Development of a Competitive Lateral Flow Immunoassay for Progesterone in Dairy Cows' Milk¹⁾

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Summary

The routine monitoring of progesterone, a hormone that plays important roles during pregnancy, has become increasingly important in dairy cows. In this study, 11 α -OH-progesterone-hemisuccinate-BSA was used to immunize Balb/c mice for the preparation of mouse anti-progesterone monoclonal antibodies (mAbs). A competitive lateral flow immunoassay for progesterone was developed with the use of purified mAbs. Standard progesterone solutions or milk samples containing different concentrations of progesterone were added to the sample pad of the test strip. A bright or weak test line represented progesterone levels < 3 ng/mL and no pregnancy; a very weak test line was indicative of 3-5 ng/mL progesterone and the need for further pregnancy diagnosis; no test line was indicative of pregnancy. The characteristics of the test strip make it ideal for early pregnancy diagnosis.

Keywords: progesterone, lateral flow immunoassay, pregnancy diagnosis, dairy cows

In the dairy industry, reproductive performance is of utmost importance. While milk yield has considerably improved, the reproductive performance of dairy cows has declined (3, 4). Currently, pregnancy in dairy cows is diagnosed by rectal palpation and ultrasound exams (11, 15). However, rectal palpation is usually performed 2 months after artificial insemination.

Progesterone, which is required for the resumption of estrous cycles and the establishment and maintenance of pregnancy (10, 16), can be used to assess pregnancy in dairy cows (7). Several researchers have reported higher blood and milk progesterone levels in pregnant dairy cows compared to their non-pregnant counterparts 20 days after artificial insemination (4). However, the current chemical methods available for detecting progesterone in biological samples have very poor sensitivity. Radioimmunoassay (RIA), which has high sensitivity, has been used for the determination of progesterone levels in milk samples (7). However, RIA requires expensive laboratory facilities and generates radioactive waste. Marcus and Hackett (13), who developed an enzyme-linked immunosorbent assay (ELISA) for serum or milk progesterone analyses, reported 92% accuracy in the assessment of pregnancy.

Comin et al. (3) developed an ELISA for whey progesterone, with a detection range of 0.05-2 ng/mL of whey progesterone. However, ELISA is time consuming and requires expensive equipment and skilled analysts. On the other hand, colloidal gold-based immunochromatographic assays are rapid, simple, and inexpensive (2, 14). In this study, a competitive lateral flow immunoassay for progesterone determination in dairy cow milk was developed and characterized.

Material and methods

Material and reagents. In this study, 11 α -OH-progesterone (11 α -OH-P₄), bovine serum albumin (BSA), dicyclohexylcarbodiimide (DCC), ortho phenylene diamine (OPD), succinic anhydride, N-succinamide (NHS), HAuCl₄·3H₂O, and sodium citrate were obtained from Sigma-Aldrich (Trading Co., Ltd., Shanghai, China). Ovalbumin (OVA), Dimethylformamide (DMF), anhydrous pyridine, and HRP-labeled goat anti-mouse IgG were purchased from Beijing Dingguo Biotechnology Development Center (Beijing, China). Nitrocellulose (NC) membranes were purchased from Whatman. Glass paper was obtained from Ahlstrom. PVC sheets, absorbent paper, and adhesive tape were purchased from Shanghai Jiening Bio (Shanghai, China). Other reagents were of analytical purity, and double distilled water was used for all experiments.

Preparation of immunoassay reagents. In this experiment, 11 α -OH-P₄-hemisuccinate-BSA (P₄-BSA) and

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11 α -OH-P₄-hemisuccinate-OVA (P₄-OVA) were synthesized according to the method by Laitinen and Vuento (8). Briefly, 100 mg of 11 α -OH-P₄ and 30 mg of succinic anhydride were dissolved in 5 mL anhydrous pyridine and transferred to a water bath for 90 min at 95°C. The mixture was dried to produce 11 α -OH-P₄-hemisuccinate. DMF (0.5 mL) with 30 mg of 11 α -OH-P₄-hemisuccinate and DMF (0.01 mL) with 8.94 mg NHS and 16.02 mg DCC were thoroughly mixed. Subsequently, 95.2 mg BSA in 7 mL PBS was added for the synthesis of P₄-BSA, and 95.2 mg OVA in 7 mL PBS was added for the synthesis of P₄-OVA. P₄-BSA was used for the preparation of monoclonal antibodies; P₄-OVA was used as an immunoassay reagent.

Preparation of monoclonal antibodies. Female Balb/c mice (6-8 weeks old; n = 3) were immunized by hypodermic injection with 50 μ g P₄-BSA emulsified in Freund's complete adjuvant, followed by 50 μ g P₄-BSA in Freund's incomplete adjuvant on days 14 and 28 (9). P₄-OVA was used as the solid-phase in indirect ELISA for the analysis of anti-progesterone activity in immunized mice serum. The immunized mice received a booster injection (50 μ g P₄-BSA) via the tail vein on day 42. Three days after the booster injection, mouse spleen cells were fused with myeloma cells to generate hybridomas (6). After 14 days, supernatants were collected and analyzed by indirect ELISA. Positive hybridomas were subcloned, cultured for 14 days, and analyzed by indirect ELISA. Subcloned hybridomas with positive indirect ELISA results were analyzed by competitive ELISA. The competitive ELISA was prepared with P₄-OVA and progesterone. To detect the subtype of the monoclonal antibody (mAbs) produced by the hybridomas, the hybridoma culture supernatants were analyzed using the Mouse Monoclonal Antibody Isotyping kit (ISO2-1KT, Sigma-Aldrich).

Purification and activity of monoclonal antibodies. To obtain purified mAbs, positive clones were expanded, hybridoma growth medium was collected, and mAbs was precipitated in 50% ammonium sulfate and purified by affinity chromatography using Protein G (GE Healthcare). Purified mAbs was initially diluted to 1 mg/mL and subsequently from 1/10⁴ to 1/1.28 $\times$ 10⁶ for indirect ELISA (9). Competitive ELISA was performed to assess the reactivity of mAbs. In this study, 100 μ g/mL oestradiol, 5-androstenedione, follotropin, corticosteroid, and corticosterone were analyzed by competitive ELISA.

Preparation of colloidal gold particles and colloidal gold probe. Colloidal gold with a particle diameter of 20 nm was prepared according to the method by Yang et al. (17). Briefly, 1.6 mL of 1% (m/v) sodium citrate was added to 100 mL of boiling water. When the mixture had boiled, 1 mL of 1% (m/v) HAuCl₄·3H₂O was added. After the color of the solution changed to wine-red (after approximately 2 min), the solution was boiled for another 10 min and allowed to cool. The resulting gold colloid was supplemented with 0.02% (m/v) sodium azide and stored at 4°C. Particle diameter was determined by transmission electron microscopy (TEM, H-7650). The optimum pH value and mAbs concentration required for the preparation of a colloidal gold probe were determined. Briefly, 20 μ g mouse anti-progesterone monoclonal antibody was mixed with 1.0 mL colloidal gold

solution at different pH values (adjusted by 0.02 M K₂CO₃) and mixed with 100 μ L of 10% NaCl. Following 60 min of constant mixing, the optimum pH value that prevented aggregation (detected at 450 nm in a microtiter plate reader) was determined. The optimum concentration of mAbs was similarly determined with 10% NaCl.

The colloidal gold solution was adjusted to pH 8.6 using 0.02 M K₂CO₃. Under constant and gentle stirring, 0.16 mg mAb was added dropwise to 10 mL of colloidal gold solution. The mixture was gently mixed for 10 min and blocked with 1.1 mL of 10% (m/v) PEG 20000. After 15 min of constant stirring, the mixture was centrifuged at 10,000 g for 30 min. The gold pellets were suspended in 1 mL dilution buffer [20 mM Tris/HCl buffer (pH 8.8) containing 1% (w/v) BSA, 3% (w/v) sucrose, and 0.02% sodium azide] (17), and stored at 4°C.

Immunochromatographic test strips for progesterone. A lateral flow immunoassay was developed to detect progesterone. The preparation and assembly of the lateral flow immunoassay test strips is shown in Fig. 1. BIODOT was used to dispense two lines on the NC membrane. P₄-OVA (0.2 mg/mL) was dispensed on the bottom section as the test line (1 μ L per 1 cm line), whereas goat anti-mouse IgG (0.5 mg/mL) was dispensed on the upper section as the control line (1 μ L per 1 cm line). The distance between the two lines was 5 mm. The prepared NC membrane was dried at room temperature for 2 h and stored under dry conditions at 4°C. The conjugate pad, which was made of glass fiber, was treated with 0.1% Tween-20 for 10 min, dried at 60°C, and saturated with the colloidal gold probe. The sample pad was saturated with 50 mmol/L phosphate-buffered saline (PBS) (pH 7.2) containing 0.3% Tween-20 and 0.5% (w/v) PVP, and dried at 37°C (15). The NC membrane, gold conjugate pad, sample pad, and absorbent pad were laminated and pasted onto the PVC plate. All strips were sealed in a plastic bag and stored at 4°C.

Each standard solution (100 μ L) containing different concentrations of progesterone was transferred to the sample pad of the test strip, and the results were read after 10 min. High progesterone levels in the sample form a complex with the colloidal gold probe in the conjugate pad and with the goat anti-mouse antibody (control line), which results in the

Fig. 1. Schematic representation of the competitive lateral flow immunoassay for progesterone detection. A: Results obtained when no progesterone or very low levels of progesterone were present. B: Results obtained when high levels of progesterone were present.

formation of red bands on the control line (Fig. 1B). On the other hand, low progesterone levels in the sample lead to the formation of two red bands on the test and control lines (Fig. 1A). Therefore, both the test and control lines are visible in samples with no progesterone or low progesterone levels. However, only the control line is visible in samples with high progesterone levels. The absence of lines would be indicative of an invalid test.

Analysis of milk samples. Milk samples from five pregnant and five non-pregnant dairy cows (examined by rectal palpation) were collected for test strip analysis. The concentration of progesterone in the milk samples was determined by a commercial ELISA (Nanjing Jiancheng Bio, China).

Results and discussion

Preparation of monoclonal antibodies. In this study, 11α -OH- P_4 was used to prepare immunoassay reagents. P_4 -BSA conjugates were used to inoculate female Balb/c mice. An indirect ELISA, prepared with P_4 -OVA, was used for the analysis of serum anti-progesterone activity. Immunized mice serum had positive results in the indirect ELISA at a dilution of $\frac{1}{2} \times 10^5$. Three days after the booster injection, splenocytes from the immunized mice were fused with SP2/0 murine myeloma cells to generate hybridomas. After 14 days, the undiluted hybridoma culture supernatants were analyzed by indirect ELISA. Hybridomas showing ELISA reactivity against P_4 -OVA were subcloned. After 14 days, the undiluted culture supernatants of subcloned cells were analyzed by indirect ELISA. Hybridomas, which had positive indirect ELISA results, were analyzed by competitive ELISA. Since culture supernatants of fused hybridomas may provide false positive indirect ELISA results, an indirect ELISA was performed for fused and subcloned hybridomas. Even though antibodies secreted from hybridomas in response to the P_4 -BSA conjugates did bind to solid-phase P_4 -OVA in indirect ELISA, only the progesterone-specific antibody bound to solid-phase P_4 -OVA was inhibited by free progesterone in competitive ELISA. The positive clones, determined by indirect and competitive ELISA, were analyzed by the Mouse Monoclonal Antibody Isotyping kit. One positive clone produced IgG1 mAbs.

The ammonium sulfate method and affinity chromatography G were used to prepare a high-purity monoclonal antibody. According to indirect ELISA results, the purified monoclonal antibody had activity at $1/4.0 \times 10^4$ dilution, and the 50% inhibiting concentration of the monoclonal antibody was 0.45 μ g/mL. The cross-reactivity analysis of mAbs did not show any cross-reactivity with oestradiol, 5-androstenedione, follotropin, corticosteroid, or corticosterone. This information was important for successful development of a lateral flow immunoassay for progesterone.

Characterization of colloidal gold particles and colloidal gold probe. TEM micrographs revealed that the diameter of the colloidal gold particles was

Fig. 2. Transmission electron microscopy (TEM) micrograph of colloidal gold and colloidal gold probe. A: Naked colloidal gold. B: Colloidal gold-PcAb conjugate

approximately 20 nm (Fig. 2A). The optimum pH and mAbs concentration for the preparation of the colloidal gold probe were determined with 10% NaCl. The optimum pH value that prevented aggregation was 8.6. At pH 8.6, the optimum concentration of mouse anti-progesterone mAbs coated on colloidal gold was 16 μ g/mL. The TEM micrographs of the colloidal gold probe are shown in Fig. 2B.

Lateral flow immunoassay for progesterone. For rapid pregnancy diagnosis in dairy cows, a competitive lateral flow immunoassay for progesterone was developed. The optimum P_4 -OVA coating concentration in the test line and the volume of the colloidal gold probe for saturating the conjugate pad were determined by the analysis of samples containing 3, 5, or 7 ng/mL progesterone. In this study, 0.2 mg/mL P_4 -OVA and 0.5 mg/mL goat anti-mouse IgG were used for coating the test and control lines, respectively. The conjugate pad was saturated with 10 μ L of the colloidal gold probe. As shown in Fig. 3, when 100 μ L of 1 ng/mL progesterone standard solution was used, two red

Fig. 3. Detection characterization of the test strip. Progesterone standard solutions at concentrations of 0, 1, 3, 5, 7, 9, and 11 ng/mL were detected.

bands were obtained at the test and control lines. When 100 μ L of 3 ng/mL progesterone was used, a weak test line and a red control line were visible. When 100 μ L of 5 ng/mL progesterone was used, there was a very weak test line and a red band on the control line. When 100 μ L of 7 ng/mL progesterone was used, only the control line was visible. Researchers have reported that 20 days after artificial insemination, higher progesterone concentrations are present in blood and milk samples from pregnant dairy cows than in those from their non-pregnant counterparts (1, 13). At this time point, milk progesterone is usually > 10 ng/mL in pregnant dairy cows and < 3 ng/mL in non-pregnant dairy cows (5, 12). Barna et al. (1), who analyzed milk progesterone levels 19-22 days after artificial insemination, reported that milk progesterone values > 4 ng/mL had a 75% accuracy in pregnancy assessment of dairy cows, whereas Marcus and Hackett (13) reported a 92% accuracy in pregnancy assessment when milk progesterone values > 5 ng/mL were used. Given the progesterone profile following artificial insemination, a competitive lateral flow immunoassay for progesterone was developed in this study. A bright or weak test line represents milk progesterone values < 3 ng/mL and therefore the absence of pregnancy; a very weak test line is indicative of progesterone values between 3 and 5 ng/mL, which warrants further diagnosis. An invisible test line suggests that the animal is pregnant.

Analysis of milk samples. Ten milk samples were analyzed by both ELISA and the competitive lateral flow immunoassay. The results revealed that when the milk progesterone value was > 7 ng/mL, only the control line was visible (Tab. 1).

In this study, a competitive lateral flow immunoassay for progesterone was developed. When 100 μ L of 1, 3, 5, or 7 ng/mL progesterone standard solution was used, the test line of the lateral flow immunoassay

was bright, weak, very weak, or invisible, respectively. The test strip represents the optimal semi-quantitative progesterone detection method for early pregnancy diagnosis. The test strip had good sensitivity for the detection of milk progesterone.

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Tab. 1. Competitive lateral flow immunoassay for analysis of progesterone in milk samples

Milk Sample	Number	ELISA (ng/mL)	Lateral flow immunoassay
Non-pregnant cow	1	1.4	—
	2	3.6	±
	3	1.2	—
	4	2.7	—
	5	2.4	—
Pregnant cow	1	15.8	+
	2	16.5	+
	3	12.3	+
	4	7.6	+
	5	9.2	+

Explanations: +: positive, progesterone concentration > 7 ng/mL; ±: doubt, very weak test line, progesterone concentration of 3-5 ng/mL; -: negative, progesterone concentration < 3 ng/mL; —: negative, a bright test line