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Cortisol levels in Puerperal Metritic Cows and Assessing Accuracy Using Receiver Operating Characteristics Analysis

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ABSTRACT

The present study evaluated cortisol levels during peripartum period in crossbred cows that did and did not develop puerperal metritis (PM). Accuracy of cortisol to differentiate the cows at the risk of developing PM from healthy cows was measured by receiver operating characteristics (ROC) analysis. Cortisol concentrations were estimated in blood plasma samples collected from pluriparous Karan Fries crossbred cows (n=20) at wk-2, wk-1, d+1, d+7 and d+14 taking the day of calving as zero. The cows that developed PM (n=8) had significantly (P<0.05) 2.34 time higher plasma cortisol than the normal cows (n=12) on d+1 of calving, however such difference was not observed on any other day of sampling. The ROC analysis revealed that plasma cortisol on d+1 could identify cows that developed PM with 79.17% accuracy (P<0.05). Optimum threshold value of plasma cortisol for early identification of PM was observed to be 27.28 ng/ml (sensitivity=75% and specificity=100%) on d+1. The results of the present study indicated that PM cows had higher cortisol levels immediately after calving, and using cortisol the PM cows could be differentiated from normal cows with moderate accuracy.

Keywords: Plasma cortisol, puerperal metritis, accuracy, crossbred cows

Puerperal metritis (PM) in dairy cows is a clinical condition in which there is enlargement of uterus beyond its normal size, red-brown fetid watery uterine discharge with fever (>39.5ºC) and systemic signs of illness such as reduced milk yield, dullness or toxemia within 21 days postpartum (Sheldon *et al*., 2006). In dairy cows, PM reduces milk production and reproductive performance significantly (Sheldon *et al*., 2009; Walsh *et al*., 2011; Kumari *et al*., 2016a). In general, under field condition per rectal examinations of uterus along with uterine discharge are considered as gold standard methods used for diagnosis of PM, but such examination required skilled personnels and most of the times go unnoticed. Therefore, early identification of ensuing PM is an important aspect for better dairy herd health management. Elevated circulating cortisol level has been observed in dairy animals around parturition (Burton *et al*., 2005; Dang *et al*., 2013; Pathak *et al*., 2015; Pathan *et al*., 2015). Elevated cortisol around parturition reduces the functional activity of circulating neutrophils (Burton *et al*., 2005; Pathak *et al*., 2015) and may increase susceptibility to uterine and udder infections immediately after calving. Previous studies reported higher circulating cortisol concentration immediately after calving in purebred high yielder exotic cows affected with uterine infections (Torres *et al*., 1997; Galvao *et al*., 2010). However, Galvao *et al*. (2010) reported lower cortisol level during 2nd week postpartum in those cows suffered with metritis within 21 days after calving. Although, some studies reported association of peripartum plasma cortisol profile with uterine infection, there is paucity of information on such association in crossbred cows affected with PM. With this back drop, present study was designed to measure the plasma cortisol profile during peripartum period in crossbred cows that did and did not develop PM.

Further, we estimated the accuracy of plasma cortisol and its optimum threshold value for early identification of ensuing PM.

MATERIALS AND METHODS

Experimental animals and management

Present study was conducted at Livestock Research Centre, National Dairy Research Institute (NDRI), Karnal and approved by Institutional Animal Ethics Committee (IAEC approval no. 11/10). The study was conducted on healthy dry-pregnant pluriparous Karan Fries crossbred cows (n=20), selected based on their body condition score (3.5-4.5 in 6 point scale, where 1=thin and 6=fat); body weight (450-550 kg); parity (2^{nd}-4^{th} parity) and previous lactation milk yield (3800-4500 kg during 305 days). Cows were maintained in a loose housing system of management and fed as per the feeding schedule followed in the dairy farm. Nutrient requirements of cows were met by seasonal green fodder (berseem, oat and mustard) and concentrate mixture (20% CP and 70% TDN). Dry pregnant cows were fed 1.5-2.0 kg/cow concentrate mixture 21 days prior to expected date of calving till calving and after that fed 1 kg concentrate mixture for every 2.5 kg milk production. The concentrate mixture consisted of maize (33%), ground nut cake (21%), mustered cake (12%), wheat bran (20%), de-oiled rice bran (11%), mineral mixture (2%) and common salt (1%).

Blood sampling and health monitoring

Blood samples were collected at two (wk-2) and one (wk-1) week prior to expected date of calving, and on d+1, d+7 and d+14 after calving using 9 ml blood collection tubes with Heparin as anticoagulant from jugular vein between 8 A.M. to 9 A.M. Blood samples were centrifuged immediately after collection at 1000 g for 20 minutes to separate plasma, and stored at -20°C till further analysis. Plasma cortisol was estimated using Bovine Cortisol ELISA test Kit (Endocrine Technologies, INC. USA). Intra-assay and inter-assay variation was 6.27% and 17.64%, respectively.

Experimental cows were monitored up to 21 days postcalving for any health problems. Uterine fluid was collected weekly twice using sterile blue sheath and universal artificial insemination syringe and assessed by transferring to a clean test tube. The febrile cows (>39.5°C) with systemic illness (decreased milk yield and dullness) and fetid uterine discharge (purulent or red-brown) within 21 days postpartum were diagnosed as PM (Sheldon *et al*., 2006).

STATISTICAL ANALYSIS

Cows were classified into two groups, either PM (n=8) or normal (n=12) after diagnosis. Plasma cortisol value was analyzed by two way analysis of variance (ANOVA) to see the effect of health (with and without PM) and period (wk-2, wk-1, d+1, d+7 and d+14) on this measure. Tukey post hoc test was used to compare all pair-wise differences in mean, difference was considered as significant if $P \leq 0.05$ and results were presented as mean±SE. Further, the time period at which plasma cortisol between PM cows and normal cows differed statistically was analyzed by receiver operating characteristics (ROC) analysis to see the accuracy of plasma cortisol to classify cows with and without PM, and to develope its optimum threshold value for early identification of PM. Statistical analysis was done using Sigmaplot 11 software package (Systat software, Inc, California, USA). In ROC analysis, accuracy of a diagnostic indicator (here cortisol) is interpreted based on area under the ROC curve (AUC) which is a two dimensional graph (Sensitivity plotted in Y-axis and 1-pecificity in X-axis, for the range of potential threshold values). Accuracy is said to be non-discriminative or non-informative if AUC is 0.5 and perfect if AUC is 1.0; less accurate if AUC is 0.5-0.7, moderately accurate if AUC is 0.7-0.9 and highly accurate if AUC is 0.9-1.0 (Swets, 1988; Patbandha *et al*., 2013).

Additionally, ROC analysis produces range of potential threshold values of a diagnostic indicator, but the value having maximum combined Sensitivity (Se) and Specificity (Sp) is called as optimum threshold value of that indicator (Patbandha *et al*., 2013). The Se of a particular threshold depends on number of true positive (TP) and false negative (FN) cases; on the other hand, Sp depends on number of true negative (TN) and false positive (FP) cases. Furthermore, both Se and Sp together define the positive likelihood ratio (LR+). In the present study, TP indicated the number of PM cows having plasma cortisol above the threshold value and FP indicates the number of normal cows having plasma cortisol above the threshold value. The TN indicates the number of normal cows having plasma cortisol below the threshold value and FN indicates the number of PM cows having plasma cortisol below the threshold value (Table 1). Here, Se was defined as the proportion of PM cows with plasma cortisol concentration above the threshold value [(TP)/(TP+FN)] and Sp was the proportion of normal cows with plasma cortisol below the threshold value [(TN)/(TN+FP)] (Table 1). The LR+ was defined as the number of times a cow with elevated plasma cortisol level above the threshold value was more likely to develop PM [LR+ =Se/(1-Sp)] compared to those with plasma cortisol level below the threshold value (Peterson *et al*., 2008; Patbandha *et al*., 2013).

RESULTS AND DISCUSSION

Plasma cortisol

Puerperal metritis (PM) is an early postpartum uterine infection which negatively affects the productive as well as reproductive performances in dairy animals. Therefore, early identification of cows that are susceptible to PM would be helpful for implementation of managemental interventions and subsequently the impact could be minimized. In the present study, after diagnosis of puerperal metritis (PM), cows were divided into two groups i.e., PM and normal, and their plasma cortisol concentrations during peripartum period were compared retrospectively. Overall mean plasma cortisol level during the experimental period, from wk-2 to d+14 did not differ statistically between PM cows and normal cows (15.07±1.99 vs. 11.67±1.63 ng/ml, Table 2, $P>0.05$). However, mean plasma cortisol of cows with PM had significantly 2.34 times higher concentration on d+1 postpartum (38.85±9.10 vs. 16.58±2.00 ng/ml, respectively, Table 2, $P<0.01$), but at other periods did not differ statistically. Higher plasma cortisol in PM cows immediately after calving (i.e., on d+1) compared to normal cows is similar to the previous report (Galvao *et al*., 2010). Higher cortisol around parturition in PM cows suppresses functional activity of neutrophils (Galvao *et al*., 2010; Huzzey *et al*., 2011; Pathak *et al*., 2015; Pathan *et al*., 2015) and may subsequently increase susceptibility to uterine infections (Hammon *et al*., 2006). Additionally, Galvao *et al*. (2010) reported lower cortisol level on 7th day postpartum which is supported by our result, but contrary to Galvao *et al*. (2010), we did not observe any significant variation of plasma cortisol concentration between PM and healthy cows on 14th day post partum.

Table 1: Confusion matrix for binary outcomes

Outcome based on diagnostic indicator test	Outcome based on gold standard test	
	Positive	**Negative**
Positive	TP	FP
Negative	FN	TN

TP, true positive; FP, false positive; FN, false negative; TN, true negative

Table 2: Plasma cortisol profile (ng/ml) during peripartum period in Normal and Puerperal metritic cows

Weeks/days relative to calving	Normal cows (n=12)	Puerperal Metritic cows (n=8)
wk-2	10.72 ± 4.35^{aA}	09.39 ± 3.16^{aA}
wk-1	09.28 ± 2.50^{aA}	13.78 ± 5.28^{aA}
d+1	16.58 ± 2.00^{aA}	38.85 ± 9.10^{bB}
d+7	14.86 ± 4.57^{aA}	05.81 ± 1.43^{aA}
d+14	06.87 ± 1.16^{aA}	07.51 ± 3.50^{aA}
Overall	11.67 ± 1.63^{A}	15.07 ± 1.99^{A}

Means with different superscript in a column (a, b) and in a row (A, B) differed significantly ($P<0.05$).

Table 3: Receiver operating characteristic analysis for optimum threshold value of plasma cortisol on d+1 postpartum

Threshold value (ng/ml)	**Se (%)**	**95% CI for Se**	**Sp (%)**	**95% CI for Sp**	**LR+**
12.28	87.5	47.35-99.68	33.33	9.92-61.11	1.31
18.90	75.0	34.91-96.81	50.0	21.09-78.91	1.50
21.06	75.0	34.91-96.81	75.0	42.81-94.51	3.00
21.90	75.0	34.91-96.81	83.33	51.59-97.91	4.49
24.60	75.0	34.91-96.81	91.67	61.52-99.79	9.00
27.28*	75.0	34.91-96.81	100.0	73.54-100.0	infinite
29.66	62.5	24.49-91.48	100.0	73.54-100.0	infinite
34.29	50.0	15.70-84.30	100.0	73.54-100.0	infinite

Se, sensitivity; Sp, specificity; CI, confidence interval. * Optimum cut off value.

Previous studies reported increased cortisol level in crossbred cows (Dang *et al*., 2013; Pathak *et al*., 2015), and zebu cattle and buffaloes (Pathan *et al*., 2015) around and immediately after parturition compared to pre- and post-partum periods. Similarly, we also observed higher level of cortisol on d+1 immediately after calving compared to pre- and postpartum periods. However, there was significant interaction of health and period (P<0.05), which indicated that in PM cows the plasma cortisol level increased significantly (P<0.05) from wk-2 to d+1 and then declined significantly (P<0.05) to d+7. Although cortisol level in healthy cows increased on d+1 compared to other periods, the difference was not statistically significant. Higher cortisol concentration around and immediately after calving in both normal and PM cows observed in the present study indicates that they experienced more stress compared to pre- and post-partum period, which may be associated with parturition stress. Further, PM cows on d+1 after calving experienced more stress (as revealed by high cortisol concentrations) compared to those cows which did not develop puerperal metritis.

Receiver Operating Characteristic (ROC) analysis

In the present study, plasma cortisol concentration of PM cows and normal cows differed statistically on d+1; hence we analyzed the results using ROC analysis to measure its accuracy to differentiate PM cows from normal cows and to identify an optimum threshold value for early identification of ensuing PM. Accuracy in ROC analysis is interpreted based on AUC and must be greater than 0.5 (Swets, 1988; Patbandha *et al*., 2013). In the present study, the AUC of plasma cortisol on d+1 was observed to be 0.791 (AUC=0.791, P=0.03). The AUC of 0.791 indicated that plasma cortisol on d+1 classified PM cows from healthy cows with 79.1% accuracy. As, AUC does not depends on the threshold value or prevalence of metritis; hence, considered as good measure of accuracy (Kumari *et al*., 2016b). Although plasma cortisol classified PM cows from healthy cows significantly on d+1, the accuracy (i.e., 79.1%) was considered as moderate.

The ROC analysis produced range of potential threshold values (Table 3), but the plasma cortisol threshold value i.e., 27.28 ng/ml was considered as optimum due to maximum combined Se (75%) and Sp (100%). The Se of 75% indicates that at optimum threshold value of plasma cortisol, 75% PM cows could be correctly identified (TP cases) but 25% PM cows may go unidentified (FN cases). However, Sp of 100% indicated that at optimum threshold value of plasma cortisol, all normal cows could be correctly identified (TN cases) and false positive cases would be zero percent. Previous study reported optimum threshold value of plasma cortisol level as 34.1 nmol/l (12.36 ng/ml) at wk-2 before calving (Huzzey *et al*., 2011). Higher plasma cortisol optimum threshold value in our study might be attributed to the time period relative to calving and the disease condition studied, as Huzzey *et al*. (2011) used plasma threshold at two weeks before calving for identification of cows those developed puerperal conditions like retained fetal membranes, displaced abomasum and ketosis.

In the present study, positive likelihood (LR+) indicates the number of time a cow with elevated plasma cortisol concentration above the threshold value is more likely to develop PM than the cows with plasma cortisol below the threshold value. Although literatures does not report direct association of plasma cortisol with PM, Huzzey *et al*. (2011) reported increased chance of puerperal conditions like retained fetal membranes, displaced abomasum and ketosis in cows with elevated plasma cortisol beyond 12.36 ng/ml. However, in our study, the chance of PM increased by 1.31 times in cows with plasma cortisol >12.28 ng/ml than those with plasma cortisol level below the threshold value, but at this threshold the false positive cases were more i.e. 66.67% (Table 3). Thus, while selecting threshold value of biological indicators for early identification of abnormal health disorder in dairy cows, the detection level of false positive and false negative should be lower at that threshold.

CONCLUSION

The results of the present study indicated that the cows, which developed puerperal metritis had higher plasma cortisol levels on day 1 after calving compared to the normal cows. The ROC analysis revealed that plasma cortisol concentration is moderately accurate to identify the cows at the risk of developing puerperal metritis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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