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ВПЛИВ АНТРОПОГЕНИХ ФАКТОРІВ НА СИНТЕЗ ФЕНОЛЬНИХ СПОЛУК ПРИ ФІТОВІРУСНІЙ ІНФЕКЦІЇ

Було показано прямий взаємозв'язок між рівнем антропогенного навантаження та рівнем інфікованості вірусом, а також вмістом фенольних сполук в цих рослинах. Також, показано, що відтворення ВТМ в рослинах цукрового буряку індукують синтез фенольних сполук які акумулюючись, можливо, починають пригнічувати накопичення вірусу.

Ключові слова: антрпогене навантаження, рівень інфікованості вірусом, фенольні сполуки.

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ВЛИЯНИЕ АНТРОПОГЕНЫХ ФАКТОРОВ НА СИНТЕЗ ФЕНОЛЬНЫХ СОЕДИНЕНИЙ ПРИ ФИТОВИРУСНОЙ ИНФЕКЦИИ

Была показана прямая взаимосвязь между уровнем антропогенной нагрузки и уровнем инфицированности вирусом, а также содержанием фенольных соединений в этих растениях. Также, показано, что воспроизведение ВТМ в растениях сахарной свеклы индуцируют синтез фенольных соединений которые аккумулируясь, возможно, начинают подавлять накопление вируса.

Ключевые слова: антрпогенная нагрузка, уровень инфицированности вирусом, фенольные соединения.

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ISOLATION OF *INFECTIOUS PANCREATIC NECROSIS VIRUS* FROM WILD-LIFE RAINBOW TROUT *ONCORHYNCHUS MYKISS* IN WESTERN UKRAINE

During routine sampling and testing of catched wild-life fish species from Siret river of Chernivtsi region, an aquatic birnavirus was isolated from diseased rainbow trout Onhorhynchus mykiss. Preliminary examination of diseased fish revealed a range of lesions particularly in pancreatic tissue. Virus isolate grew in fish cell line RTG-2, FHM and EPC. IPNV caused morphological changes, such as vacuole enlargements and cells rounding. Subsequently cells scaled from a surface and characteristic cytopathic effect (CPE) of virus on cells was visible. Examination by electron microscopy demonstrated that the isolated virus was ultrastructurally similar to IPNV. In addition after amplification of viral RNA incorporating three different primer pairs the IPNV specified PCR products were visible on agarose gel stained with ethidium bromide. The nucleotide sequences of amplified fragments were analysed and the prevalance of Ukrainian isolate, which was named "Karpaty", to Sp strain was revealed. The comparison of sequences of IPNVs VP2 and NS genes from NCBI and amplified fragments of IPNV strain "Karpaty" confirmed the high identity of 95-99% with Sp strain, firstly isolated in Denmark. Among the isolates of Sp strain the most related to IPNV "Karpaty" were viruses found in Great Britain, Norway, France, Turkey and Iran.

Key words: Infectious pancreatic necrosis virus, cell culture, RT-PCR, sequence analysis.

Introduction. Infectious pancreatic necrosis virus (IPNV) belongs to the family *Birnaviridae* and is an agent of an acute, contagious fish disease causing high mortality not only in juvenile salmonids but also in non-salmonid fishes. Members of the family *Birnaviridae* are icosahedral viruses of approximately 65 nm in diameter composed of five polypeptides and two strands of double-stranded RNA [1].

In young salmonid fish, IPNV can cause high mortalities followed by a life-long, chronic infection in the survivors. Persistently infected fish are asymptomatic that have virus in many visceral organs and can shed live virions [2]. The IPNV is widely distributed in Europe and there are severel new reports about virus isolation in neighboring countries of Ukraine such as Poland, Czech Republic, Slovakia and Russia [3,4]. Since salmonids breeding are mainly located in the west region of Ukraine the IPNV is an economically important fish pathogen for all Ukrainian trout farms.

The **aim** of present study was to isolate IPNV from salmonids in Ukraine. Therefore the goals of the present study were to provide preliminary characterization of isolated strain in cell culture and electron-microscopy, to select valid oligonucleotide primers and test it in PCR assay; to provide sequencing of amplified products in way of verification of target amplification; and to accomplish the phylogenetic analysis of Ukrainian IPNV strain.

Materials and methods. During June 2011, a total of 14 fish samples (body weight 0.4-0.7 kg) were continuously collected from a diseased wild-life rainbow trout catched in Siret river, west region of Ukraine. Each fish was dissected, and the samples of pancreas, kidney and spleen were removed from individual fish and placed into a 1.5 ml microcentrifuge tube. Samples were transported on ice to the laboratory and processed immediately.

RTG-2, FHM and EPC cell lines were maintained in DMEM medium (SIGMA) supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% fetal bovine serum (FBS). The samples of kidney and spleen of tested fish were homogenized with DMEM and filtred through the 0,45 μm membrane (Millipore). Then the virus suspension was inoculated onto 24-hours cell monolayers growing in 25 cm^2 flasks. After adsorption for 60 min at 20°C, DMEM medium supplemented with 2% FBS was added to the monolayers. When a complete viral cytopathic effect (CPE) was evident, the tissue culture supernatant was harvested and centrifuged at 2500 × *g* for 10 min at 4°C to remove cell debris. The 50% tissue culture infective dose ($TCID_{50}$ ml^{-1}) of the resulting supernatant was determined [5].

The IPNV was purified both from collected organs and the tissue culture supernatant by the method of ultracentrifugation. Briefly, after cell debris was separated by centrifugation at 2500 × *g* for 10 min at 4°C the pellet was discarded and the supernatant was centrifuged in ultracentrifuge Beckman L5-50B in a rotor SW-40 for 60 min at 70500 × *g* at 4°C. The virus pellet was suspended in TNE (50 mM Tris-HCl, 150 mM NaCl, 1 mM disodium ethylene diaminetetracetic acid [EDTA], pH 7.5) and centrifuged at 2500 × *g* for 5 min at 4°C. Then the virus suspension was used for electron-microscopy investigation and viral RNA extraction [6].

For electron-microscopy investigation the viral suspension was stained with 2% uranyl acetate and studied in electron microscopy EM-125.

Genomic viral RNA was extracted from collected fish organs, viruses-infected cells culture supernatant and purified virus suspension using GeneJET™ RNA Purification Kit (Fermentas) as described in manufacturer's protocol. After elution of the RNA in DEPC-treated water, it was kept at -20°C until required. The cDNA synthesis was conducted using RevertAid™ Premium First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer's instructions and subjected to PCR amplification.

Three sets of virus specific-primers (one-step PCR) were used for amplification of fragments of viral dsRNA targeting the IPNV NS and VP-2 proteins [7-9]. PCR amplification was conducted with a pre-dwel cycle 50°C for 15 min and an initial cycle 95°C for 2 min and followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 60 sec. A final extension step was conducted at 72°C for 7 min. The PCR products were analyzed by 2.0% agarose gel electrophoresis.

For extraction of DNA from agarose gel the Silica Bead DNA Extraction Kit was used. Sequencing was performed on a 3130 Genetic Analyzer and analyzed using BLASTN, Vector NTI 10 and MEGA version 5.2 software.

Results and Discussion. This study reports the isolation of IPNV from wild-life rainbow trout in Siret river Chernivtsi region. During June 2011, a total of 14 fish samples were continuously collected from a diseased wild-life rainbow trout catched in Siret river, west region of Ukraine. External signs of disease in catched trouts included uncoordinated spiral swimming, violent flexing of the body and developing of anaemia. Diseased fish were notably darker in colour and appeared weak and lethargic. Internally, the alimentary tract was empty of food but moderately distended and filled with greyish mucus. The liver was dark and inflamed and the kidney and spleen were pale and swollen.

Between 4 and 7 days post infection (d.p.i.), viral CPE was evident in the RTG-2, FHM and EPC cells. All three cell lines were sensitive to virus. IPNV caused morphological changes, such as vacuole enlargements and cells rounding. Infected cells became filamentous in the early stage of infection. Subsequently cells scaled from a surface and characteristic cytopathic effect (CPE) of virus on cells was visible. For cell lines of RTG-2 and FHM the complete destruction of monolayer was noted on 7-8 day after infection (d.a.i.). For culture of EPC characteristic CPE and complete destruction of cell monolayer were marked on 10-12 d.a.i. Infectious titer of IPNV "Carpathians" in studied cell lines was following for EPC $10^{5,5-5,8}$ $TCID_{50}$/ml, and for the lines of FHM and RTG-2 $10^{6,2-6,5}$ and $10^{6,9-7,4}$ $TCID_{50}$/ml respectively. The low infectious titer of virus in cells of EPC can be related to its slow reproduction in this culture. The greatest infectious titer was observed for the culture of RTG-2, that is fully appropriately, as this cell line was derived from a rainbow trout – natural IPNV reservoir. That is why the RTG-2 is the most appropriate cell lines for accumulation of the Ukrainian isolate IPNV "Carpathians". But for diagnostics of the Ukrainian isolate IPNV "Carpathians" all three cell cultures of RTG-2, FHM and EPC can be used.

Results of our electronic-microscopy researches of purified viral particles revealed basic for birnaviruses morphology and ultrastructure characteristics. Virions of the Ukrainian isolate of IPNV "Carpathians" had a hexagonal form, their diameter was 70 ± 5 nm. Viral particles were non-enveloped (Fig. 1).

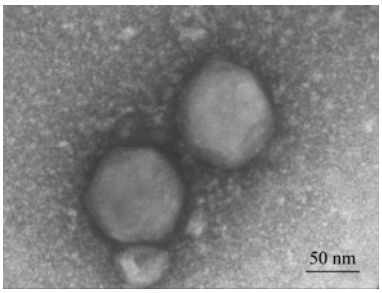

Fig. 1. Electron-microscopy of IPNV particles after purification (×80000)

For rapid diagnostic of Ukrainian IPNV strain the method of PCR was developed. Three sets of primers targeting NS and VP2 genes were used for virus identification and the parameters of PCR cycling were optimized. It was shown that WB primers are the most efficient for virus diagnostic, however the IPN and PrD primers also can be used. Amplified fragments were in size of 200 base pairs (bp) for WB primers, 620 and 175 bp for IPN and PrD primers respectively. In case of low concentration of target RNA only WB primers were enabled to identify the virus. It was noted that annealing temperature of 60^0C was the most suitable for all primer sets.

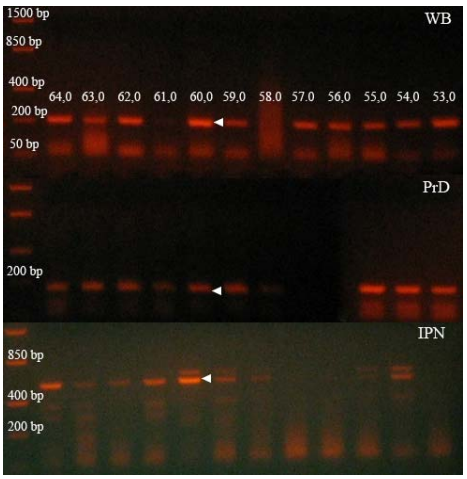

Fig. 2. Amplification in PCR of VP2 (IPN and WB primers) and (PrD primers) NS gene fragments of IPNV strain "Karpaty". The best result is shown ◄; left side DNA ladder FastRuler™ (Thermo Scientific); range of annealing temperature 53-64 ^{0}C

The nucleotide sequences of amplified fragments were analysed and the prevalance of Ukrainian isolate of IPNV "Karpaty" to Sp strain was revealed. The comparison of sequences of IPNVs VP2 and NS genes from NCBI and amplified fragments of IPNV strain "Karpaty" confirmed the high identity of 95-99% with Sp strain, firstly isolated in Denmark. Among the isolates of Sp strain the most related to IPNV "Karpaty" were viruses found in Great Britain, Norway, France, Turkey and Iran.

Thus the selected primers and developed PCR assay can be used for IPNV diagnostic in salmonids cultivated in fish-farming or native ponds of Ukraine. For rapid virus identification in PCR method the WB primers should be used. The complete monitoring of IPNV in Ukraine has to result in total data of virus distribution in Ukraine and also to identify another strains which are widespread in Europe. It will be the subject of our future research.

Infectious pancreatic necrosis can cause a significant economic impact on salmonids with a worldwide distribution. IPNV occurs in most major salmonid-farming countries and it is believed to be spread through the importation of salmonid fishes and their eggs. The virus is vertically transmitted; therefore, the detection of virus in broodstock, even in the absence of the disease, often means the destruction of these valuable fish species [10]. There will be a need to screen the farmed populations of trout near the river Siret in order to confirm of IPNV presence.

Rapid and accurate diagnosis of IPNV infection is critical to the control of the virus because trout surviving infections as juveniles may become life-long carriers and shed live virus. Therefore the screening of samples using RT-PCR would potentially be more sensitive than tissue culture, however suitable cell lines also can be used for surveillance of wild fish for IPNV. Additionally for Ukrainian strains of IPNV the moleqular technics are required and always must be used in purpose of serotype and genotype determination.

Conclusion. The water birnavirus was isolated from wild-life trout in Siret river of Chernivtsi region, Western Ukraine. Preliminary characterizaton of isolated virus revealed its relatedness to IPNV, which was nemed "Karpaty" strain. The nucleotide sequences of amplified fragments were analysed and the prevalance of "Karpaty" isolateto Sp strain was revealed. Among the isolates of Sp strain the most related to IPNV "Karpaty" were viruses found in Great Britain, Norway, France, Turkey and Iran.

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ВИДІЛЕННЯ ВІРУСУ ІНФЕКЦІЙНОГО ПАНКРЕАТИЧНОГО НЕКРОЗУ З ДИКИХ ПРЕДСТАВНИКІВ РАЙДУЖНОЇ ФОРЕЛІ *ONCORHYNCHUS MYKISS В ЗАХІДНІЙ УКРАЇНІ*

В роботі представлено результати дослідження вірусу інфекційного панкреатичного некрозу, виділеного від форелі з річки Сапет, Чернівецької області, Західна Україна. Досліджено репродукцію українського ізоляту IPNV "Карпати" в перевивних культурах клітин риб RTG-2, FHM та EPC. Всі три клітинні лінії виявились чутливими до вірусу. IPNV призводив до морфологічних змін, таких як вакуолізація цитоплазми та округлення клітин. Згодом клітини відшаровуватись від поверхні і проявлялась характерна цитопатична дія (ЦПД) вірусу на клітини. Результати електронно-мікроскопічних досліджень очищеної вірусної суспензії показали характерну для бірнавірусів морфологію та ультраструктуру. Віріони українського ізоляту IPNV "Карпати" мали гексагональну форму, їхній діаметр складав 70 ± 5 нм. Підібрано олігонуклеотидні праймери, специфічні до фрагментів генів VP2 та NS, та проведено оптимізацію постановки ПЛР. Аналіз нуклеотидних послідовностей ампліфікованих фрагментів IPNV "Карпати" свідчить, що український ізолят належить до штаму Sp. Ампліфіковані фрагменти кДНК на 95-99% ідентичні з послідовностями генів NS та VP2 інших ізолятів штаму Sp. Серед ізолятів штаму Sp найбільш спорідненими до українського ізоляту IPNV виявились віруси, виділені у Великобританії, Норвегії, Франції, Турції та Ірані.

Ключові слова: вірус інферкійного панкреатичного некрозу, культури клітин риб, бірнавіруси.

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ВЫДЕЛЕНИЕ ВИРУСА ИНФЕКЦИОННОГО ПАНКРЕАТИЧЕСКОГО НЕКРОЗА У ДИКИХ ПРЕДСТАВИТЕЛЕЙ РАДУЖНОЙ ФОРЕЛИ *ONCORHYNCHUS MYKISS* В ЗАПАДНОЙ УКРАИНЕ

В работе представлены результаты исследования вируса инфекционного панкреатического некроза, выделенного из форели реки Сапета, Черновицкой области, Западной Украины. Исследовали репродукцию украинского изолята IPNV "Карпаты" в культурах клеток рыб RTG-2, FHM и ЭДС. Все три клеточные линии оказались чувствительными к вирусу. IPNV приводил к морфологическим изменениям таким, как вакуолизация цитоплазмы и округление клеток. Впоследствии клетки отслаиваються от поверхности и проявляется характерное цитопатическое действие (ЦПД) вируса на клетки. Результаты электронно-микроскопических исследований очищенной вирусной суспензии показали характерную для бирнавирусив морфологию и ультраструктуру. Вирионы украинского изолята IPNV "Карпаты" имели гексагональную форму, их диаметр составлял 70 ± 5 нм. Подобраны олигонуклеотидные праймеры, специфичные к фрагментам генов VP2 и NS, и проведена оптимизация постановки ПЦР. Анализ нуклеотидных последовательностей амплифицированных фрагментов IPNV "Карпаты" свидетельствует, что украинский изолят относится к штамму Sp. Амплифицированные фрагменты кДНК на 95-99 % идентичны с последовательностями генов NS и VP2 других изолятов штамма Sp. Среди изолятов штамма Sp наиболее родственными к украинскому изоляту IPNV оказались вирусы, выделенные в Великобритании, Норвегии, Франции, Турции и Иране.

Ключевые слова: вирусинфекционного панкреатического некроза, бирнавирусы, культуры клеток рыб.