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Lateral Flow Immunoassay for Rapid Detection of Potato Ring Rot Caused by *Clavibacter michiganensis* subsp. *sepedonicus*[1]

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Abstract—A lateral flow immunoassay for the rapid detection of *Clavibacter michiganensis* subsp. *sepedonicus* bacteria causing potato ring rot was developed. Multimembrane composites (test strips) containing polyclonal antibodies against the bacteria and gold nanoparticle-antibody conjugates were used for the analysis. The test strips are suitable for the analysis of potato tuber and leaf extracts within 10 min; the detection limit of bacteria is 4×10^5 cells/mL. No cross-reactivity with strains of *Clavibacter michiganensis* subsp. *michiganensis*, *Pectobacterium carotovorum* subsp. *carotovorum* and saprophytes of healthy potato plants was detected. The results of analysis of 26 potato samples by the developed tests were compared with those obtained by the PCR method and using the commercial enzyme immunoassay kits. The results of lateral flow immunoassay were confirmed in 96.2% of cases, which supports the high correlation with other analytical approaches. The developed immunoassay may be considered as a promising means of phytosanitary control.

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Infectious diseases of potato are important factors reducing the yields of this crop. Thus, only bacterial pathogens cause the 10–25% loss (during epiphytotics, up to the 80% loss) of the harvests [1–3]. The ring rot is one of the most common and harmful bacterial diseases of potato [2, 4, 5]. The causative agent of potato ring rot, the bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (**Cms**), is included into the A2 list of the European Plant Protection Organization as an object of international quarantine control [6]. The bacterium may localize in stems and tubers of potatoes in a hidden (latent) form during a long time, without pathological changes of the tissues. Due to the lack of effective protocols for the mass treatment, the main agrotechnical approach is based on the rejection of infected plants and the selection of healthy plants. In this regard, the detection of Cms is essential for the effective protection of plants, especially in the production of seed potato [1, 7]. Thus, the diagnostics of the infection is very important for the production of potato seed tubers without Cms [2, 8, 9].

Nowadays, different techniques are applied for the diagnostics of plant infections, including immunochemical, genetic, biochemical techniques, etc. [10–13]. However, these laboratory techniques are time-consuming and require complex equipment and skilled personnel. The widespread monitoring of potato infestation could be accomplished using simple, reliable, highly specific, and sensitive analytical methods suitable for the field diagnostics without special equipment and skills. The lateral flow immunoassay (**LFIA**), also called the immunochromatographic assay, complies with all mentioned requirements [14, 15]. The application of antibodies makes LFIA sensitive, specific, and simple, whereas the immunochromatographic principle provides the rapid analysis under out-of-laboratory conditions [16, 17]. All reactants are applied to the test strip beforehand, and its contact with a test sample initiates specific interactions finalizing by a visible result, i.e. the presence or the absence of coloration in certain areas of the strip. The only preparation for the detection of plant pathogens by LFIA is the simple and rapid homogenization of the tissue in the appropriate extracting solution [18–20]. Immunochromatographic assays are included into the national and international regulations for agrotechnical control [6, 21]. However, the majority of available test strips are developed for the viral infections control [18–20]. Therefore, the development of LFIA tests for bacterial phytopathogens is an urgent problem.

The aim of this study was to develop LFIA for the rapid detection of potato ring rot caused by Cms. The study includes the characterization of the test components, the analysis of plant samples, and the comparison of these results with those obtained by alternative analytical methods.

[1] The article is published in the original.

Abbreviations: Cmm, *Clavibacter michiganensis* subsp. *michiganensis*; Cms, *Clavibacter michiganensis* subsp. *sepedonicus*; ELISA, enzyme-linked immunosorbent assay; GNP, gold nanoparticles; IgG, immunoglobulin G; LFIA, lateral flow immunoassay; PBS, phosphate buffered saline (50 mM K-phosphate buffer, pH 7.4, 0.14 M NaCl); PBST, PBS with 0.05% Triton X-100; PCR, polymerase chain reaction.

The polyvalence of the Cms bacterium as antigen with plurality of surface-exposed epitopes allows us to develop LFIA based on the methodological approaches that we have proposed and successfully implemented in LFIA of plant viruses in our previous studies [22, 23].

MATERIALS AND METHODS

Reagents. Goat anti-rabbit IgG antibodies (Arista Biologicals, USA), peroxidase conjugate of streptavidin (Imtek, Russia), peroxidase conjugate of anti-rabbit antibodies (Medgamal, Russia), tris(hydroxymethyl)aminomethane (Tris), Tween 20, Triton X-100, 3,3',5,5'-tetramethylbenzidine dihydrochloride, sodium azide, biotinamidohexanoic acid N-hydroxysuccinimide ester, protein A–Sepharose CL-4B, Freund's adjuvant (Sigma, USA), chloroauric acid (Fluka, Germany), Triton X-100, bovine serum albumin (**BSA**), sodium citrate, dimethylsulfoxide (MP Biomedicals, UK), GeneRuler^{TM} 100 bp DNA Ladder (Thermo Fisher Scientific, USA), glycerol, NaCl, K_2CO_3 (DiaM, Russia), Na_2CO_3, $NHCO_3$, KH_2PO_4, and KOH (Khimmed, Russia) were used for the study. All chemicals were of analytical or chemical grade.

All solutions for the production of gold nanoparticles (**GNP**) and their conjugates were prepared using water deionized by a Milli-Q system (Millipore, USA). ELISA was carried out using 96-well transparent polystyrene microplates, Costar 9018 (Corning Costar, USA). For LFIA tests components produced by Advanced Microdevices (India) were used: lateral-flow nitrocellulose membrane CNPC-12μ, conjugate release matrix PT-R5, sample pad GFB-R4 (0.35), absorbent pad AP045, and laminate MT-1.

Preparations of bacterial samples. The Cms 204, Cms 12, Cms M1, Cms M2, and Cms R77 strains as well as strains of *Clavibacter michiganensis* subsp. *michiganensis* (**Cmm**) : Cmm 1214 and Cmm 1208 were from the collection of phytopathogens of the All-Russian Potato Research Institute. The DSM 30168 strain of *Pectobacterium carotovorum* subsp. *carotovorum* was from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Extracts from leaf and tuber tissues of pathogen-free potato plants were used as sources of saprophytic bacteria. Yeast Dextrose Carbonate agar was used for growing the bacteria [24], the cultures were incubated at 27°C during 48 hours.

Production of polyclonal antibodies. Isolates of the Cms 204 and Cms 12 strains were used as antigens for antisera producing. Chinchilla rabbits (4–5 months) were immunized according to the scheme that included two subcutaneous and four intramuscular injections with complete and incomplete Freund's adjuvant at weekly intervals. Total dose for the immunization was 5×10^9 bacterial cells per animal. Blood was collected on 7th–12th day after last injection. Titer of the obtained antisera determined by indirect ELISA was equal to 1 : 250000.

Immunoglobulins were isolated from the antisera by affinity chromatography on protein A-Sepharose according to the manufacturer's instructions.

Biotinylation of antibodies. The antibodies were dialyzed against a 1000-fold volume of 50 mM potassium phosphate buffer, pH 7.4, with 0.14 M NaCl (**PBS**), for 4 h at +4°C. Then, biotinamidohexanoic acid *N*-hydroxysuccinimide ester (10 mg/mL, in dimethyl sulfoxide) was added to the antibodies in 15 : 1 molar ratio. The mixture was incubated for 1 h at room temperature and then dialyzed against PBS.

Synthesis of gold nanoparticles (GNP). One milliliter of 1% $HAuCl_4$ was added to 95 mL of deionized water, heated to boiling, and 4 mL of 1% sodium citrate was added with stirring [25]. The mixture was boiled for 25 min, cooled, and stored at 4–6°C.

Transmission electron microscopy. Preparations of GNP were applied to 300-mesh grids (Pelco International, USA) coated with a support film of poly(vinyl formal) dissolved in chloroform. The images were obtained with a JEM CX-100 electron microscope (JEOL, Japan) operating at 80 kV. The digital images were analyzed with the Image Tool program (University of Texas Health Science Centre at San Antonio, USA).

Enzyme-linked immunosorbent assay (ELISA). Immunoglobulins were immobilized from a 100 μL volume in microplate wells at +4°C overnight using solutions at a concentration of 2 μg/mL in a PBS. The microplate was washed four times with a PBS with 0.05% Triton X-100 (**PBST**). Then portions (50 μL) of bacteria solution (the concentrations varied from 10^8 to 500 cell/mL) and antibodies-biotin conjugate solution (4 μg/mL) in PBST were added into the microplate wells. The microplate was incubated at 37°C for 60 min and washed as described above. Then portions (100 μL) of the peroxidase conjugate of streptavidin (in the 1 : 5000 dilution of the commercial preparation in PBST) were added and incubated at 37°C for 60 min. The microplate was repeatedly washed, and the peroxidase activity was determined. The substrate (100 μL of 0.4 mM 3,3',5,5'-tetramethylbenzidine solution in 40 mM sodium citrate buffer, pH 4.0, containing 3 mM H_2O_2) was added to each microplate well. After the incubation at room temperature for 15 min, the reaction was terminated by adding 1 M H_2SO_4 (50 μL), and OD_{450} was measured.

Synthesis of gold nanoparticle-antibody conjugates. The antibodies were dialyzed against a 1000-fold volume of a 10 mM Tris-HCl, pH 9.0, at +4°C for 2–3 h. The pH of the GNP was adjusted to 9.0 with 0.2 M K_2CO_3 and then GNP was added to a solution of antibodies at the concentrations of 20 μg/mL. The mixture was stirred at room temperature for 60 min, and then BSA was added to the final concentration of 0.25%. Gold nanoparticles with immobilized antibodies were separated by centrifugation at 20000 g for 30 min and re-suspended in PBS containing

0.25% BSA, 0.25% Tween 20, and 1% saccharose. For the long-term storage, NaN_3 was added to the sample to the final concentration of 0.02%.

Preparation of test strips. Membrane compounds and multi-membrane composite were prepared using approaches described in [22]. The GNP-antibody conjugate was deposited onto CNPC-12μ membranes from a solution that had optical density equal to 10 at $\lambda = 520$ nm; the conjugate load was 16 μL per cm of strip width. The test zone was formed by anti-pathogen IgG; the control zone—by goat anti-rabbit IgG. Both loading solutions were at concentrations of 1.0 mg/mL in PBS; they were applied to 2 μL per cm of strip width. After the assembly of membranes, the multimembrane composite was cut into strips of 3.5 mm width by Index Cutter-1 (A-Point Technologies, USA), which were hermetically packed into bags composed of laminated aluminum foil and containing silica gel as desiccant, using an FR-900 continuous band sealer (Wenzhou Dingli Packing Machinery, China). Cutting and packing were carried out at 20–22°C in a separate room with relative humidity no more than 30%.

Testing of seed material. Testing of potato for latent potato ring rot infection was performed on 26 samples (see Table 1) obtained by testing laboratory of All-Russian Potato Research Institute. Potato tubers and leaves were used to prepare extracts.

The tested lots included 200 tubers for samples 1–14, 15 tubers for samples 15–24, and 10 tubers for samples 25 and 26. Segments (5 × 5 × 10 mm) were cut from each lot of tubers and placed into 250 mL conical flask. Then 40 mL of autoclaved 0.05 M phosphate buffer, pH 7.0, was added and the resulting mixture was incubated at 5–10°C overnight. The extract was filtered through white ribbon filter paper. The resulting filtrate was centrifuged at 10000 g during 10 min at 5–7°C. The pellet was re-suspended in the buffer of samples for ELISA and LFIA, or in sterile 0.01 M Na-phosphate buffer, pH 7.0, for PCR.

The leaf samples were triturated with pestle in a porcelain mortar with 0.05 M Na-phosphate buffer, pH 7.0, at 1 : 20 ratio. The extract was filtered, and all further operations were carried out as for tubers.

Lateral flow immunoassay. The assay was performed at room temperature. The test strip was vertically submerged into the tested sample for 1.5 min and then it was taken out and placed on the horizontal surface. The qualitative results were estimated visually after 10 min. The visual limit of detection of the assay was defined as the minimum concentration giving rise to the band at the test zone.

The color intensity was quantified by densitometry after the complete air-drying of the strip using a Reflekom portable photometric detector (Okta-Medika, Russia) [26]. The registered 0.5 arbitrary unit of colour intensity accords to the threshold of reliable visual detection.

Table 1. Studied samples of potato

No	Region of growing (Russia)	Variety
1	Voronezh region	Impala
2	″	Impala
3	″	Kibits
4	″	Saturna
5	″	Phasan
6	″	Bonus
7	″	Hermes
8	Tula region	Monaliza
9	″	Red Scarlet
10	″	Salin
11	″	Agatha
12	Voronezh region	Picasso
13	″	Nevsky
14	″	Impala
15	Moscow region	Agria
16	″	Ariel
17	″	Ariel
18	″	Arosa
19	″	Belaroza
20	″	Jelly
21	″	Eurostar
22	″	Zhukovsky early
23	″	Courage
24	″	Red Scarlet
25	″	Lady Claire
26	″	Unknown

Samples 1–14 accord to certified grade, samples 15–24—to basic grade, samples 25, 26—to pre-basic grade of seed material.

ELISA using commercial kits. Kits for Cms from Loewe (Germany) were used according to the manufacturer's instructions.

PCR analysis. Bacterial DNA was isolated from plant extracts using the Sample-GS kits (DNA Technology, Moscow). Thermal Cycler (Nyx Technik, USA) was used for the PCR. The analysis was performed with PSA 1/PSA-R primers described in [27]. The given primers are specific to the intergenic spacer

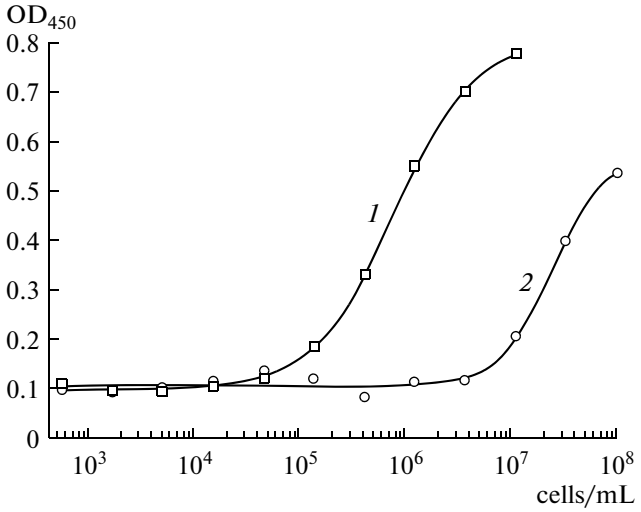

Fig. 1. Optical density versus Cms (cell/mL) for sandwich ELISA. Curve *1*, the Cms 204 strain; curve *2*, the Cms 12 strain.

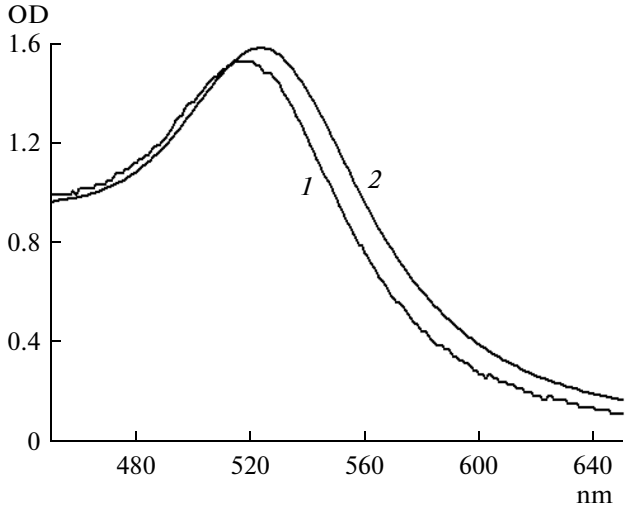

Fig. 2. Adsorption spectra of gold nanoparticles; curve *1*, unconjugated GNP; curve *2*, the antibody-GNP conjugate.

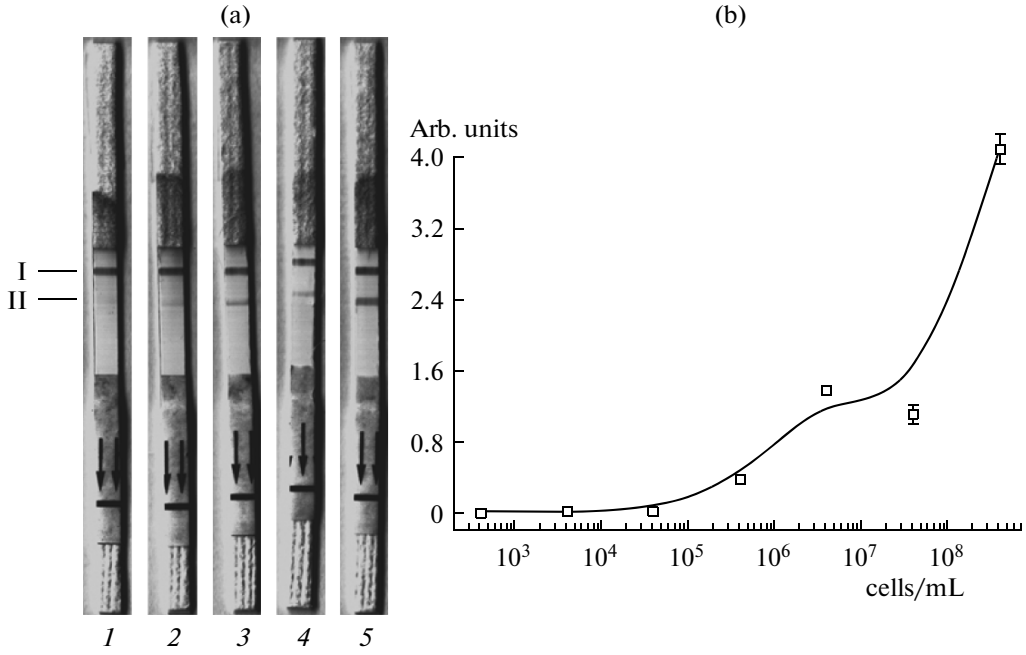

Fig. 3. LFIA of Cms 204 in the buffer solution: (a) test strips after the assay (I, the control zone; II, the test zone); *1–5*, the concentrations of Cms are 4×10^4, 4×10^5, 4×10^6, 4×10^7, and 4×10^8 cell/mL; (b) the color intensity in the test line (arb. units) versus the concentration of Cms (cell/mL).

of 16S-23S rRNA of Cms and give an amplification product with size of 502 bp. The reaction mixture (25 μL) included 20 pmol of each primer, 5 μL of PCR buffer 5x 1.5 mM $MgCl_2$, 200 pmol of a mixture of dNTP, and 5 units of Smart Taq DNA polymerase (Dialat, Moscow). Total DNA of the R77 strain of Cms was used as a positive control, and total DNA of healthy leaves or tubers of potato plants—as a negative control.

To visualize the amplification results, electrophoresis in 1.5% agarose gel with addition of ethidium bromide was performed [27], using a horizontal camera from Helicon (Russia).

RESULTS AND DISCUSSION

Characterization of antibodies specific to Cms. Initially the specificity of the polyclonal antibodies was

tested by ELISA. The antibodies specific to Cms were screened by this method. Their characteristics were quite different depending on the strain used (Fig. 1). Thus, antibody preparations with the lowest limits of detection (for the Cms 204 strain) in the range of 10^4–10^5 cells/mL were selected (Fig. 1, curve *1*).

Gold nanoparticles and their conjugates with antibodies. An average diameter (20 ± 3 nm) and the form factor (the maximum to minimum axis ratio) (1.12 ± 0.08) of the synthesized GNP have been estimated from the transmission electron microscopy data. The absorption maximum of the synthesised GNP is about 520 nm (Fig. 2, curve *1*). The results given above meet the criterion of a narrow size distribution of GNP [25]. A colloidal gold solution is stable for months.

Cold nanoparticles were conjugated with specific polyclonal antibodies (IgG). The absorption maximum of the conjugate was observed at 524 nm (Fig. 2, curve *2*). The conjugate concentrations were determined using OD_{520} taking into consideration that the immobilization of the antibody on the GNP surface leads to an approximately 10% change in the absorption [22].

Development of the lateral flow immunoassay. Since bacteria are polyvalent antigens, the sandwich format of the LFIA was used based on the formation of immobilized antibody—antigen—labeled antibody complexes. The LFIA was optimized by choosing the adequate concentrations of reactants (IgG, IgG-GNP conjugates) to achieve the maximum color intensity in both the test and control zones, as well as the lowest detection limit in the absence of the background staining. The selected conditions are presented in the section "Materials and Methods".

Anti-rabbit specific antibodies (1 mg/mL) were immobilized onto a nitrocellulose membrane as the control line and antibodies specific to Cms (1 mg/mL) were immobilized as the test line. The OD_{520} of the IgG–GNP conjugate solution used for the application in LFIA was 10.0.

According to the digital recording data, the maximum colorations in the test and control zones were observed 10 min after the assay had started.

Analytical characteristics of the developed LFIA. Figure 3a shows the test strips after the detection of Cms at different concentrations. According to the principle of the sandwich immunoassay, the color intensity of the test zones gradually grows with increasing analyte concentration in the sample. Thus, the color development and dependence may be controlled either visually (see Fig. 3a) or using the quantitative data describing the coloration intensity (Fig. 3b). Figure 4 shows the detection of Cms in leaf (curve *1*) and tuber (curve *2*) extracts.

The developed assay is characterised by the detection limit of 4×10^5 cells of Cms 204 per mL, which is comparable with that obtained by ELISA. For other pathogenic strains of Cms, the detection limit varies

Table 2. Results of LFIA for preparations of different Cms strains

Concentration of Cms, cell/mL	Color intensity at the test line, arb. units*
Cms M2	
4.7×10^9	2.50 ± 0.10
4.7×10^8	1.80 ± 0.10
4.7×10^7	1.50 ± 0.06
4.7×10^6	0.50 ± 0.10
4.7×10^5	0.40 ± 0.11
4.7×10^4	0
4.7×10^3	0
4.7×10^2	0
Cms R77	
4.0×10^9	2.17 ± 0.06
4.0×10^8	2.23 ± 0.06
4.0×10^7	1.93 ± 0.15
4.0×10^6	0.50 ± 0.10
4.0×10^5	0
4.0×10^4	0
4.0×10^3	0
4.0×10^2	0
Cms M1	
5.6×10^9	2.20 ± 0.01
5.6×10^8	0.50 ± 0.10
5.6×10^7	0.60 ± 0.06
5.6×10^6	0.80 ± 0.03
5.6×10^5	0.10 ± 0.17
5.6×10^4	0
5.6×10^3	0
5.6×10^2	0
Cms 204	
4.1×10^9	1.63 ± 0.06
4.1×10^8	4.10 ± 0.17
4.1×10^7	1.10 ± 0.10
4.1×10^6	1.40 ± 0.06
4.1×10^5	0.37 ± 0.06
4.1×10^4	0
4.1×10^3	0
4.1×10^2	0

* For three repetitions.

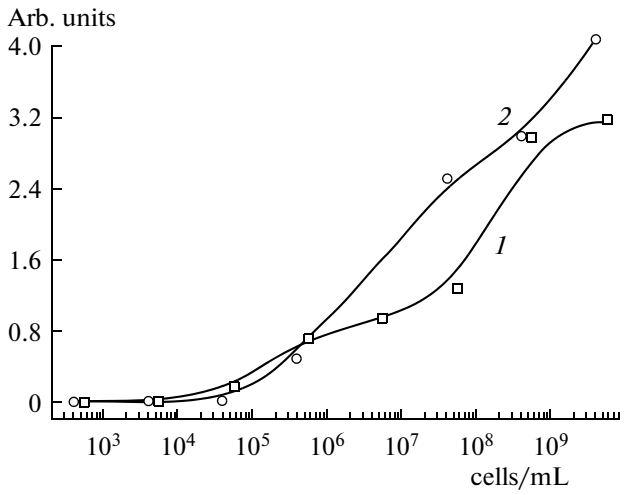

Fig. 4. Dependences of the colour intensity of the test line of the lateral flow test system (arb. units) on the concentration of Cms (cell/mL) obtained for potato extracts; curve *1*, strain Cms M1 in the leaf extract; curve *2*, strain Cms 204 in the tuber extract.

from 4×10^6 to 5×10^6 cell/mL (Table 2). The obtained values correspond to a the usual accumulation of Cms tubers of infected potato plants [2].

It was shown that there is no cross-reactivity with the Cmm 1214 and Cmm 1208 strains related to the other subspecies of the studied bacteria, namely, *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). The tests did not give false positive results for *Pectobacterium carotovorum* subsp. *carotovorum*, the DSM 30168 strain (causing blackleg of potato and vegetables) and saprophytes from tubers and leaves of healthy potato plants isolated from pure cultures (five samples).

Examination of test strips. Twenty-six tuber extracts (Table 1) were analyzed for the presence of Cms by LFIA using the developed tests and two alternative methods, such as ELISA (Loewe kits) and PCR.

A latent ring rot infection was found in samples 1, 8, 9, and 13 (Table 3) by all methods; in sample 14, by LFIA only. A few examples of test strips after the examination (samples 7, 8, and 9) are given at Fig. 5a. The amplification with primers PSA 1/PSA-R gave amplicons with a size of 502 bp for samples 1, 8, 9, and 13 (Fig. 5b). These samples were positive in ELISA testing. As can be seen from Table 3, the LFIA correlates well

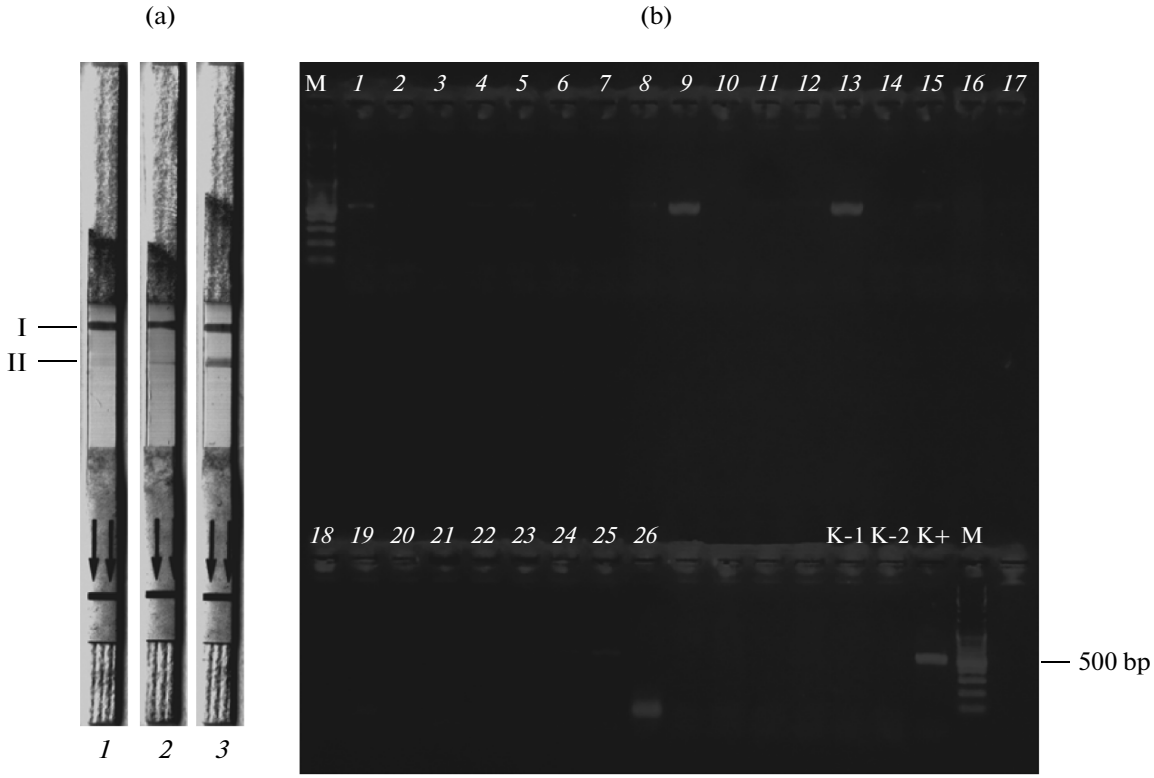

Fig. 5. Testing of seed material: (a) an example of test strips after their use in the testing (I, the control zone; II, the test zone); *1–3*, the numbers of samples are 7, 8, and 9. (b) Electrophoretic analysis of amplification products with PSA 1/PSA-R primers; M, GeneRuler^{TM} from 100 to 1000 bp; 1–26, potato samples; K-1, DNA from the tuber extract of healthy plants; K-2, DNA from the leaf extract of healthy plants; K+, total DNA of the Cms R77 strain.

Table 3. Results of ELISA, LFIA and PCR for potato tubers extracts

No	ELISA		LFIA		PCR	Conclusion about Cms presence
	OD_{405}	Assay result	Color intensity at the test line, arb. units	Assay result	Assay result	
1	0.21	+/–	0.70	+	+	Yes
2	0.06	–	0.25	–	–	No
3	0.01	–	0.20	–	–	No
4	0.04	–	0.00	–	–	No
5	0.03	–	0.25	–	–	No
6	0.01	–	0.40	–	–	No
7	0.01	–	0.10	–	–	No
8	0.16	+/–	0.53	+	+	Yes
9	3.05	+	2.80	+	+	Yes
10	0.04	–	0.10	–	–	No
11	0.01	–	0.00	–	–	No
12	0.07	–	0.37	–	–	No
13	3.32	+	1.06	+	+	Yes
14	0.03	–	0.50	+	–	No
15	0.01	–	0.00	–	–	No
16	0.02	–	0.00	–	–	No
17	0.01	–	0.00	–	–	No
18	0.02	–	0.37	–	–	No
19	0.01	–	0.20	–	–	No
20	0.01	–	0.00	–	–	No
21	0.02	–	0.00	–	–	No
22	0.02	–	0.00	–	–	No
23	0.02	–	0.00	–	–	No
24	0.02	–	0.10	–	–	No
25	0.01	–	0.00	–	–	No
26	0.01	–	0.00	–	–	No
Negative control	0.10	–	0.00	–	–	No
Positive control	1.06	+	0.80	+	+	Yes

with the other methods; the results of the assay were confirmed by 96.2%.

The results of the examination showed that the developed lateral flow immunoassay is suitable for the rapid diagnostics of plant infections, including the application in field and out-of-laboratory conditions.

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