PEER REVIEWED

Choosing the Right Cutoff Level of Milk Progesterone to Determine Pregnancy Status of Dairy Cows on Day 21 Post-Breeding

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Abstract

A field study was conducted between February and November 1995 in three Holstein dairy herds (193 inseminations) to determine the overall accuracy and usefulness of the skim milk progesterone pregnancy test on day 21 post-breeding, and to examine the effects of using different cutoff values and conception rates on predictive values of this pregnancy test. Results of the milk progesterone test were compared to pregnancy diagnosis after 25 days post-breeding using transrectal ultrasonography and palpation as a non-perfect gold standard. The conception rate of the study population based on transrectal palpation was 42%. The relative sensitivity, specificity, and positive and negative predictive values of the milk progesterone test at a cutoff level of 1 ng/ml were 90%, 67%, 66% and 90%, respectively. These results confirm that in a herd population with a conception rate of 42%, the milk progesterone test is not a good predictor of pregnancy because the proportion of false positive results was high at 19% (37/193). The capacity of the test to detect open cows is acceptable with an overall proportion of false negative tests of 4% (8/193). Because 8 out of 83 cows tested negative were subsequently diagnosed pregnant by transrectal examinations, this milk progesterone test would be expected to result in a 10% probability of misdiagnosing pregnant cows as non-pregnant. If this test was applied in the field, the 1 ng/ml cutoff value would be expected to result in optimal probabilities (>90%) in predicting non-pregnancy in dairy herds with a conception rate of less than 50%. Herds with a conception rate over 50% would not be expected to benefit as much from the test since the predictive value of a negative test is less than 90%. Finally, considering that a false negative diagnosis is more costly than a false positive, the optimal cutoff value was also calculated at a three to one ratio in favor of finding fewer false negatives. In this case, the optimal cutoff value for the herd with a 42% conception rate was 1.2 ng/ml. This study shows that the usefulness of the progesterone test relies on assessing the right cutoff level for the milk progesterone pregnancy test which relies not only on the measurement itself, but also on the expected conception rate and the ratio between false negative and false positive test results.

Introduction

In industrialized countries, it is very costly when dairy cows are open for more than 90-100 days.[5,8] For profitable lifetime production, the calving interval for dairy cows should be approximately 12 to 13.5 months. To achieve this objective, detection of non-pregnant cows as soon as possible following insemination is necessary

in order to decide whether to shorten the next ovulatory cycle and reinseminate, or alternatively to cull the cows from the herd.

Physical detection of the gravid uterine horn by transrectal palpation and more recently visualization of the embryo by ultrasonography have been the methods of choice to confirm pregnancy between days 25 and 45 post-insemination.[6,19] Prior to day 25, because the embryo cannot be easily detected by ultrasonography, the estimation of serum or milk progesterone concentrations between 21 and 24 days post-insemination is still the most practical test to determine early pregnancy of dairy cows.[11,13]

Progesterone is not a pregnancy-specific hormone *per se*. The main purpose of measuring progesterone concentrations between days 21 and 24 post-insemination is not to detect the pregnant cows. It is to identify cows that have failed to conceive or had early embryonic death before maternal recognition of pregnancy, and have thus undergone luteolysis. In a recent field study conducted on 472 cows to determine the usefulness of a whole milk progesterone pregnancy test at 21 days post-breeding, Rajamenhendran *et al*[13] reported a test specificity of 57.5% based on a cutoff level of 1 ng/ml. At a conception rate of 53%, the predictive value of a negative result (progesterone < 1 ng/ml) was 94% and in accordance with other studies.[7,11] The low specificity of the test can be attributed, in part, to non-pregnant cows experiencing a later decline in progesterone due to estrous cycles being longer than 21 days, and to a proportion of cows experiencing early embryonic losses.[6,7,13]

There is considerable variation in the literature concerning the concentration of milk progesterone used as a cutoff level for pregnancy diagnosis. Cutoff values of 1, 1.5 and 3 ng/ml have been used.[2,11,13] Higher values were proposed when the assay for milk progesterone was first developed twenty-five years ago.[9,12] Several factors can explain these differences, such as the collection and processing of the milk sample itself, the laboratory assay method used, and finally the choice of a cutoff level to optimize the predictive value of the diagnostic test. Progesterone is soluble in fat and therefore the absolute concentration is higher in whole milk than in skim milk.[12] Whole milk progesterone concentrations increase with the increase in fat content of evening milkings compared to morning samples.[9] Similarly, content of fat and progesterone in whole milk is higher in composite bulk milk and hand-drawn post-milking strippings compared to hand-drawn fore-milk samples.[9,12] When producing skim milk samples, the progesterone content decreases if the temperature of the whole milk kept at 39°F (4°C) is allowed to increase to room temperature prior to centrifugation. This is due to a reversible physical phenomenon of temperature-dependent solubility of progesterone in the milk fat fraction.[4,10] The precision of the laboratory assay method used also has an effect. For instance, the use of more popular direct assay kits developed for human serum overestimate the absolute concentrations of progesterone in cow milk compared to the more cumbersome extraction procedure.[16] For the reasons mentioned above, the type of milk sample analyzed as well as the progesterone assay method used need to be standardized to enable the bovine practitioner to make the best use of the test.

Finally, the choice of an appropriate cutoff value of progesterone to optimize the predictive values of the diagnostic test will also have an impact on the interpretation of the test result. The sensitivity and specificity of the test, which are absolute properties of the test, depend on the cutoff value and the validity of the gold standard.[15] The positive and negative predictive values of the pregnancy test, which are more important to the practitioner, are relative, varying in response to changes not only in test sensitivity and specificity, but also in response to changes in the conception rate of the study population (or prevalence).[15] For all these reasons, it is very important for the bovine practitioner to better understand what can affect milk progesterone levels and to be more critical about interpretation of the test results.

Objectives of the present field study were to 1) evaluate the relative sensitivity, specificity and predictive values of a quantitative milk progesterone assay performed at Day 21 post-insemination on pregnancy status confirmed by transrectal ultrasonography and palpation after Day 25, 2) examine the effect of using different cutoff values of progesterone on pregnancy test performance and 3) evaluate the applicability of such a test in relation to different simulated herd conception rates in dairy cattle.

Materials and Methods

Herd selection and experimental protocol

Three dairy herds followed on a monthly herd health program (Ambulatory Clinic of the Faculty of Veterinary Medicine, Université de Montréal, Québec, Canada) were used in the study. These herds had a 305d equivalent (305ME) milk production average of 21,450 lb (9750 kg). One hundred and ninety three artificial inseminations (AI) were performed between February 13, 1995 and November 14, 1995, and cows were followed intensively according to the schedule presented in Figure 1. At Day 21 post-insemination, dairy producers were asked to collect morning post-milking samples in plastic tubes containing potassium dichromate as a preservative. On a weekly basis, the participating farms were visited by the same bovine practitioner who performed pregnancy examinations and brought the milk samples back to the laboratory. Samples were kept at

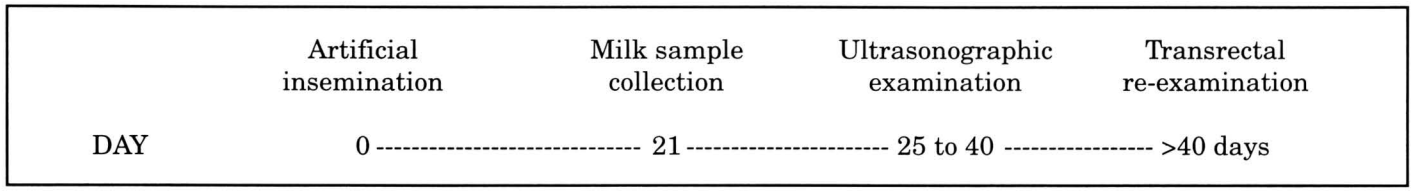

Figure 1. Schedule of events following artificial insemination of dairy cows in order to evaluate the milk progesterone pregnancy test on Day 21 post-breeding.

the farm at 39°F (4°C) for up to one week. They were brought back to the laboratory and kept at 39°F (4°C) until centrifugation at 3000 RPM for 10 minutes at 39°F (4°C). Following centrifugation, the fat layer was removed by aspiration. The skim milk was kept at -4°F (-20°C) until assayed for progesterone. Progesterone was measured by solid phase radio-immunoassay[a] following the manufacturer's specification, adapted according to Srikandakumar *et al* using human serum standards diluted 1:3 in a pool of low progesterone skim milk.[16] Reference standards contained 0.025 to 10 ng/ml. Samples were measured in duplicate with a mean within assay coefficient of variation of 4%. Four quality control samples were measured in each assay and the average values ± 1 standard deviation were 0.34 ± 0.09, 1.28 ± 0.23, 1.73 ± 0.32, 3.21 ± 0.47 ng/ ml, and their respective coefficients of variation were 27%, 18%, 19% and 15%. The absolute sensitivity (lowest detectable value) of the assay was 0.02 ng/ml.

Farmers were asked to maintain a written daily log of any physical signs to determine estrous and breeding dates. Transrectal ultrasonographic examinations were performed between days 25 and 40 post-insemination with a portable ultrasound scanner[b] using a 5 MHz rectal probe. The ultrasound examination was then followed by a transrectal palpation pregnancy diagnosis performed after day 40 post-insemination unless cows were rebred in the interim.

Definitions

Cows were assumed to be non-pregnant if they demonstrated signs of heat, were rebred and/or confirmed open by transrectal ultrasonography and palpation. On the other hand, cows were declared pregnant to a given AI if they were confirmed pregnant by the veterinarian with transrectal ultrasonography or palpation. Hereafter, the transrectal pregnancy examinations refer to the ultrasonography and palpation results of the pregnancy outcome definitions described above.

Statistical Analysis

Statistical analysis was performed using SAS statistical software.[14] A contingency 2 by 2 table was created to compute the progesterone test relative sensitivity, specificity, positive and negative predictive values using the pregnancy outcome definitions described above as the non-perfect gold standard. The term relative is used to describe the test sensitivity and specificity because the gold standard is only an estimation of the true conception rate since it does not take into account early embryonic mortality that could have occurred prior to ultrasonography.

The formula described below was used to find the cutoff value according to the economic weight pertaining to the cost of a misdiagnosed pregnant cow (false negative) compared to a misdiagnosed non-pregnant cow (false positive). Once the false negative to false positive ratio and the probability of pregnancy (i.e conception rate) are given, the sensitivity becomes a function of specificity. The calculations were done using a 1:3 ratio with pregnancy probabilities of 28% and 42%.

$$\frac{False(negative)}{False(positive)} = \frac{Prob(pregnancy)*(1-Sensitivity)}{(1-Prob(pregnancy))*(1-Specificity)}$$

Results and Discussion

Results of the 21-day milk progesterone test compared to transrectal pregnancy examinations are presented in Table 1. The apparent conception rate of the study population based on transrectal palpation was 42% (81/193). At this prevalence, the relative sensitivity, specificity, positive and negative predictive values of the milk progesterone test at Day 21 using a cutoff level of 1 ng/ml were 90%, 67%, 66% and 90%, respectively. The high sensitivity of the test indicates that 90% of the 81 transrectally diagnosed pregnant cows had a milk progesterone value equal to or greater than 1 ng/ml on Day 21 post-insemination. However, the specificity of 67% indicates that only 2/3 of the transrectally diagnosed non-pregnant cows had progesterone concentrations below 1 ng/ml, and thus 1/3 of non-pregnant cows had progesterone values above this threshold and were wrongly declared pregnant. These false positive results can be attributed, in part, to non-pregnant cows experiencing a later decline in progesterone due to estrous cycles being longer than 21 days

and/or to a proportion of cows experiencing early embryonic losses. According to the literature, early embryonic losses before 42 days post-insemination should range between 10 and 20%.[6,7,17,18] Because the relative sensitivity and specificity assessment for milk progesterone at Day 21 post-insemination are in relation to a non-perfect gold standard measured after this time,[3] it is not possible to determine precisely if false positive progesterone test results are due predominantly to cows experiencing long estrous cycles, to cows that have had early embryonic mortality or to cows that had been bred 21 days previously at the wrong time on misdiagnosed estrous signs by the dairyman. To evaluate the risk of embryonic mortality in the absence of a gold standard we have proposed the use of the Gibbs sampling method.[1]

With a positive predictive value of 66%, 2/3 of cows with a milk progesterone level equal to or greater than 1 ng/ml were correctly diagnosed as pregnant, but 1/3 were misdiagnosed as being pregnant. These findings are in general agreement with other studies that have reported a positive predictive value ranging from 72 to 77%[11,13] and confirm that the milk progesterone test is not a very good indicator of pregnancy. On the other hand, the negative predictive value of 90 % means that 75 out of 83 cows tested negative (i.e. with milk progesterone levels below 1 ng/ml) were correctly diagnosed as non-pregnant with the remaining 8 cows misdiagnosed as being open. Previous studies have reported a negative predictive value of milk progesterone tests ranging from 94 to 97%.[7,11,13] Apparent differences between the present study and those previously reported are discussed below after examining the effect of changing the cutoff value.

Table 1. Results of the milk progesterone test performed on Day 21 post-breeding compared to transrectal pregnancy examinations (ultrasonography and palpation) performed after 25 days post-insemination.

	Pregnant (+)	Non-Pregnant (-)	Total
Progesterone (+) ≥ 1 ng / ml	73	37	110
Progesterone (-) < 1 ng / ml	8	75	83
Total	81	112	193

Apparent prevalence or conception rate is 42%
Test sensitivity = 90% (73/81) and specificity = 67% (75/112)
Predictive value of a positive test = 66% (73/110)
Predicitive value of a negative test = 90% (75/83)

It is clear that comparing pregnancy test performance between studies using milk progesterone determinations at Day 21 post-breeding requires that all the diagnostic test parameters, including the conception rates, are available, as well as the sampling and assay methodology. In this respect, the study of Rajamahendran *et al*[13] can be compared to the present study. Both studies used a cutoff value of milk progesterone of 1 ng/ml. However, Rajamahendran *et al*[13] used whole milk and the direct assay using human standards only, whereas we used skim milk and human standards diluted in bovine milk. It would be reasonable to say that based on previous findings[12] the 1 ng/ml cutoff value of the present study using skim milk is equivalent to a higher cutoff value measured in whole milk in the study by Rajamahendran *et al*.[13] Therefore, in relative terms, the 1 ng/ml cutoff of their study would correspond to a lower cutoff value than that of our study. The presumed lower cutoff value is supported by the test characteristics which show a higher sensitivity (97% versus 90%) but lower specificity (57% versus 67%) in their study compared to the present study. Therefore the results of both studies are similar scientifically and any differences are likely due to a combination of many factors, including different absolute cutoff values associated with the nature of the milk sample used (whole milk versus skim milk), different conception rates, and use of different non-perfect gold standards.

The effect of changing the cutoff levels of progesterone from 0.8 to 3.0 ng/ml are shown in Table 2. Increasing the cutoff level decreases the sensitivity of the test but increases the predictive value of positive tests from 65 to 76%. It is apparent from this table that the positive predictive value is inversely related to the negative predictive value. Choosing the optimal progesterone cutoff value is an economical issue where the cost of a false positive needs to be weighed against the cost of a false negative. However, it is reasonable to say that losses for a cow that is pregnant, but declared open (false negative), are higher than losses for a cow that is open, but declared pregnant (false positive). Some additional costs of a false negative would be due to hormone treatments to return to estrus, heat detection, rebreeding, plus additional feed costs. Therefore we hypothesized that a false negative could be three times more costly than a false positive and calculated the adjusted cutoff value according to the formula described in the materials and methods. Using a probability of pregnancy of 42% in the study population of the present study and a three to one ratio in favor of finding a false positive rather than a false negative, it was calculated that a cutoff value of 1.2 ng/ml should be used. By comparison, using the same ratio in favor of declaring more false positives but in herds experiencing a lower conception rate of 28%, the cal-

Table 2. Effects of changing the progesterone cutoff values on relative sensitivity, specificity and predictive values of the milk progesterone test compared to transrectal pregnancy examinations (ultrasonography and palpation) performed after 25 days post-insemination in herds having an overall conception rate of 42%.

Progesterone Cutoff Concentration (ng/ml)	Relative Sensitivity	Relative Specificity	Positive Predictive Value	Negative Predictive Value
0.8	91%	64%	65%	91%
0.9	91%	64%	65%	91%
1.0	90%	67%	66%	90%
1.1	89%	68%	67%	89%
1.2	88%	71%	68%	89%
1.4	83%	74%	70%	86%
1.5	82%	78%	73%	85%
2.0	68%	82%	73%	78%
3.0	36%	92%	76%	67%

culated cutoff value should be adjusted to 1.5 ng/ml. Therefore, for any given conception rate and economically determined ratio of false negative to false positive, it is possible to find an appropriate cutoff value that would make the test useful.

Furthermore, it has been shown that prevalence affects the diagnostic test parameters.[15] For example, when using a fixed cutoff value of 1 ng/ml in herds with different simulated conception rates, the positive and negative predictive values of the milk progesterone test performed at Day 21 are shown in Table 3. This simulation shows that a 1 ng/ml cutoff value would be best suited to improve detection of non-pregnant cows in herds where the conception rate is less than 50%, whereas herds with a conception rate above 50% would not benefit as much from the progesterone test for early detection of open cows. This again shows the importance of adjusting the cutoff value to the expected conception rate of a given herd.

All of these factors are difficult to consider together and many of them are dynamic and change over time. This might explain why the milk progesterone test is not used extensively in the field by bovine practitioners in order to determine pregnancy status in dairy cows.

Conclusions

This study illustrates the importance of assessing the right cutoff level for the milk progesterone pregnancy test which relies not only on the measurement itself (type of milk sample submitted, assay method used), but also on the conception rate, and the economic weight of the decision in favor of a balance between false negative and false positive results.

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Footnotes

[a]Coat-A-Count, Diagnostic Products Corp, Mississauga, Ontario, CANADA

Table 3. Positive (PPV) and negative (NPV) predictive values of the milk progesterone test based on a cutoff value of 1 ng / ml, a test sensitivity of 90%, a test specificity of 67% and different simulated conception rates (CR).

CR	25%	30%	35%	40%	45%	50%	55%	60%
PPV	48%	54%	60%	64%	70%	74%	77%	81%
NPV	96%	94%	94%	91%	90%	87%	86%	82%

[b]Echo 900, Alliance Medical Inc., Montréal, Québec, CANADA

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Abstract

Bovine Viral Diarrhoea Virus and Ovarian Function in Cattle

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Bovine viral diarrhoea virus (BVDV) is endemic throughout the world and is acknowledged as a major cattle pathogen responsible for a spectrum of symptoms, including reproductive failure. The endemic nature of BVDV means that susceptible cattle are under constant threat of infection through direct contact or the use of contaminated semen, embryos, bovine sera etc. In this article we review some experimental data which investigated the distribution of BVDV antigens within ovarian tissue recovered from 3 BVDV infected heifers and one BVDV naïve animal. In a separate study, the effects of an acute infection 2 days before oestrus on oestradiol, progesterone and prostaglandin secretion was analyzed.

BVDV antigens were localized in ovarian sections by indirect immunofluorescence using a monoclonal antibody, raised against the non-structural protein NS3. BVDV was widely distributed within the ovarian stroma, the follicular cells and oocytes. Detectable levels of BVDV antigen were present in 18.7% of the oocytes. The proportion of antigen positive oocytes did not differ ($P>0.05$) between the primordial 227/1247 (18.2%), primary 122/630 (19.4%) and secondary 13/62 (21.0%) follicle populations.

To assess the effects of BVDV infection on ovarian/endometrial function oestradiol, progesterone and PGF2α metabolite (PGFM) levels were analyzed in plasma samples collected from 14 cows during a synchronized oestrous cycle. Seven cows were challenged with non-cytopathogenic BVDV so that peak viraemia occurred during the initial period of luteal development. The remaining 7 cows served as control animals and remained BVDV negative throughout the study. Leucopenia, viraemia and BVDV neutralizing antibodies were detected in the 7 cows challenged with BVDV. In addition, the BVDV challenge significantly ($P<0.01$) reduced plasma oestradiol levels between Day 4 and Day 9 post oestrus (6 to 11 days post challenge) although the plasma concentrations of progesterone and PGFM did not differ from the controls.

From these experiments we conclude that bovine follicular cells and oocytes are permissive to BVDV infection at all stages of development and that BVDV can transiently suppress oestradiol secretion. These data highlight two potential routes by which BVDV may reduce fertility in the cow, namely impairment of oocyte quality and disruption of gonadal steroidogenesis.