Talanta 132 (2015) 685–689

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Pretreatment-free lateral flow enzyme immunoassay for progesterone detection in whole cows' milk

J.V. Samsonova [a,*], V.A. Safronova [a], A.P. Osipov [b]

[a] Department of Chemical Enzymology, Chemistry Faculty, M.V. Lomonosov Moscow State University, 1-11 Leninskie Gory, Moscow, 119991 Russia
[b] National University of Science and Technology "MISiS", 4 Leninsky Ave., Moscow, 119049 Russia

ARTICLE INFO

Article history:
Received 19 August 2014
Received in revised form
12 October 2014
Accepted 18 October 2014
Available online 28 October 2014

Keywords:
Progesterone
Lateral flow enzyme immunoassay
Horseradish peroxidase
Whole cows' milk

ABSTRACT

New rapid method of lateral flow enzyme immunoassay (LFEIA) for progesterone detection in whole cows' milk was developed. The test system utilized horseradish peroxidase as a label along with the substrate solution containing 3,3',5,5'-tetramethylbenzidine and dextran sulfate to obtain an insoluble blue colored product of the enzyme reaction on a surface of analytical membrane (test and control lines). Several aspects of LFEIA were optimized: time of the signal detection, membrane materials and assay conditions. Resulting competitive LFEIA can be performed within 15 minutes with the limit of progesterone detection of 0.8 ng/ml. Progesterone concentration in whole milk samples was determined by LFEIA and enzyme-linked immunosorbent assay (ELISA). The results obtained were in good correlation (R=0.97, n=46). Thus new sensitive LFEIA can be successfully used for on-site monitoring of oestrus status of cows' reproductive system and for early none-pregnancy detection. The method is fast, easy to perform and needs no preliminary sample preparation.

1. Introduction

Nowadays lateral flow immunoassay (LFIA) is a convenient method for semi-quantitative and quantitative detection of important biologically active substances performed on-site, point-of-care places or for personal testing [1]. The approach is widespread in medical and veterinary diagnostics, food control and many other areas due to short time of analysis (10–15 min) and easy procedure. LFIA test strips contain all necessary reagents in dry form; so to perform a test it is sufficient to add a liquid sample (extract) which migrates along overlying membranes and results in the formation of specific immunocomplexes in different areas of a test strip. The result of analysis is usually presented in a form of colored lines in the test and control zones of analytical membrane. A range of labels is used in LFIA including colloidal gold, latex particles, enzymes, quantum dots etc. [1–3]. It was reported that enzyme labels provided up to 10–30 times higher assay sensitivity compared to conventional LFIA labels such as colloidal gold [3–6]. Enzyme immunochromatography as a form of quantitative immunoassay was pioneered by Zuk et al. in 1985 [7]. The authors used paper test strip totally covered by specific antibodies and the assay was based on the measurement of the height of colored bars formed on strips as a result of immunoreaction with the following enzyme substrate saturation. Later enzyme immunoassay was realized in conventional LFIA format with specific reagents immobilized in a form of narrow bands [8]. In enzyme LFIA the most often used label is horse radish peroxidase (HRP) [3–6], however alkaline phosphatase [8,9] and cholinesterase [10] were also employed. As far as HRP is concerned, colorimetric [3–6], chemiluminescent [11,12] and electrochemical [13] detection modes were reported. Substrate solution contained 3,3',5,5'-tetramethylbenzidine (TMB) was predominantly employed for colorimetric detection in HRP-based LFIA [4,6], however 4-chloro-1-naphthol was also used in few studies [5,7].

In veterinary practice one of important biomarkers which should be checked on-site is sex hormone progesterone (P4). Thus P4 concentration in cows' milk is used to monitor cyclicity and confirm oestrus behavior as well as to perform non-pregnancy diagnosis on day 19–21 after artificial insemination [14,15]. Milk sampling is a convenient way of sample collection because probes are readily available. Moreover P4 concentration in milk is few times higher than in blood and they are closely correlated [16,17]. On day 19–21 (end of the oestrus cycle) P4 level in milk of non-pregnant cow is low (around 1–2 ng/ml) whereas for pregnant cow it is high (> 7 ng/ml) and remains high throughout gestation. Milk P4 test is the earliest proven method of identification of non-pregnant cows with high sensitivity [15].

* Corresponding author. Tel.: +7 495 9393407; fax: +7 495 939 2942.
E-mail addresses: jvs@enz.chem.msu.ru, jvsamsonova@gmail.com (J.V. Samsonova), capreace@inbox.ru (V.A. Safronova), aposipov@mail.ru (A.P. Osipov).

http://dx.doi.org/10.1016/j.talanta.2014.10.043
0039-9140/

Despite the fact that a variety of LFIA–based test systems is in use for a range of applications worldwide the practically important lateral flow immunoassay to measure the level of cows' milk P4 within the required concentration range hasn't been developed yet. Just a few attempts were taken to develop rapid LFIA for P4 [18–21]. The first paper established LFIA for a hapten where P4 was used as a model antigen was published in 1996 [18]. Finnish researchers showed that P4 can be detected with the plain LFIA test strip prepared of nitrocellulose membrane. A sample (milk) was first added and soaked into the membrane followed by the colloidal gold-labeled P4-protein conjugate. The detection limit was from 5 ng/ml (visual detection) to 2 ng/ml (photometric analysis). Later the influence of some LFIA parameters on the performance characteristics was investigated but sensitivity and variability of the method remained a problem [19]. Another LFIA system with an enzyme label was just able to detect mean differences between 5 and 50 ng/ml of P4 but due to large variability the limit of detection could not be determined [20]. A colloidal carbon was also used as a label in another modification of LFIA for P4 [21]. To increase the sensitivity of the assay high coating concentration of a P4-protein conjugate in combination with low concentration of specific antibody was used. As a result an IC_{50} equal to 0.6 μg/l P4 was achieved. However the assay was developed in buffer and did not work in whole cows' milk.

In our previous work the main approaches to a new P4 test based of lateral flow principle and utilized an enzyme as a label were established [22]. The objective of the present study was to develop quick and sensitive lateral flow enzyme immunoassay (LFEIA) for P4 determination in whole cows' milk without preliminary sample preparation.

2. Material and methods

Dextran sulfate MW 8000 (w/v) and P4 were purchased from «Sigma» (USA). Inorganic salts, acids and organic solvents were obtained from «Chimmed» (Russia). Tween 20 was from «MP Biomedicals» (France). Ready-to-use substrate solution containing TMB and H_2O_2 was supplied by "Immunoved" (Russia).

The following buffers were used: 0.01 M K-phosphate (K_2HPO_4-KH_2PO_4), 0.15 M NaCl, pH 7.4 (PBS) and 0.01 M K-phosphate (K_2HPO_4-KH_2PO_4), 0.15 M NaCl, 0.05% Tween 20, pH 7.4 (PBST).

P4 standard solutions were prepared by dilution of the stock solution (1 mg/ml in ethanol) with PBST.

Polyclonal rabbit antiserum obtained against 11α-hydroxy-progesterone hemisuccinate and the synthesis of a conjugate of 3-O-carboxymethyl oxime progesterone (3-CMO-P4) with HRP were described elsewhere [22].

The following membrane materials were used to prepare LFEIA test strips: analytical nitrocellulose membranes CNPC (15 μm), 150CNPH, CNPF (10, 8 and 5 μm) (MDI, India); absorbent pad AP045 (MDI, India) and sample pads MAPDS-0300 (Arista Biologicals, USA), FR1 (0,35; 0,6), R4, R7 and WF1 (MDI, India).

Whole cows' milk samples were kindly provided by SE «Ermolino» (Kaluga region, Russia) and stored at 4 °C.

2.1. Preparation of LFEIA test strips

Test strips (75*4 mm) were assembled according to the scheme shown in Fig. 1. Sample pad and absorbent pad were 27 mm long and overlain the analytical membrane by 2 mm. The specific antibody solution in PBS as test capture reagent was dispensed on the analytical membrane using BioJet Quanti 3000 and BioDot XYZ- 3050 dispensing platform (USA). To form a control zone, the antibodies against HRP were dispensed onto the strip at a distance of 5 mm from the test zone. The following parameters of the pump were applied: droplet size – 30 nl, step – 0.3 mm, speed – 50 mm/s. The strips were dried for 24 hours at 37 °C.

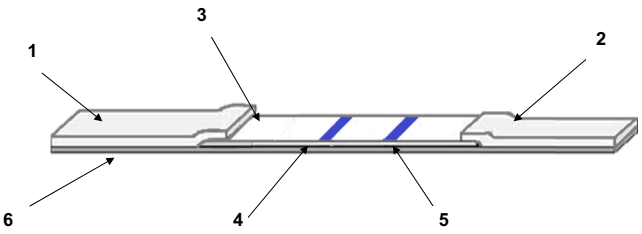

Fig. 1. Construction of LFEIA test strip. 1–sample pad; 2–absorbent pad; 3–analytical membrane; 4–test line; 5–control line; 6–plastic base.

2.2. Sample preparation

Whole milk samples were incubated for 30 min at 37 °C and vortexed before use.

2.3. ELISA procedure

"ELISA-progesterone-milk" kit (Immunoved, Russia) was used to perform P4 measurement in whole cows' milk samples. Briefly, aliquots of 10 μl P4 standard solutions/samples were added to wells of a mictotiter plate followed by 100 μl enzyme tracer (P4-HRP). After incubation and plate washing the substrate solution (100 μl) was added to each well. The color reaction was stopped after 10–15 min with 100 μl stop solution. Result of the reaction was evaluated on spectrophotometer at 450–620 nm ("Molecular Devices", USA). The values of optical densities were converted to $\%B/B_0$ values according to the formula: $B/B_0=(I/I_0) * 100\%$, where I is a value of optical density for a sample, I_0 is the value of optical density for 'zero' standard.

2.4. LFEIA procedure

A test strip was placed on a horizontal surface. The mixture of 10 μl 3-CMO-P4-HRP and 120 μl standard solution or sample was added onto the sample pad, and the solution migrated toward the absorbent pad. In 10 min the test strips were placed into tubes containing 2 ml of substrate solution supplied with 10% dextran sulfate and incubated for 5 minutes while stirring. After staining test strips were dried for 15 minutes at room temperature. Dried strips were scanned (Epson Perfection V700 Photo, Seiko-Epson, Japan) with a resolution of 600 dpi in 24-bit color (RGB). Analysis of the digital images and quantification of color intensity of test and control lines was performed using Scion Image software. Line intensity was corrected by the background subtraction and converted to $\%B/B_0$ mode as described earlier.

3. Results and discussion

In present work HRP with colorimetric detection of an analytical signal was used. As it was shown in our previous article colloidal gold label in LFIA of P4 did not provide required assay sensitivity [22]. So the approach called LFEIA (lateral flow enzyme immunoassay) consisting of the combination of the sensitivity of enzyme immunoassay with the rapidity of lateral flow assay was proposed. Enzyme-based lateral flow immunoassay with visual generation of results in test and control zones requires an additional procedure – coloring of a test strip by substrate solution. In our study to visualize the result of immunoreaction in LFEIA HRP was used in combination with the substrate solution contained

TMB and dextran sulfate. It was found earlier that dextran sulfate dramatically lowers the solubility of the TMB oxidation product which concentrated in the membrane zone where an enzyme conjugate bounded and that allows to develop highly sensitive technique for the detection of HRP [3,23]. TMB coloring can face a problem of background noise and line diffusion [6]. It was reported that after a completion of immunoreaction by vertical flow the best way to pass TMB substrate solution over the test strip in terms of uniformity of signal color and the background noise is the horizontal direction of substrate solution flow [4,6]. For this approach special substrate pads were applied to provide flow across analytical membrane. In our study P4 conjugated to HRP was mixed with a sample (standard solution) and the resulting mixture was applied onto a sample pad and migrated along the test strip (Fig. 1). To perform staining the test strip was immersed in a tube containing a HRP coloring precipitating substrate solution (TMB). This simple approach provided uniform coloring pattern and reduced diffusion of test and control lines. In this case the visual detection can be carried out in 5 minutes.

To develop accurate LFIA for P4 with HRP as a label the color intensity of the precipitating product of TMB oxidation should be proportional to the amount of the enzyme within immunocomplexes concentrated in the test line of analytical membrane, moreover the measured signal (end-point measurement) should be stable during time. Considering these conditions the dependence of the color intensity formed on the surface of analytical membrane during substrate reaction at different concentrations of HRP was studied. The experiment was conducted as follows: HRP solution was spotted on a surface of analytical membrane; after drying a piece of membrane was incubated in TMB solution for 5 minutes then the membrane was taken from substrate solution and quantification of colorimetric analytical signal was carried out by a scanner within 60 min (Fig. 2). The results have shown that the analytical signal (color intensity) remains stable for at least an hour after staining (Fig. 2A). The signal was also proportional to the concentration of HRP absorbed on the membrane in a form of saturation curve (Fig. 2B). Some reduction of signal intensity during initial 15–20 min after staining could be attributed to a membrane drying and to the fact that the background signal increased within the first 10–15 minutes (data not shown). Therefore, visual detection of LFEIA results can be performed immediately after staining and further instrumental detection – in 15 minutes after staining.

In current work direct competitive format of LFEIA was employed where specific antibodies were immobilized on a surface of analytical membrane. Sensitivity of the competitive immunoassay strongly depends on the label concentration, sensitivity of signal registration and antibodies affinity. The next step of the work was the selection of optimal membrane components and reagents concentration for sensitive competitive LFEIA. Five types of commercial analytical membranes with different pore size (15, 10, 8, 5 μm) and capability to bind proteins (CNPF – lower, CNPC – higher and CNPH – the highest protein binding) were considered. A range of lateral flow assay characteristics (assay time, sensitivity etc.) depends on the pore size of analytical membrane used. For example, while the pore size increase the sensitivity of the assay decrease, however the analysis time significantly reduces. Also the visual form of the stained line on the analytical membrane looks different. As shown in Fig. 3 for membranes with larger pore size the test line became less clear and diffused that can complicate visual detection of the results. It was also found that the membrane with a smaller pore size required 3 times less amount of P4 labeled with HRP to produce a line color of the same intensity, thus LFEIA sensitivity can be increased and background signal reduced. On the basis of the above characteristics a membrane with pore size of 8–10 μm was considered as optimal.

To develop lateral flow immunoassay in whole cows' milk a few aspects should be addressed – assay should detect P4 in the required concentration range (1–10 ng/ml) and can be applicable to such complicated matrix as undiluted whole milk. Few sample pads were checked in a combination with analytical membranes to fabricate a test strip. The LFEIA sensitivity (Fig. 4A) and the ability of whole cows' milk to migrate along a test strip were considered (Fig. 4B). As shown on Fig. 4, assay sensitivity was affected by a type of a sample pad used. For example, percent of signal inhibition at P4 concentration equal to 10 ng/ml varied from 80% to 20%. This could be attributed to different components which are used by membrane material producers for the pretreatment of sample pads and quite often are unknown. Among them are detergents, wicking agents etc. The zone of a test strip reached by the front of a whole milk sample also depended on a particular combination of membranes (Fig. 4B). Our aim was to find such combination which allows milk sample to flow up to absorbent

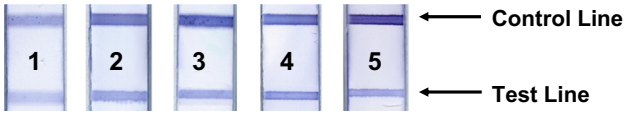

Fig. 3. Appearance of test and control lines on different type of analytical membrane in LFEIA: 1–CNPC 15 μm, 2–150CNPH, 3–CNPF 10 μm, 4–CNPF 8 μm, 5–CNPF 5 μm.

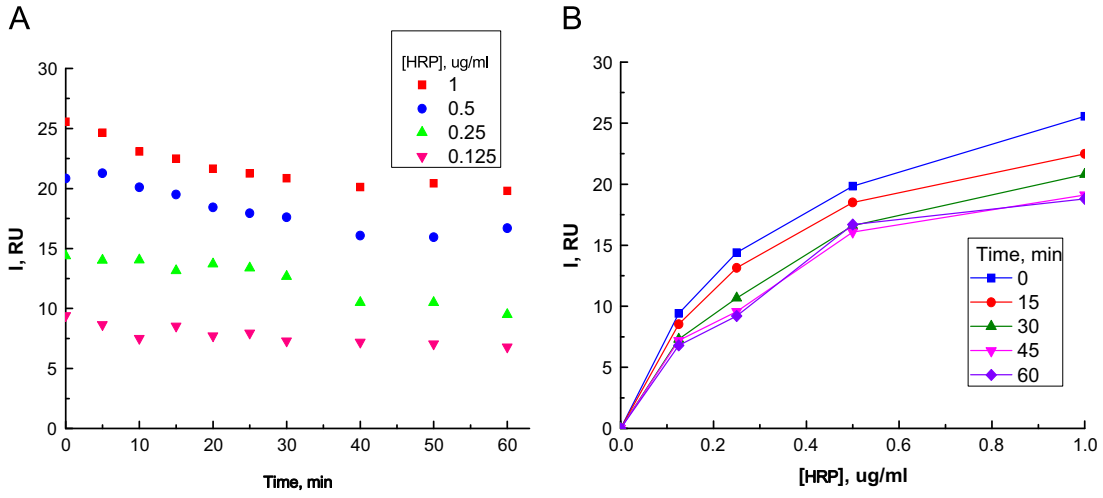

Fig. 2. (A) The dependence of the signal intensity on measuring time and (B) the enzyme concentration (HRP) on the membrane surface after 5 min staining in TMB solution. I, RU – color intensity of the zone with immobilized HRP, relative units.

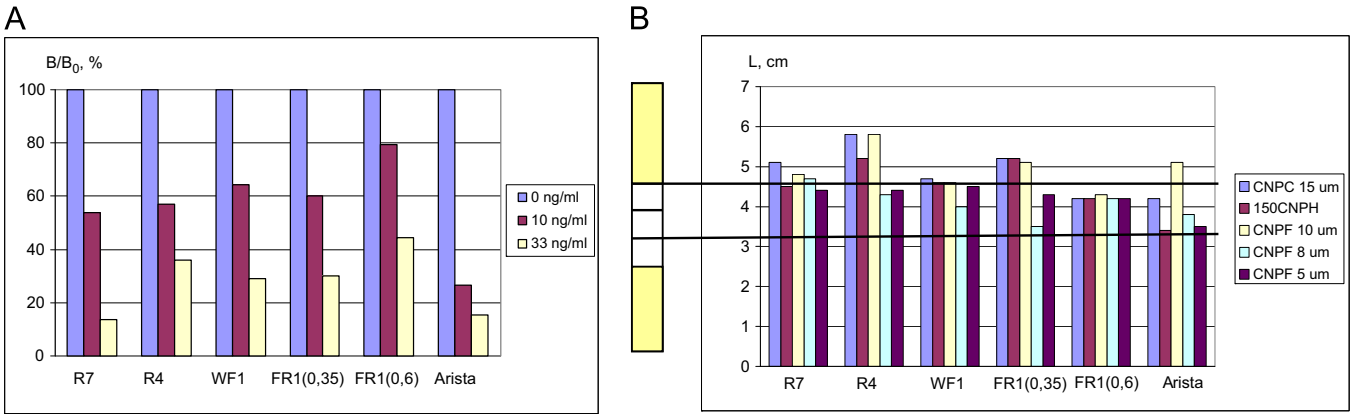

Fig. 4. (A) Analytical signal inhibition at different P4 concentrations (analytical membrane CNPF 10 μm) and (B) length of milk front migration on LFEIA strip (b) for a range of combinations of analytical membrane and sample pad (bottom row).

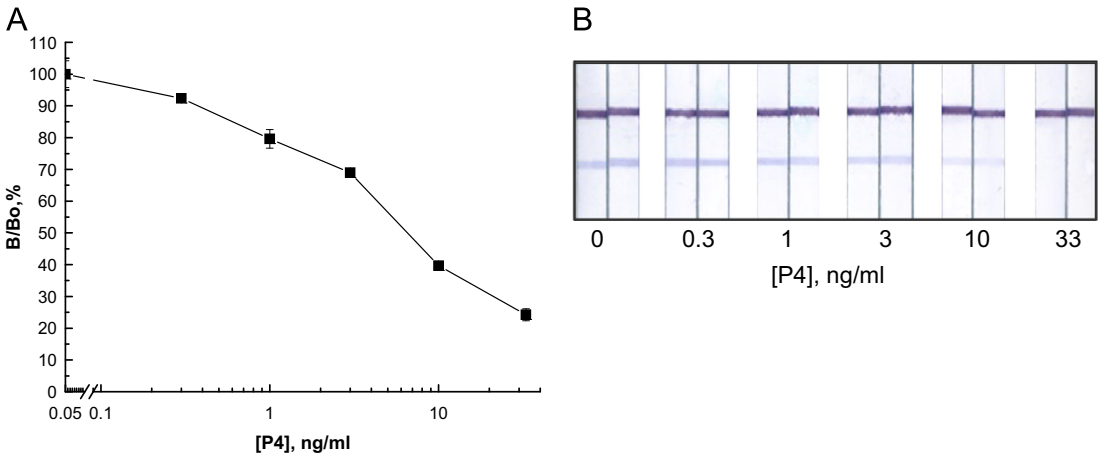

Fig. 5. LFEIA P4: calibration curve and its visualization after substrate staining.

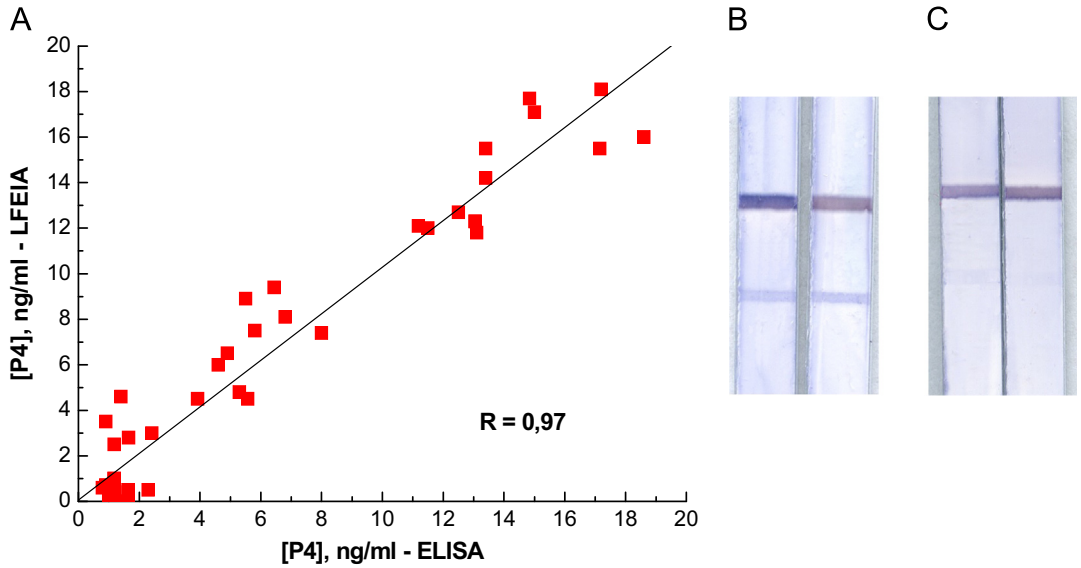

Fig. 6. (A) Correlation of the results of P4 detection in whole cows' milk obtained by ELISA and LFEIA (a) and example of signal visualization for (B) non-pregnant and (C) pregnant cow.

pad and at the same time provides necessary LFEIA sensitivity. On the base of above factors an optimal combination of the sample pad and analytical membrane was chosen for sensitive LFEIA of P4 in whole milk.

As a result of above study rapid LFEIA was designed and carried out as follows: aliquots of standard solution/sample and HRP-labeled P4 were mixed and applied onto the sample pad. When the solution was absorbed, the membrane was immersed in a test

tube with substrate solution and incubated for 5 minutes. Then the strip was taken out and visual result was registered. The instrumental detection was carried out in 15 min. An example of calibration curve and visual appearance of test and control lines are presented on Fig. 5. The detection limit of LFIA P4 was 0.8 ng/ml. Variation of results did not exceed 10%. It could be seen that concentration of P4 about 7–10 ng/ml and higher caused significant color change of a test line and its almost full disappearance.

To evaluate the applicability of this method for whole cows' milk, 46 samples from pregnant cows as well as from non-pregnant animals taken on different days of oestrus cycle were analyzed by the developed LFEIA and ELISA. The results were in good correlation ($R=0.97$) (Fig. 6). Moreover analysis of whole milk samples taken from pregnant cows caused almost complete disappearance of a test line whereas milk samples from non-pregnant cows taken at the beginning or end of oestrus cycle were identified as non-pregnant by visual and quantitative detection. Thus, this method can be used to quantify the level of P4 in whole cows' milk and can be applied for early determination of cows' non-pregnancy on a dairy farm without any special equipment. Quantitative detection of P4 with the help of an available portable device can give valuable information about status of a cow's reproductive system.

4. Conclusions

Here the applicability of LFEIA approach to P4 detection in particular concentration range in such complicated matrix as whole cows' milk was demonstrated. The pretreatment-free test can be used for rapid P4 detection while monitoring status of cows' reproductive system and for early non-pregnancy diagnosis on day 19–21 after artificial insemination. Development of rapid P4 milk test for on-farm use was a complicated task for years [14,21] and LFIA-based test was just recently commercialized. Using enzyme labels in LFIA is new and to some extent underestimated direction of research. "Enzyme immunochromatographic assay", "enzyme-based dry-reagent strip biosensor", "ELISA-on-a-chip", "enzyme-linked immuno-strip biosensor", "lateral flow immunosensor" – these are all names of the same assay principle which combines sensitivity of the enzyme immunoassay with advantages of lateral flow immunoassay (rapidity, low-cost and disposability) [4–6,9,13]. The approach can be considered as rapid ELISA performed in the form of lateral flow format or as an LFIA utilizing an enzyme label. This approach could be a potential substituent of a conventional ELISA as soon as it will be realized in rapid form with minimal manipulation required, particularly in cases when the sensitive on-site detection of a target analyte is needed. For instance, a delayed-release effect of an enzyme substrate by the asymmetric polysulfone membrane described recently by Joung et al. [12] provided one-step mode of chemiluminescent enzyme LFIA. Rapid and sensitive colorimetric, chemiluminescent or electrochemical detection of enzyme labels in visual or quantitative mode with the help of portable devices can be applied for a wide range of enzyme flow immunoassay applications in different areas.

Acknowledgments

The authors gratefully acknowledge dairy farm SE "Ermolino" (Kaluga region) for providing whole milk samples.

References

[1] G.A. Posthuma-Trumpie, J. Korf, A. van Amerongen, Anal. Bioanal. Chem. 393 (2009) 569–582. http://dx.doi.org/10.1007/s00216-008-2287-2.
[2] D.G. Bruno, Pathogens 3 (2014) 341–355. http://dx.doi.org/10.3390/pathogens3020341.
[3] C. Parolo, A. de la Escosura-Muniz, A. Merkoci, Biosens. Bioelec 40 (2013) 412–416. http://dx.doi.org/10.1016/j.bios.2012.06.049.
[4] J.H. Cho, E.H. Paek, I.H. Cho, S.H. Paek, Anal. Chem. 77 (2005) 4091–4097. http://dx.doi.org/10.1021/ac048270d.
[5] A.N. Kawde, X. Mao, H. Xu, Q. Zeng, Y. He, G. Liu, Am. J. Biomed. Sci. 2 (2010) 23–32. http://dx.doi.org/10.5099/aj100100023.
[6] C. Zhang, Y. Zhang, S. Wang, J. Agric. Food Chem. 54 (2006) 2502–2507. http://dx.doi.org/10.1021/jf0531407.
[7] R.F. Zuk, V.K. Ginsberg, T. Houts, J. Rabbie, H. Merrick, E.F. Ullman, M.M. Fischer, S.N. Sizto, D.J. Litman, Clin. Chem. 31 (1985) 1144–1150.
[8] H. Watanabe, A. Satake, M. Matsumoto, Y. Kido, A. Tsuji, K. Ito, M. Maeda, Analyst 123 (1998) 2573–2578.
[9] T. Ono, M. Kawamura, S. Arao, H. Nariuchi, J. Immunol. Methods. 272 (2003) 211–218.
[10] D. Du, J. Wang, L. Wang, D. Lu, Y. Lin, Anal. Chem. 84 (2012) 1380–1385. http://dx.doi.org/10.1021/ac202391w.
[11] I.H. Cho, E.H. Paek, Y.K. Kim, J.H. Kim, S.H. Paek, Anal. Chim. Acta. 632 (2009) 247–255. http://dx.doi.org/10.1016/j.aca.2008.11.019.
[12] H.A. Joung, Y.K. Oh, M.G. Kim, Biosens. Bioelec. 53 (2014) 330–335. http://dx.doi.org/10.1016/j.bios.2013.10.004.
[13] K. Inoue, P. Ferrante, Y. Hirano, T. Yasukawa, H. Shiku, T. Matsue, Talanta 73 (2007) 886–892. http://dx.doi.org/10.1016/j.talanta.2007.05.008.
[14] G.A. Posthuma-Trumpie, A. van Amerongen, J. Korf, W.J. van Berkel, Trend. Biotechnol 27 (2009) 652–660. http://dx.doi.org/10.1016/j.tibtech.2009.07.008.
[15] G. Purohit, Webmed Central Reproduction 1: WMC001305 (2010) http://www.webmedcentral.com/article_view/1305.
[16] J.A. Laing, R.B. Heap, Br. Vet. J. 127 (1971) 19–22.
[17] H. Dobson, S.E. Midmer, R.J. Fitzpatrick, Vet. Rec. 96 (1975) 222–223.
[18] M.P. Laitinen, M. Vuento, Acta Chem. Scand. 50 (1996) 141–145.
[19] M.P. Laitinen, M. Vuento, Biosens. Bioelec 11 (1996) 1207–1214.
[20] K. Sananikone, M.J. Delwiche, R.H. BonDurant, C.J. Munro, Trans. ASAE. 47 (2004) 1357–1365.
[21] G.A. Posthuma-Trumpie, J. Korf, A. van Amerongen, Anal. Bioanal. Chem. 392 (2008) 1215–1223. http://dx.doi.org/10.1007/s00216-008-2362-8.
[22] V.A. Safronova, J.V. Samsonova, V.G. Grigorenko, A.P. Osipov, Moscow Univ. Chem. Bull 67 (2012) 241–248. http://dx.doi.org/10.3103/S0027131412050045.
[23] J.L. McKimm-Breschkin, J. Immunol. Method. 135 (1990) 277–280.