
SYSTEM FOR ENSURING VIROLOGICAL PURITY OF VETERINARY IMMUNOBIOLOGICAL DRUGS IN THE CONTEXT OF PESTIVIRUS CONTAMINATION

Napnenko Oleksandr, Solomon Vyacheslav, Midyk Svitlana
DOI <https://doi.org/10.30525/978-9934-26-714-7-14>

INTRODUCTION

Control and prevention of infectious diseases of domestic animals and poultry have historically been a key vector for ensuring epizootic well-being in livestock farming. Significant progress in the elimination and containment of the spread of viral nosoforms has been achieved primarily due to the widespread introduction of live (attenuated) vaccines into the practice of veterinary medicine.

Veterinary immunobiological agents (VIA) produced by the industry from animal raw materials are products of cell life, individual components of endocrine-enzymatic and special raw materials. VIA from animal raw materials differ from each other in the nature of the active base: proteins, peptides, amino acid derivatives, nucleic acids, steroids, etc., which determines different methods of their use. The collection and primary processing of endocrine-enzymatic and special raw materials of animal origin are carried out mainly at meat industry enterprises. The quality of the raw materials at this stage is determined only organoleptically, which does not allow objectively assessing its suitability for the production of therapeutic and prophylactic agents. Therefore, the guarantee of the quality of the raw materials is, first of all, strict compliance with the sanitary requirements for its collection and primary processing.

Biotechnological features of their production are associated with the use of complex biological substrates. As cell systems and components of nutrient media, primary and subcultured mammalian and avian cell cultures, chicken embryos, as well as biological additives of animal origin (in particular, blood serum) are traditionally used. These substrates and components are characterized by a high degree of risk regarding the presence of hidden (latent) pathogens, which defines them as potential sources of biological contamination at all stages

of the production cycle – from the initial biotechnological raw materials to the final immunobiological product^{1, 2}.

A separate type of biological raw material is precisely the strains of microorganisms: bacteria, viruses, mycoplasmas, fungi, etc. Their biological purity is one of the keys to the production of a high-quality, effective drug^{3, 4}.

The absence of contamination of biological means of animal protection is one of the most important indicators of its safety. Despite the use of a number of antimicrobial drugs in the production of biological means of animal protection, contamination of raw materials and products with bacteria, fungi, yeast, mycoplasmas, viruses is quite often found. The production process may also pose a threat of contamination with foreign antigens, nucleic acids.

The most difficult to detect contamination is latent and persistent viruses, which can be unrecognized for a long time in the absence of cytopathic effect (CPA), hemagglutinating and hemadsorbing properties. The ability of viruses to overcome species barriers is associated with their persistence in cell cultures, which in turn can lead to contamination of drugs.

All contaminating viruses can be divided into three groups according to their significance for vaccinated people and animals:

- 1) viruses with established pathogenicity;
- 2) viruses that do not harm the vaccinated population;
- 3) viruses whose pathogenicity has not been proven.

However, the lack of such evidence does not yet indicate their absolute harmlessness to vaccinated people.

Among the possible contaminants of biological raw materials and products, viruses occupy the lion's share. The largest risk group for contamination is cell cultures, especially primary trypsinized ones. Virus contaminants vary greatly in virulence, and under the influence of a biological object and environmental factors, they can mutate and change their biological properties.

¹ Яворський М. І., Коваленко В. І., Литвиненко Л. Ю., Юрченко П. А. Ендокринно-ферментна та спеціальна сировина і лікарські препарати на її основі. Довідник. Асоціація виробників натуральної продукції і лікарських засобів «Вітафарм». Київ : Урожай, 1995. 219 с.

² Напненко О. О. Стандартизація умов розроблення та виробництва ветеринарних імунобіологічних засобів. Матеріали XI міжнародного конгресу спеціалістів ветеринарної медицини (3–4 жовтня 2013 р., м. Київ, Україна). Київ, 2013. С. 95–96.

³ Напненко О. О., Головка А. М., Ареф'єв В. Л., Гордієнко О. І. Створення системи оцінювання ефективності діяльності колекцій штамів мікроорганізмів в Україні. Матеріали Міжнародної науково-практичної конференції «Біотехнологія та її роль в забезпеченні здоров'я людей і тварин». Ніжин : ПП Лисенко М. М., 2023. С. 168–169.

⁴ Напненко О., Головка А., Іванченко П. Система контролю за контамінацією сторонніми агентами ветеринарних імунобіологічних лікарських засобів. Фармаком, 2021. С. 89–91.

The most significant place among viral contaminants is occupied by viruses of the Flaviviridae family of the Pestivirus genus, as evidenced by numerous data from various scientists^{5, 6}.

Of particular interest as contaminants are bovine viruses, which are detected in CK and blood serum used in virological and medical practice. The most common among contaminants is bovine diarrhea virus.

Due to serum contamination, cell culture lines (CK) of various origins were found to be chronically infected with bovine diarrhea virus. These included transfected cell cultures of cattle (lines: MDBK, GBK, BKD, BK4, etc.), horses, pigs, sheep, rabbits, cats and monkeys. The produced series of bovine serum are usually controlled for the absence of viral contamination in primary cell cultures of chicken embryos, calf kidneys and in HeLa cell cultures. In addition to examination under a microscope, cell cultures are checked for hemadsorbing properties with human, chicken and guinea pig erythrocytes.

The fact of the spread of contamination requires the development of modern methods for detecting contaminants, a system for preventing their entry into biological raw materials and finished products.

The provision on the inadmissibility of any foreign agents entering vaccines is generally recognized. By decision of the World Health Organization, the production of medical vaccines on biological systems contaminated with foreign viruses, especially oncogenic ones, is prohibited in all countries^{7, 8}.

Data from literature sources indicate a significant diversity of viral contaminants in biological raw materials and products. There are a large number of methods for detecting contaminating viruses. However, these methods do not always allow us to establish contamination, especially with persistent viruses and in the latent phase. Molecular genetic methods are only being introduced in Ukraine and are used mainly for the purpose of diagnosing animal and poultry diseases, and not for quality control of drugs. Most of the data provided in the literature relate to medical devices, and in the veterinary bioindustry, the introduction of the quality indicator of drugs “absence of contamination with foreign viruses” as a mandatory requirement has only occurred since 2003.

⁵ Напненко О. О. Система запобігання та виявлення контамінації ветеринарних імунобіологічних препаратів пестівірусами : автореф. дис. ... канд. вет. наук : 16.00.03; Одеський державний аграрний університет, 2012. 18 с.

⁶ Test for sterility and freedom from contamination of biological materials. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). Publisher: Paris : OIE, World Organisation for Animal Health, 2024. https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/1.01.09_TESTS_FOR_STERILITY.pdf.

⁷ Державна фармакопея України. Державне підприємство «Український науковий фармакопейний центр якості лікарських засобів», 2-ге видання. Доповнення 3. Харків : Державне підприємство «Український науковий фармакопейний центр якості лікарських засобів», 2018. 416 с.

⁸ European Pharmacopoeia. Sixth Edition. Edition of the Council of Europe, Strasbourg, France, 2022.

Latent and persistent viruses, which are difficult to detect in the material under study, pose a particular danger. During persistence in biological material or in the animal body, these viruses can change their biological properties. It is especially difficult to detect non-cytopathogenic types of viruses.

Virological methods for detecting contaminants require significant time and costs, and also have a low sensitivity threshold. While molecular genetic methods make it possible to detect the presence of proteins, nucleic acids of microorganisms in the material in a short time and in low titers. Therefore, on the way to solving the problem of viral contamination of biological products, the development of a contamination prevention system and diagnostic test systems based on the polymerase chain reaction is an urgent and extremely necessary task.

1. Research Materials and Methods

The study was conducted at the laboratories of the State Scientific and Control Institute of Biotechnology and Strains of Microorganisms (DNKIBSM). Separate studies were performed at the National Scientific Center “Institute of Experimental and Clinical Veterinary Medicine” and production laboratories of Kherson and Sumy biological factories.

Taking into account the data from literature sources, we identified risk groups for contamination with viruses, which included cell cultures, production strains of microorganisms, preparations from animal blood or with its addition, live culture vaccines, as well as antigens of microorganisms that do not undergo the inactivation stage during the production process.

Veterinary biological preparations for research were selected directly from the warehouses of manufacturers or from the network of pharmacies of the corporation “Ukrzoovetprompostach”, as well as preparations that were received for the purpose of passing state quality control. 119 batches of veterinary immunobiological preparations were studied.

The raw materials from which the preparations are made were selected directly at the production site: cell cultures, embryos, biotechnological sera, strains of microorganisms, biological material from donor animals, trypsin solutions, etc. The following were studied: 18 cell culture lines, 144 strains of production microorganisms, 50 commercial batches of bovine blood serum.

The research methods met the requirements of the European Pharmacopoeia, the recommendations of the International Epizootic Bureau and the regulatory documents in force in Ukraine: standards, technical conditions, technological regulations, etc. Each preparation, biological material was studied using three to four methods, in particular in cell culture, on laboratory animals, by hemagglutination reaction (HAR), hemadsorption reaction (HAR),

immunofluorescence reaction (IFR), neutralization reaction (NR) and polymerase chain reaction (PCR)⁹.

Cell culture studies were performed to detect contamination of cell cultures themselves and contaminants of various products. Results were recorded based on the intensity of cytopathogenic effect (CPA), immunofluorescence reaction results, and hemadsorption reaction^{10, 11}.

Control of manufactured virus vaccines for contamination by foreign viruses began with determining the neutralization of the vaccine virus by specific serum. Based on data on the virus titer in the biological product and the neutralizing titer of the specific serum, the corresponding volumes of the biological product and specific serum were mixed. Cell cultures were contaminated with a sample of the product after exposure for 45–60 min at a temperature of 37 °C.

The viral suspension of the main seed culture in an amount of 1.0 cm³ was distributed into monolayers with a growth area of 75 cm² cells. At least two monolayers used in the test were maintained as uninfected controls.

The cell culture (experimental and control) was cultivated for 21 days (3 subcultures) at a temperature of (37 ± 0.5) °C, controlling under a microscope for the presence of cytopathic changes. At the end of the observation period, the culture was tested in the hemadsorption and immunofluorescence reactions, as indicated above. The material was considered to have passed the control if there were no cytopathic changes in the experimental and control cultures and a negative hemadsorption result. If there were cytopathic changes and (or) hemadsorption in the control cultures, the experiment was repeated on another batch of cell cultures. For the study, samples of cell cultures obtained from various enterprises of the biological industry of Ukraine and the DNAIBSM cell culture bank were selected. Before the study, the cell cultures were stored in liquid nitrogen¹².

Studies on the detection of possible viral contaminants were performed by conducting three consecutive subcultures and by reverse transcriptase polymerase chain reaction, since it is the most sensitive method for detecting bovine diarrhea virus.

⁹ Напненко О., Головка А., Безвін Є. Організація серійного контролю якості ветеринарних імунобіологічних засобів в Україні. *Біологія тварин*, 2024, Т. 26, № 3. С. 96.

¹⁰ Блоцька О., Головка А., Кузьмич Г., Напненко О. Біотехнологія. Методи попередження контамінації культур клітин : ДСТУ 8115:2015. Введено в дію 01.01.2017. Київ : ДП «УкрНДНЦ», 2018. 9 с.

¹¹ Code of Federal Regulations. Animal and Animals Products. Title 9, Parts 1.199. The Office of the Federal Register, National Archives and Records Administration: US Government Printing Office, Washington DC, USA, 2024. <https://www.govinfo.gov/content/pkg/CFR-2024-title9-vol1/pdf/CFR-2024-title9-vol1.pdf>.

¹² Напненко О. О., Головка А. М., Ареф'єв В. Л., Гордієнко О. І. Створення системи оцінювання ефективності діяльності колекцій штамів мікроорганізмів в Україні. Матеріали Міжнародної науково-практичної конференції «Біотехнологія та її роль в забезпеченні здоров'я людей і тварин». Ніжин : ПП Лисенко М. М., 2023. С. 168–169.

During the work, 18 cell culture lines of more than 50 different passages were studied: SNEV (SPEV), VNK-21, FLK, HeLa, A-72, CV-1, KST, Marc, RK-13, PK-15, Na, McCoy, FEK, Vero, PO-2, CHO-KI, KF, TrT¹³.

Laboratory animals were used to detect virus contamination and monitor the activity of control virus strains: white mice weighing from 16 g to 20 g; in the amount of 1750 heads; rabbits weighing from 1.5 kg to 2.5 kg; in the amount of 12 heads; guinea pigs weighing 40–50 kg, in the amount of 18 heads.

In order to study the spread of viral diarrhea among cattle, seromonitoring of this disease was carried out in different regions of Ukraine. The neutralization reaction was performed according to the classical method in test tubes and using the micromethod in culture plates.

Theoretical calculation of primers during the development of a diagnostic kit for the detection of pestivirus RNA by PCR was carried out by analyzing nucleotide sequences published in GeneBank, EMBL, DDBJ¹⁴.

The obtained research results were statistically processed using digital indicators of the Microsoft Excel for Windows XP program on a personal computer.

Interlaboratory commission tests were conducted with the participation of representatives of the National Scientific Center “Institute of Experimental and Clinical Veterinary Medicine” (NSC “IEKVM”) and the State Research Institute for Laboratory Diagnostics and Veterinary and Sanitary Expertise.

2. Analysis of Contamination Risk Groups

In order to develop a system for preventing and detecting contamination of VIP with pestiviruses, viral contamination risk groups were identified. The following risk groups were identified:

- animal blood serum;
- cell culture production lines;
- virus production strains;
- live viral vaccines, primarily cultured;
- therapeutic and prophylactic animal blood serums;
- diagnostic antigen and serum kits.

¹³ Barone P., Wiebe M. E., Leung J. C., Hussein I. T. M., Keumurian F. J., Bouressa J., Brussel A., Chen D., Chong M., Dehghani H., Gerentes L., Gilbert J., Gold D., Kiss R., Kreil T. R., Labatut R., Li Y., Müllberg J., Mallet L., Menzel C., Moody M., Monpocho S., Murphy M., Plavsky M., Roth N. J., Roush D., Ruffing M., Schicho R., Snyder R., Stark D., Zhang C., Wolfrum J., Sinskey A. J., & Springs S. L. Viral contamination in biologic manufacture and implications for emerging therapies. *Nat. Biotechnol.* 2020. Vol. 38. P. 563–572.

¹⁴ U.S. Dept. of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Biologics Evaluation and Research. Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications; Availability. Federal Register. 2010. Vol. 75, No. 42. <https://www.govinfo.gov/content/pkg/FR-2010-03-04/pdf/2010-4553.pdf>.

3. Development of a Test System for Detecting Contamination

At the time of the beginning of this dissertation work, there were no registered domestic or imported diagnostic kits in Ukraine aimed at detecting the genome of pestiviruses¹⁵.

The theoretical basis for the development of such a diagnostic kit was literature data and the results of our own research, which were conducted using imported test systems for the detection of pestiviruses, which indicate the prospects of PCR for the detection of pathogens of the genus Pestivirus¹⁶.

The first and main stage in the development of a diagnostic kit is the selection of sequences for oligonucleotide primers. In order to solve the problem, a large number of nucleotide sequences of pestivirus genomes were analyzed: classical swine fever virus (CSFV), bovine diarrhea virus (BDV), sheep border disease virus, published as a web page on the database server of the European Molecular Biology Library (EMBL), the Japanese Database of Nucleotide Sequences (DDBJ) and PDB sequences.

Determination of optimal conditions for PCR. One of the factors affecting the results of PCR is the creation of optimal temperature conditions for primer annealing. Experimental verification of the effect of temperature was carried out using three annealing temperature regimes, namely 58 °C, 60 °C and 62 °C¹⁷.

According to the results of the detection of the reaction products, which was carried out by electrophoresis in a 1.5% agarose gel stained with ethidium bromide, using Tris-borate buffer with a voltage gradient of 10 V/cm and was evaluated by taking into account the results after electrophoresis using a transilluminator under UV light for the presence (or absence) of red-orange DNA fragments. The first pair of primers reacted weakly with the CSF virus strain "K" (ASV) at temperature regimes of 58 and 62 °C, a barely noticeable amplification product was observed at a temperature of 60 °C. Clear luminescence of the amplification product on the transilluminator was established with the cattle diarrhea virus strain Oregon C24V at temperature regimes of 58, 60, 62 °C (Fig. 1).

In the second pair of primers PestF2 and PestR2, at annealing temperatures of 58 °C, 60 °C and 62 °C, clear specific reaction bands were detected in PCR with both CSF virus and bovine diarrhea virus, which indicates the specificity

¹⁵ Стегній Б. Т., Герілович А. П., Лиманська О. Ю. Полімеразна ланцюгова реакція у практиці ветеринарної медицини. Харків : ННЦ «ІЕКВМ». 2006. 110 с.

¹⁶ Levings R. L., Wessman S. J. Bovine viral diarrhea virus contamination of nutrient serum, cell cultures and viral vaccines. *Dev Biol Stand*, 1991. Vol. 75. P. 177–81.

¹⁷ Erickson G. A., Bolin S. R., Landgraf J. G. Viral contamination of fetal bovine serum used for tissue culture: risks and concerns. *Dev Biol Stand.*, 1991. Vol.75. P. 173–175.

and sensitivity of the developed primers. This pair of primers detected both pestivirus genotypes (Fig. 2).

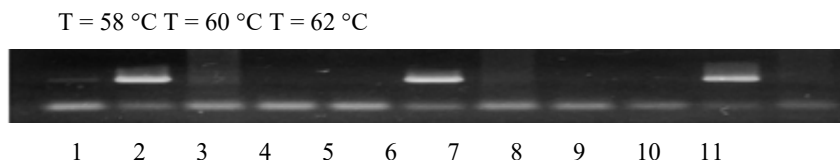


Fig. 1. Results of amplification with primers PestF1 and PestR1 obtained at different annealing temperatures in the range of 58–62 °C
 (1, 5, 9 – blood of rabbits infected with CSF virus strain “K” (ASV);
 2, 6, 10 – culture of TrT cells infected with cattle diarrhea virus strain Oregon C24V; 3, 7, 11 – culture of TrT cells; 4, 8 – negative reaction control)

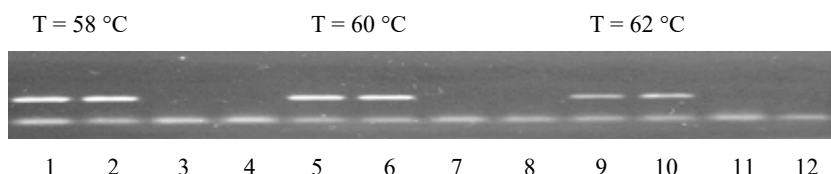


Fig. 2. Results of amplification with primers PestF2 and PestR2 obtained at different annealing temperatures in the range of 58–62 °C
 (1, 5, 9 – blood of rabbits infected with CSF virus strain “K” (ASV);
 2, 6, 10 – culture of TrT cells infected with cattle diarrhea virus strain Oregon C24V; 3, 7, 11 – culture of TrT cells; 4, 8, 12 – negative reaction control)

The control primer pair, which was taken from the article by Barbara Wirz, showed clear specific reaction bands with both CSF virus and bovine diarrhea virus in PCR, which indicates the specificity and sensitivity of the designed primers. This primer pair detected both pestivirus genotypes (Fig. 3).

The obtained results give grounds to conclude that the first pair of primers is specific to cattle diarrhea virus, and the second pair of primers is specific to both representatives of the pestivirus family. The control pair of primers also reacted with both representatives of the pestivirus genus.

Taking into account the obtained research results, the optimal annealing temperature of the primers was determined to be 60 °C, at which amplification products were formed. At temperatures of 58 and 62 °C, amplicons were formed in small quantities or were not detected at all. Of the two developed primer

pairs, the second pair PestF2 and PestR2 was chosen for the diagnostic kit – with a number of 310 nucleotide pairs, since it turned out to be more sensitive and specific compared to the others during the detection of pestivirus RNA.

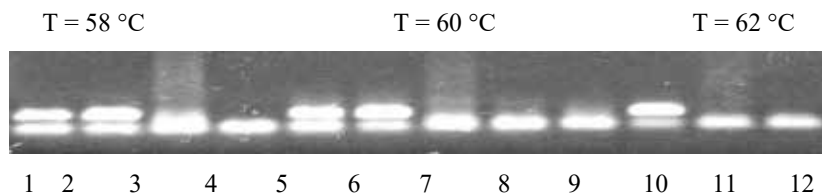


Fig. 3. Results of amplification with Barbara Wirz primers obtained at different annealing temperatures in the range of 58–62 °C
(1, 5, 9 – blood of rabbits infected with CSFV strain “K” (ASV);
2, 6, 10 – culture of TrT cells infected with cattle diarrhea virus strain Oregon C24V; 3, 7, 11 – culture of TrT cells; 4, 8, 12 – negative reaction control)

4. Determination of Sensitivity and Specificity of the Test System

In the process of studying the sensitivity and specificity of the “PCR Pestivir” test system, RNA of bovine diarrhea virus and CSF virus, DNA and RNA of viruses of other families, bacteria, cell cultures, as well as samples of biological material from patients with CSF were studied.

In the process of determining the specificity, reference strains of diarrhea virus “Oregon C24V”, “VK-1”, CSF virus “K”, “Shi-Min” and “Washington” were analyzed in a comparative aspect with strains of viruses causing Aujeszky's disease, parainfluenza-3 of cattle and enteroviruses, as well as pathogens of escherichiosis, pasteurellosis of pigs and erysipelas; pathological material from a pig sick with CSF, TrT cell culture.

The choice of these samples was due to the fact that pestivirus infections quite often occur in association with other diseases and have similar clinical signs.

A characteristic amplification product was observed in positive samples of reference strains of diarrhea virus “Oregon C24V”, “VK-1”, CSF virus “K”, “Shi-Min” and “Washington” and material from a pig sick with CSF. At the same time, in other samples corresponding to bacterial and heterogeneous virus strains, TrT cell culture, the amplification product was absent, which indicated the specificity of the developed set. The sensitivity of the primers was determined by studying the classical swine fever virus strain “Shi-Min” and the cattle diarrhea virus strain “Oregon C24V” in different dilutions with

a step of 10. The initial titer of the viruses was: 6 lg LD₅₀/cm³ – strain of CSF “Shi-Min” and 6.5 lg TCD₅₀/cm³ – strain of cattle FD “Oregon C24V”. It was found that in the studied dilution ranges of CSF virus from 10⁻¹ to 10⁻⁹ and for bovine diarrhea virus from 10⁻¹ to 10⁻⁸, RNA amplification products were detected. Clear specific bands on the trans illuminator were detected at the level of CSF virus from 10⁻¹ to 10⁻⁶, and for bovine diarrhea virus – at the level of 10⁻¹ to 10⁻⁷. Barely noticeable bands were at the level of other dilutions. The results obtained indicate high sensitivity of the primers (Table 1).

The results of the study showed that the use of the drug makes it possible to detect pestivirus in a titer of 0.1 lg, which is sufficient for quality control of the drugs.

The positive result of the dossier examination for the developed diagnostic kit gave the right to conduct interdepartmental commission tests of the “Diagnostic kit for the detection of pestiviruses PCR-PESTIVIR”. The tests were conducted at the DNAIBSM with the participation of employees of the National Scientific Center “Institute of Experimental and Clinical Veterinary Medicine” and the Research Institute for Laboratory Diagnostics and Veterinary and Sanitary Expertise. The results of interdepartmental commission tests confirmed the high sensitivity and specificity of the developed diagnostic kit.

5. Comparative Study of the Effectiveness of the Methods

In order to determine the effectiveness of the polymerase chain reaction, it was studied in comparison with the neutralization reaction (RN) of the virus in cell culture and the immunofluorescence reaction (IFR).

For this purpose, bovine blood serum was taken and artificially contaminated with the bovine diarrhea virus cytopathogenic strain “Oregon C24 V”. Lyophilized virus with an activity of 6.5 lg TCD₅₀/cm³ was dissolved in sterile phosphate buffer solution to the original volume (2 cm³) and tenfold dilutions were prepared in the same solution. To contaminate serum samples, the virus was added in dilutions of 10⁻² (4.5 lg TCD₅₀ of the virus), 10⁻⁴ (2.5 lg) and 10⁻⁵ (1.5 lg). To each serum sample of 4.5 cm³, 0.5 cm³ of the appropriate virus dilution was added. In this way, we kept serum samples containing 3.5 lg, 1.5 lg and 0.5 lg of virus, respectively.

The obtained serum samples were examined using a transfected culture of calf tracheal cells (TpT). For the study of each serum sample, eight tubes with cell culture were taken and eight tubes were left intact. After 15 minutes of contact of the samples with cells, 4 tubes from each group of tubes were examined in the neutralization reaction and 4 tubes in the RIF. Control cell cultures were contacted with blood serum without the addition of virus.

At the same time, serum samples containing different amounts of virus were examined by the reverse transcriptase polymerase chain reaction method. Each sample was examined in four replicates.

To avoid false negative and false positive PCR results, four controls were set. As a positive control, a virus-containing culture fluid containing the bovine diarrhea virus Oregon C 24 V strain undiluted with an activity of 6.5 lg TCD₅₀/cm³ and the same fluid in a dilution of 10⁻⁶ were used. As a negative control, we used bovine blood serum free from virus contamination, and we also set a blank control, which is a reaction mixture containing all reacting components, except nucleic acid.

Studies have shown that the polymerase chain reaction has a sensitivity higher than the neutralization reaction and the immunofluorescence reaction, which makes it possible to use it in further work as a promising reaction for quality control of VIP.

The reliability of the result obtained by PCR is confirmed by reaction controls. Negative results of the blank control indicate the purity of the reaction mixture.

The results of the study are shown in the table.

Table 1

**Efficiency of Different Methods for Detecting Pestiviruses
in Bovine Serum**

Serum virus titer	Samples examined	Of them responded positively					
		Number				%	
		neutralization reaction (PH)		immunofluorescence reaction (IFR)		polymerase chain reaction (PCR)	
3.5 lg	4	4	100.0	4	100.0	4	100.0
1.5 lg	4	4	100.0	4	100.0	4	100.0
0.5 lg	4	1	25.0	2	50.0	4	100.0

Studies have shown that PCR is the most effective and sensitive method for determining serum contamination with pestiviruses.

When comparing the effectiveness of PCR and IFR for detecting possible contamination of biological systems when cultivating production strains of different types of viruses, we conducted an experiment on artificial contamination of cell culture in which the bovine parainfluenza-3 virus was cultivated with the bovine diarrhea virus of the Oregon C24V strain in different titers. The studies were accompanied by the establishment of a negative control – uncontaminated cell culture and a positive control – bovine diarrhea virus.

The results of the study showed that the contaminant virus in the biological system of the production strain of parainfluenza-3 virus was detected by PCR

in titers of 3.5 lg, 1.5 lg and 0.5 lg of the virus in 100.0%, while in IFR – in titer of 0.5 lg in 50.0%, which confirms the superiority of PCR for detecting contaminant pestiviruses.

6. Research on Pestivirus Contamination of Biological Raw Materials and Products

The research of various raw materials and products made from them was carried out using the developed PCR test system and generally accepted methods.

One of the main types of raw materials used in the production of VIP is animal blood serum, most often cattle. In Ukraine, most biological industry enterprises do not have specialized animal husbandry farms to meet production needs. Therefore, the source of blood serum is commodity farms, which do not always control the infection of the organism with pestiviruses^{18, 19}.

This prompted us to study the spread of the diarrhea virus among cattle in different regions of Ukraine. This work was carried out in close cooperation with the staff of the National Center “IEKVM”. Seromonitoring of the presence of specific antibodies in the serum to this pathogen in the RN was carried out. Blood was collected from cows that were not vaccinated against viral diarrhea of cattle.

We established the presence of antibodies to the diarrhea virus in 23.41% of animals, which indicates a significant spread of the virus in the cattle population.

During the work, blood donor cows belonging to the Kherson State Enterprise – Biological Factory, Zhytomyr National Agroecological University and cows (village Kalynivka), which are blood donors for the needs of the company “Ukrvak”, were examined. Blood serum samples were examined in PCR by the nested method using the PCR-Pestivir kit. The level of contamination of blood serum from donors was 10%.

50 batches of native bovine blood serum without preservatives were tested by PCR, 7 (14.0%) were positive for pestivirus contamination. In order to confirm the PCR results, samples from seven batches suspected of contamination were tested at the IFR – of these, 5 (10.0%) confirmed contamination with diarrhea virus. Contaminated batches were discarded.

¹⁸ European Commission (2015). The Rules Governing Medicinal Products in the European Union. Eudralex. Vol. 4–9. European Commission, DG Health and Food Safety, Public health, EU Pharmaceutical information, Eudralex. Available online at: http://ec.europa.eu/health/documents/eudralex/index_en.htm (accessed 25 July 2023).

¹⁹ World Health Organization (WHO). 2012. WHO Expert Committee on Biological Standardization. World Health Organization Technical Report Series, Report No. 964. World Health Organization, Geneva, Switzerland. Available online at: https://apps.who.int/iris/bitstream/handle/10665/89142/9789241209779_eng.pdf (Accessed 25 July 2023).

7. Study of Pestivirus Contamination of Cell Cultures

We studied samples of different QC lines that were in the biological materials bank of the National Center for Microorganism Strains DNAIBSM.

QC studies in PCR were performed using two kits: “Diagnostic kit for the detection of pestiviruses PCR-Pestivir” and “Test system for the detection of RNA of bovine diarrhea virus “BOVI-RNA-TEST-BVDV”.

By virological research methods: by the manifestation of CPD and RGAd, contamination with viruses was not detected, although in several cases the result was questionable. In the latter case, a delay in cell proliferation was observed, filling the monolayer by 60–75%.

IFR was performed using a kit for the diagnosis of viral diarrhea in cattle. Positive PCR results were confirmed by positive IFR results.

Of the 18 studied cell culture lines, 7 (36.0%) were found to be contaminated with pestiviruses. Of the 26 isolates, only 3 (11.5%) had a cytopathogenic effect in cell cultures.

Research on pestivirus contamination of production virus strains in cell cultures

For the study, samples of strains stored in the depository of the National Center for Microorganism Strains DNAIBSM and samples of strains received for the purpose of deposit were selected, special attention was paid to those virus strains for the cultivation of which cell cultures are used.

We studied 144 virus strains, most of which were found to be uncontaminated by foreign hemadsorbing, hemagglutinating and cytopathogenic viruses. As a result of the studies, the following strains were found to be contaminated: attenuated strains of porcine enteroviruses PS 27 (SMEDI A) serogroup 8; PS 026 (SMEDI B) serogroup 3; PS 37 (SMEDI E); Bereznyansky 652; Teshen disease virus Perechensky 642, Chernihiv 2372; Aujeszky's disease virus strains “Arsky” and “18b UNDIEV”; bovine coronavirus VS-1; bovine parainfluenza virus-3: “ZKSM” and infectious rhinotracheitis “Moldavian”, bovine rotavirus “Tiverval”. When studying the above strains, signs of CPD of viruses were revealed, which were manifested by rounding of cells, their destruction, or delayed proliferation.

The above virus strains were additionally examined in PCR using commercial kits for the detection of pestiviruses, in particular the diagnostic kit for the detection of pestiviruses “PCR-Pestivir” and the kit developed at the NSC IEKVM “Test systems for the detection of bovine diarrhea virus RNA “BOVI-RNA-TEST-BVDV”. The PCR results were confirmed by setting the IFR.

The results of the studies showed that the level of contamination of the broodstock of production strains of viruses used in the production of VIP for the pig industry ranges from 22.0 to 45.0%.

The level of contamination of the broodstock of production strains of viruses used in the production of VIP for the cattle industry ranges from 33.0 to 50.0%. The production strains of bovine coronavirus were contaminated in 100.0%.

Pestivirus contamination study of diagnostic kits

Among the diagnostic kits that were tested for contamination with foreign viruses, the majority were kits for diagnosing bovine leukemia in the immunodiffusion reaction.

21 series of antigen specific to bovine leukemia virus and 14 series of specific sera were tested, produced by the following companies: NPP “Leukonad”, Poltava; SE “Veterinary Medicine”, Kharkiv and the Institute of Epizootology of the NAAS of Ukraine, Rivne.

The absence of contamination of sera and antigens with pestiviruses was confirmed by IFR and PCR. It was found that 52.0% of antigen series and 50.0% of blood serum series were contaminated.

Among other virological kits, in particular for diagnosing Aujeszky's disease, coronavirus infection of cattle, and enterovirus infections of pigs, no contaminated series were detected. With a small sample of VIP samples (1–2 series), it cannot be stated that the production technology provides a 100.0% guarantee of the release of uncontaminated products.

Pestivirus contamination studies of live viral vaccines

For the study, vaccines from various manufacturers, both from Ukraine and abroad, were selected, in particular, Kherson State Enterprise – Biological Factory, Sumy State Biological Factory, Pokrovsky Plant of Biological Products, Stavropol Biological Factory, Bio-Test Laboratory, Hipra Laboratory, Intervet International.

Vaccine series not contaminated with bacteria and fungi were taken into the study, which was confirmed by studies in accordance with DSTU 4483.

The absence of contamination of vaccines with pestiviruses, in particular with the cattle diarrhea virus, was studied by PCR, special attention was focused on the study of culture vaccines.

Among the vaccines studied, live culture vaccines against infectious diseases of pigs and cattle prevailed, since pestiviruses pose a threat to them. The study found that the degree of contamination of culture vaccines varied from 2.0 to 60.0% in different groups of vaccines. We found that no contamination was found in drugs imported from countries where the GMP system has been implemented in the production of VIP. Contaminated VIP batches were found in batches of drugs manufactured in Ukraine.

Development of a system for preventing and detecting contamination with VIP viruses

The search for the causes and sources of contamination of finished products turned out to be difficult and in many cases ineffective. An obstacle to resolving this issue was the lack of requirements regulated by law for the registration of production process protocols. Thus, only in 40.0% of cases of contamination of cell cultures were the causes established.

Our studies confirm that the main source of contamination of veterinary biological products with pestiviruses is cattle blood serum.

Taking into account the results we obtained, we developed a system for preventing and detecting contamination with VIP viruses. This system includes not only control of raw materials and products, but also control of production conditions, control of the human factor. The basis of this system was the scheme for controlling medicines, approved by the World Health Organization and interpreted for use in the practice of manufacturing veterinary drugs.

The developed system provides for measures to prevent contamination and clear documentation of all actions and their results; self-control during research; research of the limits of permissible deviations and double control of each sample at each stage of production.

The result of work in this direction was the development and implementation of state standards of Ukraine: DSTU 4517:2010 and DSTU 5832:2008.

The implementation of the developed regulatory documents and the implementation of the developed system for the prevention and detection of viral contamination of veterinary immunobiological preparations in the practice of VIP production allowed to significantly reduce the level of contamination. Thus, during 2004–2005, the level of contamination of commercial batches of blood serum reached 62.0%, cell cultures – 36.0%, passages of individual lines of cell cultures had a significantly higher level of contamination: SNEV – 75.0%, TrT – 100.0%. While in 2011 the level of contamination of cattle blood serum decreased to 10.0%, and cell cultures – to 8.0–12.0%. Due to the fact that uncontaminated passages of cell cultures were taken to obtain production virus broodstocks, uncontaminated production virus broodstocks were obtained. Due to the provision of uncontaminated raw materials to the enterprises of the biological industry of Ukraine over the past two years, no contaminated batches of VIP were detected.

CONCLUSIONS

Based on the conducted virological and molecular biological, serological studies, the situation regarding the contamination of veterinary immunobiological preparations with pestiviruses was studied, a diagnostic kit was developed

for their detection and identification by PCR, and a system of measures was proposed to detect and prevent contamination of VIP with foreign viruses.

Based on the results of bioinformatic analysis of the gene sequences of pestiviruses, in particular, the bovine diarrhea virus and the classical swine fever virus, the nucleotide sequence of two pairs of primers Pest1F and Pest1R and Pest2F and Pest2R was calculated, which in PCR (33 amplification cycles, 2.0 mM Mg²⁺, 2 U of polymerase, annealing temperature 60 °C) form amplification products with a length of 310 and 350 bp, respectively.

A diagnostic kit for the detection of pestiviruses based on PCR using genus-specific primers has been developed and implemented, which allows for reliable detection of the virus in biological material at a minimum titer of 0.1 lg.

The main contaminant of veterinary immunobiological preparations is pestiviruses and, in particular, bovine diarrhea virus, which contaminated up to 50.0% of the studied series of VIP.

Isolates of bovine diarrhea virus, which contaminated cell cultures only in 11.0% of cases showed CPD.

The main factor of VIP contamination is bovine blood serum, which is used in the production of cell cultures and virus strains. The level of contamination of commercial series of bovine blood serum is on average 10%.

At enterprises with a low level of implementation of measures to prevent contamination of veterinary immunobiological drugs with viruses, its level can reach 60.0–80.0%.

The main reason for contamination of finished products is improper control of input raw materials used for the production of drugs. Before the introduction of the system for preventing and detecting contamination with pestiviruses of VIP, its level reached 62.0% of the studied series of blood serum of cattle, up to 36.0% – passages of cell culture lines, up to 30.0% – broodstock of production strains of viruses.

During the period of implementation of the system for preventing and detecting contamination of VIP with pestiviruses, the level of contamination decreased in cell cultures from 36.0% in 2004 to 12.0% in 2011, blood serum for the production of VIP from 62% to 10%; The use of uncontaminated cell cultures and blood serum allows the risk of contamination of production virus broodstock to be reduced to zero.

SUMMARY

Situations were studied in relation to distribution of the phenomenon of contamination of veterinary immunobiological preparations of Pestivirus; it is set that basic contaminant is virus of Bovine Virus Diarrhea. A diagnostic set is worked out for an exposure of the Pestivirus method of polymerase chain

reaction (PCR). The basic source of contamination is blood sera of Bovine, which is used for the production of veterinary immunobiological preparations. It is set that most isolates which contaminant preparations were does not own a cytopathic effect that testifies to absence for them of potential pathogenicity. The system of measures, which allows substantially bringing down the risk of contamination the viruses of products, is worked out.

Bibliography

1. Яворський М. І., Коваленко В. І., Литвиненко Л. Ю., Юрченко П. А. Ендокринно-ферментна та спеціальна сировина і лікарські препарати на її основі. Довідник. Асоціація виробників натуральної продукції і лікарських засобів «Вітафарм». Київ : Урожай, 1995. 219 с.
2. Напненко О. О. Стандартизація умов розроблення та виробництва ветеринарних імунобіологічних засобів. Матеріали XI міжнародного конгресу спеціалістів ветеринарної медицини (3–4 жовтня 2013 р., м. Київ, Україна). Київ, 2013. С. 95–96.
3. Напненко О. О., Головка А. М., Ареф'єв В. Л., Гордієнко О. І. Створення системи оцінювання ефективності діяльності колекцій штамів мікроорганізмів в Україні. Матеріали Міжнародної науково-практичної конференції «Біотехнологія та її роль в забезпеченні здоров'я людей і тварин». Ніжин: ПП Лисенко М. М., 2023. С. 168–169.
4. Напненко О., Головка А., Іванченко П. Система контролю за контамінацією сторонніми агентами ветеринарних імунобіологічних лікарських засобів. *Фармаком*, 2021. С. 89–91.
5. Напненко О. О. Система запобігання та виявлення контамінації ветеринарних імунобіологічних препаратів пестівірусами : автореф. дис. ... канд. вет. наук : 16.00.03; Одеський державний аграрний університет, 2012. 18 с.
6. Test for sterility and freedom from contamination of biological materials. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). Publisher: Paris: OIE, World Organisation for Animal Health, 2024. https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/1.01.09_TESTS_FOR_STERILITY.pdf.
7. Державна фармакопея України. Державне підприємство «Український науковий фармакопейний центр якості лікарських засобів», 2-ге видання. Доповнення 3. Харків : Державне підприємство «Український науковий фармакопейний центр якості лікарських засобів», 2018. 416 с.
8. European Pharmacopoeia. Sixth Edition. Edition of the Council of Europe, Strasbourg, France, 2022.

9. Напненко О., Головка А., Безвін Є. Організація серійного контролю якості ветеринарних імунобіологічних засобів в Україні. *Біологія тварин*, 2024, Т. 26, № 3. С. 96.

10. Блоцька О., Головка А., Кузьмич Г., Напненко О. Біотехнологія. Методи попередження контамінації культур клітин : ДСТУ 8115:2015. Введено в дію 01.01.2017. К.: ДП «УкрНДНЦ», 2018. 9 с.

11. Code of Federal Regulations. Animal and Animals Products. Title 9, Parts 1.199. The Office of the Federal Register, National Archives and Records Administration: US Government Printing Office, Washington DC, USA, 2024. <https://www.govinfo.gov/content/pkg/CFR-2024-title9-vol1/pdf/CFR-2024-title9-vol1.pdf>.

12. Напненко О. О., Головка А. М., Ареф'єв В. Л., Гордієнко О. І. Створення системи оцінювання ефективності діяльності колекцій штамів мікроорганізмів в Україні. Матеріали Міжнародної науково-практичної конференції «Біотехнологія та її роль в забезпеченні здоров'я людей і тварин». Ніжин: ПП Лисенко М. М., 2023. С. 168–169.

13. Barone P., Wiebe M. E., Leung J. C., Hussein I. T. M., Keumurian F. J., Bouressa J., Brussel A., Chen D., Chong M., Dehghani H., Gerentes L., Gilbert J., Gold D., Kiss R., Kreil T. R., Labatut R., Li Y., Müllberg J., Mallet L., Menzel C., Moody M., Monpoeho S., Murphy M., Plavsic M., Roth N. J., Roush D., Ruffing M., Schicho R., Snyder R., Stark D., Zhang C., Wolfrum J., Sinskey A. J., & Springs S. L. Viral contamination in biologic manufacture and implications for emerging therapies. *Nat. Biotechnol.* 2020. Vol. 38. P. 563–572.

14. U.S. Dept. of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Biologics Evaluation and Research. Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications; Availability. Federal Register. 2010. Vol. 75, No. 42. <https://www.govinfo.gov/content/pkg/FR-2010-03-04/pdf/2010-4553.pdf>.

15. Стегній Б. Т., Герілович А. П., Лиманська О. Ю. Полімеразна ланцюгова реакція у практиці ветеринарної медицини. Харків, ННЦ «ІЕКВМ». 2006. 110 с.

16. Levings R. L., Wessman S. J. Bovine viral diarrhea virus contamination of nutrient serum, cell cultures and viral vaccines. *Dev Biol Stand*, 1991. Vol. 75. P. 177–81.

17. Harasawa R., Mizusawa H. Detection of Pestiviruses from Mammalian Cell Cultures by the Polymerase Chain Reaction. Proceedings of 3rd Internet World Congress on Biomedical Sciences, 1996. Vol. 12. P. 9–20.

18. Erickson G. A., Bolin S. R., Landgraf J. G. Viral contamination of fetal bovine serum used for tissue culture: risks and concerns. *Dev Biol Stand.*, 1991. Vol. 75. P. 173–175.

19. European Commission (2015). The Rules Governing Medicinal Products in the European Union. Eudralex. Vol. 4–9. European Commission, DG Health and Food Safety, Public health, EU Pharmaceutical information, Eudralex. Available online at: http://ec.europa.eu/health/documents/eudralex/index_en.htm (accessed 25 July 2023).

20. World Health Organization (WHO). 2012. WHO Expert Committee on Biological Standardization. World Health Organization Technical Report Series, Report No. 964. World Health Organization, Geneva, Switzerland. Available online at: https://apps.who.int/iris/bitstream/handle/10665/89142/9789241209779_eng.pdf (Accessed 25 July 2023).

Information about the authors:

Napnenko Oleksandr,

Candidate of Veterinary Sciences, Senior Research Fellow,
Associate Professor at the Department of Veterinary Hygiene
named after A. K. Skorokhodko
National University of Life and Environmental Sciences of Ukraine
15, Heroyiv Oborony Street, Kyiv, Ukraine

Solomon Vyacheslav,

Candidate of Veterinary Sciences,
Head of Department of Veterinary Hygiene
named after A. K. Skorokhodko
National University of Life and Environmental Sciences of Ukraine
15, Heroyiv Oborony Street, Kyiv, Ukraine

Midyk Svitlana,

Candidate of Veterinary Sciences, Senior Researcher,
Associate Professor at the Department of Veterinary Hygiene
named after A. K. Skorokhodko
National University of Life and Environmental Sciences of Ukraine
15, Heroyiv Oborony Street, Kyiv, Ukraine