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THE MACROPHAGES ACCUMULATION IN CHICKENS VACCINATED AGAINST AVIAN MYCOPLASMOSIS

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Summary. The goal of our investigations was determination of the dynamics and intensity of macrophages accumulation in the immunocompetent organs of chickens vaccinated against Avian mycoplasmosis.

For creation of experimental series vaccines we have applied two technologies. In the first series as an antigenic bases used formaldehyde inactivated bacterin of production strain Mycoplasma gallisepticum VK (VB); in the second series – ultrasound disintegrated bacterial mass of cells of the same strain (VS). Experiments were carried out on chickens. Birds of first experimental group (n = 30) were immunized intramuscularly twice at an interval of 30 days by vaccine VB (VB group). Birds of second experimental group (n = 30) were immunized at the same scheme by vaccine VS (VS group). Birds of control group (n = 30) was not vaccinated.

On the 7th, 10th, 14th and 21st days after the second injection of vaccines 5 individuals from each group were euthanized; from birds were taken lungs, trachea, spleen and caecum tonsil. Preparations were stained by immuno-histochemistry method using labeled streptavidin-biotin. Presence and percentage of cell populations macrophages into organ samples account in the process of smear microscopy.

Macrophages are actually the first link of cellular immunity. After immunization the activation occurs in a relatively short time after injection of immunizing substance. It is this process we observed in the study of the internal organs of immunized chickens.

It was found that injection of inactivated vaccines in chickens promoted stimulation for primary link of cellular immunity. The population of macrophages increased rapidly during the first 10 days after the second injection of both vaccines. The highest value of this indicator was recorded in the spleen and lungs of birds (24.125 % and 22.280 % in the VB group; 21.010 % and 20.333 % in the VS group). Over the next 11 days, their number gradually decreased and on 21st day almost reached the level of the Control group. However, in VB group, this process was more intense, as evidenced by high values recorded during the study.

Keywords: inactivated vaccine, avian mycoplasmosis, macrophages

The main condition for the successful conduct of poultry industry is the prevention of infectious diseases, which cause significant economic losses, these include avian mycoplasmosis (Georgiades, 2002; Olanrewaju, Collier and Branton, 2011). Effective method to prevention of this infection is vaccination by inactivated vaccines (Kleven, 2008; Hussein et al., 2007; Ferguson-Noel et al., 2012; Branton et al., 2000). A clear indicator of immune reactivity of bird is intensity of macrophages accumulation in the immunocompetent organs. However, the dynamics and intensity of this process varies and depends on many factors, particularly important for determining the level of activation of the immune system is considered the first three weeks after vaccination (Bolotnikov and Konopatov, 1993; Parker et al., 2002; Halvorson, 2011).

The goal of our investigations was determination of the dynamics and intensity of macrophages accumulation in the immunocompetent organs of chickens vaccinated against avian mycoplasmosis.

Materials and methods. For creation of experimental series vaccines against avian mycoplasmosis we have applied two technologies. In the first series as an antigenic bases used formaldehyde inactivated bacterin of production strain Mycoplasma gallisepticum VK (VB); in the second series – ultrasound disintegrated bacterial mass of cells of the same strain (VS). Vaccines contained 30 % of antigenic substrate (3×10^7 CFU) and 70 % adjuvant (Mantanide ISA 70 VG).

Experiments were carried out on 3 groups of chickens. Birds of first experimental group (n = 30) were immunized intramuscularly twice at an interval of 30 days (at the age of 30 and 60 days, respectively) by vaccine VB (VB group). Birds of second experimental group (n = 30) were immunized at the same scheme by vaccine VS (VS group). Birds of control group (n = 30) was not vaccinated.

On the 7th, 10th, 14th and 21st days after the second injection of vaccines 5 individuals from each group were

euthanized; from birds were taken lungs, trachea, spleen and caecum tonsil.

Organ samples were fixed in 10 % neutral formalin solution, and pouring paraffin carried, histological sections prepared by standard methods. Preparations were stained by immuno-histochemistry method using labeled streptavidin-biotin. Presence and percentage of cell populations macrophages into account by using the «Video Test Morphology - 5» in the process of smear microscopy using a microscope Axiskop 40 / 40FL (Carl Zeiss).

Statistical processing of the data was performed using the program SPASS Statistics 17.0.

Results. Macrophages (mononuclear phagocytes) are the population of "long lifetime phagocytes". The role of macrophages in shaping the immune response is important, they provide phagocytosis of heterogenous protein components, processing and presenting antigens for T-lymphocytes. This is unique group of so-called "antigen-presenting cells". However, they have two important features: the ability to form complex antigenic peptide with molecules I and II MHC class, serving as the first signal to the proliferation and differentiation of T-lymphocytes; ability to initiate expression of co-stimulants to ensure the passage of the second signal to activate of T-lymphocytes (Lam, 2002).

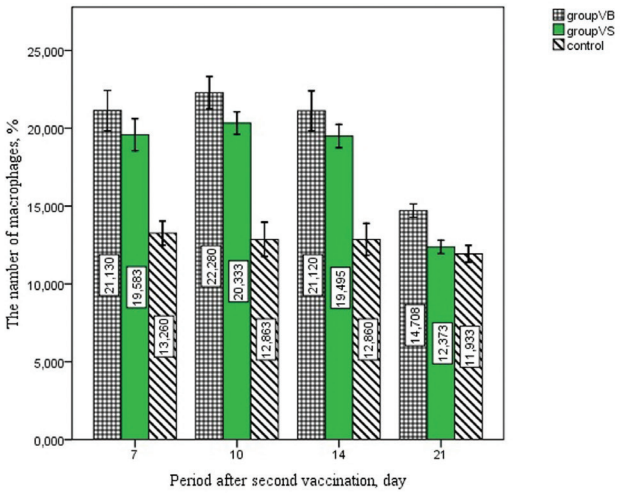

Figure 1 – Changes in the number of macrophages in the chicken lungs

Thus, macrophages are actually the first link of cellular immunity. After immunization the activation occurs in a relatively short time after injection of immunizing substance. It is this process we observed in the study of the internal organs of immunized chickens.

The number of macrophages in the lungs of chickens VB group grew rapidly in the first 10 days after the second vaccination. On the 7th day it reached 21.130 %, and the 10th day acquired the highest value – 22.280 %, which is almost twice as analog in Control group. Then the number of these cells began to gradually decrease and 14th day nearly equal to the level that was recorded on the 7th day.

A week later indicator value decreased to almost 14.708 % in this period exceeded the reference value only 2.775 %.

In the VS group observed similar changes, but the number of macrophages in the lungs of chickens was smaller. On the 10th day it was equal to 20.333 %, which is higher than the value in Control group of almost 1.6 times, but was lower than in VB group in 2 %. Further population of these cells decreased and on 21st day almost reached the level of the Control group (12.373 % against 11.933 %, respectively). This is lower than in the VB group on 2,335 %.

In trachea of both groups of birds macrophages accumulated by a similar scheme, but this process was not as intense as in the lungs, is shown in Fig. 2.

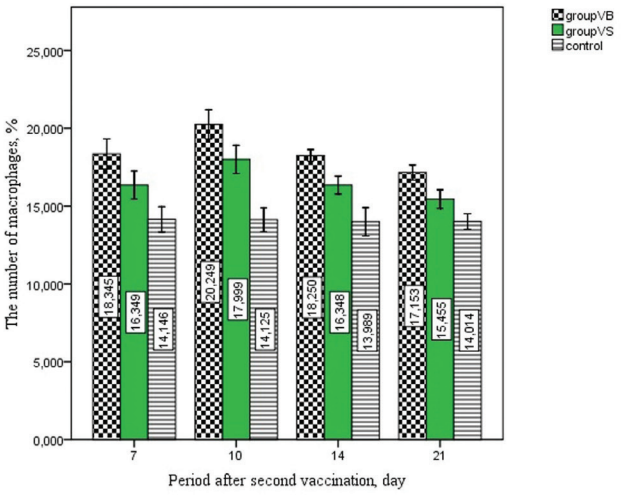

Figure 2 – Changes in the number of macrophages in the chicken trachea

On the 7th day the number of macrophages in the trachea of chickens VB group exceeded analogue for the Control group to 1.3 times in VS group this difference was 1.15 times.

The maximum value of this index in both groups reached 10th day and reached for the VB group – 20.249 % and for VS group – 17.999 % (against 14.125 % in Control group). Then we observed a gradual decline and at the 21st day in VB group the population of these cells was equal to 17.153 %, which was on 3.139 % higher than in the Control group and in the VS group the difference was smaller – 1.441 % (15.435 % vs. 14.014 %, respectively).

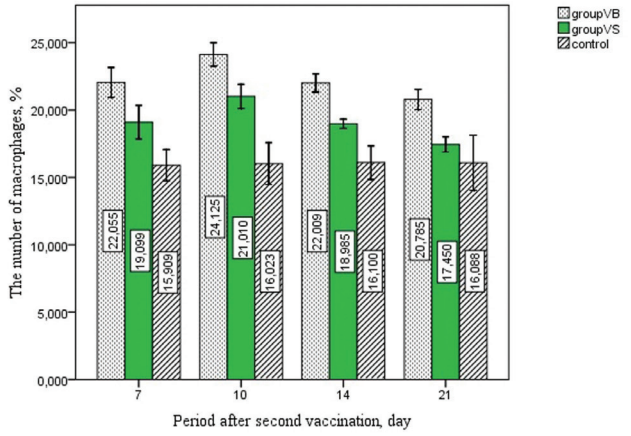

Figure 3 – Changes in the number of macrophages in the chicken spleen

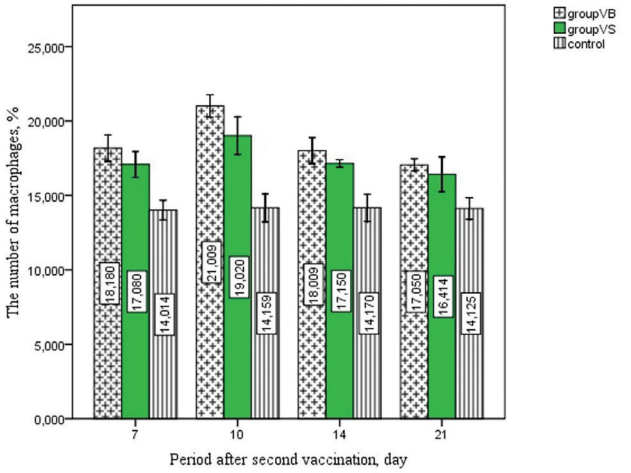

Figure 4 – Changes in the number of macrophages in the chicken caecum

The most striking and intense was the dynamics of accumulation of macrophages in the spleen of immunized birds. Thus, on the 10th day in VB group population of these cells were 24.125 % of the other cells, in the lungs compared to this period there were 22.280 %, and in the trachea - 20.249 %. Reducing the number of macrophages was recorded on the 14th and 21st day (up to 22.009 % and 20.785 %, respectively). In the VS group maximum value was also on 10th day, but their number was lower (21.010 %). Later their value decreased to 17.450 % on 21st day (against 16.088 % in the Control group). In caecum tonsils macrophage accumulation occurred at a high level, but they showed a slightly lower number than in the spleen. Thus, the highest value of this indicator was found in both experimental groups revealed on the 10th day after the second vaccination in the number of 21.009 % and 19.020 %, respectively (Fig. 4).

At the 14th day noted the decrease in the number of these cells almost to the level of the 7th day. The declining trend in the population of these cells was observed by us and over the next week. On 21st day the number of it in the BV group amounted to 17.050 %; in VS group – to 16.414 %, which is higher than the analog in the Control group only on 2.925 % and 2.289 %, respectively.

Thus, it was found that injection of inactivated vaccines in chickens promoted stimulation for primary link of cellular immunity. The population of macrophages increased rapidly during the first 10 days after the second injection of both vaccines. The highest value of this indicator was recorded in the spleen and lungs of birds (24.125 % and 22.280 % in the VB group; 21.010 % and 20.333 % in the VS group). Over the next 11 days, their number gradually decreased and on 21st day almost reached the level of the Control group. However, in VB group, this process was more intense, as evidenced by high values recorded during the study.

Conclusions. It was found that level of macrophages in chickens increased rapidly during the first 10 days after the second injection of inactivated vaccines against avian mycoplasmosis. The highest value of this indicator was recorded in the spleen and lungs of birds treated by vaccine with Mycoplasma gallisepticum bacterin (24.125 % and 22.280 %, respectively). In group treated by vaccine with subunit Mycoplasma gallisepticum antigen this process was less intense. Generally it was shown that injection of inactivated vaccines against avian mycoplasmosis in chickens promoted stimulation for primary link of cellular immunity.

References

Bolotnikov, I. and Konopatov, Yu. (1993) *A practical immunology of poultry [Prakticheskaya immunologiya sel'skokhozyaystvennoy ptitsy]*. Sankt-Peterburg: Nauka. ISBN 5-02-25816-4. [in Russian].

Branton, S. L., Lott, B. D., May, J. D., Maslin, W. R., Pharr, G. T., Bearson, S. D., Collier, S. D. and Boykin, D. L. (2000) 'The effects of ts-11 strain Mycoplasma gallisepticum vaccination in commercial layers on egg production and selected egg quality parameters', *Avian Diseases*, 44(3), pp. 618–623. doi: 10.2307/1593101.

Ferguson-Noel, N., Cookson, K., Laibinis, V. A. and Kleven, S. H. (2012) 'The efficacy of three commercial Mycoplasma gallisepticum vaccines in laying hens', *Avian Diseases*, 56(2), pp. 272–275. doi: 10.1637/9952-092711-reg.1.

Georgiades, G. K. (2002) 'Detection of antibodies against Mycoplasma gallisepticum and Mycoplasma synoviae in day-old broiler chicks and broilers', *Journal of the Hellenic Veterinary Medical Society*, 53(1), pp. 33–38. Available at: http://www.jhvms.com/sites/default/files/JHVMS%202002%2053%281%29%2033-38%20GEORGIADIS.pdf.

Halvorson, D. A. (2011) 'Biosecurity on a multiple-age egg production complex: a 15-year experience', *Avian Diseases*, 55(1), pp. 139–142. doi: 10.1637/9580-101710-case.1.

Hussein, A.-D., El-Shaib, T., Saoud, S., Shalaby, N., Sultan, H. and Ragab, A. (2007) 'Protective immune response of Mycoplasma gallisepticum vaccines in poultry', *Egyptian Journal of Immunology*, 14(2), pp. 93–99.

Kleven, S. H. (2008) 'Control of avian Mycoplasma infections in commercial poultry', *Avian Diseases*, 52(3), pp. 367–374. doi: 10.1637/8323-041808-review.1.

Lam, K. M. (2002) 'The macrophage inflammatory protein-1β in the supernatants of Mycoplasma gallisepticum-infected chicken leukocytes attracts the migration of chicken heterophils and lymphocytes', *Developmental and Comparative Immunology*, 26(1), pp. 85–93. doi: 10.1016/s0145-305x(01)00053-2.

Olanrewaju, H. A., Collier, S. D. and Branton, S. L. (2011) 'Effects of single and combined Mycoplasma gallisepticum vaccinations on blood electrolytes and acid-base balance in commercial egg-laying hens', *Poultry Science*, 90(2), pp. 358–363. doi: 10.3382/ps.2010-01006.

Parker, T. A., Branton, S. L., Jones, M. S., Peebles, E. D., Gerard, P. D., Willeford, K. O., Burnham, M. R. and Maslin, W. R. (2002) 'Effects of an s6 strain of Mycoplasma gallisepticum challenge before beginning of lay on various egg characteristics in commercial layers', Avian Diseases, 46(3), pp. 593–597. doi: 10.1637/0005-2086(2002)046[0593:eoasso]2.0.co;2.

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PHYLOGENETIC AND MOLECULAR GENETIC STUDIES OF THE ANIMALS ARTERIVIRUSES

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Summary. This study was aimed to provide the phylogenetic characteristics of arteriviruses of the different species, and to perform PCR-based surveillance of the porcine diseases, caused by PRRS virus and its associates.

The study was conducted based on the molecular genetics methods, including PCR, phylogenetic analysis of sequences of the different arteriviral species.

The phylogenetic relationships of the porcine reproductive and respiratory syndrome virus circulating in different geographical regions were studied. The availability of the phylogenetic analysis for genotyping was performed and microorganism molecular markers were demonstrated. The monitoring of the pathogen PRRS spread in the farms of Eastern Ukraine was conducted.

Variability of genes encoding glycoproteins GP2, GP3, GP4 and GP5 of the arteriviruses of animals was demonstrated and allowed to recommend these for PRRSV molecular epidemiology study. The monitoring spread of the PRRS virus in the farms in the Eastern Ukraine demonstrated the possibility of PRRS virus association with porcine circovirus type 2.

Keywords: porcine reproductive and respiratory syndrome virus, real-time polymerase chain reaction, phylogenetic analysis.

Introduction. The most common event in the molecular evolution of biomacromolecules (DNA and RNA) are nucleotide substitutions, that accumulated by the independent evolution of sequences from a common ancestral form. The average number of the nucleotide substitutions for two homologous sequences of two type biological molecules of the organisms to the one nucleotide site determines the evolutionary distance. Setting the evolutionary distance is to find the differences of the genetic material and its relationship at the evolution. In the future, this knowledge makes it possible to build the phylogenetic trees, to determine the time of taxon divergence based on the comparison of the primary structures of the genetic biomacromolecules, to reconstruct the history of the biota, to study the recent changes, evolution of genes in the space and the time (Riddle, 1996; Hewitt, 2001). If the replacing is rare (or the evolution time is small), we can assume that the number of substitutions is directly proportional to the time of their evolution in a pair of sequences.

The topology of the constructed phylogenetic trees, based on the evolutionary distances, allows obtaining the reliable information, particularly, about the genotypic characteristic of the pathogen, spectrum of the infectious agent isolates circulating in a particular area.

These processes complicate greatly the genotyping of infectious agents and, therefore, require the careful study by the experts of veterinary medicine and molecular biology, this is important for the development of the livestock industry of the Ukraine, creation of the modern means for veterinary support of pig farming.

One of the most common and developed industries is the swine livestock today. A lot of swine viral diseases causing a negative impact to the animal reproduction system, including porcine reproductive and respiratory syndrome (PRRS) and porcine circovirus desease, caused by porcine circovirus type II (PCV-2) were described in Ukraine (Gerilovich et al., 2011). Diseases caused by these pathogens as monoinfection and in the associations, cause significant economic losses to pig production. In addition, the PRRS agent has hypervariable organization of genetic material that causes the interest of genotyping and studying genetic markers of the virus origin and pathogenicity.

The disease caused by a PRRS virus was discovered in 1967 in North Carolina (USA) and Canada, and a few years later – in Europe (1990 – in Germany, 1991 – in the UK) (Wensvoort et al., 1991). Today this contagious viral infection is widespread in a lot of countries in Southeast Asia, Europe and America. PRRS virus was first isolated in primary cultures of pig alveolar macrophages (PAMs) in 1991 (the Lelystad strain) (Lurchachaiwong et al., 2008).

There are two PRRS genotypes (each has its subtypes) – European (type I) and American (North American) (type II). Their genomic RNA was characterized by only (55-70) % similarity and may have different ways of the evolutionary development (Stadejek et al., 2006; Nelson et al., 1993. It is believed (Martínez-Lobo et al., 2011) that pathogens of both genotypes differ on some biological properties, including pathogenicity.

In particular, showed that type II isolates causing more severe respiratory disease than type I isolates (Martínez-Lobo et al., 2011). But numerous studies concerning the comparison of both genotypes isolates never carried out. These genotypes have permanent structural difference related concerning the amino acids ORF7 (open reading frame) at positions 123 and 127, and the differences in replication concerning ORF1a and 5-noncoding region of genomic RNA. In addition, the genotypes of the PRRS virus differ in serological cross-reactions (Murtaugh et al., 1998).

Other arterivirus – arteritis virus – often found in populations of horses in different countries, but information concerning the genetic variability of this virus is almost absent. Only in 1999 was suggested the possible existence of separate geographical groups of the virus by studying of 22 strains of EAV from North America and Europe (Stadejek et al., 1999).

The aim of this work is to study the phylogenetic relationships of the animal arteriviruses and the monitoring of the PRRS and PCV-II viruses spread in the swine livestock in the eastern region of Ukraine by real-time PCR.

Materials and Methods. Mega 4, ver. 4.0.2 (Tamura et al., 2007); POWER, ver. 1.0 (PhylOgenetic WEb Repeater (POWER), 2005); PhyML ver. 3.0 program were used for the phylogenetic analysis (Dereeper et al., 2008). To build a traditional dendrograms based on gene sequences and genomic RNA paramyxoviruses used remote-matrix method – a method of binding neighbour joining and maximum parsimony. To test the reliability of the received dendrogram topology used bootstrap.

We have selected fully and partially sequenced genomic RNA sequence and basic arteriviruses gene of the genus Arterivirus for the phylogeographic studies of the arteriviruses from the farm animals: 1) porcine reproductive and respiratory syndrome virus (PRRSV); 2) equine arteritis virus (EAV). All sequences obtained in the FASTA (*.fasta) or GenBank (*.gb), that allowed to use modern bio-molecular software (including on-line) for pair and multiple, local and global alignment to determine conserved and variable fragments of genes and insertions, mutations and deletions, to build the dendrograms and to appropriate phylogenetic analysis (Abramson, 2007; Lukashov, 2009). Clinical material from pigs of different gender and age groups of livestock has been collected in the eastern region of Ukraine and pathological material from dead animals or aborted fetus during 2013-2014. Viral RNA and DNA extraction was performed by affinity sorption. Reverse transcription reaction was performed by using «First Strand cDNA Synthesis Kit» (Thermo SCIENTIFIC, USA).

For the elaboration of specific DNA and cDNA was used real-time PCR with the commercial kit «Maxima SYBR Green / ROX qPCR» (Thermo SCIENTIFIC, USA). For setting reaction was used the primers system (Kleiboeker, 2004). Amplification was performed on Thermocyclers DT lite («DNA technology», Russian Federation) at the next time and temperature parameters:

For PCV-2
1 step: denaturation – 94 °C – 2 min– 1 cycle;
2 step: denaturation – 94 °C – 15 sec;
annealing – 58 °C – 1 min,
synthesis – 72 °C – 20 sec,
detection – 72 °C – 12 sec;
} 40 cycles

For PRRS virus:
1 step: denaturation – 94 °C – 2 min – 1 cycle;
2 step: denaturation – 94 °C – 15 sec;
annealing – 60 °C – 1 min,
synthesis – 72 °C – 20 sec,
detection – 72 °C – 12 sec.
} 40 cycles

Results and Discussion. Arteriviral genome presented by single-helix non-segmented RNA molecule with positive polarity, length is about 15 thousand nucleotides, that encodes the virus structural proteins, including four glycoproteins (GP2, GP3, GP4 and GP5), two non-glycosylated structural nvelope proteins (E and M), non-structural proteins Nsps, which are crucial for viral replication and immune modulation, and nucleocapsid protein N (Chen et al., 2011; Li et al., 2011). Multiple aligned arteriviruses genomic RNA sequences circulating in different geographical regions and represented in the international databases, demonstrated the least conservative genes are genes that encode proteins GP3 and GP5. This is the basis for using sequences of these genes for genotyping based on the results of the phylogenetic analysis.

The most conserved were genes that encode the proteins M and N, this agrees with the data of works (Snijder and Meulenberg, 1998; Grebennikova et al., 2004). Phylogenetic analysis based on genes sequences encoding protein M, demonstrated high level of similarity for different arteriviruses (Fig. 1, cluster 1), that was the porcine respiratory reproductive syndrome virus, which circulates in the Canada (ES- 437 022 isolate) and in the China (HN-09 strain), and equine arteritis virus, which circulates in the United States (S4216 isolate) and in the France (strain F62).

The topology analysis of the phylogenetic tree based on the sequences of genes encoding N protein, showed the impossibility of the porcine respiratory reproductive syndrome two main virus genotypes - North American and European differentiation. Because, the representatives of the different pathogen genotypes belonging to the same cluster, and branches that correspond to isolate of the North American PRRS virus genotype, localized within the cluster (Fig. 2).

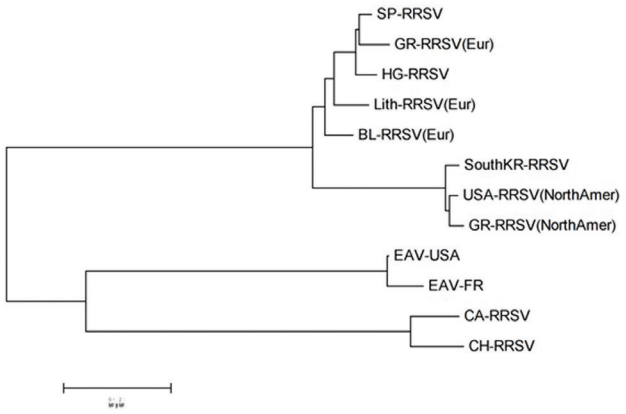

Figure 1 – Phylogenetic tree based on genes sequences encoding a M protein of the arteriviruses, which circulates in the different countries (USA – United States; BL – Belarus; CA – Canada; CH – China; SP – Spain; GR – Germany; HG – Hungary; Lith – Lithuania; SouthKR – South Korea; FR – France; Eur – European genotype; NorthAmer – NorthAmerican genotype)

Figure 2 – Phylogenetic tree based on genes sequences encoding a protein N of the arteriviruses, which circulates in the different countries (USA – United States; BL – Belarus; CA – Canada; CH – China; GR – Germany; HG – Hungary; Lith – Lithuania; SouthKR – South Korea; FR – France; IT – Italy; ROM – Romania; SLV – Slovakia; GB – United Kingdom; SL – Slovenia; SB – Serbia; AU – Austria; RU – Russian Federation; CZ – Czech Republic; TL – Thailand; PL – Poland; SP – Spain; BU – Bhutan; HR – Croatia; VN – Vietnam; TR – Turkey; Eur – European genotype; NorthAmer – NorthAmerican genotype)

This topology of the phylogenetic tree indicates a high level of similarity of the genes sequences encoding protein N, and therefore a large number of conservative structures in this protein that consistent with the results of work (Grebennikova et al., 2004b).

To study the phylogenetic relationships of the arteriviruses we have selected 16 fully sequenced PRRS virus genomic RNA sequences, 3 – equine arteritis virus circulating in a different geographical regions and sequences of genes encoding glycoproteins GP2, GP3, GP4 and GP5 of the arteriviruses, which role in joining the virus to permissive cells, causing a viral pathogenesis, an apoptosis, increased antibody depends is not been fully elucidated. The linear topology of the phylogenetic tree showed on the Fig. 3 indicates the origin of known PRRS virus genotypes from a common ancestor, that is consistent with the results of work (Grebennikova et al., 2004a), whose authors studied the primary structure of the pathogens genome of the both genotypes circulating in Poland and Lithuania.

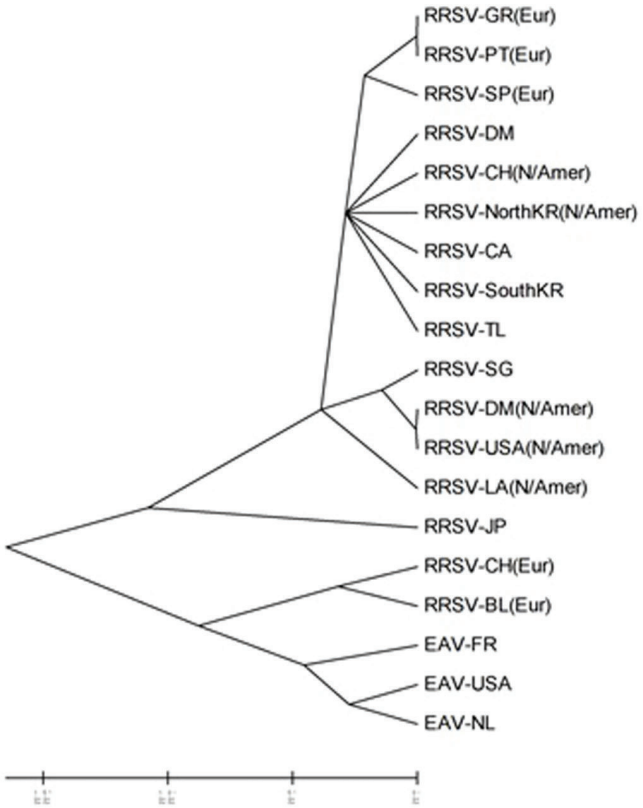

Figure 3 – Phylogenetic tree based on fully sequenced genome RNA sequences of the arteriviruses, which circulates in the different countries (USA – United States; BL – Belarus; CH – China; GR – Germany; South KR – South Korea; FR – France; TL – Thailand; SP – Spain; PT – Portugal; DM – Denmark; North KR – North Korea; CA – Canada; SG – Singapore; LA – Laos; JP – Japan; NL – Netherlands; Eur – European genotype; N / Amer – North American genotype)

The presence of two clusters for members of the European PRRS virus genotype and two clusters (Fig. 3) for members of the American pathogen agent genotype indicates the possibility of the existence at least two subtypes for each genotype. PRRS virus isolates that make up a particular subtype, have not only a high level of similarity of the genomic RNA primary structure, but perhaps have the common immunobiological properties that were the object of study in recent years (Kukushkin et al., 2004).

According to the conducted phylogenetic analysis of the arteriviruses we can conclude the phylogenetic proximity of the equine arteritis virus which isolates form separated cluster on the phylogenetic tree (Fig. 3) to one of the subtypes of the European genotype PRRS virus.

The most significant for the genotyping arteriviruses of the animals is a gene encoding a protein GP5. This glycoprotein is the major coat protein of the PRRS virus with molecular weight of 26 kDa, consisting of about 200 aminoacids residues. It is known (Kapur et al., 1996; Andreyev et al., 1997) that the GP5 protein is highly polymorphic, being under the constant pressure of selection due to its open position on the virions outer surface (Meulenberg et al., 1995). Due to its polymorphic nature the GP5 protein is considered as the main molecule in creating subunit vaccines. The aminoacid sequences of the ORF5 open reading frame have two hypervariable regions, one of which is localized in the signal peptide. Thanks GP5 polymorphism, the gene encoding this protein is highly informative regarding the evolution and origin of different PRRS strains and considered as a target for the analysis of genetic diversity not only PRRS virus, but the equine arteritis virus.

The complex nature of the encoding the protein GP5 gene, allows to consider this gene as the main subject in the study evolutionary relationships of the arteriviruses. However, the results of the arteriviruses phylogenetic analysis (circulating in different geographical regions) based on sequences of genes encoding proteins GP2 (Fig. 4), GP3, GP4, that were established, and compare them with the results of the phylogenetic analysis based on the sequences of the gene encoding glycoprotein GP5, convincing the possibility of the arteriviruses differentiation and PRRS virus genotyping (type determination) based on these genes. It is important that each PRRS virus genotype, as the equine arteritis virus isolates, forms separated cluster on the dendrogram.

The early diagnosis of the swine viral diseases is a necessary condition for the effective development of a pig production as one of the promising sectors of Ukraine livestock. It is important to timely identification of patients and latently sick animals, in that it affects the effectiveness of a livestock treatment. One of the modern and fast methods for the detecting infectious agents in animals, even in the early stages of the disease, is the real-time polymerase chain reaction (RT-PCR).

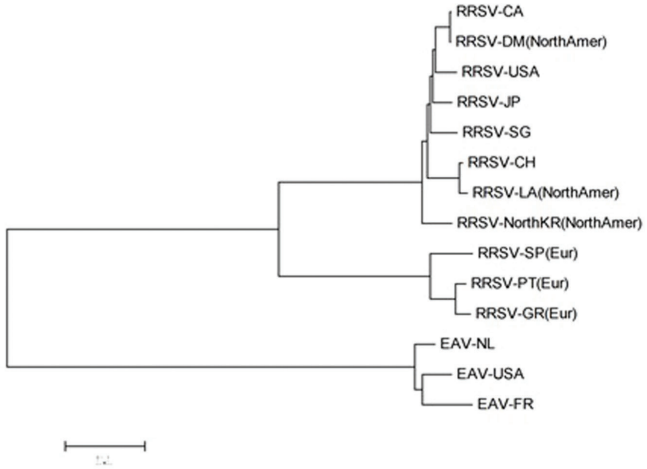

Figure 4 – Phylogenetic tree based on genes sequences encoding G2 protein of the arteriviruses, circulating in the different countries (USA – United States; CH – China; GR – Germany; PT – Portugal; DM – Denmark; North KR – North Korea; CA – Canada; SG – Singapore; LA – Laos; JP – Japan; NL – Netherlands; FR – France; SP – Spain; Eur – European genotype; N / Amer – North American genotype)

The results of our studies suggest the prevalence of respiratory reproductive disorders in pigs in the Ukraine caused by PRRS virus, PCV-2 or their associations (Gerilovich et al., 2011). Clinical signs of disease were extremely diverse in the study of epizootic state of farms, where more often met PRRS associations and PCV-2 associations, respiratory disorders appeared in the young animals, while adult cattle recorded the disorders of the reproductive system.

126 samples of various materials were tested by real-time PCR during 2013-2014. We observed the fluorescence signal for 65 samples only with primers that flank the specific PRRS virus fragment (Fig. 5); 23 samples contained only PCV-2 genetic material (Fig. 6).

We observed the fluorescence signal for 17 samples with primers system, which the target was the PRRS virus fragment, and with a primers system flanking specific fragment of the PCV-2. Therefore, these samples contained genetic material of both pathogens, and diseases of animals, which samples were selected, due to the association of these viruses.

number of wells	nomekr tube	Cp. Fam	Cp. Hex	results
A1	sample 1	17,3		+
A2	sample 2	20,5		+
A3	K+	16,8		+
A4	K-			-

Dependence of the FAM channel fluorescence from cycle number

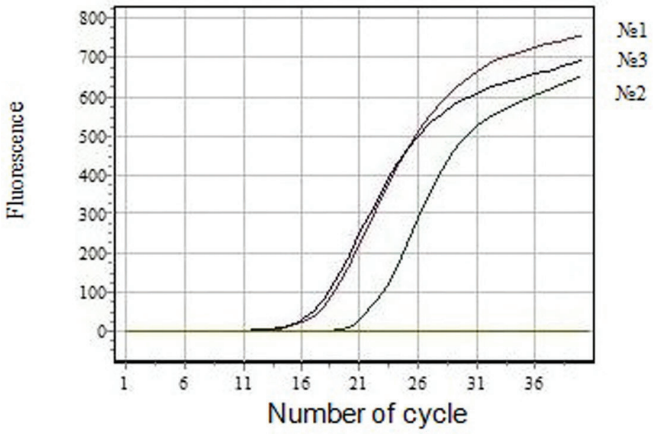

Figure 5 – Amplification curves of specific cDNA fragment of the PRRS virus with using the intercalating dye SYBR – Green. № 1, № 2 – amplification curves of specific cDNA fragment PRRS virus from samples taken from animals; № 3 – amplification curve of the positive control.

number of wells	nomekr tube	Cp. Fam	Cp. Hex	results
A1	sample 1	16,5		+
A2	sample 2			-
A3	K+	21,1		+
A4	K-			-

Dependence of the FAM channel fluorescence from cycle number

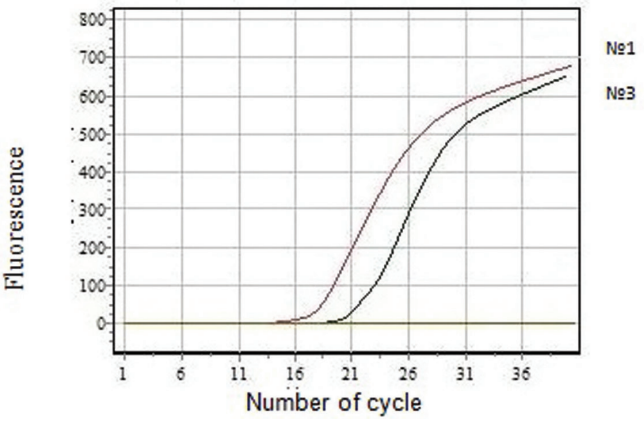

Figure 6 – Amplification curves of specific cDNA fragment of the PCV-2 virus with the intercalating dye SYBR – Green using. № 1, № 2 – amplification curves of specific cDNA fragment PRRS virus from samples taken from animals; № 3 – amplification curve of the positive control.

Thus, it was proved the variability of genes encoding glycoproteins GP2, GP3, GP4 and GP5 of the arteriviruses of animals. The phylogenetic analysis of the arteriviruses demonstrated the possibility of differentiation and, in particular, the PRRS virus genotyping based on these genes. The monitoring spread of the PRRS virus in the farms in the Eastern Ukraine was held, the possibility of PRRS virus association with porcine circovirus type 2 was shown.

References

Abramson, N. I. (2007) 'Phylogeography: results, issues and perspectives' [Filogeografiya: itogi, problemy, perspektivy], *Bulletin of Vavilov Society of Geneticists and Breeders [Vestnik Vavilovskogo obshchestva genetikov i selektsionerov]*, 11(2), pp. 307–331. [in Russian]. Available at: http://www.bionet.nsc.ru/vogis/pict_pdf/2007/t11_2/vogis_11_2_04.pdf.

Andreyev, V. G., Wesley, R. D., Mengeling, W. L., Vorwald, A. C. and Lager, K. M. (1997) 'Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field strains based on sequence analysis of open reading frame 5', *Archives of Virology*, 142(5), pp. 993–1001. doi: 10.1007/s007050050134.

Chen, N., Cao, Z., Yu, X., Deng, X., Zhao, T., Wang, L., Liu, Q., Li, X. and Tian, K. (2011) 'Emergence of novel European genotype porcine reproductive and respiratory syndrome virus in mainland China', *Journal of General Virology*, 92(4), pp. 880–892. doi: 10.1099/vir.0.027995-0.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M. and Gascuel, O. (2008) 'Phylogeny.fr: robust phylogenetic analysis for the non-specialist', *Nucleic Acids Research*, 36(suppl. 2), pp. W465–W469. doi: 10.1093/nar/gkn180.

Gerilovich, A., Bolotin, V., Rudova, N., Sapko, S. and Solodyankin, A. (2011) 'Etiological structure of circovirus-associated diseases of pigs in the Eastern region of Ukraine' [Etiolohichna struktura tsyrkovirus-asotsiiovanykh khvorob svynei v hospodarstvakh Skhidnoho rehionu Ukrainy], *News of Agrarian Sciences [Visnyk ahrarnoi nauky]*, 1, pp. 34–36. Available at: http://agrovisnyk.org.ua/files/van1_11.pdf. [in Ukrainian].

Grebennikova, T. V., Alexeev, K. P., Gibadulin, R. A., Musiyenko, M. I., Tsibezov, V. V., Bogdanova, V. S., Zaberezhny, A. D., Aliper and T. I., Nepoklonov, E. A. (2004a) 'Synthesis of recombinant proteins of the American and European viruses of the porcine reproductive and

respiratory syndrome made in the baculovirus system and their antigen properties' [Sintez v bakulovirusnoy sisteme rekombinantnykh belkov amerikanskogo i evropeyskogo tipov virusa reproduktivnogo i respiratornogo sindroma sviney i ikh antigennye svoystva], *Problems of Virology [Voprosy virusologii]*, 49(2), pp. 37–42. [in Russian].

Grebennikova, T. V., Zaberezhny, A. D., Vlasova, A. N., Musiyenko, M. I., Sokolov, M. A., Grabovetsky, V. V., Tsibezov, V. V., Bogdanova, V. S., Orlyankin, B. G., Aliper, T. I. and Nepoklonov, E. A. (2004b) 'Genetic variability of the nucleocapsid protein of the virus of the porcine reproductive and respiratory syndrome' [Variabel'nost' gena belka nukleokapsida virusa reproduktivnogo i respiratornogo sindroma sviney (RRSS)], *Molecular Genetics, Microbiology and Virology [Molekulyarnaya genetika, mikrobiologiya i virusologiya]*, 2, pp. 37–40. [in Russian].

Hewitt, G. M. (2001) 'Speciation, hybrid zones and phylogeography - or seeing genes in space and time', *Molecular Ecology*, 10(3), pp. 537–549. doi: 10.1046/j.1365-294x.2001.01202.x.

Kapur, V., Elam, M. R., Pawlovich, T. M. and Murtaugh, M. P. (1996) 'Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the Midwestern United States', *Journal of General Virology*, 77(6), pp. 1271–1276. doi: 10.1099/0022-1317-77-6-1271.

Kleiboeker, S. B. (2004) *Development of Real-time, multiplex PCR/RT-PCR assays for improved PRDC pathogen detection.* Research Report NPB #03-114. National Pork Board. Available at: http://old.pork.org/filelibrary/researchdocuments/03-114-kleiboeker.6-28-04.pdf.

Kukushkin, S. A., Baibikov, T. Z., Kurman, I. Ya., Baborenko, E. P., Pylnov, V. A., Kanshina, A. V. and Teterin, I. A. (2004) 'The immunological-and-biological properties of viral isolates of the porcine reproductive and respiratory syndrome' [Immunobiologicheskie svoystva izolyatov virusa reproduktivnogo i respiratornogo sindroma sviney], *Problems of Virology [Voprosy virusologii]*, 49(2), pp. 42–46. [in Russian].

Li, B., Fang, L., Guo, X., Gao, J., Song, T., Bi, J., He, K., Chen, H. and Xiao, S. (2011) 'Epidemiology and evolutionary characteristics of the porcine reproductive and respiratory syndrome virus in China between 2006 and 2010', *Journal of Clinical Microbiology*, 49(9), pp. 3175–3183. doi: 10.1128/jcm.00234-11.

Lukashov, V. V. (2009) *Molecular evolution and phylogenetic analysis [Molekulyarnaya evolyutsiya i filogeneticheskiy analiz]*. Moscow: BINOM. Laboratoriya znaniy. ISBN 978-5-9963-0114-0. [in Russian].

Lurchachaiwong, W., Payungporn, S., Srisatidnarakul, U., Mungkundar, C., Theamboonlers, A. and Poovorawan, Y. (2008) 'Rapid detection and strain identification of porcine reproductive and respiratory syndrome virus (PRRSV) by real-time RT-PCR', *Letters in Applied Microbiology*, 46(1), pp. 55–60. doi: 10.1111/j.1472-765X.2007.02259.x.

Martínez-Lobo, F. J., Díez-Fuertes, F., Segalés, J., García-Artiga, C., Simarro, I., Castro, J. M. and Prieto, C. (2011) 'Comparative pathogenicity of type 1 and type 2 isolates of porcine reproductive and respiratory syndrome virus (PRRSV) in a young pig infection model', *Veterinary Microbiology*, 154(1-2), pp. 58–68. doi: 10.1016/j.vetmic.2011.06.025.

Meulenberg, J. J. M., Besten, A. P.-D., De Kluyver, E., Moormann, R. J. M., Schaaper, W. M. M. and Wensvoort, G. (1995) 'Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus', *Virology*, 206(1), pp. 155–163. doi: 10.1016/s0042-6822(95)80030-1.

Murtaugh, M. P., Faaberg, K. S., Laber, J. L., Elam, M. and Kapur, V. (1998) 'Genetic Variation in the PRRS Virus', in Enjuanes, L., Siddell, S., and Spaan, W. (eds) *Coronaviruses and arteriviruses*. New York: Springer Science+Business Media, LLC (Advances in Experimental Medicine and Biology, 440), pp. 787–794. doi: 10.1007/978-1-4615-5331-1_102.

Nelson, E. A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J. E. and Benfield, D. A. (1993) 'Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies', *Journal of Clinical Microbiology*, 31(12), pp. 3184–3189. Available at: http://jcm.asm.org/content/31/12/3184.full.pdf.

PhylOgenetic WEb Repeater (POWER) (2005). National Health Research Institutes. Available at: http://power.nhri.org.tw/power/home.htm.

Riddle, B. R. (1996) 'The molecular phylogeographic bridge between deep and shallow history in continental biotas', *Trends in Ecology and Evolution,* 11(5), pp. 207–211. doi: 10.1016/0169-5347(96)10032-x.

Snijder, E. J. and Meulenberg, J. M. (1998) 'The molecular biology of arteriviruses', *Journal of General Virology*, 79(5), pp. 961–979. Available at: http://vir.sgmjournals.org/content/79/5/961.full.pdf.

Stadejek, T., Björklund, H., Ros Bascuñana, C., Ciabatti, I. M., Scicluna, M. T., Amaddeo, D., McCollum, W. H., Autorino, G. L., Timoney, P. J., Paton, D. J., Klingeborn, B. and Belák, S. (1999) 'Genetic diversity of equine arteritis virus', *Journal of General Virology*, 80(3), pp. 691-699. Available at: http://vir.sgmjournals.org/content/80/3/691.full.pdf.

Stadejek, T., Oleksiewicz, M. B., Potapchuk, D. and Podgórska, K. (2006) 'Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes', *Journal of General Virology,* 87(7), pp. 1835–1841. doi: 10.1099/vir.0.81782-0.

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.

Wensvoort, G., Terpstra, C., Pol, J. M. A., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J. M., Moonen, P. L. J. M., Zetstra, T., de Boer, E. ., Tibben, H. J., de Jong, M. F., van 't Veld, P., Greenland, G. J. R., van Gennep, J. A., Voets, M. T., Verheijden, J. H. M. and Braamskamp, J. (1991) 'Mystery swine disease in the Netherlands: The isolation of Lelystad virus', *Veterinary Quarterly,* 13(3), pp. 121–130. doi: 10.1080/01652176.1991.9694296.

UDC: 573.6: 578.2'21: 616.98:578.833.3:619

CREATION MOLECULAR-GENETIC CONTROL SYSTEM OF PESTIVIRUS CONTAMINATION IN BIOTECHNOLOGY OBJECTS

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Summary. This study aimed on (i) creation molecular-genetic control system of pestivirus contamination in biotechnology objects, (ii) identification of persistently infected with bovine viral diarrhea virus (BVDV) animals and (iii) genetic typing of selected BVDV isolates.

RNA extraction, cloning, polymerase chain reaction (PCR), real-time PCR, enzyme-linked immunosorbent assay, serum neutralization test, sequencing.

It was shown that we had constructed the recombinant plasmids with insertion E^{rns} gene fragment (826 base pair) of BVDV-1 and BVDV-2. Also we had developed and optimized parameters of duplex PCR for the simultaneous indication Mollicutes DNA and BVDV RNA, with the possibility of nested PCR for further identification of BVDV genotypes. Specific BVDV antibodies were detected in 725 of 1042 (69.6 %) analyzed samples. In this study 5 persistently infected with BVDV animals were detected in farms B and C of Kharkiv region. The genetic typing of viral isolates revealed that only BVDV-1 viruses were present. The phylogenetic analysis confirmed two BVDV-1 subtypes, namely b and f and revealed that all viruses from the farm B of Kharkiv region and from biotechnological objects were typed as BVDV-1b, but virus from the farm C of Kharkiv region and farm of Kherson region were typed as BVDV-1f.

The obtained recombinant plasmids can be used as a positive control for PCR and test-system for control of pestivirus contamination in biotechnology objects. Our results indicated that the BVDV infection is widespread in cattle herds in the eastern Ukraine, that requires further applying of new approaches to improve the current situation.

Keywords: bovine viral diarrhea virus, pestivirus contamination, cloning, pTZ57R/T, restriction enzyme digestion analysis, ELISA, SNT, PCR, real-time PCR, genotyping, phylogenetic analysis.

Introduction. Bovine viral diarrhea virus (BVDV) belongs to *Pestivirus* genus of Flaviviridae family (Heinz et al., 2000). The agent is represented by two genotypes – 1 and 2 which are designated as BVDV-1 and BVDV-2. In particular BVDV-1 viruses are very heterogenic, with at least 13 subgroups, whereas two subgroups are differentiated in the more homogenous BVDV-2 viruses (Jackova et al., 2007).

BVDV is present in the cattle population worldwide (Nettleton and Entrican, 1995). The success of BVDV rests on its capacity to establish persistent infection. Viral persistence is established during a "window of opportunity" early in gestation and associated with immunotolerance to the infecting viral strain. Different from persistent infections by herpesviruses and lentiviruses, persistent infected (PI) animals remain free of antibodies to BVDV (Chase, Elmowalid and Yousif, 2004), which calls for detection of viral antigen or viral RNA as the sole methods for diagnosing persistent infection. Although transiently infected animals maybe capable of transmitting virus to susceptible cattle to a limited extent, only PI animals are responsible for viral persistence in the host population. Typically, about one percent of the cattle population is PI and some 60 percent are seropositive when the infection has reached equilibrium (Houe, 1999; Hessman et al., 2009).

Calves has been born by the seropositive cows receive colostral antibodies against BVDV (Peterhans et al., 2010). These antibodies decrease in titer overtime and the calves become susceptible for infection. The time span of colostral protection depends on the antibody titer and the level of infectious pressure to which the animals are exposed. Older animals are more likely to be seropositive, due to a longer time during which the animals are at risk of being exposed to PI animals. In contrast, many heifers may still be seronegative during their first pregnancy. When exposed to PI animals during the critical period of development, fetuses may be infected to become PI, thereby assuring viral persistence in the next generation.

Pestiviruses and mycoplasmas also are potential contaminants of biological products produced in bovine or porcine cell or manufactured which are produced with the use of animal raw materials such as bovine sera. These materials are widely used in diagnostic laboratory in cell culture and vaccine production as a cell growth promoting factor. Therefore, any viral contaminants or antibodies present in bovine serum may hamper proper diagnosis and efficient application of the vaccines. Vaccine contamination may not only influence on the results of vaccination but also lead to new infections, causing serious economic problems in a herd.

The control methods for sterility, in particular, eliminate viral contamination of animal origin raw materials and ready preparations are important for today.

The aim of this work was to creation molecular-genetic control system of pestivirus contamination in biotechnology objects, identification of persistently infected animals and genetic typing of selected BVDV isolates.

Material and methods. Reference samples. In conducting the research reference samples of BVDV-1b (strain Osloss), BVDV-1a (strain Oregon), BVDV-2 (strain Kosice) and *Mycoplasma orale* N-I, *Mycoplasma hyorhinis* BTS-7 and *Mycoplasma bovis* PG45T were used.

Cattle and sample collection. 274 veterinary immunobiological preparations, 2437 samples of biotechnological raw materials and 746 samples of clinical materials from cattle origin were used for the monitoring of pestivirus contamination. 1042 sera samples of cattle from 3 different farms in North-East territory of Ukraine were used for the detection of PI animals using molecular-genetic screening and serological monitoring. The samples were collected from November 2011 to June 2012. Animals were selected of different ages beginning from the newborns. A detailed questionnaire was completed for each herd with the owner's support. The variables of interest related to individual animals as well as to the herd and comprised the type of farm, animal movements, general management, feeding, prophylactic health measures, disease incidence, and BVDV disease awareness.

Assay reagent. Extraction of DNA and RNA, reverse transcription, amplification, electrophoresis, ligation, restriction enzyme digestion analysis, transformation, enzyme-linked immunosorbent assay (ELISA), serum neutralization test (SNT) and sequencing were carried out using reagent manufactured by Ltd. Lab. Isogene (Russian Federation), Boehringer Mannheim, JenaBioscience, QIAGEN (Germany), Fermentas, GeneJET (Lithuania), Applied Biosystems, Kirkegaard and Perry Labs, Promega, Serva, Sigma-Aldrich Ltd. (USA), IDEXX Laboratories (Switzerland).

Extraction of RNA was performed using silica-based extraction method (Boom et al., 1990).

Amplification of nucleic acid was carried out using classical polymerase chain reaction (PCR) and real-time PCR with the following primer sets: Pan_324/326 (Vilček et al., 1994), P1/P2, TS3/P2 and TS2/P2 (Sullivan and Akkina, 1995) – for the detection and genotyping of Pestiviruses (5' UTR and E^{rns} genes), GPO-1/MGSO (Van Kuppeveld et al., 1992) – for the detection Mollicutes (16S rRNA gene), M13/pUC_F/R – for the screening transformed bacterial colonies on the presence of recombinant plasmids.

PCR Optimization. PCR protocol was optimized by the determination of appropriate thermal (denaturation, annealing and elongation temperatures) and time parameters of amplification cycles and reaction components.

Construction of vector systems with insertion fragment of the BVDV E^{rns} gene 826 bp in length were carried out using the commercial kit «Ins TA clone PCR Cloning Kit» (Fermentas, Lithuania) in accordance with the manufacturer's instructions. The recombinant ampicillin resistant Escherichia coli cells were obtained by transformation of plasmid vector pTZ57R/T with target gene into the competent *E. coli* DH10B cells.

Antibodies capture ELISA test was performed by the commercially available ELISA Kit HerdChek BVDV Ab Test (IDEXX Laboratories, Switzerland) for the detection of total antibodies to BVDV antigens and by the protocols of Institute of Veterinary Virology, University of Bern (Switzerland) for the detection of IgG antibodies to BVDV antigens (Canal et al., 1998).

Serum neutralization test was carried out in case of a discrepancy between the results of both ELISA by the common method (Steck et al., 1980).

Phylogenetic study. Samples determined as positive in PCR were studied with sequencing based on the classic chain termination method (Sanger, 2001). Phylogenetic analysis in 5'-UTR (245 bp fragment) was used for the genetic typing of BVDV isolates into subgenotypes. Phylogenetic trees were constructed by Neighbor Joiningand Maximum likelihood, Minimum evolution algorithms. All phylogeny trees buildings and analyses were done with modules of MEGA 5.2 SeqManII, AmplifX 1.0, Clone Manager 7.0, BLAST on-line and other software.

Statistical analysis. Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp. One Microsoft Way, Redmond, WA, USA) for analysis using chi-square (χ^2) and Student's (t) distribution. Creating databases and the calculation was performed by NCSS 07.1.21 statistical software (NCSS, LLC, Kaysville, Utah, USA).

Results and discussion. ***Development of the molecular diagnosis tools for the detection of BVDV genetic materials.*** With the aim of obtaining the recombinant positive controls for Bovine Viral Diarrhea diagnostics by polymerase chain reaction, the construction of plasmid vectors with insertion E^{rns} gene fragment (826 base pair) of BVDV-1 and BVDV-2 was conducted.

At the first stage of the work conducted RNA of BVDV-1 (strain Osloss) and BVDV-2 (strain Kosice) was used as a matrix for obtaining cDNA by reverse transcription assay followed by cDNA amplification by PCR. The PCR products were checked by agarose gel electrophoresis, specific bands of an estimated length of 825 bp indicative of E^{rns} gene fragment were observed in agarose gel (Fig. 1).

Figure 1 – Electrophoregram with BVDV cDNA amplification results using primers P1/P2: M – molecular weight marker (100 bp DNA Ladder, Ltd. Lab. Isogene, Russian Federation); 1 – BVDV-1; 2 – BVDV-2; 3 – negative control

The PCR products were extracted from agarose gel and subsequently inserted into the corresponding site of cloning vector pTZ57R/T. The constructed plasmids pTZ57R/T-VD1 and pTZ57R/T-VD2 (Fig. 2) had a E^{rns} gene fragment, a selective β-lactamase gene (bla(ApR)) responsible for ampicillin resistance, nucleotide sequence complementary to M13/pUC primers for the subsequent selection of cells containing recombinant plasmids.

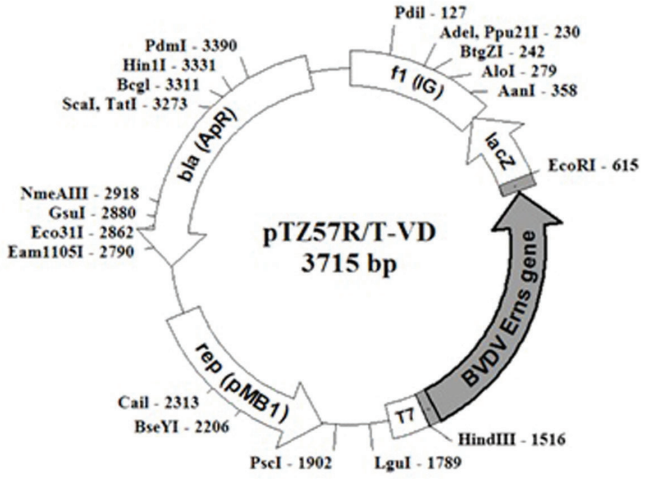

Figure 2 – pTZ57R/T-VD plasmid restriction site map

Two recombinant plasmids were cloned in *E. coli* DH10B cells. Screening of recombinant *E. coli* colonies was performed by inoculating the colonies into the selective medium containing ampicillin. To exclude artifact DNA structures and incorporable amplicon complexes, as well as potential plasmid DNA structure damages commonly observed at cloning, PCR screening of all *E. coli* colonies with acquired resistance to ampicillin was performed. Thus, eight recombinant colonies were screened using P1/P2 and M13/pUC_F/R primers and the insertion was confirmed in all colonies (Fig. 3).

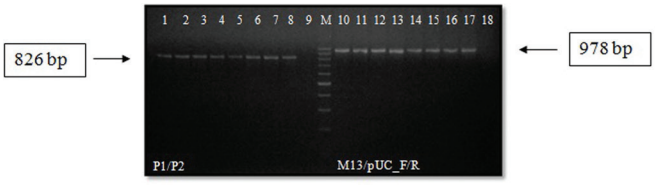

Figure 3 – Electrophoregram with amplification results using P1/P2 and M13/pUC_F/R primers in agarose gel: 1–4, 10–13 – recombinant colonies carrying BVDV-1 E^{rns} gene insertion; 5–8, 14–17 – recombinant colonies carrying BVDV-2 E^{rns} gene insertion; 9, 18 – negative control; M – molecular weight marker (100 bp DNA Ladder, Ltd. Lab. Isogene, Russian Federation)

The insertion location in all recombinant colonies was confirmed by restriction analysis using restriction endonucleases EcoRI and HindIII cleaving the plasmid in the corresponding restriction sites. After restriction two fragments of expected length of 2814 and 901 bp were observed in agarose gel (Fig. 4).

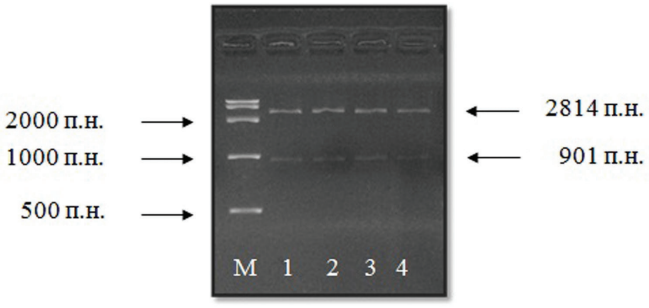

Figure 4 – Electrophoregram of pTZ57R/T-VD plasmid restriction analysis: 1–2 – recombinant colonies carrying BVDV-1 E^{rns} gene insertion; 3–4 – recombinant colonies carrying BVDV-2 E^{rns} gene insertion; M – molecular weight ladder (Fast Ruler High Range DNA Ladder, Fermentas, Lithuania).

The culture of *E. coli* cells including the recombinant plasmids with insertion fragment of the E^{rns} gene was created. The constructed plasmids were involved in subsequent studies as positive controls for development of the diagnostic kit «Bovi-Multi-Test-Myc-BVDV» aimed for the detection *Mollicutes* DNA and BVDV RNA by duplex PCR with following the identification of BVDV genotypes.

During the development of test system the selection of optimal primer annealing temperature, concentration of primers and number of amplification cycles were conducted. After set of the experiments it was found that the optimum parameters of amplification for the simultaneous detection *Mollicutes* DNA and BVDV RNA are the temperature annealing at 55 °C and 40 cycles of amplification.

Panel with 10x dilutions (final dilution – 10^{-6}) of plasmids pTZ57R/T-VD and *Mycoplasma bovis* PG45T DNA were used for determine the analytical sensitivity of the test system. After amplification of the pathogens genetic materials were detected up to dilution 10^{-4}, corresponding to a concentration of 10 pg/ml (Fig. 5).

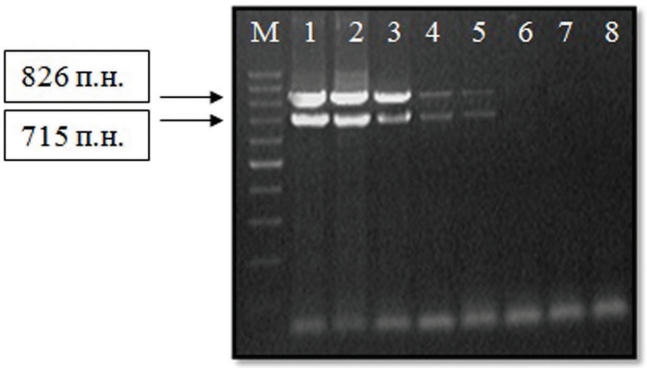

Figure 5 – Analytical sensitivity of the test system for detection Mollicutes and BVDV genetic materials by the duplex PCR: 1 – undiluted positive control; 2 – 10-fold dilutions of positive control; 3 – 100-fold dilutions of positive control; 4 – 1000-fold dilutions of positive control; 5 – 10^4-fold dilutions of positive control; 6 – 10^5-fold dilutions of positive control; 7 – 10^6-fold dilutions of positive control; 8 – negative control; M – molecular weight ladder (100 bp DNA Ladder, Ltd. Lab. Isogene, Russian Federation).

After series of laboratory studies and inter-laboratory testing of the designed test system appropriate sensitivity, specificity, reproducibility and the absence of cross-reactions were shown.

During the ongoing work have been developed and optimized parameters of duplex PCR for the simultaneous indication *Mollicutes* DNA and BVDV RNA, with the possibility of nested PCR for further identification of BVDV genotypes (Fig. 6).

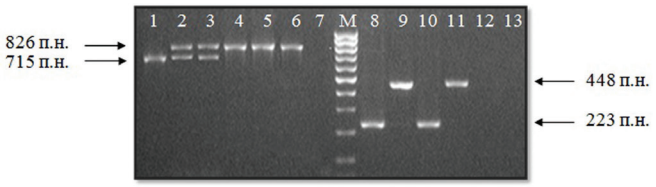

Figure 6 – The results of the detection Mollicutes DNA and BVDV RNA by duplex PCR with following the identification of BVDV genotypes. First round of nested PCR: 1 – *M. bovis* PG45T; 2 – *M. bovis* PG45T and BVDV-1 (strain Osloss); 3 – *M. bovis* PG45T and BVDV-2 (strain Kosice); 4 – BVDV-1; 5 – BVDV-2; 6 – classical swine fever virus (strain LK-M); 7 – negative control. Second round of nested PCR (samples 2-7): 8 – BVDV-1; 9 – BVDV-2; 10 – BVDV-1; 11 – BVDV-2; 12 – classical swine fever virus; 13 – negative control; M – molecular weight marker (100 bp DNA Ladder, Ltd. Lab. Isogene, Russian Federation).

Compared with existing systems of the separate detection viral and bacterial contamination (Studer, Bertoni and Candrian, 2002; Uphoff and Drexler, 2002; Sung et al., 2006; Kong et al., 2001; Bolin et al., 2009) developed duplex PCR can detect several pathogens in one reaction, allowing to significantly reduce the time and material costs and to prevent contamination of biotechnology objects on time.

Screening of bovine clinical material samples by the developed test system. The study of cattle farms of different Ukrainian regions found that the genetic material of the BVDV contained in 94 samples out of 746 (12.6 %) analyzed bovine clinical material samples. Genetic analysis of BVDV according to our methodology showed that all detected viruses belonged only to genotype 1. BVDV circulation was established in 10 among 35 investigated farms of different Ukrainian regions. This indicates a widespread of BVD in Ukraine.

Application of the developed test system for studies biotechnology materials. 2437 samples of bovine serum and 274 samples of veterinary immunobiological preparations were tested for the presence of BVDV RNA. The results of the studies found that BVDV was present in 224 samples out of 2711 (8.5 %) analyzed.

According to studies found that 214 serum samples to the appropriate filtration treatment were contaminated with BVDV. Serum that containing BVDV were re-examined

after decontamination, and the results showed the absence of contaminants genetic material. In 10 out of the 274 (3.6 %) studied veterinary immunobiological preparations the presence of BVDV was found. 9 (6.8 %) contaminated preparations were found in 2011, 1 (1.2 %) – in 2012 and none contamination of biological product was found in 2013. It can be concluded that the involvement of molecular genetics techniques to the biotechnology industry reduces the incidence of viral contamination by selecting only high quality raw materials that are confirmed in 2012 ($K = 216$, $t_f = 2,79$, $t_{st} = 2,58$, $p < 0,01$).

The obtained results of the raw materials for the manufacture of biological products and ready products contamination with BVDV are common for the production that observed in studies Vilcek et al. (2004), Kennedy (2006), Charleston et al. (2001).

All viruses detected in positive samples were genotyped by the developed test system and appropriated only to the genotype 1. Several samples were used in further phylogenetic studies of BVDV cDNA.

Using the developed test system for the identification persistently infected animals. With the aim of the detection persistently infected cattle by molecular-genetic screening and serological monitoring, 1042 blood samples collected from three cattle farms in the Kharkov region

As the first step of our study BVDV specific antibodies were detected by ELISA using the commercially available ELISA Kit HerdChek BVDV Ab Test (IDEXX Laboratories, Switzerland) and in house-protocol (Institute of veterinary virology, University of Bern, Switzerland) (Tabl. 1).

Table 1 – Comparative results of different ELISA protocols at the different cattle age group and farms n=1042)

Age	Farm A			Farm B			Farm C		
	analyzed samples	positive samples by IDEXX ELISA	positive samples by in house ELISA	analyzed samples	positive samples by IDEXX ELISA	positive samples by in house ELISA	analyzed samples	positive samples by IDEXX ELISA	positive samples by in house ELISA
< 1 year	158	41 (26 %)	4 (2,5 %)	230	178 (87,7 %)	198 (97,5 %)	150	138 (92 %)	120 (80 %)
1-5 years	56	1 (1,8 %)	0	176	144 (81,8 %)	163 (92,6 %)	93	75 (80,6 %)	85 (91,4 %)
> 5 years	63	10 (15,9 %)	6 (9,5 %)	87	78 (89,6 %)	82 (94,3 %)	56	44 (78,6 %)	51 (91,1 %)
Total	277	52 (18,7 %)	10 (3,6 %)	466	400 (85,8 %)	443 (95,1 %)	299	257 (85,9 %)	256 (85,6 %)
Statistics	df = 2, $\chi^2_f = 227$, $\chi^2_{st} = 13,82$, $p < 0,001$			df = 2, $\chi^2_f = 2,48$, $\chi^2_{st} = 13,82$, $p > 0,001$			df = 2, $\chi^2_f = 4,8$, $\chi^2_{st} = 13,82$, $p > 0,001$		

BVDV specific antibodies were detected by the both ELISA protocols in 709 of 1042 samples analyzed (68 %). This number is in agreement with findings in many cattle herds around world. However the number of positive samples differed in the herds.

In order to refine the mismatched results using IDEXX and in house protocols, serum neutralization test was carried out as a "gold standard" of serological methods for the detection of BVDV antibodies. Considering the results obtained by SNT and both ELISA protocols BVDV specific antibodies were detected in 725 samples out of 1042 (69.6 %). While 10 samples out of 277 (3.6 %) were identified in farm A, 438 out of 466 (94 %) and 277 out of 299 (92.6 %) animals were positive in farms B and C.

As the second step of our study seronegative animals with BVDV RNA were revealed with the involvement PCR. The PCR assay detected BVDV RNA in 140 of 1042 samples analyzed (13.4 %) (Tabl. 2).

With the aim of the genotyping revealed viruses the developed test system was used. According to the obtained results all detected viruses belonged to genotype 1.

Animals that were virus-positive in the PCR but antibody-negative in ELISA and SNT were considered to be persistently infected. Based on these criteria, the results obtained with the antibody detection method and the PCR were concordant in 40 of the 1042 animals. The literature refers to the fact that PI animals are the main source of infection within the infected herd, because they shed virus in very high concentrations in all bodily fluids throughout their life (Ståhl and Alenius, 2012; Larson et al., 2004). Therefore samples of these animals were further

investigated by quantitative real-time PCR. 5 positive samples out of 40 (12.5 %) were found in the second and the third herd by quantitative PCR. The genetic materials of BVDV were not found in the first herd. All 5 virus-positive samples were serological negative. Consequently, 5 of these 1042 (0.48 %) animals were persistently infected (Tabl. 3). The 5 viruspositive animals were 2, 4, 5 and 8 month old.

Table 2 – Comparative results of PCR at the different cattle age group and farms (n = 1042)

Age	Farm A		Farm B		Farm C	
	analyze	positive	analyze	positive	analyze	positive
< 1 year	158	22 (13,9 %)	203	34 (16,7 %)	150	16 (10,7 %)
1-5 years	56	4 (7,1 %)	176	28 (15,9 %)	93	9 (9,7 %)
> 5 years	63	8 (12,7 %)	87	12 (13,8 %)	56	7 (12,5 %)
Total	277	34 (12,2 %)	466	74 (15,8 %)	299	32 (10,7 %)
Statistic	k = 2, χ^2_f = 2,35, χ^2_{st} = 13,82, p > 0,001		k = 2, χ^2_f = 0,29, χ^2_{st} = 13,82, p > 0,001		k = 2, χ^2_f = 0,37, χ^2_{st} = 13,82, p > 0,001	

Table 3 – The results of molecular-genetic and serological studies on the presence of BVDV and it antibodies (n = 1042)

Farm	PI animals	Seropositive animals
A	0	10 (3,6 %)
B	4 (0,86 %)	438 (94 %)
C	1 (0,33 %)	277 (92,6 %)
Total	5 (0,5 %)	725 (69,6 %)

It is important, that PI animals were found in farms with high amount of seropositive animals. These data are typical for circulation of persistent infection that was observed in the work of other researchers (Houe, 1999; Hessman et al., 2009).

Phylogenetic study. The genetic typing of viral isolates revealed that only BVDV type 1 viruses were presented. The phylogenetic analysis confirmed two BVDV-1 subtypes, namely b and f (Fig. 7) and revealed that all 4 viruses from farm B were typed as BVDV-1b and all of them were absolutely identical in 5'-UTR, but virus from farm C were typed as BVDV-1f.

The genetic diversity, demonstrated in the study, releases the belonging of characterized viruses to BVDV-1b strains with the distance not more 2-4 %. This is typical in the current genetic studies of worldwide characterized viruses. Allocated viruses of this subtype are truly same inside this clad of Ukrainian viruses.

Another detected subtype was 1f. This group of BVDV-1 was also detected in several countries of the Central and Western Europe, so they are not unique. Characterized isolate had 4.5 % differences among subtype-belonged related viruses of BVDV-1f genotype.

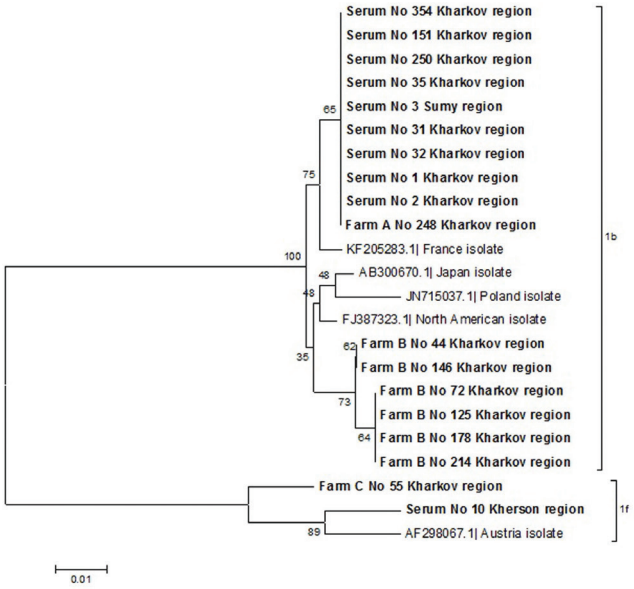

Figure 7 – Genetic typing of BVDV isolates in the 5'-UTR region

Current scientific literature explains the significant role of the BVDV-1 in the epidemiology of bovine viral diarrhea all over the World. It demonstrates distribution in all European countries, only several countries have been eradicated this disease by the implementation of the eradication strategies based on PI animals elimination and/or vaccination of susceptible animals.

Viral genetic divergence studies allows to study the molecular diversity of virus for the creation of effective prevention means, and gives the opportunity to determine viral origin and source for recognition of the epidemiology of bovine viral diarrhea and its eradication strategy development.

Conclusion. The analyses conducted allowed to confirm the presence and correctness of recombinant pTZ57R/T-VD plasmid insertion in E. coli DH10B cells. The work resulted in the recombinant plasmids carrying 826 bp fragment of BVDV E^{rns} gene. The obtained recombinant plasmids can be used as a positive control for PCR.

During the ongoing work have been developed and optimized parameters of duplex PCR carrying out for the simultaneous indication of the genetic material represented by Mollicutes and BVDV, with the possibility of nested PCR for further genotyping of BVDV.

Our results concluded that the involvement of molecular genetics techniques to the biotechnology industry reduces the incidence of viral contamination by selecting only high quality raw materials.

High seroprevalence levels for BVDV (69.6%) were demonstrated in the cattle herds. The PCR assay detected BVDV RNA in 13.4% of cattle have been tested. 0.48% animals were persistently infected.

The genetic typing of viral isolates revealed that only BVDV-1 was present. The phylogenetic analysis confirmed two BVDV-1 subtypes, namely b and f and revealed that all 4 viruses from the farm B were typed as BVDV-1b and all of them were absolutely identical in 5'-UTR, but virus from the farm C was typed as BVDV-1f.

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References

Bolin, S. R., Lim, A., Grotelueschen, D. M., McBeth, W. W. and Cortese, V. S. (2009) 'Genetic characterization of bovine viral diarrhea viruses isolated from persistently infected calves born to dams vaccinated against bovine viral diarrhea virus before breeding', *American Journal of Veterinary Research*, 70(1), pp. 86–91. doi: 10.2460/ajvr.70.1.86.

Boom, R., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M. and van der Noordaa, J. (1990) 'Rapid and simple method for purification of nucleic acids', *Journal of Clinical Microbiology*, 28(3), pp. 495–503. Available at: http://jcm.asm.org/content/28/3/495.full.pdf.

Canal, C. W., Strasser, M., Hertig, C., Masuda, A. and Peterhans, E. (1998) 'Detection of antibodies to bovine viral diarrhoea virus (BVDV) and characterization of genomes of BVDV from Brazil', *Veterinary Microbiology*, 63(2-4), pp. 85–97. doi: 10.1016/s0378-1135(98)00232-6.

Charleston, B., Fray, M. D., Baigent, S., Carr, B. V. and Morrison, W. I. (2001) 'Establishment of persistent infection with non-cytopathic bovine viral diarrhoea virus in cattle is associated with a failure to induce type I interferon', *Journal of General Virology*, 82(8), pp. 1893-1897. Available at: http://vir.sgmjournals.org/content/82/8/1893.full.pdf.

Chase, C. C. L., Elmowalid, G. and Yousif, A. A. A. (2004) 'The immune response to bovine viral diarrhea virus: a constantlychanging picture', *Veterinary Clinics of North America: Food Animal Practice*, 20(1), pp. 95–114. doi: 10.1016/j.cvfa.2003.11.004.

Heinz, F. X., Collett, M. S., Purcell, R. H., Gould, E. A., Howard, C. R., Houghton, M., Moormann, R. J. M., Rice, C. M. and Thiel, H.-J. (2000) 'Family Flaviviridae' in van Regenmortel, C. M. F. M. H. V., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R. and Wickner, R. B. (eds) *Virus taxonomy: Seventh report of the International Committee on Taxonomy of Viruses*. San Diego, CA: Academic Press, pp. 859–878. ISBN 978-0-12-714181-7.

Hessman, B. E., Fulton, R. W., Sjeklocha, D. B., Murphy, T. A., Ridpath, J. F. and Payton, M. E. (2009) 'Evaluation of economic effects and the health and performance of the general cattle population after exposure to cattle persistently infected with bovine viral diarrhea virus in a starter feedlot', *American Journal of Veterinary Research*, 70(1), pp. 73–85. doi: 10.2460/ajvr.70.1.73.

Houe, H. (1999) 'Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections', *Veterinary Microbiology*, 64(2-3), pp. 89–107. doi: 10.1016/s0378-1135(98)00262-4.

Jackova, A., Novackova, M., Pelletier, C., Audeval, C., Gueneau, E., Haffar, A., Petit, E., Rehby, L. and Vilcek, S. (2007) 'The extended genetic diversity of BVDV-1: Typing of BVDV isolates from France',

Veterinary Research Communications, 32(1), pp. 7–11. doi: 10.1007/s11259-007-9012-z.

Kennedy, J. A. (2006) 'Diagnostic efficacy of a reverse transcriptase–polymerase chain reaction assay to screen cattle for persistent bovine viral diarrhea virus infection', *Journal of the American Veterinary Medical Association*, 229(9), pp. 1472–1474. doi: 10.2460/javma.229.9.1472.

Kong, F., James, G., Gordon, S., Zelynski, A. and Gilbert, G. L. (2001) 'Species-specific PCR for identification of common contaminant mollicutes in cell culture', *Applied and Environmental Microbiology*, 67(7), pp. 3195–3200. doi: 10.1128/aem.67.7.3195-3200.2001.

Larson, L. R., Grotelueschen, D. M., Brock, K. V., Hunsaker, B. D., Smith, R. A., Sprowls, R. W., MacGregor, D. S., Loneragan, G. H. and Dargatz, D. A. (2004) 'Bovine Viral Diarrhea (BVD): Review for Beef Cattle Veterinarians', *Bovine Practitioner*, 38(1), pp. 93–102. Available at: http://www.aabp.org/members/publications/2004/prac_feb_04/feb04_14.pdf.

Nettleton, P. F. and Entrican, G. (1995) 'Ruminant pestiviruses', *British Veterinary Journal*, 151(6), pp. 615–642. doi: 10.1016/s0007-1935(95)80145-6.

Peterhans, E., Bachofen, C., Stalder, H. and Schweizer, M. (2010) 'Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction', *Veterinary Research*, 41(6), pp. 44–57. doi: 10.1051/vetres/2010016.

Sanger, F. (2001) 'The early days of DNA sequences', *Nature Medicine*, 7(3), pp. 267–268. doi: 10.1038/85389.

Ståhl, K. and Alenius, S. (2012) 'BVDV control and eradication in Europe — an update', *Japanese Journal of Veterinary Research*, 60(suppl.), pp. S31–S39. Available at: http://hdl.handle.net/2115/48530.

Steck, F., Lazary, S., Fey, H., Wandeler, A., Huggler, C., Oppliger, G., Baumberger, H., Kaderli, R. and Martig, J. (1980) 'Immune responsiveness in cattle fatally affected by bovine virus diarrhea-mucosal disease', *Zentralblatt für Veterinärmedizin Reihe B*, 27(6), pp. 429–445. doi: 10.1111/j.1439-0450.1980.tb01790.x.

Studer, E., Bertoni, G. and Candrian, U. (2002) 'Detection and characterization of Pestivirus contaminations in human live viral vaccines', *Biologicals*, 30(4), pp. 289–296. doi: 10.1006/biol.2002.0343.

Sullivan, D. G. and Akkina, R. K. (1995) 'A nested polymerase chain reaction assay to differentiate pestiviruses', *Virus Research*, 38(2-3), pp. 231–239. doi: 10.1016/0168-1702(95)00065-x.

Sung, H., Kang, S. H., Bae, Y. J., Hong, J. T., Chung, Y. B., Lee, C. K. and Song, S. (2006) 'PCR-based detection of Mycoplasma species', *Journal of Microbiology*, 44(1), pp. 42–49. Available at: http://www.msk.or.kr/inc/download_FIDX.asp?FTYPE=5&FIDX=2338.

Uphoff, C. C. and Drexler, H. G. (2002) 'Detection of mycoplasma in leukemia–lymphoma cell lines using polymerase chain reaction', *Leukemia*, 16(2), pp. 289–293. doi: 10.1038/sj.leu.2402365.

Van Kuppeveld, F. J., Van der Logt, J. T., Angulo, A. F., van Zoest, M. J., Quint, W. G., Niesters, H. G., Galama, J. M. and Melchers, W. J. (1992) 'Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification', *Applied and Environmental Microbiology*, 58(8), pp. 2606–2615. Available at: http://aem.asm.org/content/58/8/2606.full.pdf.

Vilček, S., Herring, A. J., Herring, J. A., Nettleton, P. F., Lowings, J. P. and Paton, D. J. (1994) 'Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis', *Archives of Virology*, 136(3-4), pp. 309–323. doi: 10.1007/BF01321060.

Vilcek, S., Durkovic, B., Kolesárová, M., Greiser-Wilke, I. and Paton, D. (2004) 'Genetic diversity of international bovine viral diarrhoea virus (BVDV) isolates: identification of a new BVDV-1 genetic group', *Veterinary Research*, 35(5), pp. 609–615. doi: 10.1051/vetres:2004036.

Part 2. Biotechnology and biology

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ANIMAL GENETIC RESOURCES OF UKRAINE: CURRENT STATUS AND PERSPECTIVES

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Summary. The objective diversity evaluation of genetic resources and genetic status of breeds in Ukraine is given. The targeted selection approach application enables a creation of the breeds range, being used in commercial purposes and for manufacturing the high-quality production. The high-priority breeds that need to be saved are determined The subjects preserving the gene pool of farm animals have to determine the gene pool for herds, farms, holdings, reserves and cryobanks of animal genetic resources. Rational form of the gene pool conservation is to preserve it within herds.

Keywords: cattle, breed, animal genetic resources

Animal industry of Ukraine has passed through the stages of profound changes, being associated with a steep decline of farm animal number and decrease of production volumes since independence time. Ukrainian cattle population counted 8378.2 thousand heads in 1990, 3635.0 thousand heads in 2005, and 2508.8 thousand heads in 2013. The milk production was estimated about 24.5 million tons in 1990, 13.7 million tons in 2005-2006 and 11.086 million tons in 2011. Nevertheless, for recent years there has been a steady tendency for agricultural production to have increased due to improved livestock productivity and modernization of production processes.The milk production has increased to 11.378 million tons in 2012 and 11.488 million tons in 2013. The dairy, pig and poultry farming remain to be traditional and economically attractive branches of the animal husbandry in Ukraine. The total numbers of farm animal species distributed between the farms of different ownership form in Ukraine are shown in Table 1.

In contrast to other European countries, the livestock of Ukraine is concentrated in large agricultural holdings being former farms of state or common ownership forms, and in small households having limited capability to meet the safety, production and storage standards of raw milk.

Animal industry modernization lagging determines relatively low or average livestock yield and production parameters for Ukrainian animals compared to European animals (Tab. 2).

About 76 % of the livestock are concentrated in rural households for today, including 52.6 % of cows reared at farms having less than 100 heads of cattle, 38.5 % at farms having 100-500 heads of cattle, and only 8.9 % cows are reared in holdings having more than 500 heads of cattle. Given the abolition of the raw milk quota in the European Union by 1st of April 2015, it raises the question of investing in large industrial complexes construction within Ukrainian agricultural holdings and development of cooperative movement oriented to improvement of milk produced at house holdings. Households own a substantial portion of other livestock species and accounted for 51 % of pigs, 85 % of sheep and goats and 42.6 % of all types of poultry.

The number of auditable livestock involved in improvement programs is comparatively small (Tab. 1). Controlled herds size is insufficient for implementation of breeding programs based on the best local gene pools, because these herds account approximately 4,6 % of the total cattle number, 4,4 % of the total pig number and 2,1 % of sheep.

Table 1 – The total number of farm animals in Ukraine for different year

Branch of animal husbandry	Species	As of 01.01.1916, thousand heads	As of 01.01.2014, thousand heads			
			Farms of all ownership forms, thousand heads *	Including		Auditable animals number **
				Agricultural holdings	Smallholdings (Private farms)	
Dairy cattle	Cow	4115.9	2508.8	565.4	1943.4	115.9
Meat cattle	Cow	-	-	33.4	-	16.3
Pig farming	Pigs, total	6469.2	7922.2	3878.9	4043.3	354.7
	including sows	-	-	252.5	-	32.4
Sheep farming	Sheep, total	6849.0	1735.2	248.5	1486.7	36.6
	including ewes and chilvers	-	-	141.6	-	21.1
Horse breeding	Horses, total	6454.5	354.2	29.1	-	3.8
	including broodmares	-	-	8.9	-	1.4
Poultry farming	All species		230204.7	132072.3	98132.4	2046.0
	including chicken			129392.4		1881.7
	geese			303.3		65.0
	ducks			480.5		79.4
	turkeys			770.3		-
	other			1126.8		19.9

* According to the Statistics Annual Report 1962, the uses of livestock in 1916 were presented by draft-cattle, meat cattle and cattle of combined use

** According to the "Condition of livestock in Ukraine: Statistical bulletin (2013)", State Statistics Service of Ukraine

*** According to the "State register of livestock breeding farms (2013)"

Table 2 – Livestock production parameters for Ukraine as of 01.01.2014

Branch of animal husbandry	Productivity index	Agricultural holdings *	Livestock breeding farms **
Dairy cattle	Milk yield, kg	4840	6116
Meat cattle	Number of calves born per 100 cows, heads	72	76
Pig farming	Average daily gain, g	474	1982
	Number of piglets born per 100 sows, heads	1936	1982
Sheep farming	Fleece per one sheep, kg	1,8	2,8
	Number of lambs born per 100 ewes, heads	76	90
Poultry farming	Average egg production per one hen, eggs	289	308

* According to the "Condition of livestock in Ukraine: Statistical bulletin (2013)", State Statistics Service of Ukraine

** According to the "State register of livestock breeding farms (2013)"

As for Ruban and Fedota (2013), the one way to manage breeding process within small herds is to identify appropriate gene panels associated with traits of economic importance or hereditary abnormalities and to create national genetic evaluation systems. Such system provides opportunity to accelerate evaluation and to improve the efficiency of livestock breeding, ensuring the livestock production of required genotypes for external and internal commerce. It is appreciated that genomic testing is based on conventional breeding approaches. From the other side, one of the cornerstone principles of these approaches is well-run breeding records system.

Conforming to the "State register of livestock breeding farms (2013)", the livestock breed composition of cattle, pigs, horses and sheep in Ukraine is characterized by the great diversity of domestic and imported breeds (Tab. 3–8). Generally, all breeds can be divided into two categories – commercial breeds, being commonly used for livestock production, and numerically small native breeds. The Table 3 shows the data on key milk production traits per cattle breed.

Table 3 – The cattle breeds composition (dairy and dual-purpose breeds for dairy and beef production) as of 01.01.2014 year *

Breed	Total population, heads	including cows, heads	Average per breed		
			milk yield, kg	%, fat	%, protein
Ukrainian Black-and-White Dairy	167967	62690	6019	3.83	3.27
Ukrainian Red-and-White Dairy	65499	26395	6103	3.80	3.29
Holstein	27406	11928	7310	3.82	3.30
Ukrainian Red Dairy	16044	6665	5843	3.85	3.25
Red Steppe	8600	3244	4164	3.86	3.74
Simmental	5387	2230	5208	3.84	3.24
Polish Red	1349	506	4098	3.83	3.17
Ayrshire	951	539	6513	3.82	3.14
Angler	300	39	4205	4.21	3.32
Lebedyn	2364	947	4445	3.80	2.94
Brown Swiss	121	100	4145	4.10	3.30
Ukrainian Brown Dairy	742	350	5463	3.89	3.02
Ukrainian Whitehead	848	300	4707	3.78	-
Total	**441578**	**115933**	**6114**	**3.77**	**3.28**

* According to the "State register of livestock breeding farms (2013)"

The Ukrainian Black-and-White Dairy is the most common dairy breed in Ukraine. The animals of this breed are raised in almost all regions of Ukraine, its intra-breed genetic diversity is low and consolidated group of animals show no significant variation in constitutional type and productivity level (Yefimenko, Burkat and Boiko, 1998).

The breed was developed by crossing the local black-and-white cattle of Dutch origin with the Holstein cattle of Canadian and American selection. It should be noted that Holstein breed is ranked third of thirteen species in Ukrainian dairy cattle population (Tab. 3), its popularity caused by import of semen from the USA and Canada sires and this breed's genetic potential affects the variety of domestic breeds.

The first cattle breed of Ukraine selection is the Ukrainian Red-and-White Dairy having been developed for over 20 years. This breed was developed by crossing the domestic Simmental cattle with imported genetic material of the Ayrshire (Finland), the Montbéliarde (France) and the Holstein (USA, Canada) breeds (Karasyk, Zubets and Burkat, 1993; Ruban, 1999). The total Ukrainian Red-and-White Dairy population within livestock farms is the second largest after population of the Ukrainian Black-and-White Dairy breed (Tab. 3).

The Ukrainian Red Dairy is a relatively young Ukrainian dairy cattle breed (Polupan et al., 2004). It was developed by the complex reproductive crossing of the Red Steppe cattle with the Angler, Danish, Red-and-White Holstein cattle.

The Red Steppe breed ranks the fifth place in number of dairy cattle, despite this breed in the early 1990s took the second place, and in the middle of the twentieth century was the most widespread Ukrainian breed. The breed was developed by the complex reproductive crossing the domestic Ukrainian Grey with the Red East Friesian, and later with some Swiss Brown and East Friesian, and was named "Red Steppe breed" in 1939.

The Lebedyn cattle on farms of different ownership account for 1 % of relative small group of brown breeds (Tab. 3, 4). The breed was developed from crossing the local breeds with the Schwyz breed. The Brown Carpathian was developed in the Trans-Carpathian region in result of crossing the Brown Swiss with the local cattle (Boyko, 1981). The Brown Carpathian and the Ukrainian Whitehead are local native breeds, being presented with low numbers in both pedigree and commercial farms (Tab. 3, 4).

The Ukrainian Whitehead was developed in Ukraine by crossing the local cattle with the Black-and-White Dutch bulls imported from the Province of Groningen, Holland in 1791 (Boyko, 1981).

The volume of beef cattle production in Ukraine is low, but has a potential to growth. The main task of breeding farms is to reproduce the breeding material from both domestic and foreign specialized beef breeds (Tab. 5).

Table 4 – The cattle breeds composition reared in farms under various forms of ownership as of 01.01.2014 year *

Breed	Total number of cows		Including					
			house holdings		Agricultural holdings			
	thousand heads	%	thousand heads	%	thousand heads	%	including breeding animals	
							thousand heads	%
Black-and-White breeds group								
Ukrainian Black-and-White Dairy	962.505	38.3	753.684	38.8	208.821	36.9	62.690	54.0
Holstein	130.090	5.2	82.847	4.2	47.243	8.3	11.928	10.3
Other	68.694	2.8	62.953	3.3	5.741	1.1	-	-
Total for five breeds	**1161.289**	**46.3**	**899.484**	**46.3**	**261.805**	**46.3**	**74.618**	**64.3**
Red-and-White breeds group								
Ukrainian Red-and-White Dairy	336.284	13.9	232.613	11.9	103.671	18.3	26.395	22.7
Simmental	202.654	8.0	151.422	7.7	51.232	9.0	2.230	2.0
Other	113.732	4.1	88.886	4.7	24.846	4.5	-	-
Total for five breeds	**652.670**	**26.0**	**472.921**	**24.3**	**179.749**	**31.8**	**28.625**	**24.7**
Red breeds group								
Ukrainian Red Dairy	40.978	1.6	24.287	1.2	16.691	2.9	6.665	5.7
Red Steppe	284.194	11.3	236.962	12.2	47.232	8.3	3.244	2.8
Other	131.561	5.3	127.530	6.6	4.031	0.8	0.526	0.5
Total for five breeds	**456.733**	**18.2**	**388.779**	**20.0**	**67.954**	**12.0**	**10.435**	**9.0**
Red breeds group								
Brown Carpathian	44.182	1.7	41.714	2.1	2.468	0.4	-	-
Lebedyn	28.888	1.1	23.576	1.2	5.312	0.9	0.947	0.9
Other	12.215	0.6	10.882	0.6	1.333	0.3	-	-
Total for four breeds	**85.285**	**3.4**	**76.172**	**3.9**	**9.113**	**1.6**	**0.947**	**0.9**
Other breeds								
Total	**152.832**	**6.1**	**106.049**	**5.5**	**46.783**	**8.3**	**1.332**	**1.1**
Total for all breeds	**2508.809**	**100.0**	**1943.405**	**100.0**	**565.404**	**100.0**	**115.957**	**100.0**

The Volinian Beef cattle herds are the most abundant, the breed was recognized as the selection achievement in 1993. The breed total number accounted 5 thousand heads, including 2 thousand heads in Volyn and Rivne regions at that time. The Volinian Beef was developed by the complex reproductive crossing of local Polish Black-and-White and the Polish Red cattle with the Aberdeen-Angus, Limousin and Hereford sires (Yanko, Burkat and Lukash (1998).

The Polissian Beef was developed by the method of complex reproductive crossing for domestic Simmental, the Ukrainian Grey and imported Charolais, Chianina and Angus (Speka, 1999). The Polissian Beef cattle are bred in Zhytomyr, Lviv and Rivne regions.

Table 5 – The beef breeds composition as of 01.01.2014 year *

Breed	Total population, heads	including cows, heads
Volinian Beef	11025	4824
Aberdeen-Angus	8926	4110
Polissian Beef	5065	2298
Southern Beef	3132	1122
Simmental Beef**	3070	1345
Ukarainian Beef	1970	831
Charolais	1047	459
Polissian Beef (Znamensk)	1033	348
Ukrainian Grey	928	351
Limousin	636	270
Blonde d'Aquitaine	399	213
Hereford	131	54
Piemontese	49	10
Total	**37411**	**16235**

* According to the "State register of livestock breeding farms (2013)"; ** new developing breed (this breed is not yet officially recognized)

The Ukrainian Beef breed is the first domestic specialized breed of cattle, having been adapted to most climatic zones of Ukraine; its population at the moment of approbation in 1993 was about 20 thousand heads, including 5 thousand cows. The breed was developed from complex reproductive crossing of the Ukrainian Grey with improver breeds – the Simmental, Charolais and Chianina (Dorotiuk, Lukash and Harmash, 1998).

The Southern Beef breed was approved in 2009 and was developed from the complex reproductive crossing of the Red Steppe (maternal basis) with the Hereford, Charolais, Santa Gertrudis using hybridization with Cuban zebu hybrids. Breed specific characteristics include zebu-shaped body, disease resistance and toleration to steppe climate (Zubets, Burkat and Melnyk, 2009). The Ukrainian Grey is one of native draft-cattle breeds. The number of the breed in 1916 and 1922 in Ukraine was 2.813 and 2.568 million heads (Zorin, 1953). The total number of the animals is diminished to 147.1 thousand heads in 1949 due to greater use of agricultural machinery for tillage and other works rather than cattle as a draft power. The Ukrainian Grey cattle population in Ukraine accounted 928 heads in 2013.

The swine rearing is a traditional branch of animal husbandry in Ukraine. The classical breeds are the Large White and the Landras (Tab. 6). The third and fifth by the number are domestic breeds. The Poltavian Meat breed is the first high-yield pig breed in Ukraine, meeting the actual market requirements in respect of the lard or meat taste, having been developed by the complex reproductive crossing of the breeds: the Large White, the Mirgorod, the Landras, the Pietren and the Wessex Saddleback (Bankovskyi, 1998).

The pedigree breeding for the Ukrainian Meat pig breed creation was carried out from 1981 to 1993, using as a basis the Poltavian Meat pigs and pigs of Kharkiv, Belarus and Ascanian selection. The breed is a complex combination of the Large White, the Mirgorod, the Landrace, the Welsh, the Pietrain, the

Wessex Saddleback and the Ukrainian Steppe White genotypes (Gerasimov et al., 2006).

The Red White-Belt Meat pig breed was created out of the Poltavian pigs, the Large White, the Landras, the Durok and the Hampshire breeds by the complex reproductive crossing method, followed by mating within hybrids. When mating the boars of the breed with sows of planned breeds the heterosis effect is about 5–15 %. The Mirgorod pig breed being created by a long-term massive selection within the Poltava pig population is of particular interest. The breeding of local short-eared pigs with the Berkshire and the Middle White pigs was started in 1880 within small group of animals, but later the large black boars and sows of the Tamworth breed were brought to Ukraine. It brought along creation of the black-and-white pig herds, which found ready market for small households (Rybalko, Buhaievskyi and Kuian, 2010).

Table 6 – The swine breeds composition as of 01.01.2014 year *

Breed	Total population, heads	including sows, heads
Large White	183910	13551
Landras	132334	15518
Ukrainian Meat	10033	801
Poltavian Meat	8980	617
Red White-Belt Meat	6444	463
Durok	3767	309
Pietren	2581	255
Mirgorod	2056	171
Large White (English selection)	1615	133
Ukrainian Steppe White	1495	283
Large Black	1076	215
Welsh	313	100
Ukrainian Steppe Black-and-White	92	21
Total	**354696**	**32437**

* According to the “State register of livestock breeding farms (2013)”

The basis of the Ukrainian White Steppe breed, being developed in the Biosphere Reserve “Askania Nova” (Kherson region), was the hybrids from crossing with the English breed boars adapted to the Southern Ukrainian regions. Later, the Ukrainian Steppe Black-and-White breed was created by crossing the Ukrainian White Steppe sows with the Berkshire and the Mangalitsa boars within Biosphere Reserve “Askania Nova”. The last three breeds belong to the minority of local breeds and need the systemic support for their conservation.

The horse livestock population of Ukraine has rapidly reduced for recent years, the population accounted 354.2 thousand heads in 2013 that is 18 times less than in 1916 - 6.4 million heads (Tab. 7). Currently, the total number of breeding horses is 3863 heads, including 1381 heads of broodmares (Tab. 7).

The creation of new domestic roadster breed has been initiated in Ukraine in 1945. When created the Ukrainian Roadster breed (Tab. 7) the horses of more than 11 breeds were used, including the Thoroughbred, the Trakenenian, the Hungarian, the Russian Trotter, less role played the Hanover, the Arabic and the Akhal-Teke breeds. However, the breed was approved only in 1990, before this it was known as the Ukrainian breeding group. Currently, work for the Ukrainian Roadster breed improving is underway within six state stud farms and 16 private stud farms amounting more than 1,000 horses (Hopka, Skotsyk and Pavlenko, 1998).

Except the Ukrainian Roadster breed, the Ukrainian national heritage includes the Hutsul breed. The Hutsul breed was formed in the Carpathian region, being first mentioned in historical documents in 1603 without any hypothesis to its origin (Golovach M. Y. and Golovach M. M., 2004). According to these authors, the number of the Hutsul horses reached in Poland – 745, Ukraine – 390, Czech Republic – 360,

Romania – 200, Slovakia – 120, Hungary – 100, Austria – 51 and Germany – 28 heads. According to several authors, the Hutsul horses are created by crossing the breeds of Tatar and Arab origin with the Tarpan, and have acquired functionally necessary traits such as good health, labor productivity, efficient feed use, fitness to work both under saddle and packs in result of exploitation of horses in the mountains.

Table 7 – The horse breeds composition as of 01.01.2014 year *

Breed	Total population, heads	including broodmares, heads
Ukrainian Roadster	1037	376
Orlov Trotter	803	289
Thoroughbred	688	223
Russian Trotter	629	228
Newolexandrian heavy draft	301	98
Westphalian	166	70
Hutsul	108	50
Trakenenian	50	15
French Trotter	32	12
Russian Heavy Draft	29	10
Torian	20	10
Total	**3863**	**1381**

* According to the "State register of livestock breeding farms (2013)"

The modern sheep breeding in Ukraine aimed at creating the dual-purpose sheep breed for wool and meat production, since this combination provides for maximum profit within this branch of livestock industry (Iovenko et al., 2006).

The list of breeding sheep breeds is headed by the Askanian meat-wool breed with crossbred wool (Tab. 8). The breed is developed out of the complex combination crossing the half-bred Lincoln-Ascanian sheep (wool quality of 48-46 grade) with the Lincoln-Tsigai ewes followed by further mating within hybrids. The Askanian crossbreed animals are large and precocious, being characterized by the strong constitution, high meatiness, high milk and wool productivity.

The history of Sokilska sheep breed in Ukraine counts about 500 years. Apparently, its pedigree includes the sheep raised for milk and fur, having been introduced by Tatarian invaders from the lower Volga. There are two versions about the origin of this sheep breed, the most probable is to be originated out of crossing a local rat-tailed sheep with the Karakul and Malich (Iovenko et al., 2006).

The Askanian Fine-wool sheep breed is derived from crossing the local Merino sheep with the Rambouillet of American selection and simultaneous improvement of animals feeding conditions.

The Ukrainian Carpathian Mountain breed is created out of the reproductive crossing the Tsakel sheep, the local rough-wool sheep breed with the half-fine-wool Tzigaia sheep (Petryshyn, 1998). The Ukrainian Carpathian Mountain sheep are reared in farms of all ownership forms in foothill and mountain regions of Zakarpattia, Ivano-Frankivsk, Lviv and Chernivtsi. The total number of breeding animals is 2.7 thousand heads (Tab. 8).

There are regional programmes for the conservation of the Askanian Fine-wool, the Ukrainian Carpathian Mountain, the Tsigai, the Romanov, the Sokilska breeds.

The poultry industry is one of the industrial and high-tech livestock industries in Ukraine through the implementation of major projects for the egg and meat production using specialized crosses.

The Ukrainian Vushanka characterized by ruggedness and durability is the breed developed in 17th century in Central and Northern parts of Ukraine. It was called due to its external exterior signs – the ear lobes, being closed dense fine feathers (Ruban, 2002).

The Romensky geese breed is developed in Sumy region, based on local breeds. To date this breed is widely spread in households of such areas as Sumy, Kiev, Chernigiv, Poltava, Luhansk and other regions (Pabat et al. , 2006).

Table 8 – The sheep breeds composition as of 01.01.2014 year *

Breed	Total population, heads	including ewes, heads
Askanian meat-wool breed with crossbred wool	8250	5146
Askanian Karakul	7748	4830
Askanian Finewool	7516	4072
Prekos	3179	1470
Ukrainian Carpathian Mountain	2760	2114
Merinolandshaft	2124	972
Tsigai	2113	1200
Romanov	1836	589
Sokilska	610	382
Latvian Blackhead	229	160
North-Caucasian	256	171
Total	**36621**	**21106**

* According to the "State register of livestock breeding farms (2013)"

The number of the Romensky geese reached 38 thousand heads, at ten years after the geese herd decreased twice. To date the number of the Romensky geese is about 22,500. Predominantly, the geese are reared in small farms.

The population of the Ukrainian White-Breasted Black ducks (Podstreshny and Bondarenko, 1998b) is developed within the Poultry Research Institute of the UAAS in the 50s of the last century out of the complex reproductive crossbreeding the local Ukrainian White-Breasted with the Pekin breed and Khaki Campbell breed ducks. The Ukrainian Grey and the Ukrainian Clay ducks are bred by the Poultry Research Institute in 40s – 60s years of the 20th century. The breed group is created by the authors under the guidance of Dahnovskij using breeding only within local gray duck breeds (Podstreshny and Bondarenko, 1998a).

Conclusion. The livestock of Ukraine is characterized by a wide variety of the species and breeds. However, there was a significant reduction in the livestock number for the last two decades followed by the decreased diversity of farm animal species.

The Ukrainian Grey, the Ukrainian Whitehead, the Brown Carpathian, the Lebedyn cattle breeds, the Hutsul horsed breed, the Sokilska and the Ukrainian Carpathian Mountain sheep breeds, the Mirgorod, the Ukrainian Steppe White and the Ukrainian Steppe Black-and-White pig breeds are threatened breeds. Mainly, the domestic poultry breeds are reared within private collection farms or households. The subjects preserving the gene pool of farm animals have to determine the gene pool for herds, farms, holdings, reserves and cryobanks of animal genetic resources. Rational form of the gene pool conservation is to preserve it within herds.

References

Anon. (1962) *The national economy of the Ukrainian SSR in 1961. Statistical yearbook [Narodne hospodarstvo Ukrainskoi RSR v 1961 rotsi. Statystychnyi shchorichnyk]*. Kyiv: Derzhavne statystychne vydavnytstvo. [in Ukrainian].

Bankovskyi, B. V. (1998) 'The Poltavian meat pig breed' [Poltavska miasna poroda svynei], in *Pedigree resources of Ukraine [Pleminni resursy Ukrainy]*. Kyiv: Ahrarna nauka, pp. 168–171. ISBN 966-540-199-8. [in Ukrainian].

Boyko, V. P. (1981) *Cattle breeds in Ukraine in the historical development and their evaluation for milk yield [Porody krupnogo rogatogo skota Ukrainy v istoricheskom razvitii i ikh otsenka po molochnoy produktivnosti]*. Kiev. [in Russian].

Dorotiuk, E. M., Lukash, V. P. and Harmash, I. O. (1998) 'The Ukrainian beef breed of cattle' [Ukrainska miasna poroda], in *Pedigree resources of Ukraine [Pleminni resursy Ukrainy]*. Kyiv: Ahrarna nauka, pp. 88–90. ISBN 966-540-199-8. [in Ukrainian].

Gerasimov, V. I., Berezovskiy, M. D., Nagaevich, V. M., Rybalko, V. P., Baranovskiy, D. I., Kovalenko, V. P. and Pelykh, V. G. (2006) *The worldwide genetic resources of pigs [Mirovoy genofond sviney]*. Kharkov: Espada. ISBN 966-7870-93-6. [in Russian].

Golovach, M. Y. and Golovach, M. M. (2004) 'The origin of the Hutsul horse breed lines and types' [Pokhodzhennia linii i typiv konei hutsulskoi porody], *News of Agrarian Sciences [Visnyk ahrarnoi nauky]*, 2, pp. 50–54. [in Ukrainian].

Hopka, B. M., Skotsyk, V. Ye. and Pavlenko, P. M. (1998) 'The Ukrainian saddle horse breed' [Ukrainska verkhova poroda], in *Pedigree resources of Ukraine [Pleminni resursy Ukrainy]*. Kyiv: Ahrarna nauka, pp. 139–141. ISBN 966-540-199-8. [in Ukrainian].

Iovenko, V. M., Polska, P. I., Antonets, O. H., Bova, V. M., Bolotova, T. H., Voronenko, V. I., Horlov, O. I., Danylenko, H. K., Zharuk, P. H. and Kalashchuk, H. P. (2006) *The sheep farming in Ukraine [Vivcharstvo Ukrainy]*. Kyiv: Ahrarna nauka. ISBN 966-540-082-7 [in Ukrainian].

Karasyk, Yu. M., Zubets, M. V. and Burkat V. P. (1993) 'The Ukrainian red-and-white dairy breed tested and approved' [Aprobovano i zatverdzheno ukrainsku chervono-riabu molochnu porodu], *News of Agrarian Sciences [Visnyk ahrarnoi nauky]*, 2, pp. 20–33. [in Ukrainian].

Pabat, V. O., Mykytiuk, D. M., Frolov, V. V., Bilous, O. V., Riabokon, Yu. O., Katerynych, O. O., Bondarenko, Yu. V., Mosiakina, T. V., Kovalenko, H. T., Hadiuchko, O. T., Hrytsenko, D. M., Bohatyr, V. P. and Liutyi, Yu. S. (2006) *A catalogue of poultry pedigree resources of Ukraine [Kataloh pleminnykh resursiv silskohospodarskoi ptytsi Ukrainy]*. Kyiv: Atmosfera. [in Ukrainian].

Petryshyn, M. A. (1998) 'The Ukrainian Carpathian mountain sheep breed' [Ukrainska hirskokarpatska poroda], in *Pedigree resources of Ukraine [Pleminni resursy Ukrainy]*. Kyiv: Ahrarna nauka, pp. 202–204. ISBN 966-540-199-8. [in Ukrainian].

Podstreshny, O. P. and Bondarenko, Yu. V. (1998a) 'The ducks of Ukrainian breed group' [Kachky ukrainskoi porodnoi hrupy], in *Pedigree resources of Ukraine [Pleminni resursy Ukrainy]*. Kyiv: Ahrarna nauka, pp. 259. ISBN 966-540-199-8. [in Ukrainian].

Podstreshny, O. P. and Bondarenko, Yu. V. (1998b) 'The Ukrainian white-brested black duck breed' [Chorni bilohrudi kachky], in *Pedigree resources of Ukraine [Pleminni resursy Ukrainy]*. Kyiv: Ahrarna nauka, pp. 260. ISBN 966-540-199-8. [in Ukrainian].

Polupan, Yu. P., Mykytiuk, D. M., Lytovchenko, A. M., Burkat, V. P. and Havrylenko, M. S. (2004) *The program of the Ukrainian red dairy breed selection for 2003-2012 [Prohrama selektsii ukrainskoi chervonoi molochnoi porody velykoi rohatoi khudoby na 2003-2012 roky]*. Kyiv. [in Ukrainian].

Ruban, B. V. (2002) *Poultry and poultry breeding [Ptitsy i ptitsevodstvo]*. Kharkov: Espada. ISBN 966-7870-10-3. [in Russian].

Ruban, S. Yu. (1999) *Methodology and system of selection for animals of the Ukrainian red-and-white dairy breed [Metodologiya i sistema selektsii zhivotnykh ukrainskoy krasno-pestroy molochnoy porody]*. The dissertation thesis for the scientific degree of the doctor of agrarian sciences. Kharkiv: Institute of Animal Breeding of Ukrainian Academy of Agrarian Sciences. [in Russian].

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. [in Ukrainian].

Rybalko, V. P., Buhaievskyi, V. M. and Kuian, N. V. (2010) 'About the status of the red white-belt meat pig breed maintain' [Pro stan sprav u vedenni ukrainskoi chervonoi bilopoiasoi porody svynei], *Effective animal breeding [Efectyvne tvarynnytstvo]*, 6, pp. 8–10. [in Ukrainian].

Speka, S. S. (1999) *The Polissian beef breed of cattle [Poliska miasna poroda velykoi rohatoi khudoby]*. Kyiv. ISBN 966-7306-18-6. [in Ukrainian].

Yanko, T. S., Burkat, V. P. and Lukash, V. P. (1998) 'The Volinian beef breed of cattle' [Volynska miasna poroda], in *Pedigree resources of Ukraine [Pleminni resursy Ukrainy]*. Kyiv: Ahrarna nauka, pp. 66–68. ISBN 966-540-199-8. [in Ukrainian].

Yefimenko, M. Ya., Burkat, V. P. and Boiko, V. P. (1998) 'The Ukrainian black-and-white dairy breed' [Ukrainska chorno-riaba molochna poroda], in *Pedigree resources of Ukraine [Pleminni resursy Ukrainy]*. Kyiv: Ahrarna nauka, pp. 46–48. ISBN 966-540-199-8. [in Ukrainian].

Zorin, I. G. (1953) *The Ukrainian grey breed of cattle [Sira ukrainska khudoba]*. Kyiv: Derzhsilhospvydav URSR. [in Ukrainian].

Zubets, M. V., Burkat, V. P. and Melnyk, Yu. F. (2009) 'The Southern meat breed of cattle – an outstanding achievement in the theory and practice of agrarian science' [Pivdenna miasna poroda velykoi rohatoi khudoby – vyznachne selektsiine dosiahnennia v teorii ta praktytsi ahrarnoi nauky], *News of Agrarian Sciences [Visnyk ahrarnoi nauky]*, 3, pp. 45–51. [in Ukrainian].

Part 3. Biosafety and biosecurity

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MODERN TURKISH APPROACHES TO SOILS' DECONTAMINATION FROM ANTHRAX' AGENT WITH ATTENTION TO UKRAINE NEEDS

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Summary. The Turkish experience on soil decontamination is based by modern conception of anthrax' driving forces – about clue role of bacteriophages in natural history of anthrax agent. This approach (as Kafkas University SOP) was proposed to approbation by Turkish-Ukrainian scientifically cooperation in framework of EU-project for soil decontamination.

Study was performed by the bacteriological (bacteria and phages isolation, identification, and cultivation, PCR) and epidemiological (surveillance with sampling, epidemiology analysis) methods. Samples were collected using basic methods from animal burial sites in Eastern Parts of Turkey (Kars, n=5) and Ukraine (Gorlovka, n=1). Soil desporulation conducted by germination of spores and elimination of vegetative *B. anthracis* with phages or peracetic acid. All procedures were standardized by SOPs of Kafkas University as in Turkish experience for soil desporulation.

The SOPs for surveys of the soil anthracic spores and phages, and soil desporulation (7 sites of sampling, 27 specimens) is effective, ecological friendly and economically low-cost. On two trials in Turkey (burial area "Külveren", S≈30m^2) and Ukraine (sample of soil from burial area "Gorlovka-2", weight 150 g) the contaminated soils (5×10^4 and 5×10^5, respectively) were de-sporulated during 24 hr. and hadn't residual viable spore in germinator presence even. Epidemiological analysis proves the Donbas region as most dangerous by anthrax' prognosis.

Turkish method is very useful to application in Ukraine, especially on the liberated territories of Donbas.

Keywords: spores of Bacillus anthracis, monitoring of soils contamination, decontamination procedure, Turkish technology, Ukrainian needs

Introduction. Anthrax to pose a dual threat – as a natural disease of all mammal species and as a biological weapon too. Therefore, attention to the problems of anthrax is growing in parallel to escalations of geopolitical situation – as such as aggressive Russian actions in Ukraine now. Over the last decade, by the efforts of research teams in the United States, Italy, Turkey and other countries, concept of anthrax' epidemiology has received important supplements and some details of this conception were even revised (Turnbull, 2002; Bouzianas, 2007; Schuch and Fischetti, 2009). To the last time, the lifecycle of *B. anthracis* often described by a short vegetative bursts in infected hosts alternating with long periods of dormancy as an environmental spore until disease is re-established. Environmental surveys show that *B. anthracis* can sporulate outside of anthrax carcasses, yielding an infectious cell type that is resistant to adverse conditions and is recoverable from the soil for long periods (Fig. 1a). To last time all events, which achieve of re-establishment of anthrax at contaminate territory, were some obscure and has a different explanations. Much more realistic and popular is Van Ness' paradigm (1971) about *B. anthracis* transformation from saprophyte into pathogen in so named "vegetative incubator area" (right part of Fig.1a). Modern dates of Rockefeller' Science Centre clear proof main factor of *B. anthracis* transformation from saprophyte into pathogen is lytic and lysogenic anthracic bacteriophages (Schuch and Fischetti, 2009). Other words, as like to "true" viruses, which are drivers of natural history of the highest forms of Earth Biota (like to animals and plants species by V. Vernadsky (1991), phages drive of behavior of the *B. anthracis* in environment. Therefore, modern view on the anthrax agent lifecycle (Schuch and Fischetti, 2009) stand the host-virus relationships at center of events of the *B. anthracis'* transformation from vegetative form to spores and back (Fig. 1b). Therefore, modern approaches to anthrax regulation may based

on promotions actions for rise of the "useful" anthracic bacteriophages activity in environmental objects of contaminate area. As on Fig. 1b show, such a "useful" phage may be ones that encoding of so-named σ factors: lysogenic *B. anthracis* with σ factor no able to sporulation (Mock and Fouet, 2001).

So to sanitation of environment especially important significance have the stimulation of phage's species that block the sporulation in mentioned vegetative incubator areas and in rhizosphere system (Fig. 1b). This principle is founded in base of decontaminate procedure that is developed in Kafkas University (t. Kars, Turkey). Turkish scientists revealed clue role of soil desporulation in its decontamination from *B. anthracis*. Therefore, regulation measures in "Turkish method" consist a both equivalent parts – 1) anthracic sporulation survey (sampling, studies of soil spores concentration, its life-ability, sporulation activity, soil phages characteristics); 2) anthracic sporulation control (provocation of soil spores growth, de-sporulation of soil by lytic phages, maintenance of soil phages and surveys quality of soil de-sporulation). Present paper summarized of initial results of "Turkish method" approbation in frame of Turkey-Ukraine scientific cooperation, which founding with European Union grant "AEDNet" (EU FP7).

Materials and methods. Bacterial & phages strains and growth conditions – The *B. anthracis* and phages strains used in this study were described in Table 1. As sources of environmental *B. anthracis* were soil of burial sites and pastures of the five agricultural holdings for cattle and/or sheep in Kars' Province (Turkey). In addition, we have taken two soil samples from old burial sites with cattle carcasses on territory of agricultural holding in Gorlovka district of Donetsk region (Ukraine) after explosions during war actions. All soil samples taken by standard "envelope" method – 5 points of the envelope at a distance of 1 m from its center point at a depth of 5–10 cm for each sample.

Bacterial cultures grown in Luria broth (LB) (Life Technologies, UK), brain-heart infusion broth (BHI) (Sigma-Aldrich, UK), or Leighton-Doi broth (Buyuk et al., 2013) according to standard protocols; plates were made by adding Bacto agar (Difco, US) to a final concentration of 1.6 %. All procedures with Turkish samples/strains conducted in Kafkas University, and with Ukrainian samples / strains – in NSC "IECVM".

B. anthracis spores isolation/titration method from soil- performed by schema above. Briefly, soil suspended

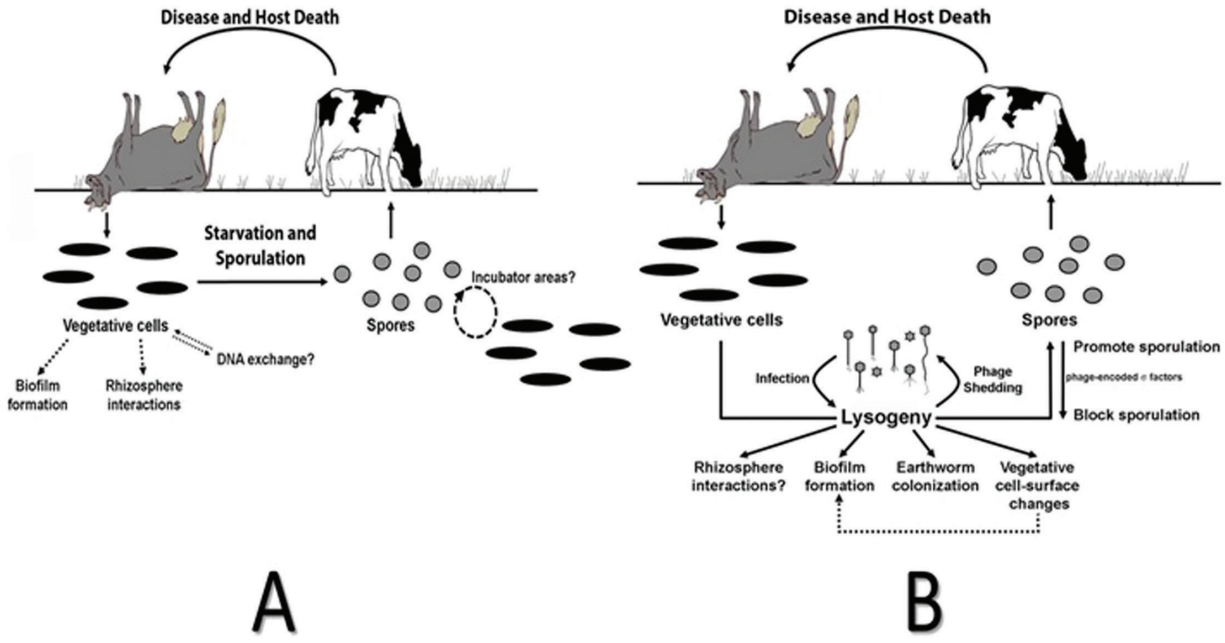
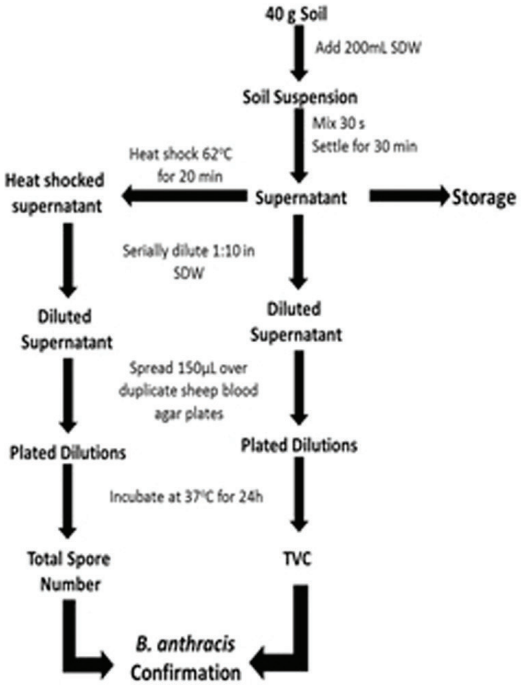


Figure – 1 The *B. anthracis* lifecycle by Schuch R1, Fischetti VA (2009) in our modification A – Former paradigm of *B. anthracis* lifecycle in which agent disease persists as a vegetative form in a model rhizosphere system, it's dormant spores (the infectious cell-type) are ingested by grazing hervibores and then germinate to produce a vegetative cell-type that causes fulminant disease in "Incubator area". B – Modern conception of *B. anthracis* lifecycle. Details – see the text



with sterile distilled water (SDW) and mixed well with shaking by hand. After incubation as drawing on schema, 2 ml of each supernatants used for isolation of both total viable cells and spores of *B. anthracis* (two portions, 1 ml of each). For bacterial cells and spores isolation were prepared 10 fold dilution on SDW. However, before bacterial spores isolation supernatant treated with heat at 62.5–63.0 °C for 15–20 min. and then dilution conducted.

All dilutions of sample was plated on blood agar plates in duplicate with a volume of 100–150 µl. Plates were incubated at 37 °C for overnight or 48 hr. at aerobic condition. Plates were checked for *B. anthracis* colony and performed the confirmation test.

Calculation of total viable spore (TVS) amount performed with considered of confirmed colonies of *B. anthracis* by next formulations:

$$\frac{\text{Colony number (confirmed as } B.\ anthracis)}{\text{Plated amount on agar plates X Dilution rate}} = \text{spore/ml}$$

The calculation method was improved with division of the spore amount in per milliliter to the value of soil amount (0,2 g) in 1 ml water to obtained total spore number in per gram soil.

Phage isolation method from soil – We used two different methods: 1 – for lytic phages isolation with a host without of inducers; 2 – total phage isolation with a host and Mitomycin C (Kyowa Hakko Kogyo Corporation, Tokyo, Japan). For lytic phage isolation, five grams of finely ground topsoil combined with 5 ml of Brain Heart Infusion (BHI) Broth (Sigma-Aldrich, US) and incubated at 37 °C for 2 hr. Followed this, 5 ml of a mid-log phase culture (the 3 to 5 hours culture of strain in BHI Broth with magnetic stirrer) of the Sterne strain of *B. anthracis* in BHI then added and the mixture incubated at 37 °C overnight. The following day top 5 ml of culture was harvested and filtered using a 0.22 µm membrane filter (Millipore, Massachusetts, US) to remove debris. The filtrate was stored at 4 °C until required. For total phage isolation, five grams of finely ground topsoil combined with 5 ml of Brain Heart Infusion (BHI) Broth and incubated at 37 °C for 2 hours. Then, 1 µl of 2 mg/ml solution of Mitomycin C added and the culture incubated for a further hour. Followed this, 5 ml of a mid log phase culture of the Sterne strain of *B. anthracis* in BHI was added and the mixture incubated at 37 °C overnight. The following day top 5 ml of culture was harvested and filtered using a 0.22 µm membrane filter to remove debris. The filtrate was stored at 4 °C until required.

Plague assay for phage activity detection – To determine the lytic activity of phage recovered using the method a plaque assay performed on Columbia Agar (CA) (Sigma-Aldrich, US) and *B. anthracis* Sterne used as host. The plaque assays carried out according to the method of T. G. Abshire (Abshire, Brown and Ezzell, 2005). Brief, host bacterial inoculum for phage propagation was prepared by transferring five to six isolated colonies from the CA culture grown overnight to 5 ml of sterile 10 mM phosphate-buffered saline (PBS), pH 7.2. CA plates were inoculated with 100 µl of the bacterial suspension, spreaded with a disposable plastic spreader until absorbed and 15 µl of the phage suspension delivered on agar surface. After fluids absorption, the plates were incubated at 37 °C overnight in inverted position. The cultures were inspected for plaque formation at regular intervals.

Phage amplification – To increase the concentration of *B. anthracis*-specific soil phages we mixed an equal volume of phage filtrate with a mid-log phase culture of the Sterne strain and incubated at 37 °C overnight. Next day the culture was spun down (8000 g, for 10 min), and supernatant filtered through 0.20 µm membrane filter. The phage titer was determined by performing a plaque assay. This process repeated until a sufficiently high-titer phage stock obtained.

Determination of the soil phages by routine test dilution (RTD) – The turbidity of an overnight culture of the Sterne strain of *B. anthracis* in BHI Broth was adjusted to a Mc Farland standard of 0.5 (1.5×10^8cfu/ml) using isotonic peptone-saline. The surface of a CA plate was 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 phage dilution was dropped onto the surface of the inoculated plates and left to dry for ten minutes. After which the plates were incubated at 37 °C and examined for the presence of plaques at 24-hour intervals. The most diluted suspension to produce complete clearing considered as the routine test dilution (RTD).

Soil desporulation procedures – These procedures were performed by two ways: 1 – on land plot that was positive on *B. anthracis* spore in Kars province; 2 – on contaminated soil sample from Gorlovka premise of Donbas region. Procedure for animal burial site in Kars province (Külveren) include the soil develop by desporulation mixture (special soil phages liquid culture in mix with *B. anthracis* germinator in standard concentration). The five soil samples were taken on land area of 1 m^2 by "envelope" method (see above) before and 24 hr. after soil developed by desporulation mixture. In second case the same desporulation mixture the same *B. anthracis* germinator in standard concentration implicated to Gorlovka' sample (5 g in three repetitions). Disinfection of vegetative forms of *B. anthracis* in Gorlovka sample conducted by peracetic acid with next neutralization of acid by Na-bisulfide [9].

Authors have considers all above procedures as Standard Operation Procedures (SOP) of Kafkas University (Turkey).

Results of study. Approbation of SOP for surveys of the soil anthracic spores and phages - In Table 1 the main results of soil anthracic spore and phages isolation and identification by Kafkas University' SOP is summarized. All soil samples contain both the *B. anthracis* spores and lytic phages. Levels of soil' contamination by spores depended from place of sampling. TVS-index of three probes (two from Turkey, one from Ukraine) was 5×10^5 and of another fours – 5×10^3 (one from Turkey, one from Ukraine), 5×10^4 and 5×10^7 (both from Turkey). *B. anthracis* bacteriophages soil-isolates showed titers < 10^{-1} RTD on first passages as on Sterne strain ("Turkish" isolates), as on "55" strain ("Ukrainian" isolates) – see numerator in RTD-column of Table 1. Nevertheless, on passages №№ 3–5 bacteriophages titers increased to 10^{-3}–10^{-5} RTD on Sterne strain ("Turkish" isolates), and to 10^{-2}–10^{-3} RTD on "55" strain ("Ukrainian" isolates) – see enumerators in RTD-column of Table 1. All of studied "Turkish" isolates had the capsules – main sign of *B. anthracis* pathogenicity.

In both Gorlovka' isolates capsule was absent, that we can explain by intensive using of live spore' and non-spore' vaccines against ruminant animal anthrax at this region of Ukraine.

On figures 2 and 3 the typical results of soil spores and phages growth and identification are presented. There are wide diversity of spore' bacteria species founding in all studied soil specimens (Fig. 2a). Main problem for this stage of work is right selection of colony with typical for *B. anthracis*' growth pattern: these are middle-size colonies with small tail and with surface with some yellowish

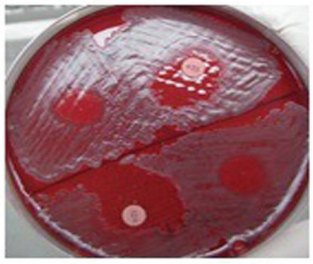
Table 1 – Results of primary analysis of soil' samples on presence of spores of the *B. anthracis*

Sample			Sites of sample	TVS	Capsula	RTD[1)]	PCR	
Code	Time of contamination	Time of sampling					PA	CAP
Dikme	2010	27.05.2014	Animal Burial	$5x10^5$	Pos.[2)]	10^{-5}	Pos.	Pos.
Selim 1	2006	27.05.2014	Animal Burial	$5x10^3$	Pos.	10^{-4}	Pos.	Pos.
Selim 2	2006	27.05.2014	Animal Burial	$5x10^7$	Pos.	10^{-3}	Pos.	Pos.
Külveren	2013	10.2013	Animal Burial	$5x10^4$	Pos.	10^{-3}	Pos.	Pos.
Subatan	2012	10.2013	Animal Burial	$5x10^5$	Pos.	10^{-4}	Pos.	Pos.
Gorlovka 1	U.N.[2)]	24.11.2014	Destroy area in Animal Burial	$5x10^3$	Neg.[2)]	10^{-2}	N.D.[2)]	N.D.
Gorlovka 2	U.N.	24.11.2014	Native area in Animal Burial	$5x10^5$	Neg.	10^{-4}	N.D.	N.D.

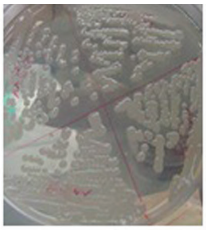
[1)]- on 3rd consecutive blind passage; in procedures with soil bacteriophages in Turkey used strain Sterne, in Ukraine - strain #55 (see text);
[2)]- U.N., un-known; Pos., positive result; Neg., negative result; N.D., not done

color. If done a right selection, on next stage of trial the bacterial colonies will growth that has sensitivity to penicillin and to *B. anthracis* gamma-phage (Fig. 2b). To *B. anthracis* pathogenic properties estimating very important to detect the capsule-formation activity of soil isolate. For this purpose, "Turkish experience " proposes the "Bicarbonat-agar" test (Fig. 2c) or PCR test with appropriate primers (not shown). Obligatory confirmation test on capsule-formation activity of soil isolates is routine develop of obtained agar cultures by any capsule staining method (Fig. 2d). In addition, for prognostic purposes, all agar cultures of soil *B. anthracis* are very important to examine of sporulation activity of originated from soil spores isolate by one of staining method (Fig. 2e). For this purpose Rakette staining is most suitable as simple and informative.

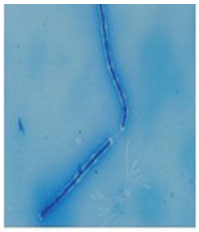
a - Soil anthracic and anthracoids spores' growth in blood agar



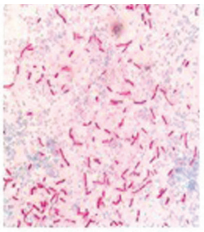
b - Confirmation test on soil-spores originated bacteria with penicillin-disk and by gamma-phage



c - Capsulated bacteria (shining colonies) from mixes of soil spores on "bicarbo-nate-agar" plate



d - Capsule staining (by Mc Fadyen) pa-ttern of soil' spore isolate Subatan of *B. anthracis*



e - Spore staining by Rakette method: pattern of soil' spore isolate Selim 1

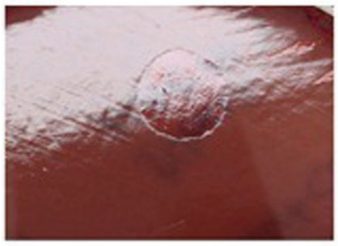
Figure – 2 Soil spores identification: routine methods' results

The specimens from different animal burial sites contained different spore concentrations – from 5×10^3 to 5×10^7 TVS. Even sampling in different places of the same animal burial site got the different results: in native place the quantity of soil' anthracis spores were much more (in 100 times) then in place of soil detonation (as explosion result, see Tab. 1 above).

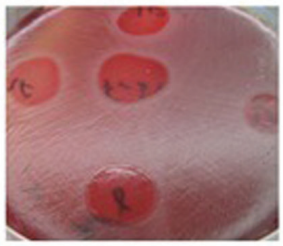
In part of soil phages' surveys, "Turkish experience" proposes the estimation of *B. anthracis* soil phages activity until and after growth on log-phase cultures of host (Fig. 3). As established by present study, the Sterne strain is the best choice of host culture (Fig. 3a, c, d); but the strain "55" is useful for preliminary investigations (Fig. 3b). Some hemolysis what we can see on Fig. 3b is caused by some anthracoid bacteria contamination of our phages isolate from Gorlovka specimens.

Soil sample № 3 (see table 1): partial lysis of Stern str. of the *B. anthracis* by phage isolate of 1st passage

Soil sample № 6 (see table 1): partial lysis of "55" str. of the *B. anthracis* by phage isolate of 1st passage



Soil sample № 3 (see table 1): full lysis of Stern str. of the *B. anthracis* by phage isolate of 3rd passage



Soil sample №№ 1–4: full lysis of Stern str. of the *B. anthracis* by phage isolate of 5th–7th passages

Figure 3 – Soil phages of *B. anthracis* growth by sequential passages in host agar-cultures: routine methods' results

Approbation of SOP through trials on soil desporulation – Trials with soil desporulation by bacteriophage-containing cocktail were effective at only environmental temperatures ≥ 16 °C (n=5). Low atmospheric temperatures (lower than 16 °C) did not provide adequate of *B. anthracis* spores germination and lytic bacteriophage activity. For successful desporulation of soil the critical significance had the level of lytic activity of the bacteriophage which used in of mentioned cocktails. As we can't achieve of lytic bacteriophage activity to ≥ 10^9 RTD in Ukraine, our attempts to use of Gorlovka' phage isolate to soil samples desporulation were partly unsuccessful: residual spore' contamination (≤ $10^{2.5}$CFU)

in soil sample dilution 10^{-2} and even 10^{-3} (in untreated sample – 10^{-5}) was registered. So for soil samples desporulation we used peracetic acid instead phage at last stages of soil desporulation. In this case additional advantage was in efficiency of peracetic acid for at low temperatures unlike to phage.

A main result of desporulation trials is summarized in Table 2 and show on figure 4.

Table 2 – The results of trials for soil anthracis desporulation

Desporulation areas					Total Viable Spores				
Code	Time of contamination	Scope of work	Time of processing	Processing method[1)]	Before	After[2)] 24hr	7 d	14 d	21 d
Külveren (Animal Burial Area)	2013	≈ 30 m²	XI-2014	A	$5x10^4$	Neg.	Neg.	N.D.	N.D.
Gorlovka 2 (Sample from Animal Burial Area)	U.N.	150 g	XII-2014	B	$5x10^5$	Neg.	Neg.	Neg.	Neg.

[1)]- A, germination and bacteriophagal lysis simultaneously; B, germination, than disinfection with peracetic acid (see text);
[2)]- Neg., negative result; N.D., not done

a – Soil spores growth control in dilution of sample 10^{-1}. Soil sample taken just after desporulation mixt application

b – Soil spores growth control in dilution of sample 10^{-2}. Soil sample taken just after desporulation mixt application

c – Soil spores growth control in dilution of sample 10^{-2}. Soil sample taken 24 hr. after desporulation mixt application

Figure 4 – Quality control of desporulation procedures (see text for details)

In both cases the similar results were obtained – application of Turkish technology allowed to full omit the *B. anthracis* spore from Külveren' animal burial area (Turkey) and from sample of soil from Gorlovka' animal burial area in (native part of area) during 24 hr. after soil processing. Absence of anthracis spores in processed objects registered in all period of quality control of trials on the SOP for decontamination: 7 days in Külveren' animal burial area (Turkey) and 21 days for sample of soil from Gorlovka' animal burial area, which exposed under 18 °C in presence of spore germinator during 24 hr. before the last examination date (21 day apart from processing start).

Analyzing the previous literature described and data above, it is possible to make the solution that Turkish method for soil desporulation in based on modern scientific conception and allow to soil release of anthrax spores and bacteria. Therefore this method is very useful for Ukrainian needs as effective, ecological friendly and economically low-cost.

On figure 5 shows the cumulative dates on anthrax outbreaks (all animal species) in Ukraine for 92-years period (from 1920 to September 2012, by Yanenko et al. (2013) with modification). As Prof. Dr. Sc. Zaviruha A.I. claims the main threats from anthrax in Ukraine are originated from hurry sites of modern and especially ancient animal burial area (Zaviriuha, Slupskaya and Yavorskaya, 2014). Agricultural processing of soil, other land uses or especially soil detonations through explosions during war actions contribute to ejection of dormant spores of burial sites on the surface of the earth. So, as we can see on animal anthrax incidence in Donbas region (see Lugansk & Donetsk oblast on map),

where arithmetic average rate is closer to the highest level in Ukraine (1046 cases for 92 years, Fig. 5), we can conclude that this region of war is most threatening about anthrax outbreaks in the nearest future. These threats increase many times because of the many possibility of terrorist use of the causative agent of anthrax – and not only in Donbas area.

Therefore we have many reasons to continuation of development and use of Turkish method for soil desporulation in Ukraine.

Conclusion. The studies based on the combination of phages and spore germinants prove the effectiveness of Turkish method for soil desporulation and show highly promising for implementation in Ukraine. And still better,Turkish scientists are ready for sharing their experiences with Ukranian scientists and cooperation.

Acknowledgements. The authors would like to thank Prof Les Baillie from Welsh School of Pharmacy, Cardiff University, UK for his scientific contributions.

Figure 5 – Map of anthrax outbreaks on all animal species for 92-years period. Donbas area marked by a dotted black (see text for detail)

References

Abshire, T. G., Brown, J. E. and Ezzell, J. W. (2005) 'Production and validation of the use of gamma phage for identification of Bacillus anthracis', *Journal of Clinical Microbiology*, 43(9), pp. 4780–4788. doi: 10.1128/jcm.43.9.4780-4788.2005.

Bouzianas, D. G. (2007) 'Potential biological targets of Bacillus anthracis in anti-infective approaches against the threat of bioterrorism', *Expert Review of Anti-Infective Therapy*, 5(4), pp. 665–684. doi: 10.1586/14787210.5.4.665.

Buyuk, F., Pottage, T., Crook, A., Hawkey, S., Cooper, C., Celebi, O., Bennett, A., Sahin, M. and Baillie, L. (2013) 'The use of peracetic acid and spore germinants to decontaminate Bacillus anthracis spores contaminating animal burial sites in Turkey', in *International Bacillus ACT Conference*. Victoria, BC, Canada, September 1-5. pp. 66–67.

Mock, M. and Fouet, A. (2001) 'Anthrax', *Annual Review of Microbiology*, 55(1), pp. 647–671. doi: 10.1146/annurev.micro.55.1.647.

Schuch, R. and Fischetti, V. A. (2009) 'The secret life of the anthrax agent Bacillus anthracis: bacteriophage-mediated ecological adaptations', *PLoS ONE*, 4(8), pp. e6532. doi: 10.1371/journal.pone.0006532.

Turnbull, P. C. B. (2002) 'Introduction: Anthrax history, disease and ecology', in Koehler, T. M. (ed.) *Anthrax*. Berlin Heidelberg: Springer-Verlag (Current Topics in Microbiology and Immunology, 271), pp. 1–19. doi: 10.1007/978-3-662-05767-4_1.

Van Ness, G. B. (1971) 'Ecology of Anthrax', *Science*, 172(3990), pp. 1303–1307. doi: 10.1126/science.172.3990.1303.

Vernadskiy, V. I. (1991) *Scientific thought as a planetary phenomenon [Nauchnaya mysl' kak planetnoe yavlenie]*. Moscow: Nauka. ISBN 5-02-003505-X. [in Russian].

Yanenko, U. M., Gudz, N. V., Tereshenko, C. M., Dorofeeva, E. V. and Yanenko, V. M. (2013) 'The analysis of the epizootic situation with anthrax in Ukraine during 1920–2013 years' [Analiz epizootychnoi sytuatsii shchodo sybirky v Ukraini za 2010-2012 roky], *Veterinary Biotechnology [Veterynarna biotekhnolohiia]*, 23, pp. 530–533. Available at: http://nbuv.gov.ua/j-pdf/vbtb_2013_23_80.pdf. [in Ukrainian].

Zaviriuha, A. A., Slupskaya, V. V. and Yavorskaya, K. V. (2014) 'Vaccines against anthrax animals and prospects of their improvent' [Protysybirkovi vaktsyny ta perspektyvy yikh udoskonalennia], *Veterinary Biotechnology [Veterynarna biotekhnolohiia]*, 24, pp. 56–63. Available at: http://nbuv.gov.ua/j-pdf/vbtb_2014_24_12.pdf. [in Ukrainian].

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SOME INTERNATIONAL PROJECTS ON INCREASING KNOWLEDGE IN BIOSAFETY AND BIOSECURITY: EFFORTS IN UKRAINE

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Summary. Life scientists have with increasing intensity been requested to recognize and take responsibility for the potential dual use implications of their work. Thus, a major challenge for today's internationally scientific community is to find effective ways to raise awareness among scientists about their social responsibility regarding the potential for the destructive use of the life science research in which they are engaged. The aim of the presented study is gathering information on the some available for Ukrainian researchers, specialists and students in life sciences educational opportunities on biosafety, biosecurity and bioethics.

Keywords: biosafety, biosecurity, education, knowledge distribution

Background. Life science research and biotechnology have offered great social benefits globally, for example in improvements to public health, agriculture and energy development. Alongside these benefits, however, the same advances also generate safety and security risks, which, while less obvious, are nonetheless real (World Health Organization, 2004).

However, in addition to the risk addressed by Biosafety containment and engineering safety standards, life science research and engineering developments can also give rise to issues of dual-use, whereby peacefully developed scientific research and engineering projects can be misused for destructive purposes, such as biowarfare and bioterrorism (Committee on Research Standards and Practices to Prevent the Destructive Application of Biotechnology, Development, Security, and Cooperation Policy and Global Affairs, National Research Council of the National Academies, 2004). But the lack of awareness of individual scientists across the globe has been clearly demonstrated (Dando and Rappert, 2005; Rappert, Chevrier, and Dando, 2006).

Thus, a major challenge for today's internationally scientific community is to find effective ways to raise awareness among scientists about their social responsibility regarding the potential for the destructive use of the life science research in which they are engaged (Committee on Advances in Technology, the Prevention of Their Application to Next Generation Biowarfare Threats, Security, Cooperation Development, Board on Global Health, Policy, Global Affairs, Institute of Medicine, National Research Council, 2006).

Life scientists have with increasing intensity been requested to recognize and take responsibility for the potential dual use implications of their work (Kuhlau et al., 2012). Scientists have an obligation to do no harm. They should always take into consideration the reasonably foreseeable consequences of their own activities. Scientists should be aware of, disseminate information about and teach national and international laws and regulations, as well as policies and principles aimed at preventing the misuse of biological research (Rappert, Chevrier, and Dando, 2006). In the current study the short brief of several International projects focused on biosafety, biosecurity and bioethics education and outreach in Ukraine are presented.

International projects activities in Ukraine. Ukrainian scientists have the opportunity to be involved in several international projects aimed at increasing knowledge in biosafety and biosecurity as well as dual-use issues. One of them is Project 3 of the European Union's Chemical, Biological, Radiogical and Nuclear (CBRN) Risk Mitigation Centres of Excellence (CoE) entitled "Knowledge development and transfer of best practice on bio-safety/bio-security/bio-risk management. Planned Project implementation period was January, 1st 2013 – December, 31st 2014 (EU CBRN CoE Project 3 'Knowledge development and transfer of best practice on bio-safety/bio-security/bio-risk management', 2013), but it was prolonged.

The strategic goal of this Project is to promote sustainable knowledge development on bio-safety, bio-security and bio-risk management and transfer of best practice through the model "train the trainers". The project also works to harmonize international bio-safety and bio-security standards among the participating countries and to strengthen regional and international collaboration and cooperation so as knowledge sharing, sustaining and enhancing networking and awareness raising on bio-related issues among the areas.

Geographical scope includes four regions: a) South East Europe, the Caucasus, Moldova and Ukraine, b) South East Asia, c) North Africa and d) African Atlantic Façade and approximately 360 persons will be trained.

The project also contributes to strengthen regional and international collaboration and cooperation as well as knowledge sharing. The project also sustains and enhances networking and awareness-raising on bio-related issues among the areas (EU CBRN CoE Project 3 'Knowledge development and transfer of best practice on bio-safety/bio-security/bio-risk management', 2013).

In a first instance, selected National Experts (NEs) were trained on bio-security, bio-safety and bio-risk management systems as well as on training methodology. Consequently, qualified and selected NEs will train National Participants (NPs), who will then become trainers at local level. An e-learning platform will provide long distance learning materials and support the training of NEs and NPs. For now two Ukrainian NEs were trained. Training for NPs will be held in January 2015.

The project "International Network of Universities and Institutes for Raising Awareness on Dual-Use Concerns in Bio-technology" is part of the European Union (EU) Chemical, Biological, Radiological, Nuclear (CBRN) Centre of Excellence (CoE) Risk Mitigation Initiative, implemented and funded by the European Commission in cooperation with the United Nations Interregional Crime and Justice Research Institute (UNICRI) (EU CBRN CoE Project 18 'International Network of universities and institutes for raising awareness on dual-use concerns in bio-technology', 2013).

The European External Action Service (EEAS) is also involved in the follow up of the initiative. The Initiative is developed with the technical support of relevant International and Regional Organizations, the EU Member States and other stakeholders, through coherent and effective cooperation at the national, regional and international level.

The Initiative aims to mitigate CBRN risks of criminal, accidental or natural origin by promoting cooperation and improving coordination and preparedness at national and regional levels.

The implementation period of the Project started in January 2013 and finished in December 2014.

The project mission is that of modernizing and internationalizing biotechnology education; improving cooperation through the sharing of international standards and good laboratory practices; and improving bio-safety of society and environment, with a special attention to the "next generations of scientists".

The Consortium of organizations working together on the implementation of Project 18 is very wide and is composed of 18 Partner Universities and Institutes in 14 countries. Palladin Institute of Biochemistry (PIB) of National Academy of Sciences of Ukraine was Local Partner Organization in the frame of the Project (EU CBRN CoE Project 18 'International Network of universities and institutes for raising awareness on dual-use concerns in bio-technology', 2013).

The members of the Consortium are faculties of Sciences and Bio-technology, but also Ecologists, Political Scientists, Lawyers, Philosophers, Bioethicists, Public Health agencies, Agriculture and Medicine faculties.

During the Project implementation a survey among professors of the local universities, to gather information on the current level of awareness, the educational opportunities, and the attitude of scientists in this field was realized. The information on current level of awareness on Biosafety, Biosecurity and dual-use in the universities of Ukraine as well as the educational opportunities was collected during the project implementation. The "Questionnaire for Educators/Faculty members" was used for gathering information. 51 people participated in the survey. All the interviewees come from academic institutions, including 9 life science universities, 10 medical universities and 7 other institutions.

The interviewees were asked several questions in order to assess their knowledge and level of awareness on a number of topics related to Biosafety, Biosecurity and dual use concerns. Interviewees have shown high level of awareness on Biosafety, Biosecurity and dual-use issues. That can be explained by previous activities of participants in seminars and conferences on studding issues before interviewing. In additional, interviewees indicated presence of Bioethics committees and absence of Biosafety committees in their institutions.

The respondents noticed that the surveyed Ukrainian institutions almost always have courses that focus primarily on Biosecurity and Biosafety. However, participants noted an above average level of availability of resources and infrastructures used for teaching on Biosafety. Thus interviewees recognize necessity of implementing a series of activities to educate the students and have some plans to change their courses or modules to accommodate such topics. For this purpose it will be very beneficial to develop and implementation of training materials and guidelines on Biosafety, Biosecurity and dual-use issues (Gergalova et al., 2014).

During the project implementations Seminars addressed to students, focusing on different aspects of biosafety, biosecurity and dual-use, according to the local priorities were held.

The Seminar "Introduction into Biosafety, Biosecurity and Dual-use Concerns in Biotechnology" targeting Master and PhD students, was organized by the PIB to ensure their biosafety and biosecurity competence (EU CBRN CoE Project 18: Seminar at Palladin Institute of Biochemistry, Kiev, Ukraine, 2014).

The Seminar was aimed to introduce students with the dual-use nature of science and technology, spectrum of biological risks and threats, biosafety and biosecurity concepts, relevant international agreements and regulations, etc. Besides the students, all those, interested in dual-use, biosafety and biosecurity issues were also welcomed to participate in the Seminar as observers.

The Seminar brought together 79 participants (students, lecturers and scientists) (Seminar for Master and PhD students 'Introduction into biosafety, biosecurity and dual-use concerns in biotechnology', 2014). Pre-seminar and Post-evaluation testing was conducted during the Seminar. It seems that many participants found some difficulties during answering the pre-seminar questions regarding biosafety/biosecurity.

The majority of the participants admitted that their previous knowledge was sufficient to follow the seminar and they had some prior knowledge about the potential "hostile misuse" of life sciences.

During the seminar participants learnt dual use/ misuse/security issues and the broader context of life science (e.g. social, ethical, legal aspects, etc) as well as acquired new skills and experience and interacted with fellow colleagues. Participants identified websites and university libraries as their best sources on information about biosafety and biosecurity issues.

The third initiative, «Education and Awareness-Raising in Ukraine» funded by the UK Ministry of Defense, began in July 2014 (Education and Awareness-Raising in Ukraine, 2014). The main objective of the project is to collect information, develop a network and to disseminate knowledge on biosafety, biosecurity and bioethics amongst life sciences experts, and specialists in the field of biotech and pharmaceutical industries. In addition, recommendations on the biosafety and biosecurity status in Ukraine will be prepared and submitted to the Government of Ukraine, relevant ministries and agencies including recommendations for the ministries with subordinated universities (at first for the Ministry of Education and Science of Ukraine) on the necessity to implement of obligatory curriculums on biosafety, biosecurity, and «dual-use» technics and materials for students studying biology, medicine and agrarian sciences.

Discussion with teachers of higher educational institutions of Ukraine and relevant local authorities for development of guidelines and a training manual for the course on Biosafety and Biosecurity is also planned during the project implementation.

One of the important tool for the Project implementation is development and further maintaining of a website http://www.bsseducation.com.ua devoted to various aspects of Biosecurity, Biosafety and Bioethics and contained all key translated materials, aimed, in particular, at lecturers and students of the Ukrainian Universities teaching life sciences in the field of biology, medicine, ecology and agriculture as well as at the relevant professional societies.

In framework of this project the Palladin Institute of Biochemistry held the first International Meeting titled "Awareness-Raising and Education on Biosafety and Biosecurity in Ukraine" in October 2014. There were 33 delegates from 20 Ukrainian universities and 5 life sciences research institutes; 9 participants from other countries attend. In November the two-day first Regional Meeting was held. During the first day 65 undergraduate and postgraduate students were involved in interactive seminars Introduction into Biosafety & Biosecurity" and "Introduction into Bioethics".

Overall, the participants were really interested in learning more about biosafety, biosecurity, bioethics aspects of modern life sciences. The second day was addressed to lecturers and dedicated new technics in teaching of biosafety, biosecurity and bioethics. During Round table the main issues on biosafety, biosecurity and bioethics education in Ukraine were discussed.

Project «Education and Awareness-Raising in Ukraine» is one of the first steps to aware Ukrainian students on biosafety, biosecurity and bioethics

Conclusions. Ukraine's efforts in promoting and engaging in biosafety and biosecurity education, and dual-use and bioterrorism issue awareness-raising are mostly recent developments. However, Ukrainian researchers, specialists and students in life sciences have the opportunity to be involved in several international projects aimed at increasing knowledge in biosafety and biosecurity as well as bioethics. In addition, all mentioned project provide possibility to collect and analyze information of the current status of biosafety and biosecurity education on in Ukraine as well as identify gaps and needs in this fields.

All these benefits can be used for improvement the current status of biosafety and biosecurity in Ukraine because of development of a nation-wide education network for these issues is a key requirement for fostering a biosafety and biosecurity culture in Ukraine.

References

Committee on Advances in Technology, the Prevention of Their Application to Next Generation Biowarfare Threats, Security, Cooperation Development, Board on Global Health, Policy, Global Affairs, Institute of Medicine, National Research Council. (2006) *Globalization, biosecurity, and the future of the life sciences*. Washington, D.C. : National Academies Press. ISBN 0-309-65754-7.

Committee on Research Standards and Practices to Prevent the Destructive Application of Biotechnology, Development, Security, and Cooperation Policy and Global Affairs, National Research Council of the National Academies. (2004) *Biotechnology research in an age of terrorism: confronting the dual use dilemma*. Washington, DC: National Academies Press. ISBN 0-309-09087-3.

Dando, M. R. and Rappert, B. (2005) *Codes of Conduct for the life sciences: some insights from UK Academia*. Bradford Briefing Paper No 16 (Second Series). Available at: http://www.brad.ac.uk/acad/sbtwc/briefing/BP_16_2ndseries.pdf.

Education and Awareness-Raising in Ukraine (2014). [Online] Available at: http://www.bsseducation.com.ua.

EU CBRN CoE Project 3 'Knowledge development and transfer of best practice on bio-safety/bio-security/bio-risk management' (2013). [Online] Available at: http://www.cbrn-coe.eu/Projects.aspx; http://icis-uninsubria.eu/index.php/programs/eu-cbrn-coe-project-3-knowledge-development-and-transfer-of-best-practice-on-biosafety-biosecurity-biorisk-management/; http://icis-uninsubria.eu/wp-content/uploads/2013/09/Summary-EU-CBRN-CoE-Pr-3_EN_08.10.2013.pdf.

EU CBRN CoE Project 18 'International Network of universities and institutes for raising awareness on dual-use concerns in bio-technology' (2013). [Online] Available at: http://www.cbrn-coe.eu/Projects.aspx; http://landaunetwork.org/index.php/eu-cbrn-coe-project-18/.

Gergalova, G., Kysil, O., Maksymovych, I. and Komisarenko, S. (2014) 'Biosafety and biosecurity education in Ukraine: current situation, gaps and necessities analysis', *Veterinary Medicine [Veterynarna medytsyna]*, 98, pp. 29–33. Available at: http://jvm.kharkov.ua/sbornik/98/1_7.pdf.

EU CBRN CoE Project 18: Seminar at Palladin Institute of Biochemistry, Kiev, Ukraine (2014). [Online] Available at: http://landaunetwork.org/index.php/2014/04/eu-cbrn-coe-project-18-seminar-at-palladin-institute/.

Kuhlau, F., Evers, K., Eriksson, S. and Höglund A. T. (2012) 'Ethical competence in dual use life science research', *Applied Biosafety*, 17(3), pp. 120–127. Available at: http://www.absa.org/abj/abj/121703Kuhlau.pdf.

Seminar for Master and PhD students 'Introduction into biosafety, biosecurity and dual-use concerns in biotechnology' (2014). [Online] Available at: http://landaunetwork.org/wp-content/uploads/2014/04/Website-Notice_full.pdf.

Rappert, B., Chevrier, M. I. and Dando, M. R. (2006) *In-depth implementation of the BTWC: education and outreach*. Bradford Review Conference Papers No 18. Available at: http://www.brad.ac.uk/acad/sbtwc/briefing/RCP_18.pdf.

World Health Organization. (2004) *Laboratory biosafety manual*. 3rd ed. Geneva: WHO. ISBN 92-4-154650-6.

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