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Lateral Flow Immunoassay for Progesterone Detection

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Abstract—A new express method based on lateral flow immunoassay (LFIA) for progesterone detection was developed. To increase the assay sensitivity an enzyme label (horse-radish peroxidase) was used instead of colloidal gold. An optimal assay format was chosen and the influence of a range of buffer supplements (detergents, proteins and sucrose) was investigated by enzyme-linked immunosorbent assay (ELISA). Linear range of LFIA was between 2 and 40 ng/mL in buffer. Limit of detection was 2 ng/mL, assay time was within 15 min.

Keywords: progesterone, lateral flow immunoassay, horseradish peroxidase

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INTRODUCTION

At present, the early diagnosis of pregnancy in cows is one of the most important tasks in agriculture. The detection of pregnancy shortly after insemination allows the service period to be significantly reduced and, therefore, is considered to be an essential tool of economically viable reproductive management strategy. Traditional rectal examination only yields reliable results on days 70–90 after insemination. Pregnancy detection using ultrasound scanners, i.e., ultrasonography, is known to achieve a reliable diagnosis using a number of different parameters on days 32–37 post-insemination; however, the implication of this approach requires the expertise of highly qualified veterinarians, as well as the use of expensive equipment. Immunochemical methods of analysis, including the enzyme-linked immunosorbent assay (ELISA) are accepted as the fastest among all known methods of pregnancy detection. ELISA allows pregnancy to be diagnosed in cows by detecting the concentration of the species-nonspecific hormone progesterone (PG) in milk and blood serum on days 19–21 post-insemination [1]. However, this method requires the use of special equipment.

Immunochemical methods of detecting pregnancy by accessing the content of PG were first developed in the 1970s [2, 3]. The diagnosis of pregnancy based on the detection of the PG concentration in milk was first described in [4]. Later, it was demonstrated that the content of PG in cow's milk correlates with its concentration in blood serum; therefore, PG can be used as a marker for detecting pregnancy in cows [5, 6]. The concentration of PG in cow milk changes cyclically [7], i.e., it is at a low level (below 2 ng/mL) during ovulation, which occurs at the beginning of the reproductive cycle, and rises to the maximum (more than 10 ng/mL) on days 13–15. When no pregnancy has occurred, the level of PG drops on days 18–20. However, if insemination was successful, PG remains at a high level throughout the whole period of pregnancy. Therefore, the detection of the PG concentration on day 21, i.e., at the end of the reproductive cycle, allows one to consider the pregnancy status of the investigated animals to be positive in the case of high PG and negative when the PG level is relatively low. A cow is considered to be pregnant if the level of PG in milk on day 21 after insemination is above 7 ng/mL.

At present, lateral flow immunoassay (LFIA) or immunochromatographic analysis is considered to be one of the simplest and the quickest methods of the semi-quantitative detection of important biologically active compounds. Since the advantages of this method are its short time (10–15 min), as well as simple and equipment-free analysis of the obtained data, the technique is widely used in medical diagnostics, agriculture, and many other areas. The analysis is carried out using special test strips that allow rapid testing to be performed outside of a laboratory (Fig. 1). The test strips contain all components needed for assay so that the reaction takes place in just one step following the loading of the analyzed sample onto a membrane. The results of analysis represents of colored strips found in both the test and control zones of the analytical membrane. There only a few works have been published so far that describe the detection of PG by LFIA [8–10]. In these works, gold nanoparticles [8], horseradish peroxidase [9], and colloidal carbon [10] were used for labeling. It should be noted that the first LFIA method for detecting a low molecular weight compound called "hapten" was actually developed for progesterone [8]. Even though ELISA kits for detecting PG in milk are commercially available both abroad and in Russia, individual rapid tests for detecting PG

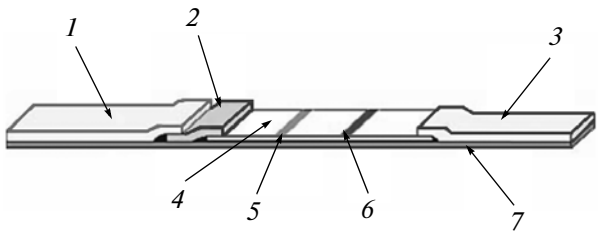

Fig. 1. Construction of a test strip for LFIA. *1*—sample pad; *2*—conjugate pad; *3*—absorbent pad; *4*—analytical membrane; *5*—test band; *6*—control band; *7*—plastic base.

in milk have been developed relatively recently. Therefore, the aim of this study was to develop a rapid LFIA-based method for detecting progesterone that can be further used in practice for the rapid testing of pregnancy in cows.

MATERIALS AND METHODS

Biochemical and chemical reagents were purchased from Sigma (USA); potassium mono- and dihydrogen phosphate, as well as sucrose, were from Helicon (Russia); tetrachloroauric acid was purchased from Fluka (Switzerland); Tween-20 and Triton X-100 were from MP Biomedicals (France); ovalbumin (OVA) was from Reakhim (Russia); ready-to-use substrate solution containing 3,3',5,5'-tetramethylbenzidin (TMB) and H_2O_2 was from NVO Immunotek (Russia); sulfuric acid and absolute ethanol were purchased from Khimmed (Russia); and horseradish peroxidase (HRP) was from Yarinvest (Russia).

The buffer solutions used in this work were as follows: PB 0.01 M, pH 7.0; PBS 0.01 M; 0.15 M NaCl, pH 7.4; PBST 0.01 M; 0.15 M NaCl; 0.05% Tween-20, pH 7.4; CB 0.01 M, pH 9.5; and BB 0.01 M, pH 8.6.

Standard solutions of PG were prepared by subsequently diluting 1 mg/mL ethanol solution of PG with PBST buffer.

Polyclonal antisera obtained via the immunization of rabbits with a conjugate of 11α-hydroxyprogesterone hemisuccinate and hemocyanin and with horseradish peroxidase were kindly donated by the Laboratory of Genetic Engineering, Department of Chemical Enzymology, Moscow State University. The immunoglobulin fraction of rabbit antiserum was isolated by double precipitation with anhydrous ammonium sulfate. The excess ammonium sulfate was removed via gel filtration using a PD-10 column (GE Healthcare, United Kingdom).

Colloidal gold preparation with the desired average size of particles was obtained as described by Frens [11].

Multimembrane test strips were prepared using analytical nitrocellulose CNPC membranes with a pore diameter of 15 μm, PT-5 conjugate pad, absorbent pad AP045 (MDI, India), and MAPDS-0300 sample pad (Arista Biologicals, USA).

Synthesis of Conjugate of Progesterone 3-O-Carboxymethyloxime with OVA

To obtain the conjugate, 2.9 mg (7.5 μmol) progesterone 3-O-Carboxymethyloxime (CMO-PG), 1.7 mg (15 μmol) N-hydroxysuccinimide (NHS), and 3.1 mg (15 μmol) dicyclohexylcarbodiimide (DCC) were dissolved in 500 μL dimethylformamide (DMF). The reaction mixture was then stirred at room temperature for 4 h and further incubated at 4°C overnight. The formed precipitate was removed by centrifugation. The supernatant (170 μL) was added to a solution of 20 mg (0.5 μmol) OVA in 1.5 mL BB with shaking and further incubated at room temperature for 4 h. The obtained solution was dialyzed against distilled water. The obtained conjugate was lyophilized and stored at 4°C until it was needed.

Preparation of CMO-PG-OVA Conjugate Labeled with Gold Nanoparticles

To label the conjugate, 1 mL of 50 mg/mL solution of the CMO-PG-OVA conjugate was added to 10 mL colloidal gold solution (pH 5.5) and mixed intensively for 15 min. To remove the excess of unbound CMO-PG-OVA conjugate, the obtained solution was centrifuged at 11000 g for 20 min at 4°C. The supernatant was then removed and the precipitate was resuspended in 1 mL PB containing 0.1% bovine serum albumine (BSA), 10% sucrose, and 0.01% NaN_3. An aliquot of the obtained solution was applied onto a strip of a conjugate pad of the 4 × 4 mm in size and the membrane was then left to dry at room temperature overnight.

Preparation of Conjugate of CMO-PG with Horseradish Peroxidase

To obtain the conjugate, 2.6 mg (6.7 μmol) CMO-PG was dissolved in 300 μL DMF followed by the addition of 0.9 mg (8 μmol) NHS and 2.7 mg (13 μmol) DCC, and the obtained solution was shaken. The reaction mixture was stirred at room temperature for 2 h and further incubated at 4°C overnight. The reaction mixture was then centrifuged, after which 100 μL supernatant were added to a solution of 2 mg HRP in 200 μL BB with shaking. The resulting reaction mixture was further stirred at room temperature for 2 h. Following the incubation step, the reaction mixture was centrifuged and the supernatant was purified by gel exclusion chromatography using a PD-10 column (GE Healthcare, United Kingdom). The optical density of the obtained conjugate was measured at a wavelength of 403 nm using a UV-1202 spectrophotometer (Shimadzu, Japan).

Preparation of Test Strip

To perform LFIA using colloidal gold as a label, specific antibodies at a concentration of 0.2 mg/mL or

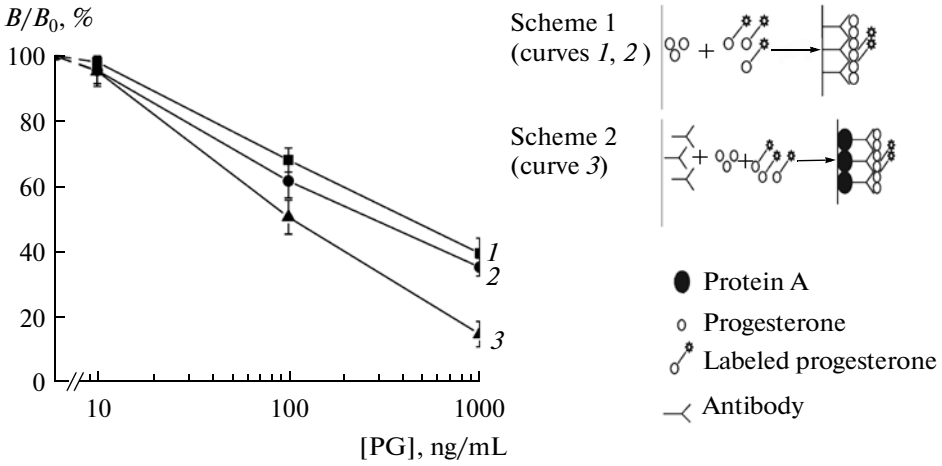

Fig. 2. Calibration curves for PG detection using LFIA employed colloidal gold as a label. Gold particles with a size 16 nm (*1*) and 35 nm (*2*, *3*) were employed in LFIA according to schemes 1 (*1*, *2*) and 2 (*3*).

protein A at a concentration of 0.1 mg/mL in PBS were applied to an analytical membrane using a programmable semi-automatic dispenser BioJet Quanti 3000 equipped with an automatic platform XYZ 3050 (BioDot, USA) to form an analytical zone. In the case of LFIA with an enzymatic label, the antibodies were applied at a concentration of 0.015 mg/mL. To form a control zone, the antibodies to HRP at a concentration of 0.025 mg/mL were applied onto the strip at a distance of 5 mm from the analytical zone. The following parameters of the dispenser pump for antibody application were used: droplet size, 30 nL; line width, 0.3 mm; and speed, 50 mm/s. The test strips were dried at 37°C for 24 h.

Procedure of LFIA Employed Colloidal Gold as a Label

The test strips (75 × 4 mm) were prepared according to the scheme presented in Fig. 1. Prior to the analysis, the test strips were placed on a horizontal surface. The application of reagents was carried out in two different ways. The first approach, which is shown in Fig. 2 (scheme 1), was employed when specific antibodies were absorbed in the analytical zone (120 μL standard PG solution was applied onto a sample pad); the second approach, which was illustrated by scheme 2 (Fig. 2), was used for protein A absorbed in the test zone of a test strip (120 μL standard PG solution and 5 μL of an antibody solution at a concentration of 0.01 mg/mL were applied onto a sample pad).

All reagent solutions were prepared in PBST buffer. After the solution loaded onto the membrane soaked in completely, the test strips were air dried at room temperature. The quantitative evaluation of the intensity of the colored control and analytical zones of the test strips was performed using an Epson Perfection V700 Photo scanner (Seiko-Epson, Japan) with a resolution of 600 dpi in the 24-bit color (RGB) setting. An analysis of the obtained digital images was carried out in the TIFF format using the Scion Image program (http://www.scioncorp.com/pages/scion_image_windows.htm). The obtained data were used to plot the calibration curves, where the color intensity of the test strips in arbitrary units or the ratio $B : B_0 = (I/I_0) \times 100\%$, where I_0 represents the stain intensity at a concentration of PG of 0 ng/mL and I is the color intensity was along the x-axis and values of concentration of a standard PG solution were along the y-axis.

ELISA procedure

ELISA was carried out according to three different schemes illustrated in Fig. 3. In the case of scheme 1, a solution of antibodies in CB at a specific concentration (150 μL per well) was absorbed in the wells of a polystyrene plate. Following the incubation of a plate at 4°C overnight, the well plate was washed three times with PBST (150 μL per well), after which 20 μL standard PG solutions followed by 100 μL CMO-PG-HRP conjugate solution at a specific concentration was added in each well. The well plate was incubated at 37°C for 1 h, the excess unbound reagents was removed by washing with PBST solution (3 × 150 μL per well) and 100 μL substrate solution was then added to each well, and the plate was incubated at 37°C for 15 min. The reaction was stopped by adding 100 μL/well of 0.2 M sulfuric acid. When scheme 2 was used, the protein A at a concentration of 1 μg/mL in CB was first absorbed in wells (150 μL per well, 2 h incubation at 37°C). Following washing with PBST (3 × 150 μL per well), an overnight incubation at 4°C with a solution of specific antibodies in PBST at a desired concentration was performed (150 μL per well). Further analysis was performed as described above for scheme 1. Analysis according to scheme 3 was carried out analogously to the one described above for scheme 2. The only difference was that, following the stage of protein A absorption, 20 μL standard PG solutions, 50 μL CMO-PG-

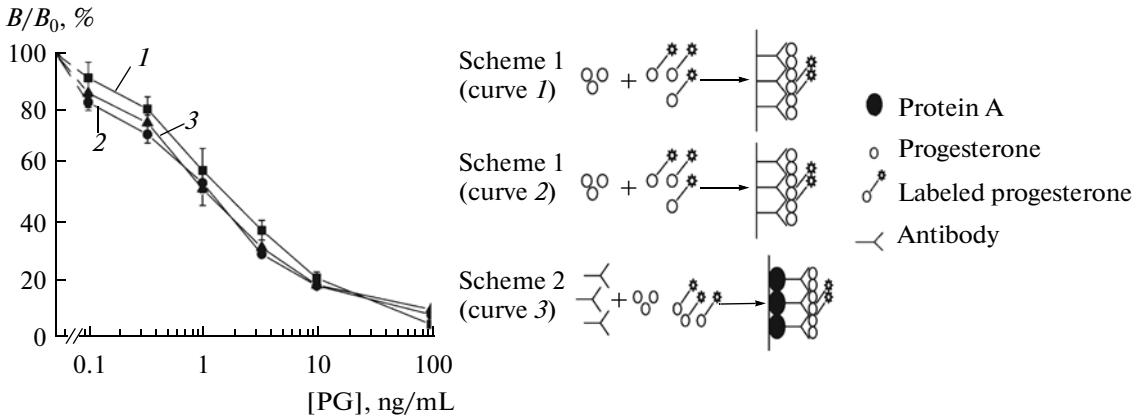

Fig. 3. Calibration dependences for detecting PG using ELISA by three different schemes.

HRP conjugate solution and 50 μL antibody solution in PBS at a specific concentration were sequentially added to wells, after which the well plate was incubated at 37°C for 1 h. The reaction results were analyzed using a vertical-beam spectrophotometer at a wavelength of 450 nm (Molecular Devices, United States). The obtained data were calculated to plot the calibration dependences where the stain intensity in optical units or the value $B : B_0 = (I/I_0) \times 100\%$ was along the abscissa of the graph and concentration values of a standard PG solution were along the ordinate.

LFIA Procedure Using Enzymatic Labeling

The test strips (75 × 4 mm) were constructed according to the scheme illustrated in Fig. 1 without using membranes to load the conjugate. To perform the assay, a mixture of 10 μL CMO-PG-HRP conjugate and 10 μL standard PG solution in PBST was applied to the analytical membrane. Following the full infiltration of the applied solution, 150 μL of PBST was applied to the very end of the membrane to load the sample. After the infiltration of the applied PBST solution, the test strip was stained via two different methods. In the first case, the analytical membrane was cut off and immersed into 500 μL substrate solution containing 10% dextran sulfate and further incubated for 5 min with shaking. In the second case, 50 μL same solution was applied to an analytical membrane. Following staining, the test strips were air dried at room temperature for 30 min. The quantitative evaluation of the obtained results was carried out as described in the case of LFIA employed colloidal gold as a label.

RESULTS AND DISCUSSION

LFIA Employed Gold Nanoparticles as Label

Progesterone is a low-molecular-weight compound; therefore, a scheme of analysis was employed based on the principle of competitive binding of labeled and unlabeled PG with the specific antibody binding sites. In this case, the resulting analytical signal is inversely correlated with the concentration of PG.

In LFIA, gold nanoparticles are often used as a label. The main advantages of the use of gold nanoparticles for antibody labeling are defined primarily by the simplicity of preparation of the predicted size particles and, secondly, by their unique optical characteristics. In the present work, we used colloidal gold nanoparticles with sizes of 16 and 35 nm. To perform LFIA with colloidal gold as a marker, the scheme according to which the PG specific antibodies were immobilized in the test zone was initially used. Gold nanoparticles were used to label a conjugate of a PG derivative with high-molecular-weight protein (OVA). The obtained calibration curves demonstrate that the use of gold nanoparticles of various sizes does not allow the reliable detection of PG in the desired range of concentration, i.e., below 10 ng/mL (Fig. 2, curves 1 and 2). Therefore, we modified the scheme of analysis and used the immobilization of the protein A in the test zone of a membrane. The main feature of the modified scheme is that all reagents, i.e., labeled (PG*) and unlabeled (PG) PG, as well as antibodies specific to PG, are simultaneously applied to a test strip. This allows one to create genuine conditions under which labeled and unlabeled reagents compete for the antibody binding sites in solution. Following this step, the binding of antibodies to the protein A molecules leads to their directed immobilization on the membrane via the Fc fragment. As can be seen in Fig. 2 (curve 3), the slope of the calibration curve for this scheme is higher than that of the calibration curve that characterizes the direct absorption of antibodies. However, this scheme of analysis also does not allow the working range of the calibration dependency to be shifted to a region of lower concentration. Therefore, the further development of a quick method of PG detection used more sensitive enzyme label. LFIA using enzyme as a label has previously been described in the literature for high- [12–15] and low- [16, 17] molecular-weight

compounds, as well as whole cells [18]. The use of an enzyme as an antibody marker allows the analysis sensitivity to be increased by 10–30 times compared to LFIA using colloidal gold for labeling [13, 15, 17]. To choose an optimal scheme of the LFIA procedure using horseradish peroxidase as a label and to study the influence of various components, which can be a part of membranes used in the analysis, the enzyme-linked immunosorbent assay was used.

Detection of PG using ELISA

Choice of an assay format. The comparative study of three different schemes of ELISA (Fig. 3) was performed. In scheme 1, antibodies were immobilized in well of plates directly, whereas in schemes 2 and 3 antibodies were absorbed directionally through the protein A. The principal difference of scheme 3 from the others is that all reagents, i.e., both free and enzyme-labeled PGs, as well as specific antibodies, are added simultaneously, which provides the genuine competition of labeled and unlabeled reagents for the binding sites of antibodies. Simultaneously, the directional binding of antibodies with protein A occurs through the Fc fragment.

The calibration dependences obtained for the above-described three assay schemes were almost identical (Fig. 3). Despite that assay according to scheme 1 requires a higher concentration of antibodies than that of both schemes 2 and 3, where the absorption of antibodies is conducted using protein A, scheme 1 prevails significantly in terms of the assay duration, i.e., 1.5 h versus 3 h in the case of schemes 2 and 3. Consequently, less complicated and much quicker scheme 1, which employs the direct absorption of antibodies, was used for further investigations.

Influence of various components on ELISA characteristics. We studied the influence of components that may be part of the commercial membranes used for LFIA. For this purpose, various components at different concentrations were added to the working buffer during the incubation stage; the calibration curves were plotted and compared for every investigated additive. We studied the influence of detergents, including Tween-20 and Triton X-100 in a concentration range of 0.0005–0.05%, as well as the effect of the addition of 0.01–10% sucrose and 0.01–1% of proteins, such as OVA, BSA, and casein. It should be noted that, among all of the investigated components, only the presence of sucrose did not result in the shift of the calibration curve to a region of higher PG concentration (data not shown). Along with the influence of single components, the effect of the simultaneous addition of two or three different compounds was studied (Fig. 4). It was shown that the addition of a detergent, along with one or two other components, results in a shift of the calibration curve into a region of higher concentration of PG. Furthermore, among all investigated combinations of various additives, only the simultaneous presence of BSA and sucrose resulted in no difference in the corresponding calibration dependency compared to that of the working buffer solution.

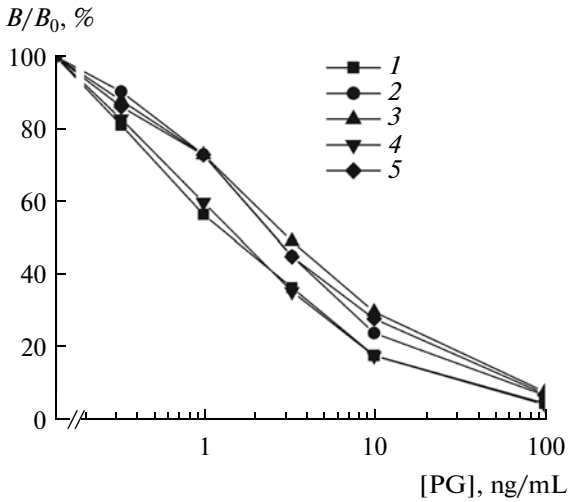

Fig. 4. Calibration curves for PG detection by ELISA using the following working buffers: *1*—PBS; *2*—PBS containing 0.05% Tween-20, 10% sucrose; *3*—PBS containing 0.05% Tween-20, 0.1% BSA; *4*—PBS containing 10% sucrose, 0.1% BSA; *5*—PBS containing 0.05% Tween-20, 10% sucrose, 0.1% BSA.

Thus, it was shown that the addition of various supplements, such as detergents, proteins, and sucrose, to a buffer system causes only a minor shift in the calibration dependency (the average IC_{50} value changes within the 1.5–2.0 ng/μL range); therefore, it does not affect the detection of PG in the range of concentration below 10 ng/μL.

The linear concentration range of the developed technique was determined as 2–40 ng/mL progesterone. The detection limit was 2 ng/mL and the duration of the developed assay was below 15 min.

LFIA Employed Enzyme as a Label

To develop LFIA employed enzyme as a label, a scheme, which features direct immobilization of antibodies on an analytical membrane, was used. The CMO-PG-HRP conjugate was used as a labeled reagent. During the development of the assay format, the sequence of applying reagents to the strip, the construction of a test strip and, finally, the method of signal registration were optimized.

Sequence of loading of reagents onto a test strip. Figure 5 presents the dependences of relative signal intensity on PG concentration for different sequences of loading of reagents onto a strip as follows:

(1) the loading of a mixture of labeled and unlabeled PG (PG + PG*) in the analytical zone of the test strip;

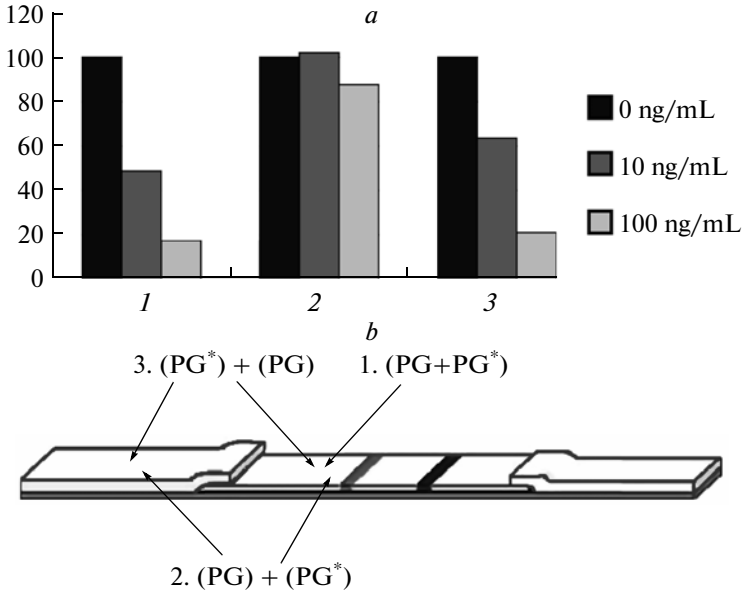

Fig. 5. Curve of color staining of analytical zone of a membrane on PG concentration for different sequences of reagent loading using LFIA employed enzyme as a label.

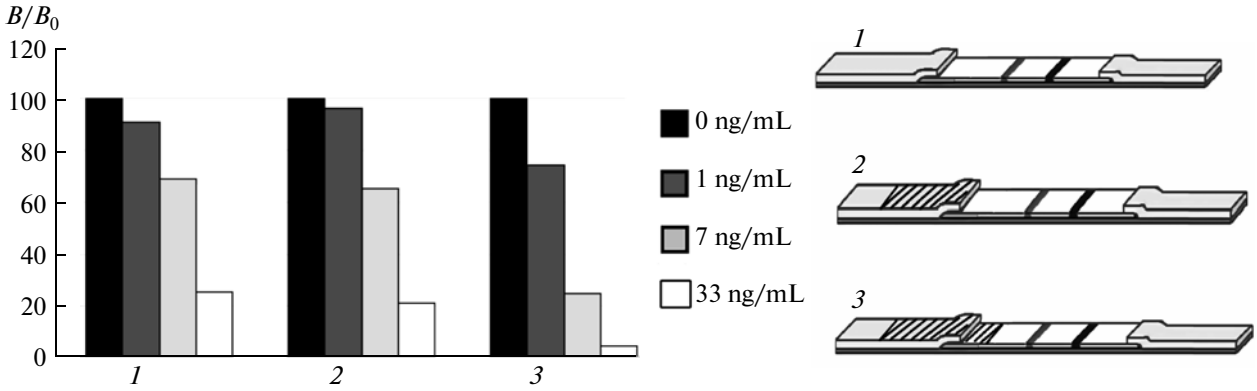

Fig. 6. Curve of color staining of analytical zone of membrane on PG concentration for different schemes employed for lamination of a test strip using LFIA with enzyme as a label. Area of lamination is patterned.

(2) the sequential loading of labeled PG* on an analytical membrane and unlabeled PG on a sample pad;

(3) the sequential loading of unlabeled PG on an analytical membrane and labeled PG*on a sample pad.

Following this, a PBST buffer solution was loaded onto a membrane as a sample in all three of the above-described cases of LFIA. Interestingly, no calibration dependency was found (Fig. 5) for loading CMO-PG-HRP conjugate solution onto an analytical membrane, followed by the application of a standard PG solution (option 2). It is likely that the CMO-PG-HRP conjugate binds to the antibodies absorbed in the testing zone with rather high affinity so that a standard PG solution, which is applied to a membrane as a sample, does not displace the conjugate in the testing zone. Options 1 and 3 are characterized by rather similar dependences. However, the amount of the conjugate used in the first case is three times lower than that of option 3. According to the obtained data, the option of loading a mixture of labeled and unlabeled PG onto an analytical membrane followed by the application of buffer solution was considered to represent the optimal sequence of reagent loading and, therefore, was used further in this work.

Lamination of the test strip. To obtain the optimal level of staining of both the testing and the control zones and to reduce the background signal during LFIA, a test strip is often laminated, i.e., covered with a specific film that features a glue-based side. This approach ensures the uniform flow of a liquid on a strip, which is extremely important in this particular type of assay. Because, in our case, the enzyme-sub-

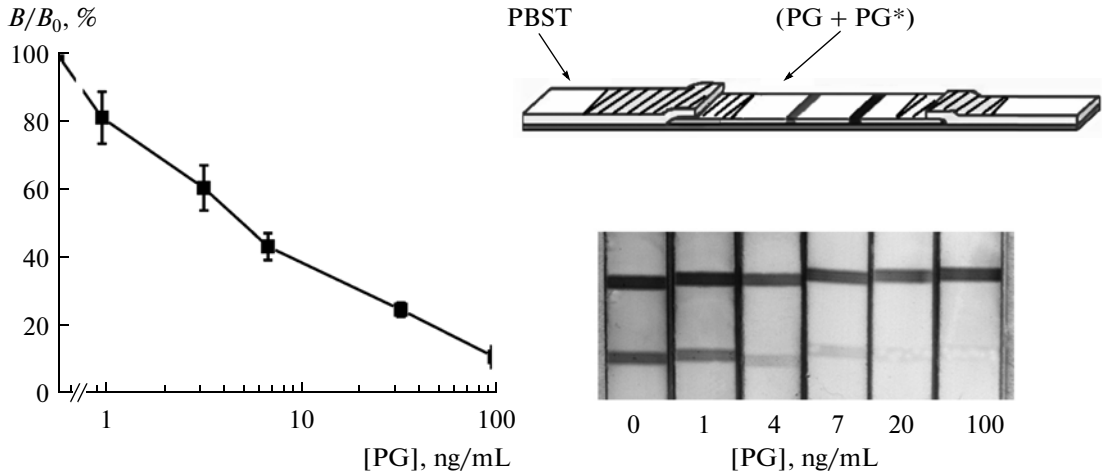

Fig. 7. Scheme of analysis, calibration curve and result of signal visualization for detecting PG using LFIA employed enzymatic labeling. Area of lamination is patterned.

strate reaction should be carried out to obtain a particular result, the test strip was only partially laminated and an area was left uncovered for loading the substrate. LFIA was performed using test strips laminated as shown in Fig. 6 (options 2 and 3) and the control test strip with no lamination (Fig. 6, option 1) according the above-described optimal scheme, and the degree of inhibition of the signal by free PG was compared at the concentrations of choice. It should be mentioned that lamination according to options 1 and 2 resulted in rather high background staining. Under these conditions, the relative value of the signal intensity corresponding to a PG concentration of 7 ng/μL was significantly higher than that of the test strip laminated according to option 3. This difference is related to the existence of a firmer contact between the membrane for sample loading and the analytical membrane, which contributes to the formation of a more uniform flow of the solution on the border between two membranes. The lamination of this zone most likely reduces the absorption of reagents loaded onto an analytical membrane by a membrane for sample loading. Thus, the lamination of a test strip using option 3 was considered to be the optimal solution for performing LFIA.

Selection of signal-detection system. LFIA with enzyme as a label is further complicated by the need to perform an additional final step, i.e., staining the test strip following the enzyme-substrate reaction. In this work, we used a substrate solution based on TMB with the addition of dextran sulfate, which reacts with the oxidized form of a product of the enzyme-substrate reaction and forms an insoluble compound on the surface of the analytical zone of the membrane. To optimize the staining step of the assay, two different options were tested, first, staining of the analytical membrane separated from other parts of a test strip was performed by immersing the membrane into a tube containing substrate solution and, second, an aliquot of the substrate solution was applied directly onto a test strip (analytical membrane). To minimize the amount of operations carried out with the test strip, staining was performed by loading an aliquot of the substrate solution in the analytical zone of a membrane. The optimized volume of the substrate solution was 50 μL. In this case, no blurring of the test or control lines was observed and the visual detection was possible shortly after 5 min of staining.

In summary, the performed investigation prompted us to choose the following LFIA format as the optimal method. A test strip was first laminated as shown in Fig. 7, after which a mixture of the CMO-PG-HRP conjugate and a standard PG solution was loaded onto an analytical membrane. The membrane was then left until the solution was fully absorbed and a buffer solution was further applied to the membrane for sample loading. The duration of this stage was 10 min. Fol-

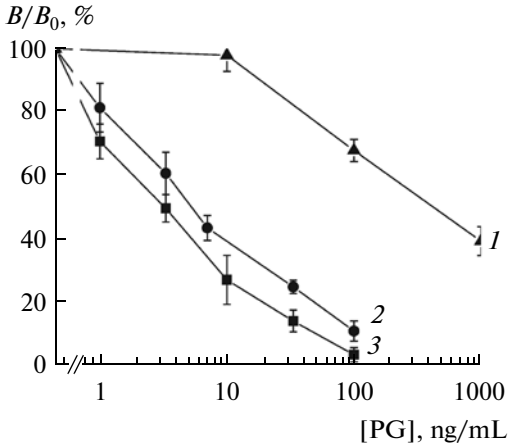

Fig. 8. Calibration dependences for PG detection using various methods as follows: *1*—LFIA employed colloidal gold as a label; *2*—LFIA using horseradish peroxidase as a label; *3*—ELISA.

Comparison of various methods used for PG detection

Analysis method	Linear concentration range, ng/mL	*IC_{50}, ng/mL	Analysis duration
LFIA (label – colloidal gold)	>30	110	10–15 min
ELISA	0.5–20	1	1.5 h
LFIA (label – horseradish peroxidase)	2–40	6	15–20 min

Note: IC_{50} is the concentration of PG, at which the signal is inhibited by 50%.

lowing this, 50 μL substrate solution was loaded in the analytical zone of the membrane and the obtained results were visually detected after 5 min of incubation. Quantitative estimate of the analysis results was carried out 30 min after LFIA was finished. The linear concentration range of the assay was determined as 2–40 ng/mL of PG, which is in the range of interest for the early diagnosis of pregnancy in cows; the limit of detection was found to be 2 ng/mL (Fig. 8, table). It should be mentioned that the visual detection of the results of LFIA benefits from the fact that band staining obtained for solutions with a threshold PG concentrations of 7 ng/mL or higher clearly differs from the intensity of staining obtained for solutions containing PG in concentrations lower than 7 ng/mL. The linear concentration range for ELISA was almost identical to that of LFIA (Fig. 8, table). However, LFIA is more advantageous in terms of the duration of assay (see table).

The method developed in this work can be used to detect PG under field conditions within the required concentration range, e.g., directly at farms, which is extremely important for the fast testing of pregnancy in cows. However, to perform rapid field diagnosis of pregnancy in cows, the detection of PG should be performed in whole milk with no additional preparation of the samples prior to testing. Milk has a complex composition and this composition, including fat content, may vary significantly in different animals [19]. The variability of investigated samples may substantially affect how analysis is carried out; e.g., the heterogeneity of the composition of a sample can prevent the flow of the sample across membranes and, therefore, complicate the analysis [20]. A number of experiments conducted by us using samples of whole, untreated milk proved that an additional optimization of LFIA conditions is required to detect PG in milk for the early diagnosis of pregnancy in cows.

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