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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-l, *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).

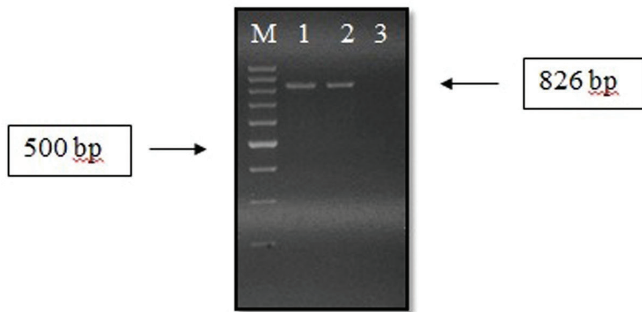

Figure 1 – Electrophoregram with BVDV cDNA amplification results using primers P1/P2: M – molecular weight marker (100 bp DNA Ladder, Ltd. Lab. Isogene, Russian Federation); 1 – BVDV-1; 2 – BVDV-2; 3 – negative control

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.

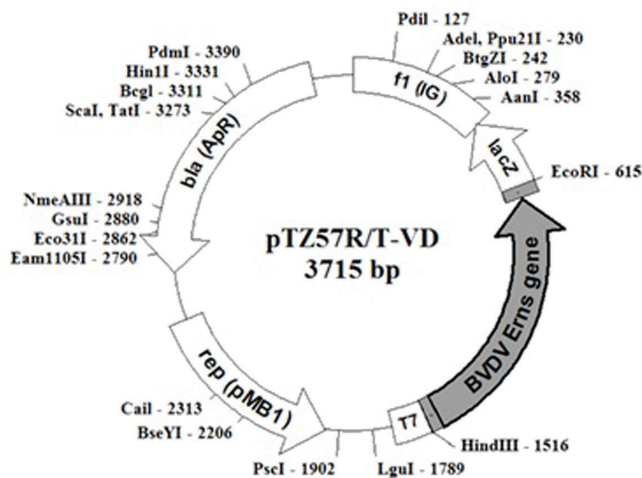

Figure 2 – pTZ57R/T-VD plasmid restriction site map

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).

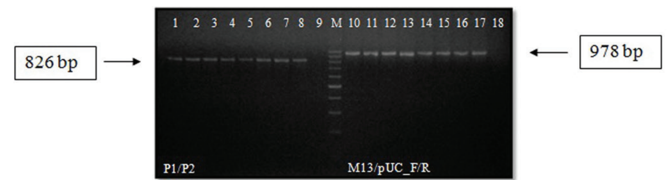

Figure 3 – Electrophoregram with amplification results using P1/P2 and M13/pUC_F/R primers in agarose gel: 1–4, 10–13 – recombinant colonies carrying BVDV-1 E^{rns} gene insertion; 5–8, 14–17 – recombinant colonies carrying BVDV-2 E^{rns} gene insertion; 9, 18 – negative control; M – molecular weight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

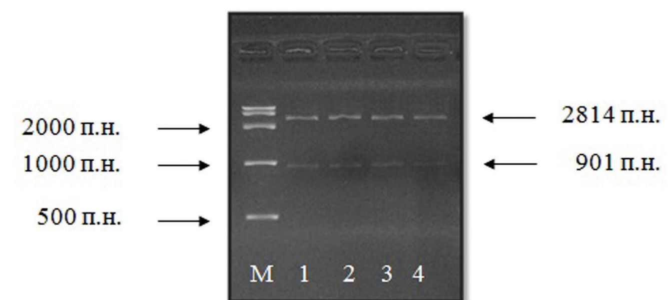

Figure 4 – Electrophoregram of pTZ57R/T-VD plasmid restriction analysis: 1–2 – recombinant colonies carrying BVDV-1 E^{rns} gene insertion; 3–4 – recombinant colonies carrying BVDV-2 E^{rns} gene insertion; M – molecular weight ladder (Fast Ruler High Range DNA Ladder, Fermentas, Lithuania).

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).

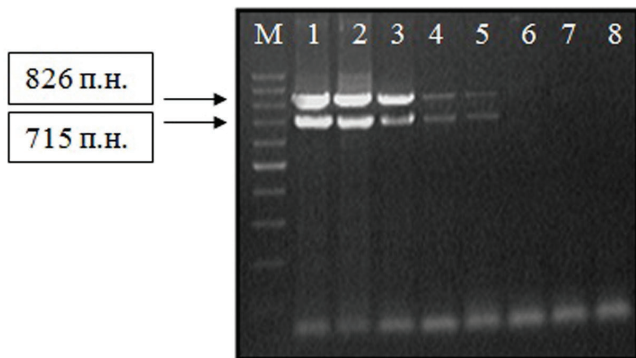

Figure 5 – Analytical sensitivity of the test system for detection Mollicutes and BVDV genetic materials by the duplex PCR: 1 – undiluted positive control; 2 – 10-fold dilutions of positive control; 3 – 100-fold dilutions of positive control; 4 – 1000-fold dilutions of positive control; 5 – 10^4-fold 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).

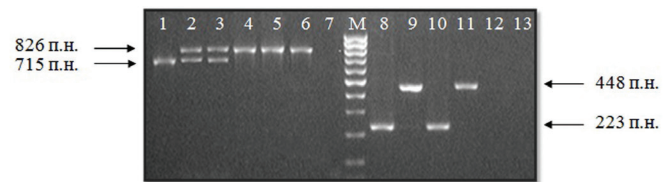

Figure 6 – The results of the detection Mollicutes DNA and BVDV RNA by duplex PCR with following the identification of BVDV genotypes. First round of nested PCR: 1 – *M. bovis* PG45T; 2 – *M. bovis* PG45T and BVDV-1 (strain Osloss); 3 – *M. bovis* PG45T and BVDV-2 (strain Kosice); 4 – BVDV-1; 5 – BVDV-2; 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.

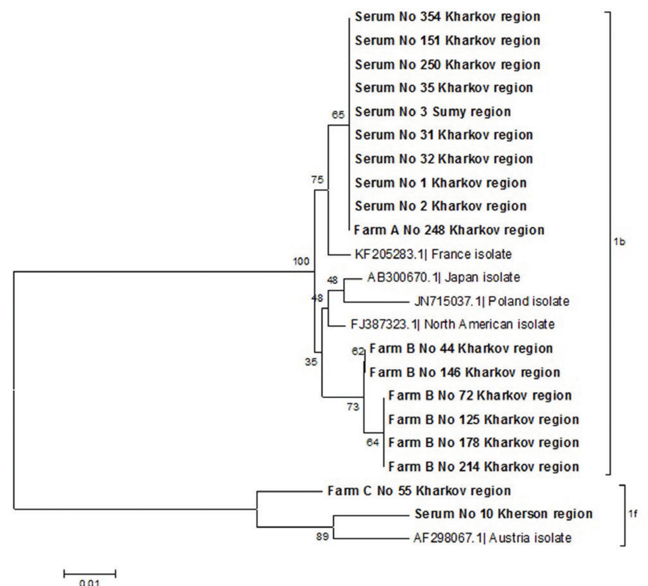

Figure 7 – Genetic typing of BVDV isolates in the 5'-UTR region

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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