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Lateral Flow Immunoassay for Rapid Diagnosis of Potato Blackleg Caused by *Pectobacterium atrosepticum*

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Potato blackleg and soft rot caused by *Pectobacterium atrosepticum* lead to significant yield losses. The early detection of *P. atrosepticum* is essential for healthy potato seed tubers. The aim of this study was to develop a method to rapidly detect *P. atrosepticum* based on the lateral flow immunoassay (LFIA) technique. Rabbit polyclonal antibodies specific to various strains of *P. atrosepticum* were obtained. Conjugates of these antibodies with gold nanoparticles averaging 20 nm in diameter were synthesized. Optimal concentrations of antibodies and conjugates deposited on membranes of the test strips were determined. The developed LFIA is suitable for analyzing potato tubers and leaves and has a visual detection limit of 2×10^5 cells/mL and a duration time of 10 min. Simple, rapid preparation of samples consists of homogenization in extracting buffer. No cross-reactivities with other potato pathogens, such as *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya dianthicola*, or saprophytes of healthy potato plants were detected. The assay was tested on 30 lots of potato tubers. The LFIA results were confirmed by ELISA (100% concurrence) and PCR (87.5% for positive samples and 95.5% for negative samples). Diagnosis of potato blackleg and soft rot by LFIA requires no equipment or training to perform, is cost effective and can be used in the field to monitor infection-causing *P. atrosepticum*.

Key words: *Pectobacterium atrosepticum*, gold nanoparticles, lateral flow immunoassay, potato diseases, potato blackleg, potato soft rot

Pectobacterium atrosepticum (previously *Erwinia atroseptica, Erwinia carotovora* var. *atroseptica*) is a necrotrophic, Gram-negative, rod-shaped bacterium from the family *Enterobacteriaceae* that causes blackleg and soft rot diseases in the potato. Worldwide, *P. atrosepticum* is largely restricted to the potato, principally found in temperate regions, in contrast to other blackleg pathogens such as *P. carotovorum* subsp. *carotovorum* and *Dickeya* spp., and has a broad host range[1]. The economic losses caused by *P. atrosepticum* can be significant, depending on the value of the crop and the severity of the attack. The potential for economic losses makes *P. atrosepticum* one of the most threatening potato pathogens. The bacterium may localize in potato stems and tubers and live there for a considerable period in latent form, without pathological changes of the tissues. *P. atrosepticum* causes characteristic decay symptoms by producing massive amounts of enzymes that degrade a plant's cell walls and the middle lamella of leaves, stems, roots and tubers. Due to the lack of effective protocols for mass treatment, the main agro-technical approach is the rejection of infected plants. Given this, the detection of *P. atrosepticum* is essential to effectively protecting plants, especially seed

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potato[2-4]. Thus, diagnosing infection is very important to the production of uncontaminated potato seed tubers[3].

Currently, various techniques are applied for to diagnose plant infection, including immunochemical, genetic and biochemical techniques[5-8]. However, these laboratory techniques are time-consuming and require complex equipment and skilled personnel. To be suitable for widespread monitoring of potato diseases in the field without special equipment or skills, diagnostics need to be simple, reliable, highly specific and sensitive. The lateral flow immunoassay (LFIA), also called the immunochromatographic assay, complies with all these requirements[6, 9, 10]. The application of antibodies makes LFIA sensitive and specific, while the lateral flow principle allows rapid analysis under out-of-laboratory conditions. All reactants are applied to the test strip before the assay, and contact between the strip and the test sample initiates specific interactions that yield a visible result: the presence or absence of coloration on certain areas of the strip. Sample preparation consists only of simple, rapid homogenization of the tissue in the extracting solution [11-15].

Currently, the list of agricultural bacteria detected by commercially available LFIA systems is small. Similarly, developments described in the literature are limited[6, 10, 14-16]. Therefore, considering the number of serious bacterial infections known to affect crops and the associated losses[17, 18], the development of LFIA tests for bacterial phytopathogens is a high priority. Furthermore, to date, there is no LFIA method for detecting *P. atrosepticum*. The most commonly used methods for detecting it are modified PCR tests[4, 19, 20].

The aim of this study was to develop an LFIA method to rapidly diagnose potato blackleg and soft rot caused by *P. atrosepticum*. The study includes characterization of the test components, LFIA development, its application in plant samples and comparison of the results obtained by the developed method and alternative analytical methods.

MATERIALS AND METHODS

Reagents and materials

The study required the use of 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), bovine serum albumin (BSA), sodium citrate, dimethylsulfoxide (MP Biomedicals, UK), GeneRuler™ 100 bp DNA Ladder (Thermo Fisher Scientific, USA), dNTP, Smart Taq DNA polymerase (Dialat, Russia), glycerol, NaCl, K_2CO_3 (DiaM, Russia), yeast dextrose carbonate (YDC) agar, Na_2CO_3, $NaHCO_3$, KH_2PO_4 and KOH (Khimmed, Russia). 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). To manufacture test strip, membrane, components produced by Advanced Microdevices (India) were used, including working nitrocellulose membrane CNPC-12µ, conjugate release matrix PT-R5, sample pad GFB-R4 (0.35), absorbent pad AP045 and laminates MT-1.

Preparation of bacterial samples

Pectobacterium atrosepticum (PaPa393, PaPa203-4 and PaPa204-3 isolates) and *Dickeya dianthicola* (D9, D17 and D33 isolates) were from the collection of phytopathogens of the All-Russian Research Institute of Phytopathology, Moscow, Russia. *Pectobacterium atrosepticum* (PaPa18077 isolate) and *Pectobacterium carotovorum* subsp. *carotovorum* (PaPa30168 isolate) were from the collection of phytopathogens of the Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures, Germany.

YDC agar was used to grow the target bacteria and saprophytic bacteria from leaf and tuber extracts[21]. The initial preparations were grown on the surface of the YDC agar medium for 48 h at 25–27 °C. The agar surface was flushed with sterile, phosphate-buffered saline (PBS; 50 mM potassium

phosphate, pH 7.4, 0.14 M NaCl) and pelleted three times by centrifugation (10,000 *g*, 20 min, 4 °C). The pellet was resuspended in PBS.

Production of polyclonal antibodies

The Pa393 isolate growing on YDC agar was used for immunization. Chinchilla rabbits (4-5 months old) were immunized according to a scheme that included two subcutaneous and four intramuscular injections with complete and incomplete Freund's adjuvant at weekly intervals. The immunization dose was 5×10^9 bacterial cells per animal. Blood was collected on days 7–12 after the last injection. Titers of the obtained antisera determined by indirect ELISA were 1:250,000. Immunoglobulins were isolated from the antisera by affinity chromatography on protein A-Sepharose according to the manufacturer's instructions.

Biotinylation of antibodies

Biotinylation of antibodies was carried out as described by Hermanson[22]. The antibodies were dialyzed against a 1,000-fold volume of 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 a 15:1 molar ratio. The mixture was incubated for 1 h at room temperature and then dialyzed against PBS. The preparation was stored at +4 °C.

Enzyme-linked immunosorbent assay (ELISA)

Immunoglobulins were immobilized from a 100-µL volume in microplate wells at +4 ºC 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, 50-µL portions of bacteria solution (the concentrations varied from 10^8 500 cell/mL) and antibodies-biotin conjugate solution (4 µg/mL) in PBST were added to the microplate wells. The microplate was incubated at 37 ºC for 60 min and washed as described above. Then, 100-µL portions of the peroxidase conjugate of streptavidin (in the 1:5,000 dilution of the commercial preparation in PBST) were added and incubated at 37 ºC for 60 min. The microplate was repeatedly washed, and the peroxidase activity was determined. For this purpose, the substrate (100 µL of 0.4 mM 3,3',5,5'-tetramethylbenzimidine solution in 40 mM sodium citrate buffer, pH 4.0, containing 3.0 mM of H_2O_2) was added to each microplate well. After incubation at room temperature for 15 min, the reaction was terminated by adding 1.0 µ of H_2SO_4 (50 µL), and OD_{450} was measured.

Synthesis of 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 on agitation[23]. The mixture was boiled for 25 min and then cooled and stored at 4-6 °C.

Transmission electron microscopy

Preparations of colloidal gold particles or their conjugates 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 Center, San Antonio, USA).

Synthesis of GNP-antibody conjugates

The conjugates were synthesized as described in Safenkova *et al.*,[15]. The antibodies were dialyzed against a 1,000-fold volume of 10-mM Tris-HCl, pH 9.0, at +4 °C for 12 h. The pH of the GNP solution was adjusted to 9.0 with 0.2 M of K_2CO_3. Then, the antibodies at concentrations of 20 µg/mL were added to the GNP solution. The mixture was stirred at room temperature for 60 min, and then BSA was added to its final concentration of 0.25%. The GNPs with immobilized antibodies were separated by centrifugation at 20,000 *g* for 30 min and resuspended in PBS containing 0.25% BSA, 0.25% Tween 20 and 1% saccharose. The conjugate was stored at +4 °C. For long-term storage, NaN_3 was added to the sample to its final concentration of 0.02%.

Preparation of test strips

Membrane compounds and multi-membrane composite were prepared using approaches described in Safenkova *et al.*[15]. The GNP-antibody conjugate was deposited onto CNPC-12µ membranes from a solution that had optical density equal to 10 at λ= 520 nm. The conjugate load was 16 µL per cm of strip width. The test zone was formed by IgG specific to *P. atrosepticum,* and the control zone was formed by goat anti-rabbit IgG. Both loading solutions were at concentrations of 1.0 mg/mL in PBS and were applied to 2 µL per cm of strip width. After the assembly of membranes, the multi-membrane composite was cut into strips of 3.5 mm in width

using an Index Cutter-1 (A-Point Technologies, USA) and hermetically packed into bags composed of laminated aluminum foil and containing silica gel as desiccant by 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 of no more than 30%.

Preparation of potato seed material for testing

The testing was performed on 30 lots that included up to 200 tubers each. Samples were obtained from various points in the Central Federal District of Russia. Samples 1-14 included 200 tubers each, samples 15-24 included 15 tubers each and samples 25-30 included 10 tubers each. Segments (5 × 5 × 10 mm) were cut from each tuber and placed in a 250-mL, conical flask. Then, 40 mL of autoclaved 50 mM phosphate buffer with pH 7.0 was added, and the resulting mixture was incubated at 5–10 °C. The extract was filtered through white ribbon filter paper. The resulting filtrate was centrifuged at 10,000 *g* for 10 min at 5–7 °C. The pellet was resuspended in the PBS for ELISA and LFIA, and in sterile 0.01M Na-phosphate buffer with pH 7.0 for PCR.

The leaf samples were triturated with a pestle in a porcelain mortar with 50 mM of phosphate buffer (pH 7.0) and 20-fold excess. The extract was filtered, and all further operations were carried out as for tubers.

LFIA

The assay was performed at room temperature. The test strip was vertically submerged in the tested sample for 1.5 min and then taken out and placed on a horizontal surface. The qualitative results were estimated visually 10 min after the beginning of the assay. 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 completely air-drying the strip using a Reflekom portable photometric detector (Okta-Medika, Russia) according to Byzova *et al.*,[11]. The registered value of 0.2 arbitrary (arb.) units of color intensity accorded with the threshold of reliable visual detection.

ELISA using commercial kits

The assay of *P. atrosepticum* was carried out using an ELISA Reagent Set (LOEWE Biochemica, Germany) according to the manufacturer's protocol. Optical absorbance of enzymatic reaction products was measured at 405 nm.

PCR analysis

Bacterial DNA was isolated from plant extracts using Sample-GS kits (DNA Technology, Moscow). Thermal Cycler (Nyx Technik, USA) was used for the PCR. The analysis was performed with ECA1f (5'-CGGCATCATAAAAACACG-3') and ECA2r (5'-GCACACTTCATCCAGGGA-3') primers described in De Boer *et al.*[24]. The given primers are specific to the intergenic spacer of the ñhromosomal DNA of *P. atrosepticum* and give an amplification product with a size of 690 bp. The reaction mixture (25 µL) included 20 pmol of each primer, 5 µL of PCR buffer 5x, 1.5 mM of $MgCl_2$, 200 pmol of a dNTP mixture and 5 units of Smart Taq DNA polymerase. Total DNA of the Pa18077 strain of *P. atrosepticum* was used as a positive control, and total DNA of healthy potato leaves or tubers was used as a negative control.

To visualize the amplification results, electrophoresis in 1.5% agarose gel with ethidium bromide added was performed as proposed by De Boer *et al.*[24] using a horizontal camera from Helicon (Russia).

RESULTS AND DISCUSSION

Characterization of antibodies

Initially, the specificity of the polyclonal antibodies was tested. The antibody obtained at immunization with the Pa393 strain was tested by ELISA in reactions with closely related strains of *P. atrosepticum* and unrelated bacteria *Dickeya* (D9, D17 and D33 isolates).

Antibody characteristics were quite similar for various strains of *P. atrosepticum* (Fig. 1, curves 1-3). The limit of detection (LOD) of *P. atrosepticum* was 1×10^5 cells/mL, which corresponds to the usual level of accumulation of the pathogen in infected potato tubers[3]. According to Czajkowski *et al.*[3], bacterial densities in symptomatic tissues are often greater than 10^6 cells/g. Specificity of the antibodies was confirmed by the low OD_{450} value the *D. dianthicola* strains (Fig. 1, curves 4-6).

Producing GNPs and their conjugates with antibodies

Transmission electron microscopy (TEM)

was applied to characterize the synthesized GNP. It showed that their average diameter was 20 ± 3 nm and that the form factor (the maximum to minimum axis ratio) was 1.12 ± 0.08. The absorption maximum of the synthesized GNP was 520 nm. These data suggest that preparation of GNP is monodisperse and does not contain aggregates.

The GNPs were conjugated with the anti-*P. atrosepticum* antibodies. The absorption maximum of the conjugate was observed at 524 nm because immobilized antibodies caused a shift in the surface plasmon resonance of the GNPs. The conjugate concentrations were determined using OD_{520}, taking into consideration that the immobilization of the antibodies led to an approximately 10% decrease in GNP absorption[25].

Developing the LFIA

The polyvalence of the *P. atrosepticum* bacterium with a plurality of surface-exposed epitopes allowed us to develop the LFIA based on the methodological approaches that we proposed and successfully implemented for LFIAs for plant

Table 1. Results of ELISA, LFIA and PCR for potato tubers extracts

Sample	Variety	ELISA		LFIA		PCR	*P. atrosepticum*
		OD_{405}	Assay result	Color intensity of the test line, (arb. units)	Assay result	Assay result	presence
1	Impala	0.11	–	0.00	–	–	No
2	Impala	0.01	–	0.00	–	–	No
3	Kibits	0.83	+	0.95	+	+	Yes
4	Saturna	0.01	–	0.00	–	–	No
5	Phasan	0.70	+	0.45	+	+	Yes
6	Bonus	1.09	+	1.57	+	+	Yes
7	Hermes	1.03	+	0.60	+	+	Yes
8	Monaliza	0.01	–	0.00	–	+	No/Yes
9	Red Scarlet	0.45	+	0.53	+	+	Yes
10	Salin	1.14	+	1.53	+	+	Yes
11	Agatha	0.01	–	0.00	–	–	No
12	Picasso	0.01	–	0.00	–	–	No
13	Nevsky	0.01	–	0.00	–	–	No
14	Impala	0.51	+	0.30	+	–	Yes/No
15	Agria	0.01	–	0.00	–	–	No
16	Ariel	0.01	–	0.00	–	–	No
17	Ariel	0.01	–	0.00	–	–	No
18	Arosa	0.01	–	0.00	–	–	No
19	Belaroza	0.01	–	0.00	–	–	No
20	Jelly	0.01	–	0.00	–	–	No
21	Eurostar	0.01	–	0.00	–	–	No
22	Zhukovsky early	0.01	–	0.00	–	–	No
23	Courage	0.01	–	0.00	–	–	No
24	Red Scarlet	0.01	–	0.00	–	–	No
25	Lady Claire	0.01	–	0.00	–	–	No
26	Unknown 1	0.01	–	0.00	–	–	No
27	Lady Claire	0.01	–	0.00	–	–	No
28	Lady Claire	0.01	–	0.00	–	–	No
29	Unknown 2	0.01	–	0.00	–	–	No
30	Lady Claire	0.01	–	0.00	–	–	No
Negative control		0.10	–	0.00	–	–	No
Positive control		2.55	+	0.97	+	+	Yes

viruses in our previous studies[25, 26]. Based on the formation of immobilized antibody-antigen-labeled antibody complexes, the sandwich format LFIA was used. The LFIA was optimized by choosing concentrations of reactants (IgG and IgG–GNP conjugates) adequate to achieve maximum color intensity in both the test and control zones, as well as the lowest detection limit in the absence of background staining. The Materials and Methods section presents the selected conditions.

The maximum color intensity in the test and control zones was achieved upon deposition of antibodies from a solution at a concentration of 1.0 mg/mL (at a deposition density of 0.2 µL/mm), and the intensity did not increase at higher concentrations. As a result, this concentration was selected for use in the optimum procedure. Anti-rabbit antibodies for control zones were applied to the membrane from a solution with a concentration of 0.5 mg/mL to give the maximum color intensity of the control 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 color intensity in both zones occurred 10 minutes after sample contact with the test strip. Therefore, a 10-minute analysis time was chosen as optimal.

Determining the detection limit of the developed LFIA

Serial dilutions of *P. atrosepticum* in PBST were tested using the developed LFIA. As shown in Fig. 2A, the color intensity of the test lines gradually grew with increasing analyte concentration in the sample (up to 10^7 cells/mL). The coloration may be controlled quantitatively (Fig. 2B). In view of the threshold of reliable visual detection (0.2 arb. units of color intensity), *P. atrosepticum* can be reliably detected by LFIA at concentrations of 2×10^4 cells/mL for Pa204-3, 3×10^4 cells/mL for Pa393 and 2×10^5 cells/mL for the Pa18077 strain (Fig. 2B). The LOD of LFIA is close

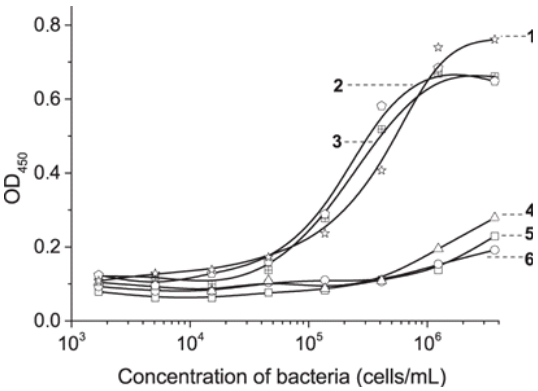

Fig. 1. Curves for sandwich ELISA with anti-*P. atrosepticum* antibody: plots of absorbance versus concentration of *P. atrosepticum* or *D. dianthicola* cells. Curves 1-6 accord to Pa393, Pa204-3 and Pa203-4 (*P. atrosepticum*); D9, D17 and D33 (*D. dianthicola*), respectively.

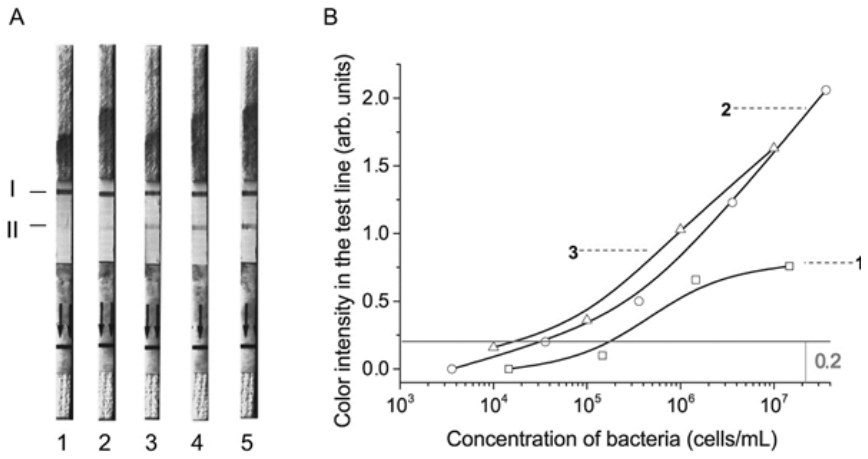

Fig. 2. LFIA of *P. atrosepticum*. A, test strips after the assay (I, the control zone; II, the test zone); 1–5, the concentrations of *P. atrosepticum* (Pa393) in PBST are 3.6×10^3, 3.6×10^4, 3.6×10^5, 3.6×10^6 and 3.6×10^7 cell/mL. B, the color intensity in the test line (arb. units) versus the concentration of *P. atrosepticum*; curves 1-3 accord to Pa18077, Pa393 and Pa204-3, respectively

in value to that obtained by ELISA.

To evaluate application of the developed LFIA to analysis of tubers and leaves, pure preparations of *P. atrosepticum* were added to extracts of healthy leaves and tubers. Figure 3 shows examples of such tests. The color intensity in the test lines was a bit higher after exposure to the leaf extracts and the tuber extracts than in the buffer solution.

Thus, the ranges of detection for *P. atrosepticum* strains by LFIA corresponded to the usual level of accumulation of the pathogen in infected potato tubers, which is greater than 10^6 cells/g for symptomatic tissues[3].

The study tested the specificity of the LFIA tests, showing that there is no cross-reactivity with the DSM 30168 strain of *Pectobacterium carotovorum* subsp. *carotovorum*. The tests did not give false positive results for the D9, D17 and D33 strains of *Dickeya dianthicola*, which cause potato and vegetable blackleg, or for saprophytes from healthy potato plant tubers and leaves isolated from pure cultures (five samples).

Validating test strips; comparing techniques for detecting *P. atrosepticum*

To examine the developed test systems, we analyzed 30 combined lots of seed potatoes (see Table 1) for the presence of *P. atrosepticum*. For this comparison, we used the developed LFIA test system, the commercial ELISA kit from LOEWE (Germany) and the PCR assay. The *P. atrosepticum* was detected in tuber extracts in samples 3, 5-7, 9 10 (Table 1) by all methods, in sample 14 by ELISA and LFIA and in sample 8 by PCR only. Fig. 4A shows examples of test strips after the examination. Electrophoretic analysis of amplification products with ECA1f/ECA2r primers shows amplicons with a size of 690 bp for seven samples infected by *P. atrosepticum* (Fig. 4B). The amplicons greater than or less than 690 base pairs indicate no presence of

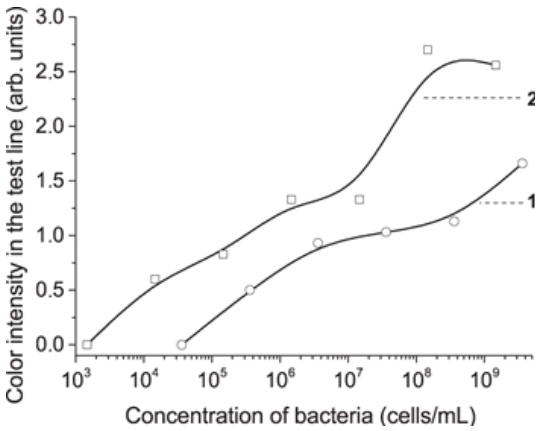

Fig. 3. Dependences of the color intensity of the test line of the LFIA system (arb. units) on the concentration of *P. atrosepticum* obtained for potato extracts; curve 1, strain Pa18077 in the leaf extract; curve 2, strain Pa393 in the tuber extract.

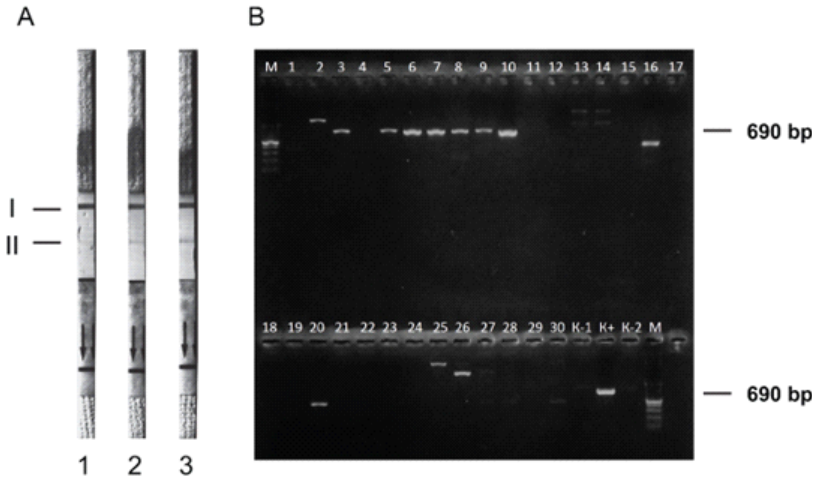

Fig. 4. Testing of seed material. A, an example of test strips after use in testing (I, the control zone; II, the test zone); 1–3, the numbers of samples are 4, 5 and 6, respectively. B, electrophoretic analysis of amplification products with ECA1f/ECA2r primers; M, GeneRuler™ from 100-1000 bp; 1-30, 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 Pa18077 strain

P. atrosepticum in the sample.

The examination showed a 100% overlap for LFIA and ELISA, for both positive and negative samples, and a high degree of overlap for LFIA and PCR, for both positive (87.5%) and negative samples (95.5%) (Table 1).

The results of this study show for the first time that using an LFIA to detect *P. atrosepticum* is feasible. This new LFIA can be used for rapid, sensitive control of bacterial cultures and symptomatic plant material and has a visual detection limit of 2×10^5 cells/mL and an assay time of 10 minutes. Furthermore, this proposed method of monitoring *P. atrosepticum* is cost effective and can be used under field conditions.

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