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# High-Sensitivity Express Immunochromatographic Method for Detection of Plant Infection by Tobacco Mosaic Virus

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**Abstract**—A highly sensitive express immunochromatography method for molecular diagnosis of plant virus infections was elaborated on the example of a model object – tobacco mosaic virus (TMV). The analysis time does not exceed 5 min, and the lower limit of TMV detection in non-clarified leaf extract (2-4 ng/ml) is comparable with the sensitivity of the enzyme-linked immunosorbent assay of the virus. A single measurement requires 0.1-0.2 ml tested solution (extract from 10-20 mg of leaf material). The sensitivity of TMV determination in the leaf tissue extract was increased by more than one order of magnitude using signal enhancement by silver and is 0.1 ng/ml. In this case, analysis time did not exceed 25 min. The simplicity of this method makes it especially convenient in express diagnosis of numerous analyzed specimens. The prototype of a diagnostic kit for serial analyses of plant viral infections both in laboratory and field conditions was elaborated.

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*Key words*: tobacco mosaic virus, immunochromatography analysis, colloidal gold, lateral flow immune analysis

Molecular diagnosis of viral infections, in particular of plant viral infections, is usually done by the enzyme-linked immunosorbent assays (ELISA) [1, 2]. Current ELISA techniques enable quantitative virus determinations in cell extracts up to the concentration of 1 ng/ml. However, they are labor and time consuming and require special training of staff and expensive equipment, which restricts their usage to specialized laboratories.

Mass express diagnosis of viral infections for virus-free plant culture industry or individual use by private individuals requires inexpensive, rapid, and simple technologies that make possible analysis without special skill and equipment even under field conditions. Besides, one of main requirements for all present-day methods of field diagnosis is the existence of analytical kits for infectiousness testing. The kit should include all necessary reagents, i.e. it should be necessary to add only the specimen under analysis. It is ideal to use in such a kit stained reagents that it make possible to reveal infection by simple visual observation.

Methods of immunochromatography analysis (IChA) (called also lateral flow immunoanalysis in the literature [3-5]) using nanoparticles of colloidal gold as a label making it possible to visually detect the compound under determination have become widespread for analytical purposes.

This work deals both with the design of an immunochromatography test system using colloidal gold as a label for express diagnosis of viral infections (on the example of tobacco mosaic virus (TMV)), the sensitivity of which is comparable with that of ELISA, and with design of a prototype of a diagnostic kit for mass analysis of viral infections.

*Abbreviations*: ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); ELISA, enzyme-linked immunosorbent assays; HRP, horseradish peroxidase; IChA, immunochromatography analysis; IgG-Au, antibody conjugate with colloidal aurum; IgG-HRP, antibody conjugate with horseradish peroxidase; TMV, tobacco mosaic virus; TPB, 0.01 M K-phosphate buffer, pH 7.4, 0.1 M NaCl, 0.1% Triton X-100.

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## MATERIALS AND METHODS

The TMV strain U1 was used as a model object. Different viral strains from infected *Nicotiana tabacum* var. Samsun-NN plants were isolated and purified by fractional salting out with ammonium sulfate followed by differential centrifugation [6].

Purified preparations of potato viruses X, S, M, Y, A, and L were obtained by methods used in the Lorkh All-Russian Research Institute of Potatoes of the Russian Academy of Agricultural Sciences [7-11]. Potato viruses X and Y were accumulated on *N. tabacum* var. Samsun-NN plants, potato viruses S and A were accumulated on the *N. clevelandii* plants, and potato M virus was accumulated on tomato plants *Lycopersicum esculentum*. The potato leaf roll virus was accumulated on thornapple *Datura stramonium* L. plants. The plants were infected using the grafting technique.

Polyclonal antisera were obtained by subcutaneous immunization with purified TMV preparation (strain U1) [2]. Globulin fraction of rabbit antiserum was isolated by precipitation with 6 kDa polyethylene glycol (Sigma, USA) or with ammonium sulfate. The IgG fraction was obtained from antiserum by step-wise ion-exchange chromatography on DEAE-cellulose (Whatman, Great Britain) with eluent 10 mM Tris-HCl, pH 8.5, 50 mM NaCl [12]. Concentration of antibodies was determined spectrophotometrically at 280 nm ($\varepsilon_{280} = 2.08 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$) [2].

Horseradish peroxidase (HRP) was used as a label for ELISA. Antibody conjugates with HRP (IgG-HRP) were obtained using the technology based on oxidation of the HRP carbohydrate component by sodium periodate [2].

Antisera were tested by indirect ELISA using polystyrene plates of maximal sorption capacity (Nunc, Denmark) with preliminarily adsorbed antigen. Enzyme-linked sandwich analysis of TMV was carried out as described elsewhere [2].

Colloidal gold preparation was obtained by reduction of $HAuCl_4$ with sodium citrate [13]. Determination of the mean size of colloidal gold particles and estimation of the gold nanoparticle polydispersion were carried out spectrophotometrically [14], by dynamic laser light scattering on a Zetasizer Nano Series apparatus (Malvern Instruments Ltd., Great Britain) at 633 nm as described in the manufacturer's instructions, and in a Hitachi HU-12 transmitting electron microscope (Hitachi, Japan) at accelerating voltage 75 keV. Carbon film supports were used for electron microscopy. Five microliters of colloidal gold preparation were applied on the film-covered grid, and solution excess was removed with filter paper.

Antibody binding to TMV in 0.01 M K-phosphate buffer, 0.1 M NaCl, pH 7.4 (100 μg/ml TMV, 0.1 mg/ml immunoglobulins) was analyzed in an atomic force microscope Nanoscope IIIa (Digital Instruments, USA) [15] in resonance regime.

Antibodies to TMV were immobilized on the surface of colloidal gold particles in accordance with the following technique: the colloidal gold solution with $A_{520} = 1.0$ was titrated with 0.1 M $K_2CO_3$ to pH 8.5, and antibody solution (0.1 mg/ml) in 10 mM Tris-HCl, pH 8.5, was added. Minimal stabilizing concentration of polyclonal antibodies was estimated by the change of the gold sol optical properties at 580 nm 5 min after addition of 10-150 μl of antibody preparation with protein concentration 0.1 mg/ml and 0.1 ml of 10% NaCl to 1 ml of colloidal gold solution. BSA was added to final concentration 0.1% for additional stabilization of the resulting polyclonal antibody complex with colloidal gold nanoparticles. To remove non-bound antibodies, the mixture was centrifuged for 45 min at 10,000 rpm and 4°C. The pellet was resuspended in 0.05 M K-phosphate buffer, pH 7.5, 0.01% $NaN_3$, 0.1% BSA. The resulting antibody–colloidal gold conjugate (IgG-Au) was stored at 4°C.

Extracts from normal and TMV-infected tobacco plants were obtained by grinding 100 mg plant material in a ceramic mortar with addition of 1 ml TPB (0.01 M K-phosphate buffer, pH 7.4, 0.1 M NaCl, 0.1% Triton X-100). The resulting homogenate (plant mass/buffer volume, 1 : 10) was centrifuged for 5 min at 10,000 rpm. The clarified extract was analyzed using the enzyme-linked "sandwich" immunosorbent assay. Clarified and non-clarified leaf extracts of normal tobacco plant were used in immunochromatography tests.

Membrane filters (MDI, India) were used in the multi-membrane composite for immunochromatography. To prepare test strips, solution of specific polyclonal antibodies to TMV in the range of concentrations 0.05-1.0 mg/ml in 0.01 M K-phosphate buffer, 0.1 M NaCl, pH 7.4, was applied onto an analytical nitrocellulose membrane to form an analytical zone. To form a control zone of the immunochromatography system, affinity-purified anti-rabbit immunoglobulins from goat antiserum (Imtech, Russia) at concentration of 0.5-1.0 mg/ml in 0.01 M K-phosphate buffer, 0.1 M NaCl, pH 7.4, were used. The IgG-Au conjugate was applied onto the 260 × 5-mm PT-5 membrane strip by immersion into the corresponding solution of this conjugate or using an AirJet Quanti 3000 automatic dispenser (BioDot, Inc, USA). The test strip components were fixed using a prepared solid base with adhesive (MDI) using an LM 100 laminator (Zeta Corporation, South Korea), and the prepared test strips were cut with an Index Cutter-I guillotine cutter (A-Point Technologies, USA). The test strips were put into plastic containers and sealed with silica gel in packets of polyethylene-laminated aluminum foil under conditions of low (30%) relative humidity using an FRG-1000 Heat Sealer semiautomatic packer (Arista Biologicals, USA).

For quantitative evaluation of results of IChA, stain intensity was detected using a Reflecom portable reflecting photometer and the Videotest program (Azamat, Russia).

The sensitivity of the IgG-Au conjugate determination and therefore of virus determination sensitivity was enhanced using the Silver Enhancement kit (GE Healthcare, Great Britain) according to the manufacturer's recommendations. To do this, the analytical part of the test strip was cut out and put for 5-20 min into a mixture of the initiator and silver enhancer solutions.

## RESULTS AND DISCUSSION

A number of methods having various efficiencies are used for research and industrial diagnosis of plant viral diseases. These methods include biological (plant-indicators) and immunological (serodiagnosis, solid-phase immunoenzyme assay) tests, electron microscopy, and methods based on molecular hybridization of nucleic acids (molecular-hybridization analysis, polymerase chain reaction). In the Russian Federation mainly two immunological tests—the dropwise serodiagnosis and solid-phase ELISA—are used for mass diagnosis of phytovirus infections [16, 17].

This work deals with elaboration of a highly sensitive and efficient immunochromatography technique for express diagnosis of plant viral infections on polycomposite test strips using colloidal gold nanoparticles as a visual label, and the prototype of a diagnostic kit for mass analyses under field conditions has been developed. The analysis is based on the use of high-affinity polyclonal antibodies purified by the stepwise ion-exchange chromatography and capable of efficient interaction with numerous protein antigenic determinants of the viral particle (the length of the TMV virion is 300 nm, radius 9 nm). The TMV capsid contains about 2130 identical protein molecules consisting of 158 amino acid residues, having molecular mass 17.42 kDa, and forming a regular helical structure with a pitch of 2.3 nm that protects the RNA. It contains 49 protein subunits per three turns of the helix [18, 19].

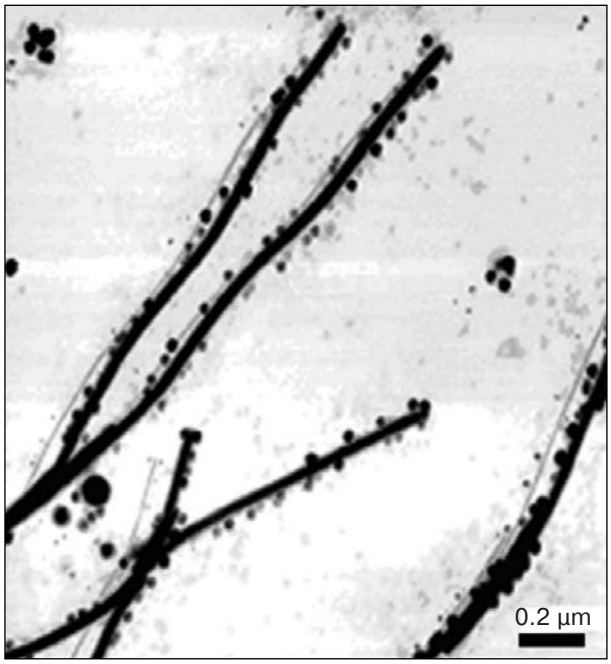


**Fig. 1.** Analysis by atomic force microscopy of antibody binding to TMV.

These facts define the high local concentration of antigenic determinants on the surface of the viral particle, and as a result, the high immunogenicity of particle. Investigation by atomic force microscopy of the gold-labeled antibody interaction with virus has shown that 5-10 antibody molecules, isolated from polyclonal antiserum, can simultaneously bind a single viral particle (Fig. 1). It should be noted that under conditions of antibody binding most TMV particles are joined by their blunt ends, thus forming concatemers of different length. The specificity of the antisera to TMV was investigated before isolation of antibodies and preparation of their conjugates for solid-phase ELISA and IChA.

The antiserum specificity (titer) towards the TMV wild strain was tested by solid-phase ELISA using purified preparations of viruses isolated in our laboratory and adsorbed on the polystyrene plate surface. The two different TMV strains (preparations of U1 strains, crTMV, isolated from leaves of tobacco, tomato, and Cruciferae family plants) and preparations of potato viruses (X, Y, M, A, S, and virus of potato leaf roll), differing in morphology, structure, and physicochemical properties, were used [20]. Antiserum to TMV strain U1 was characterized by high titer (over $1 \cdot 10^6$), which made possible to design highly sensitive and specific analytical immunochromatography test systems. Cross reactivity on the studied TMV strains isolated from tomato and Cruciferae leaves was 0.1% of the titer determined on the original material (TMV U1 from tobacco). Comparative testing of anti-TMV antiserum on heterologous potato viruses has shown even lower cross-reaction. The high specificity of the anti-TMV antiserum was also confirmed by a similar serum testing in the case when the juice of normal and infected leaf material was adsorbed on the plate (tobacco leave juice in dilution 1 : 10 in 0.01 M K-phosphate buffer, pH 7.4, 0.1 M NaCl was used).

Figure 2 shows calibration dependence for TMV (strain U1) determination by the solid-phase ELISA in clarified tobacco leaf extract. Sensitivity of the method was 8 ng/ml, and variation coefficient did not exceed 8-10% ($n = 10$) over the studied interval of optical density calibration dependence on concentration of the virus under determination. It was also found that endogenous components of plant juice had practically no effect on main parameters of virus detection compared to TMV determination in buffer solution.

The time required for the solid-phase sandwich ELISA exceeds 4 h, which is characteristic of this analy-

sis and is due to the time required for heterophasic immunochemical reactions caused by external diffusion restrictions of mass transfer due to the necessity of the antigen or conjugate molecules to migrate from the solution volume onto the solid phase surface for participation in the reaction [2]. A possible way to solve this problem is to reduce the unstirred boundary diffusion layer near the solid body surface, the liquid inside being considered as motionless so that mass transfer of the immunochemical mixture components to the solid body surface is caused only by molecular diffusion [21]. This can be realized by using mobile-phase lateral diffusion along the porous carrier surface [22]. It is reasonable to suppose that the thickness of the unstirred liquid layer within the narrow membrane pores becomes significantly lower in the course of lateral migration of the analytical test system components (antibodies, conjugates, and antigens). In this case the rate of antigen binding to immobilized antibodies upon immunochromatography increases by one order of magnitude compared to traditional dot-analysis, and the kinetics of antigen complex formation with antibodies during lateral mass transfer along the porous membrane surface is close to the kinetics in solution [23].

Nanoparticles of colloidal gold were used as a marker of anti-TMV antibodies in the immunochromatographic test system developed in our laboratory. The advantage of colloidal gold as an antibody marker is first defined by the simplicity of obtaining the predicted size nanoparticles for optimal protein/gold ratio in conjugate synthesis; second, by the possibility of drying the antibody–colloidal gold conjugate without its inactivation; and third, the high sensitivity of visual detection of the label based on the gold sol [24].

We prepared colloidal gold by reduction of chloroauric acid using sodium citrate. Analysis of the preparation by dynamic laser light scattering showed that the bulk of particles have diameters in the range of 20 ± 5 nm. Independent analysis by transmission electron microscopy confirmed the mean diameter of most of the spherical colloidal gold particles determined by laser light scattering.

In this work, polyclonal antibodies have been used whose molecules can differ significantly in chemical composition and immunochemical and physicochemical properties. To obtain colloidal gold conjugates with heterogeneous protein molecules of polyclonal antibodies, optimal synthesis conditions were chosen such as pH and ionic strength of solution and protein (antibody preparation) concentration in the sol (Fig. 3). Minimal stabilizing concentration of polyclonal antibodies was estimated by the change in optical properties of stabilized gold sol at 580 nm after addition to the colloidal gold solution of various amounts of antibodies and NaCl solution. Non-stabilized colloidal gold solution changed its color in the range of antibody concentration of 0-5 μg/ml due to coagulation of sol particles. Figure 3 shows that in the studied range of sol loading by antigens of 1-15 μg/ml sol, the minimal stabilizing concentration of immobilized antibodies was 6 μg/ml, which is revealed by the plateau at 580 nm of the colloidal system optical density dependence on concentration of immobilized antibodies. In this case the gold sol stabilized by antibodies did not change its color after addition of electrolyte solution. Finally, antibody concentration for immobilization on the colloidal gold particles was chosen according to the dependence of the colloidal solution optical density at 580 nm on antibody concentration, based on the criterion of 10% excess over the level providing for the plateau of optical density (Fig. 3). To fulfill this requirement, the antibody concentration 7 μg/ml colloidal gold solution was chosen. Minimal load of immobilized antibodies, determined on the basis of concentration dependence of colloidal gold binding to antibodies and providing for maximal sol resistance to the electrolyte coagulating effect, was then used upon obtaining the polyclonal antibody conjugates with nanoparticles of colloidal gold.

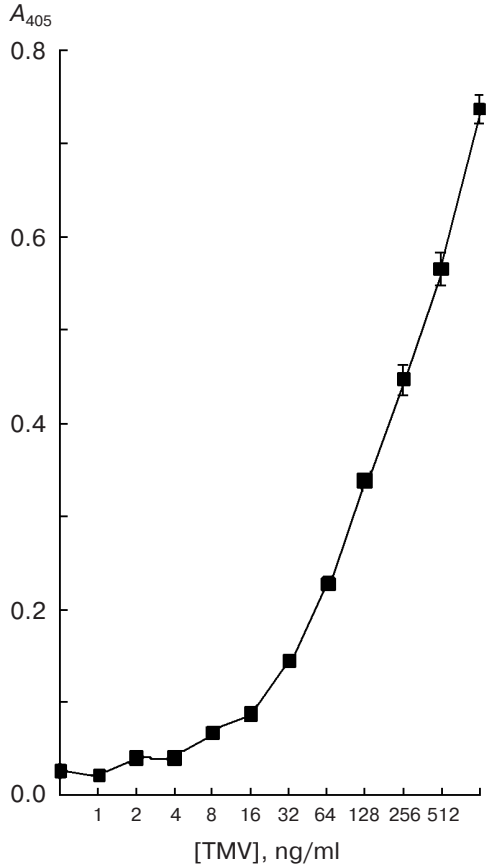


**Fig. 2.** Calibration dependence for TMV (strain U1) detection in tobacco plant sap (1 : 10 TPB) by the solid-phase immunoenzyme "sandwich" technique (sorption of antibodies (2 μg/ml) for 12 h at 4°C in 0.02 M carbonate buffer, pH 9.5, on polystyrene plates; interaction with IgG-HRP (1 nM by the marker enzyme) for 60 min, 37°C, TPB). Ordinate axis, optical density $A_{405}$ of the product of enzymic reaction (30 min, 20°C).

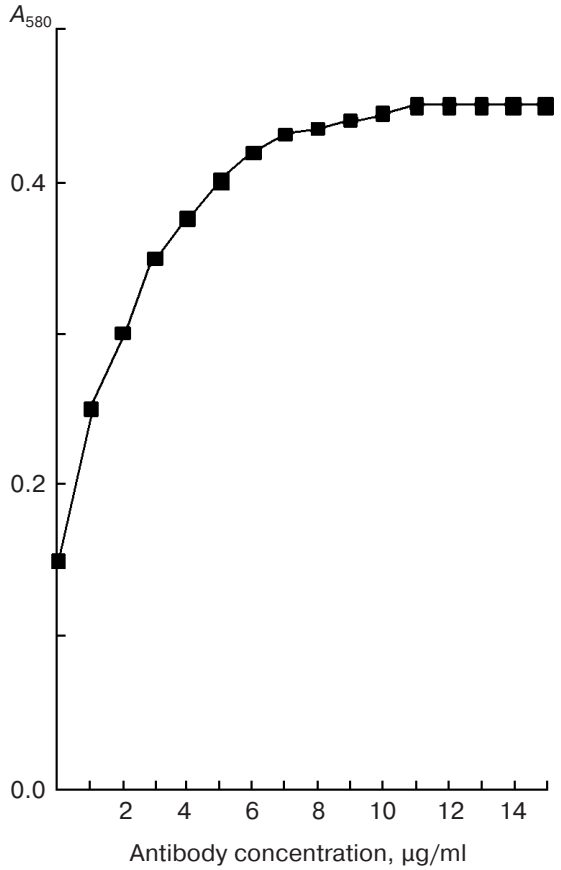


**Fig. 3.** Change in optical properties of stabilized gold sol at 580 nm after addition of different amounts of polyclonal anti-TMV antibodies and NaCl solution to colloidal gold solution.

The observed effects can be explained by the fact that the adsorbed protein macromolecules containing polar groups are well hydrated and form around colloidal gold particles powerful hydrated envelopes that prevent adhesion of nanoparticles (the adsorption-solvate factor) [25]. Besides, only in the presence of protein stabilizers the gold sol can be applied onto a porous support like that of glass fiber, dried to dryness, and then the dry residue can be completely transformed into colloidal solution. The latter circumstance is especially important in immunochromatography when all used components are applied onto various membranes and dried. Antibodies labeled by colloidal gold are dissolved in the course of analysis, which provides for their quantitative and rapid interaction with the antigen under study during combined lateral migration with the analyzed solution along the surface of the porous membrane. At the same time, introduction of too low amounts of high molecular weight substances into the gold sol solution can result not in protection, but on the contrary, in the loss of the colloidal solution stability. One of the factors responsible for such behavior of a colloidal system is adsorption of different regions of the same protein macromolecule on the surface of different colloidal nanoparticles, after which particles of colloidal gold agglutinate, thus forming large precipitating agglomerates.

Three antibody preparations with colloidal gold as marker in IChA are applied onto the multi-membrane composite test strip: 1) antibodies to the antigen under determination (in our case it is a virus) conjugated with particles of colloidal gold. These antibody conjugates are applied near the part of test strip immersed into the solution under study (plant extract); 2) antibodies to the antigen under study (virus) which are immobilized on analytical membrane in the test zone; 3) secondary (antispecies) antibodies to primary (antivirus) antibodies that are immobilized on analytical membrane in the control zone of the test strip.

Upon test strip immersion into the investigated cell extract, containing the virus under study, virus particles, stimulated by capillary forces, begin to migrate along the membrane surface together with the front of the liquid. Moving along the membrane, the virus forms immunochemical complexes with the colloidal gold-labeled antibodies that continue further migration together with the front of the solvent. After reaching the zone of the membrane-immobilized primary antibodies, the virus and its complex with the colloidal gold-labeled antibodies interact with antibodies immobilized on the porous membrane; in this case there are formed both the triple immunocomplex of immobilized antivirus antibodies with virus and colloidal gold-labeled antivirus antibodies and the complex of the virus with the membrane-immobilized antibodies. Thus, in the analytical zone of the immunochromatography membrane the stained triple complex of immobilized antibodies with the virus and the colloidal gold-labeled antibodies is formed as a narrow band easily registered visually or by instrumental methods of stain intensity detection. The intensity of staining formed in the analytical zone during analysis is proportional to the amount of the virus in the test sample (Figs. 4 and 5). In the absence of virus in the analyzed plant extract, the colloidal-gold-labeled antibodies easily pass the zone of membrane-immobilized antivirus antibodies and no stained band is formed in the analytical region of immunochromatography membrane; this band is formed only in the control zone. The stained band in the control zone is indicative of the efficiency of the test strip.

Drying thin polymeric porous carriers with preliminarily applied antibody solutions provides for the interaction of immunoreagents in very high concentrations during analysis, because re-dissolution of the immunoreagents takes place in a very small volume of mobile phase that is defined by properties of the porous carrier. Since microvolumes of reagents are applied onto the strip, their consumption in a single IChA is low, which makes the technology of test strip production economical.

Figure 4 shows calibration dependences of registered signal in the analytical zone of an immunochromatogra-

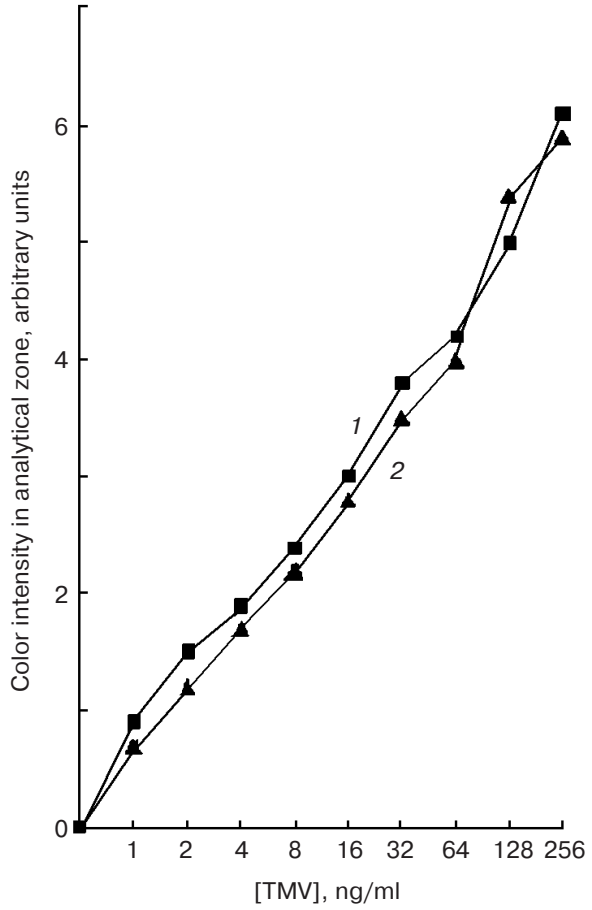


**Fig. 4.** TMV (strain U1) determination in TPB solution (*1*) and in tobacco plant juice (1 : 10 TPB) (*2*) by immunochromatography. Five microliters of Au-IgG ($A_{520}$ = 3.0) were applied onto the PT-R5 membrane and IgG (0.25 mg/ml) was applied onto a CNPF-SN-12-L2-H50 nitrocellulose membrane; the lower and upper AP-045 absorbing membrane and GFB-R4 membrane for specimen application were also used. Analysis time was 2-5 min.

phy membrane in the case of determination of TMV (strain U1) purified preparation in TPB solution and in unclarified leaf extract of normal tobacco plant (dilution 1 : 10 in TPB) to which different amounts of the virus were added in advance. Sensitivity of TMV detection in TPB solution was 1 ng/ml; analysis time was 2-5 min.

The developed express system of immunochromatography of TMV detection makes it possible to reveal virus infection with a lower limit of detection 1-2 ng/ml, comparable to the similar parameter for one of the most sensitive methods for this virus – solid-phase ELISA (Fig. 2). In this case, the time of analysis is reduced from several hours to several minutes. It should be noted that if there is no studied virus in the buffer solution, practically no signal is revealed in the analytical zone of the immunochromatography membrane, which is extremely important for visual evaluation of results in non-laboratory and field conditions. As the concentration of determined virus in the buffer solution increases, the registered signal in the analytical zone is proportionally enhanced (Figs. 4 and 5).

In the case of TMV determination in unclarified leaf extract of normal tobacco plant to which known amounts of virus preparation were added in advance, no significant effect of endogenous micro- and macrocomponents of plant juice on results of measurements was registered (Fig. 4). Colored pigments of the tobacco leaf juice are practically completely retained on the absorbing membrane and do not interfere with instrumental or visual evaluation of the analysis results. Despite increased viscosity of analyzed solution compared to the original buffer solution, the virus detection sensitivity in this case was 2-4 ng/ml, while the time of analysis did not increase from 5 min. Thus, in this variant of analysis investigation of even unclarified leaf homogenate in TPB (1 : 10) is possible, which makes sample preparation significantly easier for studying in non-laboratory and field conditions.

After analysis, membranes retain the original color in the analytical and control zones for a long time and can be scanned (Fig. 5) for quantitative evaluation during field-testing using a reflectance photometer. Within a single series, coefficient of variation of photometric measurements for control and analytical zones of the immunochromatographic composite in the working interval of TMV detection does not exceed 15% ($n$ = 10). As in solid-phase ELISA, in this case from 0.1 to 0.2 ml tested solution (10-20 mg leaf material) is required for one measurement, which makes this method especially efficient in analysis of small amounts of tested plant material.

Comparison of our immunochromatography test system with an analogous system of Agdia (USA) has shown that their sensitivities are similar. Under our conditions the sensitivity of TMV (strain U1) detection in TPB buffer on Agdia immunochromatographic test strips was 8 ng/ml and analysis time was 15-30 min. The repro-

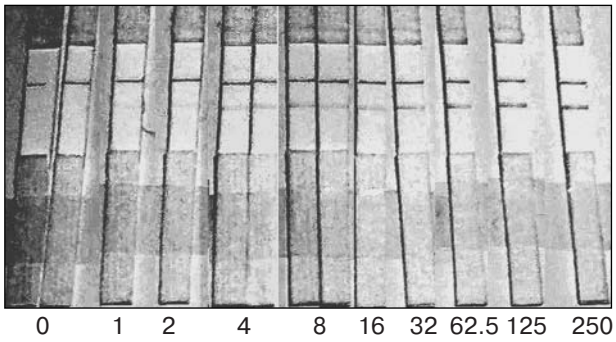


**Fig. 5.** TMV determination by immunochromatography in TPB solution. Figures below corresponding test strips point to final concentration of TMV U1 added to the aliquot of normal plant leaf extract.

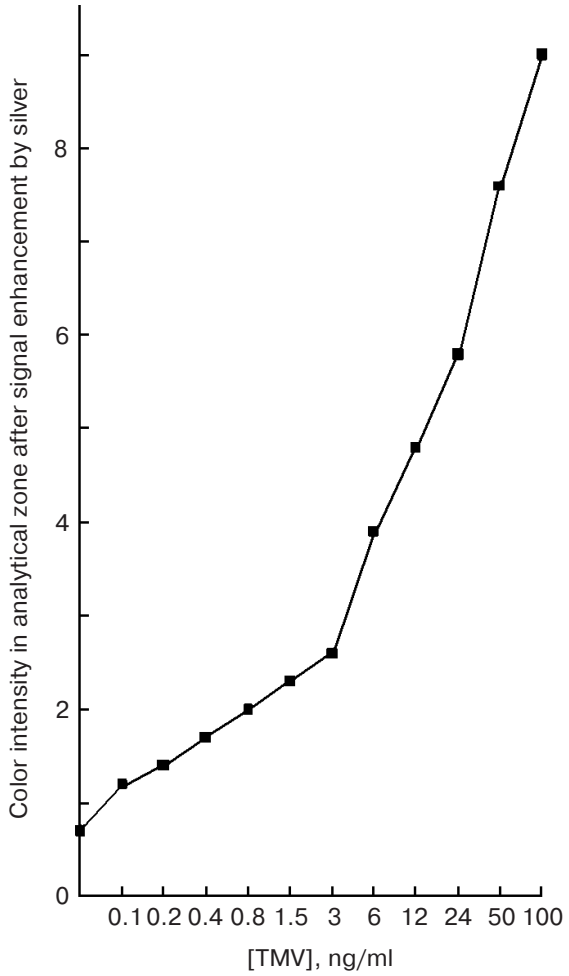


**Fig. 6.** Determination of TMV U1 in tobacco leaf juice (1 : 10 TPB) by immunochromatographic technique using silver enhancer. Two microliters of Au-IgG ($A_{520}$ = 3.0) were applied onto PT-R5 membrane, IgG (0.1 mg/ml) was applied onto CNPF-SN-12-L2-H50 nitrocellulose membrane, and absorbing lower and upper AP-045 membrane and GFB-R4 specimen application membrane were also used. Time of analysis was 10 min.

ducibility of the imported test strips and those described in this work is comparable, because the coefficient of variation did not exceed 14-15% ($n$ = 10) in the studied range of virus concentrations.

The system of enhancement by silver salts of intensity of spots or bands stained by colloidal gold was elaborated for immunochemical reactions on synthetic membranes as in dot or blot detection of antigens. As far as we know, we are the first to use such enhancement in immunochromatographic detection of plant viruses. To increase the IChA sensitivity, we used a commercial Silver Enhancement system (GE Healthcare). As seen in Fig. 6, this results in increase in the TMV (molecular mass 39 MDa) detection in the leaf tissue extract for by one order of magnitude – reaching 0.1 ng/ml (~$2.5 \cdot 10^{-15}$ M).

The elaborated highly sensitive express method of TMV infection diagnosis by immunochromatography on porous membrane carriers is a basic technique. We have used it as the basis for elaboration of a prototype of a kit for diagnosis of economically important potato virus infections (in preparation). Its further development depends on possible increase in sensitivity, productivity (by simultaneous detection of several viruses on a single test strip), and certainly, on expansion of its application for agricultural and medical diagnosis of infections and environmental protection for determination of a broad circle of plant, animal, and human viruses.

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