Full Length Article

MELATONIN RECEPTOR GENE (*MTNR1A*) POLYMORPHISM AND ITS ASSOCIATION WITH AGE AT FIRST CALVING IN BUFFALOES

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ABSTRACT

A total of 203 blood samples were collected along with data on age at first calving from Murrah / graded Murrah buffaloes maintained at different organized farms. Genomic DNA isolation and Polymerase Chain Reaction (PCR) were performed to amplify the PCR products and were digested with HpaI restriction enzymes overnight. Population genetic indices was calculated based on basic statistical tools. At the MTNR1A / HpaI locus, three genotypes viz., CC, CT and TT identified, with frequencies of 0.225, 0.505 and 0.270 respectively. The allele frequencies of C and T alleles were 0.478 and 0.522 respectively. The overall means of age at first calving was 1385.76 ± 24.31 days. Individuals with the CT and TT genotypes (1356.78 ± 33.03 and 1378.38 ± 37.66 days) had shorter age at first calving than CC genotype (1422.14 ± 53.01 days) and the differences were statistically significant (P<0.05).

Keywords: *MTNR1A* polymorphism, PCR –RFLP, AFC

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INTRODUCTION

The Livestock plays a significant role in the agricultural economy of many developing countries including India by providing milk, meat and draught power (Gordon, 1996). The reproductive performance of buffaloes is commonly affected due to late maturity, poor expression of estrous, anestrous, inactive ovaries, prolonged postpartum interval, seasonal cyclicity and silent estrous (Brar and Nanda, 2004). The reproductive performance of buffaloes is affected by many hormones viz. Estrogen, Melatonin, Follicle-stimulating hormone and Luteinizing hormone (Sarla *et al*., 2015, Yi *et al*., 2012, Terzano *et al*., 2012).

Elevated secretion of melatonin is responsible for the induction of ovarian cycle in buffaloes. Melatonin (*MTNR*1*A*) gene has been repeatedly proposed as a candidate gene and seems to play a key role in the control of circadian concentrations of melatonin (Dubocovich, 1988 and Weaver *et al*., 1996)

In Italy, however, where buffaloes are fed with a constant balanced diet, a distinct seasonal reproductive pattern is also found, and the inference from a series of studies is that seasonality is influenced by photoperiod, mediated by melatonin secretion (Zicarelli, 2010). In India, where summer heat stress is severe, high prolactin secretion has been identified as a factor contributing to acyclicity and poor fertility by lowering progesterone secretion during the summer months (Roy and Prakash, 2007).

Messer *et al*. (1997) reported that identification and characterization of genes responsible for reproduction traits may be a powerful tool in animal breeding programme. On realising the importance of the functions played by melatonin hormone receptor gene in reproduction, the present study was undertaken to reveal the association of polymorphic variants of *MTNR*1*A*, gene with age at first calving of buffaloes.

MATERIALS AND METHODS

Experimental animal and DNA extraction

Blood samples were collected from 203 Murrah / graded Murrah buffaloes maintained at different organized farms and farmer's herd (Table 1). About 5 ml of blood samples were collected in the vacutainer containing 8 % EDTA.K3 solution (Improvacuter®, Germany). The reproductive performance data viz. date of birth (DOB) and age at first calving (AFC) of buffalo was collected from farm register / pedigree sheets. Genomic DNA was extracted by using the standard high salt method as described by Miller *et al.* (1988).

Polymerase Chain Reaction

A 824 bp fragment of exon 2 of *MTNR*1*A* gene was amplified by using a set of forward (5′ TGT GTT TGT GGT GAG CCT GG 3') and reverse (5′ ATG GAG AGG GTT TGC GTT TA 3') primers (Carcangiu *et al*., 2011). PCR was carried out in 25 µl volume containing 12.5 µl of 2 X PCR master mix, 9.5 µl of nuclease free water 1 µl of each primer (10 pM) and 1 µl of genomic DNA. PCR cycle conditions were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles denaturation at 94°C for 1min, annealing at 62°C for 1 min, extension at 72°C for 1 min and final extension 72°C for 10 min (Bio-Rad T^{100}).

Restriction Fragment Length Polymorphism

The PCR products were digested with *Hpa*I restriction enzyme at 37 °C overnight with a final reaction volume of 15 µl (Takara Bio USA, Inc.). The restriction fragments (10 µl) were subjected to electrophoresis in two per cent agarose gel in 1 X TAE buffer containing ethidium bromide to determine the genotypes. The gels were visualised under UV and the images were documented (Bio-Rad Gel Doc™).

Statistical analysis and association studies

The allele and genotype frequencies were calculated by direct counting method. The expected heterozygosity (H_e) was measured as per the formula given by Nei, (1973). Effective number of alleles was calculated as per the formula given by Kimura and Crow (1964). The polymorphism information content (*PIC*) was calculated using the formula explained by Botstein *et al.* (1980).

Testing of Hardy – Weinberg Equilibrium was done by Chi-square test **(**Falconer and Mackay, 2009). The allele frequency, effective number of alleles, Hardy – Weinberg equilibrium proportions, heterozygosities and heterozygote deficiency or excess (F_{IS}) were drawn using POPGENE 32 (Version 1.32) programme by Yeh *et al.* (2000). The effect of genotypes and different locations were worked out using least squares analysis of variance as described by Harvey (1986) for non-orthogonal data.

RESULTS AND DISCUSSION

PCR - RFLP

The mean yield of DNA isolated was 450.88 ng/µl. Digestion of *MTNR*1*A* PCR product with *Hpa*I restriction enzyme revealed three genotypes *viz., CC, CT* and *TT*. The resulted fragments were 824, 745 and 79 bp and the genotypes observed were *CC* (745 and 79 bp), *CT* (824, 745 and 79 bp) and *TT* (824 bp) (Figure 1).

Similar to the present findings, presence of *CC*, *CT* and *TT* genotypes were reported in Mediterranean Italian buffaloes by Paludo *et al.* (2011); Luridiana *et al.* (2012) and Brazil buffaloes by Zetouni *et al*. (2014); Barbosa *et al.* (2016). Pandey *et al*. (2019) and Gunwant *et al*. (2018) also reported the same three genotypes in Indian water buffaloes. Cheema *et al*. (2016) revealed three genotypes using three different set of primers and restriction enzymes (*Mn*lI*, Rsa*I *and Hpa*lI) in Indian Murrah buffaloes. In the present study, *C* and *T* alleles were equally contributed in the Indian buffalo population and were comparable to the values reported in both Indian and Mediterranean buffaloes. In many locations, higher frequencies of *CT* genotype similar to present study was reported in Indian and Mediterranean buffaloes.

Population genetic Indices

The observed genotype and allele frequencies along with their expected genotypic frequencies for *MTNR*1*A*/*Hpa*I gene were presented in Table 2. The *C* and *T* allele were equally contributed in the buffalo population. Analysis revealed, no significant differences between observed and expected genotype frequencies in the studied population and was consistent with Hardy-Weinberg equilibrium (P>0.05). The distribution of genotypic

Table 1. Details on the location and number of blood samples collected

S. No	Location of farm	No. of blood samples
1	Post Graduate Research Institute in Animal Science (TANUVAS), Kattupakkam, Tamil Nadu.	34
2	Buffalo Research Station, Venkataramanna Gudem, S.V.V.U, West Godavari District, Andhra Pradesh	86
3	Saraswathi Krishi Vigyan Kendra, Karur District, Tamil Nadu.	19
4	Central Cattle Breeding Farm, Alamadhi, Chennai, Tamil Nadu.	20
5	Farmers herd, Namakkal, Tamil Nadu	44
	Total	203

Table 2. Distribution of genotypes and allele frequencies at *MTNR1A / Hpa*I locus in buffaloes

Sample size	Observed Genotype frequency			Allele frequency		Expected Genotype frequency			χ^2 value	P value
	CC	*CT*	*TT*	*C*	*T*	*CC*	*CT*	*TT*		
203	0.227 (46)	0.502 (102)	0.271 (55)	0.478	0.522	0.229 (46.38)	0.499 (101.30)	0.272 (55.31)	0.0097^{NS}	0.9215

Figures in parentheses are the number of observations; NS: Not significant (P>0.005)

Table 3. Heterozygosity statistics and genetic diversity at *MTNR1A / Hpa*I locus in buffaloes

Sample size	Observed homozygosity	Observed heterozygosity	Expected homozygosity	Expected heterozygosity	N_e	PIC	F_{IS}
203	0.498	0.502	0.501	0.499	1.996	0.499	-0.006

Ne = Effective number of alleles; PIC = Polymorphic information content; F_{IS} = Fixation index

Table 4. Location and genotype effects on age at first calving in buffaloes for *MTNR1A* / *Hpa*I gene

Main effects / subclass	Age at first calving
Overall Mean	1385.76 ± 24.31 (57)
Location	*
Central Cattle Breeding Farm (CCBF), Alamadhi, Chennai	$1514.26^{b} \pm 38.09$ (20)
Buffalo Research Station (BRS), Andhra Pradesh	-
Post Graduate Research Institute in Animal Science (PGRIAS), Kattupakkam	$1335.18^{a} \pm 47.01$ (17)
Saraswathi Krishi Vigyan Kendra, Karur District	$1307.86^{a} \pm 40.72$ (20)
Genotype	*
CC	$1422.14^{b} \pm 53.01$ (12)
CT	$1356.78^{a} \pm 33.03$ (24)
TT	$1378.38^{ab} \pm 37.66$ (21)

( ) = No. of observations

Table 5. Means (±S.E) of age at first calving for *MTNR1A* / *Hpa*I genotypes in buffaloes

Reproductive traits	Genotype		
	CC	***CT***	***TT***
Age at first calving	$1467.33^{b} \pm 59.02$ (12)	$1361.25^{ab} \pm 36.48$ (24)	$1340.43^{a} \pm 34.16$ (21)

( ) = No. of observations

Table 6. Means (±S.E) (in days) of age at first calving for *MTNR1A* / *Hpa*I genotypes in buffaloes at different locations

Locations	Genotype			Over all mean
	CC	*CT*	*TT*	
Central Cattle Breeding Farm, Alamadhi, Chennai	1553.57 ^{a} ± 85.38 (7)	1432.44 ^{a} ± 71.61 (9)	1556.75 ^{a} ± 57.31 (4)	1499.70 ± 45.56 (20)
Post Graduate Research Institute in Animal Science, Kattupakkam	1405.50 ^{a} ± 60.50 (2)	1312.75 ^{a} ± 65.24 (8)	1287.29 ^{a} ± 54.98 (7)	1313.18 ± 38.08 (17)
Saraswathi Krishi Vigyan Kendra, Karur District	1307.33 ^{a} ± 40.57 (3)	1325.14 ^{a} ± 35.74 (7)	1291.10 ^{a} ± 32.05 (10)	1305.45 ± 20.57 (20)

( ) = No. of observations

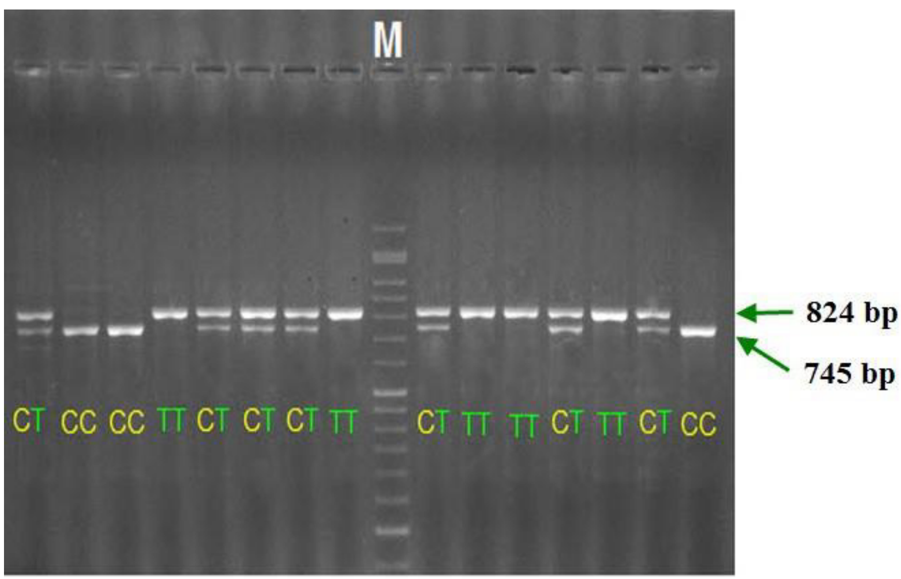

Fig. 1. PCR-RFLP patterns of *MTNR1A* / *Hpa*I gene in buffaloes.

frequencies revealed a heterozygosity value of 0.505 which was almost equal to the expected heterozygosity (0.499) and effective number of alleles (*Ne*) was 1.996. Estimated Polymorphic information content (PIC) for the gene was 0.4990 and it falls in the medium range of 0.25 to 0.5. Fixation index (*F*IS) values were negative and showed an excess of heterozygosity that was strongly accentuated in *MTNR*1*A* / *Hpa*I locus (Table 3).

Association of *MTNR*1*A* / *Hpa*1 Polymorphism with age at first calving

The least-squares means of age at first calving for *MTNR1A* / *Hpa*1 genotypes and non-genetic effect are presented in Table 4. The mean age at first calving for different genotypes is presented in Table 5. Significant effect of location (p<0.05) was observed in age at first calving. Individuals with the *CT* and *TT* genotypes had shorter age at first calving and the differences were statistically significant (P<0.05). In contrary to present findings, Paludo *et al.* *(*2011) and Luridiana *et al.* (2012) reported non-significant effect on age at first calving.

Significantly (P<0.05) higher age at first calving was noticed in CC genotypes than TT geneotypes. Zetouni *et al*. (2014) reported a shorter age at first calving for *TT* genotype and longer period for *CC* and *CT* genotypes however the differences were not statistically significant. Generally, individuals with the *CC* genotype had more age at first calving in all the government organized farms however, the differences were not statistically significant (Table 6).

In conclusion, in the present study there was an association found to exist between the MTNR1A gene polymorphism and age at first calving in buffaloes. Based on association with small sample size, suggestion could not be made that the polymorphism might be considered as a genetic marker to identify reproduction capacity in buffaloes. To improve the reproduction performance of Indian buffaloes, more studies are warranted with large numbers of animals in different Indian buffalo populations and may lead to genetic variation and that can be used for selection of animals for reproduction.

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