India is home for 15% of world cattle population and ranks second in the world's cow milk production (FAOSTAT 2010). Despite the overall higher milk production, the productivity is lower owing to the harsh tropical climate and higher incidence of reproductive disorders. Retention of fetal membranes (RFM) has been found to be the single largest postpartum complication in the bovine species and is defined as the failure to expel placenta even after 12–24 hrs of calving (Leblanc, 2008; Mohamed and Amer, 2009); whereas in India, it ranged from 4.5 to 28.2% (Dongre et al., 2011). RFM has a multifactorial etiology (Hanafi et al., 2011) and it adversely affects the fertility by delaying the uterine involution and increasing the time to first service, services per conception and days open as the condition causes metritis and endometritis (McDougall et al., 2001). RFM cows are also at high risk of suffering from ketosis and mastitis (Beagley et al., 2010). Therefore, any tool in predicting the occurrence of RFM may help in initiating prophylactic measures, thus preventing and alleviating its negative consequences on fertility. During pregnancy, the maternal immune response towards the fetus bearing paternal allo-antigens are modulated rather than suppressed for a successful pregnancy outcome. There is partial down regulation of the expression of MHC antigens on placenta (Low et al., 1990); production of immuno-suppressant molecules like Interferon-τ (Thatcher et al., 1986; Spencer et al., 2007) and also a shift from Th1 to Th2 type immune responses (McCracken et al., 2004). Th2 cells promote antibody responses which are more conducive for pregnancy and produce anti-inflammatory cytokines—IL–10, TNF–α and IL–6 would, in part, reveal the immune changes taking place during advanced pregnancy and aid in predicting the RFM. Accordingly, the levels of IL–10, TNF–α and IL–6 were estimated in pre-partum serum samples of 81 cows by bovine specific Enzyme linked immunoabsorbant assay (ELISA). Out of 81 cows studied, 8 developed RFM and the rest calved (n=73) normally. IL–10 and TNF–α concentration was found to be significantly higher in RFM cows than their normal counterparts (P<0.01), whereas the IL–6 concentration was higher (P<0.01) in normal cows than the RFM group. This study for the first time demonstrates that these cytokines are associated with the development of RFM and, therefore, their significant level of detection could serve as a predictive tool. However, studies with large number of RFM cows are needed to ascertain the utility of these cytokines as markers.


**ARTICLE HISTORY**

Received: 2013–08–20
Revised: 2013–08–30
Accepted: 2013–08–30

**Key Words**: Cow; Cytokine; Pregnancy; RFM; Marker

**ABSTRACT**

India, by virtue of its huge cattle population ranks second in world cow milk production, however the per–capita availability of milk is less owing to the lower production potential of cows. This could be attributed to the harsh tropical climate and high incidence of reproductive disorders. Among the various postpartum reproductive disorders, the incidence of retention of fetal membranes (RFM) is highest. Identifying the RFM cows ante–partum would help in the better management and minimize its adverse consequences on the post–partum fertility. During the peri–partum period, a cytokine mediated immune mechanism operates at foeto–maternal interface for the successful placental separation and any failure or delay in this process precipitates RFM. Hence, we hypothesized that the estimation of serum level of cytokines—IL–10, TNF–α and IL–6 would, in part, reveal the immune changes taking place during advanced pregnancy and aid in predicting the RFM. Accordingly, the levels of IL–10, TNF–α and IL–6 were estimated in pre–partum serum samples of 81 cows by bovine specific Enzyme linked immunoabsorbant assay (ELISA). Out of 81 cows studied, 8 developed RFM and the rest calved (n=73) normally. IL–10 and TNF–α concentration was found to be significantly higher in RFM cows than their normal counterparts (P<0.01), whereas the IL–6 concentration was higher (P<0.01) in normal cows than the RFM group. This study for the first time demonstrates that these cytokines are associated with the development of RFM and, therefore, their significant level of detection could serve as a predictive tool. However, studies with large number of RFM cows are needed to ascertain the utility of these cytokines as markers.


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The collagenase activity that culminates in the dehiscence of foetal cotyledon from the maternal caruncle (Beagley et al., 2010).

TNF–α and IL–6 are the pro-inflammatory cytokines with authoritative role in wide range of inflammatory, infectious, autoimmune and malignant conditions; produced in response to infection primarily by monocytes/macrophages and T and B lymphocytes (Apostolaki et al., 2010; Scheller et al., 2011). Sustained levels of increased TNF–α in the serum could reflect persistent uterine bacterial infection. IL–6 operates by two signaling pathways out of which classic signaling mediates regenerative and anti-inflammatory activities whereas trans-signaling brings about pro-inflammatory responses (Scheller et al., 2011). IL–10 is a general suppressive cytokine which inhibits pro-inflammatory responses from innate and adaptive immunity and has a key role in the resolution of inflammation (Filippi et al., 2008). This study was aimed to predict RFM cows by determining the prepartum serum levels of pro and anti-inflammatory cytokines during peri-partum period.

A total of 81 healthy advanced pregnant cows between 2nd and 4th parity which had no history of reproductive abnormalities during the previous gestation were selected, randomly at 240 days of pregnancy, from a herd of cross bred cows maintained at an organized dairy farm, IVRI, I zadnagar. The animals were maintained under uniform feeding and managemental conditions. The pregnancy diagnosis was performed routinely in the above mentioned farm at 50–60 days post insemination. The expected date of calving was fixed at 280 days post insemination based on artificial insemination data supported with record of pregnancy diagnosis. Five ml of blood was collected from the jugular vein at 15 days before the expected date of calving using a vacutainer and the serum was separated. The serum was aliquoted and stored at −20°C till analysis for assay of cytokines—IL–10, TNF–α and IL–6.

The cows were closely observed on the day of calving for the occurrence of any complications and the time of expulsion of placenta was recorded. The cows which expelled placenta within 24 hrs of calving were categorized as Non–RFM (n=73) and those which retained placenta for more than 24 hrs were grouped as RFM (n=8). The serum concentration of IL–10, TNF–α and IL–6 was estimated using commercially available bovine specific ELISA kits (Blue Gene, India). The ELISA kit works on the principle of competitive enzyme immunoassay technique, making use of a monoclonal anti-cytokine antibody and a cytokine–HRP conjugate.

All the kit components and samples were brought to room temperature (20–25°C) before start of assay and performed as per manufacturer’s instructions. The optical density was measured immediately at 450 nm using a microplate reader. A standard curve was obtained by plotting the concentration of the standards (0, 50, 100, 250, 500 and 1000 pg/ml) against their optical densities. The value of unknown samples was interpolated from the standard curve and was presented in pg/ml.

Statistical analysis was performed using SPSS software version 16. Independent sample t– test was used to compare the values between cows with and without RFM.

The IL–10 concentration was found to be significantly (P<0.001) higher in RFM cows than those without RFM (1798.5 ± 143.44 Vs 944.28 ± 129.25 pg/ml). Similarly, the cows with RFM had a significantly higher (P<0.001) levels of TNF–α (911.35±64.69 pg/ml) as compared to those which did not experience RFM (675.54±83.33 pg/ml). However, a lower serum level of IL–6 was observed in RFM (1139.4 ± 95.90 pg/ml) than Non–RFM cows (1657.2 ± 77.97 pg/ml) (Table).

The exact cause responsible for this pre–partum rise in IL–10 levels in cows which subsequently developed RFM is still not clear. The higher IL–10 levels might have suppressed the T cell activation and macrophage activity, thus inhibiting the mechanisms responsible for placental separation. The placental separation, an immune mechanism by itself, might have failed to occur in presence of a strong anti–inflammatory environment created by IL–10.

Further, the significantly higher levels of TNF–α in RFM cows are also in agreement with Islam (2012) who observed a similar pattern of TNF–α in cows with RFM and CM at approximately 15 days before the due calving. Experiments in murine models have detected a rise in the plasma levels of pro-inflammatory cytokines viz., TNF–α, IL–6 and IL–1β even in normal pregnancy during late gestation and assumed it as a normal regulatory or immune mechanism taking place during the periparturient period (Orsi et al., 2006). A higher concentration of TNF–α was observed after lipopolysaccharide (LPS) injection in ewes (Dow et al., 2010). LPS induced abortion in cows (Foley et al., 1993) and also in LPS stimulation of whole blood from cows (Rontved et al., 2003).

TNF–α is a potent pleiotropic pro-inflammatory cytokine with definitive role in inflammatory, infectious, autoimmune and some malignant conditions (Apostolaki et al., 2010). It is the end product of NFkB signaling pathway which is initiated by binding of pathogen associated molecular patterns (PAMP) on microbial organisms to their respective pattern recognition receptors on host cells (Fitzgerald et al., 2000; Bryant et al., 2001).
2010). How this pathway gets activated during the periparturient period, especially in the RFM cows, is yet to be determined. Bacterial contamination of the immune suppressed uterus or whether any genetic predisposition is behind it needs to be ascertained. The placental retention occurring even in the high TNF–α milieu suggest that this cytokine may only be a contributory factor in placental separation rather than having a direct effect.

The significantly higher serum levels of IL–6 in normally-calved cows than those of RFM cows are also in line with the findings of Ishikawa et al. (2004) who reported lower prepartum serum IL–6 concentrations in cows which subsequently developed RFM. They also reported a higher prepartum IL–6 levels compared to post–partum. The low level of IL–6 in RFM cows would have compromised the reaction to the allograft (placenta) and eventually resulted in RFM (Ishikawa et al., 2004). Further, IL–6 levels in cervico-vaginal fluid of women and in dried blood spot specimens from preterm neonates were found to be increased during spontaneous preterm birth, when compared to full–term infants (Wei et al., 2010). Serum IL–6 level monitoring proved useful for detecting the acute rejection of hepatic allografts in studies conducted with monkeys and higher peaks was observed in recipients who rejected grafts (Ohzato et al. 1993). All these studies suggest the important role of IL–6 in maintenance of pregnancy and the initiation of labour.

Thus it can be opined that higher serum levels of anti-inflammatory cytokine IL–10 in RFM cows would have contributed to the decreased levels of IL–6 in the present study. IL–6 being a pro-inflammatory cytokine is an essential component of immune mechanisms and a fall in its level might have detailed the normal immune mediated separation taking place at the foeto-maternal interface and precipitated RFM.

A significantly higher pre-partum serum concentration of IL–10 and TNF–α was observed in cows which developed RFM, whereas IL–6 concentration was lower in RFM cows than those of normally calved ones. This suggested that an immune mechanism involving cytokines is of importance in placental separation. The present study demonstrated that these cytokines are associated with the development of RFM and, therefore, could serve as a predictive tool. However, further studies with large number screening of RFM cows is needed to ascertain their utility as markers.

Acknowledgement: This study is part of the MVSc research work conducted by the first author. The authors thank ICAR and Director IVRI for the financial assistance.

Conflict of Interest: The authors have no conflict of interest to declare.

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ISSN: 2307–8316 (Online), ISSN: 2309–3331 (Print)
