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DETECTION OF PORCINE CIRCOVIRUS TYPE 2 AND PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS USING PCR AND IMMUNOHISTOCHEMISTRY IN THE TISSUES OF ABORTION FETUSES

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Abstract. *As a result of this study, the PCR approach was shown as the appropriate tool for detection Porcine Circovirus type 2 and Porcine Reproductive and Respiratory Syndrome Virus in the tissues of abortion fetuses. Besides, the frequency of detection PCV2, PRRSV and co infection of these two pathogens had been calculated from the results of PCR. The analyzed material include samples of abortion fetuses, collected in the period from 2007 to 2013 year. Moreover, the efficacy of immunohistochemistry test had been proved as a method for detection PCV2 and PRRSV antigens in myocardium tissues, lungs and lymph node of abortion fetuses.*

Keywords: *Porcine Circovirus, PCV2, Porcine Reproductive and Respiratory Syndrome Virus, PCR, immunohistochemistry (IHC), abortion fetuses*

Accordingly information by 2014-2015, Porcine Circovirus type 2 (PCV2) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) are the most circulated pathological agent of pigs. Moreover, because of economic losses, which correlate with those pathogens, they belong to the most studied pathogens in the industrial breeding in the world [7]. Thus, because of high cost of vaccine and antimicrobial agents needed for treatment secondary infections that complicate PCV2 and PCV2-assocciated diseases, rate of loss from these diseases in 2012-2013 exceeded 14 million euros in Europe and 80 million dollars in the USA, Canada and Mexico [7, 14, 22].

Also, there are very interesting, but at the same time difficult studying and understanding the pathological processes, which caused by common infection PCV2 and PRRSV [23]. First, it is related, that these viruses have common targets cells, which they affect and where replicated - macrophages, lymphocytes and dendritic cells. In these infected cells, pathogens interact with each other at the molecular level, which leads to increased virulence of both pathogens agents [5, 10]. Second, those target cells are components of the immune system, therefore their lesions causes random infringement by the normal immune response to these viruses and other potential pathogens. Besides, there are known fact that co-infection PCV2 and PRRSV causes incorrect immunity formation after vaccination. This fact is confirmed in many experiments, and determined that provided PCV2 and PRRSV co-infection, adequate humoral immune response to vaccination would not developed. Furthermore, vaccination could lead to complications clinical sings and uncontrolled progression of the disease [4, 7, 14, 23, 25].

Moreover, the most interesting and difficult by studying is a phenomenon when PCV2 and PRRSV, and some bacteria (ex. *Mycoplasma hyopneumoniae*) infected one cell, what associated same target cell. This interaction lead by increase of PCV2 in tissues and development of other PCV2-assocciated diseases [24]. The positive effect of co-infection by replication PCV2 and progression of PCV2-assocciated diseases, have been shown on the gnotobiotic animals. Thus, after infection of gnotobiotic pigs used material lymphatic tissue from animals with acute manifestations PCV2-assocciated diseases, patients had similar clinical symptoms. However, study of pathological materials, which selected for infection, were detected not only PCV2, but also Porcine Reproductive and Respiratory Syndrome Virus. Later was confirmed presence of mixed infections PCV2 and VRRSS in causes of pigs-associated systemic infection and circovirus-associated reproductive disorders [8, 9, 21, 24].

The first cases of reproductive disorders in pigs which associated with Porcine Circovirus type 2 and PRRSV, were registered in Canada in 1999 [15]. There were described the increased of rate of abortion fetuses, stillborn pigs [14, 27] and fetal mummification at different stages of development. During the morphological and pathological analysis the main feature of PCV2 associated lesions is non-purulent necrotic or fibrotic myocarditis of stillbirths or weak neonatal porcine. The etiology of the myocarditis was confirmed by the presence of high concentrations of the PCV2 antigen in immunohistochemical analysis [1, 14]. While for the pathology caused by the influence of VRRSS were observed abortions outside normally developed fetuses during the last trimester, and hyperaemia of the spleen, thymus and lung during dissection.

During the experimental infected fetuses by Porcine Circovirus type 2 it was described a powerful virus accumulation in the tissues of the fetus and there was found that the heart is the primary organ for its replication [2]. At intrauterine entering of the virus at 57, 75 and 92 days of gestation, there was found that the level of virus replication of infected fetuses at 57 day is significantly higher than in infected fetus at 75 and 92 days. And at research of the tissues of this fetuses at 21 day after infection there was determined that damage (such as swelling, hyperaemia of the liver and other organs) were observed only among fetuses infected at 57 day [2]. But in researches of Johnson et al. [6] there was shown that from 37 examined fetuses of 3 animals that were infected at 86, 92 and 93 days of gestation, 24 were without pathology, 7 - mummified, and 6 - stillborn. The following data indicate that infecting the fetuses by PCV2 is possible at the last days of gestation and also leads to reproductive problems [6]. At the same time, there was confirmed by multiple researches that the infection with PRRS virus is possible at any stage of animals gestation and in all cases it lead to disturbed of development of the fetuses, but with varying degrees of pathological manifestations [1, 6, 14].

Proceeding from the above, the aim of our study was detection Porcine Circovirus type 2 and Porcine Reproductive and Respiratory Syndrome Virus in

the tissues of abortion fetuses that were taken during the period from 2007 to 2013 using PCR method and immunohistochemistry.

Materials and methods. The objects of the study were isolates PCV2 and VRRSS, taken from tissues of aborted fetuses at the 2nd and 3rd trimesters of gestation. Analysis of morphological and biological condition of selected for the research animals was carried out in the laboratory of pathological anatomy SPE CSR "Bio-Test Lab." For this there were performed dissection of fetuses, description and photograph of pathological changes in tissues and collecting the samples of lungs, myocardium, thymus and intestine for further molecular genetics and immunological researches [17, 18].

At the first stage of the research, the isolation of DNA and RNA from the selected samples was carried out by generally known method using ready-filtration columns for the purification of nucleic acids «Tissue RNA / DNA» (Machery Nagel, Germany). Then carry out the reaction of reverse transcription using a set of reagents «First Strand DNA synthesis Kit» (Thermo, USA). Making of polymerase chain reaction was carried out separately for detecting RNA of PRRSV virus and DNA for PCV2 virus using fabricated reagents for PCR (Thermo, USA), and two pairs of specific primers with the following nucleotide sequence and characteristics:

№ n/p	Primer	The orientation of primers	The nucleotide sequence of primer	Position in the genome
1	Cir_orf2_F	forward	5’-ATGGTTACACGGATATT GTAGTCC -3’	1086-1110
2	Cir_orf2_R	reverse	5′- TCCCGCACCTTCGGATA TACTG -3′	1567-1588

For immunohistochemically research were taken the samples of lung, myocardium, and thymus from aborted fetuses that according to preliminary laboratory tests were co-infected with PRRSV and PCV2. The histological sections with 5 μm of thick were made with kriomikrotom Microm HN 505 N. Immunohistochemically reaction was carried out using the previously published

methods [12] with the use of monoclonal antibodies PCV VP2IHQ ORF2 (Ingenasa, Spain) and PRRSV NIHQ ORF7 (Ingenasa, Spain). Visualization of antigens was carried out using peroxidase and secondary polyclonal antibodies (Sigma, USA). Microscopic of the samples under 200x and 400x magnification was carried using the relevant microscope lenses of Axioskop 2 plus microscope (CarlZeiss). The photographing of samples for further analysis was performed using the camera on a fourfold optical zoom.

Results. For primary diagnosis and determine the causes of sows abortions certain clinical and pathological-anatomical features are generally used that`s why on the first stage of the work the description and analysis of pathological changes of aborted material. There is well-known fact that the fetuses become immunocompetentstarting from 70 days (about 17 cm in length), and after this become permissive to PRRSV. This figure is used as the primary selection criteria for diagnosis in RRSSV and PCV-2. Because of the transmission of the RRSS virus through transplacental barrier in the third trimester of gestationfor, the aborted material for research was taken in this term.

Overall, as a result of this work, we had examined 1156 animals from 65 farms of 24 regions of Ukraine and Crimea. Due to pathoanatomical analysis it was found that the examined aborted fetuses had hemorrhages in the myocardium (9% of the total number of tested animals) and lung (14%), anemia (3%), icteritiousness (2%), pulmonary hyperemia (11%), thymus (9%), spleen (3%), pulmonary edema (9%) and thymus atrophy (7%) (Fig. 1). The presence of up listed symptoms inherent mainly for infection and RRSSV and PCV-2.

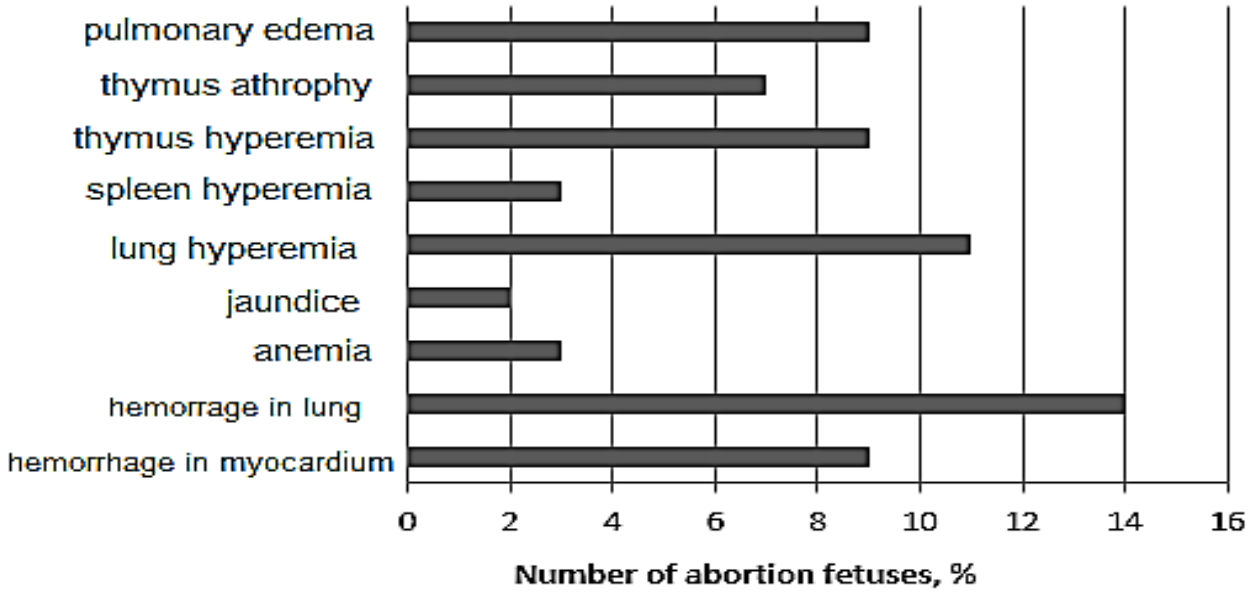

Fig. 1. Pathophysiological changes, associated with PCV2 and PRRSV, detected in samples of abortion fetuses

Due to obtained results, the next phase of work was carried with identify of genetic material of PCV2 and PRRSV by polymerase chain reaction (PCR). For the biomolecular researches was carried the selection of unified samples (lung, myocardium and thymus),the samples of the selected material were numbered and systematized according to the place of taking of aborted material. Since the PRRS virus quickly destroyed after the death of aborted fetuses, the identify its genetic material by PCR in the aborted material is quite complicated. To identify RRSSV in aborted fetuses in view of data of Raymond R.R. Rowland et al. thymus and lungs were selected [20].

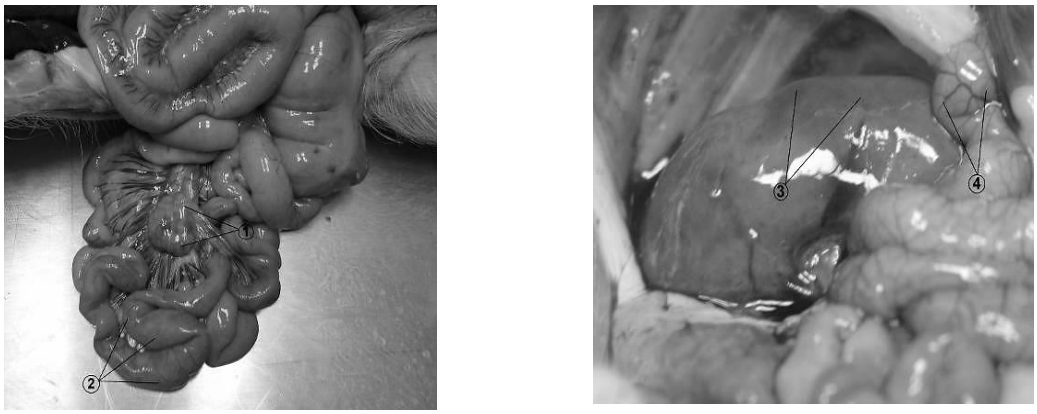

Fig. 2. Pathological changes in organs of tested animals 1 - hyperemia of the mesenteric lymph nodes; 2 - catarrhal enteritis of small intestine; 3 - pinpoint hemorrhages on the kidneys; 4 - hyperemia of a small intestine

Overall there were found 192 positive PCV2 samples abortion material that is about 17% of the all samples (Figure 2). PRRSV was found in 125 samples (11%) (Figure 2). We had samples with both pathogens in 17 clinical cases, that is about 2% of all samples (Figure 2). In these cases, the presence of co-infection PRRSV and PCV2 complicated the expression of reproductive problems in sows.

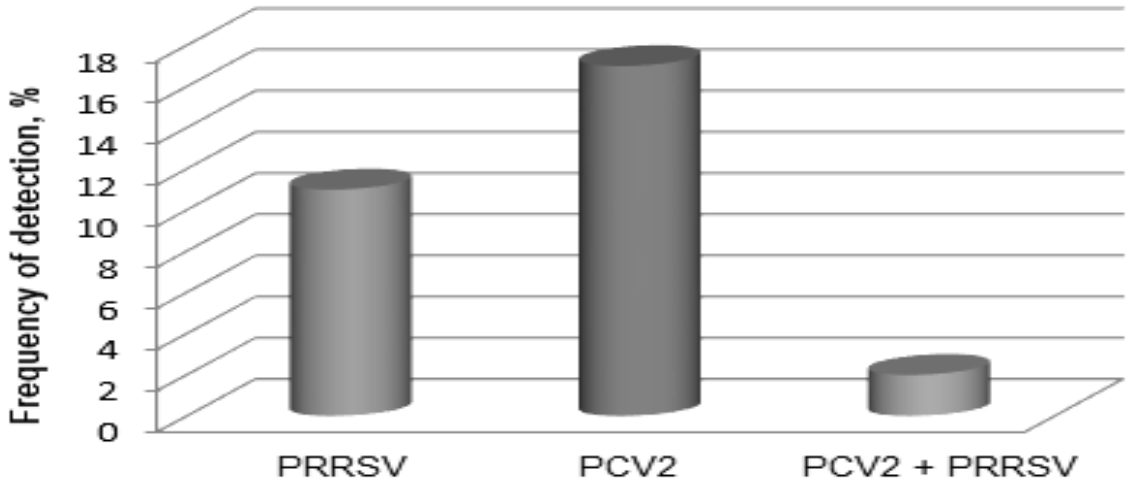

Fig. 3. Frequency of detection PCV2, PRRSV, and co-infection in the tissues of abortion fetuses on 3th trimester of gestation

The next stage of our research was to identify the antigen PRRSV and PCV2 in the tissues of aborted fetuses, in which, based on the results biomolecular research, was detected genetic material of both pathogens (PRRSV and PCV2). According to the results of immunohistochemical investigation the antigen to PRRSV frequently and in larger amounts was found in the thymus, while the PCV2 antigen was more often in the lungs, and considerably less in the myocardium of aborted fetuses (Fig. 3). Raymond R.R. Rowland et al. showed that the largest number PRRSV virus at research of tissues of the fetus are present in the thymus, while in the lungs - significantly less [20], which is fully consistent with our results

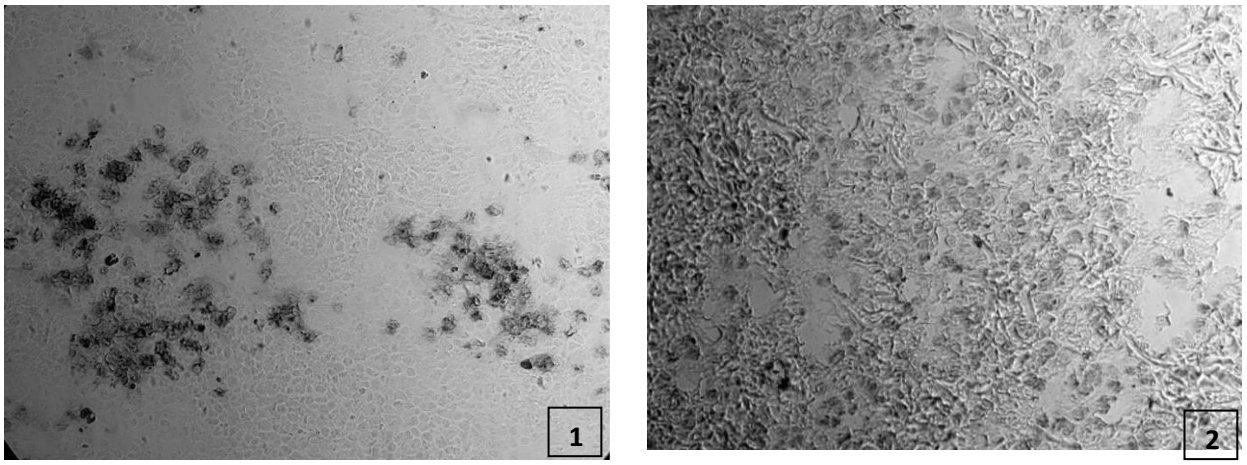

Fig. 4. Thymus tissue (1), lung tissue (2); aborted fetus infected with PRRSV and PCV2. Intracytoplasmic coloring of PRRSV antigen macrophages of the thymus (1) and lungs (2). Immunohistochemical coloring, x400

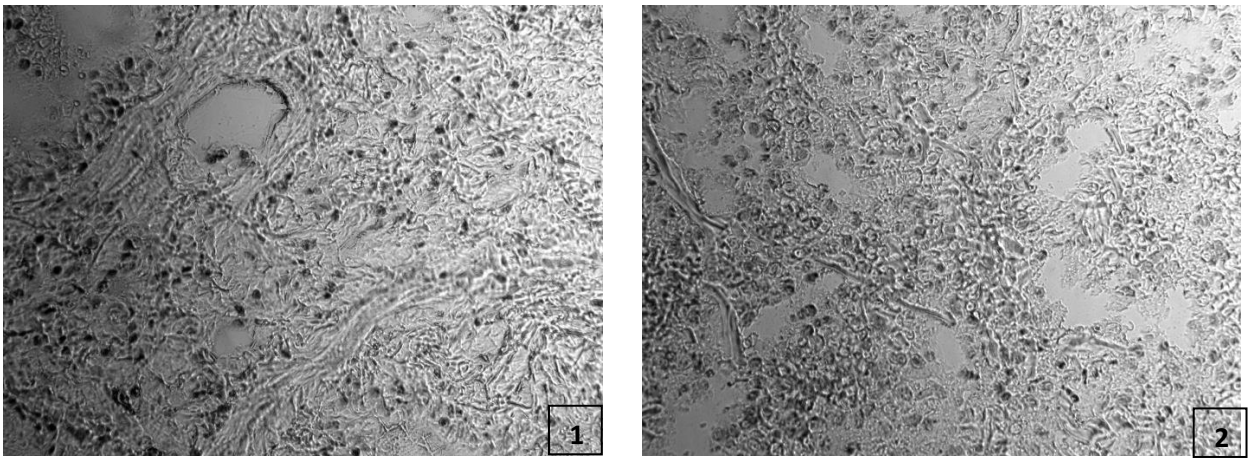

Fig. 5. Myocardial tissue (1), lung tissue (2); aborted fetus infected with PRRSV and PCV2. Intracytoplasmic coloring of PCV2 antigen in the myocardium (1) and lungs (2). Immunohistochemical coloring, x400.

Therefore, using immunohistochemistry method to detect antigens of PRRS and PCV2 viruses, there were investigated samples of tissue of 17 aborted fetuses, which had previously identified genetic material of both pathogens. The PRRSV antigens were found in 14 samples, while PCV2 antigen was detected in all 17 samples. The results are point to weaknesses of using immunohistochemical method in research for diagnose PRRSV. The efficiency of immunohistochemistry for detection of the PRRSV antigen depends on the genetic characteristics of pathogen and quality of aborted material (the degree of autolysis of tissues). Using of immunohistochemical method to diagnose PCV-2 is important and revealing, because we did not get incorrectly negative results even in case of a minor viral load supplementing the previously published data [12].

Conclusions

Thus, the result of the work we have studied 1,156 aborted fetuses from 65 farms of 24 regions of Ukraine and Crimea for these 7 years. As a result, it was found 334 of the total sample were affected at least by one of the two viral pathogens (PRRSV and PCV-2). Generally, our results indicate the prevalence of PRRSV and PCV-2 in Ukraine and the significant role of the above pathogens in the occurrence of reproductive problems of sows. Necessary to add that we found a tendency to

increase the number of infected PRRSV and PCV-2 of aborted fetuses during the last 3 years what also show increasing importance of these pathogens especially for industrial pig farming in Ukraine.

Over these 7 years was discovered only 17 clinical cases (2% of the total) co-infection of the fetus and PRRSV and PCV-2. However, given the above significant of pathogens distribution in Ukraine and world [11, 13, 16, 19, 26] the number of co-infections in could increase tenfold. Since because of the co-infection PRRSV and PCV-2 there are observed complications of manifestations of both diseases, there is an urgent need to implement effective solutions to the existing problems and ways of blocking PRRSV and PCV-2 through Ukraine.

One of the effective ways of combating with those pathogenic is their fast and accurate detection. Since the work with aborted material is complicated by rapid tissue decomposition and elimination of PRRSV immunohistochemical method of diagnostic of PRRSV, is less indicative compared with efficiency of use of PCR. PCV-2 is showing greater resistance to the environment, in view of this there was no problems with detection of the pathogen by IHC and PCR.

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ОБНАРУЖЕНИЕ ЦИРКОВИРУСА СВИНЕЙ ТИПА 2 И ВИРУСА РЕПРОДУКТИВНОГО РЕСПИРАТОРНОГО СИНДРОМА СВИНЕЙ С ПОМОЩЬЮ ПЦР И ИММУНОГИСТОХИМИИ В ТКАНЯХ АБОРТИРОВАНЫХ ПЛОДОВ

Л. Дудар, О. Иващенко, В. Полищук

Аннотация. *В результате нашего исследования показано, что метод ПЦР является подходящим инструментом для обнаружения цирковируса свиней типа 2 (PCV2) и вируса репродуктивного и респираторного синдрома свиней (PRRSV) в тканях абортированных плодов. Кроме того, частота обнаружения вирусов отдельно и при совместной инфекции этих двух патогенов могут быть рассчитаны, исходя из результатов ПЦР. Анализируемый материал включает образцы абортированных плодов, собранных в период с 2007 по 2013 год. Кроме того, эффективность иммуногистохимического теста была доказана для использования в*

качестве метода для обнаружения PCV2 и PRRSV антигенов в тканях миокарда, легких и лимфатических узлов абортов плодов.

Ключевые слова: *свиной цирковирус, PCV2, вирус репродуктивно-респираторного синдрома свиней, ПЦР, иммуногистохимия*

ВИЯВЛЕННЯ ЦИРКОВІРУСА СВИНЕЙ ТИПУ 2 ТА ВІРУСУ РЕПРОДУКТИВНОГО РЕСПІРАТОРНОГО СИНДРОМУ СВИНЕЙ ЗА ДОПОМОГОЮ ПЛР ТА ІМУНОГІСТОХІМІЇ В ТКАНИНАХ АБОРТОВАНИХ ПЛОДІВ

Л. Дудар, О. Іващенко, В. Поліщук

Анотація. *В результаті дослідження було показано, що метод ПЛР є підходящим інструментом для виявлення цирковіруса свиней типу 2 (PCV2) і вірусу репродуктивного та респіраторного синдрому свиней (PRRSV) в тканинах абортованих плодів. Крім того, частота виявлення PCV2, PRRSV та спільної інфекції цих двох патогенів можуть бути розраховані за результатами ПЛР. Аналізований матеріал включає зразки абортованих плодів, зібраних в період з 2007 по 2013 рік. Крім того, була доведена ефективність імуногістохімічного тесту в якості методу для виявлення PCV2 і PRRSV антигенів в тканинах міокарда, легенів і лімфатичних вузлів абортів плодів.*

Ключові слова: *свинячий цирковірус, PCV2, вірус репродуктивного респіраторного синдрому свиней, ПЛР, імуногістохімія*