Full Length Article

EXPRESSION PROFILE OF EARLY PREGNANCY MARKER GENES (OAS-1 AND MX-2) AND SERUM CONCENTRATION OF MX-2 PROTEIN IN JERSEY CROSSBRED COWS

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ABSTRACT

In the present study, the expression pattern of OAS-1 and MX-2 gene in peripheral blood mononuclear cells (PBMC) was associated with the early pregnancy in cattle. A total of 60 animals were selected and divided into 2 groups, treatment (50) and control (10) group and synchronized using double $PGF_{2\alpha}$ protocol by 11 days apart followed by insemination at 72 and 96 hrs after second dose of $PGF_{2\alpha}$. The cows were subjected to blood collection on day 0, 14, 18, 20 and 25 post insemination and Peripheral Blood Mononuclear Cells (PBMC) were harvested using Histopaque® solution, followed by RNA isolation and cDNA synthesis. A significantly ($P \leq 0.01$) higher expression of OAS-1 and MX-2 gene was observed on days 18 and 20 post oestrum by quantitative real-time PCR and concentration of MX-2 protein in serum were significantly higher ($P \leq 0.05$) on day 18, 20 and 25 in pregnant cows when compared with that of non-pregnant cows. Hence, the present study is concluded that the expression of OAS-1 and MX-2 genes and their encoded proteins may be used to develop a marker for early pregnancy diagnosis in cattle.

Key Words: Early pregnancy, Cattle, qPCR, OAS-1 and MX-2 genes

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INTRODUCTION

Fertility in dairy cows has declined over the past five decades as milk production per cow has increased (Walsh *et al*., 2011). Reduced fertility leads to an increased number of days open, prolonged calving intervals, and more services per conception before a successful pregnancy can be established. Per-

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rectal pregnancy diagnosis approximately 45 days post breeding is the most common method under field conditions (Arthur *et al*., 1996). Moreover, trans-rectal ultrasonography is an accurate method for the diagnosis of pregnancy as early as day 25-30 post-breeding; however, this necessitates expensive equipment and expertise (Kastelic *et al*., 1991). Some molecules like early pregnancy factor (EPF) (Ghaffarilaleh *et al*., 2008) and Pregnancy Associated Glycoprotein (PAG) (Silva *et al*., 2009) have been documented as earliest indicators of pregnancy but have major limitations. Another pregnancy proteins such as interferon-tau (IFNT), and genes stimulated by IFNT, viz. ubiquitin-like modifier (ISG15), the 2,5-oligoadenylate synthetase 1 (OAS-1) and myxovirus resistance genes (MX-1 and MX-2), were found to be upregulated during maternal recognition of pregnancy in sheep and cattle and may be considered to be early pregnancy marker in cattle (Pugliesi *et al*., 2014). The conceptus secretes IFNT as a maternal recognition factor and acts in the uterus around day 16 of post breeding or insemination and prevents luteolysis by inhibiting $PGF_{2\alpha}$ release, resulting in the maintenance of corpus luteum function thus leads to establishment of pregnancy (Thatcher *et al*., 2001).

IFNT induces the synthesis and secretion of IFNT stimulated genes (ISG) such as 2, 5 oligoadenylate synthetase (OAS-1), IFN regulatory factor 1, ISG 15, MX1 and MX-2 not only in the uterus but also in blood cells in cows (Gifford *et al*., 2007). A higher expression of OAS-1 was observed on day 20 and was increased on day 8, 12, and was in peak on day 20 in pregnant cows as compared with non-pregnant cows (Shirasuna *et al*., 2012). Thus the present study was undertaken to study the expression pattern of OAS-1 and MX-2 genes and the serum concentration of MX-2 protein during early pregnancy in Jersey cross bred cattle to understand the clue for identification of early pregnancy markers.

MATERIALS AND METHODS

Collection of Samples

Sixty normal cyclic Jersey crossbred cows maintained at Post Graduate Research Institute in Animal Sciences, Kattupakkam, Tamil Nadu, India, between 2 and 8 years of age and in good body condition were selected based on the breeding history and rectal examination for this study. All the selected cows were synchronized by adopting double $PGF_{2\alpha}$ protocol. Each animal received two doses of 500 µg Injection Cloprostenol sodium (Pragma, Intas) 11 days apart followed by insemination at 72 and again at 96 hrs after second dose of $PGF_{2\alpha}$. The cows were then randomly divided into two groups viz Group I consisted of 10 cows which were synchronized but not bred/inseminated (control) and group II consisted of 50 cows which were synchronized and inseminated. The day of AI was designated as 0 d, and

blood was collected from the jugular vein in EDTA vacutainers on 0, 14, 18, 20 and 25 days after post insemination. Peripheral blood mononuclear cells (PBMC) were harvested using Histopaque-1077® solution as per manufacturer's instructions and stored in RNA latter solution at -80°C until further process.

Total RNA was isolated from whole blood using TRIzol® LS reagent as per manufacturer's instructions. All RNA samples were quantified by Nanodrop and the purification of RNA with A260/A280 ratio of 1.8 and above were considered for cDNA synthesis (reverse transcription) using oligo dT primers with initial concentration of 500 ng of total RNA from each sample using the High capacity cDNA synthesis kit (Applied Biosystems, USA) based on manufacturer's instructions of 20 µl final volume.

Published oligonucleotide primers were used for real time PCR, viz. Beta actin Forward CTGGACTTCGAGCAGGAGAT and Reverse GGATGTCGACGTCACACTTC (Liu *et al*. 2016). The oligonucleotide primer for OAS-1 is Forward TAGGCCTGGAACATCAGGTC and Reverse TTTGGTCTGGCTGGATTACC (Shirasuna *et al*., 2012). The oligonucleotide primer for MX-2, Forward GCACAGCAAGCTCTCTACGA and Reverse GCAAAACCCAGAAGGACTGC was designed using NCBI primer design software. The primers were standardized using conventional RT-PCR and used in Real time PCR to amplify OAS-1 and MX-2 gene along with endogenous control Beta actin.

Reverse Transcriptase PCR for OAS-1 and MX-2 gene

The PCR conditions for the genes OAS-1, MX-2 and Beta actin used were Uracil-N-Glycosylase (UNG) inactivation at 50 ºC for 2 min, Initial denaturation at 95 ºC for 10 min, denaturation at 95 ºC for 1 min, annealing at 59.5 ºC for 45 sec, extension at 72 ºC for 45 sec and final extension at 60 ºC for 1 min. Confirmation of OAS-1, MX-2 and Beta actin genes amplification was done by agarose gel electrophoresis.

qPCR

The real time PCR was carried out using SYBR green based method for OAS-1 and MX-2 genes in Applied Biosystem qPCR master cycler using SYBR Premix Ex Taq II (TliRNase H Plus, Takara Bio, Japan) using 10µl reaction mix volume. In the negative control reactions, instead of cDNA, 1µl of nuclease free water was added. The real time PCR machine was run with the above mentioned cyclic conditions. After each PCR cycle, melting curves were obtained to ensure single product amplification. The assay was designed based on ΔΔCT method and comprised of samples collected at different time point interval along with negative controls, having technical replicate wells (2 each) with endogenous control as Beta actin.

Relative quantification of OAS-1 and MX-2 genes

The Ct values were recorded for all target and the internal control genes and the melting curve analysis was carried out to check the specificity of the products. The relative quantification and expression pattern of OAS-1 and MX-2 genes on different days in pregnant and non-bred control experimental animals were analysed keeping Beta actin as endogenous control and 0 day as calibrator. ΔΔCt method was used to analyze the relative expression. $2^{-\Delta\Delta Ct}$ value gave the relative quantification of expressed genes.

Serum MX-2: ELISA test

Serum MX-2 concentrations were determined with a quantitative sandwich immune-enzymatic assay (Bovine interferon induced GTP binding protein MX-2 ELISA kit, Cat no: E1381134, Sincere Biotech Co., Ltd, Beijing, China). The detection range is from 0.295 ng/mL to 20 ng/mL and assay had a sensitivity of 7.81 pg/mL. The micro-wells were pre-coated with specific antibodies providing a solid phase for capture of MX-2 in the serum. After incubation of standards and samples for 30 min at 37°C in the dark, the wells were thoroughly washed to remove all components other than those bound to antibodies. Pregnancy protein antibodies coupled to horseradish peroxidase (HRP) were then added to the wells and allowed to bind the immobilized pregnancy protein during a second incubation (for 30 min in dark place at 37°C). After washing the wells to remove unbound conjugate, HRP substrate was added to the wells and allowed to react during final incubation step for 15 min. Thereafter the stop solution was added. The optical density of the well was measured at 450 nm 15 min after the addition of the stop solution using a spectrophotometer.

Statistical analysis

Statistical analysis of data was carried according to the method described (Snedecor and Cochran, 1967). The data pertaining to relative expression of OAS-1 and MX-2 genes were analysed by ANOVA using SPSS statistics 20.0 (International Business Machine (IBM) corporation, Chicago, USA). Data with respect to serum concentration of MX-2 were analysed by independent sample t-test using SPSS statistics 20.0.

RESULTS AND DISCUSSION

In the present study, the (Mean±SE) relative expression of OAS-1 in control, non-pregnant and pregnant animals is presented in Fig. 1. The relative expression of OAS-1 in the PBMC significantly increased from days 14 to 20 and reached peak by day 20 of post insemination when compared to day 0 in the pregnant cows. The results are in agreement with Sahatpure and Patil (2008) who observed a gradual increase in the expression profile of OAS-1 was observed from day 14 onwards, with peak at day 18 and then a gradual decline from days 20, 22 to 24 in buffalo and

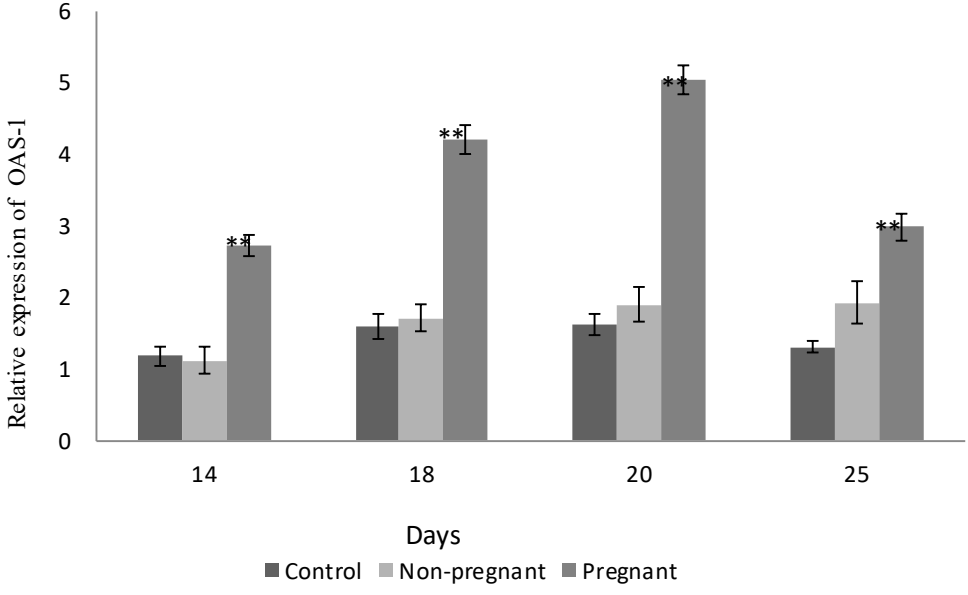
Shirasuna *et al*. (2012) who observed that OAS-1 mRNA increased on day 8, 12 and it was peaked on day 20 in pregnant Holstein cows as compared with non-pregnant Holstein cows. Similarly, a gradual increase in OAS-1 mRNA and protein was noticed in the liver hepatocytes suggestive that IFNT induces ISG in the parenchymal part of the liver in early pregnancy of cattle (Ruhmann *et al*., 2017). OAS-1 gene expressed to a maximum of 5 folds on day 20 which is agreement with the findings of Rahul *et al*. (2021) who reported 5.4 folds expression of OAS-1 gene on day 20 in pregnant Jersey crossbred heifers.

In control animals, there was no significant difference was observed in the relative expression of OAS-1 between different days. In pregnant animals, the OAS-1 expression was significantly ($P \leq 0.01$) higher on days 14, 18 and 20 and 25, post-insemination which is corroborating to the findings of Rahul *et al*. (2022); however, on days 14 and 25, fold changes were similar. In the present study, the OAS-1 expression was significantly higher on day 18 and 20 of post-insemination. Pugliesi *et al*. (2014) reported that the OAS-1 expression increased in pregnant cows compared to non-pregnant cows with a functional CL on day 20 and in pregnant cows, a progressive increase in the abundance of ISGs from day 15 to day 20, a sharp decrease by day 22, and a further decrease to the basal levels from day 30 through day 60 post-insemination. Increased activity of OAS-1 mRNA in endometrium during early pregnancy is probably due to the action of the conceptus interferons, an intracellular OAS-1 dependent endoribonuclease is involved in cleaving viral and cellular RNAs, thereby preventing synthesis of proteins required for replication of viruses within host cells as well as cellular proteins (Taylor and Grossberg, 1990).

Expression profile of MX-2: The relative expression (Mean±SE) of MX-2 in control, non-pregnant and pregnant animals is presented Fig. 2. The Interferon Tau enhance the expression of MX-1 and MX-2 genes in peripheral blood mononuclear cells and signals the maternal recognition of pregnancy in ruminants (Matsuyama *et al*., 2012). The MX genes were found to be expressed in the uterus of pregnant cow, sheep, pig and mare (Shirozu *et al*., 2016). In early pregnancy diagnosis, about 80% accuracy can be achieved by the estimation of ISG-15 and MX-2 levels on granulocytes during days 20-22 of gestation (Yoshino *et al*., 2018). In the control animals of present study, no significant difference was observed in the relative expression of MX-2 between different days whereas in pregnant animals, significantly ($P \leq 0.01$) higher relative expression of MX-2 was observed on days 14, 18, 20 and 25, post-insemination, which is in agreement with the findings of Shirozu *et al*. (2016). Thus, the MX-2 expression was significantly higher on 18 and 20 day post-insemination in pregnant animals when compared to non-pregnant cows. This higher expression of MX genes during early pregnancy is due to increase in their levels of mRNA and proteins, secreted by the trophoblast cells at the time of implantation

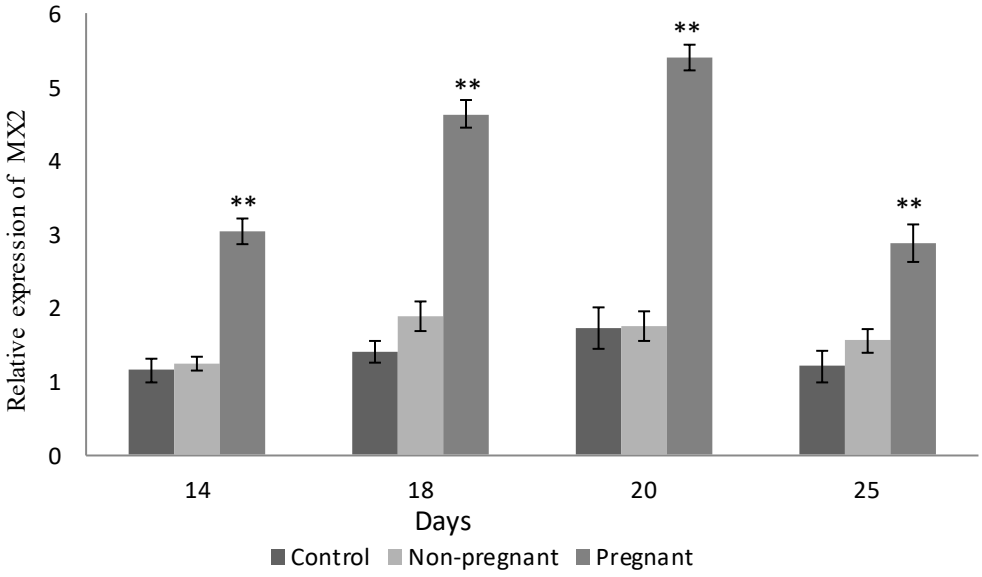
which involves in the uterine reception of embryo.

Quantification of serum MX-2 protein: The (Mean±SE) concentration of MX-2 protein (pg/mL) in non-pregnant and pregnant animals is presented in Fig. 3. The proteins such as MX-1 and MX-2 proteins have anti-viral properties and play a vital role in the regulation of endometrial secretion, uterine remodelling and anti-luteolytic activities (Hicks *et al*., 2003). In the present study, significantly (P≤0.01) higher concentration of MX-2 protein was observed in pregnant animals on days 18, 20 and 25 post-AI as compared to control and non-pregnant cows, which is in agreement with the findings of Buragohain *et al*. (2016) who reported marked upregulation on day 14, reaching maximum on day 28 and diminution on day 35. Though the concentration of MX-2 protein in non-pregnant cows was significantly differed between day 18 and 20, significant difference was not observed as compared that of pregnant animals. Buragohain *et al*. (2016) reported nearly 10 times higher MX-2 protein concentration on day 14 and 14 times higher MX-2 protein concentration on 28 post-AI in pregnant animals. The reason might be due to the GTPase activity of MX proteins which impart maternal neutrophils to migrate to the developing embryo (Pantarelli and Welch, 2018) or to enhance the dam's innate immunity during pregnancy in order to protect against viral infections (Panda *et al*., 2020).



**P< 0.01

Fig. 1. Relative expression of OAS-1 gene on different days post oestrum in non-bred control, non-pregnant and pregnant in Jersey cross bred cows



**P< 0.01

Fig. 3. Serum MX-2 protein concentration (pg/ml) on different days post-insemination in non-pregnant and pregnant Jersey cross bred cows

CONCLUSION

In the present study, upregulation of OAS-1 and MX-2 genes and MX-2 protein s were observed at early pregnancy and their down regulation in later stages suggests its abundance in early pregnancy and its applications in early pregnancy diagnosis. Serum concentration on MX-2 protein can also be correlated with MX-2 transcript level and estimation of MX-2 protein during early pregnancy may helpful for the early pregnancy detection in cattle thus improves the reproductive efficiency.

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