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Heat identification by 17β-estradiol and progesterone quantification in individual raw milk samples by enzyme immunoassay

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Abstract

Background: There is a substantial decline in first-service-pregnancy-rate in dairy cows. In this regard, future prospects are to measure milk hormones on-farm and progesterone levels in milk are not enough to precise ovulation unless connected to other data. The objectives of this study were to investigate whether 17β-estradiol could be measured from individual cow milk samples using a commercially available non-radiolabelled enzyme immunoassay kit (EIA) with no previously reported milk application, and whether those detections could precisely illustrate 17β-estradiol pre-ovulation peak in spite of its limited concentration and short manifestation in milk. **Results:** Milk sample treatments for progesterone and 17β-estradiol EIA measurements are described. Hormonal profiles from daily milk samples of six different cows were reported and 17β-estradiol pre-ovulation peak was visualized in all cases. Heat detection was possible by EIA using one every 2 days milking samples in almost all studied cases. Only in one case, morning and afternoon milking samples were required to visualize the 17β-estradiol pre-ovulation peak. **Conclusions:** 17β-estradiol EIA quantification in raw milk is a reliable, rapid, economic and a precise method to describe cow heat along with EIA progesterone determination.

Keywords: cattle, estrous, heat, hormone, insemination

INTRODUCTION

Dairy cow fertility is getting lower with increasing milk yields (Lucy, 2001). High milk production increases the number of silent heats making more difficult to correctly detect estrous and, consequently, to successfully artificial inseminate dairy cows (Harrison et al. 1990; Dobson et al. 2008).

In cattle, prior to ovulation, 17β-estradiol arises in blood mainly secreted by the developing follicle and progesterone concentrations drop in absence of gestation. The levels of both progesterone and 17β-estradiol in milk are strongly correlated with those found in blood (Meisterling and Dailey, 1987; Górecki et al. 2004). The determination of progesterone concentration alone is not sufficient to predict ovulation because there is a large variation in the timing of progesterone decrease relative to ovulation among animals (Roelofs et al. 2006). Alternatively, the determination of the 17β-estradiol peak precisely indicates pre-ovulation. In dairy cattle, insemination should take place within a 12 hrs window (between 6 and 18 hrs before ovulation) to achieve high fertilization rates.

There are many commercially available enzyme immunoassay (EIA) kits for progesterone measurements. However, the range of concentration of 17β-estradiol in milk is very low (pg mL^{-1}) and usually requires pre-concentration by organic solvent extraction from the original sample. Up to date, radio immunoassay (RIA) continues to be the most commonly reported technique for measuring 17β-

estradiol. Many studies describe estrogen quantification in skimmed milk but few in raw milk and whenever reported it has been conducted by RIA (Górecki et al. 2004; Pape-Zambito et al. 2007; Yamanaka et al. 2007). Although there are few commercially available EIA kits for 17b-estradiol detection in human samples, none of them have been yet described for use in raw milk samples. Here in, we report the use of two commercially available EIA kits for progesterone and 17β-estradiol to directly detect those hormones in raw milk. The obtained results allowed heat identification using daily individual milk samples in all studied cases.

MATERIALS AND METHODS

Sample pre-treatment experiments were conducted using raw tank milk from a dairy farm in Barcelona, Spain. For sexual cycle hormone determination, duplicate milk samples were collected morning and afternoon from six third-lactation Holstein cows from a dairy herd in Girona, Spain. Samples were manually taken during 29 days from the four quarters, discarding the first milk drops. Samples were kept at -20ºC until processing.

Milk weights and health status were daily recorded on farm. Visual observation of estrous behaviour was daily recorded identifying signs like restlessness and increase in physical activity, licking and sniffing of the genital area, and mounting activity.

Milk sample pre-treatments consisted on raw milk (RM), with additive (Add) and skimmed milk (SM), at 4ºC or -20ºC. 10 mL of tank RM samples were kept at 4ºC or -20ºC with or without additive (Add) (potassium dichromate, LacTabs Mark II, Thomson and Capper Ltd, England) (1 tablet per 10 mL of milk sample) during 18-20 hrs prior analysis. Defrosting was carried out at 4ºC. SM was obtained after centrifugation at 20,000 Xg, 20 min at 4ºC. Supernatant was carefully recovered aspirating it with a glass Pasteur pipette without taking any adherent fat. Each sample pre-treatment was processed by duplicate.

Progesterone was measured using the Progesterone EIA kit of Euro-Diagnostica B.V. (Arnhem, The Netherlands) following manufacturer's instructions. 17β-estradiol was measured with the Estradiol EIA kit of Cayman Chemical Company (Ann Arbor, MI, USA) using the provided protocol without modifications. Samples were analyzed by triplicate. Raw milk samples were defrosted at 4ºC and conscientiously homogenized using a 10 mL syringe and a 18 $GI_{1/2}$ needle prior analysis. Hormone concentrations were expressed as the arithmetical mean value and significance was determined using a T-test.

RESULTS AND DISCUSSION

Progesterone values from RM tank samples ranged within 2,14 ± 0,16 to 3,23 ± 0,12 ng mL^{-1} (Figure 1a). There were significant differences in progesterone content between fresh or frozen RM samples with (Add) and without additive (P = 0.03 RM at 4ºC and -20ºC and P = 0.05 Add at 4ºC and -20ºC). In both cases, frozen samples showed an increase in progesterone concentration of 11% and 14% (with and without additive, respectively) compared to fresh ones. There were also significant differences in progesterone concentration between SM samples in relation to RM, at 4ºC and -20ºC. In all cases progesterone values in SM were lower than in fresh RM (P = 0.01 RM and SM at 4ºC and P = 0.03 RM and SM at -20ºC). A second batch of analyses was carried out and the results confirmed these differences (data not shown). Additive itself did not affect progesterone determinations (P = 0.17 RM and Add at 4ºC and P = 0.46 at -20ºC).

17β-estradiollevels in milk tank ranged between 26,75 ± 8,63 to 52,91 ± 12,99 pg mL^{-1} (Figure 1b). There were no significant differences between fresh or frozen samples (P = 0.279). SM samples had lower levels of 17β-estradiolthan RM samples (P = 0.05 RM and SM at 4ºC, P = 0,005 RM and SM at -20ºC). There were also significant differences in 17β-estradiol levels between fresh RM samples (4ºC) with or without additive (P = 0,002).

Progesterone and 17β-estradioldiminished in SM compared to RM, at 4ºC and -20ºC. This might be due to the fact that both hormones are lipophilic molecules and subsequently their concentration is

greater at the fat fraction of milk (Pape-Zambito et al. 2007). Also, the addition of the potassium dichromate tablet as a preservation agent is not recommended for 17β-estradiol determination since detected levels drop significantly in its presence (Figure 1b). Milk thawing did not interfere with 17β-estradiol measures but significantly increased values of progesterone. However, this increase was similar and consistent in all tested samples. Sample freezing was required due to the amount of samples to analyze. Results indicated that both hormones could be detected and quantified directly from raw milk by respective EIA and no previous specific pre-treatments were required.

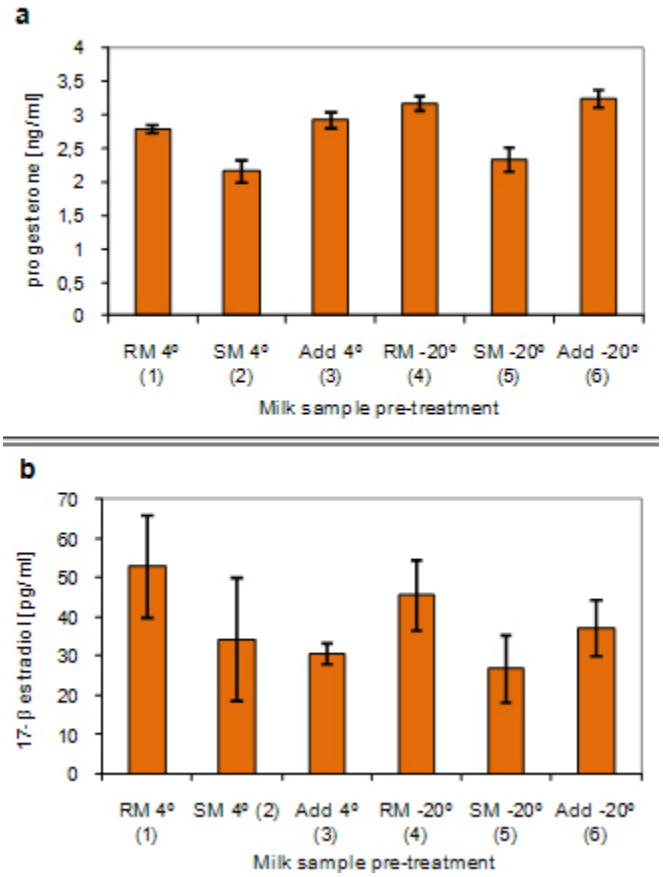

Fig. 1 a) Progesterone concentration (ng mL^{-1}) in milk according to sample pre-treatment and standard deviation. b) 17β-estradiol concentration (pg mL^{-1}) in milk according to sample pre-treatment and standard deviation. RM 4º: raw milk at 4ºC; SM 4º: skimmed milk at 4ºC; Add 4º: raw milk with additive at 4ºC; RM -20º: raw milk at -20ºC; SM -20º: skimmed milk at -20ºC; Add -20º: raw milk with additive at -20ºC.

In order to monitor progesterone content and identify 17β-estradiol pre-ovulation peak in milk individual daily milking samples were taken. Initially, just one every 2 days morning-milking sample was analyzed (Figure 2, graphics a, b, c, d, e, f). Results indicated that 17β-estradiolpre-ovulation peak was detected in all cases and levels were greater than previously reported in milk and variable within the six cows (33,58 up to a 184,73 pg mL^{-1}). Only in one case morning and afternoon milking samples of the same day were required to identify the peak (Figure 2f). Identification of the specific dates when further 17β-estradiol analyses were required was possible by means of description of the progesterone pattern.

Figure 2e, however, corresponds to a cycle where progesterone levels were unexpectedly low while 17β-estradiolpeak was detectable at previously described levels (100 pg mL^{-1}). The EIA measurements proved to be an affordable, simple and trouble-free method for hormone determination in raw milk and consequently heat identification with reliable results.

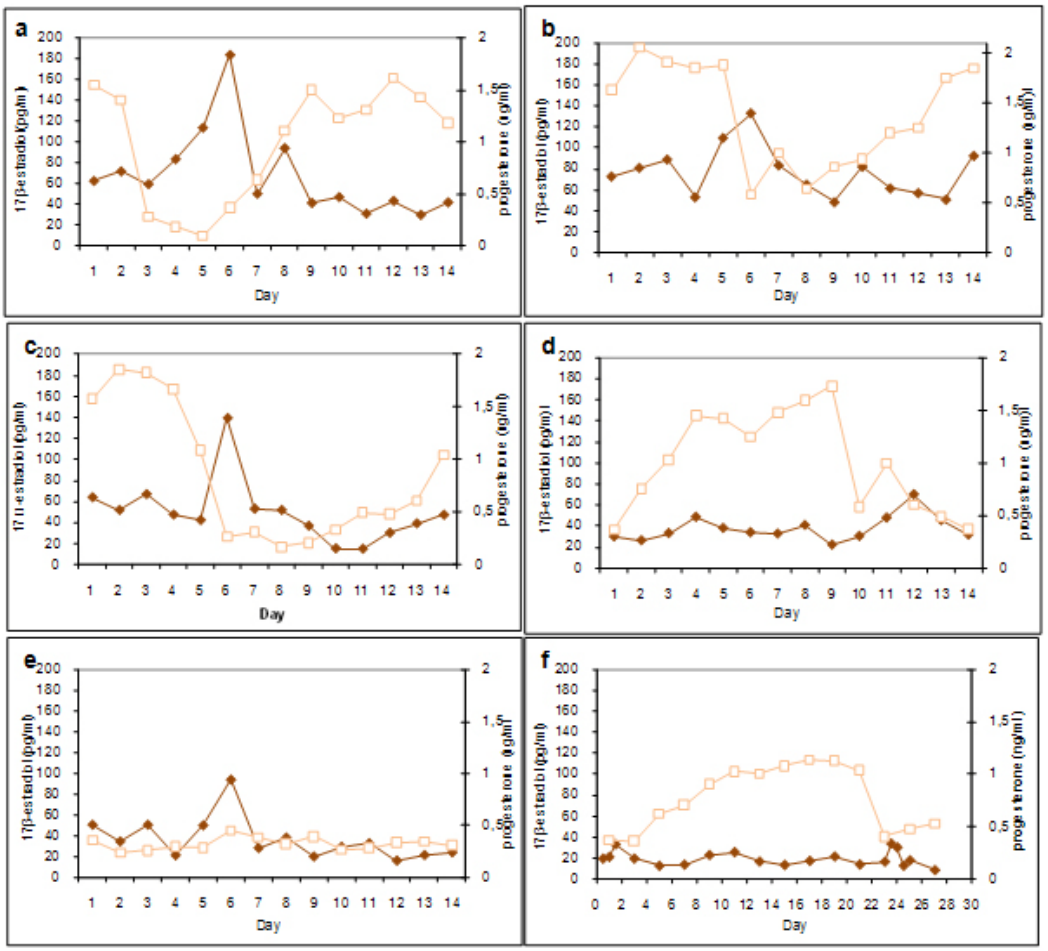

Fig. 2 Hormonal profiles of progesterone (clear symbols) and 17β-estradiol (brown symbols) of 6 different cows (a, b, c, d, e, f) along one sexual cycle. Black arrows indicate on-farm visual heat identification.

On-farm heat identification by behavioural means was detected in four of the six cows corresponding to the hormonal cycles of graphics a, c, e and f of Figure 2. Heat was not detected by behavioural signs in cows corresponding to graphics b and d of Figure 2 despite of showing a typical hormonal profile. Discordances in heat identified dates were up to 8 days except for Figure 2f where heat was detected within an optimal 12 hrs window of the pre-ovulation peak.

In conclusion, it is possible to quantify 17β-estradiolcontent in individual raw milk samples using a non-radioactive immunoassay kit with no previously milk application reported. Precise cow heat identification is possible by means of detection of the pre-ovulation progesterone decrease and 17β-estradiolincrease in individual milk samples. The EIA methods described herein are reliable and simple and require no specific or expensive equipment. In addition, they could represent a useful tool for potentially monitoring heat identification.

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