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MX2 gene mRNA expression as potential biomarker for early pregnancy diagnosis in cattle

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ABSTRACT

Early pregnancy diagnosis is vital for economic sustainability of dairy farms and maintaining the reproductive efficiency of the herd. There are many techniques including progesterone assay, pregnancy specific proteins and interferon stimulated genes have been explored for early pregnancy diagnosis but, they are associated with varying level of efficacy. In the present experiment, interferon stimulated gene (Myxovirus resistance gene 2/*MX2*) expression pattern was used as a potential biomarker for early pregnancy in cattle. The association of *MX2* gene expression in relation to progesterone assay was studied to explore its potential use as biomarker of early pregnancy. The plasma progesterone concentration in conceived animals on day 7 (2.26±0.19 ng/ml), 17 (5.42±0.35 ng/ml) and 21(6.38±0.39 ng/ml) was recorded to be significantly higher as compared to respective values in non-conceived animals, i.e. 1.55±0.09 ng/ml, 4.14±0.14 ng/ml and 0.81±0.06 ng/ml. The sudden decrement in plasma progesterone concentration after day 17th discriminates conceived and non-conceived animals. *MX2* expression levels were observed to spike in blood due to release of interferon tau (τ) after implantation of embryo. The relative mRNA expression of *MX2* gene showed a 9.5 to 28.64-fold higher expression on 17 days post insemination in pregnant animals as compared to non-pregnant animals. Thus, *MX2* gene can be used as a reliable biomarker for the early detection of pregnancy.

Keywords: Interferon tau, *MX2* gene, Pregnancy diagnosis, Progesterone

Early pregnancy diagnosis in cattle has emerged as an important tool to improve poor reproductive management in India. Improper reproductive management results in longer inter-calving period and increased proportion of dry animals in the herd thus affecting the economic sustainability of dairy cattle farming. For early pregnancy diagnosis estimation of plasma concentration of estrone sulphate and progesterone have been in practice (Balhara *et al*. 2013). Low progesterone concentrations during 18-24 days post-insemination may accurately depict non-pregnancy, but high progesterone concentration during this period do not accurately depicts pregnancy or viability of fetus due to great variations in progesterone concentration during oestrus cycle among individual animals and cystic corpora lutea in non-pregnant animals (Balhara *et al*. 2013). This showed the requirement of a conceptus specific marker to improve the accuracy of pregnancy diagnosis methods within 21 days of cycle. After implantation the conceptus is known to secrete a substance called Interferon τ during peri-attachment period to facilitate maternal recognition of pregnancy (MRP) which can be detected as early as day 13 post insemination (Kim *et al*. 2013, Kose *et al*. 2014, Pugliesi *et al*. 2014). The Interferon τ in blood, up regulates the expression of interferon stimulated genes (ISG) in peripheral blood mononuclear cells (Gifford *et al*. 2007). The Myxovirus resistance gene (*MX2*) (an ISG) in peripheral blood mononuclear cells can be used as a biomarker for early pregnancy diagnosis (Matsuyama *et al.* 2012, Buragohain *et al.* 2016). The *MX2* gene transcript can be detected in blood of pregnant animals as early as Interferon τ secretion starts, i.e. from 13-14 day of pregnancy in cattle (Buragohain *et al.* 2016). In cattle, the concentration of plasma progesterone and increased expression level of *MX2* gene coincide during pregnancy. This association can eliminate the false positive and negative results obtained from plasma progesterone hormone analysis and establish the *MX2* gene as a potent biomarker for early pregnancy diagnosis. Thus, present study was undertaken to explore the expression of *MX2* gene in cattle using real-time PCR to explore the possibility of establishing it as a potent biomarker for early pregnancy diagnosis and its correlation with serum progesterone level in pregnant animals.

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MATERIALS AND METHODS

Ethical approval: The trial was ethically approved by the Institutional Animal Ethical Committee (IAEC) (11011(13)/16/2021-CPCSEA-DADF dated 23-09-2021), Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut.

Selection and breeding of animals: A total of 20 cows between 3rd to 6th parity of approximately similar body weight and maintained under isomanagerial conditions at livestock farm of Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh and Gaushala, Khatauli, Uttar Pradesh, were inducted into the study. They exhibited no clinical signs of any disease during the whole study period. The experimental animals were synchronized for oestrus using Ovsynch protocol by administering receptal 20 microgram (buserelin acetate) on day 0, clostenol (500 microgram cloprostenol) on day 7 and receptal (20 microgram buserelin acetate) on day 9 by intramuscular route. Cows showing signs of oestrus at day 10th of protocol were inseminated with frozen semen after 12 h of onset of oestrus.

Blood sample collection: Blood sampling of experimental animals was done on day 0 (day of insemination/ oestrus), 7, 17 and 21 by jugular veni-puncture in EDTA vials.

Estimation of progesterone concentration: Quantitative determination of progesterone concentration in plasma samples was done using Enzyme Linked Immuno Sorbent Assay (ELISA) kit (Xema immunodiagnostics, Moscow), as per the manufacturer's instructions.

RNA isolation and reverse transcription: Total RNA was extracted from 400 µl of freshly collected blood using 1ml of TRIzol reagent (Thermofischer, USA). The mixture was vortexed for 15 sec and the sample was allowed to stand at room temperature for 5 min. To the mixture, 0.2 ml of chloroform was added and the mixture was again vortexed for 15 sec and incubated at room temperature for 15 min. Mixture was then centrifuged at 12000 rpm for 15 min at 4°C temperature. After centrifugation, colourless aqueous phase was separated in separate tube to which equal amount of isopropyl alcohol was added and the mixture was then incubated at -20°C for overnight. The reaction mixture was again centrifuged at 12000 × g for 15 min at 4°C and the supernatant was discarded. The pellet was washed with prechilled 70% ethanol, air dried and dissolved in nuclease free water (NFW). The isolated RNA was immediately reverse transcribed and stored at -20°C for further use.

The cDNA was prepared from the RNA isolated after measuring their concentrations using nanodrop (Thermo Scientific, USA). In PCR tubes, 1 µl RNA (500 ng/µl each sample) were mixed with 1 µl of Random hexamer and NFW to make the total volume of 10 µl. The RNA primer-complex was vortexed and heated at 70°C for 5 min. To this mixture master mix (Promega, USA) consisting of 5× Reaction Buffer (4 µl), RNase Inhibitor (1 µl), 10 mM dNTP Mix (2 µl), RevertAid M-MuLV RT (1 µl), Nuclease Free water (2 µl) were added and applied to thermal cycler (Applied Biosciences, USA). Reaction mixture was incubated at 25°C for 5 min and then at 42°C for 60 min. The reaction was inhibited at 70°C for 5 min. Concentration of the cDNA product obtained was measured using nanodrop and it was stored at -20°C until further processing.

Expression analysis of MX2 gene by real-time PCR (RT-qPCR): Expression analysis of *MX2* gene transcript by RT-qPCR was carried out on cattle cDNA using EvaGreen qPCR Mastermix-low ROX (Applied Biological Materials, Canada) in a ABI 7500 Real-Time PCR System (Applied Biosystems, USA) with published primers (Buragohain *et al.* 2016) (Table 1) following manufacturer's recommendations. The endogenous control *GAPDH* and target *MX2* gene reactions were carried in triplicate of each sample in which negative controls (non-template control, NTC) were also included in the reaction. The average threshold cycle (CT) was obtained for *MX2* and *GAPDH* gene so that the comparison could be established. The data were subjected to comparative CT method (ΔΔCT method) for analysis of expression levels of target gene (*MX2*) and an endogenous control. The reaction mixture was prepared by adding EvaGreen qPCR Master mix-low ROX (10.0 µl), Forward Primer (10 µM) (0.5 µl), Reverse Primer (10 µM) (0.5 µl), Template (3.0 µl) (500 ng/µl), Nuclease free water to make total volume of 20 µl. The three stage PCR protocol was set for real time amplification of both *MX2* and *GAPDH* cDNA for all the samples. The protocol involved initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min, final dissociation step was set at 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. The data obtained was subjected to comparative Ct method (Livak and Schmittgen 2001) analysis for the study of expression levels of targeted *MX2* gene.

Table 1. Primers for amplification of *MX2* and *GAPDH* gene in real-time PCR (Buragohain *et al*. 2016)

Gene	Primer sequence	Size (bp)
MX2	F: 5'GTCCACCTGAACGCATAC3'	155
	R: 5'GAAGCAGCCAGGAATAGTG3'	
GAPDH	F: 5'GCAACAGGGTGGTGGACC3'	91
	R: 5'ACTCTTCCTCTCGTGCTCC3'	

Statistical analysis: The obtained RT-qPCR data were analyzed by two-way ANOVA. The least significant difference approach was used to analyze differences between means, and ($P<0.05$) was considered statistically significant. The average Ct (threshold cycle) data were analyzed for fold change in expression of the target gene. The comparison of progesterone concentration in conceived and non conceived groups was done using paired t-test.

RESULTS AND DISCUSSION

The estimation of progesterone concentration alone for pregnancy diagnosis in cattle may give false positive results in non-pregnant animals with cystic corpora lutea and those in diestrus phase of cycle (Balhara *et al*. 2013).

Thus, determination of a pregnancy biomarker along with plasma progesterone estimation becomes a reliable method of early pregnancy diagnosis.

In present study, plasma progesterone concentration was estimated on day 0 (day of oestrus), 7, 17 and 21. No significant difference in plasma progesterone concentration was observed between the animals of conceived (0.55±0.50 ng/ml) and non-conceived (0.52±0.83 ng/ml) groups at day 0 (day of oestrus) and they remained below 0.6 ng/ml. The mean progesterone concentration in conceived animals was found significantly (P<0.01) higher as compared to non-conceived animals at day 7, 17 and 21 (Fig. 1). The results obtained showed a significant increase (P<0.01) in amount of progesterone concentration in conceived animals on ascending days while, sharp decline was observed in the progesterone concentration after day 17 of insemination in non-conceived animals indicating the initiation of luteal regression (Henericks *et al*. 1970). During luteal phase, the plasma progesterone levels were observed to spike till day 16 in both pregnant and non-pregnant animals and started declining after day 16 in non-conceived animals.

The plasma progesterone reached to a basal value (0.81±0.06 ng/ml) in non-conceived animals on day 21 but the concentration in conceived animals (6.38±0.3 ng/ml) remained of increasing trend (Fig. 1). The fairly high concentration of plasma progesterone in conceived animals may be because of maternal recognition of pregnancy and the presence of a fully functional corpus luteum (Silva *et al*. 2018). It has been suggested that in conceived cattle, the progesterone level from corpus luteum recorded an increase due to presence of a viable embryo and its direct or indirect impact on progesterone secretion (Henericks *et al*. 1970, Balhara *et al*. 2013) which is suggested by the apparent fall of plasma progesterone on day 6 of the cycle (Plotka *et al*. 1967, Wolf *et al*. 2003). Henericks *et al*. (1970) also recorded a high correlation (r=0.89) between plasma progesterone concentration and luteal functions which supports the findings that plasma progesterone showed significantly lower values in non-conceived animals and recorded a sharp decline after day 17 of oestrus, which corresponds to the time of luteal regression. Further, the peak plasma progesterone concentration (4.14±0.14) in non-conceived animals on day 17 corroborates with the findings of Henericks *et al*. 1970, showing a sharp decline after day 17 and return to basal value suggestive of absence of luteal tissue on the ovary. The ascending increase in conceived cattle even after day 17 and an apparently higher value on day 21 (6.38±0.39) indicates maintenance of corpus luteum due to the establishment of pregnancy.

Estimation of levels of progesterone in serum between days 17 and 21 after insemination (Shemesh *et al*. 1973, Sasser *et al*. 1987, Prvanovic *et al*. 2009) along with the expression levels of *MX2* gene can be used as diagnostic tool for pregnancy diagnosis. The maternal recognition of pregnancy in cattle is observed to occur between day 13-day 16 (from the day of artificial insemination) (Pugliesi *et al*. 2014, Kimura and Matsuyama 2014). It occurs after releases of Interferon τ (IFN- τ) (Kim *et al*. 2013, Kose *et al*. 2014, Pugliesi *et al*. 2014) which triggers the expression of IFN-stimulated genes present in peripheral blood cells (Bott *et al*. 2010). Myxovirus resistance gene (*MX2*) is one of the IFN-stimulated gene which is associated with the process of maternal recognition of pregnancy among cattle (Kim *et al*. 2013, Kizaki *et al*. 2013, Kose *et al*. 2014, Pugliesi *et al*. 2014).

Previous study suggested that level of *MX2* gene transcript (mRNA) increases in the pregnant cows in comparison to nonpregnant cows on day 16, 18 and 20 post AI (Gifford *et al*. 2007, Green *et al*. 2010). Hence, high level expression of *MX2* mRNA in peripheral blood leukocytes (PBL) of cattle might be a suggestive parameter of pregnancy detection at 21 days post AI (Kizaki *et al*. 2013). However, the *INF*-τ secretion is purely transitory and it reaches its maximum level by 20 to 24 days and is completely vanished by day 30 of pregnancy (Roberts 1985).

The amplification plots of both the genes (*MX2* and *GAPDH*) were found similar, confirming the similarity in kinetics of Real-Time PCR (Fig. 2 and Supplementary Fig. 1). The dissociation curves of the amplified products of *MX2* gene and *GAPDH* gene were also generated which showed the single peak confirming the specificity of the amplification (Fig. 3 and Supplementary Fig. 2). The fold changes of expression of *MX2* gene between non-pregnant and pregnant animals at day 17 was also estimated and presented as Table 2.

Following conception, interaction between the embryo

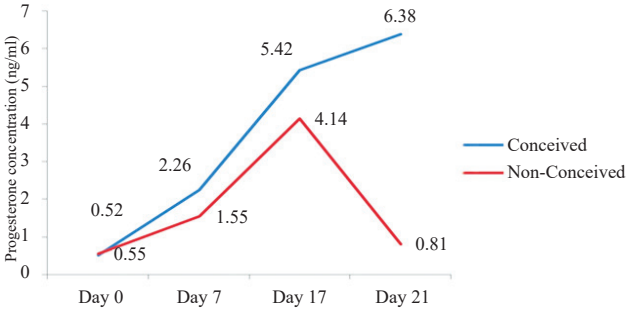

Fig. 1. Plasma progesterone concentration (ng/mL) in conceived and non-conceived cows at different time intervals post insemination.

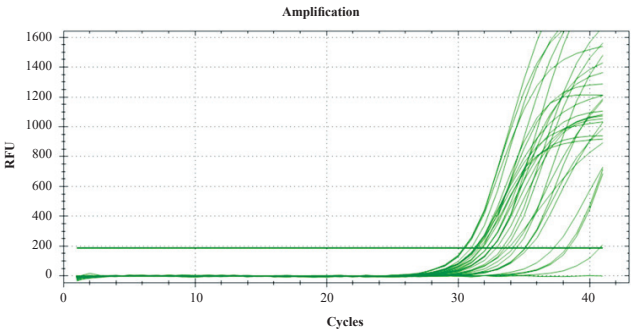

Fig. 2. Amplification plot of *MX2* gene transcript in Real-Time PCR.

Table 2. mRNA expression of *MX2* gene in conceived cows

Sample	Avg CT (*MX2*)	Avg CT (*GAPDH*)	ΔCT (*MX2*-*GAPDH*)	ΔΔCT (ΔCT Target-ΔCTNP)	Fold change inexpression of *MX2* ($2^{-\Delta\Delta CT}$)
S40 (NP)	36.27±0.18	30.38±0.13	5.89±0.19	0.00±0.19	01.00±0.19
T8	32.72±0.15	31.62±0.23	1.10±0.21	-4.79±0.21	27.66±0.21
T6	31.08±0.11	30.03±0.26	1.05±0.17	-4.84±0.17	28.64±0.17
459	32.08±0.45	30.17±0.33	1.91±0.41	-3.98±0.41	15.78±0.41
S10	31.80±0.11	29.79±0.27	2.01±0.23	-3.88±0.23	14.72±0.23
S32	32.88±0.22	30.24±0.24	2.64±0.21	-3.25±0.21	9.51±0.21

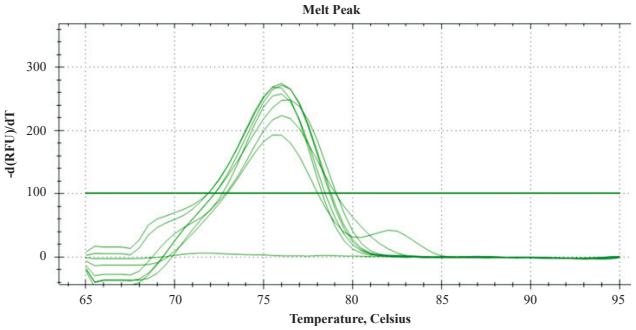

Fig. 3. Dissociation curve of *MX2* gene transcript in Real-time PCR.

and uterus leads to the secretion of *IFN*-τ which then enters the uterine vein and up-regulates the expression of Interferon stimulated genes in both peripheral blood cells and corpus luteum (Bott *et al*. 2010). The up regulation of these genes, especially *MX2* mRNA may be used to detect early pregnancy and embryo viability (Matsuyama *et al*. 2012, Kizaki *et al*. 2013). It is a potent biomarker for early pregnancy diagnosis between days 14-21 among conceived animals (Matsuyama *et al*. 2012, Kizaki *et al*. 2013).

The mRNA expression of *MX2* gene was significantly high in pregnant animals in comparison to non-pregnant animal (S40) at day 17 post AI (Table 2). In pregnant animals T6, T8, 459, S10 and S32, the mRNA expression of *MX2* gene was found as 28.64, 27.66, 15.78, 14.72, and 9.51-fold higher ($P<0.05$), respectively than non-pregnant animal (S40) (Table 2). Green *et al*. (2010) and Serrano-Pérez *et al*. (2019) reported higher expression of *MX2* gene along with other genes, viz. *OAS1*, *ISG15* and *MX1* in pregnant cows in comparison to non-pregnant cows on 19 days post AI. It was observed that *MX2* mRNA levels in non-pregnant cows showed 1 to 1.5-fold increase on days 16, 18, and 20. However, it increased to 2, 4 and 5-fold on days 16, 18, and 20 in pregnant cows (MacMicking 2004). Buragohain *et al*. (2016) reported a sixteen-fold higher expression of *MX2* gene in pregnant buffalo blood using qRT-PCR analysis as compared to inseminated and non-inseminated non-pregnant animals on day 14-28 post AI.

However, our study showed much higher (9.51 to 28.64-fold) increase in *MX2* gene expression at day 17 post AI in cattle. Several researchers had also revealed that semiquantitative RT-PCR and qPCR showed higher level of expression of *MX2* transcript during 17-21 days post AI in blood of pregnant cattle (Kizaki *et al*. 2013) and buffalo (Buragohain *et al*. 2016). Batra *et al*. (2018) reported nearly five and three-fold higher expression in *MX2* transcript in the blood of pregnant animals (*Bubalus bubalis*) in comparison to non-pregnant animals on days 18 and 21 ($P<0.05$) post AI, respectively. The increased level of mRNA expression was also found positively correlated with serum progesterone in pregnant animals. The plasma progesterone values observed in pregnant cattle on day 17th post AI were found to be greater in conceived than non-conceived cattle, which was found to coincide with the *MX2* gene expression in them. Thus, *MX2* gene expression on day 17 along with progesterone assay on day 21 can be used as a potent biomarker for early pregnancy diagnosis in cattle.

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