene of bovine leukemia virus circulating in different geographical regions

The same value of divergence of the BLV *env* gene, circulating in the Kharkiv region, characterized proviral DNA sequences circulating in western Europe (Table 1). The smallest divergence observed for the *env* gene of European isolates of bovine leukemia virus. In general, the results supported that the BLV *env* gene is highly conserved, and its primary structure does not change depending on the habitat of the causative agent.

Table 1 — The degree of similarity and divergence of the *env* gene of BLV circulating in different geographic regions, calculated relative to the corresponding sequence from isolate fragments of Kharkiv region

	AF111171 (Poland)	**AF399704 (Brazil)**	**AF503581 (Belgium)**	**AY078387 (USA)**
The number of nucleotides that do not coincide	14	15	14	19
Divergence, %	1.8	2.0	1.8	2.5
The degree of similarity, %	98.2	98.0	98.2	97.5

To establish phylogenetic relations between isolates of bovine leukemia virus circulating in Ukraine, and their phylogenetic relationships with isolates of other regions of the world (Europe, Asia, North and South America), was constructed the phylogenetic tree, that illustrated proximity of BLV isolates circulating in Ukraine to isolates of European and Asian subgroups (Fig. 2, *1*).

BLV isolates, that proviral DNA was extracted from peripheral blood of animals from farms in Rivno, Poltava and Kharkiv regions, are closer to the European subgroup (Austria isolate). Bovine leukemia virus, circulating in the farms of Crimea, is closer to Asian subgroup (Zanjan, Tehran isolates). Isolates from the America form a separate, American, subgroup (Fig. 2, *2*).

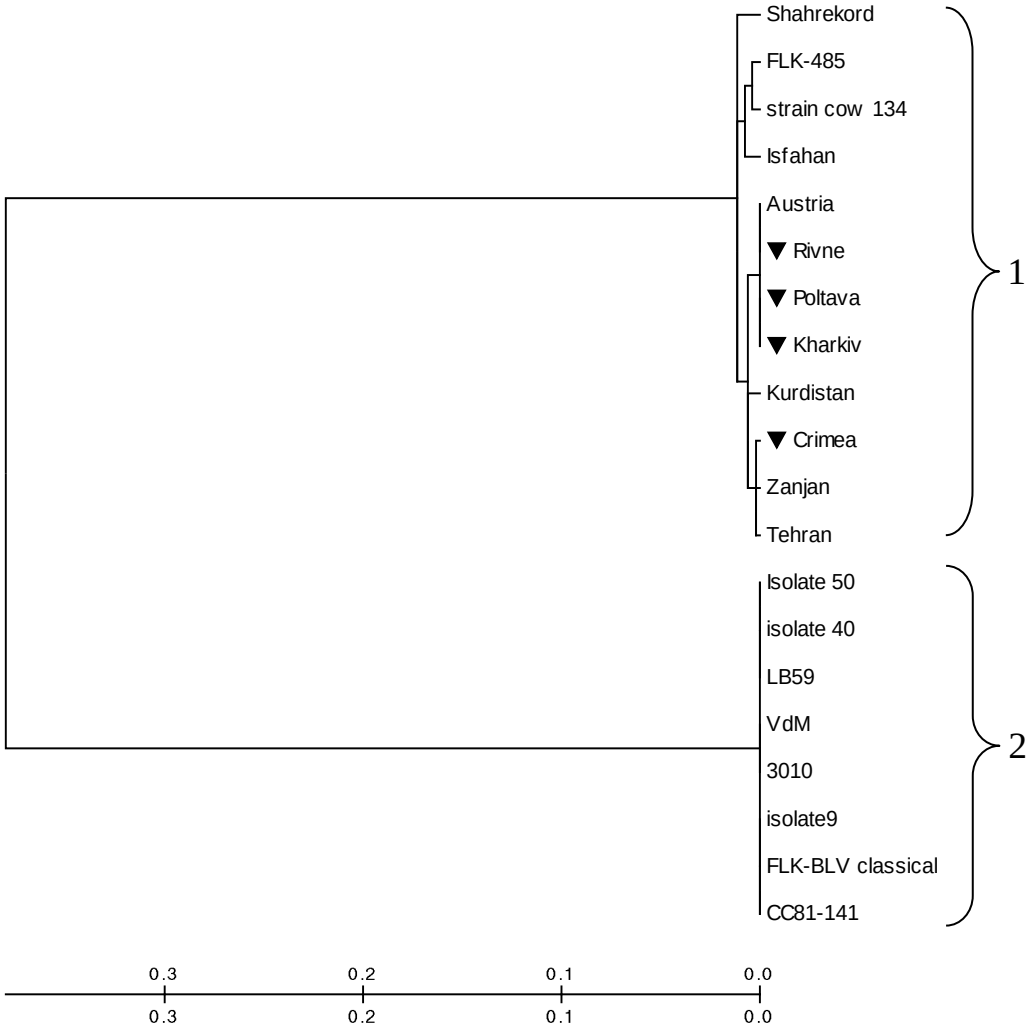

Figure 2. Dendrogram, based on *env* gene fragment of proviral DNA of bovine leukemia virus isolates from different geographical regions

Thus, the results of phylogenetic studies can be used to identify and study possible subgroups (or genotypes), to create the basis for the genes search, that determine the high biological activity of viruses.

To study the phylogenetic relationships of bovine leukemia virus was created databases of sequenced gene sequences and their fragments, isolated in different geographical regions and represented in international databases GenBank and EMBL: the bovine immunodeficiency virus, the Jembrana disease virus — lentivirus, that causes severe acute disease of cattle characterized by lymphopenia and lymphadenopathy; syncytial virus; bovine leukemia virus.

Phylogenetic analysis, based on retroviruses *env* gene sequences (Fig. 3) and completely sequenced proviral DNA sequences (Fig. 4), showed firstly that isolates of Jembrana disease viruses, immunodeficiency and syncytial viruses form separate branches; secondly, membership of bovine leukemia virus isolates to one cluster; thirdly, the evolutionary closeness of the leukemia virus and bovine syncytial virus.

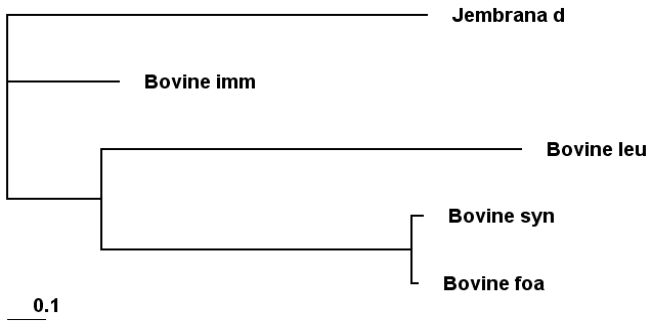

Figure 3. Dendrogram, based on *env* gene fragment of proviral DNA of bovine retroviruses isolates

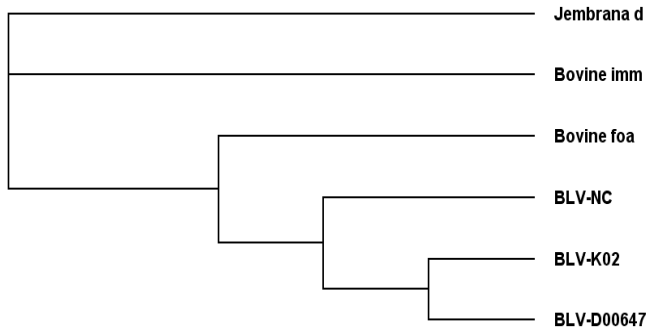

Figure 4. Dendrogram, based on complete sequences of proviral DNA of bovine retroviruses isolates

Conclusions. Established that the BLV *env* gene is highly conserved, its primary structure has significant changes depending on the habitat of the causative agent. The greatest degree of similarity observed for *env* gene sequences of European BLV isolates. It was shown, that bovine leukemia virus isolates, circulating in farms of different geographic regions in Ukraine, are closer to the Euro-Asian subgroup. Based on phylogenetic analysis, proved the genetic proximity of leukemia virus and bovine syncytial virus, immunodeficiency virus and Jembrana diseases virus.

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DEVELOPMENT OF PCR TEST SYSTEMS FOR SPECIES DIFFERENTIATION OF CHLAMYDIAL AGENTS IN BIRDS

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Summary. According to the modern classification adopted by the 2nd European Meeting on Animal Chlamydioses and Zoonotic Implications (EMAC-2), the genus *Chlamydia* contains three species of bird chlamydial agents — *C. avium*, *C. gallinacea* and *C. psittaci*. Three PCR test systems have been developed for indication and species differentiation of these bacteria. The bases for the developed diagnostics are three pairs of oligonucleotide primers designed and synthesized to flank the DNA fragments specific for each of the three bacteria species of the *Chlamydia* genus, which are pathogenic for birds. Analytical specificity of the developed PCR test systems was confirmed by the results of amplification of 18 biological materials samples, among them 13 being *Chlamydia*-containing, one of them contained *C. avium*, one — *C. gallinacea*, and 11 — *C. psittaci*. In addition *Leptospira* and *Babesia* DNA samples were tested. The developed PCR test systems for indication and species differentiation of bird Chlamydiosis agents permit to reliably study certain aspects of chlamydial infection.

Keywords: Chlamydioses, birds, MOMP, PCR test system, species differentiation, *Chlamydia avium*, *Chlamydia gallinacea*, *Chlamydia psittaci*

Introduction. *Chlamydia* is a group of infectious diseases caused by Gram-negative intracellular bacteria of the Chlamydiales order. According to the current classification adopted at the 2nd European Meeting on Animal Chlamydioses and Zoonotic Implications (EMAC-2), 8 families (3 of them having a candidate status) are represented by 13 genera (5 with a candidate status) and 25 species (among them 7 microorganisms having a candidate status).

The mammals' and birds' pathogens are the bacteria from *Chlamydia* genus, namely: *C. abortus*, *C. avium*, *C. caviae*, *C. felis*, *C. gallinacea*, *C. muridarum*, *C. pecorum*, *C. pneumoniae*, *C. psittaci*, *C. suis* and *C. trachomatis* (Sachse, 2013; Ksyonz and Liubetskyi, 2014).

Bird Chlamydioses are caused by three bacteria species: *C. avium*, *C. gallinacea* and *C. psittaci*. In particular, *C. avium* causes diseases in pigeons and Psittaciformes (Sachse et al., 2014). *C. gallinacea* is the causative agent for of Galliformes (chickens, turkeys) diseases (Guo et al., 2016). *C. psittaci* causes Chlamydiosis in over 170 species of domestic, decorative, synanthropic and wild birds. This agent is also capable of causing disease and asymptomatic infection in various mammals, including humans (Lagae et al., 2013; Vanrompay, Ducatelle and Haesebrouck, 1995).

Currently, Ukrainian scientists and veterinary medicine practitioners do not have available diagnostic tools capable of differentiating agents of birds' chlamydial infections by species. However, such a tool is necessary both to study various aspects of the identified infections, and to develop the effective strategies for the treatment measures. The purpose of our work was to develop three PCR test systems for indicating and species differentiation of *C. avium*, *C. gallinacea* and *C. psittaci*, i.e. etiological factors of birds' Chlamydiosis.

Materials and methods. The study was carried out in the Laboratory of Animal Health and Genetics of the Institute of Pig Breeding and Agroindustrial Production of the National Academy of Agrarian Sciences of Ukraine.

184 primary sequences of the gene encoding the main outer membrane protein (MOMP), belonging to three bacterial species of the *Chlamydia* genus pathogenic for birds, namely *C. avium*, *C. gallinacea* and *C. psittaci* were obtained from the nucleotide sequences databases GenBank (USA) and analyzed to construct the primers for the species-specific PCR test systems.

The nucleotide sequences of the gene encoding MOMP of the three designated species of *Chlamydia* genus were aligned using the MEGA v. 4 and MEGA v. 7 software (Tamura et al., 2007). To develop the design of oligonucleotide primers, DNA fragments specific for each species of the *Chlamydia* genus bacteria have been selected.

Sequences of oligonucleotide primers with their annealing temperature parameters were obtained using the FastPCR software (Kalendar, Lee and Schulman, 2014). Among the calculated primers, a single pair (the direct primer and the reverse one) was selected for each species of the *Chlamydia* genus bacteria pathogenic to birds.

Based on the designs developed, synthesis of the oligonucleotide primers was ordered at 'Thermo Electron Corporation' (Germany). The resulting synthesized primers were diluted with sterile deionized double-distilled water to the stock concentration of 100 pM/μl and then to the use concentration of 20 pM/μl.

In addition to the primers, the test systems used reagents for the PCR manufactured by 'Fermentas UAB' (Lithuania), namely: deionized water, PCR buffer, $MgCl_2$, deoxyribonucleoside triphosphate solution (dNTP), and Taq-polymerase.

Polymerase chain reaction using the developed PCR test systems for indication and species differentiation of *C. avium*, *C. gallinacea* and *C. psittaci* was carried out in 0.6 cm^3 polypropylene microcentrifuge tubes using the 'Biometra TRIO-Thermoblosk' thermocycler (Germany) in the reaction volume of 25 μl.

The ratio of the reaction mixture and the amplification program were adjusted experimentally and practically to obtain the most distinct bands on electrophoregrammes.

Fractionation of the amplification products was performed by the method of horizontal electrophoresis in the 2.0% agarose gel in the 'Cleaver Scientific Ltd.' (UK) electrophoretic chamber with a visual assessment by means of the UV transilluminator manufactured by NVO 'Progress' (Ukraine), after staining with bromine ethidium.

Restricted vector DNA *pUC19/MspI* ('Fermentas UAB', Lithuania) was used as a DNA marker.

DNA isolation from the biological samples under study was carried out using the commercially available reagents set 'PROBA-RAPID' manufactured by LLC 'NPO DNA Technology' (Russian Federation).

Materials for testing the experimental PCR test system parameters were samples of *C. avium*, *C. gallinacea* and *C. psittaci* control DNA received from the Chlamydiosis Laboratory of Friedrich Loffler Institute (Jena, Germany); DNA samples isolated from the field chlamydia isolates from domestic, decorative, synanthropic and wild birds and DNA samples isolated from the *Babesia* stored in the Laboratory of Animal Health at the Institute of Pig Breeding and Agroindustrial Production of NAAS; DNA samples isolated from *Leptospira* received from the Museum of Microorganisms of the Leptospirosis Laboratory at the Institute of Veterinary Medicine of NAAS.

Results. Bioinformational studies of 831 primary sequences of various genes belonging to the *Chlamydia* genus bacteria (16S rRNA, RNase P RNA, MOMP) have determined the highest level of nucleotide sequence variability (97.1%) in the gene encoding the main outer membrane protein (MOMP). When aligning the fragments of the *C. avium*, *C. gallinacea*, and *C. psittaci* MOMP gene, the level of nucleotide sequences' variability was 76.2%. That is why the primary sequences of this gene received from the international bases have been used in designing oligonucleotide primers for PCR test systems for indicating and differentiating *C. avium*, *C. gallinacea*, and *C. psittaci*.

By means of the 'FastPCR' computer software, oligonucleotide primers were designed. Among a large number of primer pairs, one was chosen for each test system, considering the size of the amplified fragment flanked by them (the most convenient for electrophoretic detection) and the optimal primers annealing temperature.

Thus, in the PCR test system for indication and species differentiation of *Chlamydia avium*, the following pair of primers was used:

ChAvMOMPL: 5'-TTCTGGTGATCCTTGCGACC-3'

CHAvMOMPR: 5'-GCTCCTAAAGTTGCACAACC-3'.

In the PCR test system for indication and species differentiation of *Chlamydia gallinacea*, the following pair of primers was used:

ChGalMOMPL: 5'-CAATACCATGAATGGCAAGC-3'

ChGalMOMPR: 5'-GAAAGTTGGGTTCCAAGCTG-3'.

The following pair of primers was used in the PCR test system for indication and species differentiation of *Chlamydia psittaci*:

ChPsMOMPL: 5'-GCACTATGTGGGAAGGTGCT-3'

ChPsMOMPR: 5'-CCATTTGCTTCTGGCTGATT-3'.

PCR-products were the fragments of the MOMP gene of the *Chlamydia* genus bacteria, having the sizes specific to each of the three *Chlamydia* species pathogenic to birds, namely: *Chlamydia avium* — 507 bp, *Chlamydia gallinacea* — 171 bp, *Chlamydia psittaci* — 208 bp.

Optimization of PCR conditions required selection of the reaction mixture composition and the temperature mode of amplification.

As a result of the PCR-protocol optimization, the following parameters of the reaction mixture were found to be optimal: 2.5 μl of 10-fold buffer (670 mM Tris-HCl, pH 8.8 at 25 °C, 20 mM BSA, 166 mM ammonium sulphate $(NH_4)_2SO_4$, 100 mM 2-β-mercaptoethanol) ('Fermentas UAB', Lithuania), 2.5 μl 2.5 mM dNTP ('Fermentas UAB', Lithuania), 2 μl 50 mM $MgCl_2$ ('Fermentas UAB', Lithuania), 2–3 units of Taq-polymerase (Thermus aquaticus) ('Fermentas UAB', Lithuania), 0.5 μl (0.1 opt. unit) of each primer and a sample of the DNA under study until the final concentration in the mixture; 1 μg/cm^3 deionized water to the volume of 25 μl. The amplification mixture was layered with 25 μl of mineral oil.

Optimal amplification parameters were:

94 °C	120 sec	
93 °C	30 sec	} 35 cycles
55 °C	30 sec	
72 °C	45 sec	
72 °C	300 sec	

To elaborate PCR parameters for the developed test systems and to test their analytical specificity, the following 18 biological materials were used: a sample of *C. avium* control DNA (1); a sample of *C. gallinacea* (2) control DNA; a sample of *C. psittaci* control DNA (3); a sample of DNA isolated from the *Chlamydia* field isolate of white Jacobin dove (*Columba livia*) from a private dovecote in Poltava (4); a DNA sample isolated from the field *Chlamydia* isolate of fantail-trumpeter (Torkun) dove (*Columba livia*) from a private dovecote in Poltava (5); a DNA sample isolated from the field *Chlamydia* isolate of shell parakeet (*Melopsittacus undulatus*) from a private parrot breeding farm in Karlovka (6); a DNA sample isolated from the *Chlamydia* field isolate of cockatiel (*Nymphicus hollandicus*) belonging to a resident of Poltava (7); a DNA sample isolated from the field *Chlamydia* isolate of yellow-collared lovebird (*Agapornis personatus*) from the private parrot breeding farm in Horyshniy Plavni, Kremenchuk district of the Poltava region (8); a DNA sample isolated from the field *Chlamydia* isolate of sparrow (*Passer* sp.) shot in the pig farm territory of LLC 'Svitanok', the Velyka Bahachka district of the Poltava region (9); a DNA sample isolated from the field *Chlamydia* isolate of greylag goose (*Anser anser*) shot in the lands of the Kobelyaky district of the Poltava region (10); a DNA sample isolated from the field *Chlamydia* isolate of greater flamingo (*Phoenicopterus roseus*) kept in the Kharkiv Zoo (11); a DNA sample isolated from the field isolate of silver pheasant (*Lophura nyctemera*) kept in the Kharkiv Zoo (12); a DNA sample isolated from the field isolate of vulture (*Gyps* sp.) kept in the Kharkiv Zoo (13); a DNA sample isolated from the field *Babesia canis* isolate of a dog belonging to a resident of Poltava (14); a DNA sample isolated from the field *Babesia bovis* isolate of a heifer at a private farm in Verkhy, Kamin-Kashyrskyi district of Volyn region (15); a DNA sample isolated from the *Leptospira* strain (LSU strain, *Louisiana* serovar, *Louisiana* serogroup) (16); a DNA sample isolated from the *Leptospira* strain (493 strain, *Poland*, *Polonica* serovar, *Sejroe* serogroup) (17); a DNA sample isolated from the *Leptospira* strain (*Hond Utrecht IV* strain, *Canicola* serovar, *Canicola* serogroup) (18).

At the first round of PCR the products electrophoregram of the 18 biological material samples was studied using the test system for indicating and differentiating *C. avium*. The specific size band of 507 bp was detected in a single track — No. 1 corresponding to the sample of the control *C. avium* DNA. In other 18 tracks, including negative control, there are no bands (Fig. 1).

At the second amplification products electrophoregram of the same 18 biological material samples were studied using the test system for indicating and species differentiation of *C. gallinacean,* a 171 bp band was detected in a single track — No. 2, which corresponds to the sample of the control *C. gallinacea* DNA. In other 18 tracks, including negative control, there are no bands (Fig. 2).

At the third PCR products electrophoregram of the above 18 biological material samples studied using the test system for indicating and species differentiation of *C. psittaci*, bands of 208 bp were detected. On 11 tracks — 3–13 corresponding to the samples of *C. psittaci* control DNA and field isolates (differentiated earlier as *C. psittaci*) from doves, Psittacidae birds, sparrow, greylag goose, greater flamingo, silver pheasant, and vulture. In other 8 tracks, including negative control, there are no bands (Fig. 3).

The results of the above 18 DNA samples studies with application of the three developed PCR test systems testify that chlamydial DNA indication occurs exceptionally in the samples corresponding to each diagnosticum's specificity: at the electrophoregrams of amplification products obtained with the help of the test system designed for the species *C. avium* differentiation, the only one band sizing 507 bp has been revealed in the track corresponding to the *C. avium* control DNA sample; at the electrophoregrams of amplification products obtained with the help of the test system designed for the species *C. gallinacea* differentiation, the only one band sizing 171 bp has also been found. In the track corresponding to the the *C. gallinacea* control DNA sample; at the electrophoregrams of amplification products obtained with the help of the test system designed for the species differentiation of *C. psittaci,* 11 bands have been detected sizing 208 bp in the tracks corresponding to the *C. psittaci* control DNA sample and to the DNA isolated from the field isolates of the above species obtained from different species of birds. There have not been found any other bands at a single electrophoregram, particularly, they were absent in the tracks of the DNA samples corresponding to *Babesia* and *Leptospira* DNA samples.

Study of the 18 above mentioned DNA samples using each of the three PCR test systems was performed in 3 replicates, with similar results (Tables 1–3). However, it should be noted, that the intensity of the bands was somewhat reduced with the concentration decrease of the DNA under study in the reaction mixture.

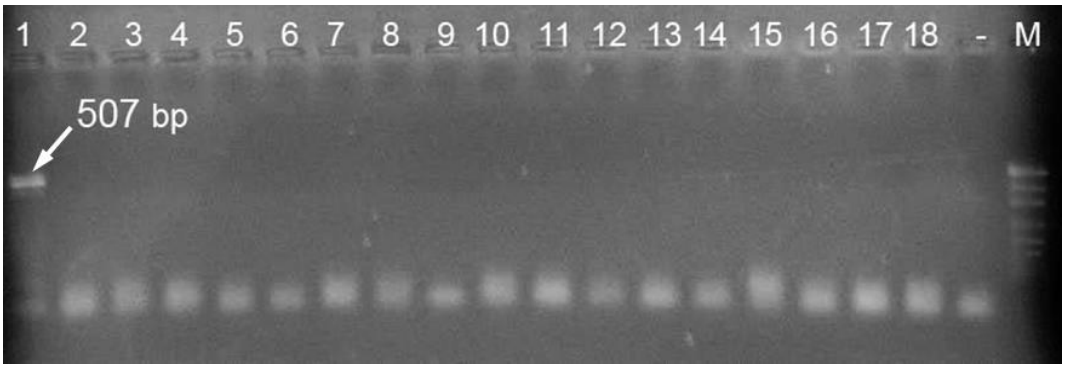

Figure 1. PCR-products electrophoregram of 18 biological material samples, studied using PCR test system for indicating and species differentiation of *Chlamydia avium* bacteria, namely: 3 control samples of *C. avium*, *C. gallinacea*, and *C. psittaci* DNA; 10 DNA samples isolated from field isolates of various bird species; 2 DNA samples isolated from the field *Babesia* protozoa isolates of a dog and cattle; 3 DNA samples isolated from *Leptospira* strains. Tracks: 1 — band of 507 bp (*C. avium*); 2–18 — negative result; «-» — negative control; M — DNA size marker *pUC19/MspI* ('Fermentas UAB', Lithuania)

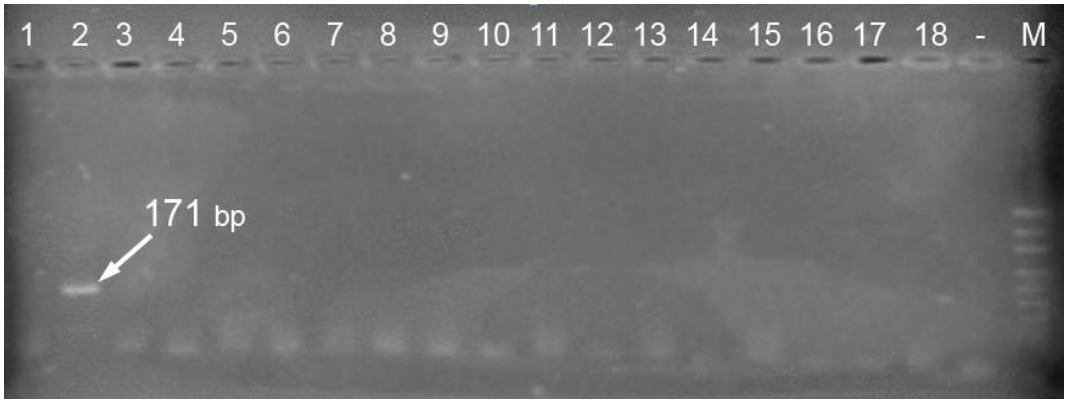

Figure 2. PCR-products electrophoregram of 18 biological material samples, tested using PCR test system for indicating and species differentiation of *Chlamydia gallinacea* bacteria, namely: 3 control samples of *C. avium*, *C. gallinacea*, and *C. psittaci* DNA; 10 DNA samples isolated from field isolates of various bird species; 2 DNA samples isolated from the field *Babesia* protozoa isolates of a dog and cattle; 3 DNA samples isolated from *Leptospira* strains. Tracks: 2 — band of 171 bp. (*C. gallinacea*); 1, 3–18 — negative result; «-» — negative control; M — DNA size marker *pUC19/MspI* ('Fermentas UAB', Lithuania)

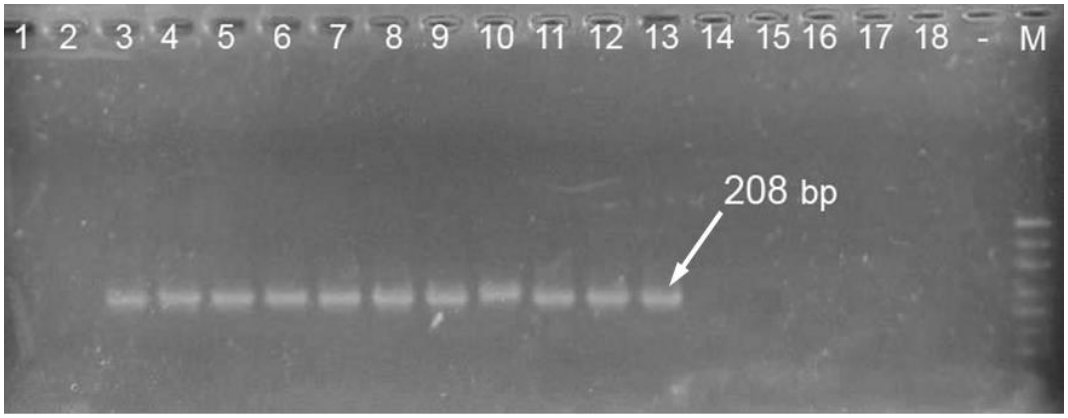

Figure 3. PCR-products electrophoregram of 18 biological material samples, studied using PCR test system for indicating and species differentiation of *Chlamydia psittaci*, bacteria, namely: 3 control samples of *C. avium*, *C. gallinacea*, and *C. psittaci* DNA; 10 DNA samples isolated from field isolates of various bird species; 2 DNA samples isolated from the field *Babesia* protozoa isolates of a dog and cattle; 3 DNA samples isolated from *Leptospira* strains. Tracks: 3–13 — band of 208 bp (*C. psittaci*); 1, 2, 14–18 — negative result; «-» — negative control; M — DNA size marker *pUC19/MspI* ('Fermentas UAB', Lithuania)

Table 1 — Testing results of the PCR test-system developed to indicate *Chlamydia avium* bacteria as to its analytical specificity and sensitivity

No.	Type of the sample studied	Study result										
		DNA amount in the reaction mixture									Band size	Agent species
		2 µl (20 µg/ml)			1 µl (20 µg/ml)			0.5 µl (20 µg/ml)				
		1 repeat	2 repeat	3 repeat	1 repeat	2 repeat	3 repeat	1 repeat	2 repeat	3 repeat		
1	Control DNA of *C. avium*	+	+	+	+	+	+	+	+	+	507	*C. avium*
2	Control DNA of *C. gallinacea*	–	–	–	–	–	–	–	–	–	–	–
3	Control DNA of *C. psittaci*	–	–	–	–	–	–	–	–	–	–	–
4	Chlamydial field isolate from white Jacobin dove (*Columba livia*)	–	–	–	–	–	–	–	–	–	–	–
5	Chlamydial field isolate from fantail-trumpeter (Torkun) dove (*Columba livia*)	–	–	–	–	–	–	–	–	–	–	–
6	Chlamydial field isolate from shell parakeet (*Melopsittacus undulatus*)	–	–	–	–	–	–	–	–	–	–	–
7	Chlamydial field isolate from cockatiel (*Nymphicus hollandicus*)	–	–	–	–	–	–	–	–	–	–	–
8	Chlamydial field isolate from yellow-collared lovebird (*Agapornis personatus*)	–	–	–	–	–	–	–	–	–	–	–
9	Chlamydial field isolate from sparrow (*Passer* sp.)	–	–	–	–	–	–	–	–	–	–	–
10	Chlamydial field isolate from greylag goose (*Anser anser*)	–	–	–	–	–	–	–	–	–	–	–
11	Chlamydial field isolate from greater flamingo (*Phoenicopterus roseus*)	–	–	–	–	–	–	–	–	–	–	–
12	Chlamydial field isolate from silver pheasant (*Lophura nycthemera*)	–	–	–	–	–	–	–	–	–	–	–
13	Chlamydial field isolate from vulture (*Gyps* sp.)	–	–	–	–	–	–	–	–	–	–	–
14	Field isolate *Babesia canis* from dog	–	–	–	–	–	–	–	–	–	–	–
15	Field isolate *Babesia bovis* from heifer	–	–	–	–	–	–	–	–	–	–	–
16	Leptospira strain LSU	–	–	–	–	–	–	–	–	–	–	–
17	Leptospira strain 493 *Poland*	–	–	–	–	–	–	–	–	–	–	–
18	Leptospira strain *Hond Utrecht IV*	–	–	–	–	–	–	–	–	–	–	–

Table 2 — Testing results of the PCR test-system developed to indicate *Chlamydia gallinacea* bacteria as to its analytical specificity and sensitivity

No.	Type of the sample studied	Study result										
		DNA amount in the reaction mixture									Band size	Agent species
		2 µl (20 µg/ml)			1 µl (20 µg/ml)			0.5 µl (20 µg/ml)				
		1 repeat	2 repeat	3 repeat	1 repeat	2 repeat	3 repeat	1 repeat	2 repeat	3 repeat		
1	Control DNA of *C. avium*	–	–	–	–	–	–	–	–	–	–	–
2	Control DNA of *C. gallinacea*	+	+	+	+	+	+	+	+	+	171	*C. gallinacea*
3	Control DNA of *C. psittaci*	–	–	–	–	–	–	–	–	–	–	–
4	Chlamydial field isolate from white Jacobin dove (*Columba livia*)	–	–	–	–	–	–	–	–	–	–	–
5	Chlamydial field isolate from fantail-trumpeter (Torkun) dove (*Columba livia*)	–	–	–	–	–	–	–	–	–	–	–
6	Chlamydial field isolate from shell parakeet (*Melopsittacus undulatus*)	–	–	–	–	–	–	–	–	–	–	–
7	Chlamydial field isolate from cockatiel (*Nymphicus hollandicus*)	–	–	–	–	–	–	–	–	–	–	–
8	Chlamydial field isolate from yellow-collared lovebird (*Agapornis personatus*)	–	–	–	–	–	–	–	–	–	–	–
9	Chlamydial field isolate from sparrow (*Passer* sp.)	–	–	–	–	–	–	–	–	–	–	–
10	Chlamydial field isolate from greylag goose (*Anser anser*)	–	–	–	–	–	–	–	–	–	–	–
11	Chlamydial field isolate from greater flamingo (*Phoenicopterus roseus*)	–	–	–	–	–	–	–	–	–	–	–
12	Chlamydial field isolate from silver pheasant (*Lophura nycthemera*)	–	–	–	–	–	–	–	–	–	–	–
13	Chlamydial field isolate from vulture (*Gyps* sp.)	–	–	–	–	–	–	–	–	–	–	–
14	Field isolate *Babesia canis* from dog	–	–	–	–	–	–	–	–	–	–	–
15	Field isolate *Babesia bovis* from heifer	–	–	–	–	–	–	–	–	–	–	–
16	Leptospira strain LSU	–	–	–	–	–	–	–	–	–	–	–
17	Leptospira strain 493 *Poland*	–	–	–	–	–	–	–	–	–	–	–
18	Leptospira strain *Hond Utrecht IV*	–	–	–	–	–	–	–	–	–	–	–

Table 3 — Testing results of the PCR test-system developed to indicate *Chlamydia psittaci* bacteria as to its analytical specificity and sensitivity

No.	Type of the sample studied	Study result										
		DNA amount in the reaction mixture									Band size	Agent species
		2 µl (20 µg/ml)			1 µl (20 µg/ml)			0.5 µl (20 µg/ml)				
		1 repeat	2 repeat	3 repeat	1 repeat	2 repeat	3 repeat	1 repeat	2 repeat	3 repeat		
1	Control DNA of *C. avium*	–	–	–	–	–	–	–	–	–	–	–
2	Control DNA of *C. gallinacea*	–	–	–	–	–	–	–	–	–	–	–
3	Control DNA of *C. psittaci*	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
4	Chlamydial field isolate from white Jacobin dove (*Columba livia*)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
5	Chlamydial field isolate from fantail-trumpeter (Torkun) dove (*Columba livia*)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
6	Chlamydial field isolate from shell parakeet (*Melopsittacus undulatus*)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
7	Chlamydial field isolate from cockatiel (*Nymphicus hollandicus*)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
8	Chlamydial field isolate from yellow-collared lovebird (*Agapornis personatus*)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
9	Chlamydial field isolate from sparrow (*Passer* sp.)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
10	Chlamydial field isolate from greylag goose (*Anser anser*)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
11	Chlamydial field isolate from greater flamingo (*Phoenicopterus roseus*)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
12	Chlamydial field isolate from silver pheasant (*Lophura nycthemera*)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
13	Chlamydial field isolate from vulture (*Gyps* sp.)	+	+	+	+	+	+	+	+	+	208	*C. psittaci*
14	Field isolate *Babesia canis* from dog	–	–	–	–	–	–	–	–	–	–	–
15	Field isolate *Babesia bovis* from heifer	–	–	–	–	–	–	–	–	–	–	–
16	Leptospira strain LSU	–	–	–	–	–	–	–	–	–	–	–
17	Leptospira strain 493 *Poland*	–	–	–	–	–	–	–	–	–	–	–
18	Leptospira strain *Hond Utrecht IV*	–	–	–	–	–	–	–	–	–	–	–

Thus, the electrophoregram results indicate the adequacy and analytical specificity of the developed PCR test systems.

Conclusion. The developed PCR test systems, included oligonucleotide primers flanking different sized DNA fragments of the gene encoding MOMP of three *Chlamydia* species, permitted the detection and identification of *C. avium*, *C. gallinacea*, and *C. psittaci* DNA, which is pathogenic to different bird species.

Indication and differentiation of *Chlamydia* by species is provided by a visual assessment of the amplified fragments due to different band sizes at electrophoregrams in 2.0% agarose gel.

Testing of the developed PCR test systems for indicating and species differentiation of chlamydial infections agents in birds demonstrated their high sensitivity and analytical specificity.

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Part 2. Biotechnology

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EFFECTS OF DIETARY BETAINE ON PRODUCTIVE TRAITS AND REPRODUCTIVE HEALTH OF DAIRY COWS

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Summary. Dietary supplementation of betaine may play an important role in productive and reproductive parameters of dairy cows. The aim of this study was to define the role of dietary betaine in dairy cows' lactation. Cows were assigned to betaine or control groups according to the 'case-control' study design. Statistical methods included Pearson's chi-squared and *t* criteria, Pearson and Spearman correlation coefficient *r* and ANOVA.

We observed positive results after betaine diet correction of milk and reproductive traits of dairy cows in the heat stress conditions. Homocysteine level in blood of dairy cows is not depend on the age of the animals (r = 0.09), on the amount of lactation (r = 0.04), on a period after calving (r = –0.07). In this period higher milk yield was observed on lower homocysteine levels in the blood plasma of animals (r = –0.32). Also we found out that more inseminations were required for animals with a higher homocysteine level in the plasma (r = 0.36).

After betaine supplementation milk fat concentration was higher in betaine-treated group of cows compared with control cows (3.05 vs 2.74%). Milk yield in betaine-treated group was in the negative correlation with milk fat (r = –0.67). An analysis of milk yields dynamics had showed that cows fed betaine had more stable and predictable milk yields per milking than controls (8.3 vs 40%) and milk yield in BET group was in a negative correlation with milk fat (r = –0,67). Negative dynamic of homocysteine level was noticed in a betaine group (27.5%) and positive (19.4%) — in the control group. More inseminations were required for cows of control group (1.8 vs 1.2).

Keywords: dairy cows, one-carbon metabolism, homocysteine, betaine, milk traits, reproduction

Introduction. One-carbon metabolism of mammals is one of the key points of metabolism and its research is a perspective direction for the development of pharmacological correction of failures. The actual figure is to assess the biochemical metabolism of dairy cows as a basis for further selection and analysis of genes, causing the reproductive traits of animals.

An adequate folate and vitamin-B status is critical to maintain the optimal efficiency of the homocysteine-methionine cycle. It is known that SNPs of one-carbon metabolism human genes are associated with — carcinogenesis (colorectal adenocarcinoma, breast cancer and ovarian cancer); cardiovascular disease (ischemic heart disease — coronary heart disease, myocardial infarction, atherosclerosis, atherothrombosis); pregnancy complications (placental insufficiency, premature detachment of normally situated placenta, late gestosis); fetal malformation (cleft neural tube, anencephaly, deformities of the facial skeleton), children born with a chromosomal abnormality, etc., because its synthesized enzymes play an important role in folate metabolism, which is an integral process for DNA and RNA synthesis and in protein methylation. So this MTHFR enzymatic activity process is lowered in subjects with MTHFR 677TT and 677CT genotypes and these individuals might require an increased intake of folate or another dietary factors to maintain or control blood levels of plasma folate or homocysteine (Maroto-Sánchez et al., 2016; Asim et al., 2015; Vanilla et al., 2015; Bertoia et al., 2014; Rai, 2011; Ciaccio, Bivona and Bellia, 2008; Forges et al., 2007; Altomare, Adler and Aledort, 2007; Doolin et al., 2002; Zetterberg et al., 2002).

Homocysteine is an intermediate sulfur-containing amino acid involved in the methionine cycle and could be converted to methionine due to the remethylation reaction requires BHMT and both folate and vitamin B_{12} as coenzymes (Maroto-Sánchez et al., 2016; Eskandari et al., 2016; Xu et al., 2016; Jia et al., 2015).

Several studies of dairy cows had shown that plasma homocysteine levels are affected by diet factors such as protein and vitamin deficiencies by genetic background and by several pathological conditions. Moreover, the homocysteine in blood could be also associated with chronic inflammatory bowel disease (Crohn's disease and

the variations of vitamin B_{12} and subacute ruminal acidosis). It was found an increase of vitamin B_{12} values in dairy cows with lower rumen pH and a decrease of homocysteine values in dairy herds with lower rumen pH (Cannizzo et al., 2012; Roblin et al., 2006).

It has been reported by Başbuğan, Yüksek and Altuğ (2015) about analysis of the diagnostic and prognostic significance of homocysteine and cardiac troponin I and routine cardiac parameters, in cows with hypocalcemia, because hypocalcemia causes a decrease in myocardial contractions, left ventricle systolic dysfunction, and thus systolic heart failure, and an increasing homocysteine levels in blood could be a signal for coronary heart disease, paralysis, peripheral vascular disease, and intravascular thrombosis.

Many articles have documented that correction and maintenance of one carbon metabolism level could be carried out with a dietary factors for human and animals, such as betaine (Saeed et al., 2017; Di Pierro, Orsi and Settembre, 2015; Jia et al., 2015; Maclean et al., 2012).

Betaine or tri-methyl glycine, is a natural compound either produced endogenously by choline oxidation or found in feed ingredients, such as sugar beet solubles and a key component in one-carbon metabolism. Betaine has two main functions in an animal's body. It is a powerful osmolyte to reduce dehydration and stabilize protein when a cell is under stress condition and it serves as a methyl donor when fed to animals (Monteiro et al., 2017; Tao et al., 2016; Zeisel, 2013; Bertolo and McBreairty, 2013; Eklund et al., 2005). Betaine reduces the lipid components in the liver and prevents fatty liver disease. The lipotropic effect is associated with the transfer of a methyl group from homocysteine to S-adenosylmethionine through methionin. In this reaction, betaine, choline and methionine supply the methyl group (Nakai et al., 2013).

It was presented by Hall et al. (2014) that betaine is a molecular chaperone and has been shown to decrease susceptibility of stress to microbial populations acts as an antimicrobial to some bacteria like *Salmonella typhimurium*, can be utilized as a nutrient and has been demonstrated to increase milk production.

Effects of betaine for animals metabolism in a heat stress conditions had been studied by different authors, because various studies indicate that prepartum heat stress of dairy cows exaggerate the dysfunctional immune system during late gestation and early lactation. The humoral immune response is also altered by heat stress during the transition period (Tao et al., 2016). Late-gestation heat stress compromises placental development, which results in fetal hypoxia, malnutrition, and eventually fetal growth retardation (Tao and Dahl, 2013). According to Tao et al. (2016) and Thompson and Dahl (2012) cows dried off in the hot months had increased incidences of mastitis, respiratory disorders and retained fetal membranes in early lactation compared with those dried in cool months.

Research of Hall (2014) had demonstrated that milk protein and lactose (%) increased with the middle dose of betaine during heat stress compared to the controls. Cows fed betaine had significantly higher milk production during thermal-neutral condition and elevated plasma glucose during heat stress. In this time nutritional supplementation of heat-stressed lactating cows has been widely studied, but the related research for dry cows under heat stress is somewhat limited, especially during the far-off period (Tao et al., 2016).

The same results were obtained after betaine supplementation for pigs and poultry (Saeed et al., 2017; Jia et al., 2015; He et al., 2015). Previous studies showed that dietary supplementation of betaine in poultry diets could positively affect nutrients' digestibility, reduce abdominal fat weight, and increase breast meat yield. Betaine may play an important role in lean meat production by positively affecting the lipid metabolism with increased fatty acids catabolism and thus reducing carcass fat deposition (Saeed et al., 2017). In the paper by He et al. (2015) about broiler betaine diet the control and betaine-supplemented broiler groups it was described higher feed consumption, body weight gain, and lower feed : gain ratio compared with the heat stress S-control group. Betaine supplementation significantly decreased triglyceride, free fatty acids, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol. Authors had shown that chronic heat stress reduces broiler production performance. However, betaine can reverse these negative effects partially and thus improve carcass composition by changing lipid metabolism.

At the same time it could be noted that the ambiguity and insufficiency of data on the effect of betaine on the productivity and reproduction of dairy cows. There are a number of factors that affect one-carbon metabolism such as age, gender, nutrition, genetics, medication, physical activity, climatic conditions and animals' husbandry conditions. That is why it is beneficial to conduct research on the Ukrainian selection breeds in the technological and fodder conditions of Ukrainian farms.

The aim of the study was to evaluate the role of dietary betaine in lactating dairy cows.

Materials and methods. A study was conducted at the State Enterprise Research Farm 'Nyva' of Institute of Animal Breeding and Genetics named after M. V. Zubets of the National Academy of Agrarian Sciencies of Ukraine and V. N. Karazin Kharkiv National University during August–early September 2016.

The type of production in SE RF 'Nyva' is organic. Cows had milk production (6,514 l), milk fat (3.65%) and milk protein (3.20%) concentration during 2016 in this farm. The base total mixed ration (TMR) was alfalfa haylage based with corn silage, corn and other silage. The system of keeping cows is traditional. Part of time animals spent in the walking areas.

To study the potential impact of supplementation of betaine containing diet, 35 cows of Ukrainian red-spotted milk and Ukrainian black-spotted milk breeds at the age of 1^{st}–7^{th} lactation after calving were randomly assigned to betaine or control groups according 'case-control' study design.

All animals had the same light/dark schedule, humidity and temperature. The air temperature at this time of year in the daytime was 22–34 °C outside, and 18–26 °C — in the cows' room.

Cows were fed individually. Betaine-hydrochloride, Beta-Key ('Orffa', Netherlands) was used as the source of studied supplement. The control group received 0 g/d, the betaine dose was fed at 40 g/d once a day. The supplement was mixed with grain mixture. A few days we spent to adapt betaine group to the new diet.

Cows were milked from 6 to 8 °a.m., and from 5 to 7 p.m. daily. Samples were individually stored and analyzed by Ecomilk-Standart ('Bulteh 2000' Ltd., Bulgaria) for fat and protein containment. The plasma homocysteine levels were analyzed using commercial ECLIA test kits. Production, reproduction and biochemical parameters were analyzed before and after betaine supplementation and compared against control to understand the effects of betaine.

Statistical analysis was performed with the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality and hypotheses — criteria t and χ^2. The relationship between traits was estimated by the Spearman and Pearson correlation analysis. Means for two groups were compared by ANOVA (Atramentova and Utevskaya, 2008).

Results and discussion. The analysis of the results obtained in the present study indicated that before experiment betaine-treated cows group (BET) and control group (CONT) had the same milk yield, milk fat and protein contents, number of inseminations for successful result and homocysteine level was little higher in one of the groups (p = 0.044) (Table 1).

Table 1 — Characteristics of CONT and BET groups

Parameters of the cows	Results of study			
	Before study		After study	
	CONT	BET	CONT	BET
Milk yield, l	21.20 ± 1.30	19.31 ± 1.42	16.10 ± 1.10	17.23 ± 0.92
Milk fat, %	3.60 ± 0.01	3.42 ± 0.08	2.74 ± 0.17	3.05 ± 0.19
Milk protein, %	2.86 ± 0.01	2.75 ± 0.9	3.07 ± 0.02	3.1 ± 0.02
Homocysteine level, Mmol/l	4.31 ± 0.26	7.91 ± 1.81	5.15 ± 0.39	5.73 ± 0.62
N	1.6 ± 0.1	1.41 ± 0.1	1.8 ± 0.1	1.2 ± 0.1

Notes: CONT — control group of cows, BET — betaine group of cows, N — number of the last successful insemination.

At the end of experiment we found statistically significant decreasing of milk fat — by 23.9% (p = 0.02) in CONT and by 10.8% (p = 0.01) in BET, milk yield — by 18.8% (p = 0.009) in CONT only, but in BET group this trait was without differences (p = 0.17). Milk protein levels increased — by 7.3% (p = 0.004) in CONT and by 12.7% (p = 0.02) in a BET group (Table 1).

Such dynamics of milk traits is typical for most Ukrainian farms under conditions of heat stress and increasing of green mass in feed during summer period.

It is important to note that we observed the statistically significant higher milk fat concentration (3.05 vs 2.74%, p = 0.000001) in the BET cows group compared to the controls one after betaine supplementation. Milk yield and milk protein were the same in the both groups. Milk yield in BET group showed negative correlation to milk fat — r = –0.67 (p = 0.037) and we found no association between these traits in control.

In accordance with the data of Tao et al. (2016) cows that received betaine diets starting at 56 d before the expected calving date had improved not only milk fat concentration (4.78 vs 4.34%), but milk production (44.2 vs 41.5 kg/d), compared with control cows. The results of Zhang et al. (2014) showed that feeding betaine to cows increased feed intake, milk yield, milk lactose, milk protein, plasma cortisol, glutathione peroxidase, superoxide dismutase, and malondialdehyde levels (P < 0.05) In contrast, it has been reported by Hall (2014) that dietary betaine increased milk yield and percent protein during the thermoneutral period. In 2016 Hall et al. conclude that dietary betaine increased milk yield during the thermoneutral conditions, but no differences

were found between betaine and control in total milk production or milk composition during heat stress, that partially consistent with our results. The research of Peterson et al. (2012) had demonstrated that supplementing 100 g/d rumen-unprotected betaine increased milk production in mid-lactation cows under a thermal-neutral condition. Monteiro et al. (2017) reports that cows enrolled at dry off, with betaine support demonstrated higher milk yield (45.1 vs 41.9 kg/d) and fat content (4.78 vs 4.34%) and elevated plasma concentrations of non-esterified fatty acids and β-hydroxybutyrate in early lactation compared to control.

The dynamics of milk yields that we observed at the end of the intake of betaine from BET cows was noted. It was from 0 to 3 liters per milking, 1.6 liters on average, in the control group cows — 0–9 liters, on average 3.3 liters. Cows fed betaine had more stable and predictable milk yields per milking than controls (8.3 vs 40%, $p < 0.05$).

It is well known that different dietary components as methionine content, riboflavin, alcohol or coffee consumption are being investigated in relation to homocysteine concentration (Varela-Moreiras, 2001). That is why the actual figure is to assess the homocysteine level in blood of dairy cows as trait of one-carbon metabolism and target for correction by betaine.

The analysis showed that the homocysteine levels in blood of animals before experiment was in the range from 2.96 to 27.9 mmol/l, reaching an average of 5.72 ± 0.73 μmol/l. Homocysteine level in blood of dairy cows (Table 2), not dependent on the age of the animals ($r = 0.09$, $p = 0.57$) or, respectively, the number of lactation ($r = 0.04$, $p = 0.81$). This is comparable to human characteristics — the level of homocysteine increases during puberty and in adults its parameters accordance to the genotype, although in adulthood it may gradually increase (Maroto-Sánchez et al., 2016; Guttormsen et al., 1996).

No association between homocysteine level in the blood of animals and a period after calving ($r = -0.07$, $p = 0.68$) was found. It is known that during normal pregnancy, the level of homocysteine in humans tends to decrease, which usually occurs at the border of the first and second trimester of pregnancy, which promotes placental circulation, but after 2–4 days postpartum, the level of homocysteine is restored (Maroto-Sánchez et al., 2016; Guttormsen et al., 1996).

Table 2 — Relationship between the homocysteine level and the signs of cow

Parameters of the cows	Parameters of study before betaine feeding		
	Homocysteine level, Mmol/l	r	p
Age of an animals, 3–9 years	2.96–27.79	0.09	0.57
Last lactation number, 1–7	2.96–27.79	0.04	0.81
The time after calving, 2–68 days	2.96–27.79	–0.07	0.68
Number of the last successful insemination, 1–4	2.96–27.79	0.36	0.05
Milk yield, 11–23 kg/d	2.96–27.79	–0.32	0.07

Notes: r — correlation coefficient, p — significance level.

Before the study of betaine diet we found a statistically significant direct correlation between homocysteine level in the blood and the number of inseminations which were carried out before successful fertilization and development of pregnancy of cows — $r = 0.36$ ($p = 0.05$). More insemination was required for animals with a higher level of amino acid in the blood plasma (Table 2).

The literature data have demonstrated that in women the lower level of homocysteine in the follicular fluid is associated with a better chance of clinical pregnancy (Ocal et al., 2012). In addition, in patients with polycystic ovary syndrome, when comparing glucose and estradiol with healthy women, the level of homocysteine in the follicular fluid was higher with a significance level of $p = 0.01$ (Eskandari et al., 2016). It was also noted that homocysteine concentration in follicular fluid in women with polycystic ovary syndrome is associated with lower quality and number of oocytes and embryos (Berker et al., 2009).

The value of milk yield of cows showed a negative correlation with homocysteine level — $r = -0.32$, at a significance level of $p = 0.07$. Higher yield observed at lower homocysteine levels in the blood plasma of animals. It is known that due to functional or genetically determined defects of enzymes, in vitamin deficient conditions, deficiency of vitamins B_{12}, B_6, B_1, folic acid, the level of homocysteine in the cells increases and enters to the extracellular space and blood plasma. Cytotoxic effect of elevated homocysteine levels is dangerous especially for endothelial cells that affect the processes of reproduction and synthesis of milk (Maroto-Sánchez et al., 2016; Ciaccio, Bivona and Bellia, 2008; Guttormsen et al., 1996).

After experiment in the BET group the level of homocysteine decreased by 27.5%, in the CONT group it increased by 19.4% and equaled in a both groups (Table 1). The number of inseminations required for successful fertilization and development of pregnancy in

the CONT group increased by 12.5%. At the same time, this trait decreased by 14.3% in the group of BET cows. More insemination was required for cows of CONT group, 1.8 vs 1.2 ($p = 0.035$).

We hypothesized that that decreasing of homocysteine level and its cytotoxic effect contributed to successful inseminations and healthy pregnancy. Besides that participation of betaine in the conversion of homocysteine to methionine probably improved fertility of cows.

It is known that betaine, by virtue of aiding in the remethylation of homocysteine, removes both toxic metabolites (homocysteine and S-adenosylhomocysteine), restores S-adenosylmethionine level, reverses steatosis, prevents apoptosis and reduces both damaged protein accumulation and oxidative stress (Kharbanda, 2009). With participation of betaine homocysteine could be converted to methionine. This is an important in a case when methionine is one of the most limiting amino acids in dairy cows (Vailati-Riboni et al., 2017).

Different works had established that cows around calving time experience a depression on immune function partially due to the marked negative energy balance, which results when cows cannot ingest enough nutrients to support dietary requirements for milk production. It has been reported that methionine plays a key role in milk protein synthesis, hepatic lipid metabolism, and immune function (Li et al., 2016; Osorio et al., 2013; Soder and Holden, 1999; Chen et al., 2007). Besides its crucial role in milk production, methionine and its derivate metabolites (e.g., glutathione, taurine, polyamines) are well-known immunonutrients in nonruminants (Vailati-Riboni et al., 2017) and well-established sources of the an oxidants (Li et al., 2016; Kim, Weiss and Levine, 2014; Luo and Levine, 2008; Moskovitz et al., 2001; Stadtman et al., 2002). So, rumen-protected methionine has been reported to increase lactation performance, health status and reproductive function of dairy cows (Hansen, 2016; Ardalan, Rezayazdi and Dehghan-Banadaky, 2010).

A role of key components of one carbon metabolism — homocysteine, methionine, betaine — in a reproduction of mammals had presented by authors from different countries. The critical relationship between perturbations in the mother's homocysteine and methionine metabolism and its impact on fetal growth and development is becoming evident (Kalhan and Marczewski, 2012). Effect of maternal methionine supplementation on the transcriptome of bovine preimplantation embryos had been described (Peñagaricano et al., 2013).

Thus, methionine has an essential role in the development of the bovine embryo from morula to blastocyst (Ikeda, Sugimoto, and Kume, 2012). Research of Luchini (2014) had demonstrated that supplementation of cows with methionine resulted in lower pregnancy losses from cows fed methionine-enriched diets, suggesting that methionine favors embryo survival, at least in multiparous cows.

Conclusions. We observed positive results after betaine diet correction of milk and reproductive traits of dairy cows in a heat stress conditions.

The relationship between productivity and reproduction traits of dairy cows — the value of milk yield and efficiency of insemination and the homocysteine level in blood of cows was shown.

After betaine supplementation milk fat concentration was higher in betaine-treated group cows compared with control cows (3.05 vs 2.74%). Milk yield in betaine-treated group was in a negative correlation with milk fat ($r = –0.67$).

Cows fed betaine had more stable and predictable milk yields per milking than controls (8.3 vs 40%).

Negative dynamics of homocysteine level was noted in a betaine group (27.5%) and positive (19.4%) in a control group. More insemination was required for cows of control group (1.8 vs 1.2).

Thus, the obtained results show the prospects for further pharmacogenetic research of dairy cows in Ukrainian farms.

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CONTROLLING OF *MYCOPLASMA BOVIS* AT A FARM IN UKRAINE AS A PART OF ERADICATION PROGRAM OF MASTITIS

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Summary. The article analyses the approaches to the diagnosis of mycoplasmal mastitis in cows. Despite decades of research in the field of etiology and pathogenesis of cattle mycoplasmosis, the diagnosis of this infection remains a topical issue in veterinary medicine. In time detection of infected cows, compliance with preventive measures in the milking parlors and in the newborncalves facilities, and the setting the order of milking are the main methods for preventing outbreaks of mycoplasmosis in farms. The necessity of proper sampling using and the order of the procedure for milking cows for the diagnosis of *Mycoplasma bovis* and other species of mycoplasmas are substantiated. Basing on the analysis, the following algorithm for determining the infectious status for *Mycoplasma* spp. and sampling for diagnosis, as well as the procedure for milking cows, was offered.

The data obtained as a result of the study indicate the necessity to plan a set of preventive and therapeutic measures in the farms to prevent the infection of healthy animals with a contagious mycoplasmal infection.

Keywords: mastitis, *Mycoplasma bovis*, contagious pathogen, cows, Ukraine, control

Introduction. Mastitis is a well-known and economically significant disease of dairy cattle. Mycoplasmosis is a common disease in cattle-breeding industry, that bring to farms many economic problems such as culling milk, spending money for treat unhealthy cows, culling animals, decreasing of milk products' cost and other. Contagious organisms are well adapted to survive and grow in the mammary gland and frequently cause infections lasting weeks, months or years (Maunsell et al., 2011; Barkema et al., 2009).

Mycoplasma species with the exception of some mycoplasmal infections that may originate in the other parts of the body and spread systemically, these three organisms gain entrance into the mammary gland through the teat canal. Thus, it is necessary to consider, that mycoplasmas can also cause inflammations of a mammary gland secretory tissue and aggravate process of treatment (Fox, Kirk and Britten, 2005). Mycoplasmosis is a disease of cattle, which is widespread in farms around the world. The most pathogenic and often diagnosed pathogen is *Mycoplasma bovis* (Pothmann et al., 2015; Lysnyansky et al., 2016; Aebi et al., 2015; Spergser et al., 2013) although there is evidence of the absence of this pathogen in the Czech Republic (Surýnek, Vrtková and Knoll, 2016).

Mycoplasmas belong to the simplest free-living prokaryotes. Their sizes are very close to viruses, but they have their own DNA replication, transcription and protein synthesis systems, are able to reproduce in conditions of an artificial nutrient medium. The size of the genome within the Mollicutes class can vary greatly, from 580 to 2,200 thousand base pairs (Stukolkina, 2005).

Mycoplasmosis has extensive clinical manifestations; it causes not only udder lesions, but also problems of the reproductive and musculoskeletal system, inflammation of the eyes and ears of animals. The carrier of the disease can be any mastitis-sick animal, which initially had a respiratory or reproductive form of mycoplasmosis because microorganisms enter the mammary gland through the circulatory system. The contagiousness and persistence of bacteria in the herd usually depend on many factors: the number of infected animals and the general immunity status, the effect of stress factors (Stipkovits et al., 2000; Houlihan et al., 2007).

The lack of a wall of Mollicutes cells makes them more sensitive to the environment, so they have a reduced ability to survive outside the animal's body (Jones and Simecka, 2003).

During the infectious process, they are mainly localized in immunocompetent cells (macrophages). *M. bovis*, entering the epithelial cells of the mammary gland, causes a cellular immune response of the body, which is manifested by an increase in the expression of mRNA of tumor necrosis factor-alpha, interleukins (IL) IL-1β, IL-6, IL-8, lactoferrin, Toll-like receptor-2, RANTES chemokine, and serum amyloid (Zbinden et al., 2015).

The chronic nature of this infection indicates that all components of the immune system can participate in response to *Mycoplasma* spp.; however, T cells are the main component of the immune response against mycoplasma infection. Progression of mastitis depends on the balance between the components of the cellular immune response, which can contribute both to an

increase in the resistance of the host organism, and cause immune-mediated pathogenesis (Rodríguez et al., 2015).

Determining the presence of *Mycoplasma* spp. in milk is an important part of the mastitis control program (Fox, 2013). Sampling, in this case, should occur in accordance with the rules of asepsis and depend on the methodology of further analysis. In the field of applied research, an urgent need is to develop cost-effective, sensitive and specific diagnostic tests that can provide accurate identification of infected animals. Data on the prevalence of pathogens are necessary in order to determine the consequences of mycoplasma infections and develop recommendations for the eradication of contagious mastitis (Rossetti, Frey and Pilo, 2010; Justice-Allen et al., 2011).

The aim of the study was to improve the diagnostics algorithm to control the epizootic situation in the farms. For this purpose, the following tasks were set: to develop a scheme for controlling herd infection using an approach for detecting the *Mycoplasma* spp. and *M. bovis* genomes in the milk of cattle by polymerase chain reaction and to approve this approach at farms in Ukraine.

Materials and methods. Research carried out in LLC 'Center of Veterinary Diagnostics' (Kyiv, Ukraine). Milk samples were taken from the different region of Ukraine and taken into sterile 30 ml phials and kept at –20 °C. Containers with selected for research samples were marked with the date of sampling, the age of the animal, the name of the farm and the area.

To identify the genetic material of bacteria of the genus *Mycoplasma* spp., primers were selected in the milk samples using the Vector NTI Advanced 11 software package ('Invitrogen', USA). Primers were tested on test strain *Mycoplasma* spp. (State Scientific Control Institute of Biotechnology and Strains of Microorganisms, Kyiv, Ukraine) selected by analyzing their level of homology to the selected DNA template of the pathogen:

F: 5'-ACTCCTACGGGAGGCAGCAGTA-3'
R: 5'-TGCACCATCTGTCACTCTGTTAACCTC-3'

The test system 'LSIVetMAX Screening Pack Real-Time PCR Kit, ruminant respiratory diseases' (France) was used for *M. bovis* detection. Amplification was conducted on a 7500 Fast Real-Time PCR-System device ('Applied Biosystems', USA).

Interpretation of the results was carried out by analyzing the curves obtained by the thermocycler, based on the presence or absence of the intersection of the fluorescence curve with the threshold line set at a certain level. A sample was considered positive if the Ct value on the FAM/Green channel was less than 45. The sample was considered negative if there was no fluorescence curve on the FAM/Green channel, and the detected fluorescence curve VIC/channel and the Ct value were less than 45 (internal endogenous reaction control).

Results and discussion. Samples of milk were selected from cows according to the clinical signs (milk was apparently with clots and containing 'flakes') and after carrying out a special test for the presence of somatic cells ('PortaChek', USA) and lactate dehydrogenases ('PortaChek', USA). It was also known in advance that the studied farms were unfavorable for mycoplasmosis (up to 30% of animals had vulvovaginitis).

The analysis of 144 milk samples obtained from 20 dairy farms for the presence of the *Mycoplasma* spp. genome revealed the presence of a pathogen DNA in 61 samples.

Positive samples were combined together into prefabricated samples according to the name of the farm, but no more than 5 samples per sample. Analysis of these samples for the presence of DNA of *M. bovis* revealed the DNA of the pathogen in 10 samples obtained from 15 farms.

Further positive samples were analyzed for the quantitative determination of the DNA content of the pathogen using real-time PCR. The results of such analysis are shown in the Tables 1 and 2.

Table 1 — DNA detection of *Mycoplasma* spp. in different region of Ukraine

Region	Number of farms examined	Number of samples examined	Number of *Mycoplasma* spp. positive samples
Poltava	5	17	4
Kharkiv	3	16	16
Sumy	1	8	8
Khmel-nytskyi	2	11	7
Kyiv	4	47	9
Vinnytsia	1	2	2
Mykolaiv	1	14	0
Cherkasy	3	19	5
Total	20	144	61

Thus, the results of a study of pathological milk for the presence of *Mycoplasma* spp. and *M .bovis* DNA showed that most cows with the identified genetic material of the pathogen in milk had also problems with infectious diseases of the reproductive and respiratory tract. The obtained data allow planning a complex of preventive and therapeutic measures in farms to prevent infection of healthy animals.

Table 2 — Results of quantitative determination of *M. bovis* genetic material in the researched cow's milk using real-time PCR (Ct value)

Region	Number of farms examined	Number of samples examined	Quantitative determination of *M. bovis*
Poltava	1	2	*Ct = 36.98
Kharkiv	3	5	Ct = 24.97
Sumy	1	2	0
Khmel-nytskyi	2	3	0
Kyiv	4	5	Ct = 37.97 Ct = 24.97 Ct = 28.97 Ct = 34.17 Ct = 22.33
Vinnytsia	1	2	0
Cherkasy	3	3	Ct = 37.97 Ct = 25.85 Ct = 32.20
Total	15	20	10 (positive)

Note: * — the value of 'Ct' is a relative indicator of the amount of the desired genetic material in the sample and is expressed in cycles from 1 to 35: the higher the cycle, the less the primary amount of material in the sample and vice versa. Ct = 16–24 — '+++', Ct = 25–31 — '++', Ct = 32–37 — '+', Ct ≥ 38–40 — '+/–'.

Basing on the analysis, the following algorithm for determining the infectious status for *Mycoplasma* spp. and sampling for diagnosis, as well as the procedure for milking cows was proposed (Fig. 1).

To control the infected status of the herd for contamination by *Mycoplasma* spp. it is necessary to collect milk samples regularly for PCR analysis from a common milk bulk tank, and it is needed to take samples from each cow with an increase in somatic cell counts and lactate dehydrogenase in a mammary gland secretion (Group № 3).

In addition, samples should be taken from all cows transferred to the milking herd immediately after calving or during lactation (Group № 2).

In order to prevent transmission of mycoplasmas through the milking machine, the newly acquired cows and calves must be quarantined for testing on *Mycoplasma* spp. before entering the herd. Cows with clinical signs of mycoplasmal infection (arthritis, conjunctivitis, vulvovaginitis) should be checked for the presence of *Mycoplasma* spp. and after confirmation of infection should be distributed to the appropriate milking group (Group № 1).

Cows that are positive for *Mycoplasma* spp. should be milked after healthy cows and after cows with mastitis of another etiology. Negative herds that regularly acquire cattle should diagnose the milk from a tank on the mycoplasma, twice a month.

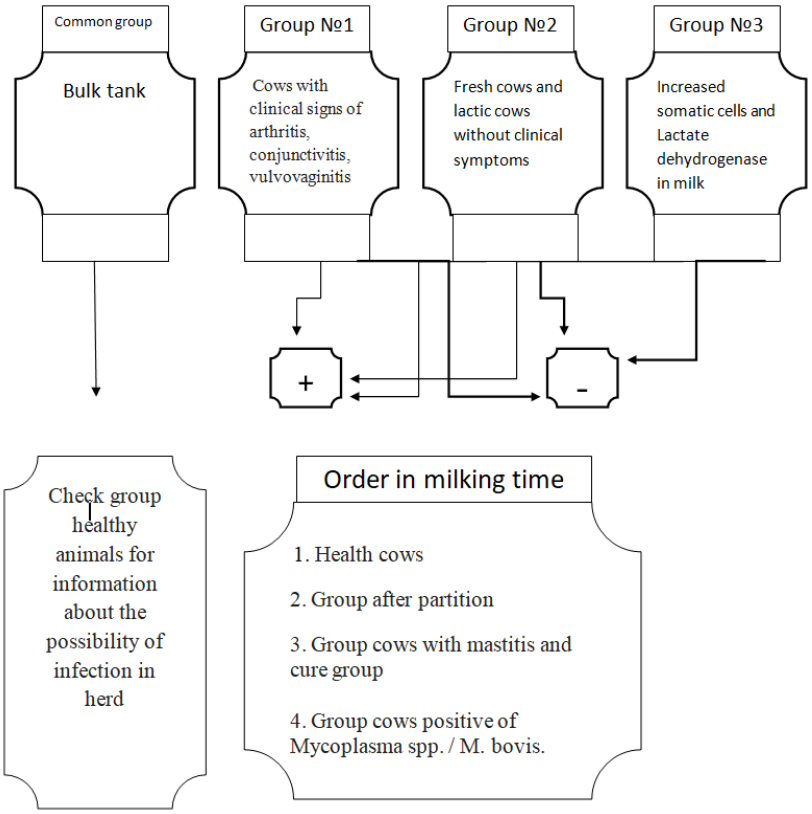

Figure 1. Algorithm for determining the status of the herd infection and sampling for the diagnosis, and the procedure milking procedure.

Conclusions. As a result of the conducted studies the present of *Mycoplasma* spp. was detected in 63 samples of all milk samples. *Mycoplasma bovis* pathogens were detected in 10 samples of milk samples. Basing on the findings, an algorithm for sampling for carrying out molecular diagnostics of mastitis of *Mycoplasma* etiology, as well as a sequence of milking cows in unfavorable farms, is proposed to prevent the spread of the disease. The results can be applied to mastitis controlling plan at farm.

The detection of infected cows, the observance of preventive measures in the milking parlor and in the rooms with new cows and the order of milking are the main methods of preventing outbreaks of mycoplasmosis.

Each farm can be exposed to breast diseases, and the number of infections is increasing if the pathogenic microorganisms associated with mastitis are not detected in time.

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THE MAIN REGULARITIES OF RAW MILK CONTAMINATION WITH *STAPHYLOCOCCUS AUREUS*

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Summary. This publication represents results of the regularities of the milk contamination process in which golden staphylococci were studied, depending on the technologies of keeping and milking cows, the level of sanitary culture of the dairy farms, the spread of morbidity of cows to mastitis, the technology of primary milk processing and transfer to the processing enterprise.

S. aureus var. *bovis* is mainly isolated from raw cow milk. Its strains are characterized by the coagulation of blood plasma of cattle and horses, formation in the medium with crystal violet of yellow and orange colonies, phage-typing of Davidson, the lack of production of lecithinase on the Chystovych's medium with 10% sodium chloride solution. It is impossible to get the milk free from pathogens because of evolution caused environmental infection of *S. aureus* var. *bovis* to the skin of the udder of cows. As an objective reality there is a certain level of contamination of milk by *S. aureus* during the milking process. Under this level, you need to understand the number of *S. aureus,* which can come from healthy glands, skin of the udder of cows and milkmaids' hands.

Keywords: *Staphylococcus aureus,* raw milk, milking machines, mammary gland of cows

Introduction. Diseases caused by *Staphylococcus aureus*, have spread so rapidly that a staph infection is right to be called the modern 'plague' or *Staphylococcus* disaster. The epidemiological significance of *S. aureus* that infects food lies in its ability to produce enterotoxins and cause food toxicities of people.

The role of milk and dairy products in the emergence of staphylococcus-associated toxicoses is constantly growing and reaches more than 30% of all cases of mass outbreaks of toxicity in people (Ladanivskyi and Dnistrian, 2000; Ladanivskiy et al., 2007; Melnychuk et al., 2003). The mammary gland of the cow when inflamed is believed to be the main source of enterotoxigenic staphylococci (Kukhtyn, 2004, 2010; Kukhtyn et al., 2016, 2017; Malanyn et al., 1994; Slobodkin, Shelkova and Levytska, 2006). A separate group of scientists believes that the microflora of the environment is constantly penetrating into the udder of cows through the milking channel. Its main mass is being destroyed by factors of nonspecific protection of the mammary gland, but the strongest part of this microflora, in particular *S. aureus* can survive in these conditions, causing various forms of mastitis (Kartashova, 1980; Kravtsiv and Maslianko, 2009; Yakubchak, 2010). This was based on their approaches to improve the level of cows non-affected with mastitis, by isolating and treatment of animals, infected with pathogenic *Staphylococcus.*

There is no comprehensive description of microorganisms of the mammary gland of cows within a microecological approach in the literature. By this time, the biotope had not been included in the scope of interest of many authoritative experts in the field of milk hygiene.

The aim of this work was to study the regularities of the process of milk contamination by *S. aureus*, depending on the technology of animal husbandry and milking, the level of sanitation of dairy farms, the distribution of the incidence of mastitis of cows, and also on the technology of primary processing of milk and transferring to a recycling facility.

Materials and methods. More than 600 composite samples of raw milk were received from dairy factories of Ukraine. They were tested for the presence of *S. aureus* within the period from 2001 to 2016. More than 1,000 milk samples from separate quarter of udder of 350 cows (17 households), more than 2,000 rinses of milkmaids' hands, swabs of cows' skin, dairy utensils and milking machines swabs were also tested. Soil samples of walking grounds and fields that had been fertilized with manure from dairy farms were also collected and tested.

Microbiological examinations of milk, swabs and soil samples were performed according to conventional methods (Bergilevich et al., 2010). Studied material and its tenfold dilutions in the amount of 1 cm^3 were made on Baird-Parker agar ('BioMérieux', France) for identifying coagulase positive staphylococci. Typical

bacterial colonies were counted on the Petri dish after 24–48 hours of incubation at 37 °C. Then pure cultures were isolated and identified according to species using the test API ID32STAPF test ('BioMérieux', France). Coccal catalysation positive cultures that were fermentable to glucose in the Hugh-Leifson's medium were ranked to the species of *Staphylococcus*. The cultures that coagulated rabbit plasma were attributed to the species of *S. aureus*. If it was necessary, additional tests (fermentation of mannithol, the ability to produce phosphatase, lecithinase) were used. Type of hemolysins was determined by S. D. Elek, E. Levy and J. Marks, A. Vaugan, the presence of DNA-ase by C. D. Jeffris, D. F. Heltman, D. Guse. The phage typing was performed using phages of Davidson (Hájek and Marsálek, 1971).

Results. It was found, that 70% of clinically healthy cows are carriers of staphylococci in the udder, where *S. aureus* occurs in 6–15% of cases. Staphylococci were allocated from the nipple skin swabs in 100% of examined cows, but only 30% of cows were carriers of *S. aureus*. Staphylococci have constantly emanated from the skin of the udder and the inner surface of the skin of the thighs and pregnant heifers, but *S. aureus* was isolated from the secret of the mammary gland of heifers only 0.8% of the tested animals. Taking into account the results of examinations of different skin areas of cows, as well as the mucous membrane of the mouth, we got a conclusion that the ecological niche of *S. aureus* in cows is the skin of the udder. Number of animals-carriers of *S. aureus* decreased in summer up to 15%, and in winter it was increased up to 80%. Cows, which udder skin swabs contained *S. aureus*, were divided into permanent carriers (up to 20% of animals) and temporary, but there was a kind of turn, when carriage appeared in some of cows and disappeared in the others.

Sanitary treatment of the udder of cows-permanent carriers with 0.25% solution of desmolux or with the solution of chlorine bleach with 0.05% content of active chlorine did not liberate the skin of the udder from *S. aureus*. Antiseptic teat treatment after milking with emulsion for three months dramatically reduced the level of infection of the skin, but also did not eliminate it.

The number of *S. aureus* in 1 cm^3 of the aseptically milked out milk of healthy cows ranged from 10 to 150. In the case of subclinical staphylococcal mastitis the content of this microorganism increased to 6–8×10^3 CFU/cm^3, and only in the presence of clinical signs, this number increased at 5–6 times or more.

S. aureus was constantly emanated in un-disinfected surfaces of milking machines and dairy utensils. *S. aureus* emanated in the floor swabs and air of barns, but it was not detected in the soil of paddocks, as well as soils that are intensively fertilized with manure from dairy farms.

Therefore, we did not find any significant spread of pathogenic staphylococci in the environment. The distribution range of *S. aureus* was limited by objects that are in direct contact with cows and milk.

S. aureus allocated on average of 50–55% of cases in swabs off the hands of the milkmaids. Usually *S. aureus* var. *bovis* and var. *hominis* in equal amounts appeared in the swabs. The intensity of infection was within 100–200 microbial cells in 1 cm^3 of swab, but that the number of microorganisms increased to 5–10×10^4 CFU/cm^3 at the presence of cracks and erosions of the skin.

The contents of *S. aureus* in the milk delivered to recycling facilities was different from dairy farms without cooling. The milk delivered to the plant non-refrigerated 3–4 hours after milking in winter period contained 2.7±0.3×10^3 CFU/cm^3 of *S. aureus*, and in summer period — 57±5×10^4 CFU/cm^3, respectively.

The rate of *S. aureus* reproduction in bulk milk depended on both temperature 2–6 °C and the amount of the initial mesophilic microflora of milk. The containing of *S. aureus* among the mesophilic microorganisms (6.0–7.0 ×10^4 CFU/cm^3), allocated from the milk, increased in 6 hours at a temperature of 37 °C by 107 times, and whole mesophilic bacteria quantity — in 122 times. Under the same conditions but with the presence of mesophilic microorganisms in milk in rate 7.0–8.0×10^5 CFU/cm^3, the number of *S. aureus* increased in 50 times, and mesophilic microorganisms — in 880 times, respectively. When the temperature of the milk was 25 °C after 6 hours the contents of the *S. aureus* increased in only 5 times, but mesophilic microflora — in 62 times. And only at a temperature of 6±1 °C, the rate of reproduction of *S. aureus* and mesole microflora equaled, their number increased in 2–3 times within 24 hours.

The experiments revealed that in the absence of milking equipment disinfection with their surfaces dispatched in 1 cm^3 of milk on average 100 CFU of *S. aureus*. If the rules for sanitary processing of milking machines and dairy equipment, pre-milking disinfection of the udder of cows and isolation in a separate group of cows with subclinical mastitis are kept, the number of *S. aureus* in bulk milk immediately after milking does not exceed 300 CFU/cm^3.

The composition of the *S. aureus* isolated from combined milk was presented by *S. aureus* var. *bovis* — up to 90% of cultures (coagulation of blood plasma of horses and cattle, formation on the medium with crystal violet of yellow and orange colonies, phage-typing of Davidson, the lack of production of lecithinase on the

Chystovych's medium at 42 °C). Others isolates belonged to *S. aureus* var. *hominis* (no coagulation of the blood plasma of cows, produced mainly α-hemolysins, lecithinase, not sensitive to phages of Davidson). *S. aureus* var. *bovis* produced basically beta gemalten on agar with blood of cattle or sheep. No culture of *S. aureus* var. *bovis* and *S. aureus* var. *hominis*, which were capable of producing β-gemalten form the zone of β-hemolysis on agar with rabbit erythrocytes.

Such peculiarity that some part of coagulase negative staphylococci isolated from breast and skin of the udder of cows, according to some tests could be attributed to pathogenic variants must be taken into account (Table 1).

Table 1 — Pathogenicity tests of coagulase negative staphylococci of breast and udder skin of cows

Hemolytic formed	Cultures tested	Produced from them, %		
		DNA-ase	Phos-phatase	Pigment golden and orange
Alfa, Beta	74	97.3	87.8	93.2
Delta	80	8.7	43.7	40.0
Non-hemolytic	116	2.6	43.1	33.6

As can be seen from the table, the coagulase-negative *Staphylococcus* cultures that are able to produce α- and β-hemolysins, in 97.3% of cases DNA-ase was formed, in 87.8% — phosphatase, in 93.2% — golden or orange pigment.

Discussion and conclusions. The microorganisms of the species of *Staphylococcus* should be considered as a part of normal micro flora of cow udder skin. They are also a part of microbiocenosis of the breast. Therefore, the presence of *S. aureus* on the skin of the udder and in the breast is a natural phenomenon. This microbiocenosis has been forming for millions of years in the process of phylogenetic development of the cattle as a species. *S. aureus* is systematically allocated from the calve skin. The presence of staphylococci, including *S. aureus*, on the skin of the udder and mammary gland, do not always lead to the disease of its master. Only under certain conditions, *S. aureus*, and coagulase positive options that have other signs of pathogenicity, manifest themselves as pathogens, causing inflammation of the breast. On the other hand, the detection of staphylococci in udder secretions of cows sick with mastitis, suggests that these microorganisms are commensally microflora of the udder. At the same time, protection factors of the gland cannot distinguish *S. aureus* from other species of the species of *Staphylococcus*, therefore, *S. aureus* can colonize gland under the same laws of formation of microbiocenosis, and others as his representatives (*Corynebacterium*, *Streptococcus*, coagulase positive species of *Staphylococcus*).

Therefore, identifying of the critical points of risk of contamination of milk with *S. aureus* in the process of its formation it is necessary to associate it with the role of this microorganism as an integral part of autosecretary of skin of the udder and mammary gland. The colonization of the skin of the udder of not even functioning breast with aureus is observed long before the first calving. *S. aureus* actively inhabits the mammary gland after calving and the start of the milking. This settling occurs with certain regularities of formation of microbiocenosis of the breast, primarily in respect of the quantitative content and the ratio of staphylococcus to other members of autoflora. Therefore, a quantitative rate of *S. aureus* in milk of healthy mammary gland does not exceed 100 CFU/cm^3.

The desire to make a full recovery of the number of cows from carriage of *S. aureus* is almost unfulfilled and is not theoretically justified. As at carrying and at the subclinical form of mastitis the *S. aureus* is primary found in the external polysaccharides biofilm (Cucarela et al., 2004; Kukhtyn et al., 2016).

Only sharp decrease in the content of this microorganism in the milk could be the characteristic of introducing of appropriate sanitary mode: culling of cows with chronic untreatable staphylococcal mastitis, effective sanitation of milking and dairy equipment, udder of cows, hand hygiene of milkmaids, milk cooling to 3±1 °C not later than two hours after milking.

As an objective reality, there is a certain natural level of contamination of milk by *S. aureus*, which is impossible to eliminate. The number of *S. aureus* that is able to enter the milk from a healthy mammary gland, skin of the udder of cows and milkmaids' hands is considered under this level.

Specific content of *S. aureus* in fresh bulk milk can serve as the indicator of the effectiveness of sanitation on a dairy farm. Approximately no more than 500 CFU of *Staphylococcus aureus* per 1 cm^3 can be considered as this indicator.

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Part 3. Biosafety

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ANTIBACTERIAL EFFECT OF VEGETABLE ESSENTIAL OILS BASED ON METAL NANOPARTICLES *IN VITRO*

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Summary. The paper presents the results of microbiological studies to determine the bactericidal activity of essential oils in relation to standard test microorganisms.

The bactericidal activity of some natural essential oils in the complex with benzalkonium chloride and silver nanoparticles has been investigated. The general characteristic of active substances of vegetable oils in relation to their influence on the organism of animals and people with the manifestation of anti-inflammatory and stimulating action has been presented.

Microorganisms, in prolonged contact with silver nanoparticles and essential oils, practically do not produce resistance to them, which is a significant advantage of essential oils in comparison with antibiotics and it may be used in human and veterinary medicine.

Keywords: essential oils, disinfection, test microorganisms, silver nanoparticles, benzalkonium chloride, broth cultures, *Escherichia coli, Staphylococcus aureus*, bactericidal activity

Introduction. There are no almost ecologically clean and safe disinfectants in veterinary practice that could be used for sanitation of veterinary supervision objects. Every year, the number of resistant and low-sensitive forms of bacteria increases significantly. In connection with this, there is a need to study new antibacterial agents that do not cause the development of bacterial resistance.

The essential oils may be used as an alternative to chemical disinfectants. Due to their properties they can effectively affect pathogenic microorganisms and show a therapeutic effect. Essential oils consist from the chemical compounds and some chemical elements. The primary elements responsible for the essential oil function are carbon, hydrogen, and oxygen. The components of essential oils are represented by various compounds, which can be arranged in the following order according to their biological properties:

Aldehydes (Melissa, lemon grass, lemon verbena, lemon eucalyptus, etc.) have anti-inflammatory, sedative and antiviral effects.

Alcohols. This is one of the most useful compound groups that have an antiseptic and antiviral effect. These include linalool (present in lavender, linaloe and rose wood), citronelol (present in rose, lemon, eucalyptus, and geraniums), geraniol (present in palmarose), as well as borneol, menthol, nerol, terpineol, farnesol, veteryrol and cetrol.

Phenols. They have a bactericidal activity and can also irritate the skin. The most common phenols of essential oils include eugenol (present in carnation and laurel), thymol (present in the thyme) and carvacrol (present in oregano and thyme).

Terpenes. Terpenic carbohydrates include limonene (an antiviral substance contained in 90% of citrus oils) and pinene (an antiseptic contained in large quantities in pine and sap oils), as well as camphene, cadinin, cariofillin, chedrine, dipentene, phellandrin, sabineen, myrcene etc. Some substances related to terpenes have pronounced anti-inflammatory and bactericidal properties.

Ketones. This is a class of chemical compounds that have a wound healing property and facilitate the secretion of mucus. Among the essential oils with high content of ketones there are also the oils of rosemary, sage, eucalyptus spherical and hyssop.

Sesquiterpenes consist of very long carbon chains. They are contained, in particular, in the essential oils of chamomile, sandy everlasting, tansy, yarrow and marigold. Sesquiterpenes have anti-inflammatory, sedative and antiviral properties, as well as bacteriostatic and immune stimulating effects.

Oxides. The most important oxide is cineol (or eucalyptol), which is presented in eucalyptus oil and has

an expectorant property. It was also found in rosemary, tea tree and cajeput oils.

Esters are the most representative group of substances found in essential oils. These include linalin acetate (present in bergamot, muscat, sage and lavender) and geranyl acetate (present in marjoram). Esters have an antifungal effect.

Lactones. This is a group of esters with an additional carbon ring. They belong to the most powerful anti-inflammatory compounds. There is a lot of lactones in arnica essential oils. Some lactones help to separate mucus even more efficiently than ketones.

Phenylpropane esters have a powerful harmonizing effect on the nervous system. They have antiseptic, stimulating, expectorant, anesthetic and diuretic properties. Cinnamon, carnation, anise, basil, tarragon and parsley oils are rich in phenylpropane esters.

Anti-inflammatory action is caused by the influence of aromatic components on vascular-tissue reactions: reduction of vascular wall permeability, optimization of vascular reactions, astringent and anti-edema activity, optimization of oxygen metabolism, stimulation of proliferation and regeneration. The most effective in acute inflammation essential oils are: tea tree, carnation, sage, oregano, muscat, basil, thyme, juniper, bergamot, myrtle, pine, schizandra, citronella, eucalyptus. In case of subacute inflammation: anise, verbena, geranium, chamomile, lavender, rose, hyssop, myrrh, marjoram, rosewood, tea tree, myrtle, muscat, oregano, spruce, pine. For chronic inflammations: orange, lemon, grapefruit, ylang, incense, neroli, petitgrain, sandalwood.

Immunostimulating effect. Aromatic carbohydrates stimulate the activity of cellular and humoral immunity, optimize the production of T- and B-lymphocytes, phagocytic activity of macrophages, destroy unbound biogenic amines, enhance the production of histaminase by intestines and kidneys — an enzyme producing an oxidative catalysis of histamine, serotonin, norepinephrine, adrenaline, tyramine, etc., resulting in a non-toxic product.

Thus, it is possible to interpret the effect of most essential oils as immunostimulating and anti-inflammatory. The most pronounced such an effect is expressed in bergamot, valerian, geranium, grapefruit, ylang, hyssop, cajeput, cedar, lavender, incense, myrtle, juniper, neroli, rose, petitgrain, rose wood, chamomile, pine, tea tree, sage, eucalyptus (Nikolaevskiy, 2000; Bergonzelli et al., 2003; Sartoratto et al., 2004; Borodina, 2004).

The aim of this study. The purpose of the research was to study the antibacterial effect of plant essential oils on the basis of nanoparticles of metals in vitro with the use of test microorganisms.

Materials and methods. There have been conducted studies of biological properties and the composition selection of a bactericidal preparation based on nanoparticles of silver, benzalkonium chloride and essential oils *in vitro* with the use of test microorganisms.

Broth cultures of *S. aureus* (strain P-209) were used in experiments. To prepare the broth culture, 25 cm^3 of the nutrient medium was poured into the flask and then 0.25 cm^3 of the overnight broth culture of microorganisms was added. A day later, broth culture was filtered through a sterile gauze-wool or paper filter. In test tubes with various dilutions of disinfectant (5 cm^3 in each), 0.5 cm^3 of overnight broth culture of tested microorganisms was added. After 10 min samples were taken from the flasks by a platinum loop and transferred to a Petri dishes with meat-peptone agar.

The indicated types of work were conducted in compliance with the conditions of sterility. After 30 min, keeping the same interval, samples were taken again and the next sowing on agar was carried out. After that, Petri dishes were placed in a thermostat with a temperature of 37 °C. The culture was viewed in 24 and 48 hours.

A similar method was also used in conducting a study to determine the bactericidal effect concerning *E. coli* (strain 1257).

For control, there was carried out the sowing of broths cultures *S. aureus* and *E. coli* according to generally accepted methods (Kovalenko, 2014; Obrazhei et al., 2008). In this case, microbiological indicative test cultures *E. coli* (strain 1257) and *S. aureus* (strain P-209) were used.

Results. In the study of bactericidal activity in the laboratory, 1.0% solution of essential oils with silver nanoparticles and benzalkonium chloride in the concentration of the basic solution 1:50 was prepared with a progressive reduction of the active substance in a solution of 1:4 by serial dilutions.

At the same time, a suspension of overnight culture *E. coli* (strain 1257) and *S. aureus* (strain P-209) in liquid media was prepared, containing microorganisms in concentration 2×10^9 CFU/cm^3, which was exposed to the investigated solution of a certain dilution at exposures of 10 and 30 min without protein and in the presence of protein (inactivated cattle blood serum). The results of the study of bactericidal activity of the drugs are presented in Tables 1 and 2.

The received data demonstrate determination of the protein index showed that in the presence of the protein substance, tested solutions 1.4 times reduced their bactericidal activity concerning *E. coli,* and 1.9 times — concerning *S. aureus.* Obtained results give grounds to assume that these preparations can be used for the sanitation of various objects.

Table 1 — Antibacterial action of preparations based on essential oils concerning *E. coli* (strain 1257)

Solutions of preparations (in the ratio)	Bactericidal dilutions Exposure, min		Average phenolic coefficient	Average protein index
	10	30		
Phenol 1:50	1:98	1:192.8	—	—
Fir+Eucalyptus+Thyme	1:192.8	1:376.5	1.96	
Fir+Eucalyptus+Thyme+Benzalkonium chloride	1:1,033.1	1:3,698.0	14.84	
Fir+Eucalyptus+Thyme+Benzalkonium chloride+Silver nanoparticles	1:3,968.6	1:5,566.0	34.68	
Fir+Eucalyptus+Thyme+Benzalkonium chloride+ Silver nanoparticles+protein	1:2,834.7	1:3,968.6	24.06	1.4

Table 2 — Antibacterial action of preparations based on essential oils concerning *S. aureus* (strain P-209)

Solutions of preparations (in the ratio)	Bactericidal dilutions Exposure, min		Average phenolic coefficient	Average protein index
	10	30		
Phenol 1:50	1:98	1:192.8	—	—
Fir+Eucalyptus+Thyme	1:98	1:137.2	0.9	
Fir+Eucalyptus+Thyme+Benzalkonium chloride	1:737.9	1:2,024.8	9.0	
Fir+Eucalyptus+Thyme+Benzalkonium chloride+Silver nanoparticles	1:2,834.7	1:3,968.6	24.06	
Fir+Eucalyptus+Thyme+Benzalkonium chloride+ Silver nanoparticles+protein	1:1,464.3	1:2,024.8	12.7	1.9

The mechanism of action of low doses of silver nanoparticles and essential oils on microorganisms consists in reducing the permeability of cytoplasmic membranes, reducing the intensity of metabolism and the activity of aerobic respiration of microorganisms, destruction of cytoplasmic membranes caused by bactericidal doses of essential oils.

Conclusions. The tested drug has high bactericidal activity concerning *S. aureus*, which 12.7 times exceeds the activity of phenol, and 24 times — in relation to *E. coli*.

The bactericide activity 1.9 times decreases growth of Gram-positive microflora and 1.4 times — Gram-negative flora in the surface protein contamination conditions.

The tested solutions of essential oils have a wide spectrum of antimicrobial action and can be used for disinfection in cases of animal diseases, when resistance of pathogens is assimilated to *E. coli*, and in cases when resistance of pathogens can be assimilated to *S. aureus* resistance.

Microorganisms, upon prolonged contact with silver nanoparticles and essential oils, practically do not produce resistance to them. That is their essential advantage over antibiotics, and is useful for their practical application in human and veterinary medicine.

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Contents