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RECEPTION OF PSEUDORABIES VIRUS ANTIGEN FOR USE IN AUJESZKY'S DISEASE DIAGNOSIS

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The authors have studied Aujeszky's disease virus (strain UNDIEV-18v), which was isolated in Ukraine. The virus was tested as a candidate for preparation of diagnostic kits. There was conducted preliminary study of cell cultures and virus in PCR concerning their contamination by outside viruses and mycoplasma. Cultural, biological and biophysical characteristics of the strain have been studied. As a result of conducted investigations there was obtained data concerning virus biological activity on the cell culture BHK-21/13 and on susceptible animals (rabbits), floating density of this strain in sucrose density gradient has been determined, virus electronic photographies have been made. Scheme of the reception of concentrated and purified antigen for diagnostic kits has been developed. The scheme includes accumulation of virus biomass by virus roller cultivation, its concentration with the help of polyethylene glycol-6000, its purification by high speed ultracentrifugation and finally, reception of pure antigen for animal immunization with the help of 1 % concentrations of sodium dodecyl sulfate and electrophoresis in polyacrylamide gel. Proposed scheme may be used as prototype for reception of concentrated and purified virus fractions for the production of diagnostic kits.

Keywords: Aujeszky's disease, Pseudorabies virus, Diagnostic kit, Swine, Ukraine

Aujeszky's disease has attained considerable importance for countries with developed swine-breeding programs due to its economic impact and difficulties in eradicating the disease. The agent causing the disease affects the central nervous system, respiratory organs, cells of immune system (macrophages, lymphocytes) causing encephalitis, pneumonia, sterility and immunodeficiency along with a complex of associated secondary pathogenic infections.

In the natural history of the development of Aujeszky's disease, there are two chronologically defined periods [1]. The first period includes the time since A. Aujeszky identified the disease as a new nozologic form in 1902 and to the beginning of the last fifty years of the past century, when sporadic outbreaks of disease of cattle, dogs, cats, rats and, rarely, pigs were registered in several countries [2]. The second period chronologically connected with the intensification of swine-breeding in European and other countries of the world in 60-70th years that was characterized by widespread Aujeszky's disease in Europe, North and South America. Since 1974, the incidence of Aujeszky's disease in the United States has increased more then 15-fold. From 1980 to 1989, Germany identified 1,093 trouble areas, while during the past 10 years the number of outbreaks has increased more than 12-fold. During the period from 1973 to 1988, Belgium registered 4,096 trouble centers for Aujeszky's disease. The epizootiologic situation concerning Aujeszky's disease stimulated the German, British and Belgian governments to adopt measures concerning the detection and registration of outbreaks of this particularly dangerous infection [3].

It is important to note that emphasis on the enzootic nature of Aujeszky's disease characterizes not only the intensity of the disease process itself but also its persistence in affected swine-breeding facilities because pigs are natural hosts and reservoir of the virus.

Current data concerning the spread of Aujeszky's disease in countries with intensive swine-breeding highlights the great potency of the agent to spread epizootically and survival in natural conditions. It also underscores the danger of underestimating the significance of the disease in countries whose economies rely heavily on swine-breeding and the potential for enormous economic losses. There is little doubt that Aujeszky's disease is one of the most dangerous viral infections for facing swine breeders [4]. In Ukraine, Aujeszky's disease usually manifests itself in the latent form, only exhibiting mild respiratory symptoms that complicate diagnosis. Uneven diagnosis can result in mass reinfection of pig populations and spread of the infection beyond the bounds of troubled farms [5, 6].

The experience of eastern European countries and United States shows that only wide serologic surveillance of pig population on farms, including farms with successful Aujeszky's disease control programs, can reveal the true epizootic extent of the disease. The diagnosis of latent and clinic forms of the disease, especially in young animals with only respiratory syndrome, enables one to determine the epizootic status of individual farms and to select the best measures for controlling Aujeszky's disease. Improvement in the health of swine herds is reached by way of vaccination, and the conduct of veterinary-and-sanitary measures using diagnostic means for detection and elimination of infected animals [7, 8, 9, 10, 11, 12]. However, even two vaccinations using inactivated vaccines does not always prevent clinical signs of disease on troubled farms, but will completely prevent weight loss and inhibit virus excretion in immunized animals [13, 14]. Oirschot J.T. van [15] has reported that following the appearance of clinical signs infected animals will discharge virus for 10–14 days after which they will enter a latent state. In those cases, vaccination does not prevent the clinical manifestations of the disease. Pensaert M.B. et al [16] have established that vaccination does not completely inhibit the excretion of Aujeszky's disease virus. That is why segregation of offspring, testing and removal from the herd of animals that show seropositive for antibodies to Aujeszky's disease virus is the most accepted measure for swine-breeding improvement and disease control [17].

Retrospective diagnosis and sero-epizootological monitoring of pig herds could be helped significantly by the availability of diagnostic ELISA-kits capable of differentiating among antibodies from naturally infected and vaccinated animals. As such, the development and scale up to production of ELISA diagnostic kits capable of making the distinction between naturally infected and vaccinated is a goal of considerable importance. Production strains of living and inactivated vaccines must be marked by deletion of a detectable glycoprotein that is inseparably connected with the diagnostic kits. It is also vitally important to investigate the role that viral proteins play in the expression of virulence since the knowledge of what differentiates vaccine from field strains of the virus will form the basis for differentiation and identification of field isolates and differentiation of postvaccinal from and post infection antibodies [18, 19, 20].

The purpose of the project was to investigate and prepare highly purified antigen from Aujeszky's disease virus, to be adapted and used in the production of monoclonal antibodies for use in diagnostic ELISA-test-systems.

The objectives of the project were achieved by way of carrying out of the following tasks:

- accumulation of Aujeszky's disease virus strain UNDIEV-18v biomass by the way of cultivation in monolayer culture of continuous cells SNEV;
- testing of virus containing cultural suspension on contamination by outside microorganisms;
- determination of authenticity of Aujeszky's disease virus strain UNDIEV-18v;
- purification and concentration of Aujeszky's disease virus;
- virus destruction by physical and chemical methods;
- investigation of specificity and antigenic activity of protein fraction of Aujeszky's disease virus;
- preparation of lyophilized sample of Aujeszky's disease virus.

Materials and methods of investigations. Investigations on preparation of Aujeszky's disease virus antigen were conducted using the epizootic strain UNDIEV-18v, which was adapted to reproduce in SNEV cell culture. The infectiveness of dried virus in cell culture was 7.0 $TCID_{50}$/ml and lethal activity - 6.5 $TCID_{50}$/ml.

Accumulation of the biomass of the Aujeszky's disease virus strain UNDIEV-18v was achieved by cultivation in monolayer cultures of continuous cells SNEV and BHK-21/13, which were grown in a mixture of equal volumes of nutrient Eagle's medium and 199 medium with 10 % nonpreserved commercial bovine blood serum, inactivated by heating at 56 ^{0}C during 30 minutes. Serum was produced by our subsidiary production unit "Veterinary Medicine", Kharkiv.

Continuous cells were cultivated in glass cultural vials (volume 1.5L) and in roller bottles (volume 3L), and in test-tubes. Cultures of continuous cells were maintained by way of multiple reseeding and cryopreservation. Cryoprotective medium was prepared using the same nutrient media in which cells had been grown along with the addition of 10 % dimethyl sulfoxide or dimexid.

The availability of cells for cultivation after long term cryopreservation in liquid nitrogen was determined by staining with trypan blue to quantitatively determine the number of living and dead cells. Cell viability was estimated by proliferative activity and the level of mitotic index.

Pathogenicity of dried and cultured Aujeszky's disease virus was studied by intramuscular injection of rabbits, Chinchilla breed, weighing between 1.8 and 2.5 kg The infectivity of Aujeszky's disease cultural virus was determined by titration in monolayer culture of continuous cells BHK-21/13. With this purpose there were prepared 10-times work solutions of UNDIEV-18v strain. Test objects (test-tubes or flasks with cell monolayer) were infected in fours. Titer of virus infectiveness was calculated by the method of Rid and Mench and was expressed in $TCID_{50}$/ml. The residual infectivity of intermediate samples of prepared antigen obtained from suspensions of cultured virus was checked by three "blind" passages in continuous cell culture.

Studies concerning the possible contamination of nutrient media, blood serum, continuous cells, dried and cultural Aujeszky's disease virus and samples on all stages of antigen preparation on the base of the strain UNDIEV-18v by outside microorganisms was conducted according to SSTC 4483:2005 "Veterinary and immunobiological preparations. Screening for detection bacterial and fungal contamination was accomplished by sowing nutrient media on meat infusion agar and meat infusion broth, thioglycolic medium and Edward and Saburo medium. Screening for possible viral or mycoplasma contamination was accomplished using the primer systems listed in Table 1.

Table 1 – Primers for detection of agents of viral origin and mycoplasma

Agent	Primers	Annealing temperature, ^{0}C	Size of the site, п.н.	Author of primers
Classical swine fever virus	CSFV	55	220	Junghyun Kim and Chanhee
Bovine viral diarrhea virus	BVDV	55	280	Gerilovych A.P., Stegniy B.T.
Swine rotavirus (types P and G)	RotaP, RotaG	52, 54	1000, 1200	Catton, J.
Swine coronaviruses	TGES/RCVS	56	1084, 240	Woods, R.D.
Porcine parvovirus	PPV	58	420	Junghyun Kim and Chanhee
PRRS virus	PRRS (Com), PRRS (Eu), PRRS (Am)	55	300, 220, 160	Stadejek, T.
Aujeszky's disease virus	AuDV	58	235	Gerilovych A.P.
Porcine circoviruses	PCV-1, PCV-2	60, 62	420, 480	Gerilovych A.P.
Mycoplasma	Myc23S	52	100-120	Sachse, K.

Investigations were conducted using classical PCR with product detection in agarose gel. RNA and DNA were extracted from investigated samples by the method of affinity absorption using commercial kits DNA-sorb-D® and Ribo-sorb-100® produced by the Central Research Institute of Epidemiology (Moscow, Russia).

Reverse transcription of RNA to produce complementary DNA was performed using the kit Reverta-L® also produced by the Central Research Institute of Epidemiology (Moscow, Russia).

Specific sites of viral and mycoplasmal DNA, complementary DNA, were determined by amplification using basic reagents like Tag-polymerase and buffer and magnesium sulfate and deionized water produced by the Central Research Institute of Epidemiology (Moscow, Russia).

As positive control samples there were taken DNA and RNA-extracts from vaccine preparations against parvo-, rota-, corona-, herpesvirus and mycoplasma swine infections produced by Intervet, Phyzer, Merial, and also DNA of the strains Am-7 and PK-15 of swine circoviruses.

Electrophoresis of PCR products was conducted in 1.5 % agarose gel at tension 15 V/sm. Result was visualized by the way of gel examination in ultra-violet rays with wave-length 280 nm.

In the case of contamination detection, investigated samples were culled and they were not used in further work.

Specificity and activity of investigated samples of Aujeszky's disease virus antigen and virus specific blood sera were tested in neutralization reaction, ELISA and radial immunodiffusion test. Neutralization reaction was conducted in cell culture BHK-21/13 by standard method using work twofold solutions of blood serum from 1:2 to 1:102 and constant virus dose (1000 $TCID_{50}$/ml).

At conducting ELISA reaction by sandwich variant, panels were sensitized by immunoglobulin. Aujeszky's disease virus antigen was detected with the help of antibodies, conjugated with horse-radishperoxidase. As antigen there were used cultural virus containing suspension and investigated samples of prepared antigen. Immunoglobulin, which was used as preparation for sensibilization of solid phase and preparation of conjugate, was isolated from specific hyperimmune blood serum of rabbits. [21].

Virus containing cultural suspension of the strain UNDIEV-18v was concentrated with the help of ammonium sulphate, polyethylene glycol-6000, ultrafiltration and high speed ultracentrifugation in sucrose and chloride caesium density gradient. With this purpose virus cultural suspension was subjected to single freezing – defrosting and after removal of cell detritus by law speed centrifugation (3000 g for 20–30 minutes), virus was precipitated by adding of different concentrations of ammonium sulphate and polyethylene glycol-6000. Mixture of virus containing suspension and solutions of these chemical substances were kept in the cold for not less than 12 hours. Obtained precipitate, washed for two-three times in FSB, after centrifugation at 3000 g for 15–20 minutes and solute in mild alkaline buffer solution in the volume 1/10 of initial suspension was used in the work.

Precipitated on filter membranes with pores 300 Å virus was gathered by FSB washing down in the volume 1:20 of initial suspension. FSB was washed for three times.

Remains of chemical substances and additional removing of denaturated and soluble proteins were removed by the way of differential centrifugation (law speed and further high speed). Later concentrated virus was purified by centrifugation in balanced density gradient and selected virus containing and virus specific protein site.

Corpuscular antigen of Aujeszky's disease virus was prepared by the method of disintegration of virus particles using detergents and ultrasound. 0,1; 0,5 and 1 % concentrations of sodium dodecyl sulfate, Twin-80 and Triton-X 100 and ultrasound current strength 20 and 50 kHz effect were tested.

At destruction of virus particles with the help of anionic detergents to 0,3 mg of purified concentrate of Aujeszky's disease virus strain UNDIEV-18v there was added 0,15 ml of proper solution of ammonium dodecyl sulfate and 0,15 ml of 0,1 M borate buffer, pH 10,5. After incubation for 40-60 minutes under the temperature 40 ^{0}C there was added 1 ml of 0,01 M natrium borate, pH 10,5 and by drops saturated solution of KCl at permanent mixing. Created under the room temperature precipitate was cooled for 30 minutes under the temperature 0 ^{0}C. Protein was precipitated by centrifugation under the same temperature and solved in 1 ml of natrium borate, pH 10,5.

At use of nonionic detergents to resuspend in cooled buffer virus concentrate (0,3 ml) there was added tried quality (0,7–2 mg) of Twin-80 or Triton X-100, exposed for 30 minutes on ice, periodically shaking, added ½ of the volume of cooled ether and energetically shaked in shuttle apparatus for 60 minutes under the temperature 4 ^{0}C. After that, mixture was centrifugated at 500 g. Remains of ether were removed by nitrogen bleeding. Lower, weakly opalescent water phase, which was additionally brightened by centrifugation at 5000 g, was selected.

Additionally samples of disintegrated virus particles were subjected to ultra sound under the conditions of controlled temperature. After that, protein fractions were separated by electrophoresis in polyacrylamide gel [22] and by gradient ultracentrifugation.

Size of virus particles was determined by investigation of concentrated samples under electron microscope.

Results of investigations. Investigations on preparation of Aujeszky's disease virus antigen adapted for using in ELISA test-systems for detection of virus specific antibodies were carried out on the model of epizootic strain UNDIEV-18v. On the base of this strain two vaccines were created in Ukraine [23, 24]. Since the virus was kept for a long time in lyophilized state, first of all its reproductive ability in monolayer culture of continuous cells SNEV has been restored. There was established that it holds infectiveness and even in the first passage it reproduced in cell culture with titer 7,0 lg $TCID_{50}$/ml. After the third passage of restoration of its properties the titer of infectiveness grew to 7,0 lg $TCID_{50}$/ml.

Simultaneously with restoration of biological properties of lyophilized strain UNDIEV-18в, testing for contamination by foreign microorganisms was carried out. Using bacteriological methods, we were able to exclude virus contamination by bacteria, fungi and mycoplasma and by the results of PCR – we were further able to exclude contamination of the virus by the agents that cause classical swine fever, reproductive-and-respiratory syndrome, transmissive swine gastroenteritis and rotavirus. At the same time based on the

results obtained by PCR, we determined that the second type (SVC II) of the continuous cell culture SNEV was contaminated by circovirus. On this basis, this line was excluded from following investigations. For accumulation of Aujeszky's disease virus biomass we used continuous cell culture BHK-21/13 which had been determined by bacteriological and molecular-genetic control methods to be free from contaminants of bacterial and viral origin.

Since cell cultures represent a key step of the biotechnological process of biological preparation and production, investigations to estimate the productivity of continuous cells BHK-21/13 were carried out. As a result of a comparative study of two methods of accumulation of their biomass, it was proved that cells growing in roller bottles are more productive compared to cells cultivated as flat mattresses (mono-layers). Roller bottle cultivation ensured more effective production of cell biomass, contributed to almost 1.5 times higher accumulation of counted cells per unit of nutrient medium. When the growth sowing surface was correlated to the volume of nutrient medium (s/v) efficiency of cell cultivation increased. In 3 L roller bottles, the correlation of s/v was 2 cm^2/ml. On the basis of these investigations it was shown that a sowing density of cells within a range of 0.45–0.57 mln/ml led to optimal reproduction and not less than a 4-fold increase of their biomass in the mixture of nutrient media 199 and DMEM from 10 % commercial blood serum of cattle. Growing cell culture BHK-21/13 characterized by stable properties concerning monolayer formation and level of biomass accumulation and cell monomorphism.

Using cytological investigative methods, it was determined that the culture of continuous cells BHK-21/13 ability to grow exceeded SNEV. Mitotic index made to 40 % and quantity of pathologic mitosis was on the level of 20 %.

Experimental grounds of basic parameters of technological process of BHK-21/13 cell biomass accumulation, which are free from contaminants, determined expediency of realization of following investigations on study of reproductive properties of the strain UNDIEV-18v and preparation of corpuscular antigen using a roller bottle system for cell and virus cultivation.

In order to exclude possible contamination by outside microorganisms while working with the virus, continuous cells and nutrient media, especially blood serum, prepared antigen samples were regularly monitored by bacteriological methods and PCR. Special attention was paid to controlling contamination by outside viruses and mycoplasma. The cell culture SNEV (8 types – 6 of the first model and 2 of the second) was probed with system Myc23S primers and mycoplasma DNA mycoplasma was not detected nor were DNA-fragments of swine parvovirus and Aujeszky's disease virus. Reverse transcriptase PCR was used cell type SNEV to look for RNA of the virus that causes porcine respiratory-and-reproductive syndrome (PRRS), swine rotavirus (SRV (P and G)), the virus of swine transmissive gastroenteritis, respiratory coronavirus of swine, and the virus of classical swine fever. The absence of these viruses was confirmed by the existence of clear strips of amplicons of calculated length in homological positive control samples: RNA rotaviruses with primers for Rota G, P, viruses, PRRS with primers PRRS (com, Eu, Am), TGES and RCVS with primers TGES/RCVS, classical swine fever with primers CSFV, and DNA of parvovirus using primers PPV (Table 2).

Table 2 – Results of investigation of mycoplasma and viral contamination of cell cultures using for preparation of PrV antigen

Cell culture	DNA mycoplasma	DNA CVS	DNA PVS	RNA VD	RNA CSF	RNA CVS	RNA RVS
SNEV-1	0/6	4/6	0/6	2/6	0/6	0/6	0/6
SNEV-2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
BHK-21	0/8	0/8	0/8	0/8	0/8	0/8	0/8

Notes: numerator – quantity of positive samples; denominator – quantity of investigated samples

However in the first sample obtained of these cells genetic material of bovine diarrhea virus (VD) that was accompanied by creation of fragment with length of 280 bp, and swine circovirus of the second type (SCV) with creation of a fragment with the length of 420 bp (Fig. 1) was detected. In cells of the second sample of types SNEV, neither RNA nor DNA of these viral contaminants was not detected.

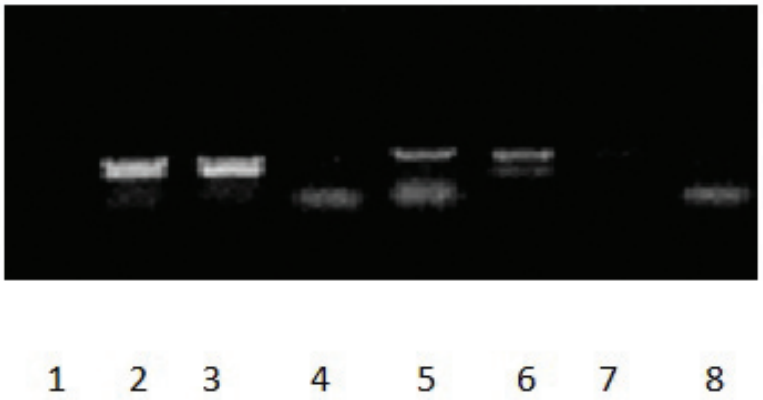


Figure 1. Investigation of contamination of SNEV culture by circovirus of type II and bovine VD: 1 – SNEV-2, 2 – SNEV -1, 3 – positive BVDV, 4 – negative, 5 – positive CVS-II, 6 – SNEV-1, 7 – SNEV-2, 8 – negative

As alternative system for accumulating PrV, we decided to use a culture of BHK-21. Upon examining 8 samples of this culture on the presence of CVS-II DNA, mycoplasma, CVS, PrV, and RNA of BVDV, RCVS, TGES/RCVS neither viral nor mycoplasma genetic material was detected. This was confirmed by the absence of amplicon creation after reaction with specific primers at existence of specific products in reactions with DNA (cDNA) of control samples.

Investigations concerning contamination by viruses and mycoplasma by PCR of cattle blood serum, native without preservatives [P. 1-10 (2007), P. 1-2 (2008)] were conducted in two stages. On the first stage after extraction of nucleic acids, amplification 280 bp of gene site of polyprotein of cattle VD was conducted. AT that, products of reaction were not determined in any investigated samples of serum. In blood test [sample P. 2 (2007)] fragment of nonspecific length was determined. However, at profound investigation using system of primers BVD2 absence of RNA virus in series of these samples was proved.

By the study of contamination of serum by mycoplasma with system of primers Myc23S specific products also were not been determined. This allowed making a conclusion about absence of material contamination by this microorganism.

On the base of registered negative reaction with primers BVDV and Myc23S further selection of nutrient substance was continued in the direction of assessment of their growing qualities

An investigation of virus strains with AuDV systems primers proved their authenticity by creating a specific fragment of genomic IgE with the length 235 bp (Fig. 2).

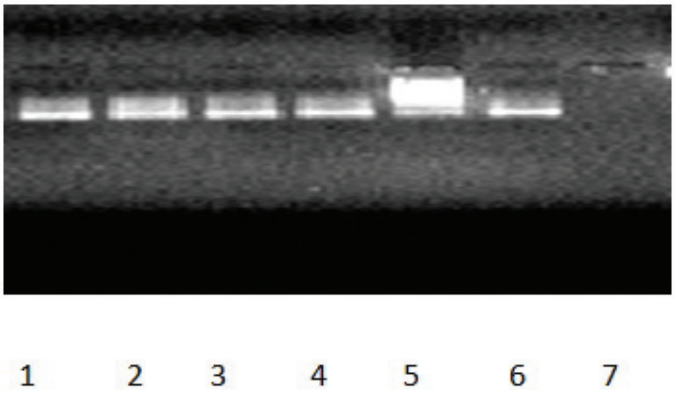


Figure 2. Investigation of PrV authenticity in breeds of the strain UNDIEV 18b – 1 – 5 – breeds of strain, 6 – positive (DNA from vaccine against PrV "BTL" LTD), 7 –negative

With the purpose of purification of the strain UNDIEV 18v from possible contamination with circo- and pestivirus, passage in rabbits was conducted. Rabbits infected by the cultural virus of Aujeszky's disease died on the second day with typical lesions at the cutaneous sites where the strain UNDIEV 18v was introduced (Fig 3).

Figure 3. Typical scratches in rabbits in the places of introduction of the strain UNDIEV-18v

The pathological material selected from necropsied rabbits (spleen, brain, blood from heart) was screened for contamination by CVS-II, mycoplasma, PCV, PRRSV, PrV, CSFV, BVDV and was found to free of any of those agents. At the same time the presence and authenticity of strain UNDIEV 18b in all analyzed samples of investigated material was confirmed.

Purified antigens of PrV were obtained by the method of polyoxyethylene glycol precipitation, by ammonium sulfate and precipitation on filter membranes and ultracentrifugation in density gradient it was determined that they are authentic, that is they contain DNA of Aujeszky's disease virus.

The results of screening of these samples with primer systems Myc23S, PCV, PRRSV, PrV, CSFV, BVDV, TGES/RCVS, RotaP and RotaG showed that purified samples did not contain DNA of swine circo-, parvoviruses, mycoplasma nor RNA of swine rota-and coronaviruses, viruses of porcine respiratory-and-reproductive syndrome and swine and bovine pestiviruses.

When preparing corpuscular antigen, great attention was paid to adaptation and study of Aujeszky's disease virus infectiveness and immunogenicity. It is known that changes of cell system for virus reproduction may influence negatively these characteristics. Aujeszky's disease virus strain UNDIEV 18v was adapted to monolayer continuous cell culture BHK-21/13 by 3 – 10 serial passages. Virus propagated in cell culture manifested cytopathic changes (Fig. 4), which stabilized during additional passages in terms of manifestation and completion.

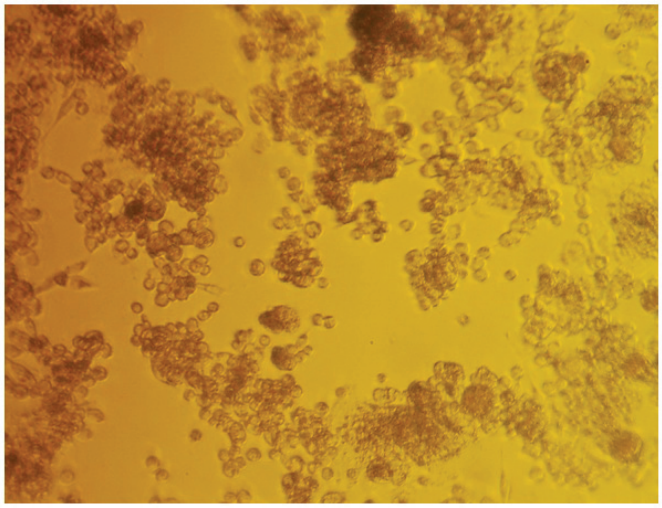


Figure 4. Cytopathic changes in cell culture BHK-21/13 on the 3rd day after infection

With serial reseeding of the virus, this term decreased and by the third passage of biomaterial selected from necropsied rabbits infected by UNDIEV 18v strain its titer of infectivity reached maximal value and in further passages it actually remained unchanged. Thus, if in the first passage in monolayer culture of continuous cells BHK-21/13 the propagated virus showed an infectivity titer of 5.0 lgTCID $_{50}$/ml, after the third passage its infectivity was raised to 7.0–7.5 lgTCID$_{50}$/ml.

A virus dose 0.001 TCID $_{50}$/cell ensured virus reproduction and CPE completion during 48–50 hours in monolayer cell culture of BHK-21/13 in roller bottles and in cultural vials – during 50–60 hours. This may be explained by the way in which the infection is propagated. In monolayer culture in mattresses, after sorption the virus spreads from cell to cell by direct contact, while in roller bottles the virus is spread by the circulation of virus particles and infected intact cells through the media which is in contact with all cells in the monolayer.

Further accumulation of virus containing cultural suspension for antigen preparation was accomplished using cell culture infection of 0,01 TCID$_{50}$/cell. (Table 3).

Table 3 – Characteristics of the parameters of reproductive properties of Aujeszky's disease virus strain UNDIEV-18v in cell culture BHK-21/13

Characteristics		Necessary parameters	
		cultural flaks	bottles
Optimal volume infection dose	**Proportion of virus volume and maintenance medium**	**1:20**	**1:30**
Virus infective activity in infection dose	TCID$_{50}$/ml	1000	1000
Multiplicity of infection	TCID$_{50}$/cell	0,01	0,01
Term of virus reproduction	hours	50-60	48-50
Titer of infectiveness of initiate raw material	lg TCID$_{50}$/ml	7,0	7,0-7,5

Investigations showed that for preparation of antigen with optimal immunogenicity "yield" of Aujeszky's disease virus from 3 to 10 passages in the culture of continuous cells BHK-21/13 is acceptable. At roller cultivation removal of viral biomass at affection of 78-80 % of cells in monolayer ensures its maximal infectiveness. By the time of cultivation it takes 40–42 hours. Further cultivation to complete CPE completion did not contribute to considerable raise of virus infectiveness.

The most optimal conditions for virus reproduction in cell culture BHK-21/13 were ensured by nutrient medium with concentration of hydrogen ions 7,0–7,2. Terms of virus reproduction decreased in weakly acidic zone (pH 6,8–7,0) for 5–6 hours.

Synchronization of BHK-21/13 cells infection by the virus in cold regime, which included tests of different doses of infection multiplicity and conducting of virus sorption on the cells under the temperature 4–10 ^{0}C for 6–8 hours, slightly decreased (for 4–10 hours) terms of CPE manifestation and contributed to raise of the infectiveness of the strain UNDIEV 18v for about 0,5 lg $TCID_{50}$/ml. But in practice this method is less suitable. At testing of the characteristics of reproduced in continuous cells BHK 21/13 there was determined that it kept virulence for animals. Virus infectiveness titer in the experiment on rabbits was 8,75 LRD_{50}/ml.

As far as Aujeszky's disease virus strain UNDIEV-18v adapted to reproduction, in continuous cells BHK-21/13 quantity of virus specific protein raised from 18,9±0,06 to 20,0±0,36 microgram/ml. But clear regularity between quantity of virus specific protein and titer of virus infectiveness in cell culture was not determined.

In our investigations on antigen preparation, Aujezky's disease virus strain UNDIEV 18v was purified by different methods before concentration, using both physical and physical-and-chemical methods. With this purpose there was accumulated 50 L of virus cultural suspension with infectiveness 7,3 lg $TCID_{50}$/ml, according to the data of bacteriological control and PCR, free from contamination by outside microorganisms. Its authenticity was confirmed in PCR.

There was found out, that centrifugation of virus containing cultural suspension at 3000 g (20–30 minutes) is ineffective because of the level of purification at the expense of removing of cell detritus was only 32,3±1,6 % by transparency to distilled water (level of purification was controlled with the help of photoelectrocalorimeter). At 10000 g (15–20 minutes) the level of purification raised to 83,9±0,6 %.

Maximal level of virus purification from ballast substances was reached by combining of high speed and isopycnic centrifugation in double gradient (caesium chloride and sucrose). With this purpose virus was layered on liner density gradient CsCl (50 %, p=1,3 g/ml) – sucrose (15 %, p=1,16 g/ml, volume to volume) and precipitated for 3 hours at 10000 g in bucket rotor of MSE centrifuge. This enabled to obtain virus concentrate.

Results of comparative concentration of Aujeszky's disease virus by precipitation with the help of ammonium sulfate and polyethylene glycol with molecular mass 6000 showed possibility of obtain 100-times concentrate. At application in tests of minimal concentrations of 10 % ammonium sulfate and 8% polyethylene glycol precipitation of correspondingly 89,29 % and 90,15 % of the virus was observed. With raising of their concentration to 20 and 30 % and to 10 and 15 % correspondingly, quality of nonexhausted virus reduced to 8,5 i 4,5 % and 3,69 i 1,95 %.

At Aujeszky's disease virus concentration using ammonium sulfate and polyethylene glycol-6000 desired level of purification was not reached. It was confirmed by electron microscopic investigation and determination of the content of total protein. Along with detection in the field of view of electron microscope of icosahedral virions, covered with capsid, size of which on average was 160–200 nm in the samples of concentrated virus at precipitation on filter membranes, and also with the help of ammonium sulfate and polyethylene glycol – 6000 there was detected content of significant quantity of foreign substances (Fig. 5).

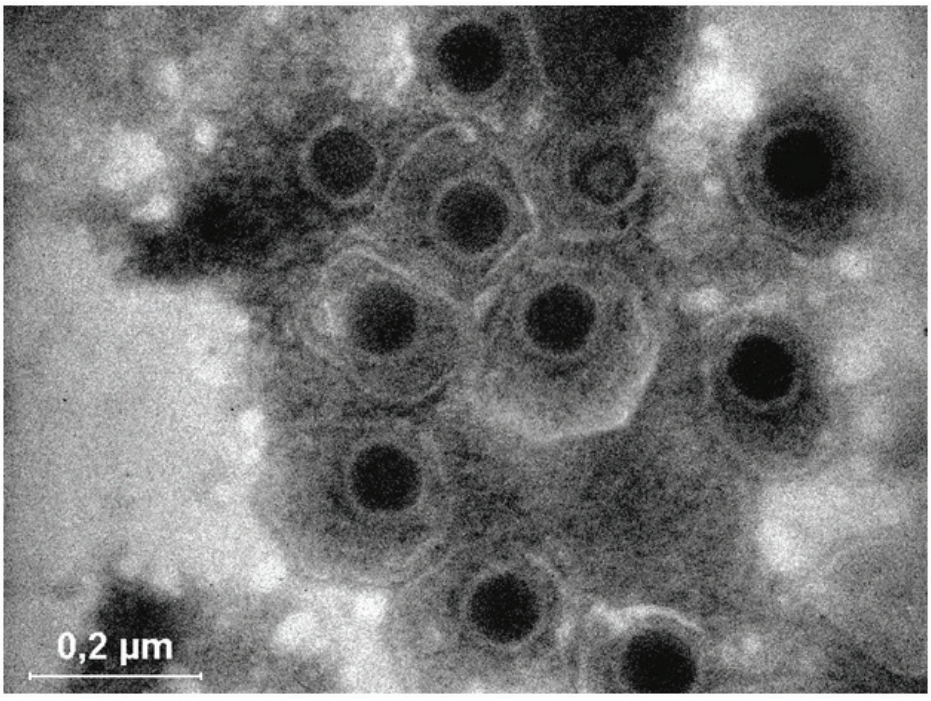


Figure 5. Electron photograph of pseudorabies virus (strain UNDIEV 18v)

Besides, in the virus samples, concentrated with the use of chemical substances, there were observed destroyed supercapside of virions (Fig. 6). Additional reprecipitation of these virus concentrates by centrifugation in caesium chloride and sucrose gradient at 100000 g for three hours enabled to obtain purified for 99,7 %virus, but density of location of virus particles in electron microscopic field of view was rather less compared with virus concentrate, precipitated on filter membranes and after purified in double density gradient.

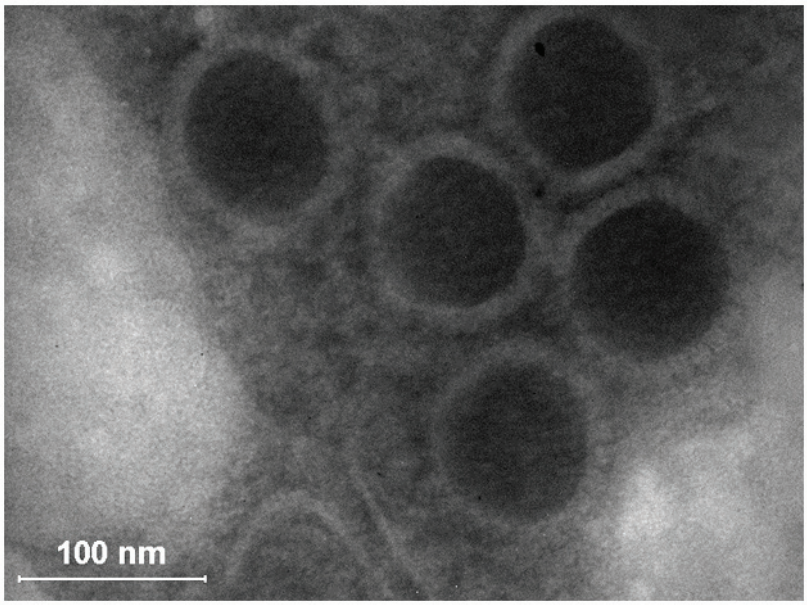


Figure 6. Electron photograph of pseudorabies virus with destroyed supercapside.

This data correlate with the results of determination of total protein. If in purified suspension of cultural virus at its precipitation in double density gradient, protein content was on the level 14,3–15 mg/ml, in concentrates, obtained by precipitation by ammonium sulfate and polyethylene glycol and additionally after purified in density gradient, it was not higher than 4,5–5,8 mg/ml. Floating density in caesium chloride gradient was 1,278 g/ml and in sucrose gradient – 1,212 g/ml (Fig. 7).

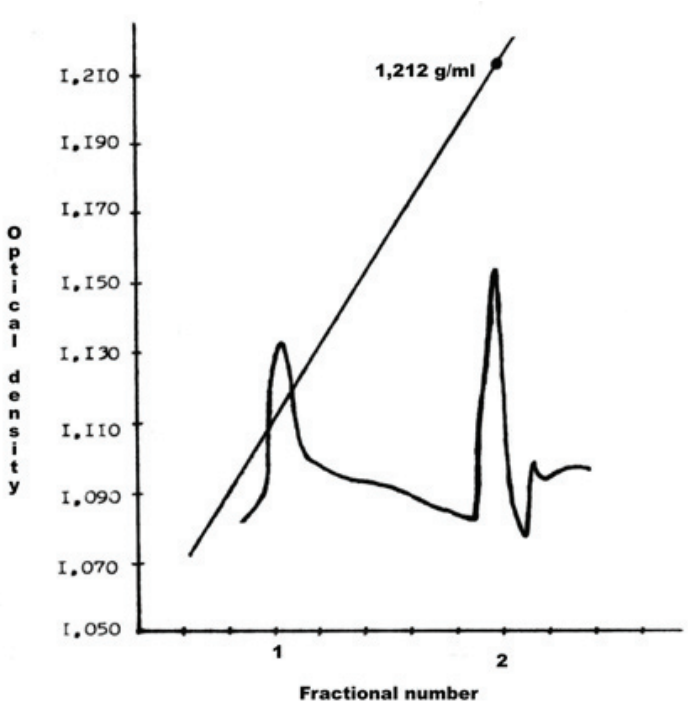


Figure 7. Floating density of Aujeszky's disease virus strain UNDIEV 18v in sucrose density gradient.

Indicators for determination of cleaning of each separate investigated sample of antigen of Aujeszky's disease virus by electrophoresis have been conformed. Most distinct electrophoretic mobility has samples of virus concentrated with use of ammonium sulfate at least at using of PEG. Moreover their electrophoretic activity have been increased with growth of concentration of these chemic substance at virus processing that connected with breach of virion safety and appearance in antigen content of considerable quantity their fragments and liberation of nucleic acid that has on 6000–7000 negative charges as much as capsule of whole virion. Refined fractions of antigen of Aujeszky's disease virus concentrated at precipitation on filter membranes and at centrifugation in gradient of chlorous cesium and

saccharose , characterized more stability, homogeneity, and electrophoretic mobility in electronic field that are showing lesser and at the same time equal contents of outside admixtures.

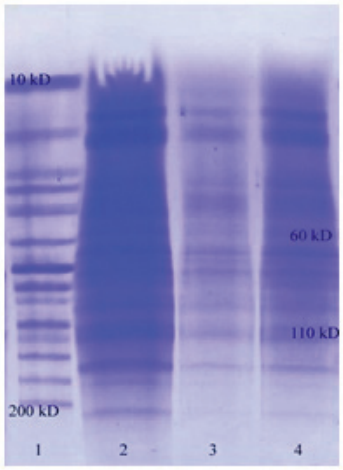
With the purpose of preparation of corpuscular antigen disintegration of viral particles of Aujeszky's disease agents, in samples of their concentrates with use in comparative aspect of 3 detergents was conducted. Both anionic (sodium dodecilsulfate) and nonionic (Twin-80 and Triton-100) detergents with concentration from 0,1 to 1 %, ensured secretion of protein of Aujeszky's disease virus. With increase of concentration their destructive activity increased, but it remained uncompleted. It was proved by investigation of samples with the help of electronic microscope. In the microscope field of vision single immobile viral particles and their fragments were observed. Additional treatment by ultra-sound with creation of local pressure and temperature of disintegration at intensity 20–25 Kc/sec during 10 minutes was conduced to full destruction of virions. More effective destruction of Aujeszky's disease virus was at processing by sodium dodecilsulfate comparatively with Twin-80 and Triton X-100. This was confirmed by less quantity of solid virions in investigated samples.

Fractionating of protein components of corpuscular antigen by gradient centrifugation and electrophoresis in polyacrylamidic gel was conducted. There was determined that additional purification by centrifugation of Aujeszky's disease cultural virus on 30 % sucrose pad did not ensure final removing of cell protein components (Fig. 8 , track 2). At centrifugation in density gradient from 12 to 55 % of sucrose there was obtained more free from foreign protein substances virus, which has 9 protein fractions with molecular mass from 15 to 120 kD (Track 3), which adjust with data of other researchers [20].

At electrophoresis of corpuscular antigen after destruction of virus concentrate there were obtained 11 protein fractions, including glycoproteins gII (110 kD) and gIII (60 kD). They are marker components for obtaining of monoclonal antibodies (Track 4).

At investigation of protein fractions there was determined that centrifugation of virus suspension on 30 % sucrose pad did not enable to get free from proteins of cell detritus (Track 2).

Virus, which we obtained by centrifugation in sucrose density gradient, is free from foreign substances and has 9 fractions with molecular mass from 15 to 120 kD (Track 3). This adjusts the information of previous researchers [20]. For obtaining of pure fraction of glycoproteins gII and gIII as marker candidates for obtaining of monoclonal antibodies, virus was additionally treated by sodium dodecyl sulfate and by ultrasound. After electrophoresis in polyacrylamide gel of this material we obtained 11 virus fractions. (track 4).



1- marker of protein molecular mass (from 19 to 200 кD);

2- protein fractions of concentrated virus, purified by centrifugation on 30% sucrose pad;

3- protein fractions of concentrated virus, additionally purified by gradient centrifugation from 12 to 55 % sucrose;

4- protein fractions after destruction of concentrated virus

Figure 8. Electrophoretic separation of Aujeszky's disease virus proteins, strain UNDIEV-18v.

As a result of these investigation there was proved that separation of protein fractions of corpuscular antigen of Aujeszky's disease virus first of all depended on their molecular mass and not on surface charge.

Because of the lack of preparative equipment for simultaneous elution at separation by electrophoresis of protein components we were not able to obtain them in sufficient for investigations quantity. This could make possible to study antigenic and immunogenic characteristics of each of them separately. In further investigations on testing of corpuscular antigen we used protein components, which were fractioned by gradient centrifugation and sandwich variant of ELISA with double titer in work solution of specific antibodies.

Ability of the strain UNDIEV-18v to induce in the animal organism virus specific antibodies was tested on rabbits. At the same time there was tested the scheme of their immunization in comparison with described above [25] with the purpose of obtaining of specific serum and preparation of immunoglobulin for conjugation with horse radish peroxidase.

Proposed by us scheme of rabbit hyperimmunization, which included three times introduction of antigen, namely concentrate of inactivated by aminoethyleneimine strain UNDIEV-18v (Vladimir, Russia) to interdigital spaces of hind limbs in the dose 2 ml, in 8–10 days - to popliteal lymph nodes for 1 ml, and on 19–25 day – intravenously 1,5–2 ml with further draining of blood in the next 10–12 days, enabled to obtain during 25 days specific, highly active, suitable for preparation of diagnostic preparation blood serum (table 4).

After testing of activity of hyperimmune serum there was isolated immunoglobulin, which was conjugated with horse radish peroxidase. By determination of activity of peroxidase conjugate, conducted by "tessellated" titration in ELISA with specific antigen, it was 1:300.

Table 4 – Activity of hyperimmune rabbit blood serum, obtained by different schemes of Aujeszky's disease virus introduction

Immunization scheme	Rabbits, heads.	Blood sera activity, log_2		
		Radial immunodiffusion test	Neutralization reaction	ELISA
By proposed method	6	7,63±0,13	7,25±0,03	8,15±0,01
By Kardi method	6	6,9±0,11	6,75±0,04	7,23±0,09

At testing of indicative activity of specific immunoglobulin there was determined possibility of disease virus detection with infectiveness from 0,5 to 3 $TCID_{50}$/ml in the organs and tissues of infected rabbits and 3,5–4 lg $TCID_{50}$/ml in cultural virus containing suspension (Table 5).

In further investigations concerning serological testing in radial immunodiffusion test there was determined, that antigenic activity of protein fractions of Aujeszky's disease virus corpuscular antigen, obtained both by gradient centrifugation and electrophoresis is not similar. It actually grew in protein fractions in direct proportion to to their molecular mass from 115) 2: kD) to 1120–30) 16: kD).

Table 5 – Results of Aujeszky's disease virus indication by ELISA

Test-object	ELISA	Isolated virus infectiveness ($TCID_{50}$/ml)
Brain	+	3,10±0,18
Lungs	+	3,07±0,09
Liver	+	4,92±0,20
Muscle	+	0,50±0,10
Submandibular lymph nodes	+	2,52±0,04
Heart	+	0,5±0,08
Virus cultural suspension (BHK 21/13)	+	4,0±0,02
Virus cultural suspension (SNEV)	+	3,5±0,10

Ability of protein fractions of Aujeszky's disease virus corpuscular antigen to detect specific antibodies actually reduced proportionally to reduce of their molecular mass. If protein fractions of corpuscular antigen with molecular mass from 30 to 120 kD in solution 1:16 enabled to identify virus specific antibodies in the titers to 9,9 log_2, fraction with molecular mass 15 kD only 1,1 log_2.

Titers of protein fractions of Aujeszky's disease virus corpuscular antigen in ELISA were higher proportionally to their molecular mass (table 6).

Table 6 – Activity of protein fractions of Aujeszky's disease virus corpuscular antigen, strain UNDIEV-18в

Protein fractions with molecular mass, kD	Solution	Activity (log_2)	
		Radial immunodiffusion test	ELISA
15	$1:2^x$	1,15	5,9
	1:4	inactive	5,0
	1:8	inactive	3,8
	$1:16^x$	inactive	3,0
60	1:2	7,33	10,85
	1:4	6,65	10,8
	1:8	6,3	10,15
	$1:16^x$	6,25	9,9
110	1:2	8,0	10,65
	1:4	7,8	10,2
	1:8	7,25	9,75
	1:16	7,1	9,35

Note: $1:2^x$ – $1:16^x$ parameters of investigated antigen titers

Both fractions of purified suspension of Aujeszky's disease cultural virus concentrate had Expressed antigen activity, which were obtained by the precipitation in binary gradient of density which both in RIF, and in IFA compaunds1:16 (maximal titer of investigated antigen). Virus specific antibodies has been developed accordingly in titer 7,65 $\log_2$ and 10,2 (1 fraction) and 8,0 and 10,9 $\log_2$ (2 fraction). However, in first fraction both in radial immunodiffusion test and in ELISA has been descended positive reaction with conjugate of specific antibodies to cells VNK-21/13 that causes necessity of protein fraction additional after purification. The second protein fraction of Aujeszky's disease virus after virion destruction has been lyophilized and used for testing.

So, results of serologic testing of different protein fractions of Aujeszky's disease virus strain UNDIEV-18в indicates that more adapted for antigen preparation with use in ELISA-reaction for indication of virus specific antibodies and for obtaining of immune splenocyte at preparation of monoclonal antibodies is:

– accumulation of initial virus inclusive raw material by the cultivation of Aujeszky's disease agent in culture of revaccinated cells BHK-21/13, which are free from contaminates of bacterial and viral origin;

– primary purification from cell detritus and large protein structures by differential low - (3000 g) and high speed (1000 g) centrifugation during 15–20 min accordingly;

– virus precipitation by the centrifugation in binary gradient of density;

– virus destruction 1 % sodium dodecylsulfate with additional treatment by ultrasound 20-25 kHz/sec during 10 min.;

– virus protein fraction by the electrophoresis in polyacrylamide gel;

– isolation by the elution of protein fractions with molecular mass 60 and 110 kD that correspond to glycoproteins g3 and g2 which are capable of evoking the creation of virus neutralizing antibodies. This technological scheme enabled us to obtain corpuscular antigen of Aujeszky's disease virus strain UNDIEV that could be used to produce virus specific antibodies that could be detected by radial immunodiffusion and ELISA tests.

Corpuscular antigen of Aujeszky's disease virus strain UNDIEV-18v was dried. Lyophilization did not influence antigen specificity, but rather reduced antigenic activity. Thus, if before lyophilization it was in radial immunodiffusion test 1:30, in ELISA – 1:100, after drying it was 1:20 and 1:80 correspondingly.

The first stage of the project was accomplished according to the work plan. Technological scheme was perfected. Sample of dried corpuscular antigen of Aujeszky's disease virus strain UNDIEV-18v, acceptable for indication of virus specific antibodies by ELISA test, and also for obtaining of monoclonal antibodies has been produced.

Conclusion. The method for obtaining corpuscular antigen of the Aujeszky's disease virus strain UNDIEV-18в, suitable for conducting of immunologic reactions, immunization of donor for the production of diagnostic sera and preparation of monoclonal antibodies, and purified by differential centrifugation, concentration and destruction of virions using chemical and physical factors has been developed.

Continuous cells BHK-21/13 have a high proliferation activity (4.8–5.2-fold increase) when grown in a mixture with equal parts of nutrient medium 199 and Eagle's medium with 10% cattle blood serum with a pH of 7.0–7.2, that ensures cell biomass accumulation in 3-liter bottles within 24–48 hours. Cells are not contaminated by bacteria, fungi, mycoplasma and viruses. Mitotic index compounds 38–40 %, quantity of pathologic mitosises does not exceed 20 %.

A monolayer culture of BHK-21/13 cells using the roller method of cultivation ensures reproduction of Aujeszky's disease virus strain UNDIEV-18v with infection titer 7.5 lg $TCID_{50}$/ml. within 48–50 hours. Cultural suspension of Aujeszky's disease virus has virulence for rabbits within 8.75 LRD_{50}/ml.

Concentration of Aujeszky's disease virus using ammonium sulfate polyoxyethylene glycol - 6000 and precipitation on filter membranes with pores to 300 Å and purification by centrifugation in a saccharose and cesium chloride density gradient results in a hundred-fold concentrate.

The protocol for immunizing rabbits using inactivated antigen of Aujeszky's disease concentrated virus, that includes oil adjuvant (Russian Scientific Research Institute of Animal Protection) ensures production of specific serum with activity in radial immunodiffusion test 7.63±0.13 $\log_2$, in VN 7.25±0.03 $\log_2$ within 29–35 days. The serum is adapted for production of diagnostic preparations particularly conjugated specific to Aujeszky's disease virus immunoglobulin with horseradish peroxidase.

There was established that more effective destruction of Aujeszky's disease virus is caused when using 1 % sodium dodecilsulfate compared to Twin-80 and Triton X-100. Absolute integration of virus proteins is reached under the influence of ultrasound at intensity 20–25 kHz/sec during 10 min.

Centrifugation of corpuscular antigen of Aujeszky's disease virus on 30 % sacrose pad revealed two protein fractions with expressed antigenic activity 1:16 (maximal titer of antigen, that was investigated) and capable of eliciting virus specific antibodies radial immunodiffusion test titers of 7.65 $\log_2$ (1 fraction) and 8.0 $\log_2$ (2 fraction) and in ELISA 10.2 and 10.9 $\log_2$, accordingly.

Electrophoresis of antigen in polyacrylamide gel after destruction of Aujeszky's disease virus ensures separation and reveals 9 protein fractions. After additional purification in a gradient of 12 and 55 % sucrose, electrophoresis shows 11 protein fractions with molecular mass ranging from 15 to 120 kD. Using serologic testing, including both the radial immunodiffusion test and ELISA it was determined that protein fraction activity grew proportionally with increase in molecular mass from 1:2 (15 kD) to 1:16 (from 30 to 120 kD).

Those protein fractions of corpuscular antigen with molecular mass ranging from 30 to 120 kD were more efficient at detecting specific antibodies to Aujeszky's disease virus. With activity 1:2 they detect antibodies in the radial immunodiffusion test 7.33–8.0 $\log_2$, and ELISA – 10.65–10.85 $\log_2$. However, if a protein fraction with molecular mass of 15 kD is used, antibody detection does not exceed 1.15 and 5.9 $\log_2$, accordingly.

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ОТРИМАННЯ АНТИГЕНУ ВІРУСУ ПСЕВДОСКАЗУ ДЛЯ ДІАГНОСТИКИ ХВОРОБИ АУЄСКІ

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Автори вивчили вірус хвороби Ауєскі (штам UNDIEV-18V), який був виділений в Україні. Вірус був випробуваний в якості кандидата для виготовлення діагностичних наборів. Проведені попередні дослідження клітинних культур і вірусу в ПЛР відносно їх контамінації сторонніми вірусами і мікоплазмами. Були вивчені культуральні, біологічні та біофізичні характеристики штаму. У результаті проведених досліджень отримані дані стосовно біологічної активності вірусу на культурі клітин ВНК-21/13 і сприйнятливих тваринах (кроликах), плаваючої щільності цього штаму у градієнті щільності сахарози, а також зроблено електронні фотографії даного вірусу. Розроблена схема концентрування та очищення антигену для виготовлення діагностичних наборів. Схема включає в себе накопичення біомаси вірусу за ролерного культивування, його концентрація за допомогою поліетиленгліколю-6000, очищення за допомогою високошвидкісного центрифугування, і, нарешті, отримання чистого антигену для імунізації тварини за допомогою 1 % концентрації додецилсульфата натрію та електрофорезу в поліакриламідному гелі. Запропонована схема може бути використана в якості прототипу для отримання концентрованих і очищених фракцій вірусу для виробництва діагностичних наборів.

Ключові слова: хвороба Ауєскі, вірус псевдосказу, діагностичний набір, свині, Україна.