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Подано експериментальні дані про особливості будови, розвитку та функціонування рослинних і тваринних організмів, флору та фауну України, одержані на основі досліджень, що проводяться науковцями біологічного факультету в галузях фізіології рослин і тварин, генетики, ботаніки, зоології, мікробіології, вірусології. Викладено також нові дані стосовно біохімічних і біофізичних основ регуляції в клітинах і органах у нормі й після впливу різноманітних фізико-хімічних факторів, наведено результати нових методичних розробок.

Розраховано на викладачів, наукових співробітників, аспірантів та студентів.

Collection of articles written by the scientists of biological faculty contains data on research in molecular biology, physiology, genetics, microbiology, virology, botanics, zoology concerning the structure, development and function of the plant and animal organisms, flora and fauna of Ukraine. Results of newly developed biophysical methods of biological research, biochemical data regarding metabolic regulation under the influence of different factors are presented.

For scientists, professors, aspirants and students.

ВІДПОВІДАЛЬНИЙ РЕДАКТОР	**Л.І. Остапченко, д-р біол. наук, проф.**
РЕДАКЦІЙНА КОЛЕГІЯ	**Т.Л. Проценко, канд. біол. наук (відп. секр.); І.В. Якубцова (техн. секр.); В.М. Войціцький, д-р біол. наук, проф.; С.В. Демидов, д-р біол. наук, проф.; М.Е. Дзержинський, д-р біол. наук, проф.; М.С. Мірошниченко, д-р біол. наук, проф.; М.М. Мусієнко, д-р біол. наук, проф., чл.-кор. УААН; В.К. Позур, д-р біол. наук, проф.; І.Ю. Костіков, д-р біол. наук, доц.; В.В. Серебряков, д-р біол. наук, проф.; М.Ю. Макарчук, д-р біол. наук, проф.; В.П. Поліщук, д-р біол. наук, проф.**
Адреса редколегії	**03022, м. Київ-22, проспект акад. Глушкова, 2, корп. 12, біологічний факультет; ☎ (38044) 521 33 67**
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V.A. Petrenko, Ph.D., D.Sc.

PHAGE NANOBIOTECHNOLOGY—A NEW TWIST IN PHAGE DISPLAY

Auburn University, U.S.A.

A new biorecognition nanomaterial—the landscape phage—emerged recently as a result of in the depth study of genetics and structure of the filamentous phage and the development of the phage display technology. This review focuses on the progress made in the development of this new nanomaterial and discusses a prospect of using the landscape phages as bioselectable robust interface in biosensors and targeting probes in gene and drug delivery devices.

Introduction. Filamentous phages f1, fd and M13 are nonlytic bacterial viruses, which structure and life cycle are encrypted in their single-stranded DNA genomes. They specifically infect male bacterial strains of *Escherichia coli* that carry an F episome and therefore express phage-specific receptors—pili. Phage virions are rod-shaped particles or filaments, approximately 900 nm long and 6 to 7 nm wide (Fig. 1). Their circular, single-stranded DNA genome is encased in a flexible tubular sheath composed of approximately 2,700 molecules of the major coat protein pVIII (the product of gene *VIII*) and several copies of four minor coat proteins, the products of genes *III*, *VI*, *VII* and *IX*. The two ends of the phage are distinguished by the presence of the minor proteins: one end contains 5 copies of the pIII and pVI proteins, whereas the other end contains 5 copies of the proteins pVII and pIX. The major coat protein dominates in the phage capsid, comprising 87% of its total mass, and 98% of its protein content.

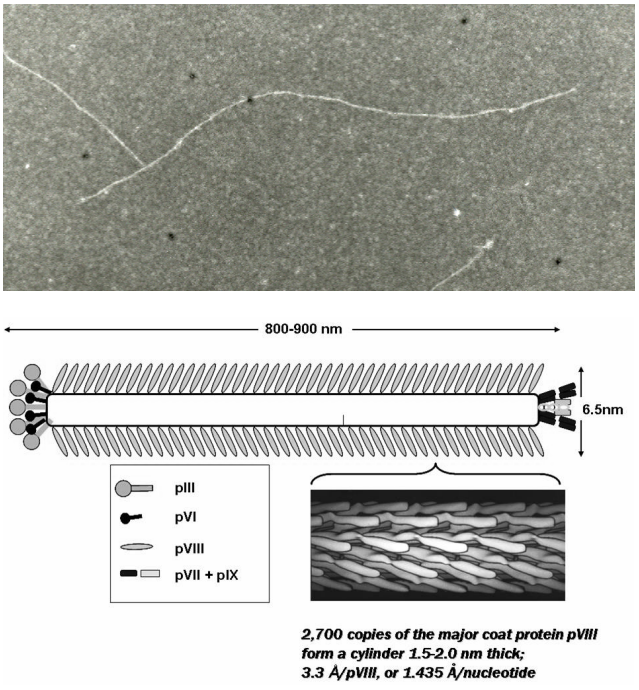

Fig. 1. Structure of filamentous phage Ff.

A. Transmission electron micrograph of phage Original magnification 57,000×).

B. Composition of the phage and arrangement of major coat protein surface domains (shown as electron densities of the proteins by Gregory Kishchenko and Lee Makowski; the image is provided by Gregory Kishchenko).

Phage display technology emerged as a synergy of two fundamental concepts: *combinatorial peptide libraries* and *fusion phage*. The first concept replaced the traditional collections of natural or individually synthesized compounds for libraries of peptides obtained in parallel synthesis as grouped mixtures (reviewed in [3]; the second—allowed displaying foreign peptides on the surface of bacterial viruses (bacteriophages) as part of their minor or major coat proteins [6, 26] (reviewed in [18, 28]). Combination of these two concepts resulted in development of *phage display libraries*—multibillion clone compositions of self-amplified and self-assembled biological particles. In particular, a paradigm of *landscape phage libraries* evolved, in which fusion phages serve not just as genetic carriers for foreign peptides, like in the traditional phage display approach, but are considered as nanoparticles (nanotubes) landscaped by thousands of random peptides, which determine emergent physico-chemical characteristics of these new nanomaterials [19]. These constructs display the guest peptide on every pVIII subunit increasing the virion's total mass by 15% (Fig. 2). Despite of the extra burden, such particles can retain their infectivity and progeny-forming ability. This review focuses on the progress made in the development of this new nonomaterial and discusses a prospect of using the landscape phages as bioselectable robust interface in biosensors and as targeting probes in gene and drug delivery devices.

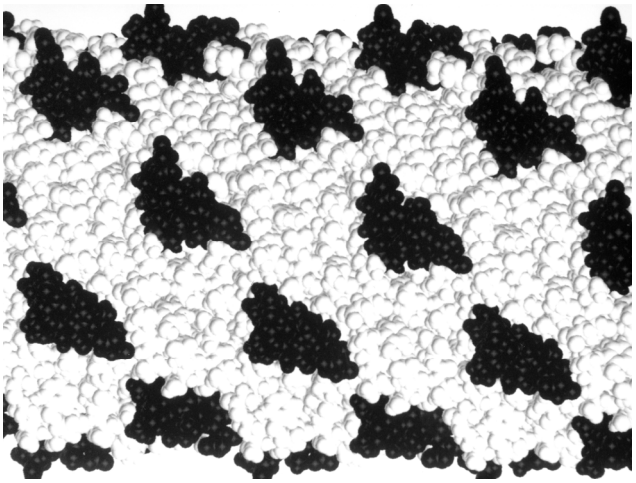

Fig. 2. Surface of the landscape phage according to the model of Marvin. About 1% of the phage length is shown. Foreign peptide inserts are pictured with dark atoms, their specific structural and positional details are entirely speculative, but their overall arrangement is presumably accurate. The distance between neighbor peptides is ~ 2.7 nm (Adapted from [19]).

Phage as bioselective nanomaterial. A *phage display library* is an ensemble of up to 10 billion different phage clones, each harboring a unique foreign DNA, and therefore displaying a specified guest peptide on the virion surface. It was shown that the foreign peptides replacing

three or four mobile amino acids at the N-terminus of the protein pVIII or replacing amino acids 12-19 in its central part don't disturb considerably the general architecture of virions. Yet, remarkably, such fusion phages can retain their ability to infect the host bacteria *Escherichia coli* and form phage progeny up to 1000 identical phage particles per bacterial cell during the doubling period. Such particles were eventually given the name "landscape phage" to emphasize the dramatic change in surface architecture caused by arraying thousands of copies of the guest peptide in a dense, repeating pattern around the tubular capsid, as illustrated by Fig. 2. The foreign peptides decorating the phage body create defined organic surface structures (landscapes) that vary from one phage clone to the next. A landscape library is a huge population of such phages, encompassing billions of clones with different surface structures and biophysical properties. The binding peptide comprising up to 20% of the phage mass and up to 50% of the phage surface may be easily prepared by precipitation from a culture of infected bacteria. Further purification, if necessary, can be readily performed by the large scale hydroxyapatite chromatography [27].

Landscape phages as substitute antibodies. *Affinity selection* is a characteristic aspect of the phage display technology that allows obtaining ligands against any receptor, including biopolymers, organic compounds or inorganic materials [29]. Therefore, the landscape phage is unique micro-fibrous material that can be selected with desired properties by the affinity binding protocol. To obtain the specific phage ligand, an immobilized target molecule or a corpuscular particle, called here "selector," is exposed to a phage display library, as shown on Fig. 3. Phage particles whose displayed peptides bind the selector are captured, while all other phages are washed away. The captured phage, generally a $1/10^8$-$1/10^7$ fraction of the initial library population, can be then eluted from the support with mild acid, alkaline or detergent solutions without affecting phage infectivity, and propagated by infecting bacterial host cells. A single round of affinity selection is able to enrich for selector-binding clones by many orders of magnitude; a few rounds suffice to survey a library with billions or even trillions of initial clones for exceedingly rare guest peptides with particularly high affinities for the selector. After several rounds of affinity selection, individual phage clones are propagated and their ability to bind the selector is confirmed.

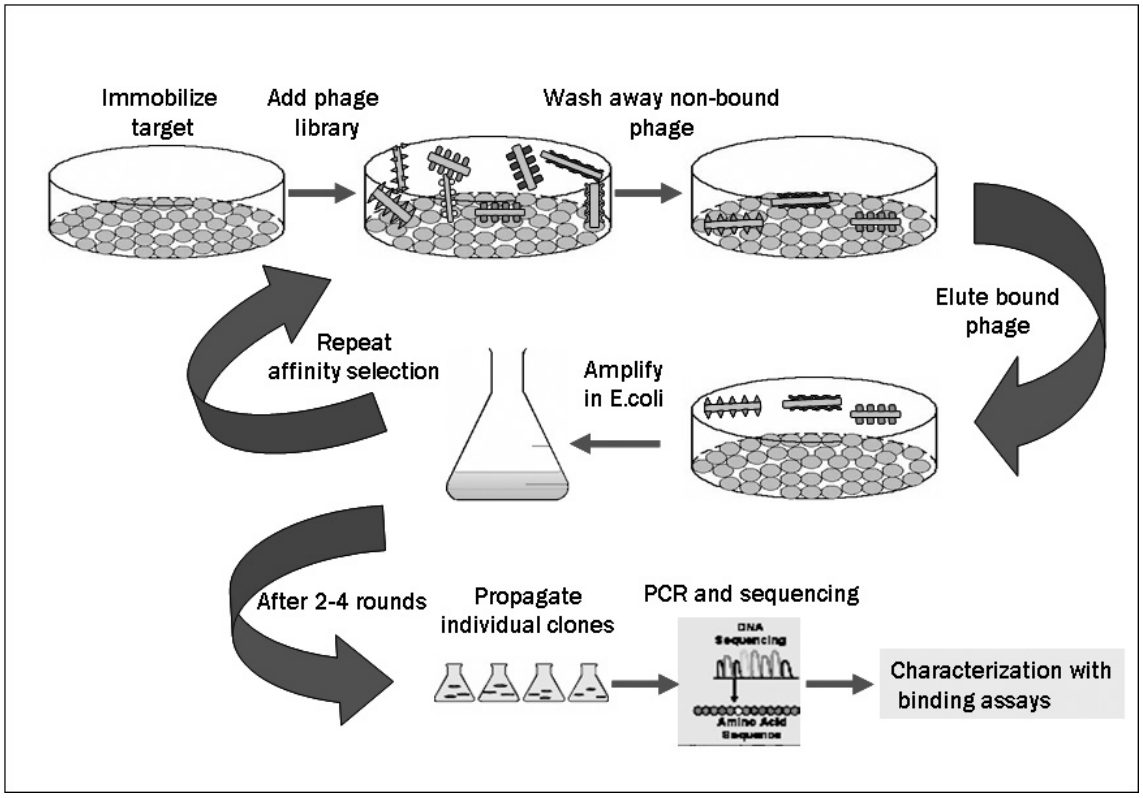

Fig. 3. General scheme of affinity selection of phage binders using immobilized targets.

Phages can be selected from landscape phage display libraries with affinities for a wide range of simple targets such as dioxin, Cibacron blue, β-galactosidase, streptavidin, neutravidin and fibrinogen [17, 19, 20], as well as for more complex targets such as prostate cancer cells [23], malignant glial cells [24, 25], or serum antibodies [7]. Landscape phages have been shown to serve as substitutes for antibodies and detection probes in enzyme-linked immuno assay (ELISA) and biosensors [16], as immunogens [11], drug- and gene-delivery vehicles [12] and affinity matrices [24].

Phage-driven biosensor devices. Analytical electronic devices that transduce a molecular recognition event into a measurable signal are generally called "sensors". Their principal components are a sensing interface that interacts with an analyte, and a signal processor that transduces the binding impulse into an observed signal. When a sensor interface is composed of biological entities, or when a sensor is designed to detect a biological agent, they are generically called "biomolecular sensors", or shortly—"biosensors". The major desired characteristics of the biosensors—sensitivity, selectivity, robustness and prompt performance—are determined mostly by the nature of the biorecognition interface. A commonly used recognition elements in biosensors are antibodies, although a variety of other bioorganic molecules have been also effectively used as an interface: peptides, enzymes, lectins, carbohydrates, nucleic acids, aptamers, recombinant proteins or molecularly imprinted polymers. In a more recent detection format, the whole cells were explores as the binding entities.

No one of these types of recognition interfaces, however, could respond well to the sensor performance requirements.

It was demonstrated that phage landscape libraries contain many potential probes for surface markers of cells, spores and bacteria. Phage probes against biological threat agents, such as *Bacillus anthracis* spores and *Salmonella typhimurium* were isolated in a nonbiased multistage selection procedure using immobilized spores or bacteria as a selector [1, 15, 29]. Binding of the selected phages to their respective targets was characterized by a precipitation test, fluorescence-activated cell sorting, enzyme-linked immunosorbent assay (ELISA), and fluorescent, optical and electron microscopy. These tests demonstrated specific dose-dependent binding of each antigen to the phage it has selected. Inhibition ELISA verified that non-immobilized synthetic peptides and peptide-bearing phages compete with immobilized phage for binding to their respective antigens [17]. These experiments with different antigens have shown that landscape phages may be used as a new type of substitute antibodies—filaments that can bind protein and glycoprotein antigens with nanomolar affinities and high specificity. It is interesting to note that representative landscape phage selected with *B. anthracis* spores in a non-biased selection scheme can bind the selector strain at a higher level than other species of *Bacillus* spores [1]. Similarly, the phage selected with *S. typhimurium* demonstrated higher affinity to the selector strain in comparison with nine other gram-negative bacteria, predominately *Enterobacteriaceae* [29]. A small amount of cross reactivity of this phage was noted with *Yersinia enterocolitica and Citrobacter freundii.* The complex of phage with bacteria was visualized by fluorescence microscopy and transmission electron microscopy (TEM) (Fig. 4) and showed a multivalent character of the phage-bacteria binding.

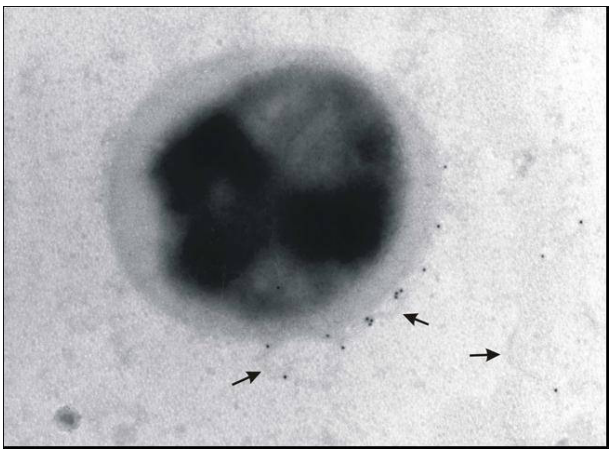

Fig. 4. TEM micrograph of bacteria-phage complex. Phage is labeled with gold nanoparticles (arrows).

The performance of the probes in detection of biological agents was illustrated by quartz crystal microbalance (QCM), in which the phages immobilized on a gold electrode of the QCM unit reacted with their analytes in solution phase [21]. Phage were immobilized onto the sensor surfaces by phage self-assemblage on Langmuir-Blodgett (LB) phospholipid by biotin/streptavidin coupling [21], or by direct physical adsorption of phage to the sensor surface [15]. In the LB method monolayers containing biotinylated phospholipids were transferred onto the gold surface of acoustic wave sensors and treated with streptavidin and biotinylated phage. The phage-loaded sensor demonstrated specific dose-dependent binding of β-galactosidase from *E.coli*. It was observed that the affinity of the complex depends on the mode of phage immobilization and type of analytical platform: 0.6 nM by acoustic wave sensor versus 30 nM by enzyme-linked immunosorbent assay (ELISA). The difference in affinities was attributed to the monovalent (ELISA) and divalent (sensor) interaction of the phage with β-galactosidase, as was indicated by the analysis of binding curves using the Hill presentation. It was hypothesized that one or another mode of interaction depends on the conformational freedom of the phage immobilized to the solid surface. Binding of the phage is quite specific because the response was reduced by 85% if β-galactosidase is preincubated with 4 nM phage. It was shown that binding of the phage to β-galactosidase is selective: presence of 1000-fold excess of bovine serum albumin in mixture with β-galactosidase did not affect the ELISA signal and reduces the biosensor signal only by 4%.
It was found that phages readily adsorb onto the gold surfaces [15], although the possible mechanism of the adsorption is still obscure [30]. When the acoustic wave sensor (Maxtek) with gold electrodes was exposed to the β-galactosidase-binding phage in suspension and then tested with gradually increasing concentrations of β-galactosidase, the sensor showed the value of EC_{50} of approximately 2 nM, comparable with results obtained by the self-assembling LB method [14]. Another biosensors specific for *S. typhimurium* demonstrated a linear dose-response relationship over six decades of bacterial concentration [15]. Bacterial binding to the sensor was confirmed by scanning electron microscopy (SEM) (Fig. 5). The sensitivity of the biosensor (-10.9 Hz) was vastly greater than the established background. The lower limit of detection based on the dose-response curve was estimated at 100 cells/ml.

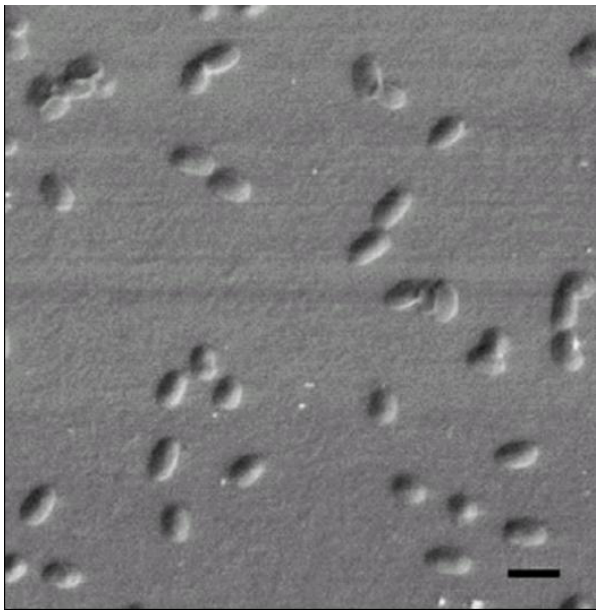

Fig. 5. Scanning electron micrograph of *S. typhimurium* binding to phage immobilized to the surface of a sensor by physical adsorption. Magnification 3000×; bar = 5 μm. Adapted from [15].

Robustness of the phage probes. Most detection devices traditionally rely on antibodies as diagnostic probes (reviewed recently in [4]). Their use outside of a laboratory, however, may be problematic because antibodies are often unstable in severe environmental conditions. Environmental monitoring requires thermostable probes, such as preselected phages that are superior to antibodies and can operate in non-controlled conditions. For example, when thermostability of a landscape phage probe and a monoclonal antibody specific for β-galactosidase was examined in parallel in the ELISA

format they were both stable for greater than 6 months at room temperature, but at higher temperatures the antibody degraded more rapidly than the phage probe [2]. At 37°C, phage degraded only slightly (half-life of phage as a probe at this temperature was 950 days), while monoclonal antibodies lost virtually all of their activity for 30-week study (half-life 107 days). At 50°C, both phage and monoclonal antibodies progressively degraded, but monoclonal antibody activity was undetectable after five weeks, while phage still retained more than 50% of its activity at the same time point. At 63°C, monoclonal antibodies were found to be completely inactivated after just 24 h. The phage probe was significantly more stable at this temperature, maintaining detectable activity for six weeks. Phage was shown to retain binding activity even after short incubations at 76°C. While a small amount of degradation was detectable after only 4 h, phage had a half-life of 2.4 days at 76°C. These results confirm that the phage probes are highly thermostable and can function even after exposure to high temperatures during shipping, storage and operation.

Phage-driven targeted gene- and drug-delivery systems. The concept of using targeted pharmaceutical nanocarriers to enhance the efficiency of anti-cancer drugs has been proven over the past decade both in pharmaceutical research and clinical setting. In particular, it is commonly accepted that selectivity of drug delivery systems can be increased by their coupling with peptide and protein ligands targeted to differentially expressed receptors. The abundance of these receptors was demonstrated recently by comparative analysis of gene expression in tumor cells and tumor vascular endothelial cells versus adjacent normal tissues [13], and their targeting is turning into a routine procedure in the most advanced laboratories due to the progress in combinatorial chemistry and phage display (a long list of different target-specific peptides identified by phage display can be found in recent reviews [22, 28]. In particular, selection protocols were developed for obtaining the phages and peptides internalizing into cancer cells, and phage homing at tumors of human patients. Selection of tumor-binding phage ligands in cancer patients, demonstrated recently by David Krag with colleagues, opened a new avenue for applications of phage technology for patient-directed cancer treatment [9].

A new challenge, within the frame of this concept, is to develop highly selective, stable, active and physiologically acceptable ligands that would navigate the encapsulated drugs to the site of the disease and control unloading of their toxic cargo inside the cancer cells. We have shown that the tumor-specific peptides fused to the major coat protein pVIII and affinity selected from multibillion clone landscape phage libraries by their ability to bind very specifically cancer cells can be converted easily into the gene-encupsulating particles, or drug-loaded vesicles. Thus, the major principle of our approach is that targeted therapeutic nanoparticles recognize the same receptors, cells, tissues and organs that have been used for selection of the precisely targeted landscape phage.

Targeted gene delivery by cancer cell-selective phage proteins. A fundamental requirement for a cancer therapeutic gene delivery device is that it has the ability to enter into a tumor compartment, target cancer cells leaving surrounding tissue unscathed, and deliver into the cancer cells a therapeutic gene that will stop their growth and cure the disease. Different structural elements of the device may be dedicated to various steps of this process. The therapeutic gene can be expressed in the cancer cell constitutively or may be activated specifically by a cancer cell product. Thus, to optimize the cancer treatment strategy, a system is required which would be versatile enough to allow using both selection and rational design for its development.

To meet these requirements, we developed a gene delivery system**,** which is based on phagemid infective particles (PIPs) encapsulated within bacteria by peptides encoded by a tumor-selected landscape phage [12]. These particles are considerably smaller than normal phage (~700 nm *vs*. ~1300 nm, but have all the elements necessary for delivery and expression of genes in mammalian cells. Their DNA can harbor a therapeutic gene, mammalian and bacterial origins of replication, as well as antibiotic resistance genes, used as markers. All the proteins required for encapsulation of the phagemid DNA, including tumor-targeting peptides, are provided by a phage helper. The strategy was justified by using a helper phage that is internalized by RG2 glioma cells selected from a landscape phage library. It was shown that the phagemid infective particles, PIPs are able to bind and penetrate into the target cancer cells and express the marker gene from within the cell. Antibody test showed that about 85% of the particles were internalized by the cancer cells. However, the low expression level of the marker GFP gene (2-3%) probably indicates a breakdown in the trafficking/expression pathway. This is not surprising since little effort was made to enhance PIPs DNA escape from the endosome. Transformation of eukaryotic cells with single stranded DNA phages can be increased if they are supplied with inverted terminal repeats of adeno-associated virus (AAV) [5].

The approach, shown for glioma cells, differs from others in that a phagemid expressing a model marker or particular therapeutic gene can be easily exchanged for a phagemid expressing a different therapeutic gene. Also, a different helper phage, selected from a phage display library can target any cell type and direct the encapsulation of any therapeutic gene encoding phagemid. Because of its versatility, the PIP system may be readily used for optimization of the gene-delivery strategies applied to specific cell and tissue targets.

Drug targeting with stripped landscape phages. The ability of the major coat protein pVIII to form micelles and liposomes emerges from its intrinsic function as membrane protein judged by its biological, chemical, and structural properties. During infection of the host *Escherichia coli*, the phage coat is dissolved in the bacterial cytoplasmic membrane, while viral DNA enters the cytoplasm. The protein is synthesized in infected cell as a water-soluble precursor, which contains a leader sequence of 23 residues at its N-terminus. When this protein is inserted into the membrane, the leader sequence is cleaved off by a leader peptidase. Later, during the phage assembly, the processed pVIII proteins are transferred from the membrane into a coat of the emerging phage. Thus, the major coat protein can change its conformation to accommodate to various distinctly different forms of the phage and its precursors: phage filament, intermediate particles and membrane-bound form. This structural flexibility of the major coat protein is determined by its unique architecture, which is studied in much detail [10]. In virions, mostly α-helical domains of pVIII are arranged in layers with a 5-fold rotational symmetry and approximate 2-fold screw symmetry around the filament axis. In opposite, in the membrane-bound form of fd coat protein, the 16-Å-long amphipathic helix (residues 8-18) rests on the membrane surface, while the 35-Å-long trans-membrane (TM) helix (residues 21-45) crosses the membrane at an angle of 26° up to residue Lys40, where the helix tilt changes. The

helix tilt accommodates the thickness of the phospholipid bilayer, which is 31 Å for *E. coli* membrane components. Tyr 21 and Phe 45 at the lipid–water interfaces delimit the TM helix, while a half of N-terminal and the last C-terminal amino acids, including the charged lysine side chains, emerge from the membrane interior. The transmembrane and amphipathic helices are connected by a short turn (Thr 19–Glu 20).

Stripped Phage Proteins. In model experiments, landscape phages were converted to a new biorecognition affinity reagent—"stripped phage" [19]. The stripped phage is a composition of disassembled phage coat proteins with dominated (98%) recombinant major coat protein pVIII, which is genetically fused to the foreign target-binding peptides. The stripped phage proteins can form bioselective vesicles decorated by target-binding peptides, which can be used for the targeted drug delivery. In our example, the stripped phages were prepared by treatment of the landscape phages with chloroform followed by conversion of resulted spheroids into the lipid vesicles by their reconstruction with phospholipids. In preliminary experiments with streptavidin- and bacterial binders it was demonstrated by competition ELISA, acoustic wave sensor and transmission electron microscopy that the stripped phage proteins retain the target-binding properties of the selected phage [19].

Fusion of the Stripped Phage Proteins with Doxil. Using intrinsic mechanism of fusion of the phage proteins with lipid membranes, we incorporated streptavidin-targeted proteins into the commercially available Doxil liposomes. The streptavidin-binding landscape phage was affinity selected from 9-mer landscape library. The phage was converted into spheroids with chloroform and incubated with Doxil to allow fusion of the phage proteins with liposome membrane. As a result of the phage fusion, the liposome acquired a new emergent property—ability to bind streptavidin and streptavidin-conjugated fluorescent molecules, as was evidenced by protein microarrays, fluorescent microscopy and fluorescence-activated cell sorting (FACS). The targeted and control liposomes were incubated with streptavidin-coated chips, washed and scanned (Fig. 6), or mixed with Texas Red-conjugated streptavidin (TRS), washed and analyzed by fluorescent microscopy and FACS. Complex of the modified Doxil with the target streptavidin demonstrated 50-fold higher fluorescence than pure Doxil and 10-fold higher fluorescence than control Doxil. The complex of the targeted Doxil liposomes with streptavidin-coated gold beads was visualized by transmission electron microscopy (Fig. 7).

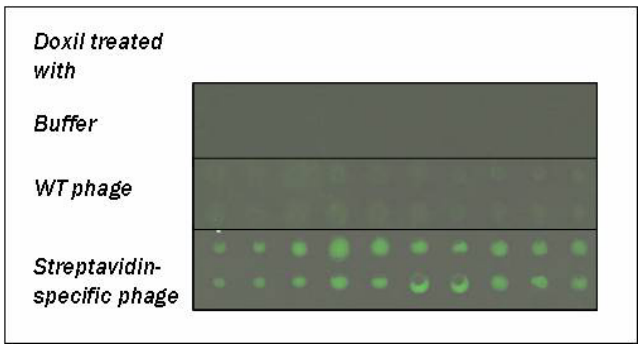

Fig. 6. Microarray test for Doxil targeting. Streptavidin-microarrayed slide was treated with unmodified Doxil, Doxil modified with wild-type phage and Doxil targeted with streptavidin binding phage.

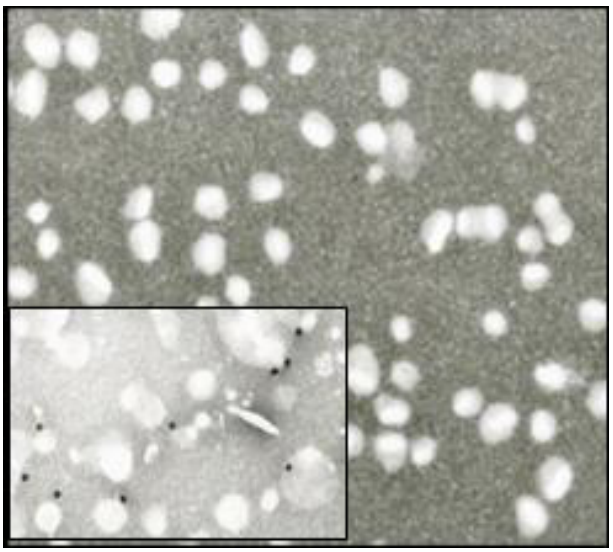

Fig. 7. Doxil liposomes (large frame), and complexes of the targeted Doxil liposomes with streptavidin-coated 20 nm gold beads (small frame). An evarage size of unmodified Doxil particles – 80 nm.

Conclusion. The presented data show that the phage engineering, which grounds on the natural mechanisms of selection, amplification and self assemblage, is a powerful and very precise technique that allows directed nanofabrication of bioselective materials, with possible applications to biosensors, nanoelectronics, biosorbents, gene/drug-delivery and other areas of medicine, technology, and environmental monitoring. The genetically driven "phage landscaping" allows the generation of libraries possessing diverse nanostructures accommodated on the phage's surface – a huge resource of diagnostic, detection and pharmaceutical probes. The new phage-based concept of bioselective nanomaterials relies on very powerful and extremely precise mechanisms of *selection, biosynthesis and self assembly*. A culture of phage-secreting cells is an efficient, convenient and discontinuous protein production system. They are secreted from the cell nearly free of intracellular components; their further purification could be easily accomplished by simple steps that do not differ from one clone to another. The major coat protein constitutes 98% of the total protein mass of the virion — the purity hardly reachable in normal synthetic and bioengineering procedures. As a normal intestinal parasite, phage itself and its components are not toxic and have been already tested for safety in preclinical and clinical trials [8, 9]. In contrast to immunization procedure, the phage selection protocol does not meet the tolerance problem, which can hamper obtaining antibodies to "self" antigens, and may require tiny amounts of a target material (thousands of tumor cells available in biopsy procedure for obtaining the tumor-specific phage ligands, affinity and selectivity of which may be controlled by exploring well developed depletion and affinity maturation procedures.

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N.O. Babii, research worker

VIRAL LOAD LEVELS OF HEPATITIS C VIRUS IN HIV/HCV-COINFECTED PERSONS

Institute of Epidemiology and Infection Diseases, Kyiv, Ukraine

The prevalence of serological mercers of HCV-infection among HIV-infected persons is investigated. The frequency of HCV activation and the genotyping structure of HCV population in HIV/HCV-coinfected patients are determined. The HCV viral loads are measured, the relationship between HCV viral load and HIV viral load, between HCV viral load and CD4 T-cell count is studied. A significant direct relationship between HCV viral load and HIV viral load and a significant inverse relationship between HCV viral load and CD4 T-cell count were found in patients who not receive HAART.

Introduction. The spreading of parenteral viral hepatitis among HIV-infected persons is connected with equal ways of these infections transmission. The percent of HCV-coinfected persons in population of HIV-infected patients in the different countries of the world is ranged from 4% to 90% depending on part of injection drug users among them [1]. HIV-positive persons have higher risk of progress of chronical hepatitis, fibrosis and cirrhosis of the liver. In spite of predominantly subclinical or asymptomatical course of viral hepatitis C, in conditions of coinfection it has more aggressive characteristics [2]. The aim of our work was investigation of frequency detection serological markers of hepatitis C and HCV viral load levels in HIV-positive individuals, investigation of relation between HCV and HIV viral load levels and CD4 cell count.

Objects and methods. Samples of serum and plasma from 425 HIV-infected patients were received from Clinic of Institute of Epidemiology and Infection Diseases; 125 weren't treated with highly active antiretroviral therapy (HAART) (I group of investigation) and 300 patients were treated (II group of investigation). Antibodies (IgG) to HCV were determined in samples of serum by ELISA (Diagnostic systems, Russia; Diaprofmed, Ukraine). HCV RNA was detected by PCR approach according to the PCR-kit protocol (AmpliSens, Russia) in HCV-seropositive samples of plasma. The HCV genotype was assessed by PCR approach with primers specific to single genotype according to the analysis protocol (AmpliSens, Russia). HCV viral load levels were determined by RT-PCR assay (AmpliSens HCV Monitor, Russia) in RNA HCV-positive samples of plasma. HIV-1 viral load levels were determined by RT-PCR assay (RealTime HIV-1 Abbott, USA). An association between HCV load and HIV load and between HCV load and CD4 T-cell count were determined by Pearson correlation coefficient.

Results and discussion. Prevalence of hepatitis C among HIV-infected patients was investigated on the first stage of the work by exposure of antibodies IgG to HCV in the samples of serum HIV-seropositive persons. The virus hepatitis C antibodies were detected in 88 (70,4±4,08%) serum samples of patients from first group of investigation and in 194 (64,67±2,76 %) among 300 samples of serum HIV-positive individuals from second group of investigation. Prevalence of hepatitis C among HIV-infected patients was analyzed against way of HIV transmission. Turn out, frequency of detection IgG to HCV in injection drug users (IDUs), infected HIV by parenteral way, was 94,34±1,42%, whereas in the group of patients, infected by HIV by sexual way, this value was 20,00±3,16%. Thus, most of cases of HIV-transmission related to injection drug using. It reduces to development in IDUs mix-infections with unpredictable consequences.

On the next stage of the work the frequency of HCV activation in seropositive patients was established by detection RNA HCV in the samples of plasma of these patients. RNA HCV was detected in 88,00±3,75% plasma samples of seropositive patients from first group, and in 81,67±4,99% plasma samples of patients from second group. So, irrespective HAART receiving, in most of HIV/HCV-coinfected patients hepatitis C is characterized by active replication of HCV.

We determined the genotyping structure of HCV population circulatory among HIV-infected persons. It is very important for determination of tactic for hepatitis C therapy, for prognostication of its clinical course. Genotype of HCV was determined in 115 RNA HCV positive samples of

plasma. According to modern classification there are 6 genotypes of HCV which in part are divided into separate subtypes. Viruses of hepatitis C with subtype 1b are most aggressive: disease induced by subtype 1b of HCV is more severe and therapy of this hepatitis with interferon is less effective, degree of liver damage is more high in comparison with disease induced by another subtypes HCV [3].

We detected the domination subtype 1b HCV among HIV/HCV co-infected persons. Furthermore, subtypes 1a and 3a HCV were founded frequently. Subtype 1b HCV was detected in 64,04±4,47%, subtype 3a – in 25,22±4,05%, subtype 1a – in 13,91±3,23% and subtype 2 – in 0,87±0,86% of plasma samples of HIV-infected patients. Thus, most of cases of hepatitis C were induced by 1b subtype of HCV.

The next stage of this work was investigation of HCV RNA level and studying of correlation between indexes of HCV and HIV viral load and CD4 cells count in HIV/HCV-coinfected patients. Data about CD4 cells count were obtained from case histories of HIV-infected persons. HIV and HCV viral loads were determined in samples of plasma of 18 persons not receiving HAART (I group) and 16 persons receiving HAART (II group). High HCV viral load (>2x10^{6}RNA copies/ml) was revealed in 87,5±8,27% patients from I group and 11,11±7,41% patients from I group. The median HCV RNA level was 3,67x10^6±2,2x10^5 copies/ml in plasma samples of patients from first group, and 7,76x10^5±3,1x10^5 copies/ml in plasma samples of patients from second group. That is, HCV viral load values in HIV-infected persons receiving HAART were higher than in non-treated HIV-positive individuals, this difference was statistically significant (p<0,005). Possibly, it may be related to hepatotoxicity of antiretroviral drugs or to increase of HCV mutability and level of HCV viral load under exposure immune press.

Levels of HIV viral load were more high in patients who not receiving HAART. So, in HIV-infected persons from I group levels of HIV viral load were from 1,2x10^3 to 7,4x10^{4}RNA-copies/ml plasma, the median value was 2,5x10^4±5,7x10^3 RNA-copies/ml plasma. In most of persons receiving HAART levels of HIV viral load were less then 75 RNA-copies/ml plasma, only 2 persons had HIV viral loads higher then 1x10^3 RNA-copies/ml plasma, the median value was 2,2x103±1,0x10^3 RNA-copies. Thus, the median value of HIV viral load in plasma of HIV-infected persons receiving HAART was in 11.4 times higher then in patients who were not treated with HAART.

The median values of CD4 cells count were not statistically different in persons from I and II groups of investigation: in patients from first group the median CD4 cells count was 496,0±53,2/mm^3 , in patients from second group this value was 414,13±17,09 /mm^3.

HCV RNA levels for HIV/HCV-coinfected patients were compared to HCV RNA levels and CD4 T-cell counts in peripheral blood. A significant direct relationship was found between HCV viral loads and HIV viral loads in patients not receiving HAART(r=0,8; m=0,15; p<0,05) (fig.1)

Thus, levels of HIV and HCV viral load are related with each other and, perhaps, by increasing of HCV viral load the increasing of HIV viral loads may occurs, and vice versa.

Moreover, a significant inverse relationship was found between HCV viral load and CD4 T-cell count in patients not receiving HAART (r= – 0, 7; m=0,18; p<0,05) (fig.2).

That is, increasing of HCV viral load is related to decreasing CD4 T-cell count in non-treated patients and vice versa. Thus, immune status plays a role in controlling of HCV replication.

At the same time, the relationship between HCV viral load and HIV viral loads (r=0,279; m=0,39; p>0,05) and between HCV viral load and CD4 T-cell count (r=0,143; m=0,37; p>0,05) in patients receiving HAART were not obtained.

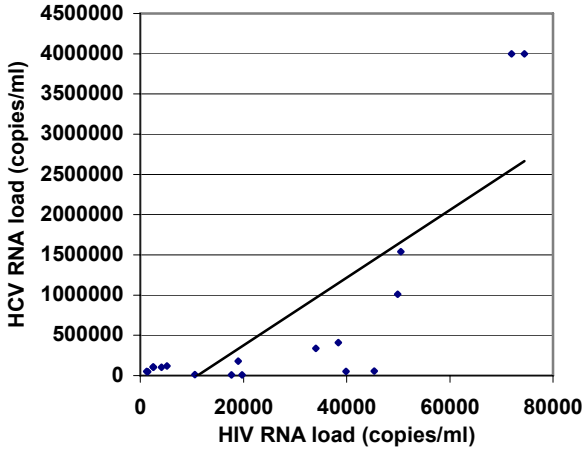

Fig.1. Relationship between HCV viral loads and HIV viral loads in patients not receiving HAART(r=0,8; m=0,15; p<0,05).

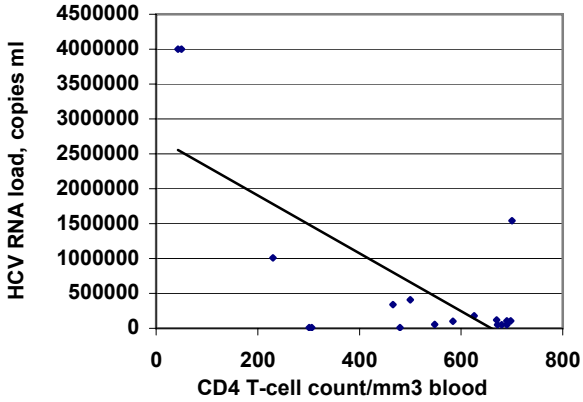

Fig.2. Relationship between HCV viral loads and CD4 T-cell count in patients who not receiving HAART (r= – 0,7; m=0,18; p<0,05).

Evidently, receiving of HAART reduces to considerable decreasing of HIV viral load, to gradual increasing of CD4 T-cell count and, perhaps, to increasing HCV viral load. All these change relationship between these indices.

Conclusions. Chronical HCV-infection is accompanied with active replication of HCV in most HCV/HIV-coinfected persons. High values of HCV viral load are affirmed this statement. Highly active antiretroviral therapy can assist in some extent for active replication of HCV and lead to increasing of HCV RNA level. According to this, it is necessary to control and carefully prescribe ARV-drugs because of possibility liver failure in HIV/HCV-positive individuals. Significant direct relationship was found between HCV viral load and HIV viral load and significant inverse relationship was found between HCV viral load and CD4 T-cell count in patients not receiving HAART, what indicate direct and immune mediated interaction between HIV and HCV at coinfection.

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S. Zagorodnya, PhD, N. Nesterova, PhD, G. Baranova, eng., A. Golovan, eng.

SEARCH OF NEW DRUGS ACTIVE AGAINST EPSTEIN-BARR VIRUS

Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine, Kyiv

Epstein-Barr virus (EBV) is the representative of the family of Herpesviridae. Infectious mononucleosis is considered clinical form of primary human EBV infection after that the virus is retained in human organism during all life with the subsequent reactivation under influencing of different factors, both internal condition of an organism, and environment that results in clinical presentations of a miscellaneous degree of complication. EBV can be the agent to cause miscellaneous lymphomas such as nasopharyngeal carcinoma, carcinoma of parotid glands, stomach adenocarcinoma and other diseases. As well as other herpesviruses EBV affects central and peripheric nervous systems.

Summarize above it is possible to conclude that a modern arsenal of antiherpetic substances is rather miscellaneous. However, despite of it herpes as ARVI remains difficult controlled infection. It is caused as genetypical features of pathogen so and long-lived persistence of virus in organism and formation of resistant strains to antiviral drugs. Therefore search for new antiherpetics is still important.

In the laboratory of viruses reproduction IMV of NASU drug screening of the miscellaneous nature with the purpose of detection of new substances with the expressed activity against EBV is carried out permanently. Among developments of the Ukrainian chemists and pharmacists it is possible to mark out numbers of substances of miscellaneous nature, which have shown rather high anti-EBV activity and low cytotoxicity, that was revealed in our investigations. There are drugs of nucleoside nature, such as 6-Azacitidin and its derivative, threecyclic nucleosides, iodine-contained drugs, such as Amizon and its derivative; drugs based on the plant extractions Proteflasid and Altabor. Antiviral activity of these investigated preparations and their new properties such as apoptosismodulatory were studied. That is the relevant feature outgoing from many-sided nature of EBV developments in clinical practice in particular its transforming capacity.

Thus, the obtained data will allow creating new antiviral drugs based on such substances for control of human diseases caused by EBV

Questions regarding chemotherapy of virus infections are central to a number of problems of infectious pathology. Obligatory intracellular parasitism of viruses substantially defines the importance of creating antiviral preparations, which would selectively inhibit virus specific processes that proceed in a cell. It is known that many inhibitors of virus-specific processes, which are known to influence the metabolism of a cell, cause a toxic action. It essentially explains the existence of only a limited set of the antiviral preparations applied in clinical practice. For the last 30 years, some tens of antiviral preparations that pertain to various groups of substances have been introduced. Interest in search of active preparations on model herpes viruses increases. Human herpes viruses are found worldwide and they are among the most frequent causes of viral infections in immunocompetent as well as in immunocompromised patients.

Viruses affect the infected cells in different ways. Along with the deep alternations of the metabolic processes and their re-direction on the synthesis of the virion components, infection may lead to the changes of the functional state and regulatory processes in the cell. Molecular processes connected with an expression of early genes often are targets for antiherpetic preparations used in medical practice. Therefore, it is important to study the influence of nucleoside preparations on virus specific enzymes of nuclein synthesis. This provides a necessary basis for the elaboration of the principles of the correction of infectious, immunosuppressive and carcinogenic impact of viruses and development of efficient ethiotropic therapy means.The Epstein-Barr virus (EBV) belongs to the family of Herpesviridae, whose characteristic feature is their ability to establish a lifelong persistent infection in human organism with further reactivation, e.g. after the change of immune status of the patient under pressure of various ecological factors or stress. EBV causes infectious mononucleosis and it is associated with several human neoplasm's – Burkitt's lymphoma, Hodgkin's disease, AIDS-associated B-lymphoma, primary CNS non-Hodgkin's lymphoma, stomachic adenocarcinoma, X-linked lymphoproliferative syndrome, nasopharyngel cancer and posttransplantation lymphoproliferative syndrome. Some representatives of Herpesviridae family, primarily EBV, herpes simplex virus, cytomegalovirus and human herpes virus type 8 pertain to AIDS-associated viruses; they take part in pathogenesis of this symptomocomplex [2-8]. The search for the novel antiviral substances active against Epstein-Barr virus (EBV) is a topical problem, EBV like other herpesviruses affects central and peripheral nervous system being involved in the pathogenesis of meningoencephalitis, arachnoencephalitis and meningitis. There are no effective drugs for treatment of different clinical forms of EBV-infection up to date. Ganciclovir, vidarabine, foscarnet, cydofovir have some anti-EBV activities. That's why the development of a new EBV inhibitor remains to be the actual problem of virology and medicine in total.

Such approaches to treatment of virus infections are known at present:

- effective antiviral chemicals include: nucleoside acyclic analogs; viral DNA polymerase and thymidine kinase are targets for them. Miscellaneous natural substances are investigsted; their mechanism of action is still insufficiently studied.
- serotherapy and seroprophylaxis, which are provided by the use of specific human immunoglobulins.
- immunomodulator drugs are active preparations with immunostimulating properties in relation to cell and humoral immunities.

Summarize above it is possible to conclude that a modern arsenal of antiherpetic substances is rather miscellaneous. However, despite of it herpes as ARVI remains difficult controlled infection. It is caused as genetypical features of pathogen so and long-lived persistence of virus in organism and formation of resistant strains to antiviral drugs. Therefore search for new antiherpetics is still important.

The role of interferon in treatment of diseases caused Epstein-Barr virus among doctors remains ambiguous as there are no works precisely proving his efficiency. Though the role of preparations of interferon in correction of immune system of the person is unconditional, at the given diseases as one of properties EBV is negative influence on system of the immune answer of an organism. Most the positive effect is observed at application of interferon of type an alpha [11].

Edward Gershburg and Joseph S. Pagano [10] in 2005 have presented the review of chemical preparations that are active against EBV. All modern spectrum of preparations existing in the world which are perspective in relation to Epstein-Barr virus is analysed in given paper. According

to their approach all preparations can be related conditionally to following groups.

The first group includes noncyclic analogues of nucleosides (Aciclovir, Ganciclovir, Penciclovir, Valaciclovir, Malgan-ciclovir, Famciclovir); noncyclic analogues nucleotides (Cidofovir, Adefovir; Foscarnet and other substances). A target of all above-mentioned substances is viral DNA-polymerase and thymidine-kinase.

Natural substances the mechanism of action of which is insufficiently investigated belong to the second group.

All above represented preparations are strong inhibitor of virus reproduction, but not all of them are perspective for clinical applications as strongly differ by toxicity, this aspect is very actual, especially concerning children and youth. All these components have a number of lacks (toxicity, insufficient bioavailability and risk of occurrence new resistant strains). So in case of heavy defeats of nervous system caused EBV, prolonged intravenous introduction ganciclovir is necessary that increases undesirable toxic action by various bodies and systems of a macroorganism [1].

It is caused by genotypic features of the activator, duration of virus persistence in an organism, and formation of strains resistant to anti-virus preparations. Therefore, search new antiherpetic preparations remains actual.

Materials and methods. *Cell and virus*. The line of lymphoblastoid B-cell Raji infected by EBV was used as a model of EBV-infection in vitro. Cultural medium of B95-8 cells producing the EBV was used as a virus-containing material after a differential centrifugation. The nutritional media for Raji cells consists of medium 1640 with supplement of 10% embryonic serum, 2 mM glutamine and antibiotics. The Raji cells were infected during 60 minutes at the temperature 37^{o} C. Upon the exposure, cells were washed and medium without serum was added.

PCR. An inhibition of reproduction of EBV in cell culture by drugs was determined by reduction of number of genome equivalents of EBV DNA on a cell in treated versus untreated cells. To determine it, a quantitative PCR was applied using primers and reagents of “AMPLY-Senc-100R” (Russia) and programme “Biotest A”.

MTT method. The CD_{50} were also determined by colorimetric method using the dye MTT (Sigma Chemical, St. Louis, Mo.). 25 µl of RPMI 1640, containing 5 mg of MTT/ml, added in each well of the microtitration plate. Incubation continued for 3 h at 37° C. The contents of each well removed (after centrifugation under 1000g during 10 min) and 200 µl of 96% ethanol added to extract the dye. After 10 min of gentle agitation at 37^{0}C the optical density (OD) at 540 nm was measured using microwell plate rider (Dynatech, Sweden). The percentage of MTT conversion in its formazan derivative for each well was calculated comparing the OD at 540 nm (OD_{540}). The line of Raji cells infected by EBV was used as a model of EBV-infection in vitro. An inhibition of reproduction of EBV in cell culture by drugs was determined by reduction of a number of genome-equivalents of EBV DNA on a cell.

Results of work. The research work was given up to studying of antiviral action of nucleosides: 6-azacytidine, 2'3'-"seco"-5-methyl-6-azacytidine, 2'3'-didezoxy-2'3'-didehydro-6-azacytidine, 2'-dezoxy-6-azacytidine to the reproduction of Epstein-Barr virus in Raji lymphoblastoid cells.

Cytotoxicical action of investigated preparations was characterized, and their doses that reduced the prolypherative activity for 50% were determined. Minimal active concentrations, which inhibited of virus reproduction, were established. Determined selectivity index allow relating these substances to potential anti-EBV preparations. It was confirmed the trustworthiness of estimations of researches results for the obtained dates by the statistics methods. The obtained dates will be using for computering modeling of an interaction between structure and biological activity of substances, which will be using to prognosis of making a new high activity antivirus medicinal preparations. 6-Azacytidine (2-β-D-ribofuranosyl-5-amino-1, 2,4-triazin-3(2H)-on; 6-AC) are an original structural cytidine analogue with the wide spectrum of biological activities (antiadenoviral, anti-HSV, anti-mycoplasmic, immunomodulating, antitumor) [9]. The objective of the present investigation was to study the activity of 6-AC against EBV, as well as its new analogues bearing. 6-Azacytidine, 6-AC acyclic derivative - 2’3’-“seco”-5 methyl-6 Azacytidine (seco 6-AC), bi- and tricyclic nitrogencontainig structures (non nucleoside protease inhibitors) are widely used as potential antiviral agents. They are used against retroviruses and some herpesviruses. 2’3’ –dideoxy-2’3’ –didehydro-6-asacytidine (№1) and 2’-deoxy-6-asacytidine (№2) are original cytidine analogues. The concentrations of 6-AC and “seco”- 6-AC which inhibited the quantity of alive cells on 50% (CC_{50}) were equal to 96 and 200 µg/ml accordingly. The minimal inhibiting concentration (MIC) of 6-AC was equal to 0,5 µg/ml, because the amount of genome - equivalents of DNA EBV on a cell was reduced with 6,0 up to 3,1. Acyclovir has shown the considerably smaller activity against EBV; in concentration of 125-500 µg/ml it reduced the quantity of DNA EBV up to 6,5-4,8 genome-equivalents on a cell (7,12 without the inhibitor). The concentrations, which inhibited the quantity of alive cells on 50% (ID_{50}), were equal to substances № 1 - 500 µg/ml and №2 – 250 µg/ml. The minimal inhibiting concentration (MIC) of №1 was equal to 4 µg/ml, because the amount of genome - equivalents of DNA EBV on a cell was reduced with 22,0 up to 10,4. MIC for № 2 was equal to 16 µg/ml (the amount of genome - equivalents were reduced with 22 up to 7) (tab.1).

Table 1. **Parameters cytotoxicity and anti-virus activity nucleosides (are synthesized in IMBG NASU)**

	6-azacytidine	2'3'-"seco"-5-methyl-6-azacytidine	2'3'-didezoxy-2'3'-didehydro-6-azacytidine	2'-dezoxy-6-azacytidine
CC 50	96 µg/ml	200 µg/ml	500 µg/ml	250 µg/ml
EC 50	0,5 µg/ml	1 µg/ml	4 µg/ml	16 µg/ml
IS	190	200	125	16

It was investigated the influence of 6-AC and acyclic derivative on CD95–mediated apoptosis in Raji cells infected by EBV as well as uninfected. It was shown that 6-AC in concentration 32 and 125 µg/ml strengthened the expression of CD 95-mediated apoptosis.

Hence, the index of selectivity (IS) was equal to 125 and 16 for 2’3’-dideoxy-2’3’-didehydro-6-azacytidine and 2’3’-dideoxy-6-azacytidine accordingly. It was investigated the influence of №1 and №2 on CD 95–mediated apoptosis in Raji cells infected by EBV.

Today drug-resistant strains of virus diseases have been documented in every country as well as multidrug-resistant strains. Thus the development of new drugs is one of the essential problems for modern chemotherapy in combating such infections. New series of Triazine Bearing Tricyclic Bases and their N-Glycoside Derivatives were synthesized as potential antiviral agents. These bases present the bioisosters of isoalloxasine chromophore of natural flavin mononucleotides.N-Glycoside Derivatives were stereospecifically prepared by glycosylation of corresponding bases with tetraacetyl ribose precursor, followed appropriate chemical modifications. Search of new effective preparations capable to inhibit herpesviruses reproduction is stipulated by their certain resistance to different groups of chemical preparations. New Triazine Bearing Tricyclic

Bases and their N-Glycosidic Derivatives structures are widely used as potential antiviral agents.

The first stage of investigation of substances was the analysis of their cytotoxicity for cell line Raji.We have studied in concentrations of 1000 to 0,1 μg/ml. The concentrations which inhibited the quantity of alive cells on 50% (ID_{50}) were equal to substances Triazine Bearing Tricyclic Bases - 750 μg/ml, N-Glycoside Derivatives №1 – 625 μg/ml and N-Glycoside Derivatives № 2 - 125. The minimal inhibiting concentration (MIC) of №1 and № 2 was equal to 1 μg/ml, because the amount of genome - equivalents of DNA EBV on a cell were reduced with 28,0 up to 14. Hence, the index of selectivity (IS) was equal to 750 and 625 for Triazine Bearing Tricyclic Bases №1 and №2 (tab.2).

***Table 2*. Parameters cytotoxicity and anti-virus activity Triazine Bearing Tricyclic (are synthesized in IMBG NASU)**

	Triazine Bearing Tricyclic Bases № 1	Triazine Bearing Tricyclic Bases № 2	N-Glycoside Derivatives
CC 50	1780 мкМ	2170 мкМ	590 мкМ
EC 50	2.7 мкМ	2.86 мкМ	4.7 мкМ
IS	625	750	125

During last decades more and more attention is given to creation of preparations for pathogenetic therapy with the polyvalent pharmacological action, which is capable essential to influence on immunity, to adjust the basic exchange processes, and also to have antiphlogistic effect. One of successful elaborations of the Ukrainian pharmacologists is the new non-narcotic analgesic Amizon with expressed antiphlogistic, antipyretic, interferon gene and immunomodulatory properties. Amizon – the derivative of isonicotinic acid (N-metyl-4-benzyl urea-pyridinit iodidum).

The objective of the present investigation was to study the activity Amizon, as well as derivative, in which structure there is no iodine, against Epstein-Barr virus. To study the cytotoxicity of investigated drugs they were entered into the culture of not infected cells in concentration from 0,1 up to 3000 μg/ml. In 48 hours there was conducted the MTT-analysis of the investigated samples. It was shown, that the concentration that oppressed proliferative activity of cells on 50 % (CD_{50}), for Amizon has compounded 840 μg/ml, and for its two derivative - 2100 μg/ml, accordingly. Drugs were investigated in concentrations of 0.1, 0.5, 1, 5, 10 μg/ml. The analysis of obtained data allowed to determine concentrations, which oppressed the replication of the virus on 50 %, that was shown by reduction of the number of genomic equivalents of EBV DNA on a cell testified. ED_{50} for Amizon has compounded 0,1 μg/ml, for it derivative - 5 μg/ml (tab.3).

***Table 3*. Parameters cytotoxicity and anti-virus activity of Amizon and his Derivative (are synthesized in IFT AMNU)**

	Amizon	Derivative № 1	Derivative № 2
CC 50	840 μg/ml	2100 μg/ml	2200 μg/ml
EC 50	0,1 μg/ml	5 μg/ml	5 μg/ml
IS	8400	400	440

Thus, the low toxicity of investigated drugs was shown and their effective doses were determined. Proceeding from the index of selectivity that is 8400 for Amizon, 400 for the its derivative, it is possible to make a conclusion about their availability for the further researches as of drugs that are active against an Epstein-Barr virus. Furthermore obtained data testify to importance of presence of iodine in structure of drug, as, apparently from the received data, the activity of derivative, not containing iodine, is below more than in 20 times.

The aim of the study was to assay the anti-EBV activity of several substances prepared from the raw material of the plant origin, namely Proteflasid. Proteflasid (Ecopharm Research and Production Company, Kyiv) represents the quercetin-containing herbal extract of wild grasses Deschampsia caespitosa L. and Calamagrostis epigeios L. The search of the novel antiviral substances active against Epstein-Barr virus (EBV) is a topical problem since the persistent EBV infection alters immune status promoting the development of adenocarcinomas and lymphoproliferative diseases. Moreover, EBV like other herpesviruses affects central and peripheral nervous system being involved in the pathogenesis of meningoencephalitis, arachnoencephalitis and meningitis. The substances were assayed within broad concentration ranges. The maximally tolerable concentrations for the cell line being assayed amounted to 1000 μg/ml for Altabor and 150 μg/ml for Proteflasid. The inhibition of EBV reproduction was assessed by PCR technique estimating the number of EBV DNA genomic equivalents. Minimal effective concentrations amounted to 0.1 μg/ml for Proteflasid.

Altabor (Borschahovskyi Chemical and Pharmaceutical Plant, Kyiv) based on the polyphenols isolated from infructescences of the alder comprises monomers and olygomers of ellongotannins (no less than 60%), melanoid polymer (about 10%), and phenolic acids (ellagic, gallic, valonic acids as well as mono- and polysaccharides). The substances were assayed within broad concentration ranges. The maximally tolerable concentrations for the cell line being assayed amounted to 1000 μg/ml for Altabor and 150 μg/ml for Proteflasid. The inhibition of EBV reproduction was assessed by PCR technique estimating the number of EBV DNA genomic equivalents. Minimal effective concentrations amounted to 2 μg/ml for Altabor and 0.1 μg/ml for Proteflasid. Therefore, the chemotherapeutic indices for Altabor and Proteflasid were estimated as 500 and 1500, respectively (tab. 4).

Table 4. Parameters cytotoxicity and anti-virus activity of plant preparations

	Proteflasid	Altabor
CC 50	150 μg/ml	500 μg/ml
EC 50	0,1 μg/ml	0,1 μg/ml
IS	1500	5000

Thus, the obtained data will allow creating new antiviral drugs for strife with human diseases caused by EBV based on such substances.

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N. Ivans'ka, PhD, C. Rybalko, PhD, T. Kalitenko, researcher, N. Nastoyashcha, PhD,
O. Kislykh, researcher, D. Fedorchenko, laborant-student

SEROLOGICAL DETECTION OF HIV-INFECTION AND ANTIGENIC MIMICRY

Lev Gromashevski Institute of Epidemiology & Infectious Diseases, Academy of Medical Sciences of Ukraine, Kyiv, Ukraine

Carrying out HIV-infection diagnostics, a lot of factors should be taken into account. The authors discuss the role of different factors and pathogens in the development of false-positive answers, that is why a continuous perfection of diagnostic test-systems is necessary to avoid interference contribution to diagnostic faults.

Test-system specificity is a problem of the greatest importance in serological diagnostics. It is especially true for infections of enormous epidemiological and social impact including the HIV-infection.

HIV-like proteins and antibodies (Abs)against them are described in patients with numerous pathologies associated with autoimmune processes [17, 21-22, 28], some of them appearing to be free from autoimmune features [6, 9-10,16]. False-positive answers (FPA) are often found in tumor-bearing patients with multiple myelomas, malignant lymph and blood conditions [23, 25], multiple warts causing antibody development against HIV core proteins [23] .

According to generally known data, there is about 70 conditions or other factors whose presence is shown to be associated with FPA for sera being studied by different serological methods including also immunoenzyme analysis (IEA) which is thought to belong to the most sensitive and specific diagnostical approaches [28]. The FPA probability becomes higher proportionally to increase of foreign antigens and factors being in contact with patients investigated. Such cases may appear due to many situations accompanied by polyclonal B-lymphocyte activation; independently on factor specificity having induced this process, a lot of Abs to different non-similar antigens raise usually and appear in blood sera [13].

Advances in molecular biology, especially the use of monoclonal antibodies (MAbs) in diagnostic field as well as study of amino acid and nucleotide sequences of enormous quantity of structures (for viruses, bacteria, and their hosts) led to publications proving immune relationship between structures of quite different origin, presence of similar or identical structures in evolutionary distant organisms including also different viruses [1, 2, 8, 14, 17-18, 20, 24, 26]. Now such phenomenon is known as molecular mimicry.

Anti-HIV antibodies cross-reacting with tissue structures may contribute to HIV-infection pathogenesis and numerous AIDS-accompanying autoimmune processes. HIV patients sera were found to contain antibodies to a lot of human cells (lymphocytes, platelets, neutrophils, erythrocytes) and their components (myelin, basic myelin protein, nucleus components, phospholipids, CD4, HLA etc [8, 18, 24, 26]. Our study deals with other compounds able to be factors of FPA when sera being screened to detect anti-HIV Abs.

An investigation of Japan researchers (Lin et al.) [19] shows a growth medium of *Streptomyces sp.* strain to contain a carbohydrate-containing biopolymer possessing neuraminin properties and inhibiting neuraminidase activity of influenza virus and paramyxoviruses. Neuraminin preparations isolated by S.Rybalko and A.Shapiro from *Staphylococcus aureus* metabolism products [4-5] was later named mimicrin due to its antigenic mimicry with viral and microbial peptides.

The main object of this study is the investigation of antigen mimicry role in the FPA development as a result of HIV serological diagnostics.

Materials and methods. *Mimicrin-producing strains of microorganisms and mimicrin preparations used. Staph. aureus* strain 392 isolated by Dr A.Shapiro in our Institute and a reference *Candida albicans ATCC* strain were taken for our investigation. Mimicrins were isolated from culture .medium after *Staph. aureus* growth (mimicrin 1), *Staph. aureus* toxin (mimicrin 2), and culture medium after growth of conditionally-pathogenic yeast-like fungus *C. albicans* (mimicrin 3).

Cultivation of mimicrin-producing strains. *Staph.aureus* and *C. albicans* strains were grown on a meat-peptone agar (MPA) produced by "Biophama' (Ukraine) supplemented by 1 % yeast extract (Olaina, Latvia).

Original culture strains were put into tubes containing meat-peptone broth (MPB) for bacteria and MPB with 1 % glucose (for *Candida*), the pH value was always supported to be 7.2. Following 18-20 h incubation (36 ± 1^0 C for bacteria and 30 ± 1^0 C for *Candida*). The cultures were sown on Petri dishes [3] to obtain colonies. Bacteria were grown at Gause medium No 2, and fungi – on Sabouraud medium containing chloramphenicol (50 mg/ml) and glucose, the incubation temperature being as earlier.

Colonies obtained were then passaged into tubes to obtain vegetative cells. In 18-20 h biomasses grown there were washed away from slanting agar surface by a buffered physiological solution, the vegetative cell concentration becoming $1x10^7$ according to turbidity standard. These cells were then taken to prepare a stock suspension – inoculate (100 ml) for mimicrin preparation. It was put into 1 l of culture medium and incubated 24 h at 37 ˚C or 30 ^{0}C for *Staph. aureus* and *C.albicans*, respectively; the stirring was 400 rpm.

*Ethanol precipitation***.** Supernatant fluid following microorganism cultivation and centrifugation (8,000rpm, 30 min) was added to ethanol (1:1.5 v/v); the mixture was incubated at 4 ˚C (16-18 h) to obtain precipitate. The pellet was washed by 60 % ethanol, run as previously; the pellet was dissolved in a phosphate buffer pH 7.2. This procedure was made twice. The last precipitate dissolved in the buffer was boiled 10 min and then pelleted. The supernatant fluid was used as a mimicrin preparation.

*Mimicrin purification by filtration on Sephadex G-200 columns***.** A sample of material was put on a Sephadex G200 column (2x30 cm) washed previously by phosphate buffer; 1 ml fractions were analyzed by biuret method to determine protein concentrations [12].

*HIV proteins***.** Oligopeptide analogs of HIV gp120 variable regions for serotypes A (KSVHIGPGQAFYATG), B (KSIHIGPG*R*AFYTTG), and C (ESVRIGPGQTFYATN) were received from the Institute of Highly Purified Biological Preparations (Russian Academy of Sciences, Sankt-Petersburg, Russian Federation). HIV gp160 protein (recombinant protein HIV1gp160LAV) was a product Protein Science Corporation (USA). Other recombinant polypeptides used here were "Diaproph-Med" company products (an Env fused protein including gp120 and gp41 a Gag fused protein including p24 and p17).

Sera, both HIV-positive and false HIV-positive ones (FPS), were tested in three alternative test-systems intended for anti-HIV Abs detection and by an immunoblot method at the Laboratory of Viral Hepatitis and HIV-Infection of our Institute. HIV-negative donor sera were taken from the Kiev City Transfusion Center.

Rabbit immunization to obtain anti-mimicrin sera was carried out using Chinchilla rabbits (about 1.5-2 kg), mimicrin preparations (1 μg/ml) being injected into retrobulbar sinus.

Solutions for antigen sorption, plate washing, sera and conjugate dilutions, substrate buffer and a chromogen, tetramethylbenzidine, as well as a stop-reagent were "Diaproph-Med" products.

Immunoenzyme analysis. All antigens including also mimicrins were diluted in a carbonate buffer (pH 9.3-9.6, 1 μg/ml), put into 96-well Nunc Maxisorp plates (100 μl per well), and incubated 16-18 h at 4 °C. Blocking procedures were made using a 2 % bovine albumin solution in phosphate buffer, pH 7.2 (1 h at room temperature). All wells were then washed by the same phosphate buffer containing 1 % Triton X-100. All samples (30 μl) were added to the solution for sera dilution (60 μl) in plate wells, incubated 1 h at 37 °C and washed again by the plate-washing buffer (4 times, 300 μl/well). Conjugate solution – peroxidase-labeled A-protein or MAbs to human immunoglobulins – were put into all wells (100 μl/well); following incubation (30 min, 37 °C) the wells were washed 6 times, and a substrate-chromogen containing solution was added to the wells. In 20-30 min the reaction was stopped by 2 M sulphuric acid solution, the sample optical density (OD) being determined using a EL_x800 reader (BioRad) in two-waved way (450/620 nm). The cutoff (CO) value was taken as OD value for negative control being + 0,1.

Test-systems used here were «New Lav Blot I» (BioRad, France) confirming the presence of anti-HIV Abs by immunoblotting and three immunoenzyme test-systems: „GENSCREEN PLUS HIV Ag-Ab" detecting simultaneously HIV-½ Abs and antigen, as well as "GENSCREEN HIV ½ version 2" (BioRad, France) and „DIA-HIV ½„ ("Diaproph-Med") detecting Abs both to HIV1 and HIV2. The sample testing was made according to user's instructions.

Resulte and discussion. The ability to interact with mimicrins was studied using IEA for sera of HIV-infected patients, FPS, and negative donor sera. The data in the Figure 1 present the data demonstrating Abs to *Staph.aureus* mimicrins in sera of donors and HIV-infected patients. It is evident no anti-mimicrin Abs were found in 37.8 % donors, 49,5 % donors contain low titers of these Abs (1:10-1:100), and in 12,3 % donors their levels are high (1:160-1:640). At the same time almost all HIV patients sera contain higher anti-mimicrin Abs titers (1:10-1:100 in 12.9 % persons; 1:160-1:640 in 13.9 % patients, and 1:1,000-1:10,000 in 73.2 % of them).

We were especially interested in sera repeatedly reacting with anti-HIV Abs. They were studied as FPS at the Laboratory of Viral Hepatitis and HIV-Infection of our Institute using three test-systems of different configuration. The Table 1 describes characteristics of these sera.

***Table 1*. Characterization of HIV false-positive sera**

Sample No	Sex, age, code	GENSCREEN, OD/CO		DIA-HIV ½, OD/CO	Immunoblot
		V2	Plus Ag/At		
1	F, 53, 108	1.5	0.8	2.4	neg
2	M, 55, 108	2.0	0.6	1.3	neg
3	M, 38, 108	1.3	0.6	1.5	p68
4	M, 54, 108	1.1	0.7	1.5	neg
5	F, 49, 104	23.2	0.8	4.2	p25
6	F, 26, 109	22.1	0.5	6.2	p25
7	F, 40,113	0.6	1.1	2.1	neg
8	F, 22,104	1.1	0.6	1.5	neg
9	F, 45,115	1.6	0.7	2.2	neg
10	F, 25, 108	0.8	8.6	1.8	p25
11	M, 29, 109	0.6	12.4	2.1	p25
12	F, 46, 108	0.6	4.1	1.6	neg
13	M, 48, 108	0.5	1.4	2.8	neg
14	M, 30, 108	0.8	4.2	1.9	neg
15	M, 46, 108	0.8	1.8	2.5	neg
16	M, 26, 108	0.6	1.6	1.2	neg
17	M, 34, 108	0.7	0.9	2.6	P31
18	F, 24, 109	1.9	0.6	1.5	neg
19	M, 36,120	10.2	0.6	30.0	neg
20	M, 37, 108	0.8	2.3	5.1	neg
21	M, 28, 108	0.6	5.6	4.9	neg
22	F, 28, 108	2.2	1.2	1.4	neg
23	M, 42, 108	8.2	0.7	1.9	neg
24	F, 51, 108	5.6	0.5	1.5	neg
25	M, 38 108	0.5	1.2	5.6	Gp120
26	M, 33, 108	5.2	0.8	1.4	neg
27	M, 31, 108	0.7	3.0	2.1	neg
28	M, 28, 108	0.5	3.9	6.2	neg
29	M, 22, 108	0.5	9.7	2.8	gp41
30	M, 30, 108	0.8	1.2	1.3	neg
31	M, 41, 108	0.4	1.5	1.9	neg
32	F, 42, 108	0.8	2.7	2.7	neg
33	M, 48, 108	0.6	1.0	1.8	neg
34	F,49, 120	0.9	5.2	1.6	neg
35	M, 50,108	0.8	1.2	1.3	neg
36	F,38, 108	10.9	1.5	3.1	neg
37	F, 25,109	1.0	2.8	1.5	p25
38	M,43,120	5.2	0.5	1.2	neg
39	M, 28,108	0.6	5.4	6.2	p31
40	M,45,108	2.2	0.5	2.1	neg

Note. Codes of persons investigated demonstrating FPA: 104 – patients with venereal diseases; 108 – donors; 109 – pregnant women; 113 – persons tested according their clinical status; 114 – confidence cabinet; 120 – preventive examination.

According to the WHO guidelines, the serum is thought to be positive following immunoblotting investigation if this serum possesses at least two bands formed with Env antigens (gp160, gp120, gp41) [7, 11]. 10 sera among 40 FPS (25 %) showed positive answer only to one of Env proteins.

Further we investigated these 10 FPS and their ability to interact with mimicrins. The Table 2 demonstrates the data obtained

***Table 2*. FPS interaction with mimicrins**

Sera No	Anti-HIV	Mimicrins (CO =0,151)					
		№ 1		№ 2		№ 3	
	OD/CO	OD	OD/CO	OD	OD/CO	OD	OD/CO
3	1,5	1,508	8,77	0,752	4,37	0,699	4,63
5	4,2	0,528	3,07	0,466	2,71	0,702	4,64
6	6,2	0,821	4,77	1,098	6,38	0,762	5,04
10	1,8	0,143	0,95	0,111	0,65	0,106	0,7
11	2,1	0,706	4,1	0,554	3,22	0,866	5,73
17	2,6	0,776	4,51	0,348	2,02	1,534	8,92
25	5,6	0,131	3,28	0,565	0,76	1,579	9,18
29	2,8	0,891	5,18	0,488	2,83	1,684	9,79
37	1,5	0,980	5,7	0,381	2,22	1,586	9,2
39	6,2	1,420	8,26	1,040	6,0	0,222	1,47
OD/CO_{mean}	3,45		4,86		3,42		5,93

Nine of 10 FPS interact with all mimicrins tested, the mean OD/CO values being 4.86, 3.42, and 5.93 for mimicrins No 1, No 2, and No 3, respectively. These data show a rather high degree of FPS Abs interaction with mimicrins. A donor serum No 10 does not react with any of three mimicrins, a serum No 25 demonstrating no interaction with the mimicrin No 2 isolated from *Staph.aureus* toxin.

We studied also interactions between mimicrins and some HIV-specific protein structures having used synthetic oligopeptide analogs of variable loop V3 region carrying gp120 sequences of HIV-1 serotypes A, B, and C (actual for Ukraine), recombinant gp160 antigen, Env, Gag and HIV-1 Env+Gag mixture. The data obtained are given in the Table 3. It is seen the FPS to react mostly with mimicrins; only four FPS samples of 10 ones react with oligopeptide B; two of them interact also with gp160 and Env forming bands with gp120 or gp41 in immunoblot test. Two other sera reacting with the Env protein form bands with p68 or p31 antigens. Five FPS react with a recombinant Gag protein having a band with p25 protein in immunoblot. A serum No 17 reacting with recombinant proteins Env and Gag gaves a band with p31antigen. The Figure 2 demonstrates a diagram comparing OD/CO ratios for FPS interacting with mimimcrins, oligopeptides, and recombinant HIV proteins.

***Table 3*. Comparative FPS interactions with mimicrins, oligopeptides, and recombinant protein HIV-1 analogs (OD/CO)**

Sample No	Mimicrins No			HIV-1 specific oligopeptides of serotypes			Recombinant HIV-specific proteins			
	1	2	3	A	B	C	gp160	Env	Gag	Env+Gag
3	8,8	4,4	4,6	0,5	0,8	0,7	0,8	2,1	0,6	1,5
5	3.1	2,7	4,6	0,3	0,6	0,9	1,2	0,9	3,4	4,2
6	4,80	6,4	5,0	0,9	0,8	0,5	0,5	0,6	2,8	6,2
10	0,95	0,65	0,7	0,7	0,5	0,2	1,4	0,9	2,5	1,8
11	4,1	3,2	5,7	0,4	0,4	0,6	0,8	0,9	2,6	2,1
17	4,5	2,0	8,9	0,5	1,1	0,4	0,6	2,9	1,6	2,6
25	3,3	0,76	9,2	0,8	3,9	0,7	5,2	8,7	0,3	5,6
29	5,2	2,8	9,8	0,9	2,2	0,6	6,1	1,5	0,6	2,8
37	5,7	2,2	9,2	0,5	0,6	0,9	0,9	0,6	4,2	1,5
39	8,3	6,0	1,47	0,6	1,2	0,8	1,9	1,6	0,6	6,2
OD/CO_{mean}	4,86	3,42	5,93	0,6	1,2	0,6	1,9	2,1	1,9	3,45

Our further aim was to use mimicrins for FPA elimination. However, the mimicrin added to FPS had not usually any effect on OD values in EIA and even sometimes led to slight increase of these values. At the same time sera of mimicrin-immunized rabbits permit to decrease the values of FPA signals. The Figure 2 shows the changes of FPS interactions with Env and Gag proteins following a rabbit serum containing Abs to mimimcrin No 3 isolated from *C.albicans* addition to these samples. It is evident the serum No 25 that had previously interacted actively with the HIV ENV protein decreased its OD value by 64 % (from 1.4 up to 0.5 O.U.). The OD value for the serum No 5 reacting with the Gag became lower by 42 %. It seems possible the isolation and purification of rabbit mimicrin-specific immunoglobulins may lead to complete neutralization of cross Abs reactivity with FPS proteins.

It is evident that it is necessary to take into consideration contributions of numerous infectious and other factors to reach true results in anti-HIV Abs detection, to avoid FPA and to perfect continuously diagnostic test-systems.

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N.S. Pukish, post-graduate

STUDYING THE RELATION BETWEEN THE START POINT OF HIV-INFECTED MOTHER'S ARV-PROPHYLACTICS AND FREQUENCY OF VERTICAL TRANSMISSION OF HIV

Taras Shevchenko` Kyiv National University, Ukraine

Correlation between the start point of HIV-infected mother's ARV-prophylactics and frequency of vertical transmission of HIV was analyzed and the most critical periods of HIV-1 mother-to-child transmission were determined.

Introduction. Despite decreasing risk of HIV-1 vertical transmission to 2% in highly-developed countries, for developing countries this is a problem of current importance [8]. Totally the number of HIV-infected children continues to grow in the world. At the end of 2006 there were 2,3 million of HIV-infected children. Only in 2006 approximately 530 000 children aged less than 14 were infected with HIV, and 90% from them have got prenatal HIV infection during HIV infected mother's pregnancy. Approximately 600 thousand of children are infected annually [3]. First case of HIV infection among children was recorded in 1988 in Ukraine and till 2006 (by 01.10.06) 13 042 children were registered with HIV infection. Near 90% of children's HIV infection take place during vertical transmission of HIV from mother to her child and half of these children and the greater part from the rest children die before there first and fifth birthday respectively [2].

Mother-to-child HIV transmission can take place during pre-natal development of fetus, during delivery and through breastfeeding [6]. To date questions of the mechanisms and exact time of HIV transmission from mother to her child are being vigorously discussed and studied [7, 4, and 6] but there is no common and reliable answer at the meantime. Determination of the most critical periods of prenatal infection of HIV is very important for choosing correct, effective and timely preventive measures for mother-to-child HIV transmission that in one's turn would cause to decreasing of HIV infection among children from their HIV positive mothers and correspondingly to decreasing of children's deaths from HIV infection/AIDS.

So, the aim of our research was to reveal the correlation between the start point of pregnant mother's prophylactic antiretroviral (ARV) therapy and the level of HIV vertical transmission and to try to estimate preliminary periods of vertical transmission of HIV against the start point of prophylactic ARV therapy for mother and her child.

Objects and methods. We have tested samples of blood for 2 447 infants aged less than 18 months that were exposed to HIV infection (were born to HIV infected mothers) from different regions of Ukraine.

Method of polymerase chain reaction (PCR) that is considered to be the most sensitive method for HIV infection determination was used. Using PCR the presence of HIV genes (proviral DNA) in children's mononuclear cells can be detected. Children's fresh whole blood (2ml) collected with anticoagulant was used for proviral DNA HIV detection.

For determination of the presence of proviral DNA HIV-1 "Biofarma-AmpiSense DNA-HIV-96M" test systems (Amplisense, Russia) with two pairs of noncompetitive primers for *pol* gene of HIV-1 and for cell gene of globin as a control were used. Analysis was performed according to the procedure of producing company.

Percentages of HIV vertical transmission from HIV infected mother to her child and the validity for their differences were evaluated using the Student's t-test; the Spearman's rank test for estimation of correlation between the start point of HIV-infected mother's ARV-prophylactics and frequency of vertical transmission of HIV was used.

Results and discussion.

To analyze our results we have divided children's samples of blood into several groups:

1 group - children's samples of blood whose mother's and themselves did not get any ARV prophylactics; 2 group - children's samples of blood whose mother's got ARV prophylactics with retrovir starting from 32-34 weeks of gestation and continuing through to delivery and newborns got retrovir for 28 days; 3 group - children's samples of blood whose mother's got ARV prophylactics with retrovir starting from 28 weeks of gestation and continuing through to delivery and newborns got retrovir for 28 days; 4 group - children's samples of blood whose mother's got ARV prophylactics with retrovir starting from 28 weeks of gestation and continuing through to delivery and single dose of nevirapine during delivery and newborns got retrovir for 28 days and single dose of nevirapine after birth.

The levels of transmission for each group are shown in table 1.

Table 1. **The level of HIV vertical transmission depending on the start point of ARV-prophylactics for HIV infected women**

Groups of investigation	Absolute number of children (n)	The level of HIV vertical transmission (P±tm)
Group 1	259	30,11% ±2×2,85%
Group 2	106	13,21% ±2×3,29%
Group 3	1455	7,42% ±2×0,69%
Group 4	54	1,85% ±2×1,83%

We have estimated dependence between the level of HIV vertical transmission and the start point of ARV-prophylactics for HIV infected mother. Having calculated the Spearman's rank coefficient it can be asserted that we have found strong direct correlation between the level of HIV vertical transmission and the start point of ARV-prophylactics for HIV infected mother.

Using the results that we have got we tried to estimate preliminary periods of vertical transmission of HIV. Because of the single circulation of the blood for mother and her fetus prenatal transmission can be possible for some viruses. Despite valuable single circulation of the blood is fully developed at the 28-th week of gestation the possibility of vertical transmission appears starting from the beginning of formation of the single circulation of the blood. HIV belongs to the group of viruses that can cross placental barrier and respectively can be transmitted from mother to her child [2]. The exact periods of prenatal HIV transmission is not determined to date. But a few groups of scientists have shown that at the 14-th week of gestation of HIV infected women HIV infection in their fetuses was not detected [4, 7]. These investigations allow deducing that till 14-th week of gestation HIV transmission from mother to child can not occur. Moreover Program of formula feeding for all HIV infected mothers is fully available in Ukraine. That is why we considered that post natal transmission of HIV through breastfeeding could not take place or could occur at very low level. So, for estimation of preliminary

periods of vertical transmission of HIV it was surmised that vertical transmission can occur starting from the period of 14 week of gestation and through delivery inclusively.

For 3 group it was established that the possibility for HIV transmission decreased by about 76% comparing to 1 group – from 30,11% to 7,42% (the difference was statistically valid, t >2). These results suggested that about three-fourths of infant HIV transmission that are prevented by this regimen must occur after the regimen is started – at the period between 28 week of gestation and delivery inclusively (fig.1).

The transmission rate can be reduced to as low as 1,85% while single dose of nevirapine was added to retrovir for mother and child (group 4) and decreased by about 96% compare to 1 group (the difference was statistically valid, t >2). These results confirm that about 20% of infant HIV transmission that are not prevented by the regimen that included just retrovir from 28 week (1 group), must occur during delivery (nevirapine influences the level of viral load of HIV in mother's blood exactly during delivery) (fig.1).

For group 2 while taking retrovir from 32-36 week gestation the rate of vertical transmission decreased by 57% compare to 1 group– from 30,11% to 13,21%. These results suggest that the greater part (57%) of the whole cases of transmission that are prevented by this regimen must occur at the late periods of fetus's development after 32-34 week of mother's pregnancy.

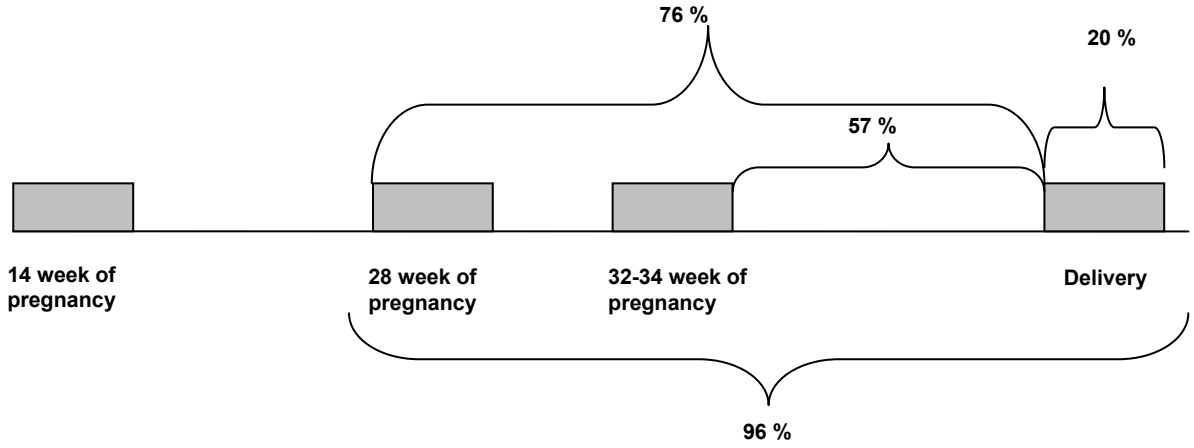

Fig.1. Estimation of preliminary periods of vertical transmission of HIV

Conclusions. First of all we have estimated strong direct correlation between the level of HIV vertical transmission and the start point of ARV-prophylactics for HIV infected mother.

It was shown that the most critical periods of vertical transmission of HIV from mother to her child are those during last weeks of gestations, starting from 32-34 weeks and through delivery. About 57% and 20% of infant HIV transmission must occur during these periods respectively.

Understanding of some peculiarities of HIV vertical transmission, using these knowledge during of elaboration of new effective medication for prevention of HIV transmission, correct motivation of HIV infected women for adherence to ARV therapy especially during crucial periods of possible prenatal transmission of virus to a child can help to influence the rate of HIV transmission from HIV infected mother to her child, decreasing the level of children's infection and as a result the mortality of children.

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O. Borodavka[1] stud, O. Deryabin[2], Head of the lab. of Molecular Biology, I. Sobko[3], Director, V. Polischuk[1], D. Sci.

DIFFERENTIATION OF INFECTIOUS BURSAL DISEASE VIRUSES USING RT-PCR AND RESTRICTION ANALYSIS OF OBTAINED AMPLICONS.

[1] Taras Shevchenko` Kyiv National University, Ukraine; [2] Institute of Veterinary Medicine, UAAS, Ukraine;
[3] Center of Modern Diagnostics, 'Bio-Test-Laboratory', Ukraine.

A method for detection of Infectious Bursal Disease Virus (IBDV) based upon the reverse transcription polymerase chain reaction, or RT-PCR has been developed. The obtained amplified DNA fragments from IBDV vaccine strains and field isolates have been analyzed by digestion with EcoRII, MboI, SspI, BspMI, SacI restriction endonucleases. The restriction analysis of obtained amplicons is able to differentiate IBDV vaccine strains and very virulent field isolates

Introduction. Infectious bursal disease (IBD) is an acute, highly contagious viral infection of young chickens of the age of 2-7 weeks which affects primarily lymphoid tissue with a special predilection for the dividing B-lymphocytes of the bursa of Fabricius. It was first recognized as a specific disease by Cosgrove in 1962 and was referred to as "avian nephrosis", because of an extreme kidney damage found in birds that succumbed to the infection. Since the first outbreak occurred in the area of Gumboro, Delaware, USA, it was also called "Gumboro disease" and it is still frequently used [8]. The economic importance of this

disease is determined by different factors. First, some virus strains may cause up to 20 per cent mortality in chickens of three weeks age and older. The second and more important manifestation is a severe prolonged immunosuppression of chickens infected at an early age. IBD is one of two of the most important diseases in poultry worldwide [9]. The disease has not only an importance in causing illness, death and reduced growth in the chickens, but also in inducing a severe immunosuppression that renders the animals susceptible for other pathogens and unable to produce protective antibodies after vaccination [4]. The total cost of IBDV infections is difficult to calculate since it depends on multiple factors such as the breed and the age of the chicken, the strain of virus, previous protection from vaccination, natural infection or maternal antibodies, the level of immunosuppression caused and secondary infections. The costs not only include the losses of dead and diseased animals, but also the costs of extra labour, veterinarians, medications and disinfection strategies. There is no doubt, however, that the disease causes serious economical losses. It is estimated that the money saved after having introduced vaccination in 1988-89 in the USA was 400 million US dollars in 1990 and 580 million in 1998 [4].

IBDV is a small non enveloped virus belonging to the genus *Avibirnavirus* of the family Birnaviridae, which is characterized by a bisegmented dsRNA genome [8]. The virion has a single capsid shell of icosahedral symmetry composed of 32 capsomeres and a diameter of 60 to 70 nm. The viral genome consists of two segments. The smaller segment B encodes VP1, a 97-kDa multifunctional protein with polymerase and capping enzyme activities. The larger

segment A encodes a 110-kDa precursor protein in a single large open reading frame (ORF), which is processed into mature VP2, VP3, and VP4 proteins [10].

There are two distinct serotypes of IBDV, designated as serotypes I and II. Viruses of serotype I are pathogenic to chickens, whereas serotype II viruses, mostly isolated from turkeys, do not replicate in the chicken bursal cells and are avirulent for chickens.

The terms 'variant', 'classical' and 'very virulent' have been used to qualify IBDV strains that exhibit a different pathogenicity. North American 'variant' IBDVs induce little if any clinical signs and no mortality but marked bursal lesions, 'classical' IBDVs induce approximately 10–50% mortality with typical signs and lesions whereas 'very virulent' IBDVs induce approximately 50–100% mortality with typical signs and lesions [5]. There has not been reported about the appearance of variant strains of IBDV in Ukraine previously. Early detection of IBDV in flocks combined with the characterization of the isolated viruses can be very useful for proper vaccination programs against IBD [8].

VP2 protein contains important neutralizing antigenic sites and elicits protective immune response, and most of the amino acid changes between antigenically different IBDVs are clustered in the hypervariable region of VP2. Thus, this hypervariable region of the VP2 is the obvious target for molecular techniques applied for IBDV detection and strain variation studies [2, 6].

Several approaches are used for the characterization of IBDV strains and isolates. Among them are the bioassays with specific pathogen free (SPF) susceptible chickens and recently developed molecular biological tools such as sequencing or the restriction analysis of the hypervariable region of the VP2 gene after amplification using RT-PCR [8, 2, 6].

The aim of the study was to develop a RT-PCR for the fast and sensitive detection of IBDV in infected chickens, combined with restriction enzyme analysis for the rapid differentiation between the two major groups of field strains, namely classical virulent and very virulent and different vaccine strains used in Ukraine poultry production.

Materials and Methods. The IBDV strains used in this study were: D78, 228E (Intervet, Netherlands), HipraGumboro GM97 (Hipra, Spain), v877 (FortDodge, Australia), MB (Tabic, Israel). Bursal tissues from sick chickens from affected by IBDV flocks have been used for the histopathology examination and the extraction of RNA. Bursas of Fabricius have been aseptically taken from chickens, cut into two halves; one of the each half of bursa has been fixed in neutral buffered formalin for the histological examination, the other one was deeply frozen at -70^{o}C to be submitted for the total RNA extraction.

The RNA from the vaccine strains have been extracted using the commercially available kits Rneasy Mini Kit (Qiagen, Hilden, Germany) as recommended by the supplier. Briefly, 10mg of each vaccine have been diluted in 50µl of distilled autoclaved water, mixed with 350µl of lysis RLT buffer, vortexed thoroughly, mixed with 350µl of 70% ethanol after what 700µl of the mix was added to the spin column. The elution of RNA from a column was performed with 30µl of DEPC-treated deionized autoclaved water. The RNA from bursal tissues was isolated by an acid guanidine thiocyanate-phenol-chloroform extraction method after Chomczynski using the kit for an RNA extraction "Amplisens", Russia. [3]. Briefly, 500mg of bursal tissues have been homogenized in a 1,5ml microtube with 500µl of sterile isotonic solution, vortexed, centrifuged during 1min at 3000 rpm, after what 100µl of the obtained supernatant have been mixed with 700µl of acid guanidine thiocyanate-phenol. The RNA was extracted according to the manufacturer's instruction. The obtained RNA has been used for the reverse transcription using the "Reverta" ("Amplisens", Russia) Reverse Transcription Kit. Briefly, 10µl of the RNA solution has been heat denaturated for 5 min at 95^{o}C and snap freezed at ice after what it has been immediately added to 10µl of RT-mix with MMLV Reverse Transcriptase. The mix has been incubated at 37^{o}C for 45 min, then for 5 min at 85^{o}C for the inactivation of the reverse transcriptase. The obtained cDNA has been used for the PCR. The oligonucleotide primers used in this work have been used as previously described by Liu et al [7] designated Bur1F (5'-TCACCGTCCTCAGCTTAC-3' nucleotide position 587-604 and Bur1R (5'-TCAGGATTTGGGATCAGC- 3' nucleotide position 1212-1229).designed to amplify the hypervariable region of VP2 gene according to Bayliss [1]. The obtained amplification product has 643 bp. To decrease the specificity and the sensitivity of the reaction a second set of primers Bur2F and Bur2R has been designed which allows the amplification of the inner region of the first amplicon obtained after the first round of the amplification using Bur1F and Bur1R primers. Two conserved regions were identified for primer annealing and the amplification of the hypervariable region of VP2 by the alignment of published IBDV serotype 1 VP2 nucleotide sequences. The resulting product has the length of 550 bp. The PCR reaction was carried out in a 25µl volume in a MasterCycler thermocycler ('Eppendorf', Germany) using the obtained cDNA as follows: 25 pM of Bur1F and Bur1R primers was mixed with sterile deionized water and dNTP mix to a final concentration of 35µM. The mix was overlaid with a melted wax which was allowed to solidify after what the 17 µl of the supermix containing 5µl of 5xPCR-buffer; 10.5µl of DNAse-free sterile deionized water, 2mM of $MgSO_4$ and 2,5U of Taq-Polymerase ("Amplisens", Russia) was added to the master mix. 3µl of the cDNA was added to the PCR mix. The first amplification was carried out using the following thermal profile: initial denaturation: 95^{o}C - 2 min, the

next 35 cycles consisted of 30s at 94°C for denaturation, 30s at 52°C for primer annealing, 30s at 72°C for elongation. 1µl of the primary amplification products has been diluted in 20µl of DNAse-free sterile deionized water and 3µl of the diluted amplicons was used for the second amplification. The second PCR mix was done as the first one except using Bur2F and Bur2R primers instead of Bur1F and Bur1R and the primer annealing temperature of 62°C. The PCR products were electrophoretically separated on the 1,5% agarose gel stained with the ethidium bromide and visualized by an ultraviolet transillumination. For the differentiation of IBDV strains and isolates the obtained PCR products have been analyzed by digestion with restriction endonucleases EcoRII, MboI, SspI, BspMI, SacI ("Fermentas", Lithuania) as recommended by a supplier. Briefly, 10µl of the obtained amplicons were mixed with the restriction buffer, 5U of the restriction enzyme and DNAse-free water in a final volume of 35-40µl and incubated at 37°C for 6-12 hours. The obtained products were analyzed on 3% agarose gel stained with ethidium bromide and visualized by an ultraviolet transillumination.

Results and Discussion. A number of RT-PCR protocols have been published for the diagnosis of IBDV infections. Restriction fragment length polymorphism analysis has been used to form six different groups of IBDV strains, so called 'molecular groups' [6]. It was previously shown that very virulent strains of IBDV have some typical amino acid substitutions in the VP2. As VP2 has major antigenic determinants, it elicits protective neutralizing antibodies and it was shown that most amino acid changes between different IBDVs are clustered within the hypervariable region of VP2. This hypervariable region was selected as the target for the differentiation of IBDV strains and isolates. In this study we have used primers binding to the conserved region flanking the hypervariable region of VP2 gene. We have optimized the reaction conditions in order to increase the specificity and the sensitivity of the reaction. The resulting PCR products have been analyzed by the restriction analysis with the restriction endonucleases EcoRII, MboI, SspI, BspMI, SacI which allows differentiating IBDV vaccine strains and field isolates. The alignment of the published serotype 1 VP2 gene nucleotide sequences has allowed to identify the cleavage sites within the obtained amplicons in different IBDV strains. It was previously shown that almost all very virulent IBDV strains have the amino acid substitution at position 222 (alanine or glutamate instead of proline) [5]. This amino acid substitution corresponds to the nucleotide mutation of the GCU codon in classical virulent strains to UCU in VP2 gene and results in a cleavage site for the BspMI. This enzyme cleaves the obtained amplicon from the very virulent IBDV in two fragments with the lengths of 100 bp and 450 bp. The alignment of the sequences of the used in the study vaccine strains and very virulent isolates has defined in all the classical virulent strains a silent mutation resulting in the cleavage site for the SacI. Cleavage of the obtained amplicons after amplification of cDNA from classical virulent strains has yielded two DNA bands of 184 bp and 366 bp (Fig.1). Neither of the studied very virulent strains had a restriction site for the SacI. The alignment of the sequences has identified the presence of the conservative valine residue in VP2 at position 256 in all studied classical virulent strains but no known restriction endonuclease can recognize specifically this site, so this can be used for the differentiation using either amplicon sequencing or other available method of single nucleotide polymorphism detection. Sequence analysis of the vaccine IBDV strain MB used in this study revealed the absence of the SacI cleavage site and the presence of BspMI cleavage site in its VP2 gene.

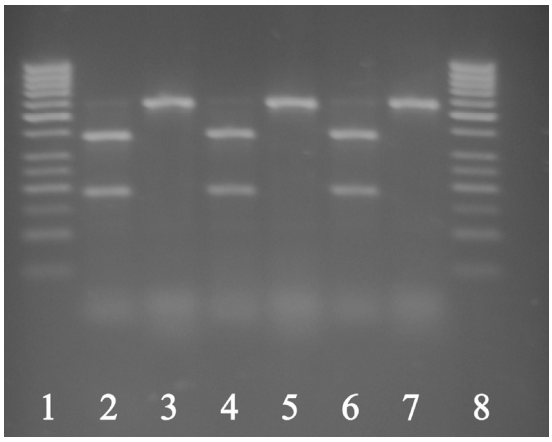

Fig.1. Agarose gel electrophoresis of DNA fragments after digestion with restriction enzymes BspMI and SacI of amplicons. Lanes 2, 4, 6 – DNA amplicons from classical virulent vaccine strains digested with SacI; 3, 5, 7 – the same amplicons digested with BspMI. 1, 8 – DNA size markers, 50 bp, ('Fermentas').-

This strain is referred as an attenuated very virulent strain and needs more molecular markers for the differentiation from other very virulent strains. The alignment of the sequences of MB strain with the sequences of very virulent strains have revealed a cleavage site for BstEII in MB absent in all other very virulent strains. The resulting DNA fragments after the cleavage of the MB amplicons with BstEII are 250 bp and 300 bp. No fragments were observed after incubation of the very virulent IBDV amplicons with BstEII (Fig.2). We have also used an SspI cleavage site for the differentiation of the very virulent and classical virulent strains. The SspI cleavage site corresponds to the nucleotide mutation in almost all previously described very virulent IBDVs which encodes isoleucine at position 299. The resulting bands after the restriction with SspI of the very virulent IBDV amplicons are 224 bp and 326 bp (Fig.2).

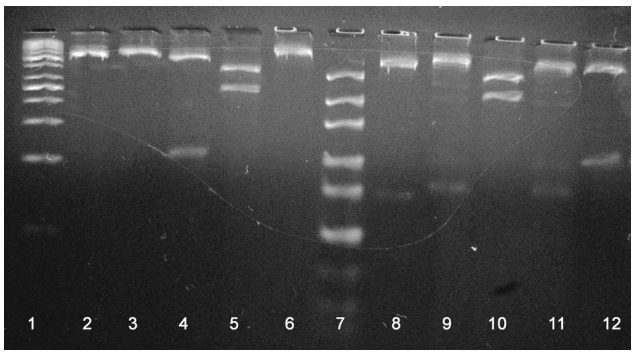

Fig.2. Agarose gel electrophoresis of DNA fragments after digestion with restriction enzymes BspMI, SacI, BstEII, SspI of obtained amplicons. Lanes: 2 – DNA amplicon, very virulent IBDV; 3- after digestion with SacI; 4 – after digestion with BspMI; 5 – after digestion with SspI; 6 – after digestion with BstEII; 8 - after digestion with MboI; 9 - after digestion with BstNI; 10 - MB strain, after digestion with SspI; 11 – MB strain, after digestion with BstEII; 12 – MB strain, after digestion with BspMI; 1, 7 – DNA size markers, 50 bp and UltraLowRange ('Fermentas').-

To determine the molecular group to which the isolated virus belongs according to Jackwood we have also used two restriction endonucleases BstNI and MboI. The restriction patterns of the vaccine strains with these enzymes corresponded to their assumed molecular groups. The restriction with BstNI of 228E and GM97 amplicons resulted in two bands approximately 475 and 75 bp; D78

207 bp; 154 bp, 112 bp, 77 bp, respectively; Restriction with MboI of 228E resulted in four DNA bands: 413 bp; 62 bp, 50 bp, 27 bp; D78: 413 bp, 62 bp, 50 bp, 27 bp; GM97: 413 bp, 71 bp, 41 bp, 27 bp. (Fig. 3).

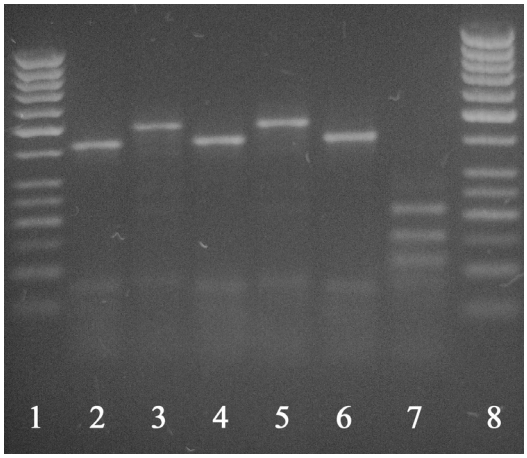

Fig.3. Agarose gel electrophoresis of DNA fragments after digestion with restriction enzymes BstNI, MboI of obtained amplicons from classical virulent vaccine IBDV strains. Lanes: 2 – DNA amplicon from GM97, after digestion with MboI; 4 – after digestion with BstNI; 5 – DNA amplicon from 228E, after digestion with MboI; 6 – after digestion with BstNI; 6 - DNA amplicon from D78, after digestion with MboI; 7 - after digestion with BstNI; 1, 8 – DNA size markers, 50 bp ('Fermentas').-

Restriction analysis of the obtained amplicons after the RT-PCR of the RNA from affected bursal tissues provides an analysis of a consensus sequence representative of the average of the global virus population, rather than the sequence of a possibly cloned quasi-species minority.

Taking into the consideration the coexistence of classical virulent and very virulent IBDV strains in the bursa of Fabricius, a restriction enzyme identification of classical virulent IBDV strains is also necessary. In the case of ambiguous results using BspMI or SspI for the identification of the very virulent strains BstNI, MboI, SacI, BstEII can also be used for further molecular characterization of IBDV isolates to avoid the misinterpretation of the results in case of any silent mutation which may result in a lack of the cleavage site of the restriction enzymes.

In conclusion, RT-PCR combined with restriction analysis as described in this communication represents a rapid and reliable tool for the differentiation of classical virulent and very virulent IBDV strains and isolates. It has been validated by the investigation of a total of 5 vaccine IBDV strains and 4 field isolates. Available nucleotide sequence data of many other IBDV strains supports the possibility of the use of this tool for the routine diagnostics and differentiation of IBDVs. The advantage of this protocol is its high sensitivity, specificity, variety of the restriction patterns of the obtained amplicons which gives the possibility to differentiate IBDV strains and to determine some vaccine strains in bursal tissues currently used in Ukraine for the vaccination of flocks. In the case of unexpected results or for the further identification and differentiation of the strain a sequencing of the PCR products can be performed. The obtained in such way information may be very useful in development of vaccination programs against IBDV and in epidemiological studies of this disease.

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A.P. Starcheus, D. Sci., V.I. Polulyah, PhD, V.A. Synycin, PhD, T.O. Sokirko, PhD, A.Y. Synycin, PhD, V.A. Evtushenko, jun. scient. employee.

HORSE EQUINE INFLUENZA IN UKRAINE - RESULTS OF SERUM MONITORING

The Institute of Veterinary Medicine of UAAS, Kyiv, Ukraine

The article gives the results of carrying out epizootological survey for equine influenza in eight regions of Ukraine in 2000-2005, as well as the data on equine influenza virus circulation among other species of farm animals

In recent years the increasing tendency of falling ill horses on virosiss diseases is traced. In particular, this is horse equine influenza - may cause to 40% lethal cases [1,3,4]. With regard that the horse equine influenza viruses have species specificity, possibility of defeat by viruses of animals of other species is not eliminated. The question of horse equine influenza viruses circulation in nature until now remains studied just a little .

Purpose of our work: analysis of serum screening results of the modern state of horse livestock in Ukraine relating to flu horses and studying the horse equine influenza virus circulation among other types of agricultural animals.

Materials and methods. We were using industrial strains of the horse equine influenza virus –

A(horse)1(Cambridge)63(H7N7) and

A(horse)2(Miami)63(H3N8).

antigens of horse equine influenza virus also were used: A(Equine)(Newmarket) 1/93, (Equine)(Praqne) 56 and (Egnine) Kentuky(1) 81. The horse equine influenza viruses titres and wheys determined by RGA and in RGGA in a method, which was modified in the virology laboratory of Institute of Veterinary Medicine [2]. Epizootological investigations on the horse equine influenza were conducted in 8 Ukraine regions. In researches were used 1149 blood horses wheys from different regions of Ukraine (20 farms), 106 cattle blood wheys (8 farms), 134 pigs blood wheys (12 farms).

Results and discussion. During 2001-2005 years serum screening of the horses livestock`s state in Ukraine comparing to horse equine influenza. Researching results in RGGA 1149 horse blood serum from 20 farms from different regions of Ukraine are listed in the table.

The wheys of blood were taken from horses, which were not vaccinated equine influenza flu, through one month after vaccination, 4 months after vaccination and 12 months after vaccination by a vaccine against horse equine influenza flu. It is discovered that in 1 month after the inoculation of horses by deactivated polyvalentive vaccine against horse equine influenza in the wheys of blood the titles of antibodies were high enough: to the horse equine influenza virus strain A(horse) 1 (Cambridge) 63 - 1:2560 -1:40960; to the equine influenza virus of strain A(horse) 2 (Miami) 63 - 1:10240 - 1:40960. Such titles of antibodies prevent possibility of disease at the contact with the virus of equine influenza.

600 tests of horses blood wheys, which belong to Kyiv State race course in 12 months after inoculation by inactivated polyvalent vaccine against horse equine influenza were investigated. Antibodies to the horse equine influenza virus were not found in blood wheys. The researches results confirm our previous information immune tension at the inoculation by vaccine against horse flu sticks up to 10 months.

In 4 months time after vaccination of horses which belong to «Koneprom» (Kiev region) with polyvalent vaccine against horse equine influenza in horses`s blood wheys antibodies not only to the viruses of culture A(horse) 2 (Miami) 63 (1:640 - 1:2560) but also to the viruses – cultures

A(Equine)(Newmarket)1/93, (1:2560 - 1:5120),

A(Equine)(Praque) 56, (1:80 - 1:320),

A(Equine)(Kentuky)1/81, (1:40 - 1:320),

which were not registered in Ukraine until this time.

High titres mean high immunogenic qualities of horse equine influenza virus culture, which are in vaccine complement: A(horse) 2(Miami) 63, A(Equine)(Newmarket)1/93, A(Equine)(Praque) 56 and isolate M 80.

In pigs blood wheys antibodies to the horse equine influenza virus were not discovered. In the cattle blood wheys antibodies to the horse equine influenza virus strain A.(horse) 2(Miami) 63 with high title (1:80 - 1:280), that confirms our information about flu virus circulation not only among horses, but also among other types of agricultural animals, in particular, cattle.

***Table*. Serological horse livestock screening for horse equine influenza in Ukraine (2001-2005)**

№	Name of organisation	Samples`s selection date	Samples, total	Horse equine influenza virus cultures			
				A(horse) 1(Cambridge)63		A(horse) 2(Miami)63	
				Positive serums	Titres in RGGA	Positive serums	Titr in RGGA
1	2	3	4	5	6	7	8
1.	Kyiv state race course Kyiv state race course Kyiv state race course Kyiv state race course Kyiv state race course	18.01.01. 21.05.02. 25.03.03. 13.02.04. 14.02.05.	20 9 300 151 300	0 0 0 0 0	0 0 0 0 0	0 9 0 0 0	0 1:640-1:2560 0 0 0
2.	Horse-sport facility Lugansk	14.02.01.	15	8	1:20-1:40	7	1:20 - 1:80
3.	CJE “Avangard” Kyiv	13.03.01	55	0	0	0	0
4.	State circus company Kyiv	14.03.01.	6	0	0	0	0
5.	CC “Dynamo” Kyiv	26.03.01.	27	0	0	0	0
6.	Horse plant “Magnat” Kyiv region	25.04.01	53	0	0	0	0
7.	CSC “Visla” Brovarsky district, Kyiv region	31.05.01.	21	0	0	0	0
8.	SCTC “Koneprom” SCTC “Koneprom” SCTC “Koneprom” SCTC “Koneprom”	15.10.02. 6.11.02. 18.11.02 2.04.03.	6 11 6 10	0 2 0 10	0 1:20 0 1:2560-1:40960	0 9 5 10	1:160- 1:1280 1:20 - 1:5120 1:10240 1:40960
9.	City veterinary laboratory	29.10.02.	2	0	0	0	0
10.	PJE Novogrygorivske Genicheskiy district, Herson region	17.12.02	1	0	0	1	1:80
11.	Institute of epizootology, Rivne	6.11.03.	12	0	0	0	0
12.	CJC “Locomotiv” Donetsk	20.03.04.	56	0	0	5	1:20 -1:80
13.	“Kleynoda” Ltd., Makiyvka, Lugansk region	25.03.04.	23	0	0	0	0
14.	Physical culture and sport development department Slavutych, Kyiv region	25.03.04.	11	0	0	0	0
15.	JCL Agrofirma in name f Shevchenko Kopachivka, Hmelnitsky region	15.10.04.	6	0	0	0	0
16.	JCL “Hliborob” Teperivka, Derajnynskiy district, Hmelnitsky region	15.10.04.	14	0	0	0	0
17.	“Agro-Meta” Ltd., Novostrilcivka, Milovskiy district, Lugansk region	28.03.05.	29	2	1:40-1:80	24	1:20-1:2560
18.	“Avtoritet” Ltd., Harcizskiy district, Donetsk region	7.06.05.	2	0	0	0	0
19.	“Rosiya” Ltd., Zlatoustivka, Volnovahsky district, Donetsk region	4.07.05.	1	0	0	1	1:80
20.	Ovrutska veterinary laboratory Zhytomirsky region	7.06.05.	2	0	0	0	0
Total			1149	22		71	

Conclusion. Thus, as a result of the annual (2001 - 2005) serum monitoring relating to the horse horse equine influenza, it was set that antibodies to the equine influenza virus were registered in the horse blood wheys of both vaccinated and not vaccinated for this disease. Circulation among horses from different farms in Ukraine of not registered before strains of the horse equine influenza virus - A(Equine)(Newmarket) 1/93 and A(Equine)(Kentuky) 1/81.It is confirmed that the horse flu virus A.(horse) 2(Miami) 63 circulates among other types of agricultural animals, in particular, cattle. Data must become subsoil for development of the system of effective epizootological measures against horse equine influenza, which are especially actual for sporting (race) horse.

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S. Dolgorukova, PhD stud., M. Vedernikova, stud., F. Demyanenko, PhD, V. Polischuk, D. Sci, prof.

STUDYING OF PLANT VIRUSES OF ANTARCTIC REGION

Taras Shevchenko` Kyiv National University, Ukraine.

Samples of the plants Deschampcia antarctica and Colobanthus quitensis that were collected in 2007 at Island Yalour, Pitterman, Galindez, cape Rassmunsen and Island Barkhan, Uruguay were analysed. Presence of viral antigens that belong to different taxonomic groups - Cucumovirus, Tobamovirus, Tospovirus in Deschampcia antarctica and antigens of CGMMV in Colobanthus quitensis using ELISA, bioassay and immunosorbent electron microscopy was estimated.

Introduction. Antarctica is one of the severest parts of the Earth that is characterized with low air temperatures, strong lingering winds, frequent snowstorms and fogs. Organic world of Antarctica's waters is not very rich. Flora of high seas consists of lots of organisms of phytoplankton, most of which are diatoms. Fauna and flora are distributed at littoral. The most numerous representatives among fauna are birds.

Antarctica is a unique object for studying. Recently different investigations were conducted here to study animal virus diseases, circulation of infectious diseases among members at Antarctic stations, features of microorganisms at Antarctic lakes and ice. Though prevalence of virus diseases is not studied enough [1, 5, 10].

There are only two species of higher vascular plants - Antarctic pearlwort *Colobanthus quitensis* and *Deshamcia Antarctica*. Both plants have the same ecological and spreading characteristics and as a rule are associated with environments, in which mosses and lichens are dominate [6, 7].

Antarctic viruses have limited host range. Antarctic Region has good conditions for high stability of organisms and modeling systems virus-host. Frozen in Antarctic ice or under icy lakes seeds could be perfect reservoirs for plant pathogens [3]. If virus could be identified at different times int ice, virus variety can be somehow interconnected with geological and climatic alterations, and it will be possible to estimate changing of genome and calculate some evolution events [1, 9].

Some ecological features of growing of Antarctic plants, features of climate that include extreme living conditions, variety of carrier of plant diseases - all this makes studying of phytoviruses in Antarctic Region very interesting [8,2].

Objects and methods. We have studied samples of two higher vascular plants *Colobanthus quitensis* and *Deshamcia Antarctica* from the Ukrainian Antarctic Station 'Academician Vernadskiy' and nearest archipelago islands – Island Yalour, Pitterman, Galindez, cape Rassmunsen, Skua, Yalour, Barselot, Uruguay that were collected by the 12-th Ukrainian Antarctic research expedition in 2007. Coordinates of the sites of collection are shown in the Table1.

The investigation for detection of plant viruses were performed for viruses that have wide range of plant-hosts *(Cucumber mosaic virus (CMV), Tomato spotted wilt virus (TSWV), Alfalfa mosaic virus (AMV), Wheat streak mosaic virus (WSMV), Barley yellow dwarf virus (BaYDV), Zucchini yellow mosaic virus (ZYMV), Tomato aspermy virus (TAV), Arabis mosaic virus (ArMV), Pepino mosaic virus (PMV), Cucumber green mottle mosaic virus (CGMMV)*) and narrow range of plant-hosts *(Brome mosaic virus (BrMV), Barley stripe mosaic hordeivirus (BSMV,) Cucurbit aphid-borne yellows virus (CABYV)* using the standard procedures for indirect and double-antibody sandwich (DAS) modifications of ELISA [4].

***Table 1*. Sites of collection**

Plant species	Location	S	W
Deshampsia antarctica	Island Yalour	65º14′139″	64º09′330″
	Island Pitterman	65º10′493″	64º08′452″
	Island Barselot	65º19′736″	64º08′613″
	Island Galindez	65º14′783″	64º14′799″
	мис Rassmunsen	65º14′819″	64º05′156″
	Island Barkhan	65º	
	Island Uruguay		
Colobanthus quitensis	Island Skua		
	Island Yalour		
	Island Barkhan		
	Island Galindez	65º14′877″ 65º14′877″	64º14′1577″ 64º14′557″

Samples that had positive results using ELISA were analyzed with immunosorbent electron microscopy:

- coated grids were deposited in the serum and incubated for 2 hours;
- washed with 100 microliter of PBS+Tween-20 three time;
- coated grids were deposited in the clarified homogenate of the plant sample in dilution 1:20 in buffer of extraction and incubated during 1 hour (0.05V PBS ph 7.2, 0.4% Na DECA, 2% PVP);
- washed with 100 microliter of PBS+Tween-20 thrice;
- grids were deposited in the serum for 1 hour;
- washed with 100 microliter of PBS+Tween-20 thrice;
- grids with applied sample were put into the 2% uranyl acetate solution for negative contrasting.

All operations were conducted under aseptic conditions.

The next step of experiment was carried out using bioassay with indicator plants that were inoculated with the clarified homogenate of the plant *D. Antarctica*.

Results and discussion. After detection of plant viruses in the samples of *D. antarctica* and *C. Quitensis* from Argentina Islands was revealed the presence of virus antigens that belong to different taxonomic groups, in particular, *BOM (Cucumovirus, Bromoviridae), TSWV (Tospovirus, Bunyaviridae), CGMMV (Tobamovirus*). These data were verified using ELISA and bioassay (the infectivity of virus extracted from *D. antarctica* was proved) (Fig. 1).

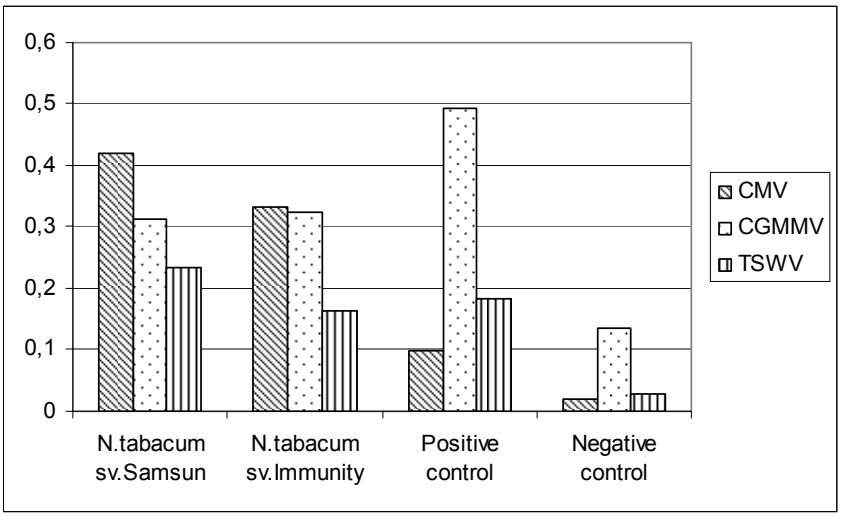

Fig.1 Detection of CMV, CGMMV, TSWV in Deschampsia antarctica, Samsun and Nicotiana tabacum sv using indirect ELISA

Serological analyses of *D. antarctica* and *C. quitensis* were carried out. Results of ElLISA confirmed the presence of viral antigens in particular *CGMMV* and *AMV* only in some plants, , other antigens were not detected (Fig. 2a and 2b).

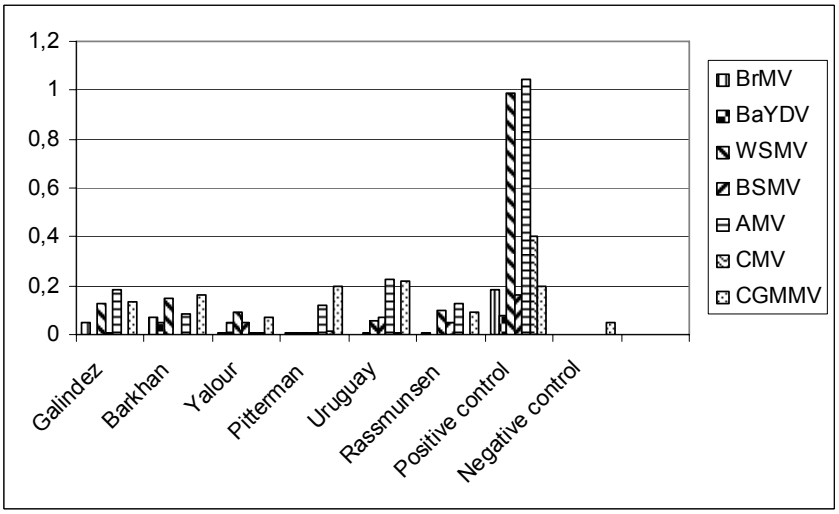

Fig.2a. Detection of plant viruses in *D. antarctica*.

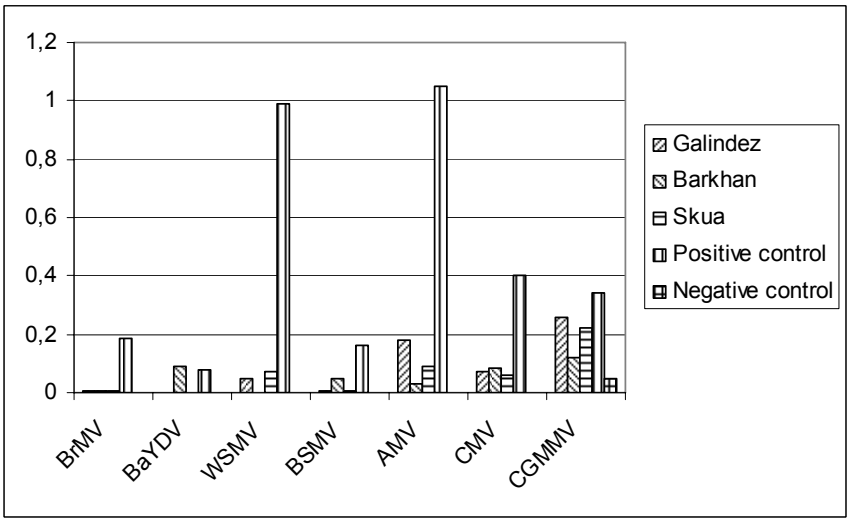

Fig.2b. Detection of plant viruses in *C. quitensis*. Results of ELISA were statistically valid and repeated

The next step was identification of virus using immunosorbent electron microscopy. Individual spherical particles were detected. This fact one more time confirm presence of viral antigens in plants. These particles had size near 85nm that according to scientific data is typical to the size of *TSWV* or serologically related virus.

Results that we have got show that grain cereal plant *D. antarctica* contains viral antigens that belong to different taxonomic groups - *Cucumovirus, Tospovirus, Tobamovirus*. According to the results of ELISA it can be assumed that *CGMMV* and *AMV* are present in *C. quitensis*.

In order to distinguish these viruses bioassay was carried out. Plants were selected in that way to avoid cross-reaction between viruses. Indicator plants for *CMV* are *Lycopersicon esculentum*, *Phaseolus aureus*, for TSWV - *Tropaeolum majus*, *Petunia hybrida* cvs Pink Beauty, for *CGMMV - Datura stramonium* and for *AMV – Pisum sativum*, *Malva parviflora.* For controls and each virus that was detected previously tree indicator plants were utilized.

Indicator plants were inoculated homogenate obtained from plant *D. antarctica* by mechanical rubbing. Results were analysed on 14-th day after inoculation. Plants showed exact visual symptoms of viral infection - Lycopersicon esculentum - mosaic of the leaves and stunting with filiform leaves to different extents, often extreme, *Phaseolus aureus* responded with small, circle necrosises with brown periphery on the leaves; *Tropaeolum majus* - inoculated leaves symptomless; in 8-12 days a systemic mosaic pattern of yellow and dark green specks develops, sometimes also with necrotic spots to *CMV* and TSWV infection of these plants respectively. Comparing to control plants all exposed plants showed positive results (Fig. 3)

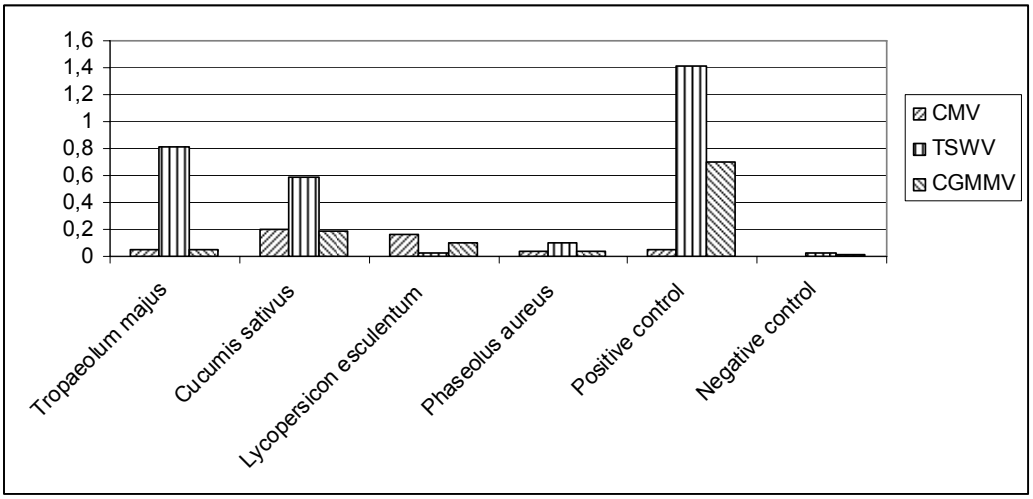

Fig.3 Detection of CMV, CGMMV, TSWV in plants Lycopersicon esculentum, Phaseolus aureus, Tropaeolum majus, Cucumis sativus using indirect ELISA

Conclusions. Detection of plant viruses in samples of plants *D. antarctica* and *C. quitensis* from Argentina Islands was performed. Using bioassay infectivity of viruses extracted from *Deschampcia antarctica* in particular CMV*, CGMMV, TSWV* was proved. Viral antigens that belong to different taxonomic groups such as *Cucumber mosaic virus* (*Bromoviridae, Cucumovirus*) and *Tomato spotted wilt virus (Bunyaviridae, Tospovirus),* Cucumber *green mottle mosaic virus* (*Tobamovirus*) were detected and in samples *C. quitensis* viral antigens of *CGMMV(Tobamovirus*) and *AMV* (*Bromoviridae*) were detected). Using immunosorbent electron microscopy spherical particles that had size similar to *Tomato spotted wilt virus* - near 85nm were detected.

Acknowledgments. The research work was supported by a Grant from Ukrainian Antarctic Centre.

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UDC 578.869

A. Gospodaryk, PhD student, I. Budzanivska, PhD, F. Demyanenko, PhD, V. Polischuk, D. Sci, prof.

DISTRIBUTION OF APPLE LATENT VIRUSES IN KIEV REGION

Taras Shevchenko` Kyiv National University, Ukraine.

Distribution of Apple mosaic virus, Apple chlorotic leaf spot virus, Apple stem pitting and Apple stem growing virus have been investigated in fruit orchards of Kiev region and the City of Kiev in 2007. The widest spread was shown for Apple chlorotic leaf spot virus (68.4%). Three different techniques for extraction of total RNA preparations from apple plant material for subsequent RT-PCR were compared. Method proposed by Kundu (2001) has been shown to be most efficient according to the quantity of obtained RNA preparation.

Introduction. Virus diseases of fruit trees inflict considerable losses in the commercial plantings of Ukraine. There are more than 40 viruses and virus-like diseases in pome fruit trees [11]; among them, the economically important virus diseases of apple trees include *Apple chlorotic leaf spot virus* (ACLSV, *Trichovirus*), *Apple mosaic virus* (ApMV, *Ilarvirus*), *Apple stem pitting virus* (ASPV, *Foveavirus*) and *Apple stem grooving virus* (ASGV, *Capillovirus*) [9,

3]. Except for ApMV, these virus diseases are typically symptomless in most commercial apple varieties and frequently occur in mixed infection. For the certification of plant material, apple plants are necessary to be tested for these 4 distinct virus diseases along with other pathogens (EPPO, 1999). Because many apple viruses are symptomless on apple plants, timely diagnostics of mother plants utilizing reliable methods is crucial to prevent the vegetative transmission of the pathogens. Today, the most widespread diagnostic techniques are woody indexing, ELISA and reverse transcription polymerase chain reaction (RT-PCR). Woody indexing, even though considered as reliable, is time consuming (minimum of several months up to 3 years), expensive, and the results are often difficult to interpret due to faint symptoms. ELISA kits have been developed for ACLSV, ApMV, ASPV and ASGV. These kits have failed on occasion due to low virus titer, or inhibitory effects of polysaccharides or phenolic compounds in tissue extracts of woody plants [4]. Highly sensitive, reliable and rapid methods for mass identification of apple viruses are needed, hence methods based on polymerase chain reaction can become an alternative.

Thus, our work primarily aimed at investigation of distribution of *Apple mosaic virus*, *Apple chlorotic leaf spot virus*, *Apple stem pitting* and *Apple stem growing virus* in fruit orchards of Kiev region and the City of Kiev in 2007 employing ELISA, and at comparison of three different techniques for extraction of total RNA preparations from the apple plant material for subsequent RT-PCR.

Objects and methods of investigation. The objects of the study were the apple viruses - *Apple chlorotic leaf spot virus, Apple mosaic virus, Apple stem pitting virus,* and *Apple stem grooving virus.* Young leaves, buds and petals of apple trees collected during May-July from orchards of Kiev region and the City of Kiev were used as material for investigation. In total, 60 samples have been tested. The samples were collected after visual diagnostics of different planting types from specialized orchards. Visual diagnostics of fruit plantings for occurrence of viral diseases was conducted early in spring. All samples were tested using DAS-ELISA [2]. Commercial test systems “LOEWE” (Germany) were used for virus diagnostics. Samples of leaf tissues, buds and petals were homogenized mechanically at 1:200 (wt/vol) dilution in coating buffer. Homogenate was centrifuged at 5000 g for 10 min. Immunological plate was covered with clarified supernatant. Passage of virus pathogens from woody plants onto indicator plants (bioassay) has been conducted by standard methods. For *Apple mosaic virus*, cucumbers (*Cucumis sativus*) cv. «Konkurent» and «Dzherelo» were employed as indicator plants; for *Apple stem grooving virus* and *Apple chlorotic leaf spot virus* – orach plants (*Chenopodium quinoa*); and for *Apple stem pitting virus* – tobacco plants were used (*Nicotiana occidentalis* cv. 37B).

Three different techniques for extraction of total RNA from the apple plant material – these were the method proposed by Kundu (2001), the method modified by Draper and Scott (Plant genetic transformation and Gene Expression, 2001), and the method by Boonham (2001) – were compared for their suitability for subsequent RT-PCR [1, 6, 7, 13].

The primers for ASPV, ACLSV, ASGV and ApMV were as previously described by Menzel [8].

Multiplex RT-PCR assays were conducted as previously described by Menzel.

The reaction mixture contained 1 µl of total nucleic acid extract, 7.5 µl 10× PCR buffer (supplied with the *Taq* polymerase, ‘Fermentas’), 0.5 µl $MgCl_2$ (25 mmol/l), 0.5 µl Tween-20 (10%), 0.5 µl dNTP mixture (each dNTP 25 mmol/l), 1 µl M-MuLV reverse transcriptase (200 U/µl, ‘Fermentas’), 0.2 µl *Taq* polymerase (5U/µl, ‘Fermentas’). For multiplex I (detection of *Apple chlorotic leaf spot virus* and *Apple stem grooving virus*), the mixture additionally contained 1.5 µl of each *Apple chlorotic leaf spot virus* primers (10 pmol/l) and 1.75 µl of each *Apple stem grooving virus* primers (10 pmol/l), and for multiplex II (detection of *Apple mosaic virus* and *Apple stem pitting virus*) – 1.5 µl of each *Apple mosaic virus* primers (10 pmol/l) and 1 µl of each *Apple stem pitting virus* primers (10 pmol/l). Sterile deionized water was added to the final volume of 50 µl. As positive control we used an internal control based on plant RNA. Internal control primers (Universal plant 5SrRNA primers): PLANT-UNI F TTT AGT GCT GGT ATG ATC GC**;** PLANT-UNI R TGG GAA GTC CTC GTG TTG CA [5]**.** The cycling parameters were: reverse transcription at 42°C for 60 min, activation of the *Taq* polymerase at 95°C for 15 min followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min. The final extension step was 72°C for 7 min.

PCR products were analyzed using electrophoresis in 1% agarose gel stained with ethidium bromide (EtBr) and viewed under UV.

Statistical evaluation of the experimental data has been conducted using parametric criteria of normal distribution for variants. Standard deviation of the mean valued was estimated via common technique [14]:

$$E = \bar{E} \pm \sigma$$
$$\bar{E} = (E_1 + E_2 + \ldots + E_i) / i$$
$$\Sigma = \left| E_{max} - E \right| = \left| E_{min} - \bar{E} \right|,$$

Where E – reliable extinction value; $\bar{E}$ – mean arithmetic value of measured extinction values, $E_1 \ldots E_i$; σ – standard deviation.

Results and Discussion. We carried out the observation of commercial plantings of apple cultivars on occurrence of visual virus symptoms in Kiev region and the City of Kiev. Apple plantings were observed in spring at the stage of 3-4 true leaves for the exposure of possible symptoms of viral diseases. Leaves of ApMV-infected plants developed bright yellow and (later) pale yellow-coloured spots and rings that expanded during spring. Afterwards these yellow patterns became necrotic. Plants affected by *Apple chlorotic leaf spot virus* exhibited line patterns and small necroses on leaves. *Apple stem pitting virus* induced symptoms on a few apple cultivars only. The most affected were cultivars Virginia Crab, Golden Delishes and Mauzen. Infected plants were stunted and developed chlorotic mottled and ribbing fruits. Plants infected with *Apple stem grooving virus* exhibited leaf chlorosis, early leaf browning. The virus also caused an increase in internodes. Leaf symptoms persisted at 20-22^{0}C and became masked at 30-32^{0}C.

Results of visual observation and analysis of literature data pointed on the infection of apple trees with ACLSV, ASPV, ASGV, and APMV. For confirmation of results we conducted testing of samples by DAS-ELISA.

Among the tested samples of apple trees, antigens of *Apple mosaic virus* (ApMV) were identified in 1.7% samples; antigens of *Apple chlorotic leaf spot virus* (ACLV) in 68.4%; antigens of *Apple stem pitting virus* (ASPV) in 23.3%; and antigens of *Apple stem grooving virus* (ASGV) in 18.3% (Fig.1). Mixed infection has also been common for most viruses: ASPV+ASGV constituted 5%; ASGV+ACLSV – 16.7%; and ACLSV+ASPV – 1.7%. We have not identified any samples infected by all three viruses simultaneously (Fig.2). Only about 6% of samples tested have been confirmed to be virus-free.

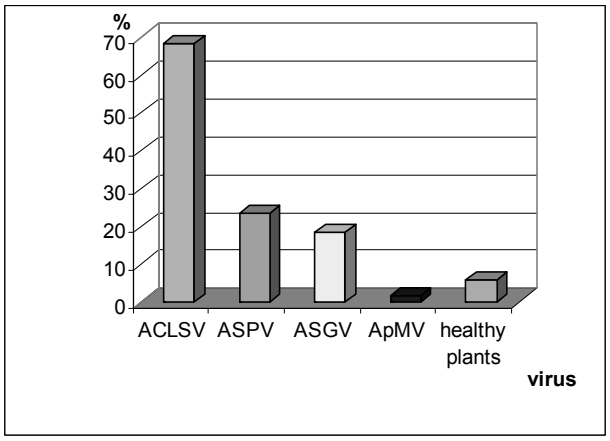

Fig.1. Occurrence of common apple viruses in samples from commercial plantings (Kiev region and the City of Kiev, 2007)

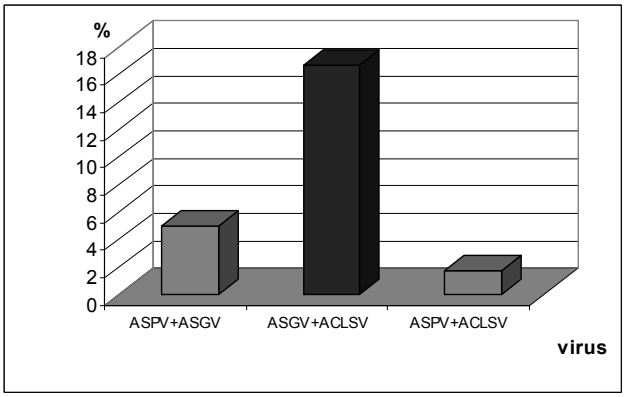

Fig.2. Occurrence of mixed virus infections in tested apple samples (Kiev region and the City of Kiev, 2007)

Further on, viruses were mechanically transmitted onto susceptible indicator plants *Cucumis sativus*, *Chenopodium quinoa*, and *Nicotiana occidentalis* 37B for virus accumulation and isolation. However, the bioassay appeared to be largely unsuccessful reflected by low rate of efficient virus passage onto assay plants, which in turn has been confirmed by ELISA (for instance, only 2 assay *C. sativus* plants from the total of 30 became infected). The external demonstration of viral infection were barely observed. The cause of this may have been high temperatures in the greenhouse during cultivation of inoculated plants (about 40^0C) which is far from optimum for the development of symptoms for these viruses.

Extraction of total RNA from fruit tree material remains a major difficulty, because woody plants contain high amounts of components like polyphenols and polysaccharides. These components usually co-purify with nucleic acids, RNA and DNA, and are believed to inhibit the sensitivity of PCR or RT-PCR [10]. It is likely that their amounts differ among different tissues at different time of the year [12]. We compared three different techniques for extraction of total RNA preparations from the apple plant material for subsequent RT-PCR. Hence, method proposed by Kundu (2001) has been shown to be most efficient according to the quantity of obtained RNA preparation (Fig.3).

Finally, we tested our apple samples (previously shown to be virus-positive in ELISA) via RT-PCR employing primers proposed by Menzel et al. (2002), however unsuccessfully (Fig.4). We believe the reason for negative outcomes maybe the difference in the nucleotide sequences of coat protein gene.

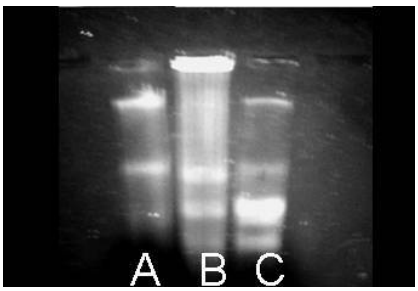

Fig.3. Total RNA agarose gel electrophoresis: A – method modified by Draper&Scott (Plant genetic transformation and Gene Expression, 2001); B – method proposed by Kundu (2001); C – method by Boonham (2001)

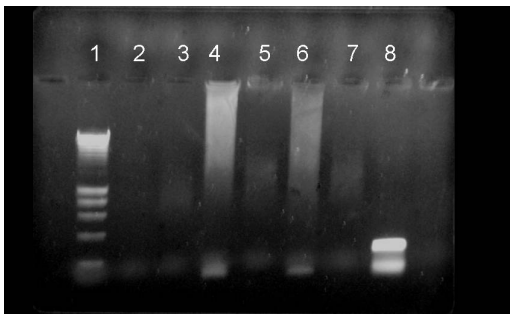

Fig.4. Agarose gel analysis of multiplex RT-PCR I, II products: 1 – DNA-ladder (HyperLadderI, Bioline); 2, 3, 4 - multiplex RT-PCR I for *Apple chlorotic leaf spot virus* and *Apple stem grooving virus* (RT-PCR products of total nucleic acid extract of infected sample); 5, 6, 7 - multiplex RT-PCR II for *Apple stem pitting virus* and *Apple mosaic virus* (RT-PCR products of total nucleic acid extract of infected sample); 8 – internal control

Conclusions. 1. Distribution of apple virus diseases (*Apple stem grooving virus*, *Apple mosaic virus*, *Apple stem pitting virus*, and *Apple chlorotic leaf spot virus*) has been studied in Kiev region and the City of Kiev. The most prevalent was shown to be *Apple chlorotic leaf spot virus* (68.4%). In addition, three different techniques for extraction of total RNA preparations from apple plant material were compared for subsequent RT-PCR. Method proposed by Kundu (2001) has been shown to be most efficient according to the quantity of obtained RNA preparation.

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UDC 578.3

A. Koreneva, PhD student, L. Mishchenko, D. Sci

VIRAL DISEASES OF VALERIANA OFFICINALIS L.

Taras Shevchenko` Kyiv National University, Ukraine.

Monitoring of cultural phytocenosises of Valeriana officinalis L. is carried out. Virus from the leaves and stems of infected valerian plants is isolated. Viral nature of disease was proved with biotesting method. The morphology and length of particles were established with electron microscopy method.

Introduction. Medicinal plants are used in pharmacological, food and parfumery industries. Valerian (*Valeriana officinalis* L.) is one of the well – known medicinal herbs, its has been used in phytotherapy roe a long time [2]. Valerian drugs reduce excitation of central nervous system, control heart functioning, raise the secretion in gastrointestinal tract, reduce intestines fermentation [9]. Valerian is applicable for insomnia and migraine [8]. Phytopreparations take part in biochemical reactions of human organism faster than synthetic drugs. Moreover, drugs made with herbs do not cause complications, especially allergy. Complex of these plant active substances possesses many-sided, varied display of pharmacological activity, promoting effective treatment.

All these facts represent a reason for obtaining significant yield in these medicinal plants. There are many problems in herbs growing, because it is needs special organizational, agrotechnical and many other cultivating methods. First of all, main reason of yield reduction is herbs diseases. It was noted that viruses are able to reduce yield and quality of medicinal plants [3, 6, 10]. It is known that various vermin, fungal and bacterial pathogens infect valerian plantations causing damage for plant quality. For instance, 60 % of valerian plants were contaminated with *Erysiphe cichoracearum* D. C. f. valerianae Jacz., *Septoria* valerianae Sacc. et Fautr. – 20 %, *Sclerotinia* Libertiana Fuck. – 15 % of plants [1]. In cultural phytocenosises, except fungi and bacteria, *Valeriana officinalis* L. can be infected with viruses. In Bulgaria it was shown that some of the herbs, including valerian, are infected with alfalfa mosaic virus (*AMV*) and cucumber mosaic virus (*CMV*) [7].

Thus, our research was focused mainly on the indication of viruses that infect valerian plantations in Ukraine.

Object and methods of investigation. *Valeriana officinalis* L. plants with symptoms of viral infection were investigated with visual diagnostics method. Samples of these plants were taken on Medicinal plants research station of agroecology Institute of Ukrainian Academy of Agricultural Sciences (Berezotocha, Ukraine).

Isolation of viruses from the infected plants has been carried out by Novikov experimental technique [4]. Homogenization of symptomatic plant leaves and stems was made in 0,05 M phosphate buffer in proportion 1:2 (w/v) with following low-speed centrifugation at 12 000 rpm for 20 minutes at 4 °C to remove the debris of plant tissues. Sedimentation of virus has been carried out with 5 % polyetilenglicol, M_r 6 000 for 2 h at 4 °C. Virus was extracted with 0, 05 M borate buffer pH 7, 6. After high – speed centrifugation (36 000 rpm for 1, 5 h) sediment was resuspended in 1 ml 0, 01 M borate buffer pH 8, 0.

Mechanical inoculation of indicator-plants with sick valerian sap was carried out for biotesting. With this purpose young *Datura stramonium*, *Nicotiana tabacum* (cv. Immune and Trapezon), *Chenopodium amaranticolor, Ch. album, Ch. quinoe* and *Phaseolus (*cv. Pinto*)* plants in stage of two true leaves were used.

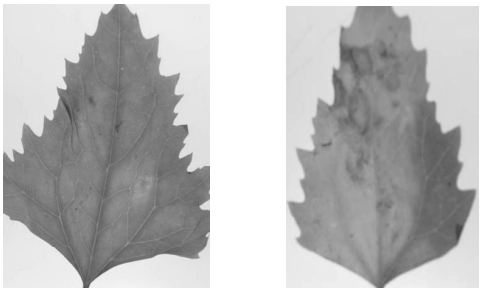
Morphology and size of virus particles were detected with electron microscopy method. Purified virus preparation was placed on the copper grid with support that has been made of 0, 2% formvar solution (“Serva, Germany). Contrasting has been made with 2% solution of phosphorus - tungstic acid pH 7, 4 for 2 minutes and then monitored on electron microscope JEM 1230 (JEOL, Japan) and EM-125 (Sumy, Ukraine) [5].

Sample analysis for presence of virus antigens has been carried out employing sandwich ELISA modification. ELISA has been carried out in polystyrol plates “Labsystem”. Results were registered using Termo Labsystems Opsis MR reader (USA), programme Dynex Revelation Quicklink at the wavelength 405 / 630 nm.

Results and Discussion. During 2006 -2007 years we were investigating valerian plant plantations of Medicinal plants research station of agroecology Institute of Ukrainian Academy of Agricultural Sciences (Berezotocha, Ukraine). Plants with symptoms of diseases were detected. These symptoms allowed supposing their viral nature of diseases. *Valeriana officinalis* L. with mosaic symptoms on the leaves, significant plants dwarfing were shown. Inflorescences of infected plants were missing. (Fig.1)

Fig. 1. *Valeriana officinalis L.* plants, infected (left) and normal (right).

Plant – indicators *Datura stramonium*, *Nicotiana tabacum* (cv. Immune and Trapezon), *Solanum nigrum*, *Chenopodium album, Chenopodium quinoe*, *Chenopodium amaranticolor, Phaseolus vulgaris (*cv. Pinto*)* in stage of two true leaves were used in biotesting method. Symptoms of disease were observed only on the *Chenopodium quinoe* and *Ch. amaranticolor* on 20 day post inoculation. There were 2 -4 brown necrosises per 1 leaf with light brown oreol (Fig.2).



A B

Fig. 2. Necrotic reaction of Chenopodium amaranticolor - A) та Ch. quinoe - B), inoculated with sap of infected valerian plants.

Next stage of our research was to study morphology of agents, causing valerian diseases, using electron microscopy method. In purified and concentrated virus preparation we observed virus-like particles, which were not detected in healthy plants sap. There are flexuous filaments, 530±10 x 11 nm in size (Fig. 3).

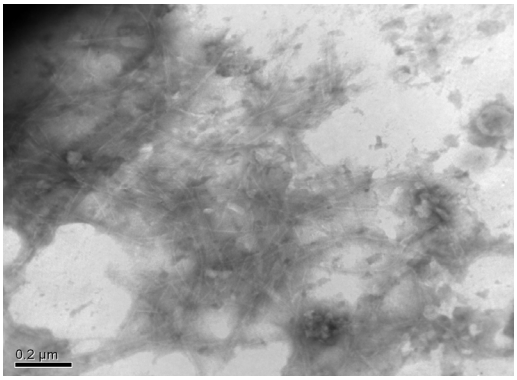


Fig.3. Electron microscopy picture of purified particles, isolated from valerian leaves.

Conclusions. As a result of phythopatological monitoring of viral infection, found on the valerian plantations, it was infected *Valeriana officinalis* L. plants were revealed. It was shown that infected plants had the symptoms as mosaic on the leaves, significant plants dwarfing and absence of inflorescences in sick plants. After inoculation with sap of infected valerian plants-indicators showed necrotic reaction. These symptoms allowed supposing their viral nature of diseases. The morphology and length of particles were established with electron microscopy method. The investigated particles were presented as filamentous virions, 530±10x11nm.

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T.P. Mudrak, stud., A.S. Bysov, PhD, T.A. Kompanets, PhD, G.V. Korotyeyeva, PhD.

INCEDENCE OF CACTUS VIRUS 2 IN COLLECTION OF UKRAINIAN BOTANICAL GARDENS

Taras Shevchenko` Kyiv National University, Ukraine.

Screening of Cactaceae plants on virus diseases in the collections of Fomin's Botanical garden of Taras Shevchenko' Kyiv National University and Botanical garden of Karasin's Kharkiv National University have been conducted. Opuntia plants were the most infected in both collections. Basing on serological, biological and morphological properties, we suggest that isolated virus is related to Cactus virus 2.

Introduction. *Virus diseases of cactaceae plants are of great significance because even when present in the latent state, the viruses could be transmitted to healthy plants and cause commercial losses.*

Specific morphology of cactus conditions difficulties in deciphering visual symptoms of virus infection. In spite of that fact, that we could predominantly diagnose virus infection basing on specific symptoms such as ring spot, mosaic and necrotic lesions, the identification of the pathogen was not possible. Sometimes *Cactacea* plant quite haven't signs of virus infection. Besides, some factors such as disbalance of mineral nutrition, non-compliance with the light regime, invasion by insects and mites, infections coursed by bacteria, mycoplasms and fungus, or genetic distortions could manifest similar to virus symptoms. This involves necessity for serological diagnostics of the collections for preservation of their commercial value.

Materials and methods. Plants of *Opuntia* sp., *Opuntia microdaysys* var. rufida, *Consolea rubenscens*, *Pereskia aculeata* v. godseffiana, *Echinocereus* sp., *Caralluma* sp. with visual virus-like symptoms from greenhouse collections of Fomin' Botanic Garden of Taras Shevchenko' Kyiv national university (Kyiv) and *Mammillaria centricirrha, Trichocereus bridgesii, Ritterocereus pruinosus* from the collection of Karazin' Botanic Garden of Kharkiv national university (Kharkiv) were the objects of this research.

Infectious nature of disorders was confirmed proved using indicators plants typical for viruses normally infecting cactuses such as *Gomphrena globosa*, *Datura stramonium*, *Nicotiana tabacum* cv. Samsun, *N. alata*. Virus identification was carried out using TAS- and indirect ELISA [3]. Same staining samples were analyzed in electron microscopy at 30,000 magnification.

Viruses from *Opuntia* sp. were purified by differential centrifugation. Highspeed centrifugation was performed for 90 min at 100,00 x g at 4C using SW-40 rotor (Beckman, Germany). Capsid proteins of the virus extracted from *Opuntia* sp. samples were analyzed by SDS-PAGE (Laemmli) [6].

Results and discussion. Virus diseases of *Cactaceae* plants aren't enough studied. Cactus virus X, Schlumbergera virus X, Opuntia virus X, Zygocactus montana X, Saguaro cactus, Sammons' Opuntia virus, Cactus virus 2 (CV2), Cactus mild mottle virus and Impatiens necrotic spot virus have been described as cactus-infecting viruses [5, 7, 8]. In most cases infection processes is symptomless [7]. Appearance of symptoms could be evidence of severe course of virus disease. Therefore we paid a lot of attention to plants in cactus collections which show abnormal colorations like mosaics and yellowing.

Symptoms of necrosis, mosaics and chlorotic spots, wrinkling and atrophy of stems in cactus collection of Fomin' Botanic Garden of Taras Shevchenko' Kyiv national university were detected. Chlorosis and stem deformation (symptoms of witches'-broom) were observed only on *Opuntia* plants. These symptoms on Opuntia could be associated with phytoplasma [2]. Although we selected these plants to detect viruses as we proposed that affection of phytoplasma could reduced plant resistance to viruses. Furthermore in this situation activation of latent virus infection and following development of symptoms could took place/occurred. It should be mentioned that different cactus species had diverse symptoms. It should be noted that *Opuntia* plants were the most affected cactus species.

It was established that the collection of cactuses of Karazin' Botanic Garden of Kharkiv national university, in general, had been in a better phytosanitary state. Only a few plants in the collection showed signs of virus disease. Symptoms of stem deformation were detected on *Mammillaria centricirrha* plants. *Trichocereus bridgesii* and *Ritterocereus purinosus* had symptoms of chlorotic spots.

To define biological properties of the pathogens, we conducted a bioassay using 6 species of indicator plants. Indicator plants were inoculated with sap obtained from cactus plants demonstrating virus-like symptoms. Results of the assay are presented on Table 1.

Table 1. **Response of indicator plants on inoculation with sap from virus-infected cactus plants**

Plant species	Symptoms on indicator plants					
	Gomphrena globosa	*Datura stramonium*	*Nicotiana tabacum* cv. Samsun	*N. alata*	*Chenopodium* sp.	*Phaseolus vulgaris*
Opuntia microdasys v. rufida	-	-	M	-	-	-
Opuntia sp.	N	N	-	M	-	-
Consolea rubescens	-	-	-	-	-	-
Pereskia aculeata v. godseffiana	-	-	-	-	-	-
Echinocereus sp.	-	-	-	-	-	-
Caralluma sp.	-	-	-	-	-	-
Mammillaria centricirrha	-	-	-	-	-	-
Trichocereus bridgesii	N	N	-	-	-	-
Ritterocereus pruinosus	N	N	-	-	-	-

- – no symptoms; M – mosaic; N – necroses.

Mosaic symptoms on *Nicotiana tabacum* cv. Samsun inoculated with sap from *Opuntia microdasys* v. rufida are not typical for any known cactus virus. On the contrary, necrotic local lesions observed on *Gomphrena globosa* and *Datura stramonium* were typical for Cactus virus X (CVX). Besides necrotic symptoms on *Gomphrena globosa* were also common for CV2.

Thus we conducted the etiology of diseases on *Opuntia microdasys*, *Opuntia* sp., *Trichocereus bridgesii* and *Ritterocereus pruinosus*. Absence of reactions on some indicator plants post inoculation with sap from diseased plants, in our opinion, couldn't be explained with non-transmittance of some viruses by mechanical inoculation or with insusceptibility of definite indicator plants to virus infection.

To determine virus nature of disease we conducted indirect and TAS-ELISA tests. Results of indirect ELISA showed positive reactions of *Opuntia* sp., *Opuntia microdasys* v. rufida, *Consolea rubescens*, *Pereskia aculeata v. godseffiana*, *Trichocereus bridgesii* and *Ritterocereus pruinosus* (Fig.1) with antiserums to Potato virus S (PVS) and Potato virus M (PVM). We deem it could indicate contamination of these plants with CV2, which is serologically related to PVS and PVM [4].

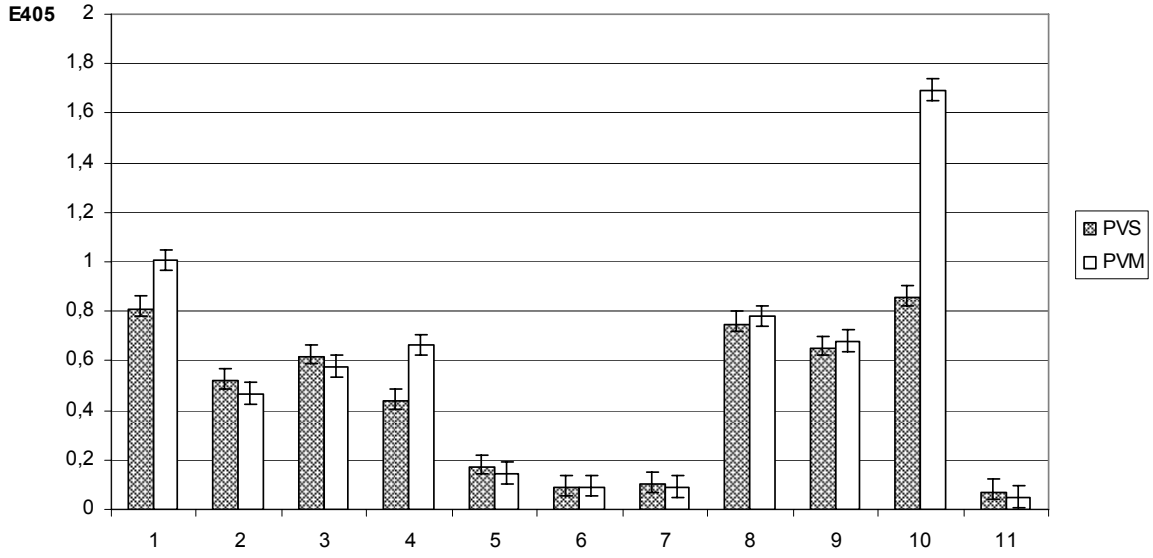

Fig. 1. Results of ELISA testing *Cactaceae* plants with antiserums to PVS and PVM: 1 - *Opuntia* sp.; 2 - *Opuntia microdasys* v. rufida; 3 - *Consolea rubescens*; 4 - *Pereskia aculeata* v. godseffiana; 5 - *Ehinocereus* sp.; 6 - *Caralluma* sp.; 7 - *Mammillaria centricirrha*; 8- *Trichocereus bridgesii*; 9 - *Ritterocereus pruinosus*; 10 – positive control, 11 – negative control (normal plant)

Results of indirect ELISA examination of the samples with antiserums to Potato virus (PVX, which is serologically related to Cactus virus X and Zygocactus montana X), and Odontoglossum ringspot virus (ORSV, serologically related to Sammons' Opuntia virus) were negative. TAS-ELISA for Impatiens necrotic spot virus was also been unsuccessful.

Comparing the outcomes of bioassay and ELISA tests we futher focused on *Opuntia* sp. and *Opuntia microdasys* v. rufida from the collection of Fomin' Botanic Garden of Kyiv National University, and *Trichocereus bridgesii* and *Ritterocereus prinosus* from the collection of Karazin' Botanic Garden of Kharkiv National University. These plants were probably infected with CV2. To confirm our assumption about CV2 infection and to study the morphology of the pathogen we carried out transmission electron microscopy.

Filamentous virus particles about 2 ± 2 nm in size were observed in the sap of the plants (Fig.2). It should be stressed that virions in sap from *Ritterocereus prinosus* were organized in stretched structures.

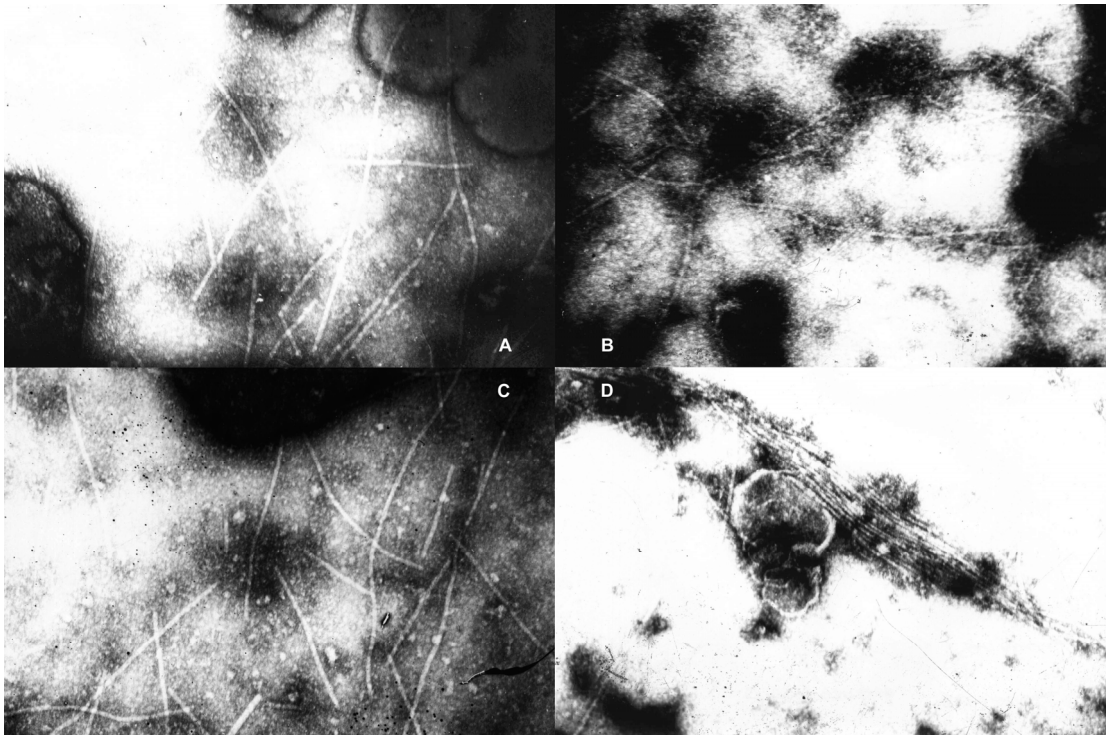

Fig.2. Electron micrograph of flexible particles after partial purification from plant material (x 30 000)
A - *Opuntia* sp.; B - *Opuntia microdasys* v. rufida; C - *Trichocereus bridgesii*; D - *Ritterocereus pruinosus*

As virus load in plant sap was demonstrated to be high, we tried to isolate the virus directly from the plant samples. *Opuntia* sp. plants were chosen for virus purification. The sap from these plants coursed different symptoms on indicator plants and provided high readings in ELISA tests. (Protein composition of the virus isolated from *Opuntia* sp. showed presence of several proteins. Two major proteins with molecular weight of 24,7 and 15,5 kD were detected (Fig.3).

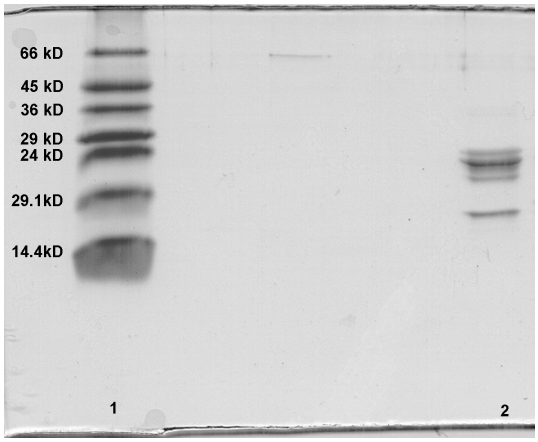

Fig. 3. Results of SDS-PAGE: 1 – Standard proteins; 2 – sample.

One capsid protein was typical for representatives of *Carlavirus* genus, such as Cactus virus 2 [4]. Presence of additional proteins (major and minor) in virus samples could be considered as the result of protein degradation or contamination by the cell proteins. Hence, virus protein content analysis requires further virus accumulation and thorough purification step to avoid possible contamination by the host components.

This work, therefore, confirmed that cactus plants in both collections are virus infected.

Virus infection, especially latent diseases, are very dangerous because of the extensive exchange of untested plants among different botanical gardens and private collections. In addition, the vegetative propagation of cactuses without virus monitoring involves uncontrolled distribution of virus infections though over all plants within the collection.

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O.G. Kovalenko[1], D. Sci., O.M. Polishchuk[1], gr., T.A. Krupodorova[2], gr., N.A. Bisko[2], D. Sci., A.S. Buchalo[2], D. Sci.

SCREENING OF METABOLITES PRODUCED BY STRAINS OF GANODERMA LUCIDUM[CURT.:FR] P.KARST AND GANODERMA APPLANATUM [PIRS.:WALLER] PAT. FOR THEIR ACTIVITY AGAINST TOBACCO MOSAIC VIRUS

[1] Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine, Kyiv; [2] Kholodny Institute of Botany NAS of Ukraine, Kyev

The higher Basidiomycetes mushrooms are impotent source of functional products which are widely used in folk medicine. During the study of mushrooms and produces of its metabolisms were found that they have antitumor, immunomodulating, antimicrobial and antiviral properties. But antiphitoviral activities have not been studied. The aim of our investigation was screening of Basidiomycetes species that can produce effective substances to plant pathogenic viruses.

We investigated two species of the genus Ganoderma, specifically G.lucidum and G. applanatum As result of experiments we selected two most active strains, which could inhibit the viral infection to 65-70% at the concentration 1000 µg/ml. The obtained results show that preparations have the highmolecular nature and can activate the plants protective mechanisms and block the virus penetration in the plant cell.

The higher Basidiomycetes are important source of functional products which are widely used in folk medicine. Study of their medical properties started not long ago [8]. It was established that there are substances among Basidiomycetes metabolism products with antitumor and immunomodulating properties. It stimulated researchers to detailed study of biological properties of active substances which were isolated from cultural liquid, fruit body and mycelium of fungi [5, 12].

Today the preparations isolated from Basidiomycetes are widely used as adaptogens and immunomodulators [5]. Such physiological activities of substances produced by fungi have been found as bioregulation, maintenance of homeostasis, treatment and prophylaxis of cancer, heart diseases, maintenance of normal cholesterol level, inhibition of clots formation, reduction of blood pressure [11]. More detailed studies have shown that polysaccharides are functionally active part of Basidiomycetes. There were selected krestin from mycelia of *Trametes versicolor*, lentinan from fruit bodies of *Lentinus edodes*, schizophyllan from *Schizophyllum commune* broth [9, 13]. But the effect of Basidiomycetes polysaccharides was investigated only using human and animal virus models [6, 7, 14]. Their antiphytoviral activities have not been studied.

At the same time it is known that polysaccharides injected to the plants tissue can inhibit the development of virus infection stirring up the mechanisms of plants protection [4]. Antiviral activities of polysaccharides depend on their structural characteristics (molecule charge, level of branching, monosaccharide composition et al. [10].

The process of resistance induction is not studied in details. The necessity of using new models for study of protection mechanisms and creation of new plants protection methods cause to search of new biologically active substances.

The aim of our study was screening of Basidiomycetes species producing metabolites effective against plant pathogenic viruses.

Materials and methods. In our work we used the cultural liquids (CL) of 14 strains of Basidiomycetes Ganoderma lucidum[Curt.:Fr] P.Karst and Ganoderma applanatum [Pirs.:Waller] Pat. grown in mineral medium containing 2,5% of glucose at the temperature 28°C with aeration (a rotary shaker, 220 rpm) for 14 days. Cultural liquids were separated from mycelium using kapron filters and then lyophilized. Antiphytoviral properties of CL aqueous solutions were tested at the model TMV (strain U_1) and tobacco plants - *Nicotiana tabacum* L.(Immune 580), N.sanderea and at the plants *Datura stramoniun*. The plants were grown in the glasshouse at the natural conditions of temperature, humidity and illumination. The plants in age of 4-6 real leaves were used in our work.

Water solutions of lyophilized cultural liquids were tested in concentrations 1000, 100, 10 and 1 µg/ml. The preparations were added to solution of tobacco mosaic virus and incubated during 30 minutes at the room temperature. Then the left parts of *Datura stramonium* L leafs were inoculated with the preparations. Right parts of leafs were inoculated with virus without inhibitors.

The degree of TMV infection inhibition was estimated from the number of local lesions (LL) in control and experimental part of leafs by formula [3]:

I=((K-D)/K) 100%, where
I - degree of virus inhibition, %;
K - number (size) of LL in control;
D - number (size) of LL in experiment.

In order to study the chemical nature of substances with antiviral activity CL dialysis against water and warming up at 100°C during 10 min has been conducted. Carbohydrates presence and concentration in the preparations were determined by the antron reaction (0,2 % solution in conc. H_2SO_4 [2]. The partially purified polysaccharides were obtained in following way: the cultural liquid was treated twice with 96% ethanol at a volume ratio 2:1. The supernatant was removed and the sediment separated by centrifugation and lyophilized [1].

The preparations were tested for their ability to induce plants resistance (IPR) to TMV. Water solutions of tested preparations in concentrations 1000-2500 µg/ml have been injected subepidermally by a syringe at the left halves of leaves. The right halves of leaves were injected with the distilled water. The TMV (concentration 4 µg/ml) was inoculated after 1, 3, 5, and 7 days. The degree of IPR was calculated using above mentioned formula. Antiviral activity of substances was tested on the model of *N.sanderea*, which is characteristic by absence of infection localization mechanism. The concentrations 1 and 2,5 mg/ml have been tested using the above mentioned method.

Results and discussion. Screening of *Ganoderma lucidum* and *Ganoderma applanatum* strains for antiviral properties of their metabolites has been shown that all of them were able to inhibit the TMV development. The activity of preparations increased with the growth of concentration. We selected two most active strains, which could inhibit the viral infection to 65-70% at the concentration 1000 µg/ml. These were strain *Ganoderma lucidum* 1900 and strain *Ganoderma applanatum* 920. Study of physical and chemical properties of active antiviral substance showed that CL from strain *G.lucidum* (Fig 1, b) was stable to the high temperature. Activity of *Ganoderma applanatum* strain has been reduced (Fig 1, a).

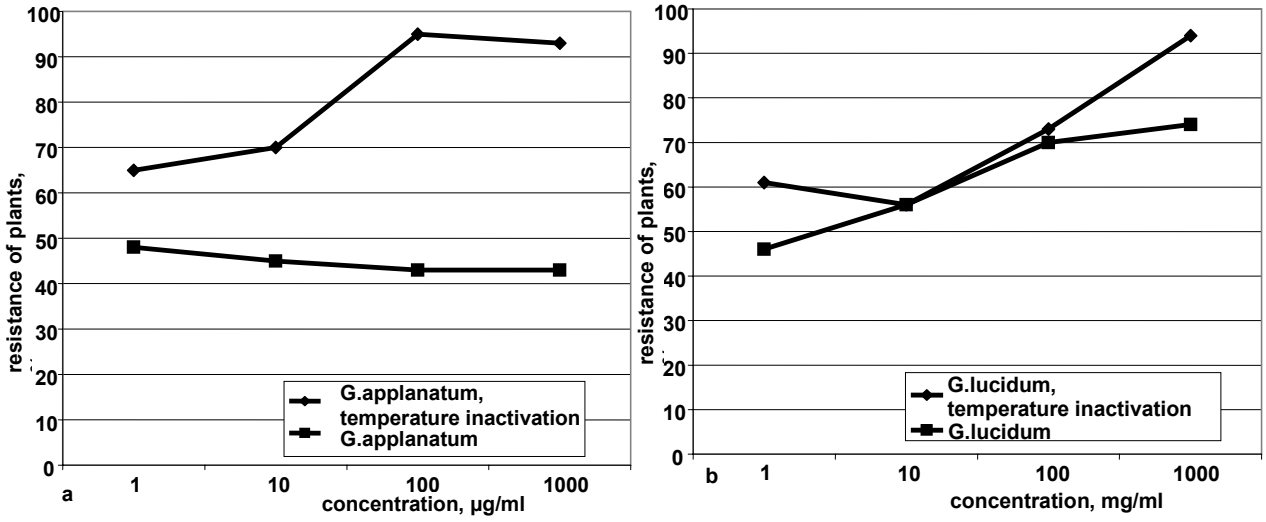

Fig 1. (a) The influens of temperature on the antiviral activity of strain Ganoderma applanatum (b) The influence of high temperature on metabolism product of G.lucidum

Investigation of dialysis products also demonstrated their antiviral properties. Antiviral activity was higher in datura than in tobacco plants. In other words these preparations activate the mechanisms of plants resistance which depend on plant genome. Dialysis did not influence the antiviral activity of isolated substances, so probably they have the high-molecular nature (Tabl 1).

Using the antron reaction with the dialysis products we have found that CL of *G.lucidum* contained 1095 µg/ml of polysaccharides and CL of *G.applanatum* – 975 µg/ml.

After isolation of polysaccharide by ethanol we have tested their antiviral properties.

As a result we have found that preparation from CL of *G.applanatum* inhibited the viral infection. The resistance induced by the preparation decreased according to the interval between the preparation injection and virus inoculation. At the first day the degree of resistance was 58%, at the third day – 43%. At the 5 and 7 days the resistance disappeared (Fig 2). The preparation from CL of *G.lucidum* did not inhibit the reproduction of virus at the concentration of 1000 µg/ml but could induce the plant resistance at concentration of 2500 µg/ml (Fig 3).

Table 1. **Antiviral property of Basidiomycetes metabolism products at the plants tobacco and datura**

Producing strain	Concentration µg/ml	Number of local lesion at the leaf		Inhibition of virus activity
		Experiment	Control	
Nicotiana tabacum, Immune 580				
G.applanatum	1	63	83.2	25^{+++}
	10	21.2	41.5	49^{+}
	100	5.6	30.6	82^{0}
G.lucidum	1	25.4	44.9	44^{+}
	10	16.3	27.2	41^{+++}
	100	9	29.8	70^{0}
Datura stramonium				
G.applanatum	1	8.4	14.1	30^{+++}
	10	6.75	38.5	83^{0}
	100	0.125	13	93^{+++}
G.lucidum	1	2.3	3	23^{0}
	10	1.03	15.4	93^{+}
	100	0.94	15.2	94^{+++}

$^{+++}$: p≤0.1%; $^{++}$:0.1<p≤1%; $^{+}$1<p≤5%; ^{0}: p>5%

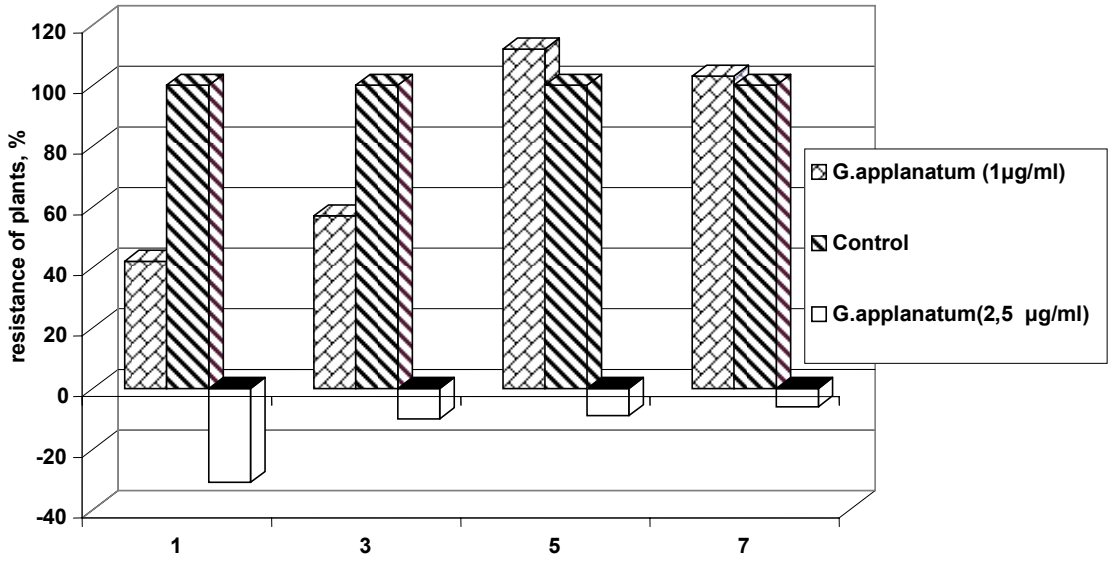

Fig 2. The influence of G.applanatum metabolism product to the resistance of tobacco to TMV

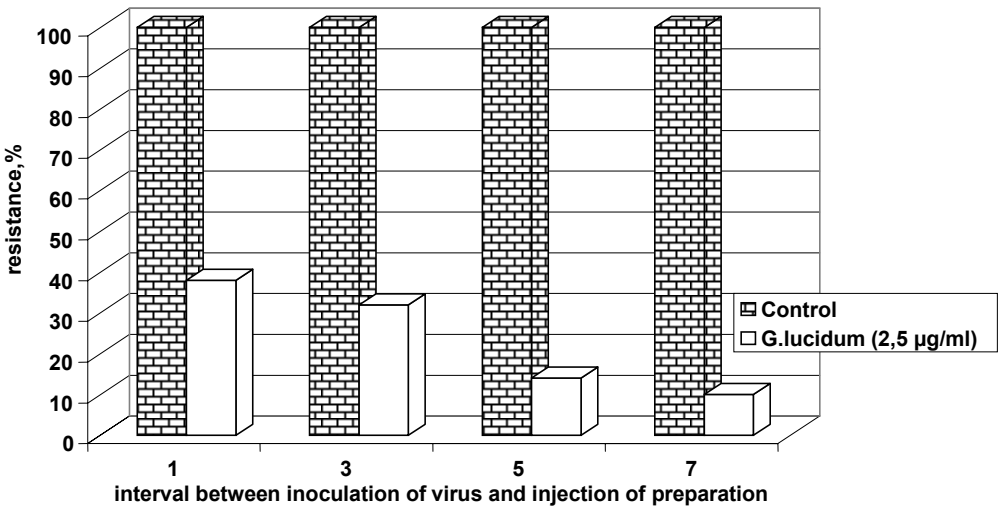

Fig 3. The influence of G.lucidum metabolism product (2,5µg/ml) on the resistance of plants tobacco to TMV

It was found by investigation at the *N.sanderea* plants that preparations could inhibit the TMV-infection at concentration 1000 µg/ml (Fig 4). The obtained results show that preparations can activate the plants protective mechanisms and block the virus penetration in the plant cell.

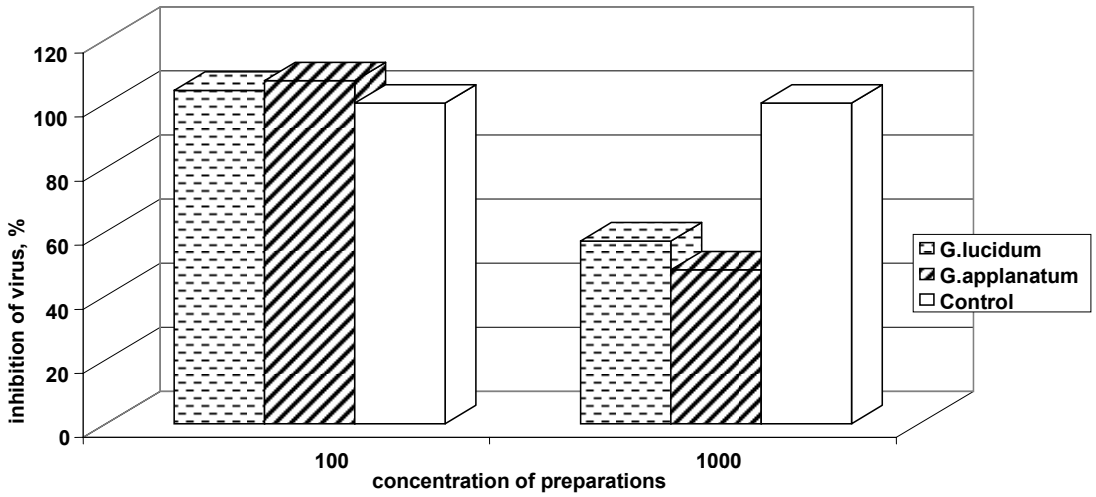

Fig 4. Antiviral properties of G.lucidum and G.applanatum strains it the plants N.sanderea

Thus as a result of screening *Basidiomycetes* strains *Ganoderma lucidum* and *Ganoderma applanatum* we have found two active strains. Their CL inhibited TMV-infection more than for 65 %. It was established that antiviral active part of preparation had the high-molecular nature, and possibly belonged to polysaccharides. The activity of *G.lucidum* preparation did not change at the influence of high temperature, the *G.applanatum* preparation activity has been reduced. Probably these active substances have different chemical structure. The antiviral properties of substances from different *Basidiomycetes* species differ one from another.

The preparation from *G.applanatum* was active in concentration 1 mg/ml; substance from *G.lucidum* inhibited the viral infection in concentration 2,5 mg/ml.

According to received results we can make conclusion that *Basidiomicetes* metabolites inhibit the plant virus infection and can induce the resistance of plants. Mechanisms of their influence are different and probably depend on their structural characteristic of active molecules which need the detailed study.

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O.G. Kovalenko, D. Sci., A.M. Kuruchenko, PhD, V.V. Shepelevich, PhD
O.V. Karpenko, PhD, R.I. Vildanova-Martcyshin, PhD, N.S. Scheglova, eng.

COMPLEX PREPARATIONS AS MEANS OF PLANTS RECOVERY AND PROTECTION AGAINST VIRAL INFECTIONS

Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine, Kyiv.

The complex antiviral preparations including YM , diacetil-DGT, DGT, cyanoguanidine, alkan-sulfatic acids and second metabolites of microorganisms have been studied and appeared to be effective as for potato plants recovering in vitro of the X-virus, so for protection of recovered and healthy plants in the field conditions and in covered soil. It has been shown that complex preparations essentially inhibited the distribution of the tobacco mosaic virus and tomato spotted wilt virus in the field conditions and their reproduction in the plant tissues in vitro and in vivo. The surfactant substances of microbial origin used in the complex preparations, had remarkable benefits due to their lower toxicity over synthetic ones and give new facilities in elaboration of antiviral active compositions for the practical usage.

One of the methods of control plants viral diseases is the use of chemotherapeutical preparations. Unfortunately, antiviral therapy still remains undeveloped . To present day any preparation is known among many chemical substances tested as inhibitors of phytoviruses [5], which could be successfully applied in viral diseases control of agricultural or ornamental plants.The main reason of this state is the specifics of virus' intracellular parasitism. At the same time, achievements of molecular biology, particularly of viral pathology of plants, give us hope on invention of the new effective antiviral preparations. The success in struggle with viral infections probably does not locate in area of traditional chemotherapy, but rather in area of non-traditional "biotherapy", which lies in looking for natural substances – modulators and inductors of natural steadiness, as well as in combining in one preparation of ecologically safe viral inhibitors with different mechanism of action.

We have shown before that the yeast mannans (YM) with the branched structure are the most effective antiviral preparations [6,2] and inductors or virus steadiness modulators [1] among microbial polysaccharides. It is also known that some surfactant substances (SS), particularly chemical analogues of lipids of cellular membranes – alkan-sulfatic acids [8], acts as inductors of virus resistance in plants and have different, than microbe glycans, nature of inducing action. Whereas analogues of uracil, especially 2-4-dioxohexahydro-1,3,5-triazine (DGT) and diacetyl-DGT, as well as cyanoguanidine inhibit early reproduction phases of phytoviruses[9].

In this work we tried to join in one preparation the virus resistance inductors, from one side, with traditional inhibitors of virus replication – from another, in order to achieve the highest possible antivirus effect.

Materials and investigation methods. The following investigation objects have been used: TMV (U_1) X-, M-, S- and other potato viruses, as well as tobacco plants (*Nicotiana tabacum* L.) of Immune 580 variety and its mutant, hypersensitive and susceptible to TMV respectively; stramonium plants (*Datura stramonium* L.) and potato of Extase and Slovianka varieties. Tested plants were grown in hothouse under natural conditions of lighting, humidity and temperature.

The branched yeast mannan (YM) preparation, obtained from *Candida maltosa* cells [2], and metabolites, mentioned above and graciously given by professor G.Shuster (Germany) have been studied. Substances were diluted (or emulsified) in water in different concentrations and applied separately or in mixtures.

The study was carried out in three stages.

At the first phase the preparations of different concentrations, their combinations and methods of use have been tested mainly in plants-indicators, which react on the viral infections with local necrosis, eg. in *D.stramonium, N.tabacum, G.globosa*, as well as in the sensitive to TMV mutant of the tobacco Immune 580 variety. Three variants of preparation use have been studied: 1) adding to virus inoculum for 30 minutes before inoculation of plants; 2) treatment of isolated leaves and leaf discs for 30-40 minutes before inoculation with TMV; 3) the same treatment of leaves and leaf discs during 30-40 minutes after inoculation. The latest variant was conditionally named as «therapy», although during that time only the deproteinization of virions took place, but the RNA replication did not happen yet [7].

Antiviral effect of preparation *in vitro* was estimated by number of local damages, which appeared on leaves on 5–7th day after inoculation. Experiments with TMV *in vivo* were made on leaf discs (ø 20 мм) or tobacco leaf-halves Immune 580 variety (sensitive to TMV lacking the gene N mutant). Tested substances were injected in the intracellular space of leaf parenchyma with the medical syringe. In control samples leaves and leaf discs were treated using the same method by water. Antiviral effect of preparation against TMV was estimated by results of determination of virus infectious titre in experiment and control samples on plants-indicators *D. stramonium*. For that aim the homogenates of tobacco leaf tissue in water (1:10) have been obtained, centrifuged (5000 rpm), supernatant liquid was put on the plant-indicators leaves by brush.

At the second phase the influence of the preparations on virus infection in potato crops *in vitro* has been studied. Potato handles of Extase and Slovianka varieties were used for microclones replication and plants regeneration. For the cultivation of handles the solid Murashige-Skoog media was used with the addition of complex preparations (experiment) and without it (control). Duration of the handles cultivation *in vitro* in different variants was 13-70 days, amount of passages was 1-3.

At the third phase the effect of preparations against TMV- and TSWV-infections in field conditions on tobacco Immune 580 variety and tomato Lyana variety has been studied. Two variants of prophylactic preparations use were tested: 1) 3-times spraying of seedlings with 7 days interval and immersing them into preparations for 30 minutes before planting; 2) 3-times spraying (with 7 days interval) of plants in field. Provocation of natural propagation of TMV-infection on tomato plants was carried out by stepchilding, periodically dipping hands in the viral suspension (40 μg/ml).

TSWV infection of plants was determined by sight, ELISA-test methods, bio-tests or it was detected by sight infection of the plants with TMV. Concentration of TMV antigene was determined by direct ELISA-test (sandwich-variant), using for this aim conjugates antibodies with peroxides or with acid phosphatase. Results of reaction were estimated with «Rider» scanner at the wavelength 490 nm.

Simultaneously the growth and development of plants were observed, taking into account phyto-toxic effects produced by the preparations, productivity of the field crops and glass-house cultures, yields structure, and other.

Results of determination of infection and titre of virus antigen were processed statistically estimating the reliability of difference between experiment and control with the Student criteria (t) [4].

Results and discussions. Using plants-indicators (*D.stramonium, N.tabacum, G.globosa*) it was chosen the most effective (by inhibition of TMV и X-potato viruses infections) combination of preparations and their concentrations /9/. Selected combinations of substances in optimal concentrations were tested against system TMV-infection in isolated leaves and leaf-discs of tobacco, sensitive to that virus (N-gene-lacked mutant of tobacco variety Immune 580). As result it was found that the most effective TMV inhibitors are yeast mannan and alkan-sulfatic acids consisting of emulsifier E-30 as for prophylactic so for therapeutic application (Fig. 1). The former were toxic for the plant tissue and damaged worked up leaves. Toxic effect of the emulsifier E-30 did not appear in the preparations mixture, where its concentration was 4 times lower, than at separate use. As a whole the complex preparation was more effective in comparison with others, especially as prophylactic preparation.

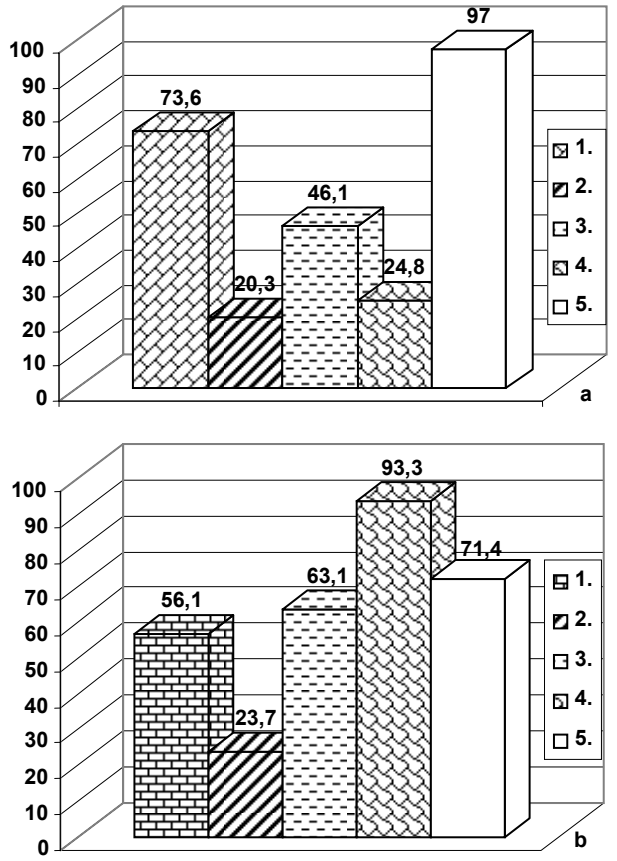

Fig1. The influence of yeast mannan and some antimetabolites on the reproduction of TMV in tobacco leaf at prophylactic (a) and therapeutic (b) investigation
Preparations: 1 – YM, 0,02%; 2 – CG, 0.1%; 3 - DA-DGT, 0,1%; 4 - E-30, 0,25%; 5 - YM (0,05%) + CG (0,025%), DA- 2 - CG, 0,1%; DGT, 0,025%, E-30, 0,0625%.

Tested preparations were also effective against system necrotic TSWV infection on tobacco, especially YM and its combinations with DGT, with DGT and cyanoguanidine. Preparations influence consisted in transformation of severe necrotizing symptomes of leaves to the light mosaics (data are not provided).

As alkan-sulfonic acids in the previous experiments were toxic to the plants tissues, we studied the second metabolites of Pseudomonas sp. (bioSS). As a standard we used in these experiments the emulsifier E-30 (alkan-sulfatic acids). Investigation of the bioSS activity against TMV infection has shown their effectivness in stramonium (Table 1). These substances were also active against X-potato viruses infection on *G.globosa* plants (data are not presented).

Comparative study of the bioSS and emulsifier E-30 against TMV on stramonium and *N.glutinosa* did not show any recognizable differences in antiviral activity of these preparations. At the same time their efficiency regarding reproductive capability of the plants was different. The bioSS did not reduce essentially the germination and germinating power of the tobacco seeds, whereas emulsifier E-30 greatly worsened these indexes.

Table 1. **The influence of microbial SS against infection of TMV *in vitro***

Preparations	Concentration%	Number of local lesion/leaf of datura		E/C, %
		Experiment (E)	Control (C)	
PS-17	1	33,9	140,4	24,1
	0,1	63,0	158,4	31,8
	0,01	50,4	72,0	70,0
	0,001	91.6	119,7	76,5
RL	1	39,5	119,6	33,0
	0,1	6,3	46,2	13,6
	0.01	139,6	180,2	77,1
	0,001	125,0	149,6	83,5
PS	1	209,3	123,0	170,0
	0,1	113,8	128,9	88,2
	0,01	137,4	142,2	96,6
	0,001	141,2	135,2	103,9

The time of TMV and preparation contact *in vitro* 30 min.

Thus second metabolites of pseudomonads may be useful as SS and can be applied as moderate viral inhibitors in complex antivirus preparations.

In previous investigations it was shown that yeast mannans and glucans contribute to recovery of potato from X-, M-, S-viruses during plants regeneration with meristem method *in vitro* (Kozar and al.,1996).

On the grounds of the previously conducted investigations it was formed two complex preparations, which compositions differed one from another: first contained the alkan-sulfatic acids as SS, second - the Pseudomonas metabolites. Other ingredients of the preparations were the same (Table 2).

Table 2. **Composition of preparations added to cultural medium for the sanitation of plants *in vitro***

Compositions	Preparation 1 (g/l)	Preparation 2 (g/l)
Yeast mannan (YM)	0,2	0,2
Diacetiyl-2-4-dioxohexahydro-1,3,5-triazine (DDHT)	0,05	0,05
Cianoguanidyne (CG)	0,05	0,05
Emulsifier E-30	0,1	–
PS-17	–	0,1

Our studies have shown that there were any remarkable deviations from the norm in the morphology of the potato plants-regenerators of Slovianka variety, obtained in the media with preparation 2 (Fig.2). At the same time there were noted remarkable anomalies in plants grown in the media with preparation 1, in particular: reduction of the root system, plants microsomia, leaves necrotizing and other. Meanwhile both preparations favoured to inhibition of reproduction and elimination of X-potato viruses infections from plant tissue. The preparation 2, which contained Pseudomonas metabolites, was more active. During continious cultivation of handles in the media with it the X-potato viruses' infections, according to ELISA-test methods results, has been completely eliminated on 49 day from the tissue and the plants brought into a healthy state (Fig.3). Concentration of the X-potato virus antigen in the plants-

regenerators has been also declined during the periodic passages of potato handles in new media with inhibitors (Fig.4). At that the concentration of the mature virions in the plants-regenerators has been decreased to the residual values, especially during the plants regeneration in the medium, which contained preparation 2.

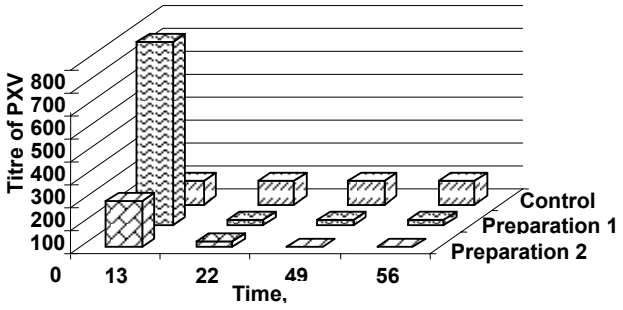

Fig 2. The influence of complex antiviral preparations on the reproduction of PXV in plants potato Slovianka during continuous cultivation with inhibitors *in vitro* (results of ELISA)

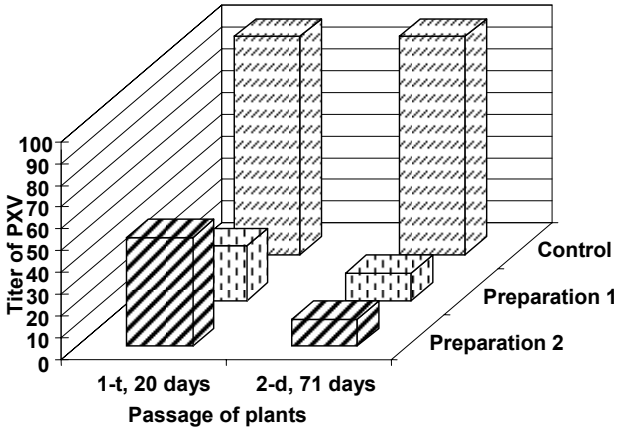

Fig 3. The influence of complex antiviral preparations on PXV antigen accumulation in plants potato sort Extas *in vitro* by passage of plants to new medium with inhibitors (results of ELISA)

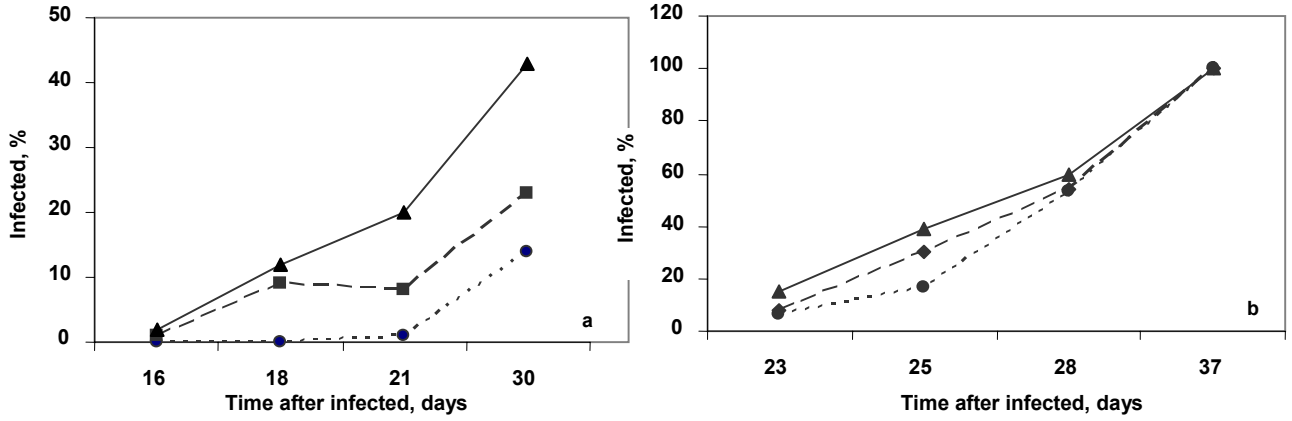

Fig 4. The influence of complex preparations to the TMV staggered tomato plants by spraying plants in field (a) and treatment of young plants (b) *(by resulte of visual account)*

Thus in described experiments the best one, by all figures among investigated preparations was that one which contained as SS microbial metabolites, but not alcansulfonic acid. This preparation, to our mind, has considerable prospect for the practical use as a remedy for the recovery of agricultural and ornamental cultures *in vitro*.

Substantial effect of complex preparations against TMV infection was shown as well at 3-times spaying of tomato plants in field with the3 days interval (Fig. 5). But treatment of seedlings was not enough for protection against infection of plants in field conditions, while antigene titre in the treated plants decreased essentially with different methods of preparations use (as during the seedlings spraying so at treating the plants in field).

We did not found important differences in the fruits reaping rape and the crop capacity of the control and experimental plants, while preparation 1, in comparison with preparation 2, slightly inhibited growth and development of plants in field.

Long-term investigations of polysaccharides bring us to thought that biologic activity of microbial glucans is conditioned on their primary structure [6]. In particular, the important peculiarity for the neutral yeast mannans is branching of molecule and considerable amount of 1-2-glicozid bonds in the side chains of polymer [2], and essential number of OH-groups at 3-d and 4-th carbonic atoms of mannopyranose, which are capable to form higher order structures with other biopolymers, particularly with lectine-like proteins, which are the base of the trigger mechanisms of protective reactions [1]. Diversity of activity of the elicitor macromolecules (like the YM) is also bound with the primary structure and gives the evidence of universality of processes of protein-carbonic detection and pathologic processes in the different host-pathogen systems.

With regard to glycolipids their biological role in complex preparations has not been clarified yet. It can be only supposed that as analogues of cellular membrane components they form rigidity of the surface of the object – the target of glicopolymer biological action. Thus glycolipids most probably prepare the field of interaction in the glycane-receptor system, stripping the targets and making them more accessible for the elicitors and other signal molecules, capable to the ligand interaction.

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T. Kalachova, stud., I. Krulko, PhD stud., A. Kharina, PhD.

STUDY OF ANTIVIRAL ACTIVITY OF METAL CONTAINING AND NON-METAL CONTAINING PORPHYRINS

Taras Shevchenko` Kyiv National University, Ukraine.

Antiviral activity of porphyrins №1 and №3, and their metal complexes №2 and №4 on model systems becteriophage-bacteria and virus-culture of plant tissues was studied. Dependence between the level of infection inhibition, substance concentration and presence of coordinate metal atom was determined. Aggregation of viral particles under the influence of porphyrins was shown. The effect of porphyrins on the extracted nucleic acid of virus was observed.

Introduction. Porphyrins are complicated heterocyclic substances, widely spread in nature (for instance, chlorophyll, hem and so on). In the cells porphyrins carry out many catalytic functions, are components of active centers of enzymes, electrons transferors. Synthetic analogues and modifications of porphyrins are actively used as anticancer, antibacterial and antiviral substances.

Some of the porphyrins have antiviral activity that makes them perspective precursors of new preparations. Among studied mechanisms of antiviral action of porphyrins is binding to the nucleic acids (degradation and block of replication) and interaction with proteins (blocking the receptors and enzymes of replication), photodynamic inactivation. Interaction with non-enveloped virions of Herpes simplex virus 1 (HSV1) is probably caused by electrostatic attraction among negatively charged capsid proteins and cationic structure of porphyrins [5]. Investigation of photodynamic inactivation of HSV1 showed the availability of virucidal and virostatic effects. Virucidal effect is connected with photosensibilization and further destruction of supercapsid of virion [6]. Virostatic activity was shown in inhibition of infective process, probably, due to binding of porphyrins to DNA with its further degradation. Direct dependence between the concentration of porphyrin and inactivation efficiency was observed.

Inhibitory activity of porphyrins on the respiratory-syncytium virus, adenoviruses, HIV was shown. One of the main mechanisms of their activity is binding to V3 loop of gp120 that blocks interaction of CD4-receptors and prevents both infecting of separate cell by virus and fusion of infected cells with healthy ones. It was also described that some of the porphyrins have the affinity to HIV protease and have the ability to specifically block its activity. DNAse activity and ability to photosensibiliize supercapsid with its further degradation was also studied for the investigated substances [7].

The activity of porphyrins on bacteriophages with both DNA and RNA genomes, with or without lipid coat was shown. Lipid layer underwent direct destruction after the interaction with studied porphyrins, as well there appeared a blocking of surface structure, but not their degradation. DNAse activity of the investigated porphyrins was shown on the extracted DNA, while DNA in the content of the virion stayed intact [8].

Object and methods of study. Activity of the porphyrins was studied on the model systems "bacteriophage-bacteria ('T4-' *Escherichia coli*») and virus-culture of plant tissues (Tobacco mosaic virus (TMV) – culture of tissues of *Nicotiana tabacum*»).

Investigated substances were presented as porphyrins and their respective metal complexes:

№1 –5-n-hexadecyl-10, 15, 20-tris (N-metyl-4-pyrydil) porphyrin tritozilat (Molecular weight (Mw)=789)

№2 – 5-n- hexadecyl-10, 15, 20-tris (N-metyl-4-pyrydil) porphyrinato Zn tritozilat (Mw=850)

№3 – 5, 10, 15 –tris (N-metyl-4-pyrydil) – 20-(n-nonil) porphyrin tritozilat (Mw=1267)

№4 – 5, 10, 15 –tris (N-metyl-4-pyrydil) – 20-(n-nonil) porphyrin Zn tritozilat (Mw=1292)

With the purpose to determine the titer of bacteriophage spot-test was held [1]. Concentration of the purified preparation of TMV that was used in work was studied using method of protein spectrophometry [3].

Mother solutions of the porphyrins had the concentration of 2mM/ml. Obtained solution was sterilized by the cold sterilization through bacterial filter.

In order to investigate antiviral activity of the substances in the system "phage-bacteria" was held the titration of phage by Grazia method with addition to *E.coli* cells culture of T4 that was previously incubated with 10, 20 and 40 μM concentrations of the substances at 4^0C during night. 200 μl of night culture of *E.coli* in LB medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) and 100 μl of phage with dilution 10^{-6} were poured into 0.7% agarized LB medium. Phage was not added to the control tubes. 0.7% agarized LB with bacteria and phage were sowed to the Petri dishes with 1.4% agarized medium and incubated during 24 hours in the thermostat conditions at 37^0C till the formation of negative colonies. In the experiment control of bacterial culture, to which phage and investiagated substance were not added and control of bacteria growth in presence of the largest concentration (40μM) of the porphyrin were used. Every investigation was held three times.

The next step was study of porphyrins effect on the process of phage absorption on the bacterial cell (method of counting of non-absorbed phage particles). 200μl of night culture of *E.coli*, 100μl of phage in the dilution 10^{-6} and 20, 10 and 5μl of mother solutions of porphyrins №1, №2, №3 та №4 (in order the final concentrations of the porphyrins were 40, 20 and 10μM) were added to 700 μl LB and incubated for 10 min at 37^0C. In the experiment control of bacterial culture to which phage and substance were not added and control of bacterial growth in the presence of 40μM concentration of porphyrin were used. Every experiment was repeated for three times.

Transmission electron microscopy was held according to the standard principles [4]. As the samples preparations of purified TMV and T4, treated with the substance №3 in the concentration of 10μM were used. As a control viruses untreated with the compound were used.

Investigation of porphyrins on plant regeneration. Fragments of leaves plates of sterile infected by TMV plants of *Nicotiana tabacum* were transferred to the hormone containing nutrient medium MS1 [2], which contained investigated porphyrins №1, №2, №3 and №4 in the concentrations 40, 20 and 10μM. As controls the explants from the plants infected with TMV and placed on the medium without substance were used. In order to study the toxic effect of porphyrins on plant tissues the explants from

healthy tobacco placed on the medium with 40μM concentration of porphyrins were used. In 30-40 days the samples for detection of virus quantity using indirect enzyme-linked immune sorbent assay (ELISA) and electrophoresis of protein in polyacrilamid gel according to Laemmle modification, were selected from the obtained callus.

For the estimation of interaction of antigen determinants of TMV and phage T4 *in vitro* indirect ELISA assay was also used. As the samples for ELISA purified preparations of T4 and TMV treated with substances №1, №2, №3 and №4 in the concentrations of 40, 20 and 10μM were used. Control samples were not treated with the porphyrins. The interaction of porphyrins with proteins of TMV *in vitro* was detected using electrophoresis of proteins in poliacrylamid gel according to Laemmli modification. As the samples for electrophoresis of proteins were used preparations of TMV treated with 40 and 10μM concentrations of porphyrins, as well as virus not treated with the investigated substances.

The activity of porphyrins on the nucleic acid was studied using the method of electrophoresis of nucleic acids. As the samples purified DNA of phage T4 treated with the substance N3 in the concentrations 40 and 10μM and T4 DNA untreated with porphyrins were used. Nucleic acid was extracted with utilization of sodium dodecyl sulfate (SDS).

The statistical data processing obtained from ELISA was held using the standard deviation [4].

The percentage of inhibition of infecting *E.coli* by phage T4 was estimated using the formula:

$$A=(1-No/Nk) \times 100\%,$$

where N_0 - quantity of negative colonies, Nk – quantity of negative colonies in control.

Results and discussion. In the work the working bacteriophage T4 titer of 10^{-5} та 10^{-6} were used. According to the results of protein spectrophotometry the concentration of TMV included 1.8 mg/ml.

Using the method of Grazia titration it was shown that the substances №1, №2, №3 and №4 are able to inhibit infection process that was shown in the reduction of quantity of negative colonies on the *E.coli* lawn to which the phage treated with different porphyrins concentrations was added. The substances №1, №3 showed the strongest inhibitory effect in the minimal concentration (10μM). In the contrast №2 – in the largest concentration (40μM), and the substance №4 showed the significant activity independently from the concentrations. For the porphyrins №1 and №2, №3 and №4 the difference in activity can be connected to the presence of coordinate metal atom. The structure of porphyrin №4 is thus that the appearance of metal in the molecule increases the inhibitory effect, hence it doesn't cause the changes of its dependence from the concentration. Reverse dependence of efficiency from the concentration for the substances №1 and № 3 can be linked to the features of aggregation of porphyrin molecules in solution (Table 1.).

Table 1. **Inhibition of viral infection by the porphyrins №1, №2, №3 and №4 in the model system phage-bacteria**

	Inhibition of viral infection in the model system phage-bacteria, %			
Concentration of substance	№1	№2	№3	№4
10μM	95±2	10±3	80±1	99±2
20μM	90±1	10±2	70±2	98±2
40μM	70±1	54±2	40±1	97±3

Absorption test showed that both non-metal containing substances №1 and №3 and metal-containing porphyrins №2 and №4 are able to inhibit the process of phage absorption on the bacterial cells. The substances №1, №3 and №4 inhibited absorption in direct proportion to the concentration; hence for the substance №2 such dependence was reverse. The effect of absorption inhibition by the porphyrins can be connected to the blocking of proper structural phage fragments that are in charge of absorption.

Electron microscopy investigations showed that the studied porphyrin №3 has the ability to aggregate the viral particles of TMV (Fig. 2).

Probably the aggregation of viruses is caused by the electrostatic interaction between the porphyrin and viral proteins (Fig.1).

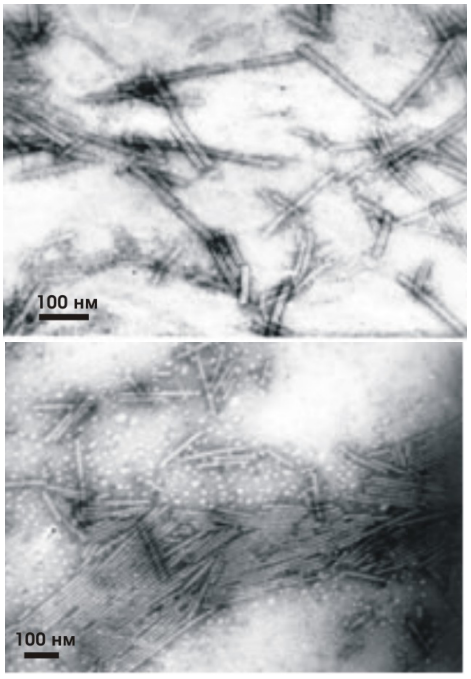

Fig. 2 Electron microscopic picture of TMV without treatment with the substance (1) and after treatment with 10μM concentration of the substance №3 (2).

While studying of porphyrins influence on the regeneration of plants *Nicotiana tabacum* from the callus tissue visual observations showed that the porphyrin №3 stimulates formation of callus in the concentration of 10μM in the contrast to larger concentrations, which mostly didn't cause the formation of callus. The substances №1, №2 and №4 stimulated the formation of callus in all three investigated substances. The porphyrins didn't show significant toxic influence on the plant material.

For the studying of quantity of virus in callus obtained from the infected with TMV explants that were grown on the medium with substances in 40, 20 and 10μM indirect ELISA was held. Results prove the antiviral activity of the porphyrins, thus the substances №1 and №2 showed the significant efficiency in all investigated concentrations, №3 showed higher activity in the concentration of 10μM, while the substance №4 – in 40μM.

In order to prove the ELISA results electrophoresis of homogenate from callus was held. Results of electrophoresis of proteins from callus obtained from the explants from infected by TMV plants on the medium in the presence of substances №1, №2, №3 and №4 show that the content of specific for TMV protein in the samples with porphyrins was significantly lower comparing to the samples without adding of porphyrins. Substances №1 and №3 showed higher activity in the smaller concentrations, while the porphyrins №2 and №4 – in higher concentrations, that matches ELISA results.

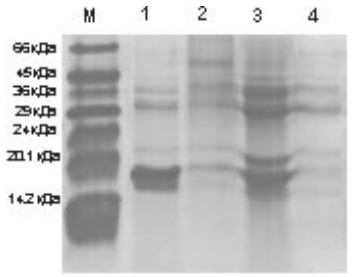

Fig.3 Electrophoresis of samples of homogenate from callus *N.tabacum* obtained on the medium in the presence and without substance №3

M – Marker proteins
1-sample from callus from *N.tabacum* infected with TMV
1-sample from callus from *N.tabacum* infected with TMV obtained on the medium with 10μM of substance №3
1-sample from callus from *N.tabacum* infected with TMV obtained on the medium with 40μM of substance №3
1-sample from callus from healthy *N.tabacum*

With the purpose to study the influence of porphyrins on antigenic determinants of TMV on bacteriophage T4 indirect ELISA was held. It was shown that the substances №1, №2, №3, and №4 cause the reduction of percentage of interaction of specific monoclonal antibodies with phage T4. The substances №2 and №3 caused more noticeable inhibition in smaller concentrations, and porphyrins №1 and №4 – in larger ones.

Most likely the porphyrins block the antigenic determinants of phage T4 that can be one of the reason of inhibition of binding of serum with phage, absorption and as a result the inhibition of infection development.

From other side ELISA results for the detection of interaction of porphyrins with TMV *n vitro* didn't show the reduction in interaction of antibodies with virus in all three concentrations. In order to investigate the influence of porphyrin on viral proteins *in vitro,* TMV was treated by two (10 and 40μM) concentrations of porphyrins, incubated at 4^0C during night and used as the samples for proteins electrophoresis in polyacrilamid gel. Results of electrophoresis show that treatment with the substances in different concentration doesn't influence the electrophoretic movement of TMV protein and doesn't cause destruction of the primary protein structure.

For the study of porphyrins activity on the nucleic acid of bacteriophage T4 extracted DNA was treated with 10 and 40μM concentrations of porphyrin №3 and undergone the electrophoresis of nucleic acids in agarose gel. While interacting of phage DNA with porphyrins in larger concentrations the reduction of electrophoretic movement of nucleic acid is observed. May be it can be connected with change of DNA charge as a result of binding to porpnyrins.

Conclusions IThe antiviral activity of porphyrins №1and №3 and their metal containing complexes №2 та №4 on model systems phage-bacteria and virus-culture of plant tissues was shown. Dependence between the level of infection inhibition, substances concentration and presence of coordinate metal atom is observed. The aggregation of viral particles under the influence of porphyrins that most likely influences the absorption and virus entry to the cell, is described. The activity of porphyins on the extracted DNA of virus was shown.

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UDC 632.38

T.B. Kastalyeva[1], PhD, K.A. Mozhaeva[1], PhD., N.V. Girsova[1], PhD, S.M. Thompson[2], J.R. Clark[2], R.A. Owens[2] PhD.

PRIMARY STRUCTURES OF FOUR RUSSIAN *POTATO SPINDLE TUBER VIROIDS*

[1] *Russian Research Institute of Phytopatology (VNIIF), Moscow Region, Russia;*
[2]*Beltsville Agricultural Research Center, Beltsville, MD USA.*

Analysis of primary structures of four Russian isolates of Potato spindle tuber viroid showed their distinctions from all those having been registered earlier in the GenBank as well as from one another. Depending on the primary structure, the isolates induced intermediate or mild symptoms on tomato plants.

Introduction. In the early 1970's, the causal agent of a potato disease known in Russia since the early 1930's as "gothic" disease was identified as *Potato spindle tuber viroid* (PSTVd) [2, 3] . Viroids are the ultimate parasites – small, highly structured, circular RNA molecules that lack the protective protein capsid and mRNA activity characteristic of plant viruses. Disease caused by PSTVd was responsible for significant losses of potato yield in Russia for a number of years. So, in the 1980's and 1990's, the yield and quality of Russian seed potatoes declined dramatically, leading to an overall loss of quality for several widely planted cultivars. PSTVd was shown to be a cause of potato degradation in many Russian regions [1]. In spite of the wide distribution of the pathogen throughout Russia, the molecular structure of Russian PSTVd isolates has remained unknown. As concerning host-plants, PSTVd infection may be latent or results in symptom disease ranging from mild to severe in intensity.

Materials and methods. PSTVd RNA was isolated from leaves of potato plants showing PSTVd symptoms and maintained as PSTVd collection for many years. Overlapping RT-PCR products [4] were generated from PSTVd isolates maintained using two pairs of primers: PSTVd180F (5′-TCACCCTTCCTTTCTTCGGGTGTC-3′) + PSTVd179R (5′-AAACCCTGTTTCGGCGGGAATTAC-3′) and PSTVd112F (5′-ACTGGCAAAAAAGGACGGTGGGGA-3′) + PSTVd359R (5′-AGGAACCAACTGCGGTTCCAAGGG-3′).

Uncloned PCR products were undergone automated sequence analysis.

Results. By 2006, collection of PSTVd isolates of Russian Research Institute of Phytopathology has included about 30 PSTVd isolates. Some of them were presented in the form of partially purified low molecular weight RNA enriched with PSTVd having been stored at -20°C since the 1980s or 1990s. The other PSTVd isolates have been maintaining in potato tubers infected with PSTVd annually reproduced in field since 1990s. In 2006, the complete nucleotide sequences of four Russian PSTVd isolates were determined and then included in the Subviral RNA database (http://subviral.med.uottawa.ca/cgi-bin/home.cgi). Simultaneously, symptoms that these PSTVd isolates induced on indicator tomato plants var. Rutgers were determined. These isolates were the following: Onega-Premier-94, Niva-95, Bugry-97 and Samara 97-3. Names were given to them in accordance with the place of their origin. So, isolate Samara 97-3 originated from Samarskaya Province (Volga-river region), and the other three isolates originated from Leningragskaya Province (Northwestern region), therefore the latter received their names in accordance with the farm's name where potato tubers infected with PSTVd were taken from (Bugry, Niva, Onega-Premier). Two ciphers following the proper isolate's name designate the last ciphers of the year when infected potato tubers began to be maintained in VNIIF. The last cipher in the name Samara 97-3 means that it was one of some isolates received from Samarskaya province in 1997 (Table 1).

Table 1. **Some characteristics of Russian Isolates.**

#	Isolates	Originating from Russian Region	Potato varieties used for viroid maintenance	Symptoms on tomato plants
1	Onega Premier-94	Northwestern	Nevskii	Intermediate-type
2	Niva-95	Northwestern	Lugovskoi	Intermediate-type
3	Bugry-97	Northwestern	Lugovskoi	Intermediate-type
4	Samara-97-3	Volga-river	Kinel'skaya rosa	Mild

Sequence of each Russian isolate was compared with that of so called Intermediate isolate from Beltsville (USA) having been registered in GenBank as PSTVd018 # V01465 (Table 2). The same was made regarding symptoms induced on tomato plants (Fig.1, 2).

Sequence of PSTVd isolate 'Onega-Premier-94' differed from the PSTVd Intermediate isolate by one substitution in conservative region of PSTVd molecule (120 A→U) and one deletion in variable region (123 A del). It was registered in GenBank under number PSTVd123 # EF 044304 (Table 2).

The second isolate, Niva-95, had the same two distinctions from Intermediate isolate, and in addition, it had insertion of T in 141a position; the registered number in GenBank was PSTVd124 # EF 044302.

The third isolate 'Bugry-97', like isolate Onega-Premier-94, had two changes in its primary structure as compared to Intermediate isolate, but as distinct from the latter, adenine in position 120 was substituted by cytosine. It had deletion of A in position 123 too.

All three isolates induced intermediate-type symptoms on potato plants after inoculation (Fig. 1,#3; Fig. 2 ##1, 2 and 3).

Primary structure of the fourth PSTVd isolate 'Samara 97-3' had some more changes than that of others. In addition to changes described for isolate Bugry-97, there were two changes in the pathogenic region in comparison to Intermediate PSTVd018 isolate and all three other Russian's isolates: substitution (310 A→T) and insertion (313a T), and one substitution C→U in position 256 in the conservative region. This PSTVd was registered as PSTVd122 # EF 044305, and gave rise to mild symptoms on tomato var. Rutgers (Fig. 2, #4).

Table 2. **Comparison of sequences of VNIIF's PSTVd isolates with that of the Intermediate PSTVd isolate (PSTVd018 GenBank # V01465, Beltsville, USA).**

#	Isolates	Changes in sequence of PSTVd			Registration in GenBank
		Region of PSTVd			
		Pathogenic	Conservative	Variable	
		47 – 73 287 - 314	74 – 120 241 - 286	121 – 148 213 - 240	
1	Onega-Premier-94	none	120 A→U	123 A del	PSTVd123 GenBank # EF 044304
2	Niva-95	none	120 A→U	123 A del, 141a U insert	PSTVd124 GenBank # EF 044302
3	Bugry-97	none	120 A→C	123 A del	PSTVd125 GenBank # EF 044303
4	Samara-97-3	310 A→ U, 313a U insert	120 A→C, 256 C→U	123 A del	PSTVd122 GenBank # EF 044305

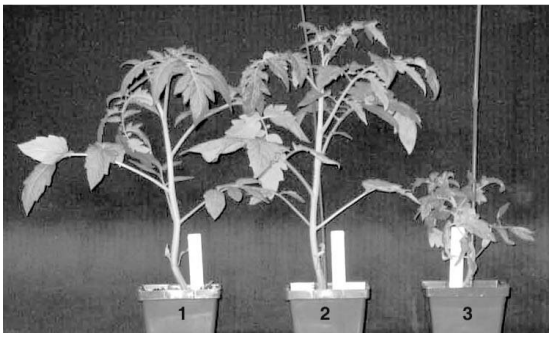

Fig. 1. Tomato var. Rutgers at 27 days after inoculation with: 1 – Control (H_2O); 2 - PSTVd Mild; 3 – PSTVd Intermediate;

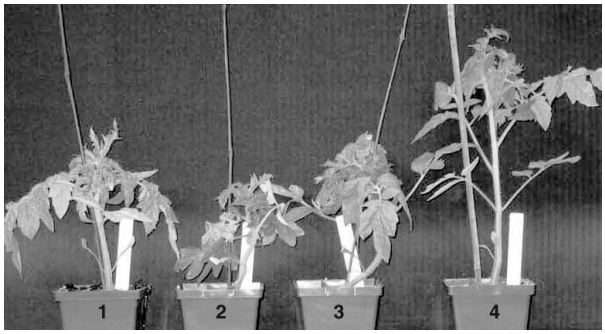

Fig. 2. Tomato var. Rutgers at 27 days after inoculation with: 1 – PSTVd isolate Niva-95; 2 – PSTVd isolate Bugry-97; 3 – PSTVd isolate Onega-Premier-94; 4 – PSTVd isolate Samara-97-3.

Discussion. There was no found full identity of primary structure among each four Russian PSTVd and any other earlier known PSTVd. All four Russian isolates have one joint distinction from Intermediate isolate: deletion adenine in position 123 (variable region). Three isolates originated from Leningradskaya province (North-western region) were closely related: All of them had substitution of adenine in position 120 (conservative region), but in the case of isolate Onega-Premier-94 and Niva-95 adenine was replaced by uracyl and in the case of isolate Bugry-97 by cytosine.

Isolate Samara 97-3 was received from the Volga-river region that is far distant from Leningradskaya province. In comparison with Intermediate isolate the isolate Samara 97-3 had the same changes in variable and conservative region of RNA molecule as isolate Bugry-97, but in addition to those, it had 2 changes in variable region. Adenine in position 310 was replaced by uracyl, and additional uracil was inserted in position 313a.

When changes affected conservative and variable regions, a character of host plants response to infection remained the same, that is, intermediate-type. When changing took place in pathogenic region (310 A→T, 313a T insert), disease induced mild symptoms. Changes in pathogenic region produced more stable double-helix structure (Figure 3).

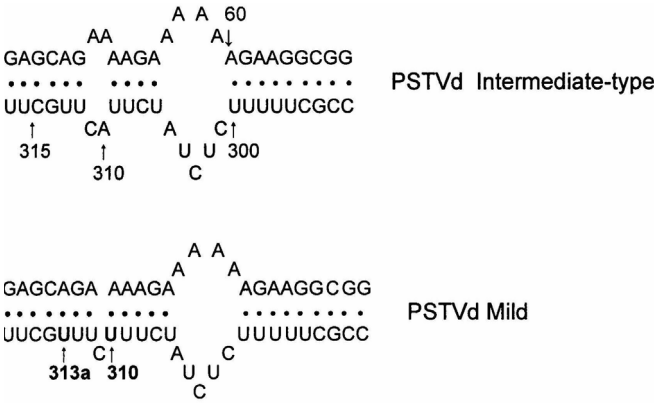

Fig. 3. Fragments of PSTVd sequence of pathogenic region (44-68, 292-317) for intermediate-type and mild strains of PSTVd (see *B. Lewin. Genes VI. Oxford – N.Y. – Tokyo, Oxford University Press, 1997. P. 145*).

When we say about severe, intermediate or mild symptoms, usually the matter concerns symptoms on tomatoes as indicator plants (cv. Rutgers), because symptoms on potato plants may differ. If PSTVd infected potato has been maintaining for many years, accumulated infection of mild PSTVd strains may induce intermediate or severe symptoms.

Additional studies with a wider range of Russian isolates of PSTVd are currently underway.

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UDC 578.85/.86

N. Birisik, H. Fidan, S. Baloglu

PLANT VIRUSES AS BIORESOURCES IN TURKEY AND ESTABLISHMENT OF TURKISH PLANT VIRUS COLECTION

[1.] Plant Protection Research Institute (PPRI), 01321.Yureğir. Adana / TURKEY
[2.] Plant Protection Department. Agr.Fac.Univetsity of Cukurova. Balcali, Yuregir. Adana / TURKEY

Turkey is known as motherland for many plants. Research/study show that number of plant viruses and virus-like agents are present in Turkey and some of them firstly have been founded in turkey such as CCDV. There is need to conserve virus and virus-like pathogen as bioresources in a suitable way and facilitate of making further study for developing continual and successful control strategy against plant viruses. An in-situ collection started to be constructed from 2006 with collecting genetic resources of virus and virus-like agents. For this aim a general survey and biological, serological, histological and molecular detection activity is performing and then the founded agent will be conserved in green and screen houses at PPRI in Adana. At the moment, more than 10 virus and virus-like agents have been founded, detected and placed into collection.

Introduction. Virus and virus-like pathogens are world wide distributed and generally cause severe loses and economical damage during plant production. The control of these diseases is based on identification and diagnosis of the causal agents. Many research activities have been taken for this reason and many virus and virus-like diseases have been reported from Turkey. There is need to conserve this type of pathogen in a suitable way and make further study for developing continual and successful control strategy.

As well known is a crucial point to have genetic recourse of the viruses for the scientific work and this source has importance from strategically point of view. There is a big need to have a virus and virus like diseases collection for Turkey because of Anatolia is not only one of the most important gene sources for plant spices but also for the plant pathogens.

Since five decade years many scientific study has been done on plant viruses and many plant viruses have been reported from different locations on different hosts in different time. Some of those reported viruses are presented at table.1.

Table 1. **The list of some plant virus diseases reported from Turkey mainly on woody hosts.**

Name of virus	Genus	Acronym	Author & date
Citrus tristeza	*Closterovirus*	CTV	Baloğlu, 1988.
Grapevine leaf roll-associated	*Closteroviruses*	GLRaV1,2,3,7	Köklü 1999.
Citrus psorosis virus	*?*	CPsV	Güllü, 1987.
Citrus chlorotic dwarf	*?*	CCDV	Korkmaz, 1994.
Christacortis	*?*	CCV	Yılmaz, 1998.
Amasya cherry diseases	*Partitivirus*	ACD	Covelli *et.* all, 2004.
Apple chlorotic leaf spot	*Trichovirus*	ACLSV	Cağlayan, 2003.
Grapevine A	*Trichovirus*	GVA	Ciğsar *et* al. 2002.
Prune dwarf	*Ilarvirus*	PDV	Cağlayan, 2003.
Prunus necrotic ring spot	*Ilarvirus*	PNRSV	Cağlayan, 2003.
Apple stem grooving	*Capillovirus*	ASGV	Birisik *at* all., 2006.
Apple stem pitting	*Foveavirus*	ASPV	Birisik *at* all., 2006.
Pepper Mild Mottle Virus	*Tobamovirus:*	PMMV	Güldür et al., 1994.
Tomato mosaic	*Tobamovirus*	ToMV	Yılmaz and Davis, 1984.
Cucumber Vein Yellowing Virus	*Ipomovirus*	CVYV	Yılmaz, 1989.
Cucumber mosaic	*Cucumovirus*	CMV	Yılmaz , 1976.
Lettuce big-vein	*Varicosavirus*	LBVV	Erkan *at* all., 1985.
Tobacco etch potyvirus	*Potyvirus*	TEV	Yılmaz *et* al. 1983.
Potato virus Y	*Potyvirus*	PVY	Ekbiç *et.,* al.,1997.
Plum pox	*Potyvirus*	PPV	Cağlayan, 2003.
Bean common mosaic	*Potyvirus*	BCMV	Güzel ve Arlı-Sökmen, 2003.
Bean common mosaic necrosis	*Potyvirus*	BCMNV	Güzel ve Arlı-Sökmen, 2003.
Watermelon mosaic 1	*Potyvirus,*	WMV-1	Yılmaz and Davis, 1985.
Watermelon mosaic 2	*Potyvirus*	WMV-2	Erdiller and Ertunç, 1988.
Zucchini yellow mosaic	*Potyvirus*	ZYMV	Yılmaz and Davis, 1985.
Eggplant mottled dwarf	*Nucleorhabdovirus*	EMDV	Palloix, *et al.,* 1994.
Cucurbit Aphid Borne Yellow Virus	*Polerovirus*	CABYV	Fidan, 1993.
Alfalfa mosaic alfamovirus	*Alfamovirus*	AMV	Fidan, 1993.
Squash mosaic	*Comovirus*	SqMV	Yılmaz and Davis, 1985.
Cauliflower mosaic	*Caulimovirus*	CaMV	Erkan *et al.,*1991.
Lettuce big-vein	*Varicosavirus*	LBVV	Döken *et al*., 1993.
Grapevine fleck virus	*Maculovirus*	GFkV	Köklü, 1998.
Tobacco ringspot	*Nepovirus,*	TRSV	Fidan, 1993.
Tomato black ring	*Nepovirus*	TBRV	Fidan, 1993.
Arabis mosaic	*Nepovirus*	ArMV	Akbaş and Erdiller, 1993
Grapevine fan leaf	*Nepovirus*	GFLV	Akbaş and Erdiller, 1993.
Strawberry latent ring spot	*Nepovirus*	SLRSV	Akbaş and Erdiller, 1993.
Grapevine Anatolian ring spot virus	*Nepovirus*	GARSV	Gökalp et al, 2003.
Cherry leaf roll	*Nepovirus*	CLRV	Çağlayan *et al*., 2004.
Grapevine virus	*Nepovirus*	GNV	Köklü, 1998.

Materials and methods. Plant virus isolates which are collected during the survey activity and some other isolates which have been accepted from other research institute and universities. Herbaceous and woody indicator plants have been used for mechanical inoculation, woody indexing and for grafting. ELISA reagent and ELISA reader used for serological detection of viruses. RNA extraction kit (Promega SV Total RNA Isolation System), PCR thermocyler, gel visualizing system for molecular assays. Screen house and green houses which are present in APPRI, for producing plant material, and to keep infected material in-situ. Climatic rooms have been used for plant production activity and maintain some virus isolates which can be spread out to nature via insect vector or some way else. Sterile growing media for indicators, Carborandum dust, grafting knife, different type of label, grafting wax, mortar, liquid nitrogen, air-conditioner, cheese clothes, nicotine, different germination media and hormones. Some insecticides, disinfectants, fertilizer, ice etc.

I. Random surveys

Random surveys were carried out during the all field visits in case of research activity and also during the pest control surveys. Samples have been taken by visual observation of the suspected plants.

II. Targeted specific surveys

Location targeted surveys have been performed according to the plants and pathogens up-to their best time for symptom expression and good time for testing. Collected samples have been taken form different part of suspected plants (Leaf, shoots, flowers, fruits) and transferred to the laboratory in appropriate conditions (into a plastic back in the ice box) for using in testing methods. Meanwhile some plant cutting included buds have been collected and conserved for grafting.

III. Provide plant seeds and indicator plants

Indicators plants and their seeds have been supplied form different research Institutes and universities such as Yalova Atatürk horticulture research institute and University of Cukurova.

IV. Breeding of seed and indicator plants

Mature plant seeds maintained in torf as one layer seed and one layer torf at 0-4.4 $C^{o,}$ for 120-130 days and then they sowed in 100x50x25x cm size boxes. When seed germinated they have transplanted to plastic bags or pots which include sterile growing media include sand, torf and soil in (1:1:1) mixture rate. Herbaceous plants have been produced in climatic rooms in conditions of 8/16 hour Light/dark period under 3000 lux supplemented light and free from any insect and mite infection.

V. Samples taking from inoculum sources

The samples taken form different site and different part (leaf, shots, bark and fruits) of suspected trees and samples will be transferred in plastic bags at 4 C^{o} to laboratory. Samples will be maintained at 4 C^{o} and then tested as soon as possible by serological and molecular means. Cuttings which have been used for biological assays, grafting for inoculation and for rooting have be wrap up with humid serviette paper and keep at the 4 C^{o} until use.

VI. DAS-ELISA

DAS-ELİSA is performed by using different commercial (Agritest, Bioreba, Loewe) antisera kits. ELISA tests done mainly according literature [4] with some differences advised by antisera producer companies. Absorbance is read after 15 min, 30 min, 60 min, and 90 min, using automatic plate reader at 405 nm absorbance (Titertek Multiskan plus MKİİ reader).

VII. Mechanical Inoculation

Sap transmissions activity has carried out using 8 herbaceous hosts belonging to 5 different families. Young or mature leaves from infeted plants, are come from the survey, are used for sap inoculation to different host plants as shown in Table 2. The leaves are extracted in 0.05 M phosphate buffer (pH 7.0). The herbaceous plants are kept at 24-27°C and daily inspected for specific symptom [20].

Table 2. **Herbaceous test plants are used in mechanical inoculation**

Nicotiana accidenatlis
Nicotiana tabacum
Phaseolus vulgaris
Vigna unguiculata
Cucumis sativus
Chenopodium amaranticolor
C. quinoa
C. album

VIII. Biyological Indexing

Indexing can be defined as any test that reproducibly assess the presence and absence of a transmissible pathogen or identify a disease on the basis the reactions induced on specific indicator plant. Woody indicator plants some other plants suitable for a certain virus diseases have been inoculated by patch and bud grafting [8].

Table 3. **List of woody indicator are used for indexing**

Mexican lime
Sweet orange
Sour orange
Prunus persiaca cv. GF305
Malus sylvestris cv. R12740-7A
Malus sylvestris cv. Virginia Crab
SPY 227
Lord Lambourne

IX. RNA extraction from the plant tissue and PCR assays

Total RNA extraction was performed by using commercial isolation kits. Isolation was done according to manufacturer advising guide (Promega SV Total RNA Isolation system) and [17]. Mainly plant phloem and leaf tissue was used. Isolated tRNAs were used in RT (Reverse transcriptase) assays in presence of M-MLV reverse transcriptase. Produced cDNA was used in PCR for amplification. PCR reactions was prepared according to each viruses based on the scientific literature. Primers were designed by IONTEK ltd. şti. All other components of the PCR such as Taq-polymerase, dNTPs, buffers were used from commercial (all from Promega).

PCR products were visualized by electrophoresis in 1.5 % agarose gel stained with 0.5 ug/ml of ethidum bromide. Basic RNA isolation and cDNA production was done according [17].

X. Maintain and observation of the infected plants in greenhouse and screen house

The viruses which are subjected to the quarantine and has potential to spread out form the collection such as CTV and PPV have been placed in the climatic rooms where have no any direct open and locked usually. Viruses have potential to spread by any natural means such pollen like PNRSV have been placed in greenhouse. If there is no any natural transmission possibility for a virus such as ASGV and ASPV they have been placed in screen house.

Rooted cutting used for some plants are suspected to be infected with any viruses such olive and fig which have no any special plant indicator for virus testing. 3-5 infected cuttings (about 30 cm long) have been taken and dipped into 3000 ppm IBA (Indol butyric aside or Naphthalene acetic aside) solution to incite rooting. Rooted cuttings transformed to plastic bags (25 x 15 cm) for adaptation to the soil.

All plants either grafted or naturally infected, they have been weekly observed for the presence of the symptoms expression after they placed in the in-situ collection.

During the weekly control, all of the plants also have been controlled for the presence of any pest and diseases. In any doubt of pest infection required pesticides have been sprayed.

Results and discussion. After two years survey more than thousand plants were visually observed and 231 suspected plants were sampled. 196 samples were tested by ELISA for several viruses of fruit trees. RT-PCR assay were proceed for 19 samples out of 231 which gave suspected result in ELISA or had no any result in ELISA even they have some symptoms indicated that virus infection. During two years surveys 7 different viruses have been founded, tested and placed in the screen house or green house according their etiology. Those are Citrus tristeza *closteravirus* (CTV), Prunus necrotic ring spot *ilarvirus* (PNRSV), Apple chlorotic leaf spot *trichovirus* (ACLSV), Apple mosaic *ilarvirus* (ApMV), Apple stem grooving *capillovirus* (ASGV), Apple stem pitting *foveavirus* (ASPV) and Fig mosaic virus disease.

Apart form the samples have been taken during activity, 29 samples have been accepted via phtytoclinc laboratory to test for any virus infection. Form those plants Prune dwarf *Ilarvirus* (PDV) have found in a local prune variety. This PDV isolate have been grafted on virus free GF-305 indicator and placed in screen house. Citrus psorosis virus (CPsV) also has been found in an infected grapefruit sapling came to institute by the grower. This CPsV isolate have been tested by ELSA and then grafted on sweet orange and both infected plants placed in screen house.

Plum pox potyvirus PPV infected buds were provided from University of Cukurova and grafted on four individual GF 305 plants and placed in screen hose under the isolation box.

In the APPRI in-situ collection 10 plant viruses are present as grafted on woody plant neither source plant nor indicator. The viruses and number of infected plant is shown at table 4. To well-care of plant and to keep them form any other infection is a very hard, but to have live infected plants is useful goods for performing detection tests and observation virus symptoms.

Table 4. **Virus diseases are present in the APPRI in-situ collection**

Virus	Hosts	Indicator	Tested by,	Number of plant
CTV	Citrus spp.	Mexican lime	1,2,3	2
CPsV	Citrus spp	Sweet orange, grapefruit	1,2	4
PNRSV	Prunus spp Rosa sp.	*Prunus persiaca* cv. GF305	2,3	5
PDV	Prunus spp	*P. persiaca* cv. GF305	1,2	2
ACLSV	Malus spp. Prunus spp.	*Malus sylvestris* cv. R12740-7A	1,2	4
PPV	Prunus spp	*P. persiaca* cv. GF305	1,2,3	4
ASGV	Malus spp.	*Malus sylvestris* cv. Virginia Crab	2,3	8
ASPV	Malus spp. Pyrus spp.	SPY 227	1,2	8
ApMV	Malus spp.	Lord Lambourne	1,2,3	3
FigMV	Ficus spp	Ficus carica	1	3

1: Detected by visual observation
2: Tested by ELISA
3: Confirmed by RT-PCR

Construction of an in-situ collection means a killing pathogen is keeping in a plant which is diseased and it may die. It needs to spend a big effort to for production of virus free indicators/hosts, for continuously re-inoculation and to care about the inoculated plant. Isolation of the virus free material and infected plants from the internal and external pathogens is very vital. Detection of the single infection in a certain individual plan is very hard for some spices which have tendency to be infected with more than one virus like grape.

If a quarantine viruses such as CTV and PPV are detected is very difficult and risky to manage and to keep it in a safe place for avoiding any diffusion. Some viruses are very latent like ASGV so that it is very hard about to be sure that artificial inoculation to the indicators is succeeded.

Viruses are pathogens and also they are biorisources from the nature. To have enough number of viruses and enough isolates of a certain viruses will give a great opportunity to work and to use them in developing detection methods, testing for cross protection and use in molecular biology.

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T. Rudnieva, PhD student

VIRUS DISEASES OF VEGETABLE CROPS IN GREENHOUSE CONDITIONS IN UKRAINE

Taras Shevchenko` Kyiv National University, Ukraine.

Spreading of viruses of vegetable group in greenhouse conditions in Ukraine were analyzed. Serological and electronic microscopy data identified 3 viruses infecting vegetable crops: Cucumber green mottle mosaic virus (CGMMV), Impatiens necrotic spot virus (INSV) and Turnip mosaic virus (TuMV). From these, two viruses: INSV and TuMV have been detected in Ukraine for the first time.

Introduction. Vegetable-growing in greenhouses is one of the most intensive forms of agriculture that gives the possibility to obtain significant yield from square unit almost all year round. Hence specific greenhouse conditions: absence of crop rotation, unchangeable utilization of soils, absence of the species and hybrids with group resistance to diseases, artificially created microclimate in greenhouse and other factors cause favorable conditions for mass development of different bacterial, fungal and nematode diseases [2]. But the most serious danger for the greenhouse is provided by viruses, as they cause about 50% of yield loss, and in some cases the yield is lost completely [3]. For today about 18 species of viruses of different taxonomic affiliations are registered on the cultures of cucumbers, peppers and tomatoes in greenhouse conditions. These viruses are transmitted by seeds, insects, soil solution and throught the contact of leaves. Lately, new observations indicate definite widening of areals for major virus infections, spread of complex and latent diseases, appearance of their novel forms with altered pathogenicity [4]. Studying of species diversity of viruses and ways of their propagation will give the possibility to limit the area of their spreading and as a result to increase the yield productivity of crops.

Thus, our research was focused mainly on the analysis of modern situation of spreading of viruses that infect vegetable grops in greenhouses conditions in Ukraine.

Object and methods of investigation. The object of the study were the cucumbers, peppers, egg-plants and tomatoes with typical viral symptoms that have been obtained from the different greenhouses of Ukraine.

Sample analysis for presence of virus antigens has been carried out employing sandwich and indirect ELISA modifications. ELISA has been carried out in polystyrene plates "Labsystem". Results were registered using 'Dynatech' reader at the wavelength 405 nm [5].

Isolation of viruses from the infected plants has been carried out by homogenization of symptomatic plant leaves in 0.1M PBS + 0.001M EDTA in proportion 1:2 (w/v), and filtra-

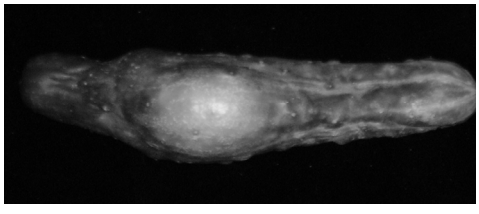
tion through a 4-layered cheese-cloth followed by low-speed centrifugation at 4000 rpm for 20 minutes at 4C using the centrifuge PC-6 (USSR) to remove the debris of plant tissues [6]. Obtained homogenetic material was used in ELISA.

Sample analysis for presence of virus antigens has been carried out with antiserums for the following viruses: rabbit antibodies and conjugates (alkaline phosphatase) were kindly provided by Prof. Herve Lecoq (INRA, France) for the following virus: *Cucumber mosaic virus* (CMV), *Cucurbit aphid-borne yellows virus* (CABYV), *Watermelon mosaic virus 2* (WMV-2), *Zucchini yellow mosaic virus* (ZYMV), *Papaya ringspot virus* (PRMV*), Squash mosaic virus* (SqMV). Rabbit antibodies were kindly provided by Prof. Tomas Kenne (Germany) for the *Cucumber green mottle mosaic virus* (CGMMV), *Alfalfa mosaic virus* (AMV), *Potato virus X* (PVX), *Tomato mosaic virus* (TMV) *and Turnip mosaic virus* (TuMV). The test systems of DSMZ (Germany) we used for detecting of *Tomato aspermy virus* (TAV), *Arabis mosaic virus* (ArMV), *Impatia necrotic spott virus* (INSV), *Tomato spott wilt virus* (TSWV) and *Pepino mosaic virus* (PepMV).

For confirmation of ELISA data, plant samples were also examined by transmission electron microscopy. Partially purified virus samples were dripped on the copper grid with support, that has been made of 0,2% formvar solution (“Serva”, Німеччина). Contrasting has been carried out with 2% solution of uranyle acetate (Serva, Germany) for 2 minutes and then monitored at 40000-60000 magnification on electron microscope (EM-125, Sumy, Ukraine) [1].

Results and Discussion. During 2001 -2007 years we have been investigating 9 greenhouses: Crimea, Dnipropetrovsk, Mykolaiv, Sumy, Kharkiv, Cherkassy, Donetsk, Poltava and Chernigiv regions**.** The cucumbers, peppers, egg-plants and tomatoes were collected for research. The plants demonstrated the following symptoms: green mottle mosaic, yellow mosaic, dark green mosaic, local necrosis, vein clearing, deformation, wartiness and decoloration of fruits (Fig.1)

а



б

Fig.1 Virus-induced symptoms on cucumber plants collected from greenhouses: *a* – leaf mosaic induced by *Impatia necrotic spott virus* (INSV); *b* – malformation of cucumber fruit induced by *Cucumber green mottle mosaic virus* (CGMMV).

Only 3 viruses were detected in greenhouse conditions in Ukraine by ELISA and electron microscopy: *Cucumber green mottle mosaic virus and Impatia necrotic spott virus* were found in cucumbers and *Turnip mosaic virus* – in tomatoes.

Cucumber green mottle mosaic virus is the most widespread pathogen in cucumbers in greenhouse conditions. This virus is not a new pathogen for Ukraine because it has been circulating 80’s. CGMMV was causing a considerable lost of yield, about 50% [3].

Nowdays CGMMV was registered in the following greenhouses of Ukraine: Crimea, Dnipropetrovsk, Mykolaiv, Sumy, Kharkiv, Cherkassy regions**.**

The widespread of this virus in the greenhouse connected with virus transmission mode. The source of infection is seeding material. Virus concentrates in the top part of seed pellicle. Virus transfer occurs via injuring of germ tissues and penetration of virus through the injured sites. Soil-mediated virus transfer occurs via soil grout. The main danger for cucumber plants is free virus, which releases into the soil after decay of plant remains, but not the remains themselves. Moreover, the virus is stable in environment [3].

Particularity of virus modes and physical properties together with failure to carry out of sanitary code in greenhouse result in total affection of plants and significant lost of yield.

As for *Impatia necrotic spott virus* and *Turnip mosaic virus* these are new pathogens for Ukraine. We were the first detected these viruses in greenhouse condition. INSV was detected in Donetsk, and TuMV- in Kharkiv rgions.

Vectors mode is a main for transmission of these viruses. Tryps transmit the INSV and aphids transmit the TuMV [7].

Constant high temperatures and humidity, absence of nature enemy result in the best condition for propagation of vermin in greenhouse [2].

Vector mode is so effective for these viruses and it can cause a significant lost of yield and in some cases it can cause the appearance of epiphytotic. So, we must use various insecticides for the control of insects in greenhouse.

Presence of described viruses in plant samples was examined by transmission electron microscopy. We have revealed rod-shaped structures of CGMMV and TuMV and icosahedral structures of INSV (Fig.2).

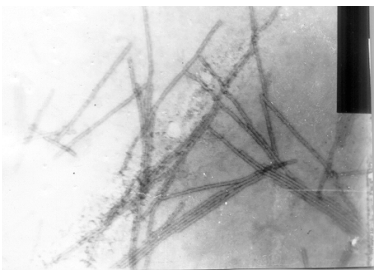


Fig.2 Electron microscopy picture of purified particles of CGMMV (magnification x 40000).

Conclusions. Only three viruses infecting vegetable crops were detected in plant samples collected from different greenhouses of Ukraine. These are *Cucumber green mottle mosaic virus, Impatia necrotic spott virus* and *Turnip mosaic virus.* We have shown that INSV and TuMV are new pathogens for Ukraine in contrast to CGMMV.

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O.I. Gordeyeichik, eng., I.C. Shcherbatenko, D. Sci.

SEARCHING FOR SIMILAR NUCLEOTIDE SITES IN GENOMIC SEQUENCES OF PLANT VIRUSES

Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine, Kyiv.

Computer database search for similar nucleotide sites in genomes of plant pathogenic viruses has been conducted by subsequent comparison of pairs of genome sequences with increasing shift value of their starting postions.

Introduction.The sequence similarity of viral genomes or some nucleotide sites is quantified as persentage of nucleotide sequence identity and widely used for the elucidation of the evolutionary histories of viruses, detecting conserved functionally importent sequences and secondary structure elements as well as comparing virus relationship and taxonomic groupings [1,9 13,14,15].

Similarity searches are commonly performed with numerous available programs by sequence alignment. Although a variety of algorithms and improvements of the widely used programs and new software applications for sequence alignment have been developed [2, 3, 6, 7, 10, 16], sequence similarity analysis still presents certain difficulties [11, 12, 17].To overcome this problem new algorithms, alternative to alignment, are of particular interest [4, 18].The objective of this study was the computational search for similar nucleotide sites in genomes of plant viruses by successive disposition of two genomic sequences with increasing displacement of their initial positions.

Materials and methods. *Sequences***.** Tests were run on 3 random nucleotide sequences and 189 genomic sequences of (+)ssRNA-containing plant viruses from Tombusviridae (Avena-, Aureus-, Carmo-, Necro-, Tombusvirus), Flexiviridae (Allexi-, Capillo-, Carla-, Fovea-, Potex-, Tricho-, Vitivirus), Luteoviridae (Luteo-, Polerovirus), Tymoviridae (Tymovirus) and Potyviridae (Potyvirus) families as well as Tobamovirus and Sobemovirus genera. All the genomic sequences used in this study were derived from the DDBJ, EMBL, and GenBank nucleotide sequence databases. The 6400 nucleotide long random sequences were generated using the random number generator: basic function int(RND^{x}4+1), producing a random number between 1 and 4 . The standard nucleotides (A, T, G and C) were assigned to 1, 2, 3 and 4, accordingly.

Similarity searches. Searches of the similar nucleotide sites in viral genomes was performed by successive disposition of two sequences with increasing displacement of their initial position: 1/1 (displacement = 0); 1/2 (displacement = +1); 2/1 (displacement = -1); 1/3 (displacement = +2); 3/1 (displacement = -2). 1/maxd; maxd/1 (maxd (maximal displacement) = genome length - site length). In all dispositions of sequences were revealing the similar nucleotide sites, which length and persentage of sequence identity were not less then preliminary user-specified. Searching results:number, length and genomic positions of the similar nucleotide sites were graphical visualizing and tabular displaing.

Software application. A simple program we have termed Similar% was written at qBASIC and tested on two random sequence, in which some similar nucleotide sites was inserted. Extensive simulation studies show that the program used: 1) is able to find all similar sites inserted; 2) the length, positions and identity persentage of finding sites are equal to that of inserting ones. The values of the mean of sites quantity and length were obtained from at least 3 to 88 pairs of genomic sequences.

Results and discussion. *Revealing of non-random nucleotide coincidence in sequences*. To determine the parameters of non-random sequence similarity the dependence between nucleotide sites length and nucleotide coincidence in random sequences was investigated. It was shown that 5-nucleotide sites with 100% identity are appeared in random sequences from 8577 to 8711 times, 10-nucleotide sites - from 8 to 15 times, 12-nucleotide sites - from 0 to 2 times, and 15-nucleotide sites are not appeared (Tabl. 1). So, parameter limits of non-random similarity of short sites are the site length not less then 15 nucleotides and nucleotide coincidence (sequence identity) not less then 100%. The limit of non-random similarity for 20-nucleotide sites is not less then 90% sequence identity, 30-nucleotide - 70%, 40-nucleotide - 60%, 50-nucleotide - 58%, 100-nucleotide - 46%, 500-nucleotide - 34%, 1000-nucleotide - 32%, 2000-nucleotide - 32. So far as non-random similarity of the short sites sharply depends on their length (15 nucleotides - 100%, 20 nucleotides - 90%), just as the long sites - on their identity persentage (1000 nucleotides - 32%, 2000 nucleotides - 30%), the 40 nucleotide site length and 60% sequence identity was chosen as starting user-specified parameter for simile sites revealing.

Table 1. **The number of similar sites in random nucleotide sequences depending on the site length and nucleotide coincidence**

Site length, nucleotides	Nucleotide coincidence, %	Pairs of random sequences *		
		rand1/rand2	rand1/rand3	rand2/rand3
5	100	8711	8577	8654
10	100	8	15	10
12	100	0	2	0
15	100	0	0	0
	70	18	14	21
20	80	0	1	0
	90	0	0	0
	60	5	11	7
30	65	1	2	0
	70	0	0	0
	54	17	28	19
40	58	1	1	1
	60	0	0	0

	52	9	17	13
50	56	0	2	0
	58	0	0	0
	42	18	17	18
100	44	5	1	3
	46	0	0	0
	30	216	178	191
500	32	11	14	8
	34	0	0	0
	28	227	237	229
1000	30	2	2	0
	32	0	0	0
	26	811	765	802
2000	28	10	7	4
	30	0	0	0

* The length of random sequences rand1, rand2 and rand3 is 6400 nucleotides

Searching for the similar nucleotide sites in viral genomes. The similar nucleotide sites was firstly searching in the genomes of viruses from 16 genera, wich had not less then 6 sequences available. Tests were carried out on 5 pairs of samples - one sequence from six available was compared with the rest five ones. (Tabl. 2). It was determined that the same genus viruses have a large clusters of similar sites varying in the mean quantity from 5.2 (Sobemovirus) to 20.8 (Allexivirus), and in the mean length - from 938.4 (Potexvirus) to 4912.5 (Foveavirus). The maximal length of similar sites reaches 1206 - 8743 nucleotides, and their maximal quantity differ from 9 to 23.

The similar nucleotide sites of viruses from different genera but the same family differ in mean size from 7.0 to 12.1 nucleotides, and in mean length - from 677.8 to 836.3 (Tabl. 3). The maximal quantity of the similar sites reaches 13 - 14, and the maximal length varying from 1509 to 5451 nucleotides.

Table 2. **The similar nucleotide sites in genomes of plant viruses from the same genus**

Virus genus	No. of sites		Site length	
	mean*	maximal	mean*	maximal
Allexivirus	20,8	23	2315,0	3391
Carlavirus	18,8	22	1399,4	1584
Tobamovirus	17,4	20	1055,8	1404
Potyvirus	17,0	19	1198,8	1424
Potexvirus	14,6	23	938,4	1206
Vitivirus	13,7	20	2972,3	6812
Luteovirus	12,0	14	2043,8	4710
Tymovirus	11,6	16	2187,0	3264
Polerovirus	10,6	13	1219,2	2197
Foveavirus	10,3	19	4912,5	8743
Trichovirus	9,4	13	3675,6	5283
Aureusvirus	8,3	11	1559,0	2692
Capillovirus	5,8	14	3948,4	6478
Necrovirus	5,6	9	1799,0	3683
Carmovirus	5,4	9	1820,4	4186
Sobemovirus	5,2	10	1027,5	1606

*- The mean values from 5 virus pairs of earch genus

Table 3. **The similar nucleotide sites in genomes of plant viruses from the different genera**

Families and genera	Virus pair		No. of site		Site length	
	No. of tested	% with similar sites	mean	max	mean	max
Tombusviridae	29	100	7,0	13	767,0	5451
Flexiviridae	69	100	12,1	24	836,3	2567
Luteoviridae	43	100	7,5	14	677,8	1509
Luteovirus/Tombusviridae	48	100	3,8	7	255,4	438
Luteoviridae/ Sobemovirus	30	100	4,4	8	281,1	407
Flexiviridae/Luteoviridae	88	58,7	0,9	5	41,4	227
Tymovirus/Flexiviridae	31	93,5	1,7	4	89,9	224
Tymovirus/Potexvirus	16	100	4,2	8	227,8	468

Comparisons of the similar sites in genomic sequences showed that the lisianthus necrosis virus from Necrovirus genus is more similar with Tombusvirus members (fig.1, samples 1,2) then with Necrovirus ones (fig.1, samples 3-8). In addition to sequence similarity the genome length and ORFs of this virus are also typical for tombusviruses (fig. 2). As a result, there are worthy arguments for taking the lisianthus necrosis virus to Tombusvirus genus.

Fig. 1. Similarity of Lisianthus necrosis virus with tombusviruses (1, 2) and necroviruses (3, 8). Tombusviruses: 1 - carnation Italian ringspot virus, 2 - tomato bushy stunt virus. Necroviruses: 3 - beet black scorch virus, 4 - leek white stripe virus, 5 - olive latent virus 1, 6 - olive mild mosaic virus, 7 - tobacco necrosis virus A, 8 - tobacco necrosis virus D

Viruses of different genera from different families usually have no similar sites, but in some cases they can have some similarity: clearly expressed clasters of a relatively long nucleotide sites located in a certain genomic region and numerous short sites scattered along the viral genome (fig. 3); diffuse clasters of a short sites located in a several genomic positions (fig. 4) and a little clasters or a single nucleotide sites located in a certain genome region (fig. 5).

Fig. 2. The ORFs of Lisianthus necrosis virus (3) and tombusviruses (1, 2) and necrovirus (4, 5. Tombusviruses: 1- carnation Italian ringspot virus, 2 - tomato bushy stunt virus. Necroviruses: 4 - tobacco necrosis virus A, 5 - olive latent virus 1. Gray lines - genome length

These experiments reveale the phylogenetic relationships among some viruses from Luteoviridae, Tombusviridae, Flexiviridae, Sobemovirus, Tymovirus and Tobamovirus. Our results are in accordance with the comparisons of the viral genomes [8], ORFs [15], coat and replications proteins [5, 6], tRNA-like structures [9] and a cap-independent translation element [14].

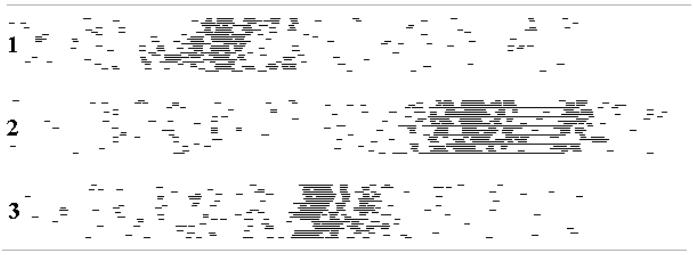

Fig. 3. Similar nucleotide sites of tombusviruses and luteoviruses (1); poleroviruses and luteoviruses (2); poleroviruses and sobemoviruses. Tombusviruses: carnation Italian ringspot virus, tomato bushy stunt virus, lisianthus necrosis virus, cucumber leaf spot virus, pothos latent virus, beet black scorch virus. Poleroviruses: beet chlorosis virus, beet mild yellowing virus, beet western yellows virus, carrot red leaf virus, cereal yellow dwarf virus, turnip yellows virus. Luteoviruses: barley yellow dwarf virus - GAV, barley yellow dwarf virus - PAS, bean leafroll virus, soybean dwarf virus. Sobemoviruses: ryegrass mottle virus, rice yellow mottle virus, cocksfoot mottle virus, southern bean mosaic virus

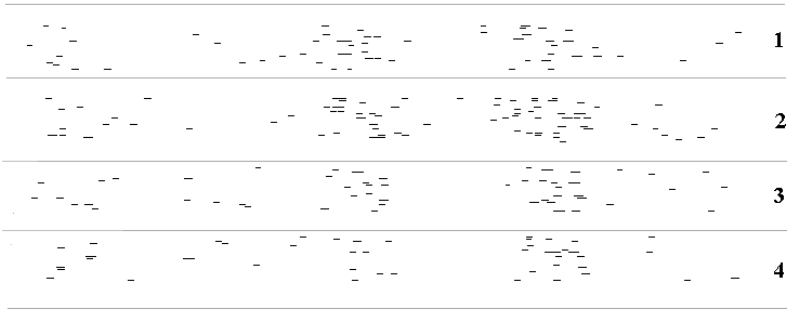

Fig. 4. Similar nucleotide sites of 22 potexviruses and anagyris vein yellowing virus (1); nemesia ring necrosis virus (2); plantago mottle virus (3); scrophularia mottle virus (4). Potexviruses: Potato Virus X, Alstroemeria virus X,Clover yellow mosaic virus, Pepino mosaic virus, Strawberry mild yellow edge virus, Scallion virus X, Foxtail mosaic virus, Potato aucuba mosaic virus, Cactus virus X, Bamboo mosaic virus, Zygocactus virus X, White clover mosaic virus(WCIMV1), White clover mosaic virus (WCIMV2), Cymbidium mosaic virus, Papaya mosaic virus, Cassava common mosaic virus, Foxtail mosaic virus, Narcissus mosaic virus, Plantago asiatica mosaic virus, Hydrangea ringspot virus,Lily virus X, Opuntia virus X

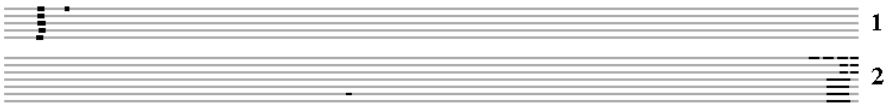

Fig. 5. Similarity of nemesia ring necrosis virus with allexiviruses (1) and tobamoviruses (2). Allexiviruses: garlic virus A, garlic virus C, garlic virus E, garlic virus X, shallot virus X. Tobamoviruses: odontoglossum ringspot virus, tobacco mosaic virus (vulgare), tomato mosaic virus, crucifer tobamovirus, tobacco mosaic virus (crucifer), ribgrass mosaic virus, turnip vein-clearing virus

Of particular interest is the location of the similar nucleotide sites in viral genomes. The distances between genomic positions of the all or the majority similar sites are equal to x.0 or x.33 or x.66 triplets (tabl. 4), which are equal to 3x, 3x+1 or 3x+2 nucleotides (tabl. 5), where x is an integer number from 0 to 300 - 500. The results suggest that the similar nucleotide sites represent an in-frame clusters of conserved sequence elements encoding some functionally important components. As such, the distances between the similar sites in the clusters may only be equal to an integer number of triplete codones, suggesting that any frameshift between similar sites is letal for virus. A large differences in the distances between the similar sites equal to 3x nucleotides might be the result of insertions/deletions/duplications/recombinations without frameshifting, as 3x+1 and 3x+2 nucleotide distances are due to additional non-integer triplete distances between starting codones of the genes containing the clusters of similar sites.

Table 4. **The distances between positions of similar nucleotide sites in genomes of two potexviruses**

Site length	Pos1*	Pos2**	Distances, nucleotides	Distances, triplets
62	815	834	19	6,3
72	1896	2704	808	269,3
65	2084	2898	814	271,3
80	2277	3091	814	271,3
60	2442	3262	820	273,3
259	2789	3612	823	274,3
344	3394	4217	823	274,3
69	4953	5820	867	289,0
65	4207	5080	873	291,0

*Pos1 - positions of similar nucleotide sites in genome of foxtail mosaic virus

**Pos2 - positions of similar nucleotide sites in genome of Scallion virus X

Table 5. **Displacement of the distances between positions of similar nucleotide sites in genomes of tobamo- and potyviruses**

Virus pair	No. of similar sites			
	All	with displacement		
		0	1	2
Tobamovirus genus				
Tobacco mild green mosaic virus / Cucumber green mottle mosaic virus	19	0	17	2
Pepper mild mottle virus / Ribgrass mosaic virus	14	2	8	4
Tomato mosaic virus / Crucifer tobamovirus	16	1	3	12
Cucumber fruit mottle mosaic virus / Odontoglossum ringspot virus	20	2	4	14
Potyvirus genus				
Potato virus A / Potato virus Y	19	0	17	2
Scallion mosaic virus / Sugarcane mosaic virus	18	1	16	1
Bean common mosaic virus / Cocksfoot streak virus	17	17	0	0
Soybean mosaic virus / Yam mosaic virus	16	1	14	1

* Displacement is equal to: or integer number of triplets (3x nucleotides, displacement 0), or 3x+1 nucleotides (displacement 1), or 3x+2 nucleotides (displacement 2)

Overall, we tested a new method for the computational search of the similar nucleotide sites in genomes by successive disposition of two genomic sequences with increasing displacement of their initial positions and revealing in all dispositions the similar nucleotide sites, which length and persentage of sequence identity were not less then preliminary user-specified.

We have determined a parameter limits of non-random nucleotide coincidence in sequences as well as the presence and localisation peculiarity of similar nucleotide sites in viral genomes. Our results also demonstrate that the lisianthus necrosis virus is the more closely related to tombusviruses then to necroviruses.

The computational search used provides an alternative sequence similarity analysis without a time-consuming aligning procedure. The method can be used as a simple, fast and obvious way for comparing sequence relatedness with graphical visualization of the similar nucleotide or aminoacid sites.

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J. Staniulis[1], Dr. habil., M. Žižytė[1], PhD student, T. Norkus[1], bacaalaureate, L. Yusko[2], PhD student, H. Snihur[2], PhD, I. Budzanivska[2], PhD

INCIDENCE OF PLUM POX VIRUS IN LITHUANIA AND UKRAINE

[1] Institute of Botany, Plant Virus Laboratory, Vilnius, Lithuania;[2] Taras Shevchenko` Kyiv National University, Ukraine.

According to the internationally agreed joint project "Investigation of distribution of plum pox virus in Lithuania and Ukraine and development of biotechnological methods of obtaining of virus-free planting material" 95 samples of plum, apricot and peach from several locations of Ukraine were collected in June 2007 and checked for the presence of PPV by DAS-ELISA. Presence of PPV was detected in all explored regions. Four isolates PPV from plum of Kyivsky and Transkarpathian regions were transferred to herbaceous plants and the presence of the virus in these plants was confirmed by DAS-ELISA and IEM investigation. The presence of PPV in samples from Lithuania was confirmed in 2 cases from 20. PPV positive samples were collected from Jonava and Sakiai regions.

Introduction. Stone fruit trees are natural hosts of several widespread viruses. Thirty virus diseases of stone and pome fruits have been described in former Yugoslavia [Šutič et al., 1999]. Plum pox virus or 'Sharka' disease agent *Plum pox potyvirus* (PPV) originally described in Bulgaria on *Prunus domestica* gradually has spread throughout European borders [Šutič et al., 1999]. PPV infects virtually all cultivated fruit tree species of the genus *Prunus*. The disease also affects some wild *Prunus* species, especially blackthorn (*Prunus spinosa*) which has been a natural source of infection in many countries. PPV is now reported in most European countries, in parts of Asia and northern Africa, and in South America. Plum

pox is the most serious disease of plums, apricots and peaches in Europe and is considered as quarantine pathogen in all continents [Roy, Smith, 1994; Šutič et al., 1999]. Plum pox symptoms can vary greatly, making the disease difficult to recognize. Visual symptoms are not always a reliable indicator of disease. In some cases, only part of the infected tree or even only a single branch exhibit symptoms. Disease symptoms are most noticeable during spring period. Then leaves can show chlorotic spots, bands, or rings, vein clearing and leaf deformation. Infected fruits can be deformed, show chlorotic spots or rings on the surface, contain internal browning of the flesh and pale rings or spots on the stones. The disease may not be visible until several months or years after the tree has been infected. Signifficant progress investigating PPV has been made after the discovery of possibility to transmit it to herbaceous plants *Chenopodium foetidum* and *Nicotiana clevelandii* [Sutic, 1961]. PPV was the first plant virus for which serological ELISA technique was created and adopted for detection in 1977 [Clark, Adams, 1977]. Using ELISA technique it was established that beginning of vegetative period when leaves are still not completely expanded virus concentration is highest and it is most suitable time for mechanical virus trasmission. With aging of leaves virus concentration gradually decreases and late in summer the virus is not detectable with electron microscope [Калашян, 1978]. PPV belongs to the most numerous filamentous mechanically and by aphids transmissible plant virus group – potyviruses. PPV virions are filamentous particles c. 660-770x12,5-20 nm [Klinkowski, 1968; Kegler, Schade, 1971]. According to electrophoretic mobility and serological properties most European isolates of PPV belong to two groups of strain – M (Marcus) and D (Dideron) [Bousalem et al., 1994; Cambra et al., 1994; Myrta et al., 1996]. Strains of D group are less aggressive and have limitted host range.

The main source of the virus is infected trees or budwood [OEPP/EPPO, 1983]. From these, PPV is transmitted either by grafting or by aphids in a non-persistent, stylet-borne manner to uninfected hosts. Furthermore, the virus can survive in the roots of rogued infected trees and spread from there by natural root grafting. The aphids acquire the virus during feeding and then transmit it to nearby healthy plants. The aphids can only transmit PPV for a short period of time after acquiring it. Long distance spread of PPV occurs through the movement and use of infected propagative materials (grafting and budding of infected material). The use of disease-free propagative material at all times is fundamental to preventing introduction to new areas. Measures to reduce the significance of plum pox in areas where it is present include the use of disease-free planting stock when planting new orchards or replenishing existing ones, removal of infected trees (including their roots), use of tolerant or less susceptible species or cultivars where possible, proper spacing of trees to reduce the rate of spread, control of aphids to slow spread, and weed control (including wild *Prunus* species) to eliminate reservoirs of either aphids or PPV [Anonymus, 1997; Nemeth, 1994; OEPP/EPPO, 1992].

First evidence for the presence of PPV in Lithuania was received in Lithuanian Institute of Horticulture by Dr. J. Stankiene who using ELISA reagents for PPV (Poland Institute of Horticulture, Skierniewice) detected the presence of the virus in some plum trees from Kaunas region in about 1995. Local isolate of PPV was transmitted to herbaceous plants in 1997 and the specific antiserum in the Institute of Botany was prepared. The Lithuanian isolate (PPV-971) appeared to be attributed to D group of strains, as was identified using monoclonal antibodies in ELISA tests conducted by Dr. D. Boscia (Centro di studio sui virus e le virosi delle colture de Bari, Italy). For molecular diagnosis and characterization of the isolate PPV-971, RT-PCR amplification of the coat protein encoding sequence by use of specific primers complementary to 5'- and 3' parts of PPV coat protein L1 was employed (GenBank accession number X81081). The specific DNA fragment, corresponding to predicted coat protein sequence size, was cloned into *E. coli* pUC57 for DNA sequencing. Amino acid sequence comparison with GenBank data indicated 98.2 % similarity with coat protein of PPV potyvirus isolated by E.Mais et al. (accession no. X81083) and 97.3 % with PPV strain Rankovic [S.Lain et al., 1989; Staniulis et al., 1998; Dargeviciute et al., 2000].

More extensive surveys on spread of PPV using ELISA were carried out after establishment of Phytosanitary Research Laboratory at Lithuanian State Plant Protection Service in 1997. Surveys for determination of PPV incidence during 1998-2002 were carried out in 14 localities of 9 administrative regions of Lithuania. Presence of PPV was confirmed in 865 tree samples of 1553 analysed by ELISA and in 66 tree samples from 150 analysed by ISEM. In 5 outbreaks of the virus all the plum trees were uprooted and burned, and in the other 9 orchards only the contaminated trees and the neighbouring trees were uprooted and burned. During 2003 7,5 hectares of plum trees were surveyed. From 91 samples checked, 9 appeared PPV positive, among them in 2 new locations. Also 206 apricot, 204 peach and 5 cherry trees were surveyed and part of them were checked for PPV presence, however with negative results for the virus. In 2004 32 samples from total 3 ha area of plum trees were checked, and 14 positive samples were detected. Of 1042 checked samples from 5873 apricot tree and 51 samples of 455 peach trees all appeared to be PPV negative. In 2005 235 samples from surveyed 6680 plum trees were checked. 3 positive samples detected were from previously defined locations. In 2006 18 hectares of stone fruit orchards were checked and two PPV infection focus were revealed. The status of PPV in Lithuania can be described as: present only in some areas.

Sharka in Ukraine was detected for the first time in 1966 in the Chernivetsky region, the size of the harmed area was 0,3 hectares. In 1970 Sharka was detected in six regions of Ukraine (Vinnitsky, Transkarpathian, Ivano-Frankivsky, Lvivsky, Ternopilsky and Chernivetsky regions with a general size of the harmed area around 57,58 hectares). The nidus of the infection in the Ivano-Frankivsky (0,105 hectares) and in the Vinnitsky (4,6 hectares) regions was timely eliminated. In spite of hard actions against the harmed area, Sharka has spread in the South (Odesky region) and in the West (Transkarpathian region) of Ukraine. Up to 1.01.2007 the biggest area of stone fruit plantings harmed with PPV was registered in the Transkarpathian region. The size of the area was around 4487,2 hectares. The harmed area in the Odesky region has decreased from 10,5 hectares to 1,5 hectares due to quarantine removal from plantings in the Ovidiopolsky district. It should be mentioned however that investigations for the PVP presence were held using visual diagnostics without confirmation of viral etiology of the disease.

For this investigation leaf samples with suspectable plum sharka disease symptoms were collected in harmed regions of Ukraine and Lithuania for laboratory analysis by DAS-ELISA, immunosorbent electron microscopy (ISEM) and for transmission to herbaceous plants.

Materials and methods. *Stone fruit tree leaf samples indicating virus-like symtoms or symptomless from Ukraine were collected in Kyivsky, Odesky and Transkarpathian regions. From Lithuania plum tree leaf samples were taken in Kaunas, Varena, Trakai, Sakiai and Jonava regions. The survey of incidence of PPV in Lithuania was performed during June in plum tree orchards in expected foci of the infection and field samples were collected. Collected field samples were assayed by DAS-*

ELISA with PPV polyclonal antibodies (BIOREBA, Switzerland or LOEWE, Germany). DAS-ELISA procedures were performed as described by Clark and Adams [1977]. In BIOREBA AB kits polyclonal antibodies were prepared against PPV isolate from the Netherlands reacting with a broad spectrum of PPV isolates from France, Greece and Switzerland. The LOEWE kits contained PPV broad range artificial mixture of antisera. Enzymatic reaction was evaluated photometrically at 405 nm using Labsystems Multiskan RC microtiter plate reader. When the absorbance values in DAS-ELISA tests were not clear-cut enough or near to the critical values of background the plum tree samples were subjected to additional analysis by immunosorbent electron microscopy (ISEM) in Plant Virus laboratory of the Institute of Botany [Derrick, 1973; Brlansky, Derrick, 1979; Kerlan et al., 1981]. For ISEM procedure antibodies prepared against Lithuanian isolate of PPV with homologous titre 1:1024 in microprecipitation reactions were used. For sensibilization of electron microscopic grids before antigen application diluted 1:50 fraction of IgG antibodies was used. Leaf tissue 1:20 (v/w) in extraction buffer (0,05M phosphate bufer pH 7,2, 0,01M DIECA, 2% PVP) clarified by centrifugation at 10 000g for 5 min. was applied as antigen. After IgG and Ag application the grids were subjected to washing by PBS-Tween-20 (0,05%). As a staining solution 2 % uranyl acetate was used. Electron microscopic preparations were examined with JEOL JEM-100S transmission electron microscope at instrumental magnification 10000-25000^x. For the mechanical virus transmission to herbaceous plants (Chenopodium feoetidum Schrad., Pisum sativum L. and some Nicotiana L. species) phosphate buffer solution 0,03M containing 1,5 % nicotine, 0,25 % Na DIECA and 0,2 % of Na_2SO_3, final pH 8,0 was used. For the PCR tests RNA extraction from plum leaves or experimentally infected herbaceous plants was carried out according to the instruction of "QuickPrep^{TM} Total RNA Extraction Kit for the direct isolation of total RNA from most eukaryotic tissues or cells" (Amersham Biosciences, UK). For general PPV detection by RT-PCR amplification of a 243 bp fragment in the coat protein (CP) coding region was carried out using the primers P1 and P2 according Wetzel et al. [Wetze et al, 1991]: P1: 5'-3' ACC GAG ACC ACT ACA CTC CC and P2: 5'-3' CAG ACT ACA GCC TCG CCA GA. Temperatures used for the cycling reaction were as follows: 30 min at 42^{0}C; 3 min at 93^{0}C, followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 54^{0}C, 30 sec at 72^{0}C; with a final extension of 5 min at 72^{0}C [Sertkaya et al., 2003].

***Results.** Typical symptoms of PPV affected plum leaves consisted of mosaic, ring pattern, vein banding with slight deformation of lamina (Fig.1), however symptoms often varied in great extent depending on many factors. Considerable part of analysed samples appeared as PPV negative.*

Fig. 1. Typical symptoms of PPV affected plum (*Prunus domestica*) leaf.

Application of antibodies for sensibilization EM grids very significantly increased quantity of captured virus particles in about 2 orders of magnitude [Brlansky, Derrick, 1979]. Without antibody coating PPV particles are difficult to be detected. Presence of specific virus particles by ISEM can be proved very evidently (Fig. 2).

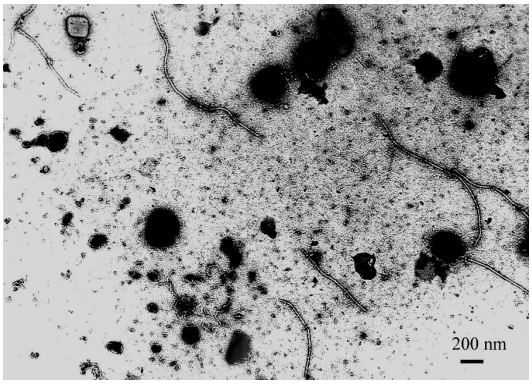

Fig. 2. PPV particles trapped in ISEM preparation from plum leaf extract. Bar represents 200 nm.

Results of tests for detection of PPV by DAS-ELISA in plum tree samples collected in some regions from Lithuania are presented in table 1 and indicate that the virus was present in two localities of Kaunas and Jonava regions.

Table 1. **ELISA test results of Lithuanian plum samples collected in June 2007.**

Tree species and location	No. samples tested	ELISA positive	ISEM positive
Garden plum – Sakiai reg., Patasyne	5	1	1
Garden plum - Trakai reg., Trakai	1	0	0
Garden plum - Varena reg., Perloja	1	0	0
Garden plum - Kaunas reg., Ringaudai	4	0	0
Garden plum - Jonava reg., Pedziai	6	1	1

Samples of stone fruite cultures collected in 3 regions were further analyzed via ELISA for PPV antigens. This study resulted in demonstration of Sharka disease in every region investigated, despite the differences in the percentage of infected plants (Fig 3).

From 40 samples tested for the Transkarpathian region, PPV has been detected in 19, which makes 45,7 % of disease rate. The virus was detected in 6 districts from 8 investigated. Regularly, PPV was observed on plum (*Prunus domestica*) and apricot (*Prunus armeniaca*), this correlates with the outcomes of visual diagnostics.

The disease rate for 2 districts in the Odesky region was shown to be 32% (10 PPV-positive samples from 31 tested). Interestingly, in in the Odesky region PPV was detected on plums, apricots and peaches.

In the Kyivsky region, the disease rate was at 13%; the virus has been detected in one district in one of three investigated plum samples (*Prunus domestica*).

Mechanical transmission to the herbaceous plants was conducted from plum leaf samples positively reacted in DAS-ELISA tests using phosphate buffer with antioxydant substances. From all ELISA positive samples inoculated *Pisum sativum* plants developed vein clearing (VC) and slight mottling (Mo) systemic symptoms (Fig. 4). During initial inoculations *Chenopodium foetidum* plants did not develop noticeable local lesions (LL) (table 2). Slight chlorotic local lesions usually appeared after few additional reinoculation. ELISA tests and electron microscopy confirmed presence of PPV in experimentally inoculated herbaceous plants.

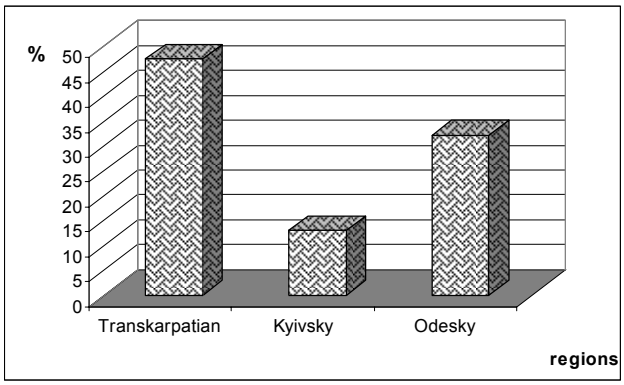

Fig. 3. Percent of PPV infected plants with positive result obtained by ELISA from different regions of Ukraine.

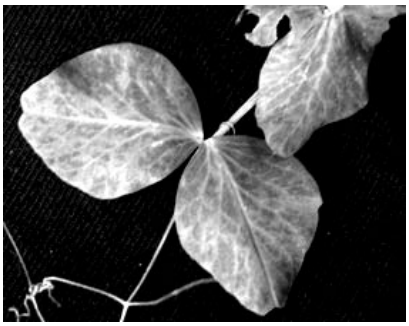

Fig. 4. Systemic mottle of leaf *Pisum sativum* infected by PPV.

Table 2. **Reaction of experimentally inoculated plants with leaf extracts from ELISA positive plum tree samples.**

Plant species	Symptoms	ELISA test results
Chenopodium foetidum	LL	+
C. quinoa	-	-
Nicotiana benthamiana	slight mottling	-
N. debneyi	-	-
Pisum sativum cv. 'Peliuškos'	VC, Mo	+
P. sativum, cv. 'Rainiai'	VC, Mo	+
P. sativum, cv. 'Žalsviai'	VC, Mo	+

The presence of PPV in plum leaves tissue and indicator plants was proved by RT-PCR, using general primers [*Wetzel, T.* 1991]. PCR reaction of all tested samples resulted in specific amplification of 243 bp fragment of the coat protein region (Fig. 5). No products were obtained from negative control sample.

Thus, we demonstrated rather high percentage of *Plum pox virus*-infected stone fruit cultures in Ukraine. The status of PPV in Lithuania can be described as: presently just in some areas. From 20 samples of plum trees collected in Lithuania the presence of PPV was confirmed by DAS-ELISA in two cases from the Jonava and Sakiai regions. Morphology of the viral parts was investigated. Four Ukrainian isolates of PPV from the plums in the Kyivsky and Transkarpathian regions were propagated on herbaceous indicator plants. The presence of PPV in plum leaves tissue and indicator plants was proved by RT-PCR, using general primers.

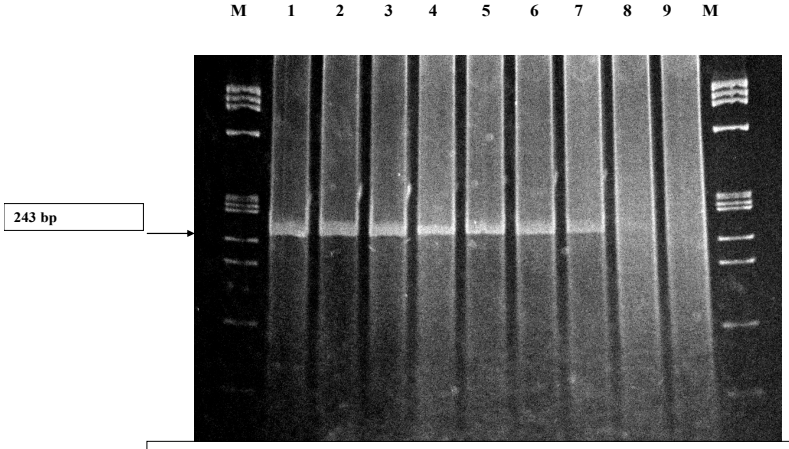

Fig. RT-PCR of PPV specific 243 bp fragment. Lanes M – DNA-ladder [*ΦX 174 DNA/BsuRI (Hae III)*], lanes 1 and 2 – Lithuanian plum samples, lanes 3-8 – Ukrainian plum samples, lane 9 – control (healthy plant). Samples: 1. Patasyne, 2. Pedziai, 3. Novosilki N3, 4. Zuliany, 5. Novosilki N4, 6. Uzgorod N1, 7. Uzgorod N6, 8. Uzgorod N11.

Fig. 5. RT-PCR of PPV specific 243 bp fragment.

Thus, we demonstrated rather high percentage of *Plum pox virus*-infected stone fruit cultures in Ukraine. The status of PPV in Lithuania can be described as: presently just in some areas. From 20 samples of plum trees collected in Lithuania the presence of PPV was confirmed by DAS-ELISA in two cases from the Jonava and Sakiai regions. Morphology of the viral parts was investigated. Four Ukrainian isolates of PPV from the plums in the Kyivsky and Transkarpathian regions were propagated on herbaceous indicator plants. The presence of PPV in plum leaves tissue and indicator plants was proved by RT-PCR, using general primers.

Acknowledgments. The research work was supported by a Grant from Lithuanian Science and Study Foundation No V-12/2008 and Lithuanian Ministry of Education and Science No SUT-268, and from Ministry of Education and Science of Ukraine No M/170-2007.

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M. Žižytė PhD student, J. Staniulis Dr. hab.

BEET NECROTIC YELLOW VEIN VIRUS: PURIFICATION AND DETECTION BY ELECTRON MICROSCOPY AND WESTERN BLOT

Institute of Botany, Plant Virus Laboratory, Vilnius, Lithuania

The best results for Beet necrotic yellow vein virus (BNYVV) purification were obtained from Chenopodium quinoa leaf tissue mechanically inoculated with the virus using slightly modified method described by Bouzoubaa [1]. The presence of BNYVV was confirmed by immunosorbent electron microscopy (IEM) and Western blot analysis. IEM with antiserum to BNYVV coat protein (CP) revealed virus particles morphology typical for rhizomania agent [8]. Purified virus suspension was used for CP evaluation and detection by Western blot.

Introduction. BNYVV (genus *Benyvirus*) causes rhizomania – destructive disease of sugar beet (*Beta vulgaris* L. ssp. vulgaris). BNYVV is rigid, rod-shaped virus, composed of particles 390, 265, 100 and 85 nm long and 20 nm wide [8]. The multipartite single-stranded RNA genome consists of four RNA species, and the 5th species is being found in some isolates (6.8, 4.7, 1.8, 1.5 and 1.45 kb) [10]. The virus is transmitted by vector, the soilborne plasmodiophorid *Polymyxa betae* Keskin, which survives in infested soil for many years [11]. The disease now has a worldwide distribution (Europe, Asia and North America) [9] and is characterized by reduced taproot size with extensive proliferation of lateral rootlets that leads to decline in root yields and sugar content [6].

Surveys to detect the presence of BNYVV have been regularly carried out in Lithuania since 1998 using DAS-ELISA test. In recent years this virus was detected in two areas of Lithuania [3, 13]. *C. quinoa* plant tissues mechanically inoculated with crude extract of infected sugar beet rootlets were used for investigation of rhizomania agent. Here we present identification of BNYVV using EM (i.e., the particle morphology) and Western blot assay (i.e., CP molecular weight).

Materials and methods. Isolation of BNYVV was performed by mechanical inoculation of *C. quinoa* using infected sap of sugar beet bearded roots. Symptomatic leaf tissue was tested for BNYVV by DAS-ELISA [2] using DSMZ immunological kit, for virus particles morphology by EM and further it was used for virus purification.

BNYVV was purified as described by Bouzoubaa with some modification [1]. Healthy *C. quinoa* extract was used as negative control. The virus was concentrated twice by PEG, NaCl. Afterwards the virus was purified by high speed ultracentrifugation through 20 % sucrose cushion and in 5-45 % sucrose density gradient centrifugation. Purified virus suspension was used for EM examination and Western blot analysis.

In attempts to examine virus particles, carbon coated grids were placed on drops of purified virus suspension, stained with 2% uranyl acetate (UA), and were viewed using JEOL JEM-100S electron microscope at x 20 000 magnification.

For BNYVV protein analysis purified virions were denatured and CP was separated by 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) in duplicate [5]. One gel was stained with PageBlue™ Protein Staining Solution. Capsid molecular mass was estimated using PageRuler™ Prestained Protein Ladder. For Western blot analysis the other gel with BNYVV CP was transferred to nitrocellulose membrane [12]. Membrane was probed with IgG to BNYVV (diluted 1:250) and the bound IgG was detected with secondary antibody conjugated to alkaline phosphatase (diluted 1:1000). Antibodies used for Western blot analysis were the same as supplied for DAS-ELISA kit. Blot was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate.

Results and Conclusions. IEM investigation of infected sugar beet rootlets revealed the presence of characteristic different lengths BNYVV particles about 20 nm in diameter (Fig. 1) [8, 10]. Tissue extracts from these rootlets was used for mechanical inoculation of indicator plant - *C. quinoa*, which leaves developed local chlorotic lesions after 5-7 days (Fig. 2).

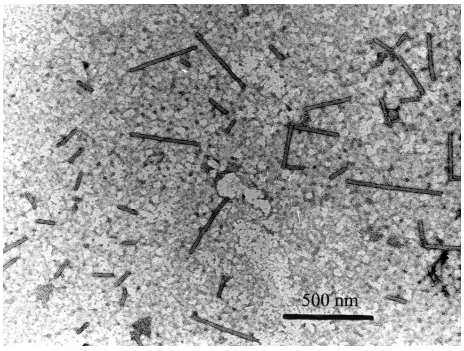

Fig. 1. IEM (instrumental magnification 20 000 x): BNYVV characteristic virus particles about 20 nm in diameter from extracts of sugar beet rootlets.

Fig. 2. Mechanical inoculations with infected plants sap: local chlorotic lesions on indicator *C. quinoa* plant.

The lesions differ in size and appearance, depending on the viral isolate. Isolates carrying RNA3 produce intence yellow lessions, which have a tendency to extend along veins [4]. Isolates carrying only RNA1 and RNA2 induce mild chlorotic local lesions and give a lower purification yield. Systemic infection hosts (e.g. spinach) did not give superior yields of BNYVV [1].

Symptomatic *C. quinoa* leaf tissue with local lesions was collected and used for BNYVV purification. An OD_{260}/OD_{280} ratio was estimated 1.11-1.17 for a pure *benyvirus* suspension. The yield of purified BNYVV preparations was 0.237-0.515 mg from 100 g infected leaves material, assuming that extinction coefficient was 3.2 [1].

EM of purified BNYVV suspension showed rod-shaped virus particles of lengths typical for rhizomania agent (Fig. 3) [8].

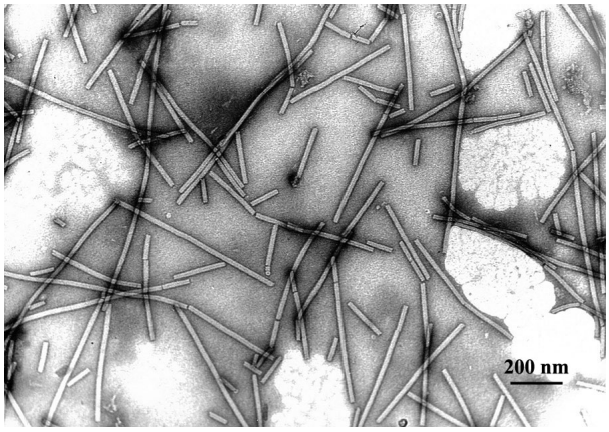

Fig. 3. EM (instrumental magnification 20 000 x): purified BNYVV particles.

The virus so obtained was stored for further examination and antisera production.

Electrophoresis of purified plant viruses can be a helpful and simple tool for characterization of their particles. For this purpose usually SDS-PAGE is used. Viruses can be separated by electrophoresis, and then they can be effectively transferred by electroblotting onto nitrocellulose membranes for further analysis. A great advantage of this technique is that it identifies the virus by two idependent properties of its coat protein – molecular weight and serological specifity [7]. The molecular mass of BNYVV coat protein was estimated by SDS-PAGE to be 21 kDa (Fig. 4). In Western blot BNYVV antiserum reacted only with the CP of purified BNYVV virions and did not reacted with any proteins from healthy *C. quinoa* extracts (Fig. 5).

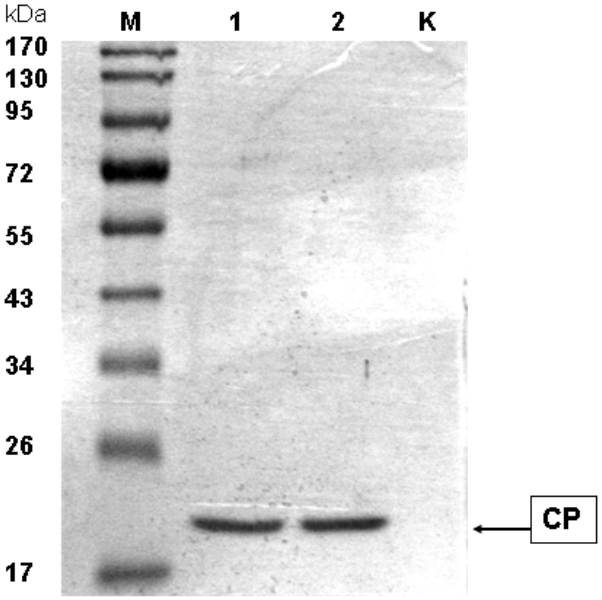

Fig. 4. Analysis of BNYVV CP in 12% SDS-PAGE stained with PageBlue™. An extract from healthy *C. quinoa* was included as control.

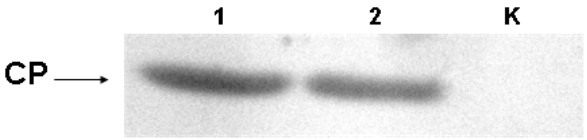

Fig. 5. Western blot analysis of BNYVV CP. An extract from healthy *C. quinoa* was included as control.

These results confirm the identification of BNYVV and so purified virus suspension could be used for antisera preparation.

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I.D. Zhun'ko, Ph.D., N.V. Limanska, Ph.D., B.N. Milkus, D. Sci.

THE SPREAD OF GRAPEVINE VIRUSES ON THE SOUTH UKRAINE

Odessa National University, Odessa, Ukraine; Nursery complex of Close Corporation "Odessa Brandy Company", Odessa, Ukraine

Screening investigations of 726 grapevine samples for the presence of grapevine leafroll-associated viruses (GLRaV-1 and GLRaV-3) grapevine fleck virus (GFkV), grapevine fanleaf virus (GFLV), grapevine virus A (GVA), grapevine virus B (GVB), Rupestris stem pitting associated virus (RSPaV) have been conducted. The grapevine material selected from vineyards of the southern regions of Ukraine was tested by enzyme-linked immunosorbent assay (ELISA-test) and reverse transcription - polymerase chain reaction (RT-PCR). Using both these methods data about the spread of latent viral infections on the vineyards were obtained. The protocol of polymerase chain reaction was optimized during the investigation. GLRaV 1, GLRaV 3, GFLV and GFkV were revealed. Our data indicate the necessity of grapevine planting material certification in Ukraine.

Introduction. Viral diseases of grapevines in Ukraine were firstly investigated and described in 1971 [11]. Since then, more viruses have been found: grapevine leafroll associated virus 3 (GLRaV-3), GLRaV-1, grapevine fleck virus (GFkV), grapevine fanleaf virus (GFLV), grapevine vein mosaic virus, grapevine vein necrosis virus, grapevine stem pitting virus.

Grapevine fanleaf virus, grapevine leafroll associated viruses 1-7, grapevine fleck virus, grapevine virus A, grapevine virus B, Rupestris stem pitting associated virus are the most harmful grapevine pathogenes. They are included by European Community to the list of viral infections that should be tested in certified grapevine planting material production [13]. Unfortunately, propagation planting material (especially regular material) may contain the pathogenic viruses.

Viral infections cause great losses to grapegrowing. Suppression of growth of root system, shoots, leafs, berries and pollination process may occur. Also viral diseases negatively affect the metabolizm and cause the pigmentation of different plant organs (Fig. 1). Viral infections often proceed in latent form [3].

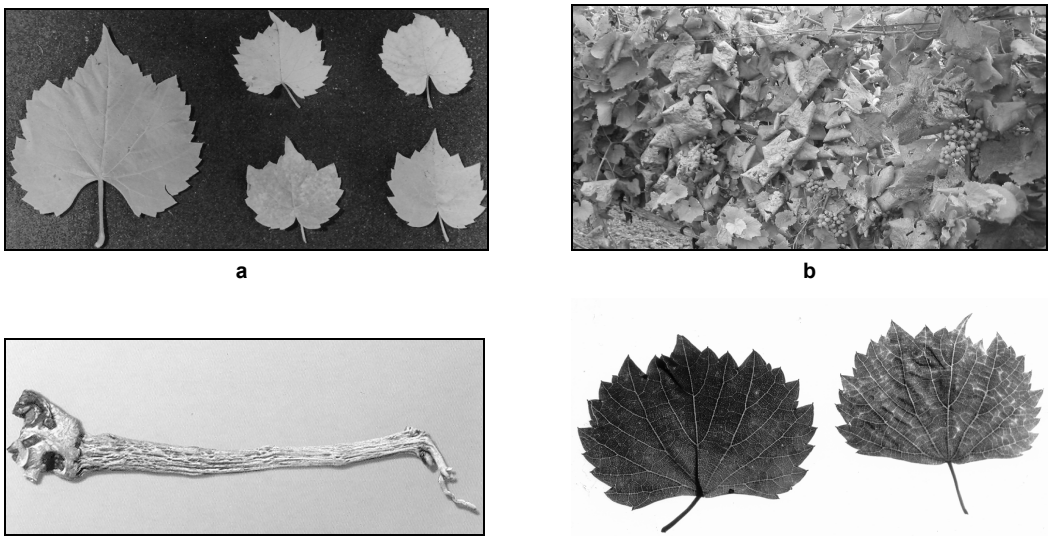

Fig. 1. Various symptoms of viral diseases of grapevine. a: Healthy grape leaf (left) and yellowed stunted leaves of *V.riparia x V.rupestris* 101-14 indicator (right) infected with grapevine fanleaf virus. b: Typical symptoms of grapevine leafroll. c: Typical symptoms of grapevine stem pitting on *V.rupestris* du Lo indicator. d: Stunted growth and vein chlorosis (right) on the leaf of *V.rupestris* du Lo indicator caused by grapevine fleck virus.

The most effective method for viral diseases prevention is a production of virus free grapevine material that can be used for the planting of new vineyards. So early diagnostics of viral diseases allows to define planting material quality quickly. The most sensitive and specific diagnostic methods are enzyme-linked immunosorbent assay and reverse transcription - polymerase chain reaction [2, 12].

Certification of grapevine planting material in Ukraine is actual now [5].

The aim of our work was to investigate the spread of grapevine viruses on the South Ukraine.

Materials and methods. The investigated vineyards are located in the southern regions of Ukraine. During the investigations the certified and regular grapevine planting material of different scion and rootstock cultivars (Pinot noir, Cabernet Sauvignon, Merlot rouge, Chardonnay, Riesling, Aligote, Sauvignon vert, Muscat tzitronny, Verdelho, Bastardo magaratchsky, Ranny Magaratcha, Perlinka, Ay-Petry, Pervenetz Magaratcha, Antey Magaratcha, Haydamack, Kafa, Muscat Livadia, Tcheurnaya apiana, Tzitronny Magaratcha, Chabache, Strashensky, Vostorg, *V.berlandieri* x *V.riparia* Kober 5BB, *V.riparia* x *V.rupestris* 101-14) were tested by enzyme-linked immunosorbent assay and polymerase chain reaction with reverse transcription.

Infected plants kindly donated from University of Bary (Italy) were used as positive controls.

Viral antigens detection was conducted by ELISA using test-systems "Agritest" (Italy). Viral RNA was detected by RT-PCR. Cambial scraping samples, leaves, and petioles were used. Templates and reaction mixtures for RT-PCR were prepared according Rowhani et al. [9]. The following pairs of primers were used: CPV and CPC for GLRaV-1 detection [8], oligo C1 and oligo V1 for GFLV detection [10], C547 and H229 for GLRaV-3 detection [7], RD1 and RD2 for GFkV detection [1], C410 and H28 for GVB detection [7], and C995 and H587 for GVA detection [6, 7] were used.The concentrations of Mg^{++} 1,3 mM for GFkV and GFLV detection and 1,5 mM for GLRaV-1, GLRaV-3, GVA, GVB detection were used. All the reagents were supplied

from “AmpliSens”, Russia, except reverse transcriptase from “Fermentas” (Latvia). Lithuania. RT-PCR was performed in termocycler “Tercik” (“DNA-Technology”, Russia). Reverse transcription was carried out during one hour at 42ºC followed by 35 cycles of PCR according to Rowhani et al. [9]. The annealing temperature for the primers to genome sequences of GLRaV-3, GLRaV-1, GVA, GVB viruses was 56 ºC [4]. For the primers to GFkV genome sequences, the annealing temperature was 62ºC, and for GFLV the annealing temperature was 61ºC.

Amplicons were analysed in 1,5% agarose gels. The marker 800-200 bp (“AmpliSens”, Russia) was used. The buffer for electrophoresis (“AmpliSens”, Russia) contained ethidium bromide. Gels were vizualised by UV transilluminator and photographed using a “Samsung” video system.

The obtained data were statistically analyzed by applied program “Statistica 6.0”.

Results and Discussion. The results of viral disease diagnostics in vineyards of Odesa region showed that 24,0 % of plant samples were latently infected by grapevine fleck virus (Fig. 2).

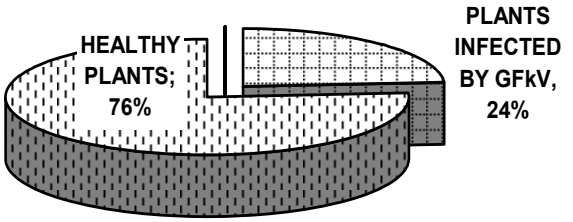

Fig. 2. Results of detection of grapevine viruses in some Odesa region vineyards.

We also revealed four latent viral infections in grapevine planting material from Crimea. The highest level of infection was registered for the grapevine leafroll associated virus: 1 – 24,6 %. Grapevine fleck virus was identified in 16,9 % of tested samples. Grapevine fanleaf virus and grapevine leafroll associated virus 3 were revealed in 2,3 % and 3,3 % correspondingly (Fig. 3).

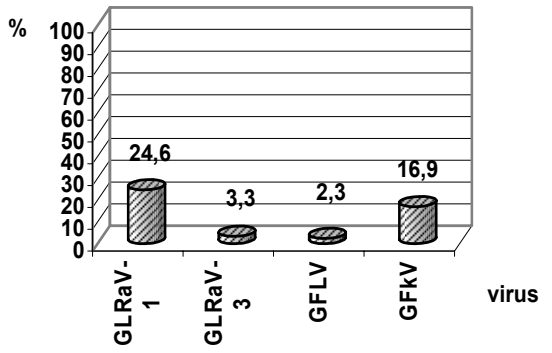

Fig. 3. Results of detection of grapevine viruses in some Crimea vineyards.

Grapevine plantations in Kherson region also were tested for the presence of grapevine viruses. During the investigations we revealed grapevine leafroll and grapevine fanleaf diseases. Grapevine leafroll associated virus 3 was identified more often than other viruses (6,7 % of tested grapevine samples were infected by this virus). Grapevine plants were infected by grapevine fanleaf virus and grapevine leafroll associated virus 1 in 3,3 % cases (Fig. 4).

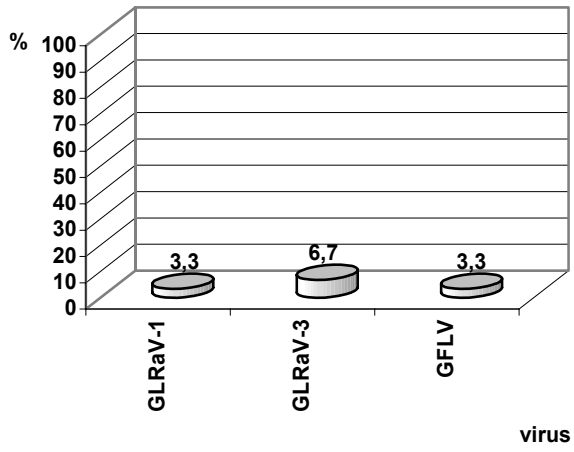

Fig. 4. Results of detection of grapevine viruses in some Kherson region vineyards.

Thus, we determined different levels of grapevine viruses contamination on the south Ukraine vineyards. In some vineyards viruses are widely spread. Such high levels of contamination are explained by non-satisfied quality of planting material.The obtained data suggest the necessity of viral diseases control and introduction of cultivar certification system in our country.

Conclusions. 726 grapevine samples from south Ukraine vineyards were tested for the presence of latent viral infections.

Enzyme-linked immunosorbent assay and polymerase chain reaction with reverse transcription have been used for harmful grapevine viruses detection. Our investigations allowed us to reveal and identify the next viruses: grapevine fanleaf virus (2,3 – 3,3 % of tested plants), grapevine fleck virus (16,9 – 24,0 %), grapevine leafroll associated virus-1 (3,3 – 24,6 %), grapevine leafroll associated virus-3 (3,3 – 6,7 %).

For the first time in Ukraine the harmful grapevine viruses were detected by PCR based technique.

Vineyards free from grapevine viruses were revealed and recommended for grapevine planting material production.

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☎ (38044) 239-32-22; (38044) 239-31-72; (38044) 239-31-58; факс (38044) 239-31-28
E-mail: vydav_polygraph@univ.kiev.ua