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Highly sensitive field test lateral flow immunodiagnostics of PVX infection

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Abstract A test system is described and expanded upon for mass field immunochromatography assay on porous membrane carriers for rapid diagnostics of potato virus X (PVX) in potato leaf tissue and sprout extracts using colloidal gold nanoparticles as a marker. Sensitivity of the assay developed for PVX identification is found to be comparable to the sensitivity of solid-phase sandwich-ELISA. Complete assay time does not exceed 15 min, and the lower limit of the PVX detection in non-clarified leaf extract is 2 ng/ml. A single measurement requires 0.1–0.2 ml (3–5 drops) of tested solution only (extracted from 10–20 mg of potato leaf tissue or sprouts). The simplicity and reliability of the method makes it especially efficient in direct rapid monitoring of many infected potato specimens in the field, as verified by field trials of 360 clones of 28 domestic and foreign cultivars of potato. A diagnostic kit for routine analyses of potato viral infections both in the laboratory and in the field is described and expanded upon.

Keywords Colloidal gold · ELISA · Molecular diagnostics · Potato virus X · Test-strip

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Introduction

More than 10% of world potato production is in Russia, where the potato is a key foodstuff. Approximately 50 potato viruses have been identified in various countries with differing climates (Anisimov et al. 2009; Loebenstein et al. 2000). Potato virus X (PVX) is an economically important potato pathogen, infecting potato cultivars in a wide range of territories. PVX gives rise to potato latent virus disease, which causes mild mosaic of potato, the symptoms of which are slight or negligible. There may be slight dwarfing of the plant or deformation of foliage, and intermediary vein mottling or mosaic is also seen. Even when there are no visible symptoms in some potato cultivars, crop failure due to PVX infection can reduce potato yield by 10–15%. Some strains of PVX are able to overcome resistance of the host, secured by potato resistance genes N_x and N_b, and induce severe necrosis at the top of the plant, resulting in up to 25% crop failure (Cockerham 1954; Jones 1985). Mixed infections of PVX with other viruses such as potato virus Y (PVY) causes more damage, increasing the disease effect (Loebenstein et al. 2000; Simakov et al. 2008).

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Major activities in the struggle against virus diseases in improving the production of potato seed include the isolation of seed-growing land, the clearing of visually infected plants, the removal of crops and early cleaning up of a potato field, and intensive disease testing via molecular diagnostics of a virus infection.

Currently, laboratory molecular diagnostics of viral infections of the potato are mainly performed through the double antibody sandwich enzyme-linked immunosorbent assay, which allows quantitative detection of potato virus in extracts of leaf material (clarified leaf juice diluted 10–20-fold with buffer solution) with a limit close to 1 ng of virus per 1 ml of extract. However, this method is time-consuming, special equipment is required, and the appropriately qualified personnel needed means that it is limited to specialized laboratories.

For general mass monitoring of potato infections, simple, reliable, highly specific, and highly sensitive rapid methods applicable to a wide range of viruses enable diagnostics to be conducted swiftly under field conditions by the ordinary user without the need for special skills and equipment. Therefore, one of the basic commercial requirements of all modern methods used in molecular diagnostics in the field (in medicine, veterinary medicine, and agriculture) is a complete diagnostic kit ready for use. Because of the high commercial importance of research in this area, data on the technological development of diagnostic kits are very limited (Danks and Barker 2000) because such research is mainly conducted by commercial firms and the published results are covered by patents or display clear self-marketing characteristics.

Ideal in these test systems is the use of tags that allow target detection by simple visual reading (colored latex, dyes, colloidal gold, liposomes containing a dye, carbon particles, etc.) without the need to add any reacting components. These requirements can be met by a rapidly developing analytical immunochromatography assay or, synonymously, lateral flow immunoassay, which displaces, because of its simplicity and speed, the traditional solid-phase ELISA for a wide range of biologically active compounds of different natures. In carrying out the assay, it is sufficient to add an analyzed plant extract only; then the immunochromatography procedure followed by formation of the detected signal in the analytical zone of the test-strip takes place.

In the immunochromatography diagnostics of viral infections with colloidal gold as a marker, three types of antibodies, applied to different areas of the test-strip, were used:

Primary polyclonal (or monoclonal) antibodies to the virus conjugated with nanoparticles of colloidal gold, which can be visually detected even in very small concentrations (instrumental detection of the signal is also possible): These antibodies are applied onto the test-strip near the site of the sample application or onto the test-strip that is then immersed in the plant extract. The preferred use of monoclonal antibodies is primarily dictated by the difficulties encountered in obtaining stable conjugates of colloidal gold nanoparticles with a heterogeneous mixture of macromolecules of polyclonal antibodies, which vary in their physico-chemical characteristics.

Primary polyclonal antibodies to the virus that are firmly but not covalently immobilized in the analytical band of the test-strip: The use of monoclonal antibodies to different epitopes of the virus is also possible.

Secondary antibodies (monoclonal or polyclonal) immobilized in the control band of the test-strip.

An immunochromatography test-strip has a complex structure and consists of several membrane strips tightly bound by two-sided transparent adhesive tape on a thin plastic backing laminate-type L-H50 (MDI, Ambala Cantt, India) so that the end of each strip continues to the next one and gives the whole design its mechanical strength. This structure of the membrane composite allows the liquid to flow freely from the membrane of one type to another, maintaining the linear front of a moving liquid.

The principle of immunochromatography analysis of plant, animal, and human viruses is common and is described as follows. When immunochromatography strips are immersed in the infected cell extract, or when applying a few drops of sample solution containing the defined virus on the absorbing sample strip, viral particles (virus, viral capsid, and viral envelope proteins) begin to migrate with the front of the fluid along the surface of the membrane because of capillary forces. Moving along the membrane composite, the virus forms very concentrated immunochemical complexes with colloidal gold-labeled antibodies to the virus and continue to migrate farther with the stream of solvent. In the meeting zone of the immobilized primary antibodies, the virus and its complex with the conjugate of primary antibodies labeled with colloidal gold nanoparticles form a stable ternary immunocomplex marking the stained analytical band, while an excess of the conjugate moves farther and marks the control band. These bands can easily be recorded visually or instrumentally. The intensity of band staining is proportional to the amount of virus in the test sample in a wide analytical range. In the absence of virus in the tested plant extracts, the colloidal gold-labeled primary antibodies pass freely through the zone of the immobilized primary antibodies, and a stained band is formed in the control band only. Thus, the testing ability of the immunochromatography strip is characterized by the control zone, where an immobilized secondary antibody binds with the conjugate of the primary antibodies and

colloidal gold nanoparticles. Therefore, a working test-strip displays two bands if the cell extract has the target virus.

As simple and effective diagnostic tools, rapid immunochromatography tests make it possible to identify and evaluate the content of various biologically active substances in a sample in a few minutes without any special skills and equipment, even under field conditions.

This work describes the elaboration of a test system for lateral-flow rapid immunodiagnostics of PVX infection using colloidal gold as a visually detectable label and the development of a diagnostic kit for conducting mass screening analysis of plant material in laboratory and field conditions.

Materials and methods

PVX isolation, immunization with PVX, and antibody isolation

Russian strain (strain Ru) PVX (Potexvirus) and a potato cultivar Krasavchik from the selection nursery of the All-Russian Potato Research Institute were used. Nucleotide sequence of the strain Ru PVX RNA is presented in Skryabin et al. (1988). Accumulation of PVX and isolation was carried out from infected *Nicotiana tabacum* cv. Samsun NN as described (Novikov et al. 1982).

Virus was detected by ELISA at a concentration of 2 ng/ml, which indicates its high antigenicity.

Immunization of rabbits was conducted intracutaneously (Novikov et al. 1982). The specific titer of antisera was $1–2\times10^6$.

Immunoglobulins were isolated by affinity chromatography on protein A–Sepharose (Institute of Epidemiology and Microbiology, Saint Petersburg, Russian Federation) according to the manual.

Immobilization of antibodies on the surface of colloidal gold particles was carried out as follows. Some 5–10 ml of antibody solution (0.1 mg/ml) in 10 mM Tris–HCl buffer, pH 8.5, was added to 50 ml of colloidal gold with $A_{520}=1.0$ in 0.1 M K_2CO_3 (pH 8.0–9.0). To stabilize the resulting complex, after 5 min of immobilization reaction, 1% bovine serum albumin (BSA) in 10 mM Tris–HCl buffer, pH 8.5, was added to a final concentration of 0.1% albumin. Unbound antibodies were removed by centrifugation of the colloidal solution at 10,000 rpm for 45 min at 4°C. Resulting conjugate of antibodies with colloidal gold (IgG–Au) was suspended in 0.05 M K–phosphate buffer, pH 7.5, containing 0.01% sodium azide and 0.1% BSA and was stored at 4°C.

Potato cell extract preparation and solid-phase immunoassay

Extracts of healthy and PVX-infected potato were prepared by rubbing 100 mg of plant material (leaves, sprouts) with capron fiber mesh in a strong plastic bag or a porcelain mortar with 1.0 ml of TPB (0.01 M potassium phosphate buffer, pH 7.4, 0.1 M NaCl, 0.1% Triton X-100). The resulting homogenates were centrifuged for 5 min at 10,000 rpm. The supernatants were analyzed both by solid-phase sandwich-ELISA using antibodies labeled with horseradish peroxidase (HRP) and by immunochromatography assay (IChA).

For the immunochromatography test, clarified (5 min at 10,000 rpm) and non-clarified plant extracts of leaves or sprouts of different varieties of potato (Bryanskiy nadeshniy, Karatop, Red Scarlett, Nayada, Malinovka, Lorch, Ladozhskiy, Nikulinskiy, Kholmogorskiy, Lubava, Rocko, Golubizna, Zhukovsky ranny, Courage, Udacha, Resurs, Krepysh, Charodey, Il'yinskiy, Rosara, Nevsky, Ramenskiy, Belarosa, Solist, Impala, Latona, Ryabinushka, and Nakra) from the selection nursery of the Russian Potato Research Institute in the Arkhangelsk region were used.

Sorption of γ-globulin fraction of antibodies on the surface of 96-well plates (Nunc, Maxi-Sorp, Langenselbold, Germany) and ELISA were carried out as described (Clark and Adams 1977; Egorov et al. 1991).

Antibody conjugate with peroxidase (IgG–HRP) with a specific activity of 900 U/mg was prepared as described (Egorov et al. 1991). Measurement of the optical density of the product of the enzymatic reaction with ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) was carried out within 30–60 min at 20°C in the wells of polystyrene plates for ELISA with an automatic absorptiometer MR-580 (Dynatech, Vienna, USA) at 405 nm.

To characterize the cross-reactivity of antisera to PVX, purified preparations of heterologous viruses, TMV (tobacco mosaic virus), PVY, PVS, PVM, and PVA (all from Collection of the potato viruses of the All-Russian Potato Research Institute of the Russian Academy of Agriculture) were adsorbed on polystyrene plates (Nunc, Maxi-Sorp, Langenselbold, Germany). In parallel, into the wells 0.1 ml PVX in 0.01 MK-phosphate buffer was added, pH 7.4, with a virus concentration of 2 mg/ml, and all samples were incubated for 12 h at 4°C.

Colloidal gold preparation

Colloidal gold with an average particle size of 15±5 nm was obtained by reduction of $HAuCl_4$ as described by Frens (1973). The size and shape of colloidal gold particles were estimated by dynamic laser light scattering with a Zetasizer (Malvern Instruments Ltd., Malvern, UK) at 633 nm and with a transmission electron microscope Hitachi HU-12 (Hitachi) at 75 kV. To obtain colloidal gold, 5 mg $HAuCl_4$ was added to 50 ml of distilled water (15 MΩ cm). The solution was brought to boil; 10 mg of sodium citrate was added, and the reaction mixture was cooled to room temperature. The resulting gold sol with $A_{520}=1.0$ was stored at 4°C.

Membrane composite, test-strip construction

The compositions of the immunochromatography test-strips and the main characteristics of the membranes (all membranes were from MDI, Ambala Cantt, India) are presented in Table 1.

Specific polyclonal antibodies to PVX in the concentration range 0.05–1.0 mg/ml in 0.01 M K–phosphate buffer, pH 7.4 (0.1 M NaCl) were applied onto a cellulose nitrate membrane using a programmable automatic dispenser BioDot XYZ 3050 (BioDot Inc., Irvine, USA) for formation of the analytical zone. Two pumps were used for simultaneous application of the primary antibodies to PVX and secondary antibodies. To form a control zone of the strip, the affinity-purified anti-rabbit immunoglobulin goat antiserum (IMTEK, Moscow, Russian Federation) at a concentration of 1 mg/ml in 0.01 M K–phosphate buffer, pH 7.4 (0.1 M NaCl), was applied at a distance of 5 mm from the analytical zone. The following parameters of the BioJet Quanti 3000 dispenser (BioDot Inc., Irvine, USA) were used for the sample application—droplet size, 30 nl; line width, 0.3 mm; and speed, 50 mm/s.

To establish the optimal quantity of the conjugate of the polyclonal antibodies to PVX with colloidal gold needed for application on the test-strip, 5×5 mm pieces of the PT-R5 membrane were saturated with a solution of the conjugate with various concentrations, then air-dried and deposited on the absorption cellulose AP045 membrane for subsequent testing of the strip. The determined optimal quantity was applied to the PT-R5 membrane of the working test-strip via the AirJet Quanti 3000 dispenser.

Prepared test trips were cut off using an Index Cutter-I-I (A-Point Technologies, Gibbstown, USA), inserted into plastic test-cassettes and kept in bags of laminated aluminum foil with silica gel in low (30%) moisture, and sealed using a FRD-1000 Heat Sealer (Arista Biologicals, Allentown, USA).

Intensity of the colored zones of immunochromatography test-strips was estimated with a portable reflection photometer, Reflecom, with VideoTest software (Reflecom, Moscow, Russian Federation).

To enhance the recorded signals, the membranes were treated with silver enhancement solutions (IntenSe BL silver enhancement system) for 5–10 min according to the manufacturer's manual (GE Healthcare Life Sciences, Little Chalfont, UK).

Shelf-time of the test-strip examination

During examination of the shelf-time for immunochromatography test-strips, no significant changes were found in their properties after storage at 37°C for 1 week or 3 months at 4°C. There was a slight decrease in sensitivity to 8 ng/ml of PVX after 6 months' storage at 4°C.

Field testing of the elaborated strips for PVX detection

Field testing was conducted for baseline clones of seed potatoes of the Bank of Healthy Potato Varieties in the Arkhangelsk region in the autumn of 2008.

Results

Previously, a solid-phase enzyme immunoassay of PVX on cellulose nitrate membranes using polyclonal antibodies labeled with HRP that allowed visual detection of up to 0.5 ng PVX in the sample over time not exceeding 15 min was described (Blintsov et al. 1997). In this analytical system, a complex of the virus with enzyme-labeled antibody was detected by immersing the porous membrane in the substrate solution, thus producing an insoluble colored product in the zone of complex localization.

In order to simplify and enhance the efficiency of this method for mass diagnostics, we devised a rapid and highly sensitive immunoassay on a polycomposite test-strip for detection of a model virus, TMV, using polyclonal antibodies labeled with colloidal gold (Drygin et al. 2009). In the last case, high-affinity polyclonal antibodies were purified by ion-exchange chromatography, thus having a close pI (isoelectric point) and an ability to interact efficiently with various antigenic determinants of the virus.

Table 1 Characteristics of membranes used for the test-strip construction

Type of membrane	Density	Pore size	Thickness	Bubble point	Binding of BSA at pH 7.4	Porosity	Strip (4 cm length) wetting
Analytical, cellulose nitrate CNPC-SS12-L2-H50.	–	15 μm	109 μm	5.9 mmHg	41 μg/cm^2	–	95 s
Glass fiber, for the IgG–colloidal gold conjugate application PT-R5	9.3 mg/cm^2	–	381 μm	–	–	19.1 μl/cm^2	35 s
Absorption cellulose, for wick AP045	12.4 mg/cm^2	–	429 μm	–	–	–	–
Sample absorbent GFB-R4	9.0 mg/cm^2	–	601 μm	–	–	54.9 μl/cm^2	57 s

Colloidal gold and its conjugate with antibody preparation and characteristics

In the present work, colloidal gold nanoparticles with an average diameter of 15±5 nm were used (Fig. 1). This particle size of the gold sol was found to be the best both in terms of high stability of the colloidal gold solution and in terms of efficient sorption of antibodies with subsequent migration of colloidal gold-labeled antibodies through the porous structure of the multicomponent membrane of the immunochromatography test-strip.

Optical density of the stable colloidal gold solution with an average diameter of spherical particles of 15±5 nm was approximately 0.9 at 520 nm in 1 cm cell (Fig. 2). The resulting hydrosol of gold was kept at 4°C throughout the year without any essential spectral changes.

Upon conjugation of the colloidal gold with chemically heterogeneous antibody molecules, optimal synthesis conditions (pH and protein concentration in solution) were taken into account, together with individual characteristics of the polyclonal antibodies to PVX. For this purpose, series of the colloidal gold solutions with various pH values (from 6.0 to 9.0) were used, and in each series, the final concentration of polyclonal antibodies varied from 0 to 15 μg/ml. After incubation for 5 min, 100 μl of 10% sodium chloride was added to these series. This changed the ionic strength of the solutions and induced aggregation of the gold nanoparticles, which was accompanied by a change in color of the solution from red to gray-blue. Minimal stabilizing concentration of polyclonal antibodies in these series was found by spectrophotometry of stable sols at 580 nm (Fig. 3). In the range of antibody concentrations 0–5 μg/ml, the non-stabilized colloid changed color, and coagulation of the sol particles was then observed. Stabilized by antibodies at a final concentration of 6 μg/ml, the gold sol did not change color after the addition of an electrolyte solution.

The final concentration of antibodies (7 μg/ml) was selected to ensure their immobilization on the colloidal gold based on the criterion of 10% excess, as recommended

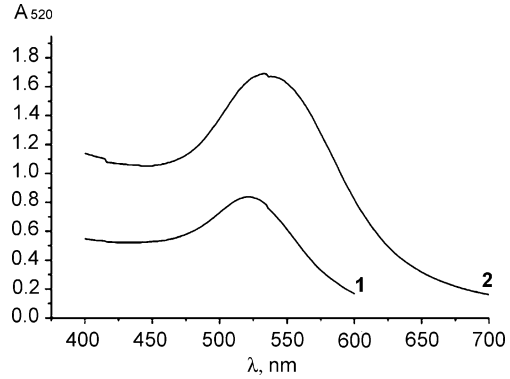

Fig. 2 Absorption spectra of colloidal gold particles with an average diameter of 15±5 nm (*1*) and the conjugate of the colloidal gold with antibodies to PVX after centrifugation and resuspension in buffer containing 0.2% BSA and 10% sucrose (*2*)

(Wong and Tse 2009) to maximize colloidal stability of the gold sol during storage and after drying. Excess of unbound antibodies was separated by centrifugation. To prevent coagulation of gold nanoparticles during centrifugation, as well as during application and while drying the conjugate on the glass fiber membrane, a sucrose solution was added to a final concentration of 10%. The stabilizing effect of sucrose is associated with the formation of protective hydration shells around the nanoparticles because of the retention of water molecules. The target conjugate was stored in solution at 4°C and remained stable for at least 3 months.

Conjugates of polyclonal antibodies with colloidal gold obtained in the pH range 6–7.5 were much less stable. After several days of storage in solution, precipitation of the conjugate was observed even in the presence of stabilizing agents. Thus, conjugates of antibodies with colloidal gold nanoparticles were prepared at pH 8.0–9.0.

Testing of antisera with ELISA

The employed antiserum to the Russian strain of PVX (Skryabin et al. 1988) was first examined by solid-phase ELISA. Antiserum to PVX was tested when leaf juice of

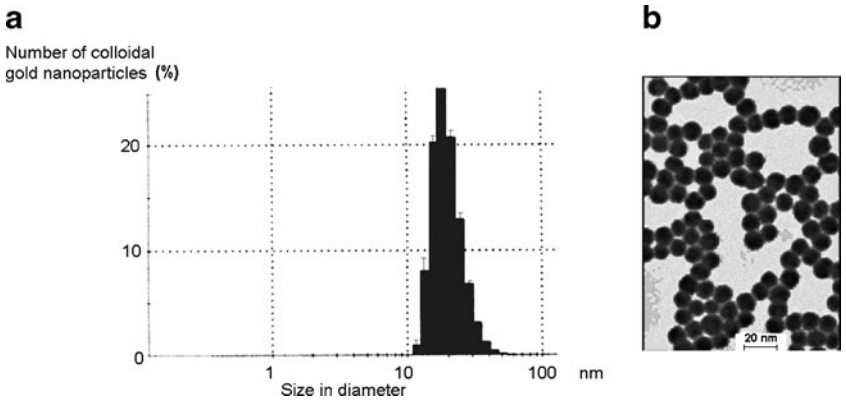

Fig. 1 Gaussian size distribution of colloidal gold nanoparticles obtained with Nanosizer (**a**) and images obtained with transmission electron microscope (**b**). *Bar* in **b** is 20 nm

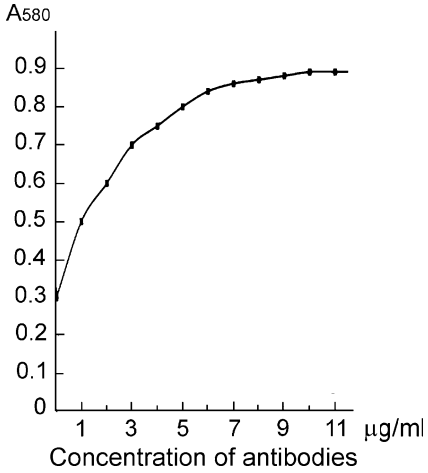

Fig. 3 Adsorption of polyclonal antibodies on the surface of the colloidal gold nanoparticles after 5 min incubation at RT. Minimal stabilizing concentration of polyclonal antibodies was found by monitoring absorption at 580 nm after adding 10–150 μl of antibody solution with a protein concentration 0.1 mg/ml to 1 ml of the colloidal gold solution (pH 8.5) and 0.1 ml 10% NaCl

healthy and PVX-infected potato diluted tenfold with 0.01 M K–phosphate buffer pH 7.4 (0.1 M NaCl) was adsorbed on plates.

Figure 4 shows calibration curves for determining PVX (Russian strain) in the clarified diluted (1:10) extract of potato leaves by sandwich-type ELISA with various concentrations of peroxidase-labeled antibodies. The sensitivity of the method was 2–8 ng/ml and depended on the concentration of the antibodies. The coefficient of variation did not exceed 8–10% in all investigated ranges of the virus determination curve.

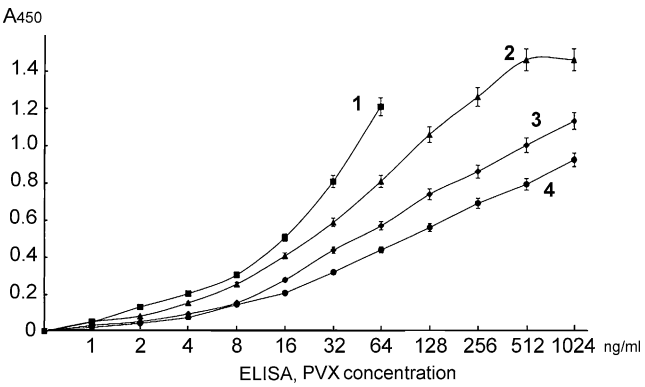

Fig. 4 Calibration curves for determination of PVX (strain PVX-Ru) by solid-phase sandwich-ELISA in the potato leaf juice diluted to 1:10 with TFB. Sorption of immunoglobulins (2 μg/ml) for 12 h at 4°C in 0.02 M Na–carbonate buffer, pH 9.5, on polystyrene plates Nunc, Maxi-Sorp. Interaction with IgG–HRP, 1.0–8.0 nM, 60 min, 37°C, TPB. *Vertical axis*: optical density of the A_{405} product of the enzymatic reaction (30 min, 20°C). *Numbers on curves* indicate concentration of conjugates of secondary antibodies with peroxidase (*1*, 1 nM; *2*, 2 nM; *3*, 4 nM; *4*, 8 nM). *Hashes* represent the range of standard deviation

As a negative control of the antiserum specificity, heterologous, highly purified TMV (Dobrov and Kust 1971) was used (Fig. 5). In addition, concentrated virus was purified by ultracentrifugation through 30% sucrose pad with SW50.1 rotor (Beckman Coulter, Moscow).

The titer of the antiserum for other heterologous virus preparations PVY, PVS, PVM, and PVA (not shown) was more than a thousand times lower also.

It was found that the antiserum to the Russian strain of PVX had a high titer (more than 4.0×10^6) and high specificity, which meets the requirements of a highly sensitive and specific analytical immunochromatography test system.

Properties of the membrane composite and construction of the test-strip

Usually, a lateral flow assay takes 2–15 min depending mostly on the type of membranes used for making a test-strip. It was found that the velocity of the liquid phase spreading along the cellulose nitrate membrane CNPC is not linear and slows with increasing distance (Fig. 6).

Therefore, the position of the analytical band with primary antibodies on the strip was chosen so that the period of immunochemical interaction of the "virus–conjugate complex" with the immobilized antibodies was sufficient for quantitative immunochemical triple complex formation. Increasing spreading velocity reduces the period of this interaction and the sensitivity of the assay. It was found that, for a 3.5 cm-long CNPC membrane, the band of the primary antibodies should be located at a distance 1.0 cm from the right of this membrane (liquid moves along the test-strip from left to right), and the control band of the secondary antibodies should be 0.5 cm to the right of the analytical band.

The ability of corpuscular antigen to interact with several molecules of antibodies simultaneously allows the virus

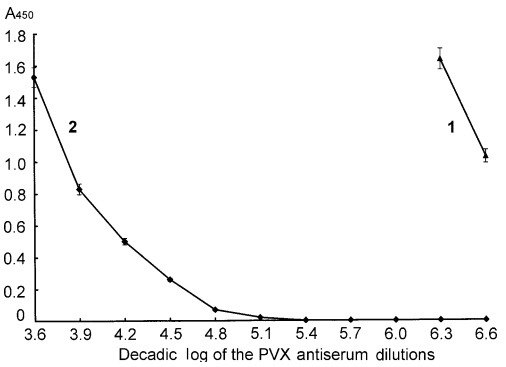

Fig. 5 Specificity of antiserum to PVX determined by titration on immobilized homologous (PVX, curve *1*) and heterologous (TMV strain U1, curve *2*) purified virus preparations. *Abscissa*: decadic logarithm of the antiserum dilutions with TPB

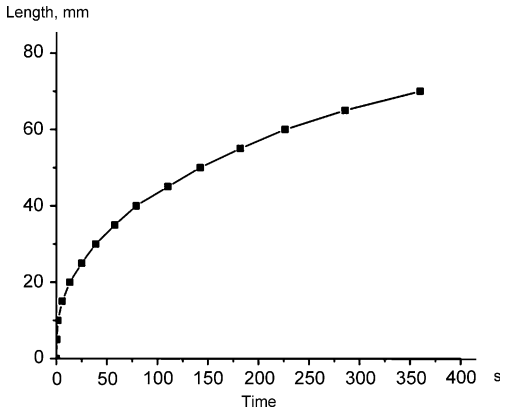

Fig. 6 Velocity of the liquid phase spreading along the cellulose nitrate membrane CNPC

particles to be fully involved in the formation of a strong signal in the presence of an excess of the conjugate and immobilized antibodies in the lateral flow.

In a comparison between two porous cellulose nitrate membranes, CNPC-SS-12-L2-H50 and 150-CNPH-(N)-SS40-L2-H50, which differ in their adsorption capacity, porosity, and pore size, macroporous membrane CNPC, which showed slightly higher values of signals in the analytical zone, was found to have a small advantage. Under the same experimental conditions, the lower limit of the virus detection with these two membranes was in the range 4–8 ng/ml of PVX. Endogenous components of the non-clarified potato leaf juice (diluted 1:10) did not affect practically the sensitivity level of the virus discovery in comparison with detection of PVX in the buffer solution (Fig. 7).

When using non-clarified diluted plant (1:10) extract of the healthy potato, a slight background signal of less than one arbitrary unit (1.0 arbitrary unit corresponds to the limit

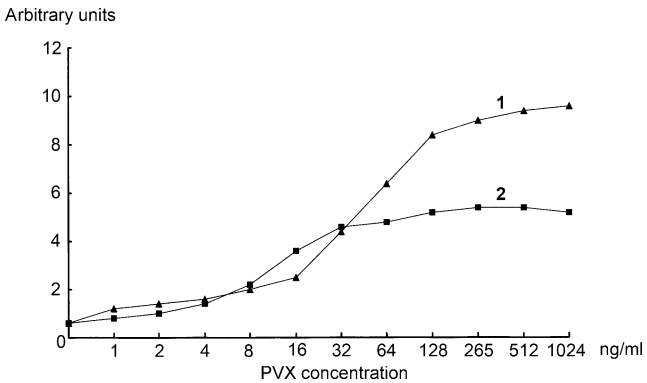

Fig. 7 Comparison of PVX determination in crude juice potato leaf diluted (1:10) with TPB using various analytical membranes: type CNPC-SS-12-L2-H50 with a pore diameter of 15 microns (*1*) and type 150-CNPH-(N)-SS40-L2-H50 with a pore diameter of 10 μ (*2*). Nine microliters of Au–IgG (A_{520}=1.7) and 1 μl of IgG to PVX (0.2 mg/ml) were brought to PT-R5 and the analytical membrane, respectively. GFB-R4 (sample application) and AP045 (wick membrane) were used

of reliable visual detection) in the analytical band was recorded with the CNPC membrane as with the CNPH membrane. Using a GFB-R4 membrane as the starting absorption membrane for fractionation of endogenous macro components of the plant extract significantly reduced the effect of colored pigments of the leaf extract on visual and instrumental evaluation of immunochromatography diagnostics.

Suppression of the background reaction

Addition of SDS (sodium dodecyl sulfate) to the extraction buffer was found to be useful in reducing further the background signal in the analytical band. Figure 8 shows calibration curves for the determination of PVX by IChA with various concentrations of SDS in TPB, which was used as the extraction buffer. When the concentration of detergent was 0.03%, a background signal in the analytical band was not detected, even instrumentally, while the lower limit of PVX detection was 4.8 ng/ml. Increasing the SDS concentration further led to a decrease in the sensitivity of the method.

Importantly, when a virus was absent in the sample, there was no coloration of the analytical zone of immunochromatography membrane, which is extremely important for visual evaluation of the results outside the laboratory under field conditions. No significant effect was noticed of endogenous micro- and macro-components of the non-clarified leaf juice of the healthy potato on the virus detection results while various quantities of PVX were added in Tris–phosphate buffer. Moreover, with increasing concentration of the virus in the sample, a proportional increase occurred in the intensity of detected signal in the analytical zone (Fig. 8). Thus, the only step needed to prepare the probe for diagnostics is the tenfold dilution of a drop of non-clarified potato juice with TPB in order to start the assay in the laboratory or under field conditions,

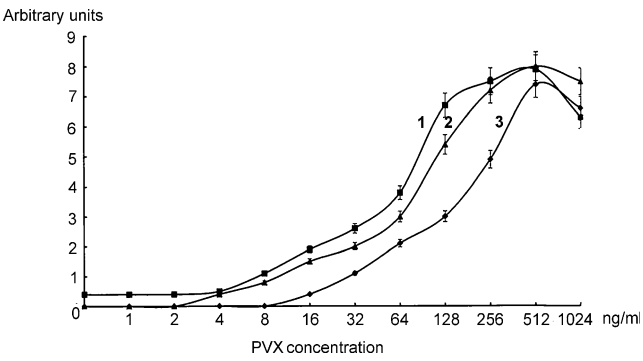

Fig. 8 Comparison of PVX determination in clarified juice potato leaf diluted (1:10) with TPB after neutralization of background noise by addition of SDS at various concentrations: *1*, no SDS added; *2*, 0.03% SDS; *3*, 0.05% SDS. The test-strip is as described in Fig. 7. *Hashes* represent the range of standard deviation

because the colored juice debris is almost completely retained on a specially selected filtering absorption membrane.

To demonstrate a possible increase in sensitivity (Drygin et al. 2009) of PVX detection by IChA, we used a commercial silver enhancement system. To suppress the background signal, SDS was added to the potato leaf extract. The sensitivity of PVX determination was increased by more than one order of magnitude at least using signal enhancement by silver in TPB in the presence of 0.1% SDS.

Shelf-time of the test-strip

During examination of the shelf-time for immunochromatography test-strips, no significant changes were found in their properties after storage at 37°C for 1 week or 3 months at 4°C. There was a slight decrease in sensitivity to 8 ng/ml of PVX after 6 months' storage at 4°C (Fig. 9).

The membrane retained the original color of the analytical and control bands for a long time after analysis and could be scanned using a reflective photometer to quantify the results of the field test. Repeated measurements of the colored bands after 2 weeks showed a slight decrease in the intensity of the signals (no more than 15%) compared with those obtained on the day of analysis.

Field testing

With the described immunochromatography test-strip, PVX infection can be detected with a lower limit of detection that is comparable to one of the most sensitive immunochemical methods—solid-phase ELISA (see Fig. 4). Coloring of the analytical zone of the test-strip appeared after 10 min for the PVX concentrations from 8 to 1,000 ng/ml. After 15 min exposition, the sensitivity increased to 2 ng/ml. Therefore, 15 min was selected for the assay as the optimal duration.

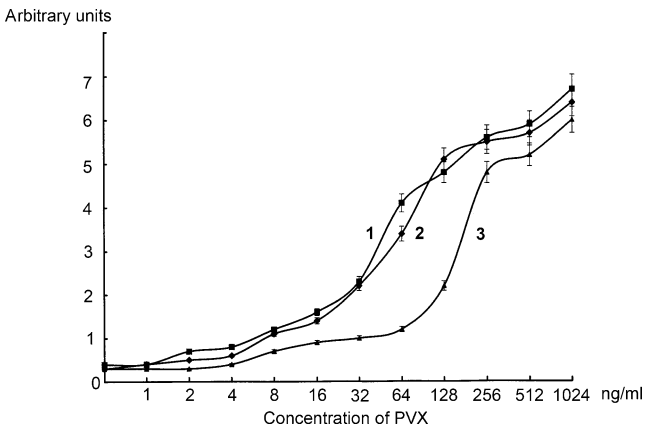

Fig. 9 Illustration of the long shelf-time of test-strips. Seven microliters of Au–IgG (A_{520}=1.9) and 1 μl of IgG to PVX (0.2 mg/ml) were brought to PT-R5 and the analytical membrane, respectively. *1* Fresh immunochromatography test-strips; *2* immunochromatography test-strips after storage for 1 week at 37°C or 3 months at 4°C; and *3* after storage for 2 weeks at 37°C or 6 months at 4°C. PVX was determined in clarified diluted (1:10) juice of the potato leaf

The probability of PVX identification in leaf extracts of various infected potato cultivars at a concentration of 10 ng/ml was ascertained to be 100%, and PVX detection in infected and healthy potato plants by ELISA and IChAwas found to be practically the same. The coefficient of variation of photometric measurements of control and analytical bands of the immunochromatography test-strip did not exceed 13% during PVX detection in the working diapason. Some 0.1 to 0.2 ml of a test solution (10–20 mg of a leaf material) is required for one assay, the same as for ELISA, which makes this method a particularly effective tool in the analysis of small quantities of plant material.

Field testing of the described immunoassay for PVX detection was conducted in the autumn of 2008 to select basic healthy clones for the Bank of Healthy Potato Varieties in the Arkhangelsk region. Using immunochromatography test-strips, PVX was detected in 18 baseline clones of different varieties of seed potatoes (Table 2).

Clones of 17 healthy varieties of potato, as ascertained by immunochromatography assay, were further analyzed by ELISA using the "potato eye test" in the laboratory (Table 3). The rate of agreement of two methods of PVX infection testing was 98.8% (see Table 3). Findings of two infected samples of the potato cultivar Ramenskiy confirmed by ELISA in the laboratory suggests that this variety of potato is wholly affected by PVX. PVX was also fully identified after testing clones of the potato variety Ramenskiy by IChA in the following summer of 2009. Simultaneously, PVX was also found by rapid field diagnostics in the clones of varieties Udacha, Ladozhskiy, Nakra, Rocko, Lorch, and Golubizna. It should be noted that PVX was found to be the most common virus among the examined potato viruses in the Arkhangelsk Bank of Healthy Potato Varieties.

It is important that the potato varieties (Karatop, Nayada, Red Scarlett, Liubava, Charodey, Il'insky), clones of which were completely free of PVX, as ascertained by immunochromatography, showed the absence of PVX after potato ripening using ELISA.

Discussion

A complete assay carried out using a solid-phase immuno technique takes more than 4 hours, which is related to the duration of heterogeneous phase immunochemical reactions, due to limitations of passive diffusion regarding mass transfer. It takes time for antigen or conjugate molecules from the bulk of the solution to migrate to the surface of the solid phase to participate in the reaction. This leads to a

Table 2 Selection of the healthy clones by the immunochromatography assay

Variety	Number of clones	Selected healthy clones	Clones rejected as infected with PVX
Belarosa	10	10	0
Bryanskiy nadezhniy	10	7	3
Charodey	10	10	0
Courage	10	10	0
Golubizna	10	7	3
Il'inskiy	10	10	0
Impala	10	9	1
Karatop	10	10	0
Kholmogorsky	10	6	4
Krepysh	10	9	1
Ladozhskiy	10	7	3
Latona	10	8	2
Lorch	10	7	3
Lubava	10	10	0
Malinovka	10	10	0
Nakra	10	8	2
Nayada	10	10	0
Nevsky	10	5	5
Nikulinskiy	10	9	1
Ramenskiy	10	2	8
Red Scarlett	10	10	0
Resurs	10	8	2
Rocko	10	8	2
Rosara	10	9	1
Ryabinushka	10	7	3
Solist	10	10	0
Udacha	70	68	2
Zhukosky ranny	60	57	3

significant increase in the time of immunochemical reactions in solid-phase analysis methods compared with homogeneous techniques (Egorov et al. 1991). One possible solution to this problem is to decrease the thickness of the steady diffusion layer near the surface of a solid boundary, within which fluid is motionless and mass transfer of components of the immunochemical mixture to a solid surface occurs only by molecular diffusion (Berezin et al. 1976). Stirring the heterogeneous mixture of immunochemicals leads only to the result that the concentration of immunoreagents outside the unsteady layer is constant. At the same time, the thickness of the steady liquid layer (Nernst layer) under normal conditions of mixing does not change significantly and is maintained at 10^{-5}–10^{-3} cm.

It was established that, in lateral flow analysis during lateral migration of the immunocomponents along the porous surface of the membrane, thickness of the non-intermixed liquid layer is significantly reduced. Therefore, the rate of antigen binding to the immobilized antibodies in immunochromatography increases in the order when compared with the traditional dot-immunochemical assay, and kinetics of the complex formation of an antigen with antibodies in the course of lateral mass transport along the surface of the porous membrane is similar to the kinetics in solution (Gorovits et al. 1993).

It is considered that the immobilization of antibodies on the cellulose nitrate membrane is sorption in origin, where the main driving forces for the binding of immunoglobulins to the membrane are electrostatic interactions, i.e., dipole–dipole interaction of strongly polarized membrane nitrate groups and carbonyl groups of peptide bonds of proteins. The amount of antibodies bound to a porous membrane is determined mainly by the internal pore surface area available for immobilization. In this case, the sorption capacity of the membrane depends on the pore size, porosity (amount of air in a three-dimensional structure of the membrane), membrane thickness, and to a lesser extent, structural features of the polymer. With other parameters being equal, the membrane surface nonlinearly decreases with increasing pore size, increases linearly with increasing thickness of the membrane, and increases non-linearly with increasing porosity.

According to Wong and Tse (2009), absorption of the IgG molecules is of the order of 50–200 μg/cm^2 for the cellulose nitrate membrane. If we take into account that the thickness of the analytical or control band on the strip is ~1 mm and the width of the strip is 0.5 cm for each line, then up to 10 μg of an antibody, and apparently the conjugate of it, can be immobilized by the surface of the band. This is an amount of antibody (or proteic antigen) 10–100 times larger than that commonly used in ELISA kits.

The advantage of colloidal gold as a marker of antibody is determined: first, by the ease of obtaining particles of given size with the optimal ratio of protein/gold; second, by the potential to dry the conjugate of antibodies with colloidal gold nanoparticles; third, by rapid and complete reconstitution of conjugated antibodies for subsequent use; and finally, by the high sensitivity of visual detection of the gold tag (Dykman and Bogatyrev 1997).

The stability of colloidal gold nanoparticles is influenced by many factors, including particle size, high temperature, exposure to light, pH, and ionic strength of the solution (see Supplementary material).

Because of orderly monolayer coverage of the surface of colloidal nanoparticles, oriented antibody molecules with maximum exposure of Fab fragments into solution maximize the stability of the formed colloidal conjugate, so there is a high degree of conservation of antigen-binding properties of the immunoglobulins during immobilization.

Adsorbed protein macromolecules contain polar groups and hence are well hydrated and form powerful hydration shells around colloidal gold particles, which prevent the

Table 3 Comparative results of clone testing in the Arkhangelsk Bank of Healthy Potato Varieties

Variety	Number of clones analyzed by strip testing	Found PVX by strip testing	Number of healthy clones found by ELISA potato eye test	Discovered PVX additionally by ELISA	Number of healthy clones
Charodey	10	0	8	0	8
Golubizna	10	1	4	0	4
Il'inskiy	10	0	5	1	5
Karatop	10	0	8	0	8
Kholmogorsky	10	0	4	0	4
Ladozhskiy	10	3	3	0	3
Lorch	10	1	5	0	5
Lubava	10	0	7	0	7
Nakra	10	1	5	0	5
Nayada	10	0	5	0	5
Nevsky	10	0	5	0	5
Nikulinskiy	10	0	5	0	5
Ramenskiy	10	8	2	2	0
Red Scarlett	10	0	7	0	7
Rocko	10	2	3	0	3
Rosara	10	0	7	0	7
Udacha	10	1	3	0	3
In total	170	17	86	3	85

adhesion of nanoparticles (adsorption–solvation factor). In addition, only in the presence of protein stabilizers can the gold sol be applied to the porous substrate, dried, and finally swiftly and fully converted into a colloid. The last is especially important in immunochromatography, where all the components used are applied on different zones of the membrane composite and dried. In the course of joint lateral migration of the sample solution and the immunoreagent along the surface of the porous membrane, fast and complete recovery of antibodies labeled with colloidal gold takes place, which affords its quantitative and rapid interaction with a viral antigen.

Highly sensitive detection of virus over a brief period of analysis appears to be associated with both a high local concentration of immobilized and labeled antibodies and the leveling of external diffusion during lateral migration of the dissolved components of the system.

As can be seen from the data presented in Tables 2 and 3, immunochromatography strip-testing is suitable for practical application in the field. In total, more than 2,000 test-strips were used for detection of the potato virus infections. Thus, the diagnostic kit for routine analysis of viral infections of plants both in the laboratory and under field conditions was expanded upon. It consists of the test-strip, extraction buffer with plastic pipette, a piece of capron net to mash a plant tissue, negative and positive control samples, strong plastic bag for mashing and kit packing. Immunochromatography test-strips were inserted in a plastic cassette device type 3 (Advanced Microdevices Pvt. Ltd., MDI) with a membrane 5 mm wide.

The high efficiency of the method was demonstrated during the selection of clones for the Bank of Healthy Potato Varieties located in the Arkhangelsk region. In total, 360 clones of 28 varieties of potatoes were analyzed, and PVX was identified in 23 clones. Selection of virus-free clones based on the results of the field immunochromatography assay of the varieties Lorch, Nakra, Rocko, Luck, and Nevsky allowed us to maintain the clones in a state free from virus infection, as confirmed via ELISA in the "potato eye test". This illustrates the effectiveness of this diagnostic system in the field in identifying one of the most widespread and dangerous potato viruses.

Further development of the presented method will depend on the potential to increase its sensitivity and improve its performance (due to the simultaneous determination of several viruses on a single practice test-strip), and of course, the expansion of its use for agricultural virology and environmental protection.

We hope that the results described in this work will be used by major producers of seed potatoes, laboratories working on potato source material, and small farm owners and recreational potato growers alike.

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