Determination of the cutoff point of milk progesterone and use it as an early indicator of pregnancy or screening of open cows in dairy farms

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Abstract
The present study was carried on a herd of Friesian cows during the period from June 2010 to May 2011, This herd Belongs to EL-Salhaya Company for Investment and Development (Dairy cattle breeding farm number 2), which is located in El-Salhaya City- Sharkia Province.

This study aimed to Calculate the optimum cutoff value of milk progesterone (P4) concentration to predict pregnancy status 19-24 days after insemination compared with diagnosis by rectal palpation performed 45-60 days of insemination and considered as the “gold standard” using a Receiver Operating Characteristic (ROC) analysis and analyze the sensitivity (Sense), specificity (Spes), positive predictive value (PPV) and negative predictive value (NPV) of the tests with examination of the differences in these values as the cutoff values were allowed to change. The cutoff point obtained by ROC curve in ELISA milk test was > 6.2 ng /ml with an area under the ROC curve (AUC) of 0.890, a result referred to as “moderately accurate”, the sensitivity % of ELISA milk test was 82.8 , specificity % was 92, the negative predictive value% was 82, the positive predictive value% was 92.3. It may be concluded that ELISA milk progesterone tests can be used as an early indicator of pregnancy and non pregnancy in cows but with some precautions.

Key words: Reproductive Performance, Pregnancy Diagnosis in Cattle, milk Progesterone test, (ROC) curve.
Introduction
Reproductive management is currently a major factor affecting profitability in the dairy industry (Oltenacu et al, 1990 and Stott et al, 1999). As dairy herd size has increased, so also has the number of cows per herdsman. This, together with increasing pressure to maximize milk yield whilst at the same time reducing production costs, has negatively affected reproductive performance of herds (Royal et al, 2000 and Lucy, 2001). Optimum management in low-fertility dairy herds requires that average calving interval be reduced, because days open could be, in the Italian and European reality, potentially expensive whenever they exceed 90–100 days (Harness et al, 1986 and Ferry, 1992). In particular, Esslemont and Kossaibati (2000) report days open in a range of 86–109 days as an excellent target, 110–120 days as adequate aim and the appearance of some problem when open days overcome the threshold of 121 days. Accurate and early pregnancy detection therefore is crucial, in order that non-pregnant cows may be re-inseminated or culled from the herd (Carriere et al, 2000).

Twenty-one days after insemination, the determination of the progesterone concentration allows one to predict whether the animal is pregnant (high progesterone concentration >5.0 ng/ml) or not pregnant (≤5 ng/ml) (Pierre and Denis 2004). The assay has to be performed between days 18 and 22 of the cycle (Kay et al, 1984).

It is known that hormone concentrations are continuous variables, so their use as a pregnancy test is based on the selection of a cutoff concentration, above or below which the studied condition is indicated. In previous studies, plasma P4 concentration ≥7 nmol/L, and PAG concentration ≥2 ng/mL have been used as cutoff values indicating pregnancy (Ropstad et al, 1999; Milner et al, 2003; Vahtiala et al, 2004; Sakkinen et al, 2005 and Akobeng, 2007). The selection of these cutoff values has been based on frequency distributions of these hormones in studied populations and on changes in their concentrations during gestation. The clinical value and usefulness of diagnostic tests can be described by diagnostic values such as sensitivity, specificity, and predictive values (Akobeng, 2007).

The present study will concentrate in the point of early detection of pregnancy and unsuccessful insemination by which will decrease the days open (D.O) which defined as the period from parturition until the successful breeding.

Materials and methods
The present study was carried out on a herd of Friesian cows during the period from June 2010 to May 2011, this herd Belongs to El-Saliya
Agriculture Company for Investment and Development (Dairy cattle breeding farm number 2), El-Salhya city- Sharkia Province.

I. Reproductive management:
After the cow had calving, the post partum period begins and the management in the farm decide a voluntary waiting period 40-50 day and then begin to examine each cow separately by rectal palpation and according to the problem, the veterinarian will solve. After that the cow may be artificially inseminated if came in estrus or making estrous synchronization to a group of cows.

II. Samples collection
Ten milliliters of milk were collected from 108 lactating cows from 19-24 day from insemination date at the parlour during milking process in falcon tubes contain 15 mg potassium dichromate and 10 mg sodium chloride as preservatives and the milk samples were preserved at -20 ºC until analytical uses (Mohan et al, 2010).

III. Analytical methods
Progesterone ELISA test
Principle of the assay
The Immunospec progesterone Quantitative test kits (of immunospec corporation 7018 Owensmouth Ave. Suite 103 Canoga Park, CA.; www.immunospec.com) we used in this experiment which based on the principle of competitive solid phase enzyme immunoassay.

IV. Statistical analysis of data and diagnostic calculations.
The progesterone cutoff value that best discriminated between pregnant and non-pregnant cows was determined using the ROC curve procedure of MedCalc1 software release 8.1.0.0 for Windows platform; and test goodness was evaluated by calculating the area under the ROC plot.
In present study the cut off point was > 6.2 ng/ml in which the values exceeding 6.2 will be considered pregnant while lower than (≤6.2) was considered negative. In previous study (Ropstad et al, 2005), the hormonal pregnancy tests were designated positive if the level of the hormone in the plasma was equal or exceeded the threshold levels set for the test. The threshold levels for detecting pregnancy with the plasma P4-ELISA test were 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 nmol/L. The 7.0 nmol/L level has been used as a cutoff value for indicating pregnancy in plasma of reindeer and in another study the cutoff value indicating pregnancy in reindeer was 5 nmol/L in plasma (Savela et al, 2009).
Note that the nmol/L is differing from the ng/ml, so for conversion from nmol/L to ng/ml we will divide the nmol/L by conversion factor which is 3.18 for progesterone.
It should be noted that the level of progesterone hormone in the milk
differ to the level in the plasma, in previous studies the level of progesterone in milk is 4 fold the level in the plasma. (Ginther et al, 1974; Ginther et al, 1976 and Manju Kamboj and Prakash 1993). From mentioned above, we can convert the values from nmol/l of plasma to ng/ml, as an example the cutoff point 7 nmol/l in plasma will be 8.8 ng/ml in milk (7/3.18 x 4) and cutoff point 5 nmol/l in plasma will be 6.3 ng/ml in milk (5/3.18 x 4) and so on. In present study the cut off point was 6.2 ng/ml. The values that obtained from the ROC curve analysis were defined as following according to Karen et al (2007) and Savela et al, (2009).

1- Sensitivity (Sens) was defined as the ability of the test to correctly detect pregnancy as the same as RP do.

2- Specificity (Spes) was defined as the ability of the test to correctly identify the non-pregnant females determined to be non-pregnant by the RP exam.

3- The positive predictive value (PPV) was the probability of a pregnant diagnosis by the test being further corroborated by the RP exam.

4- The negative predictive value (NPV) was the probability of non-pregnant diagnosis by the test being corroborated by the RP exam.

RESULTS AND DISCUSSION:

This experiment was conducted to evaluate utilization of progesterone concentration in whole milk to predict pregnancy status in cows by ELISA method, and to highlight the principal factors that influence the predictive power. Pregnancy diagnosis was performed 45–60 days post-insemination in order to compare test results to the gold standard following normal cyclicity (two cycles). The acceptability of rectal palpation as gold standard was emphasized by Paisley et al (1978), in that diagnosis errors are assumed to be equally likely for pregnant and non-pregnant cows and may vary from 0 to 5%. ROC analysis allows the calculation of an optimal cutoff value, based on examination of all possible specificity —sensitivity combinations; the area under the ROC curve, that assumes values between 0.5 (inaccurate) and 1.0 (perfect) and is a good measure of accuracy (Obuchowski, 2003). The results of ROC analysis were summarized in Table 1 and the ROC plot for sensitivity versus 100 minus specificity is shown in Fig. (1), in which the upper-left tract of the plot yields a sensitivity equal to 82.8% (95% CI = 70.6 - 91.4%) and a specificity equal to 92% (95% CI = 80.8 - 97.8%). These two values correspond to a cutoff of >6.2ng/mL progesterone in whole milk. The dot plot in Fig. (2) depicts distributions of the two pregnancy-outcome groups classified according to the >6.2
ng/mL cutoff value. Area under the ROC plot was 0.890 [95% CI = 0.815 to 0.942], a result referred to as “moderately accurate” (Greiner et al, 2000), the value of AUC in this study was similar or some what higher than to the value reported by Faustini et al (2007), which was 0.859 (95% CI = 0.835–0.880). The optimal P4 cutoff point was set, by the ROC method, to > 6.2 ng /ml in this study was equal to the cutoff value determined by Savela et al (2009), they calculate a cutoff value of 5 nmol/l progesterone plasma which equal to 6.3 by conversion to ng/ml in whole milk as previously discussed in the material and methods section. This value was lower than the value determined by (Ropstad et al (1999); Milner et al (2003); Vahtiala et al (2004); Sakkinen et al (2005) and Akobeng et al (2007) they reported plasma P4 concentration ≥7 nmol/L, and higher than values determined by Rajamahendran et al (1993) who used progesterone concentration in whole milk on day 21 after insemination. Those authors reported a P4 cutoff of 1 ng/ml but with lower specificity (Spec = 57.5%), but in our study specificity was 92%. P4 cutoff value of >6.2 ng /mL did not differ significantly from rectal palpation sensitivity and specificity. At all other cutoff values, either sensitivity or specificity was significantly lower compared with those of rectal palpation. These results are similar to those reported by Savela et al (2009). The sensitivity%, specificity%, positive predictive value%, negative predictive value% according to the results of ROC analyses and cutoff value of >6.2 ng/ml, summarized in table (1), the sensitivity % was 82.8 (95% CI = 70.6 - 91.4%), this value is lower than value reported by Faustini et al (2007) (Sens = 98.2 %), and Savela et al (2009) (Sens= 96.4%) and this due to the number of true positive cases is smaller than reported by those author. The specificity % was 92 (95% CI = 80.8 - 97.8%)., this value is higher than value reported by Faustini et al (2007) (Spec = 70.9 %), and Savela et al (2009) (Spec = 85.2%) . Those results mean that the a P4 was better at identifying the non pregnant animals than at identifying those that were pregnant and these results was not agreed with those reported by Savela et al (2009) in which the lower specificity of the test came from the high number of false positive which lead to decrease specificity (Spec = 85.2%) . Even though sensitivity and specificity are central measures of the accuracy of pregnancy tests, the predictive values are important in revealing the probability by which a test will give a correct or incorrect diagnosis (Altman and Bland, 1994 and Karen et al, 2003). Because the test result is known at the time when culling decisions are made, the negative predictive value is especially important for the clinical value of
pregnancy detection (Savela et al, 2009). Note that Predictive values are affected by the prevalence of the condition (Akobeng et al, 2007), and care should be taken when predictive values calculated for a certain population are applied to another population with a potentially different prevalence. In the current study, the negative predictive value% of P4 was 82 (95% CI 69.5 - 91.2) which mean that if 100 cow is diagnosed non pregnant by P4, 18 cow would actually be pregnant. This was due to the high number of false negative cases and these results in our study may attributed the lower conception rate of the group of cows that used the conception rate are important factors subsequently influencing the rate of cows pregnant at routine rectal examination. These results is in accordance with those reported by Savela et al (2009) (NPV = 80%), and was not in agreement with those of Faustini et al (2007) (NPV = 96.4 %) in which the false negative cases is smaller than reported in the present study. In this study the high number of false negative cases is due to the lower concentration of P4 and these cases are pregnant and this results agreed with those of Friggens et al (2008) where there was a false negative cases which is due to lower concentration of progesterone hormone, these decreases were in some cases indistinguishable from those one would detect if the cow was returning to oestrus following in this study and part of these cows sampled and examined in the summer season and the farm was affected by outbreaks of three day sickness which affect the percent of conception in the farm, also group of cows that used in the experiment had reproductive problems and in this time was treated fore these reproductive problems which affect the percent of true positive in this experiment , this explanation is agreed with Francos (1999) who concluded that Progesterone concentrations measured by milk examinations performed three weeks after insemination and the interaction between the rate of true positives and early embryo loss (e.g. a sudden decrease to < 10 ng / ml) but these cows remained pregnant and subsequently calved, given these types of cases, it is hard to envisage being able to substantially improve pregnancy detection without simultaneously increasing the number of false negatives, however, of greater important to the end user is detecting true pregnancy failure. So the test here is useful in this case if we combined the P4 determination with rectal palpation examination and treat the cases that is pregnant and have lower concentration of P4, so the ability to correct diagnose the non pregnant cases by P4 determination is lower than the ability of correctly diagnose the pregnant cases because the PPV was higher ( 92.3 % (95% CI 81.5 - 97.9 ) than the NPV, this
92.3% PPV mean that if 100 cow diagnosed pregnant by the P4, 7.7 would actually be not pregnant. Those results in accordance with those of Savela et al (2009) (PPV = 97.6 %) and was not in agreement with those of Faustini et al (2007) (PPV = 81.7 %) which have high number of false positive cases which is may be due to due to persistent corpora lutea or to early embryonic losses (Carriere et al, 2000; Hommeida et al, 2004). The resulting high number of false negative diagnoses practically rules out the use of P4 as a diagnostic method but may be used if combination the P4 determination with rectal palpation examination and treat the cases that is pregnant and have lower concentration of P4 cited by Faustini et al (2007). To correctly benefit the increase of the PPV and decease of NPV, we should make the following, concerning the high PPV in which the corporation of positive cases by the test is low, we should stay until making rectal palpation and sure that the positive P4 is positive by rectal palpation and the incorrect cases (false positive) can be easily discovered and reinseminated while the decrease of NPV in this situation can be managed by reexamination of the negative cases by rectal palpation and if there was no corpus luteum structure on the ovary and beginning of ovarian follicle, so the cow is not pregnant and should be reinseminated, but if have corpus luteum and low P4, we should repeat the test after period. This recommendation is cited by Faustini et al (2007).
Table (1) Determination of cut off level of progesterone hormone of high accuracy from different cut off levels which determined from Roc curve analysis.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Sens</th>
<th>95% CI</th>
<th>Spec</th>
<th>95% CI</th>
<th>Pos</th>
<th>95% CI</th>
<th>Neg</th>
<th>95% CI</th>
<th>Pos</th>
<th>95% CI</th>
</tr>
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<tr>
<td>&gt;4.6</td>
<td>86.21</td>
<td>74.6 - 93.9</td>
<td>66.0</td>
<td>51.2 - 78.8</td>
<td>74.6</td>
<td>62.5 - 84.5</td>
<td>80.5</td>
<td>65.1 - 91.2</td>
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<td></td>
</tr>
<tr>
<td>&gt;4.8</td>
<td>86.21</td>
<td>74.6 - 93.9</td>
<td>78.0</td>
<td>64.0 - 88.5</td>
<td>82.0</td>
<td>70.0 - 90.6</td>
<td>83.0</td>
<td>69.2 - 92.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4.9</td>
<td>82.76</td>
<td>70.6 - 91.4</td>
<td>86.0</td>
<td>73.3 - 94.2</td>
<td>87.3</td>
<td>75.5 - 94.7</td>
<td>81.1</td>
<td>67.9 - 90.6</td>
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<td></td>
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<tr>
<td>&gt;6.2 *</td>
<td>82.80</td>
<td>70.6 - 91.4</td>
<td>92.0</td>
<td>80.8 - 97.8</td>
<td>92.3</td>
<td>81.5 - 97.9</td>
<td>82.1</td>
<td>69.5 - 91.2</td>
<td></td>
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<tr>
<td>&gt;6.6</td>
<td>65.52</td>
<td>51.9 - 77.5</td>
<td>92.0</td>
<td>80.8 - 97.8</td>
<td>90.5</td>
<td>77.4 - 97.3</td>
<td>69.7</td>
<td>57.1 - 80.4</td>
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<tr>
<td>&gt;6.7</td>
<td>65.52</td>
<td>51.9 - 77.5</td>
<td>94.0</td>
<td>83.5 - 98.7</td>
<td>92.7</td>
<td>80.1 - 98.5</td>
<td>70.1</td>
<td>57.7 - 80.7</td>
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<tr>
<td>&gt;11.6</td>
<td>32.76</td>
<td>21.0 - 46.3</td>
<td>94.0</td>
<td>83.5 - 98.7</td>
<td>86.4</td>
<td>65.1 - 97.1</td>
<td>54.7</td>
<td>43.5 - 65.4</td>
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</tbody>
</table>

Area under the ROC curve (AUC) | 0.890

Standard Error<sup>a</sup> | 0.0347

95% Confidence Interval<sup>b</sup> | 0.815 to 0.942

z statistic | 11.225

Significance level P (Area=0.5) | <0.0001

Variable
Classification variable | Progesterone test

Conception rate (%) | 53.7

Sample size | 108

Positive group : Rectal palpation = 1 | 58

Negative group : Rectal palpation = 0 | 50

<sup>a</sup> DeLong et al., 1988  
<sup>b</sup> Binomial exact
**Fig. (1)** ROC plot (solid line) and 95% confidence interval (broken lines) for the progesterone cut off level. [AUC = (0.890)]

**Fig. (2)** Dot plots for the distribution of progesterone in pregnant or non-pregnant cows. Horizontal bar marks the cut point level reported in ROC plot.
Conclusion:
In general, from the results obtained, it may be concluded that ELISA milk progesterone tests can be used as an early indicator of pregnancy and non pregnancy (open cows) in cows but with some precautions. concerning the high PPV in which the corroboration of positive cases by the test is high, we should stay until making rectal palpation and sure that the positive P4 is positive by rectal palpation and the incorrect cases (false positive) can be easily discovered and re inseminated, while the decrease of NPV in this situation can be managed by re examination of the negative cases by rectal palpation and if there was no corpus luteum structure on the ovary and beginning of ovarian follicle, so the cow is not pregnant and should be re inseminated, but if have corpus luteum and low P4, we should repeat the test after period.

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تحديد نقطة القطع المثلى لهرمون البروجسترون في اللبن واستخدامها كمؤشر مبكر للحمل أو عمل مسح للابقار داخل الفترة المفتوحة في مزارع الحلاب

أجريت هذه الدراسة على قطيع من الأبقار الفريزيان خلال الفترة من يونيو 2010 إلى مايو 2011. هذا القطيع تابع لشركة الصالحة للإستثمار والتنمية (محطة ابقار التربية والالبىان 2)، والتي تقع في مدينة الصالحة الجديدة التابعة لمحافظة الشرقية. تهدف هذه الدراسة إلى حساب قيمة القطع المثلى لتركيز هرمون البروجسترون في اللبن للتنبؤ بحالة الحمل بعد 01-02 يوما من التلقيح بالمقارنة بتضخيم الحمل من خلال الجسد بعد 05-06 يوما من التلقيح. وتعتبر "معايير الذهب" باستخدام تحليل استقبال خاصية التشغيل (ROC) والحساسية، الخصوصية، القيمة التنبيهية الإيجابية (PPV) والقيمة التنبيهية السلبية (NPV) للاختبارات في هذه القيم مع اختلاف قيمة القطع للهرمون. كانت نتائج تحليل منحنى استقبال خاصية التشغيل (ROC) للحساسية مقدار 100 مطروحا منها الخصوصية تكشف على أن الجهد العليا اليسرى من المنحنى يعطى حساسية تساوي 87,8% وخصوصية للاختبار تساوي 93%.

تم تحديد نقطة القطع المثلى لهرمون البروجسترون في اللبن عند > 0.2 نانوغرام / مليلتر. وكانت المنطقة تحت المنحنى 0.89، ويشير إلى هذا الرقم بأنه معدل الدقة. وكانت الحساسية والخصوصية للاختبار عند هذه النقطة (0.2 نانوغرام / مليلتر) ليس لها اختلاف معنوي مع نتائج جس الأبقار، ولكنها وجدت من نقاط القطع الأخرى ليس لها اختلاف معنوي مع نتائج جس الأبقار ولكن كانت بنسبة أعلى بكثير من نقطة القطع المثلى (> 0.2 نانوغرام / مليلتر).