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3. Authors make sure there are no typographical errors in the manuscript

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(e). Summary in English (between 200 to 300 words), which should be included: the aim of the work, materials and methods, the results of the work, conclusions

(f). Keywords (up to 8)

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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 to viruses of human T-cell leukemia. BLV is a very convenient model for studying the pathogenesis of human leukemia. Genomes of retroviruses have high variability levels 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 objectively 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, to establish phylogenetic relationships between isolates sequenced bovine leukemia virus, which circulates in farms of different regions in Ukraine, with other animals retroviruses. The sampling of clinical material from cattle farms was conducted in different geographical regions in Ukraine and extracted proviral BLV DNA. Totally 831 samples of peripheral blood were collected and tested from cattle farms in Kharkiv region, 10 samples — Kirovohrad region; 10 samples — Donetsk region; 41 samples — Crimea, Simferopol region; 10 — samples of Poltava region. Sequenced fragments of env gene of bovine leukemia virus proviral DNA, circulating in different geographical regions in Ukraine were analyzed. 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 the one of the retroviruses representatives. 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., 1993; 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 the 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 of the existence of point and tandem mutations (Milos, 2009). Effectiveness and objectivity of molecular phylogenetic studies depend 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 DNA-analyzer ABI PRISM 311D using the technology of ABI (Applied Biosystems, USA).

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

Results. The sampling of clinical material of cattle was performed in farms of different geographical regions in Ukraine and proviral DNA of BLV was extracted. There were analyzed 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).

Multiple alignment and comparison of BLV *env* gene sequences circulating in the Kharkiv region and other geographical regions was created to determine the possible divergence and the genetic variability of bovine leukemia virus. 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 1548 bp, and partially sequenced fragment with length 960 bp from Poland (AF111171). Fragment of the multiple alignment of selected sequences demonstrated in Fig. 1 (nucleotides that are not the same in this position with other nucleotides of selected sequences are underlined).

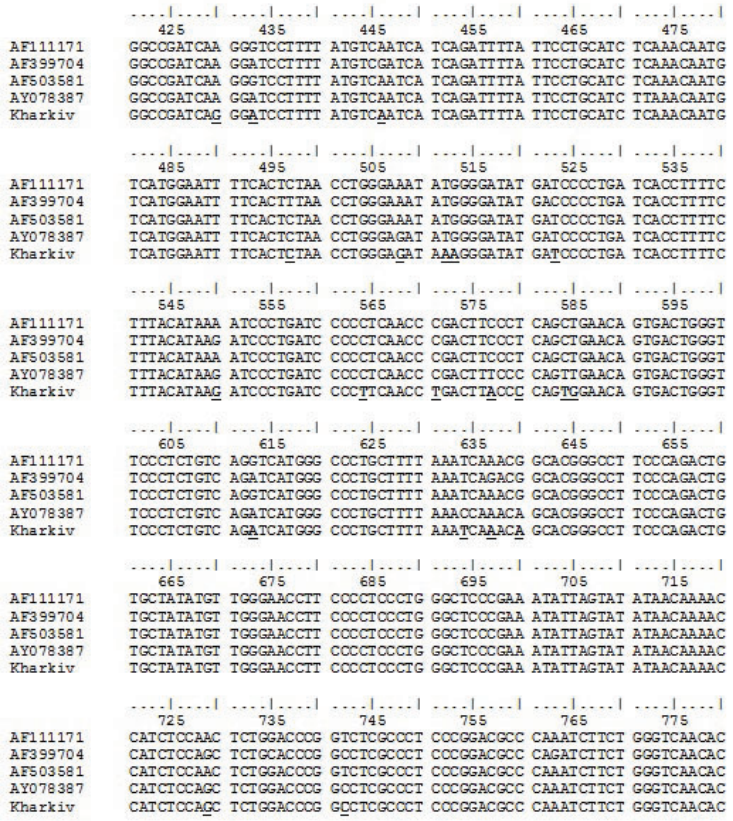

Figure 1. Fragment of multiple sequence alignment of *env* gene of bovine leukemia virus circulating in different geographical regions

The same value of divergence of the BLV *env* gene, of agent circulating in the Kharkiv region, characterized proviral DNA sequences from the agents circulating in western Europe (Table 1). The smallest divergence levels were 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

	AF 111171 (Poland)	**AF 399704 (Brazil)**	**AF 503581 (Belgium)**	**AY 078387 (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	98.2	97.5

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

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.

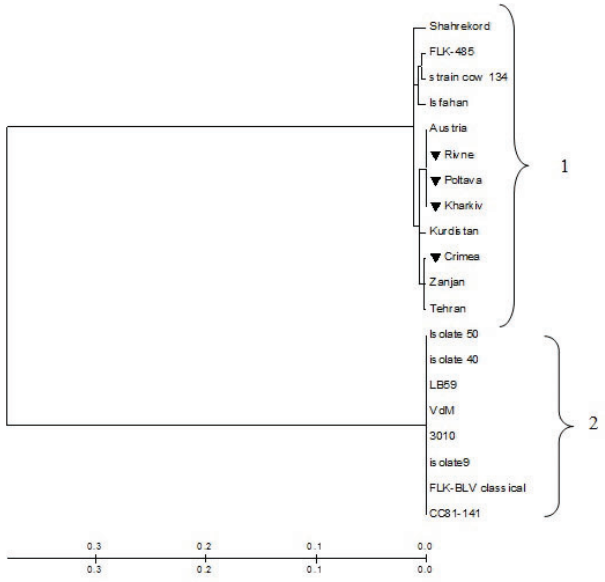

Figure 2. Dendrogram, based on env gene fragment of proviral DNA of bovine leukemia virus isolates from different geographical regions

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.

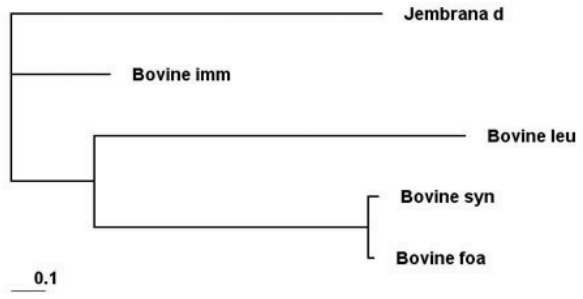

Figure 3. Dendrogram, based on env gene fragment of proviral DNA of bovine retroviruses isolates

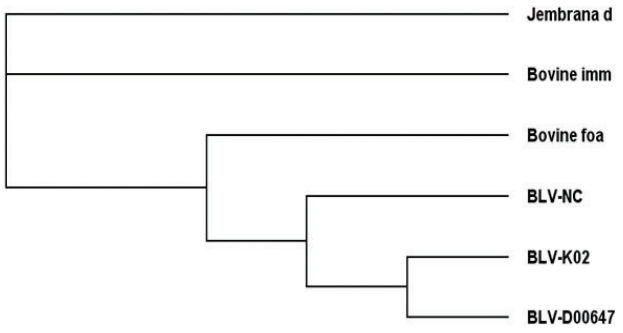

Figure 4. Dendrogram, based on complete sequences of proviral DNA of bovine retroviruses isolates

Conclusions. The BLV env gene is highly conserved, its primary structure has significant changes depending on the habitat of the causative agent of bovine enzootic leukosis. 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.

References

Caraguel, C., Stryhn, H., Gagné, N., Dohoo, I. and Hammell, L. (2009) 'Traditional descriptive analysis and novel visual representation of diagnostic repeatability and reproducibility: Application to an infectious salmon anaemia virus RT-PCR assay', *Preventive Veterinary Medicine*, 92(1–2), pp. 9–19. doi: 10.1016/j.prevetmed.2009.07.011.

Chang, Z., Jin, M., Liu, N., Xie, H., Cui, S., Zhang, Q. and Duan, Z. (2009) 'Analysis of epidemiologic feature and genetic sequence of Sapovirus in China', *Chinese Journal of Virology*, 25(2), pp. 113–116. doi: 10.13242/j.cnki.bingduxuebao.001979.

Darlix, J.-L. and Spahr, P.-F. (1983) 'High spontaneous mutation rate of Rons sarcoma virus demonstrated by direct sequencing of the RNA genome', *Nucleic Acids Research*, 11(17), pp. 5953–5967. doi: 10.1093/nar/11.17.5953.

Dube, S., Bachman, S., Poiesz, B. J., Ferrer, J. F., Esteban, E., Choi, D., Love, J. and Spicer, T. (1997) 'Degenerate and specific PCR assays for the detection of bovine leukaemia virus and primate T cell leukaemia/lymphoma virus pol DNA and RNA: Phylogenetic comparisons of amplified sequences from cattle and primates from around the world', *Journal of General Virology*, 78(6), pp. 1389–1398. doi: 10.1099/0022-1317-78-6-1389.

Giammarioli, M., Pellegrini, C., Casciari, C., Rossi, E. and De Mario, G. M. (2008) 'Genetic diversity of bovine viral diarrhea virus 1: Italian isolates clustered in at least seven subgenotypes', *Journal of Veterinary Diagnostic Investigation*, 20(6), pp. 783–788. doi: 10.1177/104063870802000611.

Katz, R. A. and Skalka, A. M. (1990) 'Generation of diversity in retroviruses', *Annual Review of Genetics*, 24(1), pp. 409–443. doi: 10.1146/annurev.ge.24.120190.002205.

Licursi, M., Inoshima, Y., Wu, D., Yokoyama, T., González, E. T. and Sentsui, H. (2003) 'Provirus variants of bovine leukemia virus in naturally infected cattle from Argentina and Japan', *Veterinary Microbiology*, 96(1), pp. 17–23. doi: 10.1016/s0378-1135(03)00202-5.

Manini, P., De Palma, G. and Mutti, A. (2007) 'Exposure assessment at the workplace: Implications of biological variability', *Toxicology Letters*, 168(3), pp. 210–218. doi: 10.1016/j.toxlet.2006.09.014.

Meyerhans, A., Cheynier, R., Albert, J., Seth, M., Kwok, S., Sninsky, J., Morfeldt-Månson, L., Asjö, B. and Wain-Hobson, S. (1989) 'Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations', *Cell*, 58(5), pp. 901–910. doi: 10.1016/0092-8674(89)90942-2.

Milos, P. M. (2009) 'Emergence of single-molecule sequencing and potential for molecular diagnostic applications', *Expert Review of Molecular Diagnostics*, 9(7), pp. 659–666. doi: 10.1586/erm.09.50.

Parvin, J. D., Moscona, A., Pan, W. T., Leider, J. M. and Palese, P. (1986) 'Measurement of the mutation rates of animal viruses: Influenza A virus and poliovirus type 1', *Journal of Virology*, 59(2), pp. 377–383. Available at: http://jvi.asm.org/content/59/2/377.full.pdf.

Steinhauer, D. A. and Holland, J. J. (1987) 'Rapid evolution of RNA viruses', *Annual Review of Microbiology*, 41(1), pp. 409–431. doi: 10.1146/annurev.mi.41.100187.002205.

Steinhauer, D. A., Torre, J. C. de la, Meier, E. and Holland, J. J. (1989) 'Extreme heterogeneity in populations of vesicular stomatitis virus', *Journal of Virology*, 63(5), pp. 2072–2080. Available at: http://jvi.asm.org/content/63/5/2072.full.pdf.

Wendel, J. F. and Doyle, J. J. (1998) Phylogenetic incongruence: Window into genome history and molecular evolution. In: Soltis, D. E., Soltis, P. S. and Doyle, J. J. *Molecular Systematics of Plants II*. Boston: Kluwer Academic Publishing. doi: 10.1007/978-1-4615-5419-6_10.

Willems, L., Thienpont, E., Kerkhofs, P., Burny, A. Mammerickx, M. and Kettmann, R. (1993) 'Bovine leukemia virus, an animal model for the study of intrastrain variability', *Journal of Virology*, 67(2), pp. 1086–1089. Available at: jvi.asm.org/content/67/2/1086.full.pdf.

UDC 619:616.98:579.842.11:636.4/.5

BIOTECHNOLOGICAL ASPECTS OF AMIXIN® APPLICATION AS AN ANTIVIRAL DRUG FOR TREATMENT OF PIGS AND CHICKEN

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Summary. Veterinary drug Amixin®, active substance dihydrochloride 2,7-bis[2-(diethylamine)ethoxy]fluorene-9-one (AMX), was tested for its antiviral activity with using of the epizootic relevance for Ukraine the infectious agents of Pseudorabies (PR), Teschovirus encephalomyelitis (TEM), classical swine fever (CSF), porcine reproductive and respiratory syndrome pigs (PRRS), 2nd type of porcine circovirus (PCV-2) and parvovirus infections (PPVI), swine (SIV) and avian influenza viruses (AIV). The influence of this drug on agents of the avian mycoplasmosis (Myc) and pasteurellosis (Past) was also learned. AMX action was tested in the concentrations of 0.5–15 mg/ml, expositions for 0.25–12 hours and at the room temperature. Under these conditions, the inactivation of 20–60% (the highest concentrations more likely) the 1000 infectious units (TCID, ELD or $PFU_{50/ml}$, respectively) of all viruses was resulted. The 5000 $ELD_{50/ml}$ of the SIV and AIV viruses were inactivated for 5 hours almost totally. Moderate toxicity of AMX was registered in it doses ≥1 mg/ml (P≤0.01) for tube cultures of PK-15 cell line and Marc-145. At the same time AMX doses ≥0.125 mg/ml inhibited by 20–75% of infective activities of agents of the PR, PRRS, PCV-2, PPVI (n=42, P≤0.01) in cell cultures Marc-145 and PK-15, respectively. The toxicity of the drug for 7–9-days-old embryos chickens began to emerge with a concentration of 1.5 mg/ml (n=18, P≤0.01). Its virostatic effect on SIV, AIV and PR agents was manifested in doses ≥0.25 mg/ml (n=24, P≤0.01). AMX was administrated in a single dose of 540 mg ana partes with sunflower oil and showed no toxicity for suckling piglets (n=4, P≤0.01) and 10-days-old chickens (n=10, P≤0.005). At the same time the antibiotic resistant Myc and Past from the blood of chicken infected by natural mixes of these agents, acquire the sensitivity to commercial food antibiotic after 5-days course of treatment by AMX. These data is interpreted the mechanism of therapeutic and preventive action of AMX through direct antivirus activity. The hypothesis of acquires the antibiotic sensitivity by pathogenic bacteria throughout its bacteriophages inhibition is proposed.

Keywords: Amixin, toxicity, antiviral activity, porcine viruses, avian agents, bacteriophages

Introduction. Antiviral activity of drugs is characterized by its virocid and virostatic activities (Mashkovskyi, 1997). It is very important to conduct of antiviral assessments with use of variants of the infectious agents that are circulated in nozoareals where target drug is planed to use (Drew, 2011; Zhu, Zhou and Tong, 2012). Most actual for Ukrainian pig farming during last 15 years were 'economical' diseases — porcine concurrent virus-bacterial infections with participation of next infectious agents: herpes-, pesti-, arterio-, circo-, tesho-, parvo- and orthomyxoviruses (Buzun, Prokhoryatova and Kolchyk, 2001).

Since 2008, the market of veterinary drugs Ukraine has a new veterinary preparation Amixin® (AMX) with synthetic substance dihydrochloride 2,7-bis[2-(diethylamine)ethoxy]fluorene-9-one. This substance is characterized with immunomodulatory and antiviral properties and produced by LLC 'InterChem' (Odessa) in slightly modified form of the well-known medical drug Amixin® IC of the same manufacturer (interchem.com.ua/en/drugs/amiksin®-Ic). Since 1990th Amixin® IC is widely used in the medical protocols to prevention and treatment of flu, herpetic, chlamydial, arboviral and other human infectious diseases (Ershov et al., 1998; Demchenko et al., 2000). Like to its American analogue — tyloron — there are huge data on the mechanism of therapeutic and preventive action of AMX substance in cells and humans and mammals (Andronati, Litvinova and Golovenko, 1999; Lyakhov and Litvinova, 2008; Zholobak et al., 2012). However, only few results of a veterinary application of this drug we have in pig breeding and poultry — unfortunately without of data on its antiviral activity against the respective porcine and poultry pathogens.

In the current paper presents the summarized results of the study cyto- and ovotoxicity and virocid and virostatic activities of veterinary AMX to determine its mechanism of action in the possible chemoprevention/treatment of Pseudorabies (PR), Teschovirus encephalomyelitis (TEM), classical swine fever (CSF), porcine reproductive and respiratory syndrome pigs (PRRS), 2nd type of porcine circovirus (PCV-2) and parvovirus infections (PPVI), swine (SIV) and avian influenza viruses (AIV). The influence of this drug on actual for Ukrainian poultry pathogens — avian mycoplasmosis (Myc) and pasteurellosis (Past) agents was learned also.

Material and Methods. ***Viruses*** — Porcine Herpesvirus I, Pseudorabies virus (PRV), strain 'Donbas-1082' (Д-1082), with infectious activity 7.0–7.5 lg rabbit-lethal doses ($LD_{50/ml}$), or 6.0–7.0 lg tissue-cytopathic infectious doses ($TCID_{50/ml}$); this agent was isolated from porcine brain in herd of small holding during PR epizootic outbreak in Chervonoarmiys'k Region of Donbas near porcine mega-complex 'Agroinvest' at 2011 and was adapted to PK-15 cell line (was used on level of 7th passage). — Teshovirus (TV), strain 'Butcha', with infectious activity 8.0–9.5 lg porcine-lethal doses ($LD_{50/ml}$), or 6.5–7.0 lg $TCID_{50/ml}$; this agent was isolated from porcine brain in herd of Poltavian back-yard holding during panzootic outbreak of TEM in 2002 and was passaged on guinea pigs and domestic pigs by per-oral route and adapted to different porcine cell lines (was used on level of 24th passage PK-15 cells). — Pestivirus, classical swine fever virus (CSFV), vaccine strain 'C', with infectious activity 4.0–4.5 lg rabbit-fever doses ($RFD_{50/ml}$), or 5.0–5.5 lg plaque-forming doses ($PFU_{50/ml}$); this agent was isolated from porcine blood in herd of Kharkiv small commercial holding on 4th day after regular vaccination at 2010 with standard vaccine manufactured on State Kherson Bioplant and was passaged on rabbits by intravenous route and adapted to PK-15 cell line (was used on level of 6th passage in this cells). — Arteriovirus, Porcine reproductive-respiratory syndrome virus (PRRSV), strain 'Vody Donbasu-08' (WD-08), with infectious activity 5.0–6.5 lg $TCID_{50/ml}$ in porcine alveolar macrophages (PAM) and 2.5–3.0 lg $TCID_{50/ml}$ in MARC-145 cell line; this agent was isolated from porcine lien in herd of Gorlovka' small commercial holding (Donbas) during PRRS outbreak at 2008 and was passaged by consecutive reproduction in suckling piglets and PAM at beginning and then adapted to MARC-145 cell line (was used on level of 9th passage in this cells). — Circovirus, Porcine circovirus type 2 (PCV-2), strain 'Ingulets'kiy-1024' (I-1024), with infectious activity 5.0–7.0 lg $PFU_{50/ml}$ in PAM and PK-15 cell line; this agent was isolated from porcine intestine lymphatic nodules in herd of Kherson farmer holding 'Dulya' in enzootic area for porcine circoviral mix-infections at 2009 and was passaged by consecutive reproduction in suckling piglets and PAM at beginning and then adapted to PK-15 cell line (was used on level of 9th passage in this cells). — Parvovirus, Porcine parvovirus (PPV), strain 'H-1', with infectious activity 5.0–7.0 lg $TCID_{50/ml}$ in PK-15 cell line; this agent was isolated from kidney of porcine embryo in herd of Sumy farmer holding in enzootic area for porcine virus-bacterial mix-infections at 2011, and was passaged by consecutive reproduction and adaptation to different porcine cell lines (was used on level of 4th passage in PK-15 cell line). — Orthomyxoviruses, swine (SIV) and avian (AIV) influenza virus, strain A/swine/Skadovs'k/01/11 (H2N?) and strain A/chick/Sivas/03/07 (H5N1), respectively. Both have infectious activity 5.0–7.0 lg chick embryo lethal doses ($ELD_{50/ml}$); SIV was isolated from lung-lien suspension of pig during epizootic outbreak in herd of pig-breeding holding (Kherson region) at 2012; AIV was isolated from brain of sick chicken during panzootic outbreak of avian flu in back-yard holding (Crimea region of Ukraine) at 2007; both agents was passaged by consecutive reproduction and adaptation to 7–9-days-old chicken embryos (was used as extra-embryonic fluids of 12th passage of the AIV and 7th passage of the SIV).

Natural Consortia (Con) of *Pasteurella multocida* type A, *Mycoplasma gallisepticum* and more then 2 species of unidentified bacteria and bacteriophages, which isolated from blood of chicken of the commercial flock in farmer holding of Kharkiv region at 2015 by traditional bacteriological methods. The consortia used in two forms: isolates 'Con A' — contain microorganisms (including the bacteriophages) which are insensitive to mix of gentamicine-tylosine on test-disks (1 mg/ml every); it was isolated from blood of 10-days-old infected chickens (n=5) before treatment with AMX; isolates 'Con B' — contain microorganisms (the bacteriophages are absent) which are sensitive to mix of gentamicine-tylosine on test-disks (1 mg/ml every); it was isolated from blood of 15-days-old infected chickens (n=5) after treatment with AMX (triple watering, once per-day every day, dose 15 mg/ml).

All of these agents are deposed, stored and maintenance in the Collection of microorganisms of NSC 'IECVM' (Kharkiv).

Systems for viruses' maintenance and titration — Cell cultures — PK-15 cell line (secondary culture of embryonic kidneys pigs) received from the Collection of cell cultures of NSC 'IECVM' and used at 3–7 passages after thawing, grown under standard operating procedures (SOP) NSC 'IECVM' in test tubes and plastic cultural vessels using a mixture of media № 199 and media Eagle MEM with 10% calf serum (Veterinary Medicine Ltd., Kharkiv). The PK-15 cell line used to work as a 2–4-days-old' monolayer. — MARC-145 cell line (the clone of MA-109 cell line secondary culture of the embryonic kidneys green monkey) received, growth and used similarly to PK-15 cell line with next differences: used at 5–11 passages after thawing, growth medium contain 10% bovine fetal serum (Invitrogen, USA). — Porcine alveolar macrophages culture (PAM) prepared from lungs of 3-month old piglets by SOP NSC 'IECVM' in test tubes and plastic cultural vessels using a mixture of Hanks' media with 15–20% homologous porcine serum (growth media); these cultures used to work as a 4–7-days-old' monolayer.

SPF-eggs received from the Department of studying avian diseases NSC 'IECVM' and used for maintenance of PR virus used 5–7-days-old CE and of CSF virus — 9–10-days-old CE.

Pasteurella lytic bacteriophages isolation. 5 ml of 48 hours culture of Con in meat-peptone broth (MPB) combined with 25 ml of MPB ('Meat Peptone' HI Media Laboratories) and incubated at 37 °C with magnetic stirrer for 4 hours. Followed this, 5 ml of a mid-log phase culture (the 5 hours culture of strain MPB with 5% of horse serum) of the 'L' strain of *P. multocida* type A and horse serum to final concentration 5% were added and the mixture incubated at 37 °C overnight without stirring. The following day top 5 ml of culture was harvested and filtered using a 0.20 μm membrane filter (Millipore, Massachusetts, US) to remove the debris. The filtrate was stored at 4 °C until required.

Work with infectious materials was carried out in boxed areas with BSL-2. Infectious activity of model viruses was determined by titration in relevant biological systems (in cell cultures grown in test tubes or embedded in them glasses or in chicken embryos, 4–5 tubes or embryos on every dilution of virus) by Reed and Munch (1938).

Determination of the *Pasteurella* lytic bacteriophages by routine test dilution (RTD) — The turbidity of an overnight culture of the 'L' strain of *P. multocida* type A in MP-Broth with 5% horse serum was adjusted to McFarland standard of 0.5 (1.5×108 cfu/ml) using isotonic peptone-saline. The surface of a MP-Agar plate then covered with 0.2 ml of this suspension and placed in a 37 °C incubator to dry for twenty minutes. Ten microliters of each dilution dropped onto the surface of the inoculated plates and left to dry for ten minutes. After which the plates incubated at 37 °C and examined for the presence of plaques at 24-hours intervals. The most diluted suspension to produce complete clearing considered as the routine test dilution (RTD).

As the main method of assay of virucidal activity of the AMX we used operating protocol to the study of anti-HIV properties of medical drugs (Erice et al., 1993) in the next modification. The cultural fluids of the PCV-2 and CSFV (1000 PFU_{50} of each virus), PRV, TV, PPV and PRRS (1000 $TCID_{50}$ of each virus) and SIV or AIV (5000 ELD_{50} of each virus) every in volume of 5 ml combined with 5 ml of AMX dilutions on solvent (sterile buffered saline with 250 OD/mg mixture of penicillin-streptomycin, pH 7.4) — experimental batch or with 5 ml of solvent without AMX — mock batch. There next AMX final concentrations were checked (mg/ml): 0.1, 0.5, 1.0, 5.0, 10.0, and 15.0. All batches were incubated under room temperature during 15, 30, 60, and 90 min and under 6 °C during 12 hours additionally. Then all batches were passed throughout Sephadex G-25 (coarse) that was packed in self-made micro-columns (high of gel 10 cm, diameter 0.5 cm, eluent — solvent as above) for each specimen (volume 0.75 ml, marker — hemoglobin, velocity of elution — 25–27 drops per min). The first fraction in volume 1.5–1.7 ml was collected in each case. This procedure was performed to liberate of specimen of experimental batch from AMX. Each specimen in volume 0.1 ml was inoculated in 4–5 tubes with mature monolayer of the respectively cell culture or in allantois of chicken eggs (n=4–5 for each specimen). A recognizing of the AMX effect was performed on results of assay of level inhibition (% of inhibition — as can see at Table 1) of viral activity in experimental batch in compare with mock batch. The presence of viruses in cell cultures was assayed by specific TCID with selective verification in immunoperoxidase method (IPM) for PRV, TV, PPV and PRRS or in IPM only for CSFV and PCV-2. The presence of SIV and AIV agents in embryos was estimated by specific signs of embryonic death and by additional titration of allantois' fluids from each embryo in standard hemagglutination (HA) with chicken erythrocytes and by identification of influenza agents in standard HA-inhibition (IHA) tests.

The three repeats of these studies were conducted with each mentioned agent.

Virostatic activity of AMX was studied after determining of its ovum-toxicity, maximal allowable dose (maximum permissible concentration) and cytotoxicity for estimation rank of doses of the drug that are not toxic for chickens, pigs, embryos and cell cultures. Ovum-toxicity was studied on 10-days-old CE and 14-days-old duck embryos. Cytotoxicity determined by inoculation of 3–4 days-old cell cultures PK-15 and AMC solutions of AMX in concentrations of 0.1–10.0 mg/ml (step of dilution — 2) on cultural medium.

The maximum permissible concentration (MPC) of the drug for poultry was determined at 1-week-old chickens, 3-days-old piglets by oral way with water suspension of AMX at doses of 35, 70, 140, 280 and 560 mg, 4 animals for each dose. The animals were observed on manifestation of clinical criteria of 'chronic toxicity' — including the measuring of average growth velocity within three weeks after AMX introduction (Stefanov, 2002).

Results and discussion. Each of the tested viruses in tested concentrations of the AMX drug demonstrated significant virocid activity (n=73, $P \leq 0.025$). The 12 hours exposition at room temperature of epizootic variant of the PRRS virus as well as vaccine strain of CSF virus without AMX (control) led to its inactivation by the 25% rate. The same inactivation degree is registered in these viruses when they were incubated 12 hours with AMX that taken in concentrations of 0.1–0.5 mg/ml (Table 1). Enhancing of the AMX concentrations

to 1.0–10.0 mg/ml led to these viruses inactivation by on 50–75% at incubation at room temperature for 0.5–1.5 hours even. There complete inactivation of PRRS virus in mixes with the AMX was during of 15 min. — incubation period at three repetitions under room temperature when the drug was used at a concentration of 15 mg/ml. Complete inactivation of CSF virus vaccine was observed under the same conditions, but when the reaction mixture incubating at least 1 hour. The most sensitivity to the AMX was revealed in PR, SIV and AIV viruses: they were fully inactivated already when AMX concentration in the reaction mixture was of 5 and more mg/ml under room temperature during 1 hour to 15 minutes: as more the AMX concentration was, then faster these viruses was inactivated.

Table 1 — Amixin® virocid activity

Amixin® concentration, mg/ml	Inactivation level of the viruses, % (high point demonstrates the hour rate)						
	PRV[c]	TD[b]	PCV-2[a]	PPV[b]	PRRS[b]	CSF[a]	TG[d]
0.0	0^{12}	0^{12}	0^{12}	0^{12}	25^{12}	25^{12}	0^{12}
0.1	0^{12}	0^{12}	0^{12}	0^{12}	25^{12}	25^{12}	0^{12}
0.5	25^{12}	0^{12}	0^{12}	0^{12}	25^{12}	25^{12}	25^{12}
1.0	75^{12}	50^{12}	50^{12}	25^{12}	$50^{1.5}$	75^{12}	$75^{1.5}$
5.0	100^{1}	50^{1}	$75^{1.5}$	$25^{1.5}$	$50^{0.5}$	$75^{1.5}$	100^{1}
10.0	$100^{0.5}$	$50^{0.5}$	100^{1}	$75^{1.5}$	$75^{0.5}$	$75^{1.5}$	$100^{0.5}$
15.0	$100^{0.5}$	$75^{0.5}$	$100^{0.5}$	75^{1}	$100^{0.25}$	100^{1}	$100^{0.25}$

Marks: [a]1000 $PFU_{50/ml}$; [b]1000 $TCID_{50/ml}$; [c]1000 $ELD_{50/ml}$; [d]5000 $ELD_{50/ml}$

The high sensitivity to the drug was also found in the PCV-2 agent of pigs: it completely inactivated during 1 hour exposure in an 'AMX-virus' mixtures with the preparation concentration 10 mg/ml and already for a 30-minute after exposure with AMX in concentration 15 mg/ml. The PPV and TV agents were the least sensitive to the contact inactivation with the AMX: their inactivation by drug at it concentrations of 5–15 mg/ml was not finished for 0.5–1.0 hours (n=24) — only when time of their exposition with the drug was raised to 12–18 hours the inactivation of these agents were inactivated completely (in Table 1 not shown).

Therefore as data above demonstrate, the veterinary drug Amixin® in wide of the concentration range hold significant virucidal contact activity against all of investigated of the modern Ukrainian variants of dangerous porcine and avian viruses — the pathogens of SIV, AIV, PR, PRRS, CSF and PCV-2, and in lower degree this relate to the TV- and PPV- pathogens.

Table 2 — Toxic effect of Amixin® in vitro, in ovo and in vivo

Amixin® concentration, mg/ml	Toxic influence in the biological systems after Amixin® inoculation					
	in vitro			in ovum	in vivo	
	PK-15	Marc-145	PAM	Chicken embryos	3-days-old piglets	3-days-old piglets (adm. with oil)
0	0/4	0/4	0/4	0/4	0/4	0/4
0.1–0.05	0/8	0/8	0/8	0/8	-	-
1.0–5.0	4/4	2/4	2/4	2/4	-	-
10.0	4/4	4/4	4/4	4/4	0/2	-
20.0	-	-	-	-	0/4	-
70.0	-	-	-	-	2/4	0/4
140.0	-	-	-	-	4/4	0/3
280.0	-	-	-	-	2/2	0/3
560.0	-	-	-	-	4/4	0/7

Table 2 summarizes the experimental data of toxicity of AMX investigation in cell cultures PK-15, MARC-145 and PAM, chicken embryos and neonatal piglet (with the addition of sunflower oil, and without it). The results show that the aqueous solution of the AMX in the concentration range from 0.1 to 0.5 mg/ml did not cause toxic damage cell cultures and embryos. In the concentration range 1–5 mg/ml some toxicity was showed for PK-15 cell culture (by signs of toxic degeneration of monolayer in all test-tubes) and embryos (delay of embryos development that are not accompanied by their death).

Processing with by AMX of MARC-145 and PAM cultures demonstrated the increasing of anisotropy of cytoplasm in the half of the used cells. This sign was not observed in control test tubes (without AMX). The dose 10 mg/ml of the AMX was toxic for all of used cultures and embryos, but the AMX was nontoxic for suckling piglets (n=6) in doses to 20 mg/ml. When we checked on piglets and chickens the suspensions of AMX in concentrations 70–560 mg/ml, we were must to use the DMSO for better dissolution of AMX. As result we observed some toxic effect of DMSO (as we believe) for piglets but not for the 10-days-old chicken (in Table 2 not shown).

Anyone from these piglets did not show signs of intoxication in the single AMX dose of 5 ml (concentration 560 mg/ml). In next three week after the watering of the drug, their average growth was 42.3±11.7 g (n=20, P≤0.01), while the control (mixture of PBS with oil) — 37.0±10.3 g (n=20, P≤0.005). It should be noted that in the chicken embryos (n=12 of 17) and embryos of ducks (n=19 of the 20, in Table 2 not shown) that survived the treatment of drug in AMX single dose of 0.2 ml (concentration 560 mg/ml, solvation with DMSO). They demonstrated no signs of teratogenicity of the AMX: all chicks and duckling in the next 3 weeks after hatching (time of observation) had no abnormalities in the development and did not differ from control nestlings (n=5) which were treated by saline in the same dose.

On the basis of determining toxicity as above, AMX virostatic action in vitro and in ovum was studied in the AMX range of concentrations of 0.125–1.5 mg/ml (Table 3). There was found that the concentration of 0.125 mg/ml drug blocks the reproduction of viruses CSF, TV, PCV-2, PPV, SIV and AIV for 24 hours, compared to control (infected cell culture without drug treatment).

Table 3 — Virostatic effects of Amixin®

Amixin® concentration, mg/ml	Time of the virostatic effect of the Amixin® in ovo/in vivo, hours						
	PRV	TV	PCV-2	PPV	PRRS	CSF	TGE
0	N	N	N	N	N	N	N
0.125	N	24	24	24	48	24	24
0.250	12	96	96	24	96	96	48
0.500	48	96	96	24	96	96	96
1.000	120	D	D	D	D	D	96
1.500	120	D	D	D	D	D	96

Marks: N — no inactivation of virus; 12–96 — time of the inactivation; D — degeneration of the monolayer of cells (cytotoxic effect)

So, after the AMX administration in the dose of 0.125 mg/ml the PAM and MARC-145 cell cultures infected with PRRS virus (1000 $TCID_{50/ml}$) in all of four test tubes TCID was observed on 48 hours later than in all mock test tubes (i. e. infected cell culture without drug treatment). The application of the drug at concentrations of 0.25–0.5 mg/ml under the same conditions caused the delay almost of on 4 days the TCID of the viruses PRRS, CSF, PCV-2 and TV (1000 $TCID_{50/ml}$), and the viruses SIV and AIV (5000 $ELD_{50/ml}$), everyone.

At the same time, the drug at a concentration of 0.125 mg/ml did not affect the reproduction of viruses Aujeszky's disease and highly pathogenic avian influenza (table not shown). From concentration of 0.25 mg/ml range as initial, the virostatic properties of the drug is extended to the maximum: we observed delay reproduction of all studied viruses in range from 12 hours (PR virus) and 24 hours (PPV) to period 48 hours (viruses SIV and AIV) and 96 hours (viruses CSF, PRRS and PCV-2). This suggests that the agents of CSF, PRRS and PCV-2 are particularly sensitive to virostatic action veterinary drug Amixin®. In all of three repeats the AMX demonstrated virostatic activity on porcine parvovirus.

The reproduction of the all studied viruses in all biological systems in vitro/in ovum was delayed as

more as the AMX dose was to increase. These directly show on a dose-dependent nature of the AMX action. With high probability, the results as above allow assuming the existence of certain range of the AMX concentrations for the each of the investigated pathogens in which this drug can have the optimal therapeutic effect. Also, it is obviously that the level of the AMX dosage for therapy of animals with u napparent infection and of sick animals may not be the same.

Because we had strong data that the AMX is effective in many cases of bacterial infections in piggery and poultry, the learning of nature of these events was launched. For it 10 chickens of 10-days-old were infected by field isolate of bacterial consortia ' Con A', which by data of preview analysis contain different microorganisms including the *Pasteurella* bacteriophage and had not sensitivity to mix of gentamicine-tylosine antibiotics. Five from those chicken were treatment with AMX by triple watering, once per-day every day, dose 15 mg/ml. 15 days apart all chickens was search on presence of antibiotic-resistant bacteria and *Pasteurella* bacteriophage in the blood samples. From chickens, which were not developed with AMX, was isolated the mixture of microorganisms (consortia) which contained *Pasteurella multocida* type A, *Mycoplasma gallisepticum* and more than 2 species of unidentified bacteria. Additionally, investigations allow estimating the presence of the lytic *Pasteurella* bacteriophage in this consortium. The same lytic *Pasteurella* bacteriophage was revealed previously in initial consortium, which used for infection of these chickens. Titer of this bacteriophage in initial and passaged consortium was the same — 10^9 by RTD: this is evidence that studied consortium is stable. The isolated *Pasteurella multocida* type A from untreated flock was insensitive to mix of gentamicine-tylosine on test-disks (1 mg/ml every) like to this bacteria in initial consortium.

At the same time from chicken, which were developed with AMX was isolated analogous consortium contained the same microorganisms with exclusion of the lytic *Pasteurella* bacteriophage. Moreover, there was revealed that isolated *Pasteurella multocida* type A from treated flock had high sensitivity to mix of gentamicine-tylosine on test-disks (1 mg/ml every) that unlike to this bacteria in initial consortium.

Based on the above results, we performed the clinical trials of veterinary drug Amixin® in complex with drugs and measures that was developed in NSC 'IECVM' to control of porcine emergent viral and bacterial reproductive and neonatal mix-infections (PRNI) in industrial pig farming.

Considering the data about AMX virucid and virostatic activity and literature data on interferonogenic activities of it and its analogues (Lyakhov and Litvinova, 2008; Zholobak et al., 2012), we can assume that the therapeutic effect of this drug can be achieved by both direct (contact) antiviral action and by mediation of interferon induction. We believe that 'antibacterial effect' of the Amixin® complex application consist in the elimination of bacteriophages that controlled the sensitivity of its bacteria-host to antibiotics. This in turn allows the infected herds to restore its self-control over the pathogens circulating in Ukraine piggery and poultry farming.

Conclusion. The results of clinical trials confirmed, that the Amixin® demonstrates considerable therapeutic and prophylactic efficacy for control of the modern epizootic situation in Ukrainian piggery. Especially useful is the Amixin® application for sanitation of herds with unapparent infections of reproductive animals with agents of PR, PRRS and with PCV-2. The clinical protocols of the porcine herds rehabilitation from PRNI without slaughtering of infected animals were included in two Guidelines that was approved by State Veterinary Inspection of Ukraine in 2010 and 2015.

References

Andronati, S. A., Litvinova, L. A. and Golovenko, N. Y. (1999) 'The oral inducer of endogenous interferon "Amiksin" and its analogs (review of the literature and our own research)' [Peroral'nyy induktor endogennogo interferona "Amiksin" i ego analogi (obzor literatury i sobstvennykh issledovaniy)], *Journal of Academy of Medical Sciences of Ukraine [Zhurnal Akademii meditsinskikh nauk Ukrainy]*, 1, pp. 53–66. [in Russian].

Buzun, A. I., Prokhoryatova, O. V. and Kolchyk, O. V. (2001) *Guidelines for control of stable form of RNPI [Metodychni rekomendatsii shchodo kontroliuvannia stiikykh form RNIS]*. Kharkiv: National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. [in Ukrainian].

Demchenko, E. V., Ivanchenko, G. F., Prozorovskaya, K. N., Bocharov, A. F. and Boykova, N. E. (2000) 'Chlamydial laryngitis: clinical picture and amixine treatment' [Klinika i lechenie khlamidioznogo laringita s primeneniem amiksina], *Vestnik otorinolaringologii*, 5, pp. 58–60. PMID: 11051861. [in Russian].

Drew, T. (2011) 'The emergence and evolution of swine viral diseases: To what extent have husbandry systems and global trade contributed to their distribution and diversity?', *Revue scientifique et technique (International Office of Epizootics)*, 30(1), pp. 95–106. Available at: http://www.researchgate.net/publication/51540841.

Erice, A., Balfour, H. H., Myers, D. E., Leske, V. L., Sannerud, K. J., Kuebelbeck, V., Irvin, J. D. and Uckun, F. M.

(1993) 'Anti-human immunodeficiency virus type 1 activity of an anti-CD4 immunoconjugate containing pokeweed antiviral protein', *Antimicrobial Agents and Chemotherapy*, 37(4), pp. 835–838. doi: 10.1128/AAC.37.4.835.

Ershov, F. I., Batkaev, E. A., Golovkin, V. I., Kisilev, O. I. and Tur'yanov, M. K. (1998) *Amixin. Application in therapy of acute and chronic viral diseases [Amiksin. Primenenie v terapii pri ostrykh i khronicheskikh virusnykh zabolevaniyakh]*. Moscow. [in Russian].

Lyakhov, S. A. and Litvinova, L. A. (2008) 'Mechanisms of the antiviral activity of amixin (review of literature). 2. Antiviral activity and influence on some biochemical processes' [Mekhanizmy protivovirusnoy aktivnosti amiksina (obzor literatury). 2. Protivovirusnaya aktivnost' i vliyanie na nekotorye biokhimicheskie protsessy], *Modern Infection [Suchasni infektsii]*, 2, pp. 112–116. [in Russian].

Mashkovskyi, M. D. (1997) *Therapeutic preparations [Likarski preparaty]*. 13th ed. Kharkiv: Torsinh. [in Ukrainian].

Reed, L. J. and Muench, H. (1938) 'A simple method of estimating fifty per cent endpoints', *American Journal of Hygiene*, 27(3), pp. 493–497. Available at: http://aje.oxfordjournals.org/content/27/3/493.full.pdf.

Stefanov, A. V. (ed.) (2002) *Preclinical studies of medicinal products: Guidelines [Doklinicheskie issledovaniya lekarstvennykh sredstv: metodicheskie rekomendatsii]*. Kiev: Avitsenna. [in Russian].

Zhu, L., Zhou, Y. and Tong, G. (2012) 'Mechanisms of suppression of interferon production by porcine reproductive and respiratory syndrome virus', *Acta virologica*, 56(1), pp. 3–9. doi: 10.4149/av_2012_01_3.

Zholobak, N. M., Kavok, N. S., Bogorad-Kobelska, O. S., Borovoy, I. A., Malyukina, M. Y. and Spivak, M. Y. (2012) 'Effect of tilorone and its analogues on the change in rat hepatocyte mitochondrial potential', *Fiziolohichnyi zhurnal*, 58(2), pp. 39–43. Available at: http://biph.kiev.ua/fiziol/2012_V.58/Fiziologichnyi Zhurnal 58(2)_2012/Fiziologichnyi Zhurnal 58(2)__2012_39-43.pdf.

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PHYLOGENETIC AND MOLECULAR GENETIC STUDIES OF PORCINE CIRCOVIRUS TYPE II

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Summary. The multiple farms all over the world are affected with porcine circovirus (PCV). This disease has been occurred in Ukraine. This work has been aimed to perform the phylogenetic and molecular genetic studies of porcine circovirus type II that circulate in Ukraine.

This study involves wide spectrum of the molecular techniques, including PCR, RT-PCR and sequencing of variable loci with the next following phylogenetic analysis.

The phylogenetic relationships of porcine circovirus type II that circulate in different geographical regions were studied. The prospect of phylogenetic analysis for genotyping and molecular marking of microorganisms was demonstrated. The porcine circovirus type II indication by the real-time PCR method was described.

Keywords: phylogenetic analysis, real-time PCR, porcine circovirus type II

Introduction. There is a significant problem of porcine circovirus infection in industrial pig farms for a number of Ukraine regions. The results of epizootic studies show its widespread not only in the territory of our country but abroad too (Cano-Manuel et al., 2014). In 1974, there was found a contaminant of the non-pathogenic viral particles in the continuous cell culture line PK-15. It was named the porcine circovirus type I (PCV-1) (Tischer, Rasch and Tochtermann, 1974). The first information about pathogenic porcine circovirus, that later was named as porcine circovirus type II (PCV-2), has been obtained after elicitation of new disease — the porcine multisystemic wasting syndrome (PMWS) in Canada (1991) (Clark and Harding, 1997). The results of porcine infectious diseases researches suggest that PCV-2 takes part in the pathogenesis of nephropathy dermatitis syndrome, proliferative pneumonia, and greasy pig disease, etc. In addition, there is an assumption that PCV-2 is able to initiate the occurrence of violations of reproductive functions in breeding sows (Chae, 2005; Opriessnig, Meng and Halbur, 2007).

The aim of this research was to study the phylogenetic relationships of PCV-2 and to develop the molecular genetic test kits based on method of real-time PCR (RT-PCR) detection of the pathogen in clinical material.

Materials and methods. The programs 4 Mega, ver. 4.0.2, POWER, ver. 1.0 and PhyML ver. 3.0 were used for the phylogenetic analysis (Tamura et al., 2007). For a traditional dendrogram building, based on gene sequences and genomic DNA of PCV-2, the remote-matrix method — a method of nearest neighbors binding (Neighbour joining) — and maximum parsimony method were used (Saitou and Nei, 1987). As a significance test of phylogenetic trees topology, the indexes of repetitions (bootstrap) were used.

For providing of the phylogeographic research of PCV-2, they were selected the completely and partially sequenced genomic DNA and major genes of the pathogen from international databases. All sequences were obtained in the FASTA (*.fasta) or GenBank (*.gb) formats. It allowed to apply the modern molecular biological software (including on-line mode) for multiple sequence alignments and to identify conserved and variable gene fragments as well as insertions, deletions and mutations, construction of dendrogram, and providing of suitable phylogenetic analysis.

The clinical material for research was taken from pigs of different gender and age groups from households of the eastern region of Ukraine. The pathological material had been taken from dead animals or abortive embryos during 2013–2015. Extraction of viral DNA was performed by affinity adsorption method.

The RT-PCR with the application of commercial kit 'Maxima SYBR Green / ROX qPCR' (Thermo Scientific, USA) was used for the derivation of specific DNA-regions. Amplification was performed by the thermocycler DT lite ('DNA technology', Russian Federation). The time and temperature parameters of the amplification are given below:

1st step:	denaturation —	94 °C —	2 min	— 1 cycle
2nd step:	denaturation —	94 °C —	15 sec	40 cycles
	annealing —	58 °C —	1 min	
	synthesis —	72 °C —	20 sec	
	detection —	72 °C —	12 sec	

Results. *Porcine circovirus* genome is presented by a single-strained closed-loop covalent circular DNA

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molecule with length of 1,767 bp. It has two main open reading frames that encode replicase (*rep* gene) and capsid protein of the virus shell (*cap* gene) (Nawagitgul et al., 2000). Using the multiple matching of the genomic DNA sequences and major genes of porcine circoviruses that circulate in different geographical regions and are represented in international databases, it was shown, that the most conservative gene is *rep* (Fig. 1).

There was found only three polymorphic sites (marked as *) in randomly selected fragment of the gene that is 59 bp in length. Analysis of the topology of the phylogenetic tree that was built from gene *rep* sequences, showed the possibility of differentiation of the two main types of PCV — type 1 and type 2, because the PCV-1 representatives belong to the same cluster, and PCV-2 isolate is were separated from the main cluster in a branch (Fig. 2).

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TW-PCV1   ATGCCAAGCAAGAAAAGCGGACCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
AUS-PCV1  ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
USA-PCV1  ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
CH-PCV1   ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
GB-PCV1   ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
HG-PCV1   ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
CH-PCV2   ATGCCCAGCAAGAAGAACGGACCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
                    *          * *
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Figure 1. Multiple alignment of PCV *rep* gene sequences fragment that was isolated in different countries (USA — United States; CH — China; GB — Great Britain; AUS — Australia; TW — Taiwan; HG — Hungary)

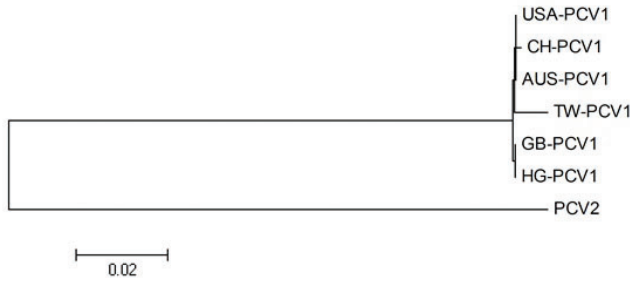

Figure 2. Phylogenetic tree based on rep gene sequences of PCV that isolated in different countries (USA — United States; CH — China; GB — Great Britain; AUS — Australia; TW — Taiwan; HG — Hungary)

However, it is not possible to differentiate genotype of PCV-1 rep gene cluster into isolates due to the high degree of similarity of the analyzed sequences, as it can be seen. The *cap* gene of the porcine circovirus was significantly less conservative: it had been found eleven polymorphic sites (marked *) for randomly selected fragment of the gene that is 59 bp in length (Fig. 3).

Exactly it was the basis for genotyping using the sequences of this gene with taking into account the phylogenetic analysis results.

The results of multiple sequences matching of PCV genes *rep* and *car* confirm to the study data (Olvera, Cortey and Segalés, 2007), where the authors showed that the *rep* gene sequence is identity for circovirus isolates in more than 93%, and identity for *cap* gene sequences is not more than 82%.

```
SWE-PCV2  GGGGCTCCAAACCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
USA-PCV2  GGGGGACCAACAAAATCTCTATACCCTTTGAATACTACAGAATAAGAAGAGTTAAGGTT
BG-PCV2   GGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
PL-PCV2   GGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
SKR-PCV2  GGGGGACCAACAAAATCTCTATACCCTTTGAATACTACAGAATAAGAAAGGTCAAGGTT
BR-PCV2   GGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
SB-PCV2   GGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
TL-PCV2   GGGGCTCAAACCCCCTCACTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
               ** *  ****** *     *
```

Figure 3. Multiple alignment of PCV-2 *cap* gene sequence fragment that isolated in different countries (USA — United States; JP — Japan; SWE — Sweden; BG — Belgium; PL — Poland; SKR — South Corea; BR — Brazil; SB — Serbia; TL — Thailand)

According to the modern classification, PCV-2 is divided into three major monophyletic groups (Wiederkehr et al., 2009). However, the dendrogram topology that based on the sequences of PCV-2 genomic DNA indicates the impossibility of genotype differentiating of the pathogen, because it is impossible to clearly distinguish clusters that confirm to PCV-2a and PCV-2b genotypes, respectively (Fig. 4).

At the same time, the presence of separate from cluster brunch with isolates of PCV 2a and 2b genotypes allows to admit an assumption about belonging of the strain RVC_UK1 (GB-PCV2 in Fig. 4) to genotype 2c.

According to the modern nomenclature proposed by Segalés et al. (2008), the dependence of PCV-2 to one or another genotype depends on the structure of distinctive motif in the protein capsid — PCV-2a, PCV-2b, and PCV-2c. In its turn, the PCV-2a isolates are second-divided into 5 clusters (2A-2E) and PCV-2b isolates are divided into 3 clusters (1A-1C).

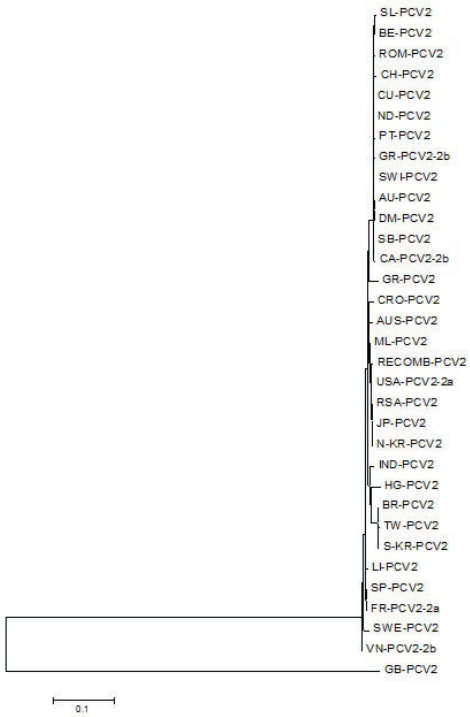

Figure 4. Phylogenetic tree, which bases on gene sequences of PCV-2 DNA isolates that circulate in different countries (SL — Slovakia; BE — Belgium; ROM — Romania; CU — Cuba; ND — Netherlands; PT — Portugal; GR — Germany; SWI — Switzerland; AU — Austria; DM — Denmark; SB — Serbia; CA — Canada; CRO — Croatia; ML — Malaysia; RSA — South Africa; JP — Japan; N-KR — North Korea; IND — Indonesia; BR — Brazil; S-KR — South Korea; LI — Lithuania; SP — Spain; FR — France; SWE — Sweden; VN — Vietnam; USA — United States; CH — China; GB — United Kingdom; AUS — Australia; TW — Taiwan; HG — Hungary)

The topology analysis of the phylogenetic tree (Fig. 5) that bases on the sequences of the *cap* gene from PCV-2 isolates that circulate in different countries demonstrates the existence of at least two subtypes for genotype 2a, two subtypes for genotype 2b, and suggests circulation of PCV-2 genotype 2c in the territory of Argentina and China. Despite the high degree of homology between PCV-1 and PCV-2 genomic DNA, which can reach 82% (Kudin and Prokulevich, 2011), we had proved the possibility of differentiating between these two types of porcine circovirus through phylogenetic analysis based on viral DNA sequences (Fig. 6). The topology of the resulting dendrogram indicates the formation of separate, explicit cluster by PCV-1 isolates. The presence of separate branch for strain PCV-1 IL that circulates in the territory of Taiwan (TW-PCV1, Fig. 5) indicates the genotypic heterogeneity of the pathogen.

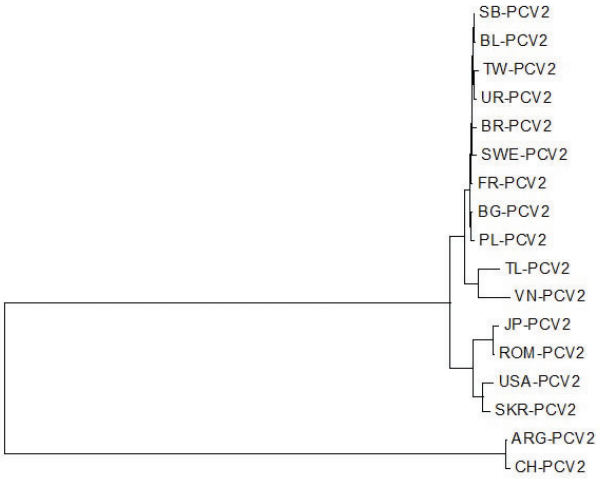

Figure 5. Phylogenetic tree that bases on sequence of PCV-2 *cap* gene from different countries (USA — United States; CH — China; TW — Taiwan; SB — Serbia; BL — Belarus; UR — Uruguay; BR — Brazil; SWE — Sweden; FR — France; BG — Belgium; PL — Poland; TL — Thailand; VN — Vietnam; JP — Japan; ROM — Romania; SKR — South Korea; ARG — Argentina)

The results of phylogenetic analysis that had been performed on the basis of genomic DNA sequences of PCV-1 confirm the possibility of existing, at least, of three genetic groups of the virus due to the existence of three clusters at the linearized phylogenetic tree (Fig. 7).

The results of the topology analysis of the phylogenetic tree that based on the sequences of different circovirus genes has showed the importance of phylogenetic analysis for genotyping and molecular marking of microorganisms once again.

The early diagnosis of viral porcine diseases is necessary for the effective development of pig farming

that is the one of the promising sectors of animal farming in Ukraine at this time. In time, detection of suspected ill animals is an important and necessary step because it affects the efficiency of livestock treatment. The polymerase chain reaction (PCR) in real-time format is one of the reliable and rapid methods for detecting of infectious agents in animals at any stage of the disease, including the early stages. Our results concern the definition of conservative and variable regions in the PCV-2 genome. This fact became the basis for oligonucleotides determining of potential primers for molecular genetic detection of the pathogen.

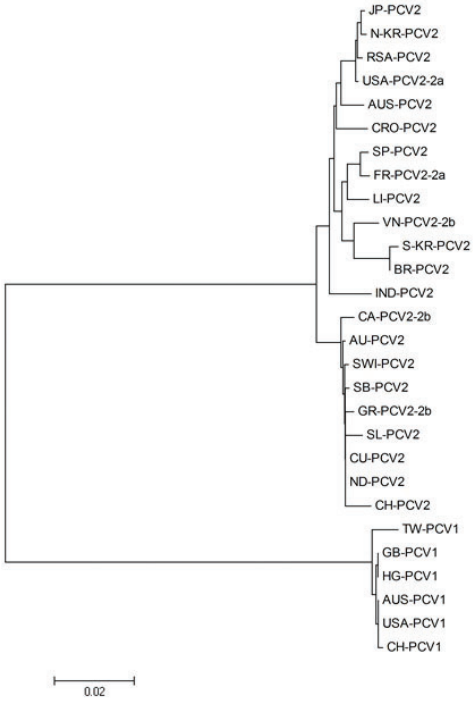

Figure 6. Phylogenetic tree that bases on gene sequences of PCV-1 and PCV-2 DNA isolates that circulate in different countries (USA — United States; CH — China; GB — United Kingdom; AUS — Australia; TW — Taiwan; HG — Hungary)

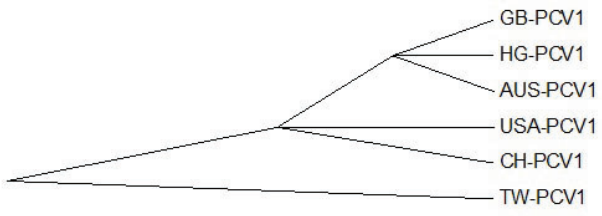

Figure 7. Phylogenetic tree that bases on gene sequences of PCV-1 DNA isolates that circulate in different countries (USA — United States; CH — China; GB — United Kingdom; AUS — Australia; TW — Taiwan; HG — Hungary)

Analysis of the sequence of selected primers for detection of PCV-2 by conventional parameters for quality check (Gerilovych, 2009; Sachse, 2003) has demonstrated their compliance with the applicable requirements and 100% complementarity to the exactly PCV-2 DNA template (Fig. 8).

RT-PCR results of protocol testing established that the optimum temperature for primers annealing for PCV-2 genomic DNA detection is 58 °C, and the threshold cycle equals 25 (Fig. 9, Table 1), that is the useful indicator in the application of this technique (Rebrikov et al., 2009).

For additional monitoring of derivation of specific DNA segments, electrophoretic analysis of the reaction products was conducted. It confirmed the amplification of the specific fragments of PCV-2 genomic DNA that is 97 bp in length (Fig. 10).

Table 1 — The results of real-time PCR analysis of material samples for the presence of genomic DNA specific fragment of PCV-2

Number of sample wells	Identify of test-tube	Threshold cycle Cp, Fam	Result
A1	Sample 1 (TEST)	25,8	+
A2	Зразок 2 (TEST)	25,9	+
A3	Sample 3 (TEST)	26,0	+
A4	Sample 4 (TEST)	25,2	+
A5	Sample 5 (TEST)	25,3	+
A6	Sample 6 (TEST)	25,9	+
A7	Sample 7 (TEST)	25,2	+
A8	Sample 8 (TEST)	27,0	+
B1	Sample 9 (TEST)	27,3	+
B2	Sample 10 (TEST)	26,0	+
B3	K + (TEST)	25,2	+
B4	K - (TEST)		-

Remark: «+» means that sample is positive and contains genetic material of PCV-2

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity
Forward primer	ATTACCAGCGCACTTCGG	18	57.80	55.56	4.00
Reverse primer	GGGTCCGCTTCTTCCATT	18	56.93	55.56	3.00

Products on target templates

>LC004734.1 Porcine circovirus-2 genes for capsid protein, replication protein, complete cds, isolate: AR-3

```
product length = 86
Forward primer  1    ATTACCAGCGCACTTCGG  18
Template        769  ..................  786

Reverse primer  1    GGGTCCGCTTCTTCCATT  18
Template        854  ..................  837
```

>KJ956690.1 Porcine circovirus-2 strain HX1, complete genome

```
product length = 86
Forward primer  1    ATTACCAGCGCACTTCGG  18
Template        493  ..................  476

Reverse primer  1    GGGTCCGCTTCTTCCATT  18
Template        408  ..................  425
```

>KJ956689.1 Porcine circovirus-2 strain BH6, complete genome

```
product length = 86
Forward primer  1    ATTACCAGCGCACTTCGG  18
Template        493  ..................  476

Reverse primer  1    GGGTCCGCTTCTTCCATT  18
Template        408  ..................  425
```

Figure 8. The results the primer system specificity verification using BLAST algorithm

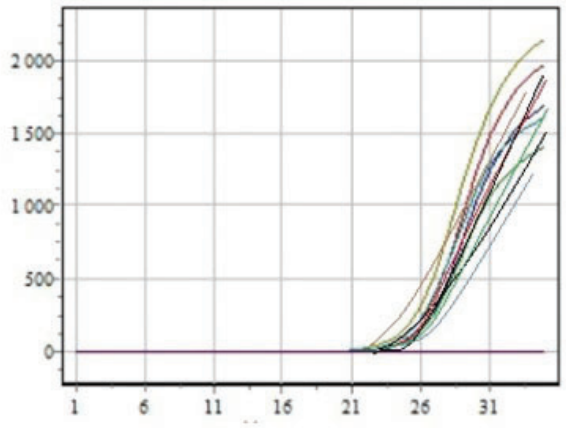

Figure 9. The amplification curves of specific DNA fragment of PCV-2 obtained with the use of SYBR Green intercalating dye

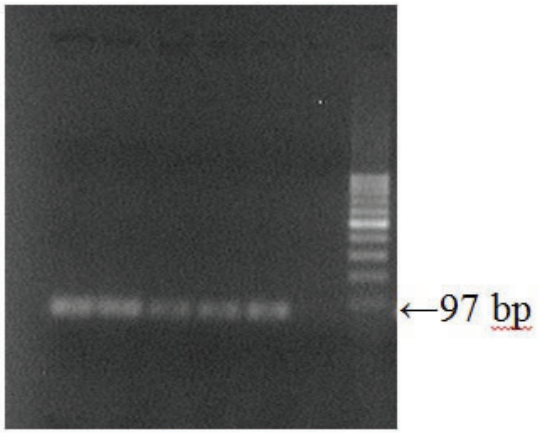

Figure 10. Electrophoretic analysis of the PCR-products obtained by amplification of the specific DNA fragment of PCV-2 with use of SYBR Green fluorescent dye

Conclusions. Thus, it was found that the most conserved gene of PCV-2 is *rep*, which encodes the replicase; and the most variable gene is *cap* that encodes a protein capsid of virus shell. The possibility of the existence of circovirus with 2c genotype for PCV-2 and the existence of at least three genetic groups of porcine circovirus type I were proved. The possibility of differentiation of porcine circoviruses type I and type II was shown due to providing of phylogenetic analysis that based on sequences of viral DNA. The method of PCV-2 indication by real-time PCR was developed.

References

Cano-Manuel, F. J., López-Olvera, J., Fandos, P., Soriguer, R. C., Pérez, J. M. and Granados, J. E. (2014) 'Long-term monitoring of 10 selected pathogens in wild boar (*Sus scrofa*) in Sierra Nevada National Park, Southern Spain', *Veterinary Microbiology*, 174(1–2), pp. 148–154. doi: 10.1016/j.vetmic.2014.06.017.

Chae, C. (2005) 'A review of porcine circovirus 2-associated syndromes and diseases', *The Veterinary Journal*, 169(3), pp. 326–336. doi: 10.1016/j.tvjl.2004.01.012.

Clark, E. G. and Harding, J. C. (1997) Circovirus — an emerging swine pathogen. In: *ISU Swine Disease Conference Proceedings.* October, 1997, pp. 63–64.

Gerilovych, A. P. (2009) 'Methodology of calculation and theoretic study of quality of oligonucleotides for nucleic acid detection of animal pathogens on base of polymerase chain reaction' [Metodolohiia rozrakhunku ta teoretychnoi perevirky yakosti olihonukleotydiv dlia vyiavlennia nukleinovykh kyslot patoheniv tvaryn na osnovi polimeraznoi lantsiuhovoi reaktsii], Veterinary Biotechnology [Veterynarna biotekhnolohiia], 14, pp. 56–69. Available at: http://www.nbuv.gov.ua/old_jrn/Chem_Biol/VBtl/texts/2009-14/statti/09GAPPCR.pdf. [in Ukrainian].

Kudin, K. V. and Prokulevich, V. A. (2011) 'The cloning of the protein capsid gene of the Belarusian porcine circovirus type 2 strain' [Klonirovanie gena belka kapsida belorusskogo shtamma tsirkovirusa sviney 2 tipa], *Vestnik BSU. Series 2: Chemistry. Biology. Geography [Vestnik BGU. Seriya 2. Khimiya. Biologiya. Geografiya]*, 2, pp. 37–41. Available at: http://www.elib.bsu.by/bitstream/123456789/13852/1/37-41.pdf. [in Russian].

Nawagitgul, P., Harms, P. A., Sorden, S. D., Morozov, I., Paul, P. S. and Bolin, S. R. (2000) 'Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein', *Journal of General Virology*, 81(9), pp. 2281–2287. doi: 10.1099/0022-1317-81-9-2281.

Olvera, A., Cortey, M. and Segalés, J. (2007) 'Molecular evolution of porcine circovirus type 2 genomes: Phylogeny and clonality', *Virology*, 357(2), pp. 175–185. doi: 10.1016/j.virol.2006.07.047.

Opriessnig, T., Meng, X.-J. and Halbur, P. G. (2007) 'Porcine Circovirus type 2-associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies', *Journal of Veterinary Diagnostic Investigation*, 19(6), pp. 591–615. doi: 10.1177/104063870701900601.

Rebrikov, D. V., Samatov, G. A., Trofimov, D. Yu., Semenov, P. A., Savilova, A. M., Kofiadi, I. A. and Abramov, D. D. (2009) Real-time PCR [PTSR 'v real'nom vremeni']. 2nd ed. Moscow: BINOM. Laboratoriya znaniy. ISBN 9785996300860. [in Russian].

Sachse, K. (2003) 'Specificity and performance of diagnostic PCR assays', Methods of Molecular Biology, 216, pp. 3–29. doi: 10.1385/1-59259-344-5:03.

Saitou, N. and Nei, M. (1987) 'The neighbor-joining method: A new method for reconstructing phylogenetic trees', *Molecular Biology and Evolution*, 4(4), pp. 406–425. Available at: http://mbe.oxfordjournals.org/content/4/4/406.full.pdf.

Segalés, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grassland, B., Baekbo, P. and Allan, G. (2008) 'PCV-2 genotype definition and nomenclature', *Veterinary Record*, 162(26), pp. 867–868. doi: 10.1136/vr.162.26.867.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) 'MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0', *Molecular Biology and Evolution*, 24(8), pp. 1596–1599. doi: 10.1093/molbev/msm092.

Tischer, I., Rasch, R. and Tochtermann, G. (1974) 'Characterization of papovavirus-and picornavirus-like particles in permanent pig kidney cell lines', *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Erste Abteilung Originale. Reihe A: Medizinische Mikrobiologie und Parasitologie.*, 226(2), pp. 153–67.

Wiederkehr, D. D., Sydler, T., Buergi, E., Haessig, M., Zimmermann, D., Pospischil, A., Brugnera, E. and Sidler, X. (2009) 'A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland', *Veterinary Microbiology*, 136(1–2), pp. 27–35. doi: 10.1016/j.vetmic.2008.10.028.

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GENETICS OF RESISTANCE TO CLINICAL MASTITIS IN COWS: A REVIEW

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Summary. The article provides an overview of data about genetics of cattle and susceptibility to infectious diseases by the example of clinical mastitis. The problems of the genetic markers associated with mastitis susceptibility of cattle in studies of scientists from different countries are analyzed.

Clinical mastitis (CM) is an inflammation of the mammary gland associated with elevated somatic cell count (SCC) and is one of the biggest problems affecting commercial milk production. In the world literature the problems of mastitis etiology were discussed and it was noted that many factors promote the development of mastitis, including the genetic background and feeding environment. The unfavorable genetic correlations between milk production and clinical mastitis are well known. The complexity of mastitis infection suggests a polygenic and multi-factorial immune response comprising many different proteins. Most studies focused on polygenic variation of the trait and genetic correlation among phenotypic traits related to mastitis such as somatic cell counts and clinical cases.

At the present time, the role of Major Histocompatibility Complex (MHC genes) in the susceptibility or resistance to intrammamary infection is well studied. Researchers have shown that investigation of genetic markers associated with mastitis susceptibility, provide initial evidence for a phenotypic association between a single nucleotide polymorphisms of CXCR2, CD18, MBL1, TLR1, TLR2, CARD15, HMGB1, ATP1A1, BoLADQA1 genes and somatic cell counts and clinical manifestations in dairy cows, as well as potential insight into specific mechanisms affected in cows more susceptible to mastitis.

According researchers currently identified differences between breeds. Dairy breeds originating from eastern France (Montbéliarde, Abondance) or central Europe (Simmental, Brown Swiss) have lower SCC and clinical mastitis frequency than Holstein. Within breed, genetic variability is also quite large. Because the disease susceptibility may be genetically dependent, in which case disease resistance could be improved by animal selection through breeding programmes.

The most economical method of reducing SCC in Ukraine, based on the experience of other countries, including in our opinion, is testing monthly milk samples from each cow after the selection of genes for mastitis resistance.

Keywords: clinical mastitis, somatic cell count, genetic markers, mastitis susceptibility, mastitis resistance, dairy breeds

Clinical mastitis (CM) is an inflammation of the mammary gland associated with elevated somatic cell count (SCC) caused by microorganisms and is one of the most frequent infectious diseases in dairy cattle with important economic losses (Heringstad et al., 2006). At the present time, mastitis is one of the biggest problems affecting commercial milk production (Hinrichs et al., 2011).

In accordance with the data presented by Looper (2012), in the United States, federal law allows milk to be sold only if the bulk tank has an SCC of fewer than 750,000 cells/cm^3. The primary reason for dairy producers to reduce SCC is because it relates to milk losses due to mastitis. The author notes that lowering somatic cell count from 600,000 to 300,000 increases milk sales by $50 per cow per year, which is a small fraction of the total benefit. If they can reduce their SCC from 600,000 to 200,000 cells/cm^3, they can decrease milk production losses by 600 pounds per cow per year. In a 100-cow herd, these losses amount to $7,500/year if milk is valued at $12.50/cwt.

The requirements in Norway and England determine 150 cells/cm^3 of SCC as the upper limit, in Denmark — 200 cells/cm^3, in most European countries — 400 cells/cm^3 (Kantsevich, Rusko and Baksheiev, 2014).

Ukrainian companies in the dairy industry must meet the high requirements for the European market. Ukraine produces more milk than eastern European countries — Romania, Czech Republic, Hungary, Bulgaria, but the proportion of raw milk that goes to industrial processing, less 41–46% in the Ukraine compare to Poland, the Czech Republic and Hungary (74–87%). The reason is the low quality of raw milk produced in the households of the population in Ukraine (> 75% of raw milk in Ukraine).

In 2011–2015 in the eastern Ukrainian farms clinical serous form of mastitis had detected in 18.3% of the cows, subclinical form — at 81.7%. In 35.7% of clinically healthy cows, SSC in milk samples is higher than 400,000 cells/ml (DSTU 3662–97) and is within 404,000–7,178,000 cells/cm^3 (Levchenko,

2015; DSU, 1997). The most numerous breed in this region is a Ukrainian Dairy Black and White breed, created on the basis of Holstein (Ruban and Fedota, 2013).

Over the last 10 years the study of mastitis and prevention are held in many countries for cattle of different breeds, for example, in Spanish Holstein (Pérez-Cabal et al., 2009) and Czech Holstein cows (Wolf, Wolfová and Štípková, 2010), in Austrian Fleckvieh cows (Koeck et al., 2010), in Dutch Holstein (Bloemhof, Dejong and Dehaas, 2009) and Norwegian Red cattle (Opsal et al., 2008), in US Holsteins (Vazquez et al., 2009) and Holstein cattle from Argentina (Baltian et al., 2012), in Holstein × Zebu (Duangjinda et al., 2008).

It was currently identified differences between breeds. According to Rupp and Boichard (2003), these genetic differences could be estimated in herds with different breeds. Dairy breeds originating from eastern France (Montbéliarde, Abondance) or central Europe (Simmental, Brown Swiss) have lower SCC and clinical mastitis frequency than Holstein. Genetic variability is also quite large within a breed. The genetic standard deviation of clinical mastitis frequency reaches about 5%. This means that in an environment with 20% average frequency of clinical mastitis, the frequencies observed for extreme genotypes range from 10% to 30%.

The authors note that many factors promote the development of mastitis, including the genetic background and feeding environment (Takeshima et al., 2008). It is known (Park et al., 2004) that such factors as environment, pathogen, and host affect susceptibility or resistance of an individual cow to bovine mastitis. According to Sæbø and Frigessi (2004), the pathogens causing mastitis are various species of bacteria, but a cow's susceptibility to the disease also depends on many other factors. It is also important to maintain the animal welfare such as sanitation, climate change and the value of the stock, among other things, as an environmental factor.

Due to the fact that the disease susceptibility may be genetically dependent, it could be improved by animal selection through breeding programs (Sæbø and Frigessi, 2004).

Breeding for mastitis resistance is becoming increasingly important because of its effect on farm economy and animal welfare. At the present time, the unfavorable genetic correlations between milk production and clinical mastitis (CM) are well known (Heringstad, Klemetsdal and Ruane, 2000; Oltenacu and Broom, 2010). Most of the countries that perform genetic evaluations for mastitis resistance lack records of CM because disease recording systems are not well developed. Thus, most commonly SCC is used as an indirect measure (Koeck et al., 2010). Wherein mastitis data recording should be carried out in early lactation because these mastitis cases are more related to the genetics of the cow than cases in late lactation, which are more affected by the environment (Hinrichs et al., 2011).

According to Baltian et al. (2012), mastitis is a complex disease that involves three major environment factors: the microorganisms as the causative agent, the cow as host, and the environment which can influence both the cow and the microorganisms. Among the major microorganisms responsible for the development of mastitis can be mentioned *Streptococcus agalactiae*, *Staphylococcus aureus* and *Streptococcus dysgalactiae* (Baltian et al., 2012). According to Ukrainian authors (Levchenko, 2015), cows with clinical and subclinical mastitis serous isolated culture *Staphylococcus aureus* (60% and 33%) *Streptococcus agalactiae* (26.6% and 20.1%), *Escherichia coli* (13.4% and 13.3%). These bacteria produce toxins that injure the milk-secreting tissues and various ducts throughout the mammary gland (Takeshima et al., 2008).

Because high SCC in milk is a response to a presence of microbes in the mammary gland, SCC can be used both as an indicator of mastitis and as a measure of response to infection. In many countries, SCC (treated homogeneously) is used as an indirect selection criterion for improving mastitis resistance and as characteristic of genotypes for the genes of resistance to mastitis (Detilleux, 2009; Heringstad et al., 2006).

Resistance to mastitis is a complex function involving various biological pathways, molecules and cells. Therefore numerous functional candidate genes could be involved in the determinism of the function as reported by Detilleux (2009). Genetic selection for increasing antibody responsiveness seems to be possible, but it should be pointed out that trends in association with clinical mastitis occurrence were not straight forward and those further investigations are needed to address genetics of immunology in mastitis (Rupp and Boichard, 2003).

Several chromosomal regions have large effects on mastitis resistance. The multitude of results reflects, to some extent, the fact that mastitis resistance is a complex function that involves many molecules and pathways that can be regulated by many different genes (Rupp and Boichard, 2003).

The complexity of mastitis infection suggests a polygenic and multifactorial immune response comprising many different proteins (Russel et al., 2012). Effective elimination of bacterial infections, such as mastitis in dairy cattle, requires four basic steps: bacterial recognition, inflammatory mediator release, leukocyte

recruitment from the bloodstream, and bacteria removal (Riollet, Rainard and Poutrel, 2000). Researchers have shown (Rambeaud and Pighetti, 2005) that investigation of genetic markers associated with mastitis susceptibility, provide initial evidence for a phenotypic association between a single nucleotide polymorphism of CXCR2 gene and neutrophil function in dairy cows, as well as potential insight into specific mechanisms affected in cows more susceptible to mastitis. Cows with the CC or GC genotype at CXCR2 +777 showed significantly lower neutrophil migration to recombinant human interleukin-8 (rhIL-8) than cows with the GG genotype. Cows with the CC genotype at CXCR2 +777 also showed decreased neutrophil migrations to zymosan-activated serum compared to these same cows. According to the authors, polymorphisms in bovine CXCR2 may potentially be used to select cows that are more resistant to disease. Of the sixteen polymorphisms in seven immune genes genotyped, presented by Beecher et al. (2010) just CXCR1–777 tended to associate with SCS, albeit only in the on-farm study (Beecher et al., 2010).

Several authors (Rupp and Boichard, 2003) focused on a mutation in the CD18 gene (BTA1) associated with bovine leukocyte adhesion deficiency (BLAD) in Holstein cattle. Cattle that are homozygous for the deleterious allele exhibit impaired diapedesis of leucocytes, extreme sensitivity in any infection and premature death. However, no difference in susceptibility to intramammary infections has been found in heterozygous carriers at the CD18 gene.

Wang et al. (2011) described three novel single-nucleotide polymorphisms of MBL1 gene in Chinese native cattle and their associations with milk performance traits**.** Among the Chinese Holstein cattle, eight different haplotypes and 19 genotype combinations were detected. Statistical analyses revealed no correlation between either g.855 G>A or g.2686 T>C and somatic cell score (SCS) however, a significant association was found between g.2651 G>A and SCS, suggesting a possible role of this SNP in the host response against mastitis. The combined genotypes of GGC/AAC with the lowest SCS, AAT/AAT with the highest protein content and AGC/AGC with the highest 305-days milk yield were favorable combinations for mastitis resistance and milk production traits. According to researchers, GGC/AAC, AAT/AAT and AGC/AGC can be used as possible candidates for marker-assisted selection in the dairy cattle breeding program.

Toll-like receptors (TLR) are important cell-surface molecules mediating immune response (Opsal et al., 2008). Investigations of whether SNPs within the bovine TLR1 gene (bo*TLR1*) are associated with clinical mastitis (CM) were presented by Russell et al. (2012). Selected bo*TLR1* SNPs were analyzed within a Holstein Friesian herd. Significant associations were found for the tagging SNP –79 T>G and the 3'UTR SNP +2463 C>T. Animals with the GG genotype (from the tag SNP -79 T>G) had significantly lower bo*TLR1* expression in milk somatic cells when compared with TT or TG animals. In addition, stimulation of leucocytes from GG animals with the TLR1-ligand Pam3 csk4 resulted in significantly lower levels of CXCL8 mRNA and protein. The authors concluded, that rapid immune response, conferred by the favorable bo*TLR1* SNP -79 TT variant, could reduce detrimental clinical manifestations of diseases via an efficient yet controlled influx of inflammatory cells to neutralise pathogens and control infection.

The results of association research of toll-like receptor 2 gene (TLR2) polymorphisms with somatic cell score in Xinjiang Brown cattle showed (Bai et al., 2012) that the SCS of AB genotype was lower than AA, the SCS of cattle with Hap5 was lower than Hap3. This suggests that Hap5 might play an important role in sub-mastitis resistance in Xinjiang Brown cattle.

Results of the study associations between CARD15 and TLR2 gene polymorphisms and milk somatic cell score in Canadian Holsteins were introduced (Pant et al., 2008). According to the authors, Toll-like receptor-2 (TLR2) and caspase recruitment domain 15 (CARD15) are important pattern recognition receptors that play a role in the initiation of the inflammatory and subsequent immune response. The hap21 (TA) in CARD15 was significantly associated with increased SCS EBV (estimated breeding values) and SNP c.3020 A>T was associated with SCS EBVs and might play a role in the host response against mastitis. In a study of Opsal et al. (2008) presented, that dense linkage maps comprising single nucleotide polymorphisms (SNPs) have been constructed for the chromosomal regions harboring TLR2 and TLR4 on bovine chromosome 17 and 8.

The most likely marker orders for both regions were compared with the corresponding human map positions and used to reorder bovine scaffolds available from the bovine genome sequence assembly (Heringstad et al., 2006). Haplotype analysis of TLR4 gene and its effects on milk somatic cell score in Chinese commercial cattle had been carried out in Chinese commercial dairy cattle including Chinese Holstein, Sanhe cattle and Chinese Simmental breeds by Wang et al. (2014). Results showed that Hap1 (30.5%) and Hap2 (30.4%) were the most common haplotypes. Hap2, Hap4 and Hap12 might negatively effect on milk SCS whereas Hap13 might positively effect on milk SCS.

Li et al. (2012) mentioned that one SNP in the 3'-UTR of HMGB1 gene affects the binding of target bta-miR-223 and is involved in mastitis in dairy cattle. According to the authors, high-mobility group box protein 1 (HMGB1) gene has a universal sentinel

function for nucleic-acid-mediated innate immune responses and acts as a pathogenic mediator in the inflammatory disease. They showed that the relative expression of HMGB1 mRNA in cows with the genotype GG is significantly higher than those in cows with the genotype AA. One novel SNP (g. +2776 A>G) in the HMGB1 3'-UTR, altering the binding of HMGB1 and bta-miR-223, was found to be associated with somatic count scores in cows. The g. +2776 A>G-GG was an advantageous genotype which can be used as a candidate functional marker for mastitis resistance breeding program.

Due to the fact that mastitis affects the concentrations of potassium and sodium in milk, presumed that polymorphism of the *ATP1 A1* gene, which encodes the bovine Na_+, K_+-ATPase α1 subunit could be associated with mastitis (Liu et al., 2012). The authors showed that the cows with genotype *CC* in *ATP1 A1* had significantly lower somatic cell scores and 305-day milk yields than those with genotype *CA*. The Na_+, K_+-ATPase activity was significantly higher in dairy cows with genotype *CC* compared to the other two genotypes, and the Na_+, K_+-ATPase activity of the resistant group was significantly higher than that of the susceptible group in dairy cows. In this regard, researchers conclude that this polymorphism has potential as a marker for mastitis resistance in dairy cattle.

Sugimoto, Uchiza and Kuniyuki (2013) have reported that the forebrain embryonic zinc finger-like (FEZL) gene promotes immune responses that are associated with mastitis resistance. According to Takeshima et al. (2008), it is preferable to genotype all known candidate genes simultaneously for estimating the susceptibility or resistance of animals to multifactorial diseases like mastitis. However, it remains difficult to genotype MHC alleles, although most candidate genes, once identified, can be readily genotyped using a method for detecting single nucleotide polymorphisms.

It is now known, that several host genes have been suggested to promote resistance or susceptibility to clinical mastitis, particularly major histocompatibility complex (MHC) genes (Takeshima et al., 2008).

According to Baltian et al. (2012), in bovine MHC (named bovine lymphocyte antigen, BoLA) some polymorphisms were associated with resistance to infectious diseases such as mastitis, enzootic bovine leukemia and others diseases. The general structure of BoLA is similar to the MHC of others mammalian species and is made up of three regions: Class I, Class II and Class III, which fulfill different roles. The class II genes encode proteins that presented processed peptides derived from extracellular antigens to CD4 cells, like DRA, DRB, DQA and DQB. In the bovine DR subregion, there are at least three BoLA-DRB loci but only the BoLA-DBR3 gene in functional. This gene is the highly polymorphic since more than 103 alleles have been reported thus far.

Many reports have documented the association of BoLADRB3 alleles with the progression of mastitis. They have also detected disease-associated BoLADRB3 alleles. These results showed that while the existence of particular BoLADRB3 alleles is important for the progression of mastitis (Baltian et al., 2012; Duangjinda et al., 2008; Sharif, Mallard and Sargeant, 2000; Takeshima et al., 2008). In the works of Takeshima et al. (2008) it was shown that heterozygosity of the BoLADQA1 gene is associated with resistance to mastitis progression.

Park et al. (2004) have presented that susceptibility to mastitis was associated with major histocompatibility complex (MHC) haplotypes that have only a single set of DQ genes. The study also revealed that susceptible cows had CD4:CD8 ratios of less than one in both their mammary gland secretions and peripheral blood. These results raise the possibility that the number of DQ genes that a cow has and/or a cow's CD4:CD8 ratio could be used as indicators of susceptibility to bovine mastitis.

According to Takeshima et al. (2008), the MHC genes are a classic example of where heterozygote advantage may apply because heterozygosity at MHC loci may enhance resistance to infectious diseases as it increases the diversity of the antigens presented to T cells, thereby generating a more diverse T-cell repertoire. Such heterozygote-favoring selection is one of the mechanisms that maintain genetic polymorphism.

Potential relationships between amino acid motifs in the antigen binding groove of various alleles of the bovine major histocompatibility complex DR (BoLA-DR) molecule and occurrence of clinical mastitis caused by *Staphylococcus* species (non-*Staphylococcus aureus*) were presented by Sharif, Mallard and Sargeant (2000). A significant association was detected between the presence of glutamic acid at position beta 74 in BoLA-DRB3.2*22, *23 and *24 alleles and occurrence of mastitis caused by *Staphylococcus* spp. with a relative risk of 11. These positions (beta 13, beta 71 and beta 74) form pocket 4 of the antigen binding groove, which plays an instrumental role in antigen binding and recognition by T-lymphocytes. The authors concluded, pocket 4 of the BoLA-DR molecule is involved in conferring susceptibility to clinical mastitis caused by *Staphylococcus* spp.

The association of the polymorphism of bovine leukocyte antigen (BoLA-DRB3) genes with resistance and susceptibility to mastitis caused by *Streptococci*, coagulase-negative *Staphylococci*, *Escherichia coli* and *Staphylococcus aureus* was investigated by Yoshida et al. (2012). The researchers concluded that in the case

of *Escherichia coli* mastitis, amino acid substitutions at the 9, 11, 13, and 30 positions had little effect, but rather substitutions at the 47, 67 positions of pocket 7, and at the 71, 74 positions of pocket 4, Tyr (47), Ile (67), Ala (71), and Ala (74), were associated with resistance and this motif was present in DRB3*1201. In 2012 the authors provided data (33), that DRB3.2*8 (DRB3*1201) and DRB3.2*16(DRB3*1501) alleles were found to be associated with susceptibility, while DRB3.2*22(DRB3*1101), DRB3.2*23(DRB3*2703), and DRB3.2*24(DRB3*0101) alleles were found to be associated with resistance.

According to de Haas et al. (2008), genetic selection on lowering the log-transformed lactation-average SCC may, therefore, be more effective in decreasing mastitis incidence than genetic selection on lowering the lactation-average of log-transformed SCC.

Looper (2012) suggests that there are several methods of reducing SCC. The first method, culling cows, is a short-term solution, which can quickly reduce SCC in the bulk tank. The second method, controlling mastitis, is a long-term solution, which should be the basis of a sound management program. The most economical method, including in our opinion, and in Ukraine, to determine SCC is testing monthly milk samples from each cow after the selection of genes for mastitis resistance.

It needs to provide further in-depth studies among Ukrainian herds with genetical analysis. We believe that due the increase in the consumption of dairy products in the world and low current exports from Ukraine. It will give an opportunity to significantly increase participation in world trade and milk producers should improve the quality of dairy products.

References

Bai, J., Lin, J., Li, W. and Liu, M. (2012) 'Association of toll-like receptor 2 polymorphisms with somatic cell score in Xinjiang brown cattle', *Animal Science Journal*, 83(1), pp. 23–30. doi: 10.1111/j.1740-0929.2011.00909.x.

Baltian, L. R.,Ripoli, M. V.,Sanfilippo, S.,Takeshima, S. N., Aida, Y., Giovambattista, G., Sanfilippo, S., Aida, Y. and Giovambattista, G. (2012) 'Association between BoLA-DRB3 and somatic cell count in Holstein cattle from Argentina', *Molecular Biology Reports*, 39(7), pp. 7215–7220. doi: 10.1007/s11033-012-1526-y.

Beecher, C., Daly, M., Childs, S., Berry, D. P., Magee, D. A., McCarthy, T. V. and Giblin, L. (2010) 'Polymorphisms in bovine immune genes and their associations with somatic cell count and milk production in dairy cattle', [Online] *BMC Genetics*, 11(99), p. 1–9. doi: 10.1186/1471-2156-11-99.

Bloemhof, S., Dejong, G. and Dehaas, Y. (2009) 'Genetic parameters for clinical mastitis in the first three lactations of Dutch Holstein cattle', *Veterinary Microbiology*, 134(1–2), pp. 165–171. doi: 10.1016/j.vetmic.2008.09.024.

DSU [State Committee of Ukraine for Standardization, Metrology and Certification] (1997) *DSTU 3662–97. Whole cow milk. Requirements for purchasing [Moloko koroviache nezbyrane. Vymohy pry zakupivli]*. Kyiv: Derzhstandart Ukrainy. [in Ukrainian].

Detilleux, J. (2009) 'Genetic factors affecting susceptibility to udder pathogens', *Veterinary Microbiology*, 134(1–2), pp. 157–164. doi: 10.1016/j.vetmic.2008.09.023.

Duangjinda, M., Buayai, D., Pattarajinda, V., Phasuk, Y., Katawatin, S., Vongpralub, T. and Chaiyotvittayakul, A. (2008) 'Detection of bovine leukocyte antigen DRB3 alleles as candidate markers for clinical mastitis resistance in Holstein × Zebu', *Journal of Animal Science*, 87(2), pp. 469–476. doi: 10.2527/jas.2007-0789.

Haas, Y. de, Ouweltjes, W., Napel, J. ten, Windig, J. J. and de Jong, G. (2008) 'Alternative somatic cell count traits as mastitis indicators for genetic selection', *Journal of Dairy Science*, 91(6), pp. 2501–2511. doi: 10.3168/jds.2007-0459.

Heringstad, B., Gianola, D., Chang, Y. M., Ødegård, J. and Klemetsdal, G. (2006) 'Genetic associations between clinical mastitis and somatic cell score in early first-lactation cows', *Journal of Dairy Science*, 89(6), pp. 2236–2244. doi: 10.3168/jds.s0022-0302(06)72295-0.

Heringstad, B., Klemetsdal, G. and Ruane, J. (2000) 'Selection for mastitis resistance in dairy cattle: A review with focus on the situation in the Nordic countries', *Livestock Production Science*, 64(2–3), pp. 95–106. doi: 10.1016/s0301-6226(99)00128-1.

Hinrichs, D., Bennewitz, J., Stamer, E., Junge, W., Kalm, E. and Thaller, G. (2011) 'Genetic analysis of mastitis data with different models', *Journal of Dairy Science*, 94(1), pp. 471–478. doi: 10.3168/jds.2010-3374.

Kantsevich, S. I., Rusko, N. P. and Baksheiev, M. M. (2014) 'Estimation of milk quality impact on economic efficiency of milk production', *The Economy of Agro-Industrial Complex*, 4, pp. 24–27. Available at: http://eapk.org.ua/sites/default/files/translate/1404kantsevich_rusko_baksheiev.pdf.

Koeck, A., Heringstad, B., Egger-Danner, C., Fuerst, C., Winter, P. and Fuerst-Waltl, B. (2010) 'Genetic analysis of clinical mastitis and somatic cell count traits in Austrian Fleckvieh cows', *Journal of Dairy Science*, 93(12), pp. 5987–5995. doi: 10.3168/jds.2010-3451.

Levchenko, A. G. (2015) *The characteristics of the manifestation of mastitis in cows in farms with various technologies and the development of a comprehensive preventive therapeutic measures [Osoblyvosti proiavu mastytu u koriv u hospodarstvakh z riznymy tekhnolohiiamy ta rozrobka kompleksnykh profilaktychno likuvalnykh zakhodiv]*. The thesis for the scientific degree of the candidate of veterinary sciences, specialty 16.00.03 — veterinary microbiology, epizootology, infectious diseases and immunology. Kyiv: State Scientific Control Institute of Biotechnology and Strains of Microorganisms. [in Ukrainian].

Li, L., Huang, J., Zhang, X., Ju, Z., Qi, C., Zhang, Y., Li, Q., Wang, C., Miao, W., Zhong, J., Hou, M. and Hang, S. (2012) 'One SNP in the 3′-UTR of HMGB1 gene affects the binding of target bta-miR-223 and is involved in mastitis in dairy cattle', *Immunogenetics*, 64(11), pp. 817–824. doi: 10.1007/s00251-012-0641-1.

Liu, Y. X., Xu, C. H., Gao, T. Y. and Sun, Y. (2012) 'Polymorphisms of the ATP1A1 gene associated with mastitis in dairy cattle', *Genetics and Molecular Research*, 11(1), pp. 651–660. doi: 10.4238/2012.march.16.3.

Looper, M. (2012) *Reducing somatic cell count in dairy cattle*. Division of Agriculture Research and Extension, University of Arkansas System, FSA4002. Available at: http://www.uaex.edu/publications/pdf/fsa-4002.pdf.

Oltenacu, P. A. and Broom, D. M. (2010) 'The impact of genetic selection for increased milk yield on the welfare of dairy cows', *Animal Welfare*, 19(S1), pp. 39–49. Available at: http://www.fao.org/fileadmin/user_upload/animalwelfare/dairy.pdf.

Opsal, M. A., Lien, S., Brenna-Hansen, S., Olsen, H. G. and Våge, D. I. (2008) 'Association analysis of the constructed linkage maps covering TLR2 and TLR4 with clinical mastitis in Norwegian red cattle', *Journal of Animal Breeding and Genetics*, 125(2), pp. 110–118. doi: 10.1111/j.1439-0388.2007.00704.x.

Pant, S. D., Schenkel, F. S., Leyva-Baca, I., Sharma, B. S. and Karrow, N. A. (2008) 'Identification of polymorphisms in bovine *TLR2* and *CARD15*, associations between *CARD15* polymorphisms and milk somatic cell score in Canadian Holsteins, and functional relevance of SNP c.3020A>T', *Developments in biologicals (Basel)*, 132, pp. 247–253. doi: 10.1159/000317167.

Park, Y. H, Joo, Y. S., Park. J. Y., Moon, J. S., Kim, S. H., Kwon, N. H., Ahn, J. S., Davis, W. C. and Davies, C. J. (2004) 'Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis-resistant and susceptible cows', *Journal of Veterinary Science*, 5(1), pp. 29–39. Available at: http://www.vetsci.org/journal/download_pdf.php?spage=29&volume=5&number=1.

Pérez-Cabal, M. A., de los Campos, G., Vazquez, A. I., Gianola, D., Rosa, G. J. M., Weigel, K. A. and Alenda, R. (2009) 'Genetic evaluation of susceptibility to clinical mastitis in Spanish Holstein cows', *Journal of Dairy Science*, 92(7), pp. 3472–3480. doi: 10.3168/jds.2008-1978.

Rambeaud, M. and Pighetti, G. M. (2005) 'Impaired neutrophil migration associated with specific bovine CXCR2 Genotypes', *Infection and Immunity*, 73(8), pp. 4955–4959. doi: 10.1128/iai.73.8.4955-4959.2005.

Riollet, C., Rainard, P. and Poutrel, B. (2000) 'Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*', *Clinical and Vaccine Immunology*, 7(2), pp. 161–167. doi: 10.1128/cdli.7.2.161-167.2000.

Ruban, S. Yu. and Fedota, O. M. (2013) 'The directions of selection organization in the dairy and beef cattle breeding of Ukraine' [Napriamy orhanizatsii selektsiinoi roboty v molochnomu ta miasnomu skotarstvi Ukrainy], *Animal Breeding and Genetics [Rozvedennia i henetyka tvaryn]*, 47, pp. 5–13. Available at: http://nbuv.gov.ua/UJRN/rgt_2013_47_3. [in Ukrainian].

Rupp, R. and Boichard, D. (2003) 'Genetics of resistance to mastitis in dairy cattle', *Veterinary Research*, 34(5), pp. 671–688. doi: 10.1051/vetres:2003020.

Russell, C. D., Widdison, S., Leigh, J. A. and Coffey, T. J. (2012) 'Identification of single nucleotide polymorphisms in the bovine toll-like receptor 1 gene and association with health traits in cattle', [Online] *Veterinary Research*, 43(17), pp. 1–12. doi: 10.1186/1297-9716-43-17.

Sæbø, S. and Frigessi, A. (2004) 'A genetic and spatial Bayesian analysis of mastitis resistance', *Genetics Selection Evolution*, 36(5), p. 527–542. doi: 10.1186/1297-9686-36-5-527.

Sharif, S., Mallard, B. A. and Sargeant, J. M. (2000) 'Presence of glutamine at position 74 of pocket 4 in the BoLA-DR antigen binding groove is associated with occurrence of clinical mastitis caused by *Staphylococcus* species', *Veterinary Immunology and Immunopathology*, 76(3–4), pp. 231–238. doi: 10.1016/s0165-2427(00)00216-6.

Sugimoto, M., Uchiza, M. and Kuniyuki, M. (2013) 'Effects of a Forebrain embryonic zinc finger-like p.Gly105(12_13) polymorphism on mastitis resistance: An embryo-transfer study', [Online] *Molecular Biology and Genetic Engineering*, 1(1), pp. 1–3. doi: 10.7243/2053-5767-1-1.

Takeshima, S., Matsumoto, Y., Chen, J., Yoshida, T., Mukoyama, H. and Aida, Y. (2008) 'Evidence for cattle major histocompatibility complex (BoLA) class II DQA1 gene heterozygote advantage against clinical mastitis caused by *Streptococci* and *Escherichia* species', *Tissue Antigens*, 72(6), pp. 525–531. doi: 10.1111/j.1399-0039.2008.01140.x.

Vazquez, A. I., Weigel, K. A., Gianola, D., Bates, D. M., Perez-Cabal, M. A., Rosa, G. J. M. and Chang, Y. M. (2009) 'Poisson versus threshold models for genetic analysis of clinical mastitis in US Holsteins', *Journal of Dairy Science*, 92(10), pp. 5239–5247. doi: 10.3168/jds.2009-2085.

Wang, C., Liu, M., Li, Q., Ju, Z., Huang, J., Li, J., Wang, H. and Zhong, J. (2011) 'Three novel single-nucleotide polymorphisms of MBL1 gene in Chinese native cattle and their associations with milk performance traits', *Veterinary Immunology and Immunopathology*, 139(2–4), pp. 229–236. doi: 10.1016/j.vetimm.2010.10.023.

Wang, X. P., Luoreng, Z. M., Gao, S. X., Guo, D. S., Li, J. Y., Gao, X., Xu, S. Z., Li, F., Chen, G. and Wang, J. R. (2014) 'Haplotype analysis of TLR4 gene and its effects on milk somatic cell score in Chinese commercial cattle', *Molecular Biology Reports*, 41(4), pp. 2345–2351. doi: 10.1007/s11033-014-3088-7.

Wolf, J., Wolfová, M. and Štípková, M. (2010) 'A model for the genetic evaluation of number of clinical mastitis cases per lactation in Czech Holstein cows', *Journal of Dairy Science*, 93(3), pp. 1193–1204. doi: 10.3168/jds.2009-2443.

Yoshida, T., Furuta, H., Kondo, Y. and Mukoyama, H. (2011) 'Association of BoLA-DRB3 alleles with mastitis resistance and susceptibility in Japanese Holstein cows', *Animal Science Journal*, 83(5), pp. 359–366. doi: 10.1111/j.1740-0929.2011.00972.x.

Part 2. Veterinary medicine

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CELLULAR AND HUMORAL MEDIATED IMMUNITY AND DISTRIBUTION OF VIRAL ANTIGENS IN CHICKENS AFTER INFECTION WITH A LOW PATHOGENIC AVIAN INFLUENZA VIRUS (LPAIV H4N6) ISOLATED FROM WILD DUCKS

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Summary. Four-week-old commercial chickens were intranasally inoculated with a H4N6 low pathogenic avian influenza virus (LPAIV) isolated from a garganey in Ukraine. The virus did not cause disease in chickens and no pathological changes were observed in inoculated birds. No virus was isolated from internal organs, but 3 chickens of 5 showed antibodies to influenza virus at intranasal infection. Cecum, spleen, lung, and trachea samples were collected from LPAIV-infected chickens from 1 to 14 days post inoculation (dpi) and examined by immunohistochemical techniques to determine the distribution of LPAIV and evaluate the host immune response using various immune markers. From 7 to 14 dpi, a sharp increase of the number of cells bearing CD4, IgM, IgG, and IgA was observed. In spleen, the number of CD4 T lymphocytes and macrophages were increased in immunohistochemical staining when compared to controls. In the lung, B lymphocytes expressing IgM (6.8±0.5%), IgG (9.4±1.3%), and IgA (8.6±0.1%) were detected in higher numbers than in controls. High levels of IFN-γ, IL-2, IL-15 were present on 7 dpi. We also found LPAIV nucleoprotein (NP) staining in the trachea observed on 10 dpi (2.7±0.4% of antibody-stained areas) as well as in the spleen on 5 dpi (3.3±0.2%). There was no NP antigen in other organs. In conclusion, although infection with a LPAIV did not cause obvious clinical disease, viral replication was seen in the trachea and spleen and both local and systemic cellular and humoral immune responses were elicited in these LPAIV-infected chickens.

Keywords: avian influenza, low pathogenic avian influenza virus, cellular immunity, humoral immunity, viral antigen, wild birds

Introduction. Avian influenza viruses (AIV) (family Orthomyxoviridae, genus *Influenza virus* A) are an important cause of economic losses in the poultry industry. Members of the order Anseriformes (i.e. ducks, swans, geese) and Charadriiformes (i.e. shorebirds, gulls, terns) are the principal reservoirs of influenza A viruses in nature and are often the focus of research and surveillance (Nemeth et al., 2010). LPAIVs produce subclinical infections in SPF chickens, but under commercial rearing conditions, these viruses can produce mild to moderate respiratory disease when there are secondary infections, environmental stress and/or immunosuppression (Costa-Hurtado et al., 2014).

When low pathogenic avian influenza viruses (LPAIVs) were first isolated from wild water birds in the 1970s, the absence of clinical signs in both naturally and experimentally infected chickens led to the conventional wisdom that LPAIV in wild water birds is avirulent, possibly owing to the adaptation of the virus to its host over many centuries (Kuiken, 2013). Neither clinical signs nor histopathological findings were observed in LPAIV-infected chickens. In addition, only a short-term viral shedding which was accompanied by seroconversion was detected in some LPAIV-infected chickens (Bertran et al., 2011).

This study was conducted to better understand the underlying host-pathogen interaction in infections with wild bird AIV in chickens. The dynamics of viral spread and local and systemic host immune responses in infected chickens were examined. The changes in cellular immune response triggered by LPAIV infection by measuring lymphocytes that express CD4, CD8, macrophages, IgM, IgG, and IgA have been

studied in chickens after infecting them with LPAIV subtype H4 isolated from wild ducks.

Reason to do this type of study is to understand infection of chickens with wild bird AIVsince it can help explain introduction or not of these viruses in poultry.

Material and methods. ***Virus and virological study.*** A low pathogenic avian influenza virus, A/Garganey/Chervonooskilske/4-11/2009 (H4N6), isolated from the cloacal swab of clinically healthy garganey in 2009 in Ukraine was used in this study. Virus was isolated and identified by the Department of Avian Diseases of National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (NSC 'IECVM'). Virus stocks were made from the first and second embryo passages of archived virus stocks which have been deposited in the collection of viral pathogens in the Department of Avian Diseases of NSC 'IECVM'. AI virus was propagated and titrated in 9-to-11-days-old embryonated specific-pathogen-free (SPF)-chicken eggs (Valo BioMedia GmbH, Germany) according to the recommendations of the OIE. Titer calculation was carried out by Reed and Mench (Dufour-Zavala, 2008). Pathogenicity of the virus was determined by intravenous pathogenicity index (IVPI) test in accordance with the methods recommended by the OIE (Dufour-Zavala, 2008; OIE, 2012).

Virus isolation from internal organ samples, cloacal and nazopharyngial swabs were performed using SPF CE as recommended by the OIE (Dufour-Zavala, 2008; OIE, 2012).

Experimental design. Forty-two 6-weeks-old chickens were obtained from Poultry Farm 'Frunze' of Southern Branch of the National University of Life and Environmental Sciences of Ukraine 'Crimean Agrarian-and-Technological University', and were used to study immunity after infection with the LPAIV. Chickens which were used for the main experiment were not SPF, but they did not have antibodies to the influenza virus both, and in AI, and ELISA. Two groups of experimental birds included: 1) Chickens experimentally inoculated with low pathogenic avian influenza virus A/Garganey/Chervonooskilske/4-11/2009 (H4N6) intranasally with a dose of $10^{6,0}$ EID_{50} per chicken, and 2) Control non-inoculated chickens. Water and feed were provided *ad libitum* and experiments were approved by the institution's animal care committee.

Inoculated chickens were monitored for 14 days after infection. On 1, 2, 4, 7, 10, and 14 days post infection birds were necropsied (3 birds per day in each group), cloacal and nasopharyngeal swabs, samples of trachea, lungs, spleen, small and large intestine tissues were collected. On these days, we collected blood samples from live birds for serological analysis.

Serological studies. Detection of antibodies to avian influenza virus subtype H4 was performed with chicken serum samples by HI test and ELISA. HI test was performed using A/Garganey/Chervonooskilske/4-11/2009 (H4N6) inactivated antigens (produced by NSC 'IECVM'). The hemagglutination (HA) assay and HI test were performed in V-bottom microtiter plates using standard protocol recommended by the OIE (Dufour-Zavala, 2008; Rath et al., 1995). Antibodies in blood samples were detected by a commercial AI ELISA kit (IDEXX).

Immunohistochemistry. Spleen, caeca, trachea and lung tissue samples collected on 1, 3, 5, 7, 10 and 14 days post AI infection were quickly frozen in the liquid nitrogen. Samples were placed in cryotubes and immersed in the Dewar vessel with liquid nitrogen. Frozen tissues were stored at — 196 °C.

Histological sections were made by cryostat microtome HM-525 MICROM (Germany) at the appropriate temperature. Sections were mounted on Star Frost glass microscope slides (Knittel Glaser), treated with L-polylysine to reduce the risk of tissue damage in subsequent stages of processing. Sections were dried at room temperature overnight, treated with a fixative liquid 10 minutes at –4 °C. They were hydrated in two portions of 0.1 M phosphate buffer saline pH 7.2 with Twin 20 for 5 min, sections were subsequently washed twice with buffer after each step of the immunohistochemical reaction. Cryostat sections of 7-μm thickness were cut and immunohistology was carried out using unlabelled primary mouse monoclonal antibodies detecting CD4 (thymocytes and T-helpers), CD8 (cytotoxic T-cells, natural killer cells, cortical thymocytes), KUL01 (monocytes and macrophages), IgM, IgG, IgA (chicken Ig or isolated lymphocytes), and FluA-NP 2C9 (recombinant influenza virus type A nucleoprotein) (Southern Biotechnology Associates, Eching, Germany) followed by a commercially available staining kit (LSAB, ChemMate Detection kit, peroxidase antiperoxidase, rabbit/mouse; DakoCytomation, Hamburg, Germany) according to the manufacturer's recommendations. Monoclonal antibodies to IFN-γ, IL-2, Il-15 (T-lymphocytes) were produced by Dr. Hyun S. Lillehoj (Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland, USA). As a negative control, slides were incubated with PBS instead of the monoclonal antibodies. Sections were counterstained with hematoxylin and mounted with Canadian balsam (Riedel de Haen AG, Seelze-Hannover, Germany).

Immunohistological tissue preparations were examined by light-microscopic image analysis ('VideoTest Morphology — 5.0'). At least 3 regions of interest of each tissue section and antibody were

scanned, the percentage of antibody-stained areas was determined, and the mean values were calculated (Berndt, Pieper and Methner, 2006).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software for Windows. All data for each group expressed as means ± SEM. The difference between the means considered at $p<0.05$.

Results. To study host immune response in chickens experimentally infected with a LPAIV, we used A/Garganey/Chervonooskilske/4-11/2009 virus (H4N6) isolated from cloacal swab of clinically healthy garganey in the Donetsk region of Ukraine in 2009. This virus was low pathogenic based on criteria of pathogenicity definition. Upon intravenous and intranasal infection with this virus, no clinical signs were observed in chickens and no pathological lesions were found at necropsy. Infection of poultry with this virus provoked an antibody response at 10 days after intranasal inoculation which ranged from 1:8 to 1:32 serum antibody titers. Only 2 of 5 chickens were positive by the HI test and 3 of 5 were positive by ELISA at intranasal inoculation. All 10 chickens were positive both and by HI test, and by ELISA. When chickens were given an intravenous infection with this virus, specific antibodies to influenza virus H4 were detected in titer ranges of 1:128 to 1:1024.

In immunohistological studies, the respiratory tract organs (lungs and trachea) showed higher level of humoral immunity (IgM, IgG, IgA-expressing cells) in the lung compared to the trachea. Also, indicators of cell-mediated immunity as measured by the CD4 and macrophage markers were higher in LPAIV-infected chickens in the lungs at 14 days post infection compared to uninfected control chickens AIV-infected chickens showed 1.5 times higher ($p<0.05$) CD4 cells than healthy chickens. The number of positive cells continued to increase until 14 dpi when it reached the maximum level as shown in Fig. 1.

Lymphocytes expressing CD8 were increased starting from 7 dpi. The chickens in the infected group showed 2 times higher levels of CD8 cells ($p<0.05$) compared to the non-infected control chickens. The time of CD8 cell increase coincided with the reduced CD4 cells which may suggest that CD8 cells play an important role in host defense against AI. Furthermore, CD8 cells declined as the number of CD4 cells increased perhaps indicating an important role of antibodies that follow cytotoxic activity of CD8 cells.

IgM cells increased in the lungs of AIV-infected chickens at 7 dpi. Level of IgM expressing cells in the lungs of AIV-infected chickens was 1.5 times ($p<0.05$) higher than in the non-infected control chickens. The level of IgM in AI-infected chickens on 3 dpi was almost 2 times higher than in the chickens of uninfected control group (4,3±0,5% vs 2,9±0,2%). From 10 dpi, less IgM cells were shown.

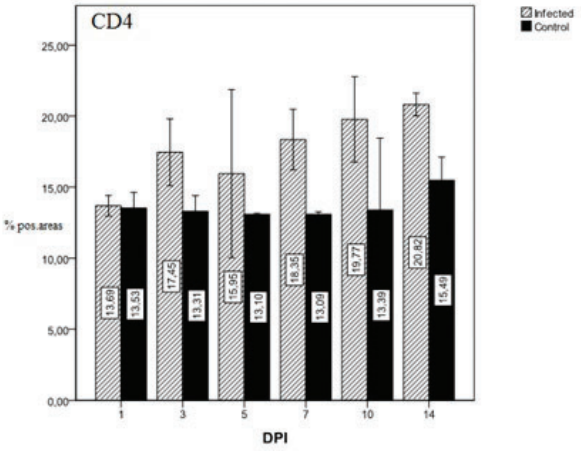

Figure 1. Dynamic response of CD4 T-cells subsets in the lung after infection with LPAIV. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 7 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

IgA cells were also seen in the lungs of infected chickens within 10 days post infection its level fluctuating until 10 dpi but reached at its maximum level at 14 dpi with AIV-infected chickens showed 2–3 times higher ($p<0.05$) IgA cells than uninfected control chickens (Fig. 2).

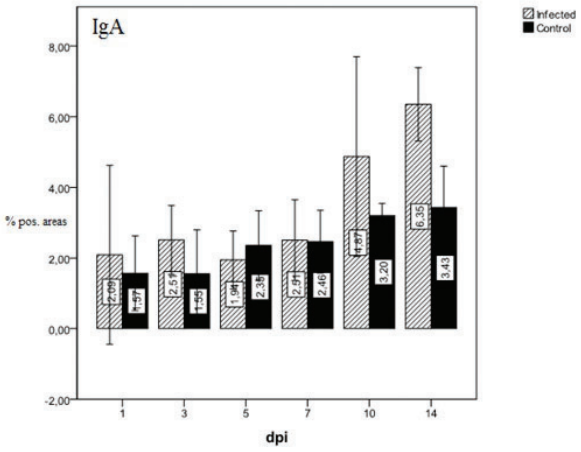

Figure 2. Dynamics of IgA changes in the lungs of AI-infected chickens. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 10 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

IFN-γ transcripts were observed in the AI-infected chickens starting at 7 dpi that coincides with the increasing level of CD4 cells. In Fig. 3 the changing levels of IFN-γ are shown.

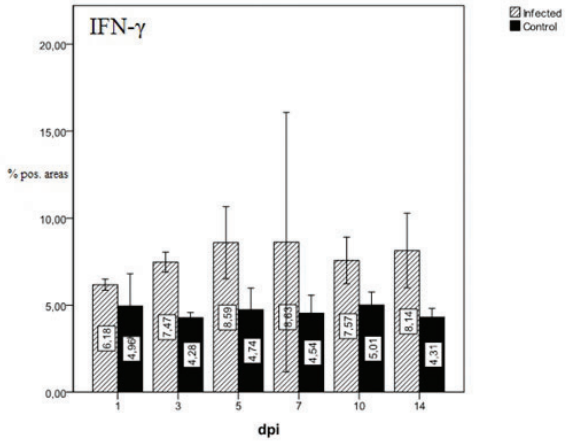

Figure 3. Dynamics of IFN-γ changes in lungs after infection with LPAIV. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 5 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

The number of lymphocytes which secrete IL-2 and IL-15 in AI-infected chickens was in general 1.5 to 2 times higher ($p<0.05$) compared to the uninfected control chickens (Fig. 4). Increased levels of these cytokines coincided with the enhanced number of CD8 cells in AIV-infected chickens.

The percentage of IgM-expressing cells was markedly increased during the first seven days post infection in the trachea. AIV-infected chickens showed 2 times higher IgM-expressing cells ($p<0.05$) compared to the uninfected control. Level of IgG-expressing cells in AIV-infected chickens increased starting from 7 dpi reaching a peak at 14 dpi (Fig. 5).

The level of cells expressing IFN-γ, IL-2, and IL-15 increased in AIV-infected chickens at 7-days after infection. The peak time coincided with a period of increasing CD8 cells. However, there was no significant difference in these cytokine levels between the AIV-infected and uninfected groups.

Interestingly, nucleoprotein-expression in the LPAIV-infected chickens was strong at 10 dpi in AI-infected chicken trachea. All chickens showed negative response in virological examination. At virological investigations, we have not identified the virus in all investigated organs. This indicates the absence of virus isolation in the environment, as evidenced by previous studies (Morales et al., 2009; Spackman et al., 2010).

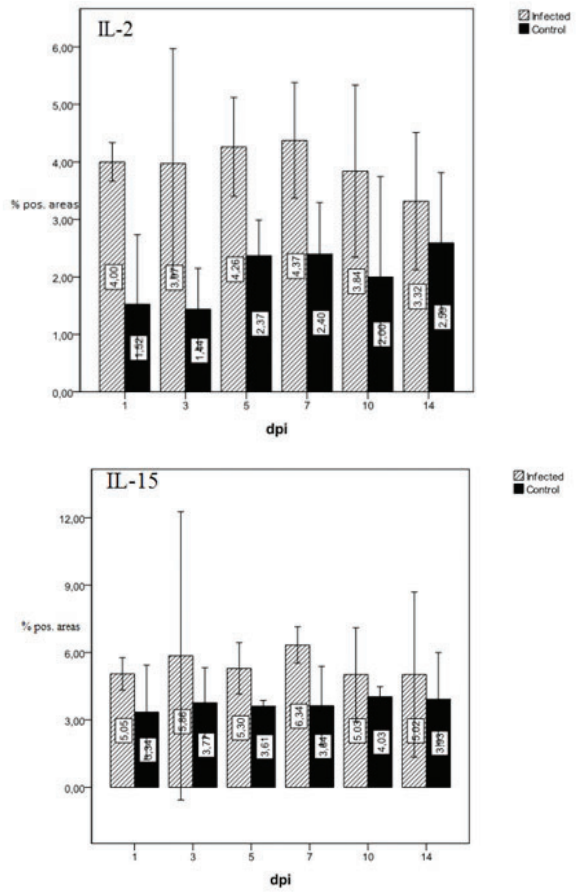

Figure 4. Dynamics of lymphocyte changes secreting IL-2 and IL-15 in the lung after infection with LPAIV. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 5 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

In the cecum, lower levels of CD4 cells were seen on 5 dpi but levels slowly increased from 7 dpi to 14 dpi following AIV infection.

In the ceca, a significant (1.5–2 times, $p<0.05$) increase in the number of cells expressing IgM and IgG was found (Fig 6).

LPAIV infection induced an increasing in macrophages and lymphocytes expressing CD4 and CD8 in the spleen throughout the period examined in this study indicating their role in host response to viral infection. Macrophage and heterophils play an important role in innate immune response in poultry and are able to ingest and kill a variety of microbial pathogens (Kogut and Klasing, 2009). The levels of macrophages in chickens of AIV-infected group were 2 times ($p<0.05$) higher than the non-infected control after the 1 dpi. This may suggest a role of macrophages

in initiating innate immune response to AIV infection. In the cecum, AIV infection enhanced lymphocytes especially the ones expressing IgG and IgA. At 10 dpi, there was a significant increase in cells expressing IgG and IgA ($p<0.05$) in infected chickens compared to uninfected control chickens.

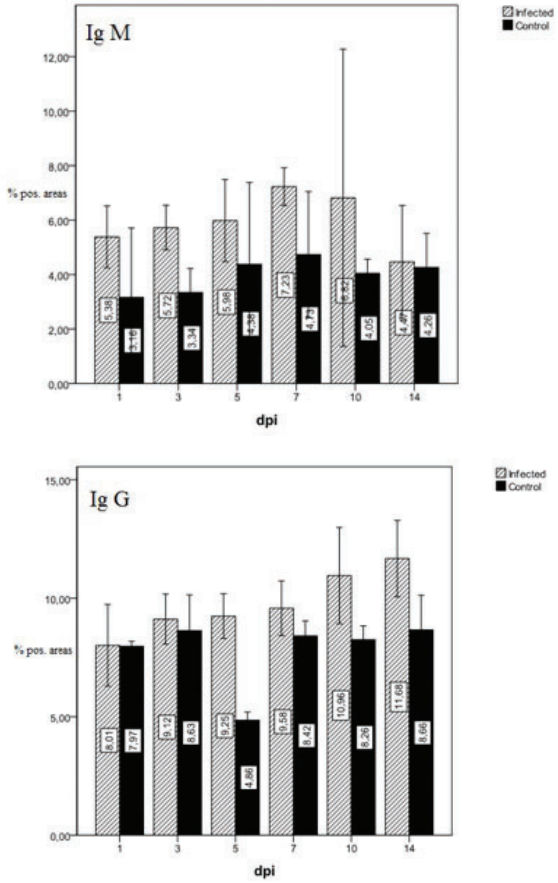

Figure 5. Dynamics of changes in lymphocytes expressing IgM and IgG in the trachea after infection with LPAIV. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 7 to 10 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

Discussion. There are no data on role of different immune cells in immune response at avian LPAI. It served as a reason to carry out current research. In this study, we assessed the clinical response, viral shedding, antibody production and immune response of chickens after infection with LPAIV A/Garganey/Chervonooskilske/4-11/2009 (H4N6) isolated from a wild bird. Our study shows that this LPAIV infection does not cause clinical signs of disease in infected chickens.

Starting at 7 dpi to 14 dpi, there was a sharp increase in the percentages of lymphocytes expressing CD4, IgM, IgG and IgA. Moreover, in the lung of AI-infected chickens, the levels of these cells were significantly higher compared to those of the noninfected control chickens. On the 3rd day after infection, the number of CD4 cells was significantly higher in the AI-infected chickens compared to the uninfected control ($p<0.05$).

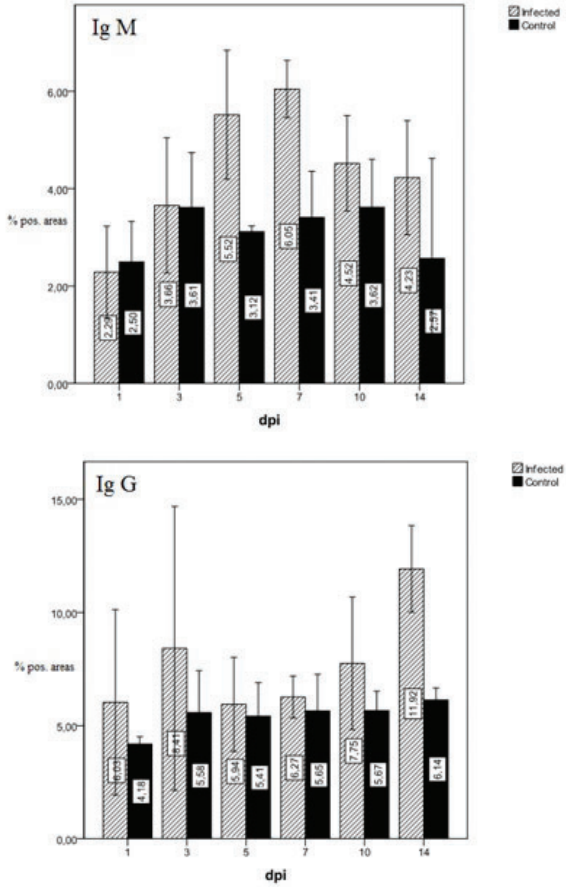

Figure 6. Dynamics of changes of cells expressing IgM and IgG in the caeca after infection with LPAIV. Each bar represents the accumulation of cells from 1 to 14 dpi. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 7 to 10 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

After AI infection, CD8 cells decreased until 6 dpi but showed a peak increase at 7 dpi (6.976±1.765 % at 3.056±0.704 %) ($p<0.05$) and then slowly declined but remained high until the end of experiment.

In general, the levels of cells expressing IgM, IgG and IgA were higher in the lung and caeca in the AI-infected chickens compared to those in the trachea. The percentages of IgM+ and IgA+ B cells increased with infection reaching almost two times higher than that of uninfected control chickens ($p<0.05$). After 7 dpi, IgG+

cells began to decrease. But then the percentage of IgG increased from 10 to 14 dpi when the levels of these cells in all experimental groups reached a maximum rate. On the 14 dpi, the percentage of IgA in AI-infected chickens was twice higher than the control group ($p<0.05$). However, on 10 dpi, there was a sharp decrease of IgM+ cells which continued until 14 dpi which may suggest that the isotype switching from IgM to IgG class antibodies may have occurred during the later phase of B cell immune response. As for cell-mediated immune response in the cecum, CD4+ cells were decreased on 5 dpi in AI-infected chickens ($p<0.05$) compared to uninfected control chickens.

Increases in cells expressing CD4 and CD8 as well as macrophages were evident in the spleen of AI-infected chickens, which indicates an enhanced AI-induced cell-mediated immunity. Macrophages were significantly enhanced on 3 dpi and persisted until 7 dpi suggesting their active role in processing viral antigens in the initiation of local immunity. CD4+ cells reached a stably high level compared to uninfected control chickens at 14 dpi ($p<0.05$).

Increased CD4+ cells and IL-2 level clearly indicate AI-induced cell-mediated immune response that can further activate CD8+ cells. IL-2 has a relatively narrow range of cells expressing IL-2 immune cells. The main ones are activated T- and B-lymphocytes and natural killer cells (NK-cells) which are induced to proliferate in the presence of IL-2. IL-2 also acts on monocytes bearing the receptor for IL-2 on its surface enhancing the generation of reactive oxygen and peroxides. Its effect can be direct or indirect. IL-2 operates at all stages of host immune response against pathogen invasion (Amrani, Mauzy-Melitz and Mosesson, 1986; Gendron et al., 1991; Klasing, 1994; Rath et al., 1995).

Following AIV infection, infected chickens showed high levels of IFN-γ, IL-2, and IL-15 expressing cells on 7 dpi when there was an increased CD8+ cells. Interferons are important cytokines in host defense against viral infections during the early phase of infection (Baron et al., 1987; Farrar and Schreiber, 1993; Grossber, 1987). Cells expressing IL-2 and IL-15 were found twice more In the lung of infected chickens, compared to uninfected control chickens especially on 7 dpi ($p<0.05$).

Infection with a low pathogenic avian influenza virus A/Garganey/Chervonooskilske/4-11/2009 (H4N6) did not cause any clinical disease at the infecting dose of $10^{6,0}$ EID_{50} when intranasally route was used. However, virus replicated in trachea and spleen inducing local and systemic cellular and humoral immune response in chickens. These results are in agreement with the previous observation (Spackman et al., 2010). No morbidity or mortality was seen when infecting doses of 10^6 or 10^8 EID_{50}/bird was administered by the upper-respiratory route suggesting that the virus is poorly adapted to chickens (Nemeth et al., 2010). Virus was shed at higher titers and spread to the kidneys in chickens when intravenous route was used (Spackman et al., 2015). We found nucleoprotein expression in the trachea at 10 dpi in the AI-infected chickens. In the spleen, NP was seen at 5 dpi. There was no NP antigen in other organs.

Conclusion. Our results indicate the potential possibility for infection of poultry with viruses isolated from wild birds. But currently, it is not completely known why some viruses from wild birds can cause infection in poultry while others can not. Further study the immune response will enable us to determine the features of the pathogenesis of low pathogenic avian influenza.

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References

Amrani, D. L., Mauzy-Melitz, D. and Mosesson, M. W. (1986) 'Effect of hepatocyte-stimulating factor and glucocorticoids on plasma fibronectin levels', *Biochemical Journal*, 238(2), pp. 365–371. doi: 10.1042/bj2380365.

Baron, S.,Dianzani, F,Stanton, G. J.andFleischmann, W. R. (eds.) (1987) *The interferon system: A current review to 1987*. Austin, TX: University of Texas Press. ISBN 029-273-845-5.

Berndt, A., Pieper, J. and Methner, U. (2006) 'Circulating γδ T cells in response to *Salmonella enterica* serovar *Enteritidis* exposure in chickens', *Infection and Immunity*, 74(7), pp. 3967–3978. doi: 10.1128/iai.01128-05.

Bertran, K., Pérez-Ramírez, E., Busquets, N., Dolz, R., Ramis, A., Darji, A., Abad, F., Valle, R., Chaves, A., Vergara-Alert, J., Barral, M., Höfle, U. and Majó, N. (2011) 'Pathogenesis and transmissibility of highly (H7N1) and low (H7N9) pathogenic avian influenza virus infection in red-legged partridge (*Alectoris rufa*)', *Veterinary Research*, 42(1), p. 24. doi: 10.1186/1297-9716-42-24.

Costa-Hurtado, M., Afonso, C. L., Miller, P. J., Spackman, E., Kapczynski, D. R., Swayne, D. E., Shepherd, E., Smith, D., Zsak, A. and Pantin-Jackwood, M. (2014) 'Virus interference between H7N2 low pathogenic avian influenza virus and lentogenic Newcastle disease virus in experimental co-infections in chickens and turkeys', *Veterinary Research*, 45(1), p. 1. doi: 10.1186/1297-9716-45-1.

Dufour-Zavala, L. (ed.) (2008) *A laboratory manual for the isolation, identification, and characterization of avian pathogens*. 5th ed. Athens, GA: American Association of Avian Pathologists. ISBN 978-09-7891-632-9.

Farrar, M. A. and Schreiber, R. D. (1993) 'The molecular cell biology of interferon-γ and its receptor', *Annual Review*

of Immunology, 11(1), pp. 571–611. doi: 10.1146/annurev.iy.11.040193.003035.

Gendron, R. L., Nestel, F. P., Lapp, W. S. and Baines, M. G. (1991) 'Expression of tumor necrosis factor alpha in the developing nervous system', *International Journal of Neuroscience*, 60(1), pp. 129–136. doi: 10.3109/00207459109082043.

Grossber, S. E. (1987) Interferons: an overview of their biological and biochemical properties. In: Pfeffer, L. M. (ed.). *Mechanisms of Interferon Action*. Boca Raton, FL: CRC Press, pp. 1–32.

Klasing, K. C. (1994) 'Avian leukocytic cytokines', *Poultry Science*, 73(7), pp. 1035–1043. doi: 10.3382/ps.0731035.

Kogut, M. H. and Klasing, K. (2009) 'An immunologist's perspective on nutrition, immunity, and infectious diseases: Introduction and overview', *The Journal of Applied Poultry Research*, 18(1), pp. 103–110. doi: 10.3382/japr.2008-00080.

Kuiken, T. (2013) 'Is low pathogenic avian influenza virus virulent for wild waterbirds?', *Proceedings of the Royal Society B: Biological Sciences*, 280(1763), pp. 20130990–20130990. doi: 10.1098/rspb.2013.0990.

Morales, A. C., Hilt, D. A., Williams, S. M., Pantin-Jackwood, M. J., Suarez, D. L., Spackman, E., Stallknecht, D. E. and Jackwood, M. W. (2009) 'Biologic characterization of H4, H6, and H9 type low pathogenicity avian influenza viruses from wild birds in chickens and turkeys', *Avian Diseases*, 53(4), pp. 552–562. doi: 10.1637/8877-041509-reg.1.

Nemeth, N. M., Thomas, N. O., Orahood, D. S., Anderson, T. D. and Oesterle, P. T. (2010) 'Shedding and serologic responses following primary and secondary inoculation of house sparrows (*Passer domesticus*) and European starlings (*Sturnus vulgaris*) with low-pathogenicity avian influenza virus', *Avian Pathology*, 39(5), pp. 411–418. doi: 10.1080/03079457.2010.513043.

OIE. (2012) *Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees)*. Vol. 1–2. 7th ed. Paris: OIE. ISBN 978-92-9044-878-5.

Rath, N. C., Huff, W. E., Bayyari, G. R. and Balog, J. M. (1995) 'Identification of transforming growth factor-β and interleukin-6 in chicken ascites fluid', *Avian Diseases*, 39(2), p. 382. doi: 10.2307/1591883.

Spackman, E., Gelb, J., Preskenis, L. A., Ladman, B. S., Pope, C. R., Pantin-Jackwood, M. J. and Mckinley, E. T. (2010) 'The pathogenesis of low pathogenicity H7 avian influenza viruses in chickens, ducks and turkeys', *Virology Journal*, 7(1), p. 331. doi: 10.1186/1743-422x-7-331.

Spackman, E., Pantin-Jackwood, M., Swayne, D. E., Suarez, D. L. and Kapczynski, D. R. (2015) 'Impact of route of exposure and challenge dose on the pathogenesis of H7N9 low pathogenicity avian influenza virus in chickens', *Virology*, 477, pp. 72–81. doi: 10.1016/j.virol.2015.01.013.

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COAGULASE-NEGATIVE STAPHYLOCOCCI AND THEIR SIGNIFICANCE FOR SUBCLINICAL MASTITIS

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Summary. Coagulase-negative staphylococci are a frequent cause of bovine intramammary infections in modern dairy herds. They have become the most common bacteria isolated from milk samples in many countries. Because of high prevalence of these intramammary infections it has a great impact on bulk tank somatic cell count. The objective of the study was to analyze the impact of coagulase-negative staphylococci intramammary infection on cows somatic cell count and on the potential of these infections to have a major impact on the bulk milk somatic cell count. The study was conducted on two dairy farms with high milk production and increased number of somatic cells in bulk tank milk. Bacteriological examination of milk samples from cows positive on CMT test pointed high prevalence of coagulase-negative staphylococci in cows with secretion disorder in both herds, up to 81.8%. Somatic cell count in these milk samples was also very high and counted over 1 million per milliliter. The highest number of somatic cells was in milk samples from cows with *Staphylococcus aureus*, but prevalence of these infections was very low.

Keywords: coagulase-negative staphylococci, intramammary infection, somatic cells

Introduction. Coagulase-negative staphylococci are a frequent cause of bovine intramammary infections in modern dairy herds. They have become the most common bacteria isolated from milk samples in many countries. Mastitis caused by coagulase-negative staphylococci in most cases remains subclinical, or the clinical signs are mild. For some reason, heifers, and primiparous cows are most susceptible to coagulase-negative staphylococci mastitis. Coagulase-negative staphylococci mastitis increases milk somatic cell count in the infected udder quarter. Very important is trait of coagulase-negative staphylococci mastitis to not causes decreasing in milk production, some authors even have found that cows with this form of mastitis had greater milk production than cows with no udder infection (Piepers et al., 2008). The increase in milk somatic cell count is usually moderate compared with mastitis caused by many other common pathogens, including *Staphylococcus aureus* and streptococci. However, high prevalence of coagulase-negative staphylococci mastitis in a herd can affect the herd bulk milk somatic cell count (Taponen, 2008).

Intramammary infections caused by major mastitis pathogens can reduce the possibility for infection with coagulase-negative staphylococci.

It seems that coagulase-negative staphylococci mastitis is a particular problem in well-managed, high-producing farms, which have successfully controlled udder infections caused by major mastitis pathogens (Myllys and Rautala, 1995).

About half of the cows with coagulase-negative staphylococci mastitis showed some clinical signs, but in most cases the signs were mild. Often only changes in milk appearance, such as clots and flakes, were detected, but sometimes also slight swelling of the affected quarters. Lack of clinical symptoms and changes in milk appearance is making diagnosis of this form of mastitis problematic and demands orderly applying of California Mastitis Test in dairy production herds.

The spontaneous elimination rate of coagulase-negative staphylococci mastitis is generally regarded as high. Some studies have demonstrated spontaneous elimination rates of as high as 60–70% for intramammary infections caused by coagulase-negative staphylococci (McDougall, 1998; Wilson et al., 1999). This process can be supported with regular applying of teat dipping and stricter hygiene on farms which allows achieving god hygienic quality of milk and an optimal number of somatic cells (Boboš et al, 2012).

Therapy of coagulase-negative staphylococci mastitis is bound with antibiotic resistance of these bacteria. Coagulase-negative staphylococci tend to be more resistant than *Staphylococcus aureus* and easily develop multiresistance. The most common resistance mechanism is β-lactamase production, which results in resistance to penicillin G and aminopenicillins. The reported percentage of penicillin resistance for coagulase-negative staphylococci isolated in mastitis was 32% in Finland (Pitkälä et al., 2004).

Materials and methods. The research was conducted on two dairy farms on the territory of Vojvodina providence. The reason for the inception of research was increased somatic cell count in bulk tank milk samples from observed farms. Somatic cell count in bulk tank

milk samples from both farms was between 400,000 and 500,000 per milliliter. Before forming of experimental group of cows a CMT test was performed on all cows in production. From cows with detected secretion disorder milk samples for bacteriology testing were taken.

Before milking cows were prepared for sampling and milk samples were collected as described.

Milk samples for bacteriology were collected aseptically. The udder and especially the teat were cleaned of dirt with a textile cloth moistened with distilled water. After that, the teat apex was cleaned with a cotton swab moistened with antiseptic solution. Samples were then stored in mobile refrigerator and transported to laboratory for further analysis.

In the laboratory, ten microlitres of milk were streaked on blood agar and incubated at 37 °C overnight (18–22 hours). Staphylococci were further identified based on colony morphology, Gram-staining, microscopy, and a catalase test.

Besides milk samples for bacteriology, cumulative milk samples for determination of somatic cell count were also obtained. These samples were conserved using 'azodiol' containing sodium azide in order to prevent decomposition of somatic cells in milk sample.

Determination of somatic cell count in cumulative milk samples was done in Laboratory for raw milk control on Faculty of Agriculture in Novi Sad, by flow cytometry.

Results and discussion. Bacteriological analysis of cumulative milk samples showed significant presence of coagulase-negative staphylococci in samples from both farms in the experiment. Results of the analysis are shown in Table 1.

Table 1 — Bacteriological findings in milk samples

Isolate	*Staphylococcus aureus*	Coagulase-negative staphylococci	Bacteriologicaly negative
First farm	2	30	21
Second farm	4	27	2

Analyzing milk samples from the first farm for presence of bacteria it can be concluded that from total 53 milk samples, coagulase-negative staphylococci were isolated in 30 samples (56.6%). On second farm from total 33 samples coagulase-negative staphylococci were isolated in 27 samples (81.8%). This result is in accordance with findings of Wilson et al. (1997), who claims that coagulase-negative staphylococci are isolated in high percent from the milk of cows with secretion disorder.

Determination of somatic cell count in milk samples showed increased number of somatic cell in milk samples with positive bacteriological findings (Table 2).

Table 2 — Somatic cell count in milk samples with different bacteriological findings

Isolate	*Staphylococcus aureus*	Coagulase-negative staphylococci	Bacteriologicaly negative
First farm	1,560,000/ml	1,135,000/ml	220,000/ml
Second farm	1,209,000/ml	1,298,000/ml	280,000/ml

The highest number of somatic cells was in samples with *Staphylococcus aureus*, but samples with coagulase-negative staphylococci also had very high values for somatic cell count, with average value 1,216,000/ml. Taponen (2008) also claimed that number of somatic cells in milk from infected cows can be highly increased up to above 1 million per milliliter.

Intramammary infections are affecting quality of milk, this can be measured through determination of somatic cell count (Radinović et al., 2014). Coagulase-negative staphylococci are very often isolated from milk and although they were not considered to be important pathogen for mammary gland, latest studies are pointing the significance of these bacteria (Taponen et al., 2006).

Coagulase-negative staphylococci are commonly considered to be teat skin opportunists that normally reside on the teat skin and cause mastitis via ascending infection through the streak canal (Radostits et al., 2007). This finding highlights significance of good udder hygiene and applying of teat dipping in order to prevent penetration of bacteria in mammary gland through teat canal. When applying therapy of coagulase-negative staphylococci mastitis, it is important to consider their resistance through β-lactamase production (Pitkälä et al., 2004). By improving cows management and hygiene, it is possible to support the process of self-healing or spontaneous elimination (MMM, 2003). The spontaneous elimination rate of CNS mastitis is generally regarded as high. Some studies have reported spontaneous elimination rates of about 60–70% (McDougall, 1998; Wilson et al., 1999). Markedly lower rates, 15–44%, have also been reported (Rainard and Poutrel, 1982; Timms and Schultz, 1987). In Finland and in the other Nordic countries, the policy is to avoid unnecessary use of antimicrobials in animal husbandry (MMM, 2003). Subclinical and mild clinical mastitis caused by CNS is usually left untreated, the rationale being that CNS will be eliminated spontaneously.

Conclusion. Coagulase-negative staphylococci are very significant cause of subclinical mastitis in dairy cows. Their effect on milk quality is measured through increasing of somatic cell count. Clinical form of mastitis is rare and decreasing of milk production is irrelevant. Good hygiene is very important for control of these agents allowing the spontaneous elimination from udder.

References

Boboš, S., Radinović, M., Pajić, M., Mihajlović-Ukropina, M., Mašić, Z and Galfi, A. (2012) 'The application of disinfection of the udder of cows in order to obtain healthy safe milk'. In: *International Conference Biological Food Safety and Quality*. Belgrade, 4–5 October 2012. pp. 110–112. Available at: http://agris.fao.org/agris-search/search.do?recordID=RS201300454.

McDougall, S. (1998) 'Efficacy of two antibiotic treatments in curing clinical and subclinical mastitis in lactating dairy cows', *New Zealand Veterinary Journal*, 46(6), pp. 226–232. doi: 10.1080/00480169.1998.36094.

MMM. (2003) *Recommendations for the use of antimicrobial agents in the treatment of the most significant infectious diseases in animals*. Memorandum 2003:9a. Ministry of agriculture and forestry, Helsinki, Finland. Available at: http://www.mmm.fi/attachments/mmm/julkaisut/tyoryhmamuistiot/2003/dE1sTO3LH/trm_2003_9a_Recommendations_for_the_use_of_antimicrobial_agents_in_the_treatment_of_the_most_significant_infectious_diseases_in_animals.pdf.

Myllys, V. and Rautala, H. (1995) 'Characterization of clinical mastitis in primiparous heifers', *Journal of Dairy Science*, 78(3), p. 538. doi: 10.3168/jds.S0022-0302(95)76664-4.

Piepers, S., Barkema, H. W., De Kruif, A., Opsomer, G., De Vliegher, S. (2008) 'Association between CNS-infections at calving and first lactation milk production and somatic cell counts in dairy heifers'. In: *Proceedings of 47th NMC Annual Meeting*. New Orleans, Louisiana, January 20–23, 2008. pp. 172–173. Available at: http://nmconline.omnibooksonline.com/47th-annual-meeting-2008-1.32419/t-004-1.32765/f-006-1.32766/a-031-1.32773?qr=1.

Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V. and Honkanen-Buzalski, T. (2004) 'Bovine Mastitis in Finland 2001—Prevalence, Distribution of Bacteria, and Antimicrobial Resistance', *Journal of Dairy Science*, 87(8), pp. 2433–2441. doi: 10.3168/jds.s0022-0302(04)73366-4.

Radinović, M., Boboš, S., Pajić, M. and Galfi, A. (2014) 'Influence of mastitis on the hygienic properties of milk' [Uticaj uzročnika mastitisa na higijensku ispravnost mleka]. In: *Proceedings and Abstracts of 25th Meeting of Serbian Veterinarians, Zlatibor, 11–14 September 2014 [Zbornik radova i kratkih sadržaja 25. Savetovanje veterinara Srbije, Zlatibor, 11–14 septembar 2014]*. Belgrade: Serbian Veterinary Society [Beograd: Srpsko veterinarsko društvo]. ISBN 978-86-83115-23-5. pp. 267–273. [in Serbian].

Radostits, O. M., Gay, C. C., Hinchcliff, K. W. and Constable, P. D. (2007) *Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats and horses*. 10th ed. Philadelphia: Elsevier. ISBN 978-0-7020-2777-2. pp. 674.

Rainard, P. and Poutrel, B. (1982) 'Dynamics of nonclinical bovine intramammary infections with major and minor pathogens', *American Journal of Veterinary Research*, 43(12), pp. 2143–2146.

Taponen, S. (2008) *Bovine mastitis caused by coagulase-negative staphylococci*. , Academic dissertation, presented, with the permission of the Faculty of Veterinary Medicine, University of Helsinki, for public criticism in Walter Hall, Agnes Sjöbergin katu 2, Helsinki, on April 11th, 2008. Available at: https://helda.helsinki.fi/bitstream/handle/10138/19035/bovinema.pdf?sequence=2.

Taponen, S., Simojoki, H., Haveri, M., Larsen, H. and Pyörälä, S. (2006) 'Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP', *Veterinary Microbiology*, 115(1–3), pp. 199–207. doi: 10.1016/j.vetmic.2006.02.001.

Timms, L. L. and Schultz, L. H. (1987) 'Dynamics and significance of coagulase-negative staphylococcal intramammary infections', *Journal of Dairy Science*, 70(12), pp. 2648–2657. doi: 10.3168/jds.s0022-0302(87)80335-1.

Wilson, D. J., Gonzalez, R. N., Case, K. L., Garrison, L. L. and Groöhn, Y. T. (1999) 'Comparison of seven antibiotic treatments with no treatment for bacteriological efficacy against bovine mastitis pathogens', *Journal of Dairy Science*, 82(8), pp. 1664–1670. doi: 10.3168/jds.s0022-0302(99)75395-6.

Wilson, D. J., Gonzalez, R. N. and Das, H. H. (1997) 'Bovine mastitis pathogens in New York and Pennsylvania: prevalence and effects on somatic cell count and milk production', *Journal of Dairy Science*, 80(10), pp. 2592–2598. doi: 10.3168/jds.s0022-0302(97)76215-5.

Contents

Part 1. Biotechnology and genetics

Part 2. Veterinary medicine

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