



Peripheral blood concentration of toll-like receptor-4 and its accuracy for prediction of postpartum performances of transition zebu (*Bos indicus*) cows

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ABSTRACT

In this study, we evaluated the peripheral concentrations of Toll-like receptors (TLR)-4 during transition period in relation to postpartum productive and reproductive performances of Deoni (zebu) cattle. Accuracy and threshold values of TLR-4 to predict the postpartum performance, were also estimated using receiver operating characteristics (ROC) analysis. Blood samples were collected at weekly intervals during transition period (from 21 days before to 21 days after calving) and TLR-4 concentration was estimated using bovine specific ELISA kits. Plasma TLR-4 concentration was significantly higher on third day of postpartum in cows that became pregnant within breeding period than the cows that remained non-pregnant (4.48 vs 1.80 ng/mL). ROC analysis revealed that the accuracy of TLR-4 for predication of ability to become pregnant within breeding period was acceptable (AUC: 0.75) with a threshold value of 2.13 ng/mL. It is concluded that concentrations of TLR-4, during transition period, could be used for predicting the possibilities of Deoni cows getting pregnant within the breeding period with moderate accuracy.

Keywords: Deoni cows, Milk yield, Reproductive performance, TLR-4 concentration, Transition period

Toll-like receptors (TLRs) are member of pattern recognition receptor family and an important component of bovine innate immunity in recognition of pathogen associated molecular pattern (PAMP) molecules present in invading pathogens. Among 10 identified TLRs in cattle, TLR-2 and TLR-4 detects gram-positive and gram-negative bacteria, respectively (Werling and Jungi 2003, Werling *et al.* 2006). Although most of the TLRs exist as transmembrane receptor forms, soluble forms of TLRs (sTLR) particularly TLR-4 were identified in human saliva (Zunt *et al.* 2009) and serum of cattle (Ma *et al.* 2011). Like sCD14 formation process, upon activation of cells (e.g. blood monocytes and neutrophils), cell-surface TLRs undergoes endocytosis and converted into sTLRs (LeBouder *et al.* 2003). Like cell surface receptors, sTLR is also believed to sequester the circulating PAMP and follows similar regulatory mechanism of innate immunity against invading pathogens (LeBouder *et al.* 2003). Ma

et al. (2011) reported that concentrations of sTLR2 and sTLR4 and expression of TLR-4 gene remain unchanged during experimentally induced *E. coli* mastitis. They also observed higher concentration of pro-inflammatory cytokines and its gene expression in those cows and indicated that it could be a protective mechanism to avoid further pro-inflammatory conditions in mastitic cows. Lack of other studies on plasma TLR-4 concentration is a limitation to understand the functionality in domestic animals. Higher expression of TLR-2 and TLR-4 in mastitis affected animals was reported by Goldammer *et al.* (2004) and Yang *et al.* (2008). Mundhe *et al.* (2018) also reported association of TLR-2 gene polymorphism with milk production in Deoni (*Bos indicus*) cows. Razak *et al.* (2015) revealed significant association of TLR-4 gene polymorphism with mastitis in Indian dairy cows. Moreover, TLRs act as double-edged swords, by executing both beneficial host defense functions or harmful disease promoting role, though it is not clearly understood when and how this happens. Collectively, it warrants further studies for better understanding the role of TLRs in dairy cattle. In this study, we estimated peripheral blood concentration of TLR-4 during transition period and its accuracy for the prediction of possibilities of getting pregnant within the breeding period in apparently healthy dual-purpose zebu (*Bos indicus*) cows.

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MATERIALS AND METHODS

Experiment animals and management: The study was conducted at LRC, SRS, ICAR-NDRI, Bengaluru, Karnataka, India. Institute Animal Ethical Committee had approved the experimental procedures (CPCSEA/IAEC/LA/SRS-ICAR-NDRI-2017-07). All the animals were apparently healthy and free from any disorders during enrollment. The experimental cows were maintained under individual tie barn housing system and provided space as per Bureau of Indian Standards (BIS). The shed used for this experiment were open from all side, concrete paved with tiled roof. All the cows were fed according to NRC (2001) recommendation using institute grown seasonal green fodders like maize, jowar, hybrid napier, paragrass, guinea grass and cowpea along with dry fodder (ragi straw) and commercially available concentrates (cow feed containing 16–18% crude protein, 70–72% TDN, 2.5–3.5% fat, 5.5–6% crude fiber, 1–1.5% acid insoluble ash and 10–11% moisture, M/s Karnataka Milk Federation, Bengaluru). The cows were fed 15–20 kg green fodder, 2–3 kg ragi straw and 1.5–2.5 kg concentrates during prepartum transition period. After calving, the cows were offered 18–22 kg green fodder, 2–3 kg ragi straw and 2.5–3 kg concentrate divided in equal proportion and fed at the time of milking during morning and evening hours based on the level of milk production of individual cow. The animals were provided with clean drinking water 4 times in a day.

Grouping of animals: A total of 29 transition Deoni cows were enrolled a month before the expected date of calving and followed till 45 days after calving. Of 29 pregnant cows, few cows were eliminated due to postpartum complications and missing sampling. Thus, 18 multiparous cows were considered for grouping based on milk yield as high ($n=6$), medium ($n=6$) and low ($n=6$) yielding cows. The overall average lactation milk yield of Deoni cows was reported as 779 kg with a lactation length of 187 days (Das *et al.* 2011). In the present study, we classified into high (HY, >600 kg), medium (MY, 300–600 kg) and low yielding (LY, <300 kg) cows. The lactation length of HY, MY and LY cows were 16–36, 15–33 and 11–21 weeks, respectively.

After a voluntary waiting period of 45 to 60 days, all the animals were routinely observed by trained personnel for estrus, twice a day. Animals identified to be in estrus were confirmed by a veterinarian for their proper stage to receive semen. Animals detected in estrus in the early morning were artificially inseminated (AI) in morning of the same day with frozen semen and those cows detected in estrus in the late evening and night were inseminated in the morning of the next day. Two inseminations were done for each cow at 12 hours interval. Pregnancy diagnosis was done 45 to 60 after AI through rectal palpation and transition cows were grouped as pregnant ($n=7$) and non-pregnant ($n=11$) after breeding period of 180 days in milk. The average parity of HY, MY and LY cows were 4.33 (range: 2–8), 3.4 (range: 3–4) and 3.4 (range: 3–4), respectively, while average parity of pregnant and non-pregnant cows were 4 (range: 2–8)

and 3.55 (range: 3–5), respectively. Average daily milk yield (kg) in these animals were 5.98 (4.84–6.67), 3.25 (2.10–3.88), 1.77 (1.0–2.16), 4.02 (3.22–4.59) and 4.18 (3.40–4.71), respectively. The total lactational yield (kg) in these animals were 956 (616–1168), 461 (406–579), 165 (108–216), 648 (108–1169) and 572 (113–1099), respectively. The average BCS (on a 5-point scale) at the start of experiment were 3.42 (3–3.75), 3.35 (3–3.5), 3.30 (3–3.75), 3.36 (3–3.75) and 3.36 (3–3.75), respectively.

Blood sampling and TLR-4 assay: Blood samples collected from 18 cows during transition period (-21 ± 2 , -14 ± 1 , -7 ± 1 , 0^{th} , 3 ± 1 , 7 ± 1 , 14 ± 1 and 21 ± 2 days relative to calving) were used for estimation of plasma concentrations of TLR-4 using ELISA kits obtained from M/s Cloud-Clone Corporation, Houston, TX (USA).

Statistical analysis: The data were analyzed by univariate method to observe interaction between two groups at each time point of intervals (i.e. from -21 to $+21$ days) and interaction within a group using statistical software package SPSS version 22 (SPSS for windows, V22.0; M/s SPSS Inc., Chicago, IL, USA). The difference of means was considered as significant if $P < 0.05$ and the results are presented as mean $\pm$ SEM. Receiver operating characteristics (ROC) analysis was done to assess the critical threshold value as per Wankhade *et al.* (2021) using Sigmaplot 11 software package (Systat software, Inc, California, USA).

RESULTS AND DISCUSSION

Metabolic inflammation plays a significant role in homeostasis of transition dairy cows (Manimaran *et al.* 2016, Wankhade *et al.* 2017). In this study, plasma TLR-4 concentration was significantly ($P=0.003$) higher on third day of postpartum in cows that conceived within breeding period compared to cows that remained non-pregnant (Fig. 1). Earlier report in same cows reported a higher plasma concentration of pro-inflammatory cytokines such as IL-1 α , TNF- α , and IL-6 and non-esterified fatty acids (NEFA) level during transition period in cows that became pregnant than that remained non-pregnant (Wankhade *et al.* 2019). Activation of TLR-4 leads to synthesis and production of pro-inflammatory cytokines such as TNF- α ,

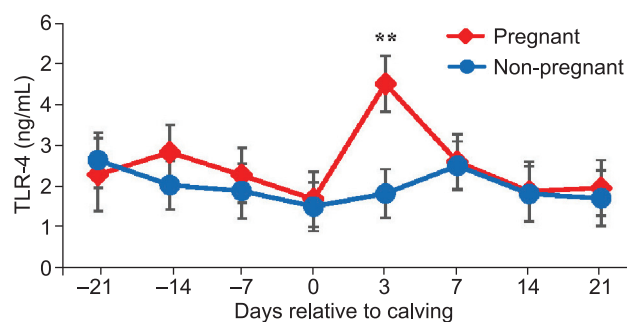


Fig.1. Plasma concentrations of TLR-4 during transition period and its relationship with postpartum pregnancy status in Deoni cows. Lines (mean $\pm$ SE) bearing asterisk differ significantly (** $P < 0.01$) between the groups at each sampling day intervals.

IL- α and IL-6 that are important in TLR signaling mechanism (Budikhina *et al.* 2021). NEFA also acts as a ligand for TLR-4 (Shi *et al.* 2006), which subsequently induce nuclear factor kappa B (NF- κ B) signaling pathway and transcriptional regulation of several pro-inflammatory cytokine genes, such as TNF- α , IL-6, and IL-1 α (Baldwin 1996). Zhang *et al.* (2018) also reported that higher NEFA concentrations activated the TLR-4 and subsequent NF- κ B signaling pathways to induce the pro-inflammatory cytokines production. Altogether, the higher TLR-4 level observed in this study, along with earlier findings, support our hypothesis that active functioning state of innate immune system is important for early conception. On the other hand, failure of innate immune response, particularly during early postpartum period due to various stressors (e.g. inadequate nutrient intake and subsequent metabolic and immune dysfunction), is one of the important reasons for low reproductive performance in dairy animals (Lucy 2019). Several studies suggested TLR-4 genes as an effective candidate molecule for assessment of reproductive performance and immune function of dairy cows (Shimizu *et al.* 2017, El-Domany *et al.* 2019).

In this study, we observed that the TLR-4 concentrations were in higher trend ($P=0.08$) among HY cows than LY cows (3.45 vs 1.77 ng/mL; Fig. 2). Although studies on TLR-4 concentration in relation to milk yield is not available, Ma *et al.* (2011) reported that concentrations of sTLR4 and its gene expression remain unchanged, but higher concentrations of pro-inflammatory cytokines (IL-6 and TNF- α) was observed in mastitis affected cows. We also found higher concentration of TNF- α and IL-6 during transition period in HY cows (Wankhade *et al.* 2018). The level of TLR-4 was also in similar range in these two studies (2.94 vs 2.2 ng/mL). Therefore, the observed condition could be a protective mechanism to avoid further pro-inflammatory state yet maintaining necessary of minimum immune response for host defense function in these cows (Ma *et al.* 2011). The variation in TLR-4 gene among the population and association of particular genotypes of TLR with milk yield (Noori *et al.* 2013, Zhou *et al.* 2017) could also be a reason for limited relationship between TLR-4 levels and milk yield. Since limited inflammatory response is essential for optimum performance of apparently healthy animals, the observed higher trend of TLR-4 along with

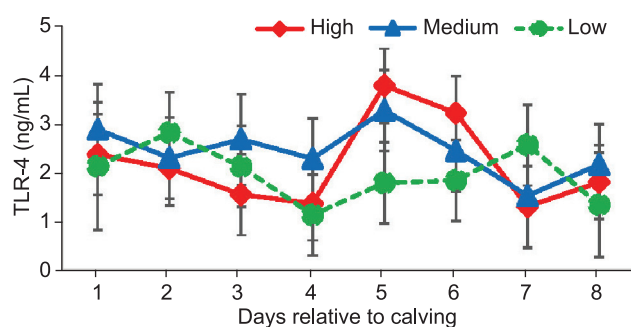


Fig. 2. Plasma concentrations of TLR-4 during transition period and its relationship with milk yield in Deoni cows.

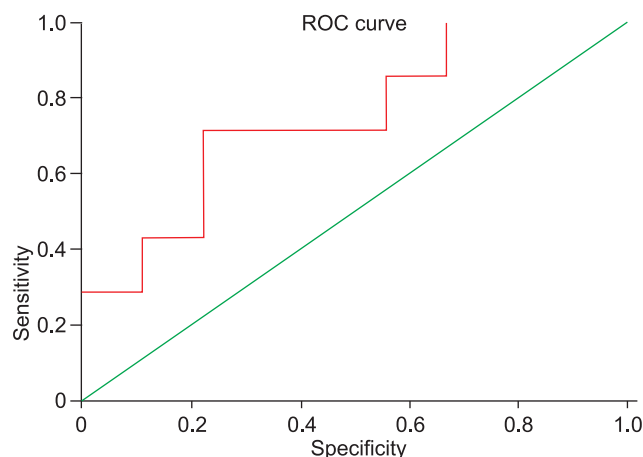


Fig. 3. Receiver operating characteristic (ROC) curve plots for TLR-4 for prediction of pregnancy in Deoni cows.

higher concentration of pro-inflammatory cytokines in these animals could be the reason for higher productivity.

ROC analysis revealed that significant and moderate accuracy (AUC: 0.75; $P=0.01$) of TLR-4 in prediction of possibilities of cows getting pregnant at an early time during breeding period (Fig. 3) with threshold value of 2.13 ng/mL, sensitivity of 71% (95% CI: 29 to 96), specificity of 78% (95% CI: 40 to 97) and likelihood ratio of 3.21. The predictive ability of inflammatory cytokines and energy indicators for pregnant and high yielding animals using ROC analysis were also reported by Wankhade *et al.* (2021). Since ROC is a step function test and we used very lesser number of samples, further studies with increased sample size are necessary to obtain true curve for better prediction of postpartum performance during transition period. Based on the earlier and present findings, it is concluded that transition period concentrations of TLR-4 could be used to predict the possibilities of Deoni cows getting pregnant at an early time during breeding period with moderate accuracy.

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