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THE EVALUATION OF FAT TO PROTEIN RATIO IN MILK AS AN INDICATOR OF CALVING TO CONCEPTION INTERVAL IN DAIRY COWS USING VARIOUS BIOSTATISTICAL METHODS

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The effect of fat to protein ratio (FPR) in milk for the prediction of calving to conception interval (CC) in dairy cows is evaluated using different biostatistics' methods.

Spearman rank correlation coefficient, a non-parametric alternative to the Pearson correlation coefficient was used to determine the correlation between reproductive parameters of the herd and the milk data record. In the time interval of 60 to 90 days postpartum the highest correlation was found between FRP and CC (r=0.415, p<0.05).

FPR was diagnostically evaluated using ROC (receiver operating characteristics) analysis which is based on completed 2x2 tables. A complete ROC analysis, including the area under the curve (AUC), provides an index of accuracy by demonstrating the limits of FPR's ability to discriminate between cows with different CC interval. The optimal cut-off value of FPR at 1.34 provided the best discrimination power according to CC of 120 days. A cut-off value of FPR at 1.1 was selected to enable over 90% correct identification of cows with CC below 120 days. On the other hand, cows with FPR above 1.44 were over 90% correctly identified as cows with CC above 120 days.

Kaplan-Meier survival curves show a significant difference in CC between cows with FPR lower and upper of 1.34. CC lower than 120 days was observed in 80 % of cows with FPR lower than 1.34 and only in 40% of cows with FPR higher than 1.34.

The FPR has been shown to be of benefit in the prediction of reproductive efficiency in dairy cows.

Key words: calving to conception interval, correlation coefficient, dairy cows, fat to protein ratio, ROC analysis, survival analysis

INTRODUCTION

Milk production curve rapidly rises in early postpartum period and reaches maximum in the first third of lactation, whereas animal consumption ability rises slowly and can not follow increased needs for the nutrients. Therefore, dairy cows enter a period of negative energy balance (NEB), which leads to mobilization of body reserves, mainly fat, to balance the deficit between food energy intake and production requirements (Huer *et al*., 1999; Opsomer *et al*., 2000). Increased fat mobilization and a decrease in dry matter and energy intake are shown in higher milk fat concentration and lower milk protein concentration in the postpartum period (Eicher, 2004; Čejna and Chladek, 2005). NEB is associated with the change in body condition score (BCS) and the rise of some metabolites such as non esterified fatty acids (NEFA) and beta-hydroxy butyrate (BHB) (Opsomer, 1999; van Knegsel *et al*., 2005; Vanholder *et al*., 2005b) and on the other hand a decrease of glucose, cholesterol and Insulin like Growth Factors-1 (IGF-1) (Nebel and McGilliard, 1993; Opsomer *et al*. 1999; Klinkon *et al*., 2001). Increased milk fat occurs in cows in NEB or ketotic ones, presumably because of the increased availability of BHB and fatty acids for milk fat synthesis (Podpečan *et al*., 2007a). In adult females, dietary intake acts at various levels within the hypothalamus-pituitary-ovarian axis to influence ovarian activity and is a key factor regulating embryo survival during pregnancy (Armstrong *et al*., 2003). The rate of body reserves mobilization is directly related to postpartum interval, first ovulation, and lower conception rate (Butler and Smith, 1989).

In our previous study we showed that fat to protein ratio in milk could be an indicator of the ability of a cow to adapt to the demands of milk production and reproductive efficiency in the post partum period resulting in a prolonged postpartum period (Podpečan *et al*., 2007b).

In this study we demonstrated the evaluation of FPR using different biostatistical approaches (Dawson-Saunders and Trapp, 1994; Petrie and Watson, 1999a). FPR was diagnostically evaluated using complete ROC analysis. The performance of diagnostic tests is usually described in terms of sensitivity and specificity (Jones and Payne, 1997). A complete ROC analysis, including AUC, provides an index of accuracy by demonstrating the limits of a test's ability to discriminate between cows with different CC (Zwieg and Campbell, 1993). Survival analysis methods are often used to analyse data from dairy herds where the outcome of interest is the interval from calving to conception. Data collected form dairy cattle often are censored because cows are lost from the study due to death or culling or because the study ends before the event is observed (Allore *et al*., 2001). Kaplan-Meier survival curves, used in our study, showed the association between portion of non-pregnant cows and duration of postpartum period.

MATERIAL AND METHODS

Animal and data records

Our study was a model for the evaluation of FPR value. We focused on pregnant cows, where different CC was observed. The following reproduction parameters were calculated from farm recording data of 51 high yielding dairy cows (25 Holstein-Freisien and 26 Brown-Swiss): calving to 1st services (CFS), calving to conception interval (CC) and services per conception (SPC).

An average lactation of the herd was 8500 L of milk in 305 days and average parity of cows, included in the study was 2.64. Voluntary waiting period of the herd was 80 days. They were inseminated by a well trained inseminator. Cows were kept in a free-stall barns system and fed total mixed ration. Basic ration was composed of hay, grass and maize silage. Basic ration was individually supplemented with protein concentrate (19% digestible raw protein), as well as roughly crushed maize grains and vitamin-mineral mixture according to the production and milk yield. All cows could access food and water ad libidum during the whole year in the stall.

Milk sampling and analysis

Milk samples were collected and daily milk yield was measured by a regular dairy control in 30 day intervals in post partum period at three stages: 0 to 30, 30 to 60 and 60 to 90 days post partum corresponding to Stage 1, 2 and 3, respectively.

Samples were conserved using sodium azide and sent at outdoor temperature to a dairy research laboratory. Protein, fat, lactose and urea were analysed in milk samples using Fourier Transform Infrared Spectroscopy (CombiFoss 6000).

Biostatistical analysis

Correlation coefficients, parametric and non-parametric tests

Non-parametric Spearman rank correlation coefficient was used to determine the correlation between reproductive parameters and milk data records.

The assumptions of the parametric tests were not satisfied due to the number of the observations or to their distribution; therefore we used the alternative type of tests, named non parametric, which do not make any distributional assumptions about the data. These tests are appropriate for small samples that are not normally distributed. The analyses in non-parametric tests are performed on the ranks of the observations instead of the original observations (Dawson-Saunders and Trapp, 1994; Petrie and Watson, 1999a).

Receiver operating characteristic (ROC) analysis

Receiver operating characteristic (ROC) analysis (Analyse-it, General + Clinical Laboratory statistics, version 1.71) was applied to evaluate FPR in the time interval of 60 – 90 days post partum.

Cows were divided into groups A and B according to criteria values of CC at 120 days post partum.

The FPR values were divided into groups of positive (T+) and negative (T-) results as follows:

- a positive test result (T+) was recorded when cows have a FPR below the cut-off value
- a negative test result (T-) was recorded when cows have FPR above the cut-off value.

Diagnostic parameters (specificity and sensitivity) were estimated for each of 51 cut-off values:

- sensitivity (Se) was calculated as the proportion of positive test results (T+) for cows with CC below the criterion value of CC at 120 days

– specificity (Sp) was calculated as the proportion of negative test results (T-) for cows with CC above the criterion value of CC at 120 days.

ROC curves plotted all sensitivity versus 1-specificity for the complete range of cut-off points (Greiner *et al*., 2000). All possible combinations of sensitivity and specificity that can be achieved by changing the test's cut-off value were summarized by a single parameter, i.e. the area under the ROC curve (AUC) (Greiner, 2000).

Selection of optimal cut-off values of FPR that best discriminates cows with different CC were based on the best balance of sensitivity, specificity and Youden index (J=Se+Sp-1) (Weiss *et al*., 2003-2004).

Survival analysis

Using survival analysis we are concerned with the time (the survival time) it takes for some critical event in the individuals (Petrie and Watson, 1999b). In our study survival time is presented with the duration of calving to conception interval, which starts with the parturition and is ended with following pregnancy in a dairy cow. In this case, the event is successfully ended. On the other hand, it could also not be ended, when the cow get not pregnant in defined time, what is indicated as "censored data". Censored data include animals which never experienced the pregnancy during the course of the clinical trial; either they are alive at the end of the study period, but not pregnant, or they are lost to follow-up, or they die during the study period from causes unrelated to the condition of interest. Kaplan-Meier survival curves are derived if the survival and censored times are known exactly. In our study the proportion of non-pregnant animals represents the survival probability, which is plotted against the time from the starting point (time from the previous parturition, i.e. duration of calving to conception interval).

RESULTS

Correlations between milk data records and calving to conception interval in a dairy herd

Milk protein concentration correlates with CC corresponding to r = -0.414; r = -0.485 and r = -0.398 in Stage 1, 2 an 3, respectively (p<0.05). Milk fat and milk yield correlated only with CC in Stage 3 (r = 0.316 and 0.297, respectively; p<0.05).

The highest correlation was observed between FPR and CC in Stage 1 (r = 0.452, p<0.05), whereas significant, but lower correlations was found in Stage 2 (r = 0.358, p<0.05), and in Stage 3 (r = 0.415, p<0.05).

Diagnostic value of FPR

ROC analysis was performed for evaluation of FPR in Stage 3 to distinguish between cows with different CC.

Area under the ROC curve (AUC) at criterion value of 120 days post partum was 0.737 (SE = 0.0777; P = 0.0012). A diagonal line in a ROC plot corresponds to a test that is positive or negative just by chance.

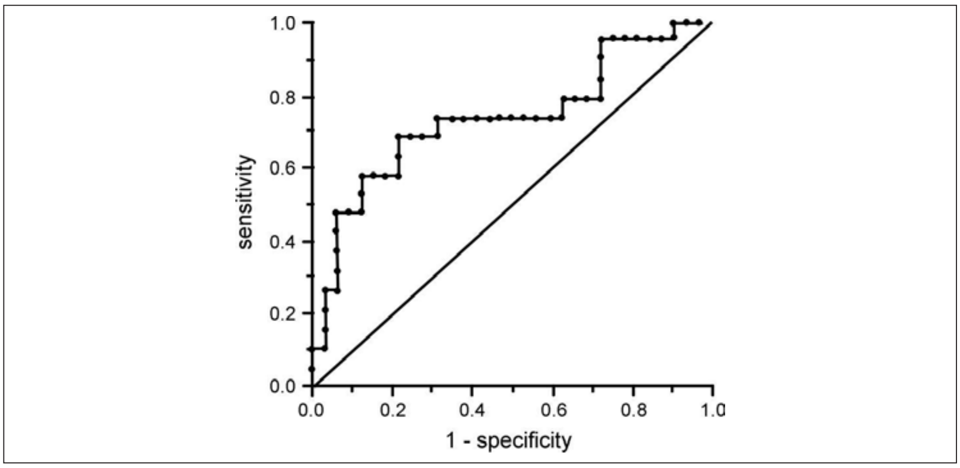

Figure 1. ROC plot of FPR for identifying cows with pre-selected criteria of CC at 120 days

Values of Youden index peaked at a cut-off point of FPR at 1.34 for pre-selected minimum of CC at 120 days (Fig 2). Sensitivity of 68.4 and specificity of 78.1% correspond to this cut-off. The test is more than 90% sensitive at FPR of 1.1 and more than 90% specific at FPR of 1.44.

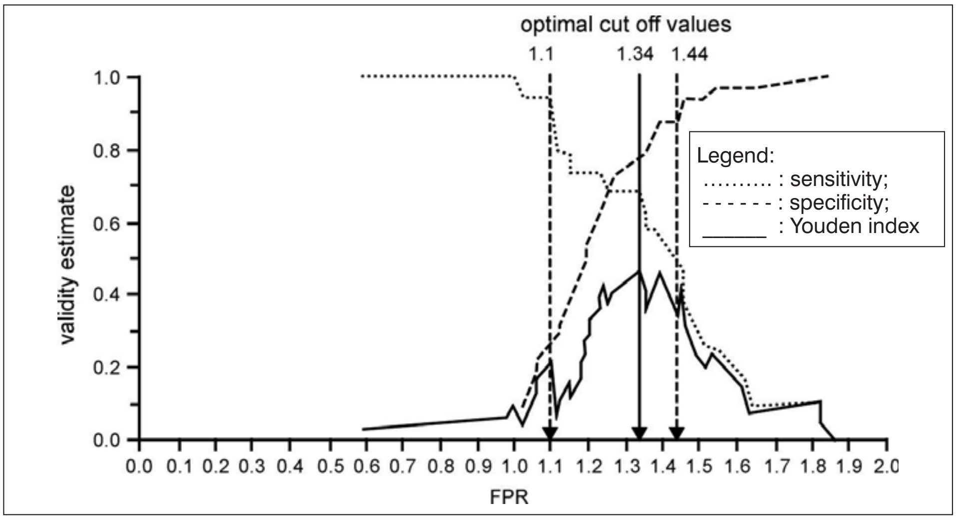

Figure 2. Plot of specificity, sensitivity and Youden index of FPR according to CC of 120 days as a function of the cut-off value of FPR

Evaluation of FPR using Kaplan-Meier survival curves

A significant difference was observed between survival curves performed by Kaplan-Meier survival analysis for groups of cows with different FPR ($P<0.05$). CC lower than 120 days was observed in 80 % of cows with FPR lower than 1.34, whereas 120 days after calving, more than 60% of cows with FPR upper than 1.34 were still not pregnant.

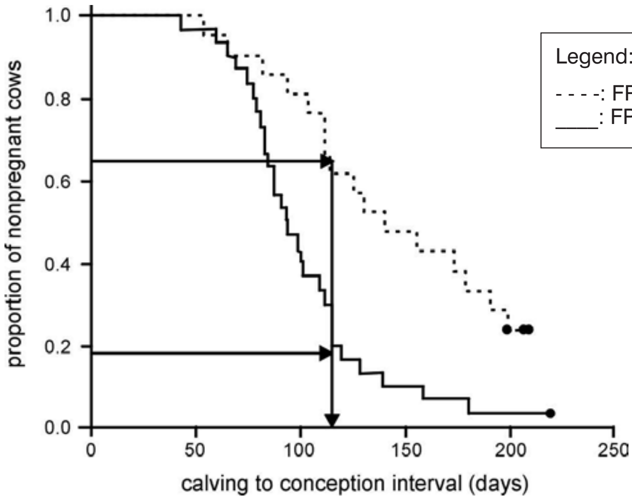

Figure 3. Kaplan-Meier survival curves for groups of cows with different FPR

DISCUSSION

During the last few decades, milk production per cow has intensively increased due to the improvement in nutrition, and genetic selection, whereas fertility in lactating dairy cows has been declining. Researchers from the field of nutrition and digestion in ruminants and reproduction are dealing with the prediction and control of NEB with the aim of diminishing the NEB influence to reproduction disorders and disease development in dairy cows (Reist *et al*., 2002; Eichner, 2004; Čejna and Chladek, 2005; Vanholder *et al*., 2005).

In early postpartum period dairy cows enter a period of negative energy balance (NEB), which leads to mobilization of body reserves, mainly fat, to balance the deficit between food energy intake and production requirements. The rate of body reserves mobilization is directly related to higher milk fat concentration and lower milk protein concentration in postpartum period (Loeffler *et al*., 1999a; Opsomer *et al*., 2000; Reist *et al*., 2002, Huszenisza *et al*., 2006; Vanholder *et al*., 2005b), as well as to the interval to first ovulation and lower conception rate (Butler and Smith, 1989). Therefore, combining protein and fat concentration in FPR may provide a better parameter for evaluating CC than assessing the mentioned characteristics independently. The aim of our study was to evaluate the FPR according to CC using different biostatistical methods.

In the statistical comparison of the results we should use appropriate tests. For example there is a question about using parametric or non- parametric tests. Animal research often results in data sets which are less than perfect according to the number of the observations or to their distribution. Therefore, we should use non-parametric tests, where no distributional assumptions about the data are proposed. These tests are also appropriate for small samples (Dawson-Saunders and Trapp, 1994; Petrie and Watson, 1999a). In our study we calculated Spearman rank correlation coefficient, which is a non-parametric alternative to Pearson correlation coefficient. Significant correlations were found between reproduction parameters and milk data records. The highest correlation was observed between FPR and CC in Stage 1, what is comparable with study of Loeffler *et al*. (1999b) who also showed significant correlations between FPR changes and fertility. They found FPR as a strong to moderate predictor of pregnancy rate. FPR changes were strongly determined by changes in the percentage of milk fat rather then protein.

We evaluated FPR in milk in Stage 3 post partum according to CC at 120 days. The criteria value, mentioned above, were based on reproductive characteristics found in Slovenian dairy herds

The performance of diagnostic tests is usually described in terms of sensitivity and specificity (Jones and Payne, 1997). Receiver operating characteristics (ROC) analysis was used to determine the optimal cut-off value and diagnostic accuracy of FPR to distinguish between cows with different CC. AUC, found to be 0.737, provides an index of accuracy by demonstrating the limits of a test's ability to discriminate between cows with different CC (Zwieg and Campbell, 1993). A plot of sensitivity, specificity and Youden index as a function of the cut-off value provides a useful visualisation and is useful in selecting optimal cut-off values of the investigated parameter. The optimal cut-off value at FPR of 1.34 provided the best discrimination power according to CC of 120 days. This value is found to be characteristic also by Duffield *et al*. (1997) who defined FPR of 1.33 as a high margin. However, in the clinical use of the investigated parameter, it is often important to correctly identify CC below or above a certain value. Therefore, a cut-off value of FPR at 1.1 was selected to enable over 90% correct identification of cows with CC below 120 days. On the other hand, cows with FPR above 1.44 were over 90% correctly identified as cows with CC above 120 days. FPR greater than 1.5 is proposed as a risk factor for metabolic disorders such a ketosis (Duffield *et al*., 1997). However, ketosis has a great influence on reproductive disorders; therefore a special attention is needed in further clinical treatment of such cows. The association between FPR and various reproductive parameters are also reported such as reduced first service per conception rate and increased CC by five days ($p<0.05$) and increased number of SPC by 0.22 ($p<0.01$) in cows with FPR above 1.5 (Heuer *et al*., 2000).

Using survival analysis we are concerned with the time (the survival time) it takes for some critical event in the individuals (Petrie and Watson, 1999b). In our study the time was presented with calving to conception interval and critical event was pregnancy. Kaplan-Meier survival curves show an excellent view on the proportion of non-pregnant cows in each day after parturition. 80% of cows with FPR lower than 1.34 have CC lower than 120 days, whereas 60% of cows with FPR upper than 1.34 were still not pregnant after 120 days after calving.

Results of the present study demonstrate that there is a significant relationship between the FPR in milk samples and the fertility data of these cows. We showed that various biostatistical methods should be used for the evaluation of FPR, because each method provides a unique view on the parameter's characteristics. The results of our study contribute to a complementary tool in the evaluation of metabolic stress in early postpartum period in dairy cows. Moreover, the results will help veterinary practitioners and farmers to analyse fertility problems in dairy herds and to improve their reproductive and productive efficiency.

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PROCENA ODNOSA IZMEĐU MASTI I BELANČEVINA U MLEKU KRAVA U PREDVIĐANJU DUŽINE INTERVALA OD TELJENJA DO KONCEPCIJE RAZLIČITIM BIOSTATISTIČKIM METODAMA

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SADRŽAJ

U radu je opisana upotreba različitih biostatističkih metoda za predviđanje dužine intervala od teljenja do koncepcije kod krava muzara na bazi procene odnosa između masti i belančevina u mleku (M/B). Za izračunavanje korelacije između parametara reprodukcije i rezultata kontrole mleka, korišćen je Spermanov koeficient korelacije ranga, koji predstavlja neparametarijsku alternativu Pearsonovom korelacijskom koeficijentu. Najveći stepen korelacije između masti i belančevina u mleku (M/B) i dužine pauze nakon porođaja je utvrđen između 60. i 90. dana po teljenju ($r=0,415$; $p<0,05$).

Odnos M/B je dijagnostički procenjivan ROC (eng. receiver operating characteristics) analizom koja se bazira na klasifikaciji rezultata u kompletnoj tabeli 2 x 2. Kompletna ROC analiza, uključujući i obračun površine ispod ROC krive (area under the curve - AUC) daje indeks poverenja pomoću koga se može proceniti M/B kao dijagnostički indikator koji omogućava razdvajanje krava na bazi različite dužine intervala od teljenja do koncepcije. Utvrđeno je da vrednost od 1,34 M/B najbolje razdvaja krave sa intervalom od telenja do koncepcije, kraćim ili dužim od 120 dana. Granična vrednost od 1,1 M/B omogućava za više od 90% pouzdanu indentifikaciju krava kod kojih je interval od telenja do koncepcije kraći od 120 dana. U slučajevima kada je M/B veći od 1,44, pouzdanost identifikacije krava koje će imati interval od telenja do koncepcije, duži od 120 dana, je veća od 90%.

Rezultati su takođe obrađeni analizom preživljavanja i krive preživljavanja po Kaplan-Meierjevoj metodi. Krive preživljavanja u podgupi krava sa M/B odnosom većim ili manjim od 1,34 se međusobno statistički značajno razlikuju. Tako je 80% krava sa M/B odnosom manjim od 1,34 imalo interval od teljenja do koncepcije kraći od 120 dana, dok je samo 40% krava koje su imale M/B odnos veći od 1,34 bilo gravidno.

M/B odnos se pokazao kao dijagnostički parametar koji može da pomogne u uspešnom vođenju reprodukcije u stadima krava muzara.