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THE EFFICACY OF MILK EJECTION INDUCED BY LUTEAL OXYTOCIN AS A METHOD OF EARLY PREGNANCY DIAGNOSTICS IN COWS

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The aim of this research was to determine the accuracy and reliability of early diagnosis of pregnancy in cows using $PGF_{2\alpha}$ analogue. Namely in the case of corpus luteum (CL) presence, the release of luteal oxytocin is induced by intravenous administration of a non luteolytic dose of $PGF_{2\alpha}$. The research was performed in 30 lactating cows, 18-22 days after insemination, and three hours prior to evening milking. A teat cannula is placed in the left fore-teat. When the cisternal milk flow ceased, Dinoprost (256 μg Dinolytic) was injected in the v. jugularis. If CL was present, the alveolar milk flow (559.4±83.7 mL) started 189±18 s later and early pregnancy could be presumed. If CL was no longer functional, the milk flow did not start again and the cow was considered not pregnant. Seventeen out of 30 cows were confirmed pregnant 35 days after insemination by ultrasound and 13 were found not pregnant. The accuracy of positive results is 100% and is identical to that obtained by ELISA of milk progesterone used as a reference method. The accuracy of negative results was lower (46.1% vs. 84.6%) ($p<0.05$), because seven out of 13 non pregnant cows has responded to the milk ejection test. In spite of this disadvantage, the test approved as being inexpensive, rapid, and easy to interpret and is therefore very applicable, especially under field conditions. It can be applied i.e. 20 days after insemination. Progesterone rapid test from milk or by milk ejection test can be used with similar accuracy.

Key words: cow, luteal oxytocin, milk progesterone, pregnancy diagnosis

INTRODUCTION

To achieve good milk and meat production results and reproductive performance, early pregnancy diagnosis is essential (Szenci *et al*., 2000; Prvanović *et al*., 2003).

The methods of early diagnosis are of particular importance due to timely detection of not gravid animals which are subjected to reinsemination or therapy in due time (Prvanović *et al*., 2009). Among other, the methods of early laboratory diagnosis based on blood or milk progesterone level determination by RIA or ELISA method are available and often used (Druckmann and Druckmann, 2005; Dobranić *et al*., 2006). Some of the rapid methods, based on milk progesterone level determination, are available for field conditions (eg Hormonost®). Unlike RIA and ELISA which are quantitative methods, rapid field tests are qualitative. The determination of progesterone concentration is the most frequently used method for early laboratory diagnostics of gravidity (Ayad *et al*., 2007; Tomašković *et al*., 2007). High progesterone level is not necessarily a reliable proof of vital embryo existence and pregnancy, but only of preserved luteal function. On the other hand a low concentration is a positive indicator of lack of pregnancy (Noakes *et al*., 2001).

Field measurements and interpretation of results on the farm is the advantage of immunoenzyme milk sample testing in relation to RIA tests of blood or milk samples which require sample transport to laboratories and thus disable prompt reinsemination. RIA, as well as immunoenzyme progesterone tests, reach satisfactory accuracy (70-75%) for the positive and almost 100% for negative cases, both after 21 day post insemination (Humbolt *et al*., 1987; Bech-Sabat *et al*., 2008). Although easy to handle, immunoenzyme kits are expensive (Mialot, 1988) and need 20 to 40 minutes to interpret the results (Tainturier *et al*., 1988) which impede their wider use in field conditions.

All these are reasons that have led us to try to establish the existence of early pregnancy CL, not by their secretion of progesterone, but by oxytocin. CL can synthesize this peptide (Labussiere *et al*., 1992; Druckmann and Druckmann, 2005), whose tissue concentrations decline earlier than those of progesterone in the case of failed fertilization (Wathes *et al*., 1984; Ivell *et al*., 1985; Gabor *et al*., 2008). Prostaglandin $F_{2\alpha}$ by positive feedback stimulates the luteal tissue to synthesise and secrete oxytocin (Spencer *et al*., 2004; Noakes *et al*., 2009). Labussiere *et al*. (1988) proved that intravenous application of 256 μg of $PGF_{2\alpha}$ doesn't bring up a risk of luteolisys, but leads to a release of luteal oxytocin and milk ejection that can be recorded by measuring the intramammary pressure or volume of milk ejected out through the teat cannula (Labussiere *et al*., 1982).

This method makes possible to distinguish pregnant from non pregnant cows 19 or 20 days after insemination. The aim of this research was to determine the accuracy and credibility of the aforementioned methods of early pregnancy diagnosis of cows under our conditions.

MATERIAL AND METHODS

Cow management

The research included 30 Simmental cows, 2 to 8 years old from one dairy farm in Bosnia. All the cows were kept in pens, milked by an automatic milking system and fed a mixture of corn and green silage, concentrate and mineral supplements.

Early diagnostics of gravidity based on milk ejection after an intravenous application of a subluteolytic dose of $PGF_{2\alpha}$

Tests were performed 18-22 days after insemination (20 days on average). The cannula was placed into the left front quarter of the udder, two to three hours before the evening milking to evacuate the cisternal milk. The quantity and time needed for the evacuation of the milk was recorded. The inserted screw cannula is a sterile metal probe, 10 cm long and 6% cone shaped. The teat was previously, very gently disinfected with swabs containing iodine soap (Vetedine or Vetoquinol) and 70% alcohol to avoid undesirable pituitary oxytocin release. After the teat cistern was emptied, a small, subluteolytic dose of $PGF_{2\alpha}$ (256 μg Dinolytic-Upjohn, USA) diluted in 2 mL 0.9% saline infusion was applied.

Over the next four minutes an option which is used in pregnancy diagnosis appears: alveolar milk ejection under the influence of luteal oxytocin. This option is considered as a positive result. Ejection characteristics (volume of ejected milk, time elapsed since application and duration of milk ejection) are recorded. The lack of new milk release is assumed as a sign of absence of functional corpus luteum. This is considered as a negative pregnancy test result.

Early pregnancy diagnostics based on milk progesterone level determination

The milk progesterone P_4 concentrations were determined at the laboratory of Veterinary station Odzak, Bosnia and Herzegovina, using rapid field test Hormonost® (Biolab, Germany). The test was performed on average 20 days after AI. The milk samples were taken from whole milk obtained after 15 jets of milk were stripped, two to three hours before the milk ejection test was performed. Further procedure was performed following the manufacturer's instructions.

Early pregnancy diagnostics based on rectal palpation and ultrasonic exam

The pregnancy diagnostics is performed 35 days after insemination by rectal palpation and ultrasonic examination of cows in their barns, without constraint. Rectal examination was performed through gentle palpation of uterine horns. The slight asymmetry of the uterine horns, differently filled uterine horns and fluctuation of the assymetric horn were considered as a positive sign of pregnancy. The ultrasonic early pregnancy diagnostics was performed by transrectal 7,5 MHz linear probe (Sonovet 2000). A finding of a live, vital embryo, 12 to 15 mm of size with detectable heartbeat was considered as positive for pregnancy. Contrary, not finding the embryo was considered as a negative finding.

The results of the performed tests were compared with the appearance of overt signs of oestrus (21 ± 2 days or more than 24 days) and also by rectal and ultrasound tests performed about 50 days after insemination.

Statistical analysis

Statistical analysis of the results was performed using the analysis of variance (ANOVA, StatSoft, Statistika, version 7.1.) and Tukey's post-hoc analysis

tests. The correlation between the obtained results was determined as well. Statistically significant results were considered those with $p<0.05$.

RESULTS

Table 1. The mean (± S.E.M.) values of studied parameters in the pregnant cow group (n=17)

VOM1 / s	KOM1 / mL	VOM2 / s	KOM2 / mL	LAB. DG.	R. P.	US
89.8±12.96	118.8±18.8	189.5±18	559.4±83.7	positive	positive	positive

Legend:
VOM1 – the time required to evacuate residual milk from the tested quarter of the udder (s);
KOM1 – the volume of residual milk evacuated from the tested quarter of the udder (mL);
VOM2 – the time elapsed after subluteolytic dose of prostaglandin was applied untill end of the milk ejection from the tested quarter of the udder (s);
KOM2 – the volume of milk ejected from the tested quarter of the udder after subluteolytic dose of prostaglandin was applied (mL);
LAB. DG. – laboratory diagnostics of gravidity in milk samples 20 days after AI by Hormonost® test (positive or negative);
R.P. – diagnostics of gravidity based on rectal palpation of the uterus 35 days after AI (positive or negative);
US – pregnancy diagnostics based on ultrasonic uterine exam 35 days after AI (positive or negative).

The positive results of Hormonost® test found in 17 cows and confirmed 35 days after AI by rectal and ultrasound examination.

Table 2. The mean (± S.E.M.) values of studied parameters in the group of open cows (n=6)

VOM1 / s	KOM1 / mL	VOM2 / s	KOM2 / mL	LAB. DG.	R. P.	US
131±32.5	189.5±49.6	0	0	negative	negative	negative

Legend: same as for Table 1.

The negative results of Hormonost® test found in 6 cows and confirmed 35 days after AI by rectal and ultrasound examination. After a subluteolytic dose of prostaglandin was applied, in this group due to the lack of corpus luteum, neither luteal oxytocin secretion nor milk ejection occurred (VOM2 and KOM2 were equal to zero).

Table 3. The mean (± S.E.M.) values of studied parameters in group A of cows considered to be pregnant (n=5)

VOM1 /s	KOM1 / mL	VOM2 / s	KOM2 / mL	LAB. DG.	R. P.	US
89.6±12.5	87.8±22.5	229.2±80.9	469.8±106.1	negative	negative	negative

Legend: same as for Table 1.

In this group of cows milk ejection occurred (VOM2 and KOM2>0) after a subluteolytic dose of prostaglandin was applied. The cows were considered to be pregnant which was disproved by the negative Hormonost® test and confirmed later by negative rectal and ultrasound exams.

Table 4. The mean (± S.E.M.) values of studied parameters in group B of cows considered to be pregnant (n=2)

VOM1 / s	KOM1 / mL	VOM2 / s	KOM2 / mL	LAB. DG.	R. P.	US
86.5±9.5	99±56	245±137	931.5±353.5	positive	negative	negative

Legend: same as for Table 1.

In this group of cows milk ejection occurred (VOM2 and KOM2>0) after a subluteolytic dose of prostaglandin was applied. The cows were initially considered as pregnant which was confirmed by positive Hormonost® test, but disproved later by negative rectal and ultrasound exams.

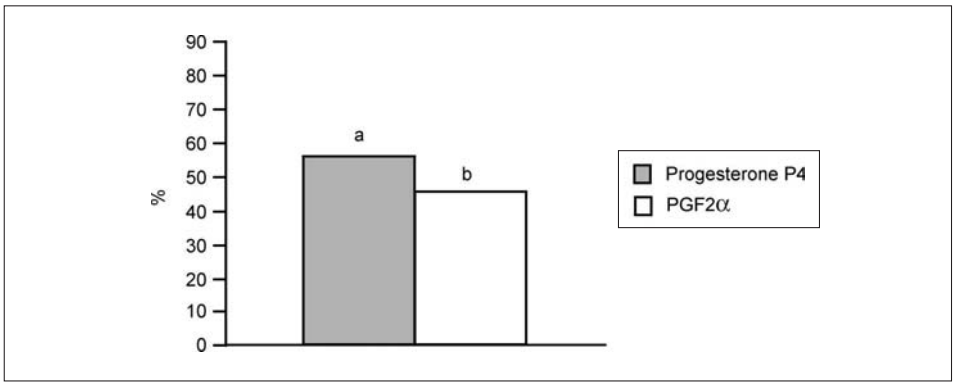

Figure 1. Percentage of animals found to be pregnant or open by rectal and ultrasound examination

The vertical axis presents the percentage. The percentage is based on the rectal and ultrasonic tests performed 35 days after AI. Seventeen out of 30 cows were found to be pregnant (56.67%), and 13 out of 30 (43.33%) were found not to be pregnant (Figure 1).

The vertical axis presents time flow in seconds and volume of milk obtained in mL. The black columns present the parameters of the pregnant cow group. The dark gray columns present in the group of open cows, white columns belongs to the group A of cows considered to be pregnant, and the pale gray ones to the group B of cows considered to be pregnant. The VOM1 for all four groups of cows was found to be 89.8, 131, 89.6 and 86.5 respectively. KOM1 values were 118.8, 189.5, 87.8 and 99; VOM2 values were 189.5, 0, 229.2, 245 and KOM2: 559.4, 0, 469.8 and 931.5, respectively (Figure 2).

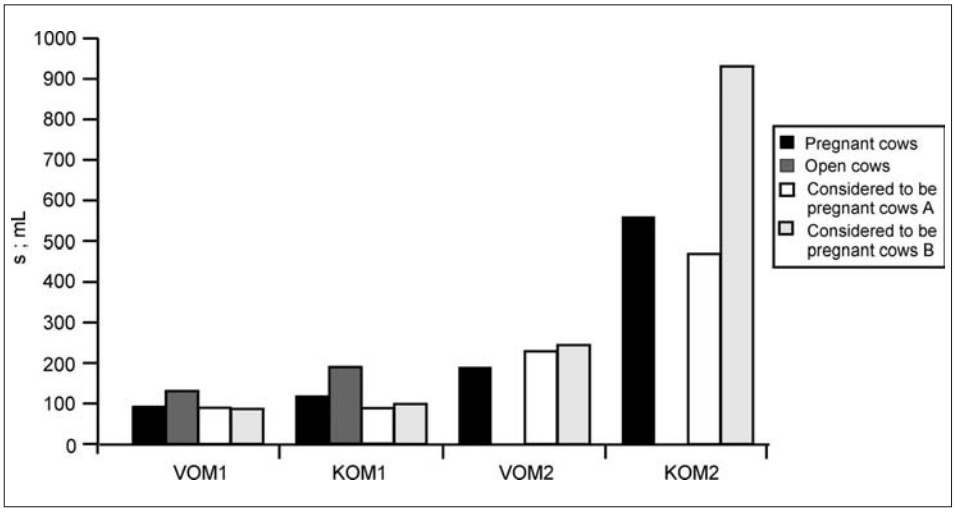

Figure 2. Comparative mean values of two tested parameters in four groups of cows

Legend:
VOM1 – the time required to evacuate residual milk from the tested quarter of the udder (s);
KOM1 – the volume of residual milk evacuated from tested quarter of the udder (mL);
VOM2 – the time elapsed after subluteolytic dose of prostaglandin was applied untill the end of milk ejection from the tested quarter of the udder (s);
KOM2 – the volume of milk ejected from the tested quarter of the udder after a subluteolytic dose of prostaglandin was applied (mL).

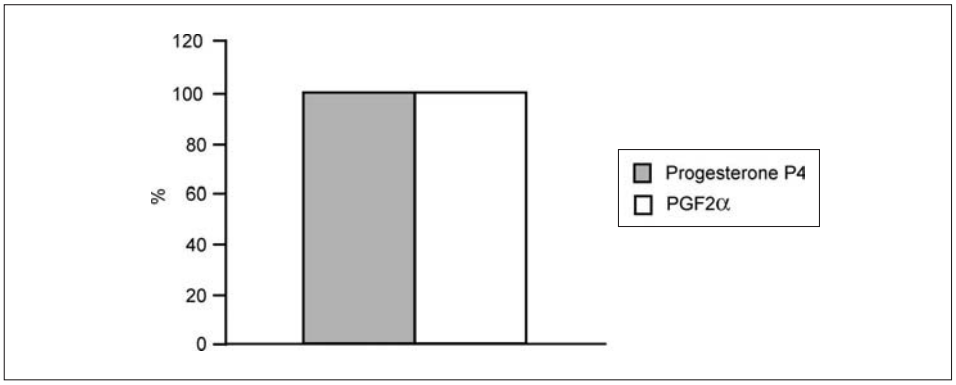

Figure 3. The accuracy (in percentage) of a positive pregnancy diagnosis made by Hormonost test® and by appearance of milk ejection from the tested udder quarter after subluteolytic dose of prostaglandin was applied

Both methods were 100% accurate which was confirmed later by rectal and ultrasound exams.

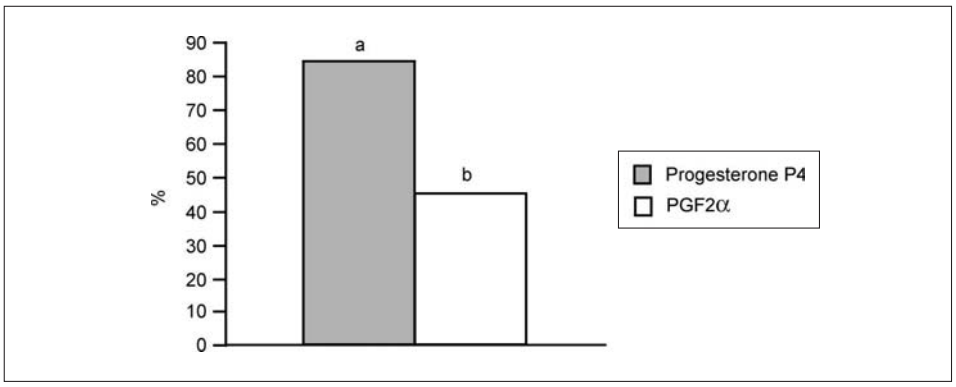

Figure 4. The accuracy of a negative pregnancy diagnosis made by Hormonost® test and by absence of milk ejection from tested quarter of the udder after application of a subluteolytic dose of prostaglandin

The negative laboratory diagnosis was accurate in 84.6% and absence of milk ejection was in 46.1% of the cases.

DISCUSSION

The rapid qualitative pregnancy diagnostic ELISA tests performed on milk samples have the advantage of direct readings on the farm (Prvanović, 2006). The RIA or ELISA laboratory quantitative tests for blood or milk samples are time consuming due to samples transport to the laboratory (Prvanović *et al*., 2009). Both RIA and ELISA tests are confidently accurate 21 days after insemination. Positive results are 70 to 75% accurate and negative very close to 100% accuracy (Humblot *et al*., 1987; Bech-Sabat *et al*., 2008). In addition, despite its obvious simplicity, immunoenzyme tests require a long reaction time (20 to 40 minutes) (Tainturier *et al*., 1988). On the other hand, most of them are expensive (Mialot, 1988), thus inhibiting their wide spread use in herds.

On the other hand, the method based on the release of luteal oxytocin induced by intravenous administration of a non luteolytic dose of $PGF_{2\alpha}$ is cheap and requires a minimum dose of Dinolytic® (Labussiere *et al*., 1988). Interpretation is simple (milk ejection is present or not) and the realization is fast (generally it takes less than 5 min) (Labussiere *et al*., 1992). This research confirmed, due to the luteal tissue present on the ovary of pregnant cows, the milk ejection occurrence soon after $PGF_{2\alpha}$ is applied. On average it lasts 189.5 seconds (max. 9 min and 4 s) for the ejection of 559.4 mL of cisternal milk (max. 1470 mL). Unlike our results, in a study of Labussiere *et al*. (1992) it took on average 90 seconds for the ejection of 230 mL of cisternal milk.

Some authors confirmed that the main advantage of the test of milk ejection is its use in early pregnancy diagnosis because of its accuracy and credibility from 20 days after the AI onward, i.e. 24-72h before progesterone detection tests

(Labussiere *et al*., 1992). However, this study did not confirm these claims due to the fact that the ELISA method for milk progesterone determination was performed the same day as the milk ejection test. However, a negative diagnosis obtained by milk ejection test allows the reinsemination in the first successive oestrus following insemination, unlike progesterone detection tests where the earliest possible performance of the test is possible 21 days after AI (sometimes even 23rd or 24th day). Some authors recommend respecting that time limit when the diagnosis is based upon detection of progesterone (Thibier, 1988; Prvanović, 2006), because Humblot *et al*. (1987) discovered two times more open cows on the 21st and 23rd than on the 19th day. This could be explained with the fact that on the 19th day many open cows could still have high progesteronemia (Humblot and Dalla Porta, 1984; Starbuck *et al.*, 2004), whereas the drop of luteal oxytocin level appears much earlier (Jones and Flint, 1988).

However, high reliability of Hormonost test® positive results was found, confirmed later on by rectal and ultrasound examination 35 days after AI.

Positive diagnosis of pregnancy based on milk progesterone determination made by Hormonost test® was found to be accurate in 100% of cases, as well as positive diagnosis based on milk ejection after subluteolytic dose of prostaglandin was applied. Negative diagnosis based on Hormonost test® was found to be accurate in 84.6% of cases whereas negative milk ejection test after an application of a subluteolytic dose of prostaglandin was reliable in 46.1% of cases.

Rectal and ultrasound exams performed 35 days after the AI found 17 out of 30 cows in our study pregnant and remaining 13 was found open. Although found to be open by rectal and ultrasonic exam performed 35 days after AI, milk ejection occurred in seven out of 30 cows. Moreover, two cows had also, besides the positive ejection milk test, positive Hormonost test® although both were found to be open by rectal and ultrasound tests performed later on.

This study confirmed that the results obtained 19 days after AI by milk ejection test were equally precise as those obtained by the Hormonost test® (100 vs. 100%). Nevertheless, Hormonost test® was overwhelmingly better in negative results detection (84.6 vs. 41.6%). These results are consistent with those of Labussiere et al. (1992) who obtained the accuracy of negative results of 100% for Hormonost test® which was significantly higher ($p<0.01$) than negative results obtained with milk ejection test (93.5%). In contrast, the accuracy of positive results was significantly higher ($p<0.01$) considering the milk ejection test (72.3%) than progesterone detection (68.2%).

Labussiere *et al*. (1992) consider the milk ejection test as a more reliable method then progesterone determination considering early diagnostic of open cows. The method allows reinsemination of cows in the very next cycle.

The reasons for unreliability of milk ejection and Hormonost test® regarding group A of cows considered to be pregnant and finally found to be open could be explained by early embryonic mortality or possibly by a persistent luteal tissue. In group B of cows, considered to be pregnant with positive milk ejection test and negative progesterone test, we assumed that alveolar milk release is more reliable than the progesterone test to detect functional corpus luteum as indicated by

Labussiere *et al.* (1992), but probably in their respective study early embryonic mortality or persistent corpus luteum occurred.

It could be concluded that the positive diagnosis of early pregnancy in cows could be obtained with same reliability using progesterone rapid diagnostic tests and by the milk ejection test based on the release of luteal oxytocin induced by intravenous administration of a non luteolytic dose of prostaglandin. Although the accuracy of milk ejection test in detecting open cows, is significantly lower, the low price and simplicity of this method in comparison with the fast progesterone tests in milk samples, allow its use in practice.

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EFIKASNOST EJEKCIJE MLEKA INDUKOVANE OKSITOCINOM POREKLOM IZ ŽUTOG TELA KAO METODE DIJAGNOSTIKOVANJA RANE STEONOSTI KRAVA

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SADRŽAJ

Cilj istraživanja je bio da se utvrdi točnost i verodostojnost metoda rane dijagnostike steonosti krava metodom koja se zasniva na praćenju otpuštanja mleka koje u slučaju postojanja žutog tela rezultira oslobađanjem lutealnog oksitocina induktovanog i/v primenom subluteolitičke doze $PGF_{2\alpha}$. Pretrage su realizovane 3h pre večernje mužnje, kod 30 krava u laktaciji, 18-22 dana nakon veštačkog osemenjavanja (prosek 19d). Kanila je postavljena u prednju levu sisu (tako da joj vrh bude u žlezdanom delu cisterne). Nakon što rezidualno cisternalno mleko iscuri, u *v. jugularis* se aplikuje 256 μg Dinolytica, analoga $PGF_{2\alpha}$. Ako

na jajniku postoji žuto telo, u naknadno potvrđenih steonih krava, nakon 189±18 sec dolazi do istecanja alveolarnog mleka (559,4±83,7 mL), na osnovi čega može da se pretpostavi da se radi o ranoj steonosti. Ako žuto telo nije u funkciji, nema alveolarnog istecanja mleka te može da se zaključi da krava nije steona. Od ukupno 30 krava, 17 ih je 35. dana nakon veštačkog osemenjavanja bilo steono, a 13 ih nije bilo steono. Točnost pozitivnih rezultata iznosi 100% te je identična onoj dobivenoj određivanjem progesterona u mleku metodom ELISA (Hormonost®) koja je služila kao kontrolna metoda (100%). S druge strane, točnost negativnih rezultata je slabija (46,1% nasuprot 84,6 %) ($p<0,05$), jer među 13 docnije potvrđeno nesteonih krava, u njih 7 je došlo do otpuštanja alveolarnog mleka. Unatoč tome, ovaj test je jeftin, brz (cca. 5 min) i jednostavan za interpretovanje (alveolarno mleko curi ili ne curi) te je stoga vrlo primenjiv, napose u terenskim uvetima. Za razliku od njega, određivanje progesterona u mleku nešto je komplikovanije za interpretovanje te je potrebno biti umešan u razlikovanju boja i njihovih nijansi. Oba testa mogu da se primene vrlo rano, već 20. dana nakon veštačkog osemenjavanja. Može da se zaključi da u ranoj dijagnostici steonosti sa sličnom pouzdanosti mogu da se koriste testovi brze progesteronske dijagnostike iz mleka kao i lutealnim oksitocinom izazvano otpuštanje mleka.