MELATONIN RECEPTOR 1A GENE POLYMORPHISM IN MURRAH BUFFALOES WITH POSSIBLE IMPACT ON SUMMER ANESTROUS

RANJNA S CHEEMA*, AMANDEEP KAUR[1], SPS GHUMAN[2] AND SHAHBAZ S DHINDSA[3]

*SENIOR PHYSIOLOGIST (REPRODUCTION), RESEARCH FELLOW, PROFESSOR, ASSITTANT PROFESSOR

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ABSTRACT

In the present study murrah buffaloes were analyzed to investigate polymorphic sites for Mnl I, Rsa I and Hpa11 using three different primers of expected size of 268 bp (1), 824 bp (2) and 856 bp (3). PCR of genomic DNA using primers 1, 2 and 3 resulted in successful amplification of bands of 824 bp, 268 bp and 856 bp corresponding to the main part of the exon II of the MTNR1A melatonin receptor gene. Polymorphism was noticeable at 50 bp and 267 / 110 bp restriction sites upon digestion of 824 bp PCR product with Mnl I and Rsa I, respectively. Genotypic frequency for the presence of 50 bp and 267 / 110 bp restriction sites was 0.62 and 0.95 / 0.33, respectively. Digestion of 856 bp PCR product with Mnl I and Rsa I also showed polymorphism at 236 / 50 bp and 320 / 50 bp restriction sites, respectively. Genotypic frequency for the presence of 236 / 50 bp and 320 / 50 bp restriction sites was 0.04 / 0.76 and 0.10 / 0.05, respectively. Our observations also revealed a difference in polymorphism of *MTNR1A* gene amid heifers and cows. Polymorphism observed in *MTNR1A* gene in Murrah buffaloes may have impact on summer anestrous and needs to be investigated.

Key words: MTNR1A, receptor, polymorphism, murrah, buffaloes

No: of Tables : 2 **No: of Figures :3** **No:of References:12**

Introduction

Melatonin is secreted by the pineal gland at night in direct proportion to a period of darkness and help to regulate circadian rhythms and reproduction changes in seasonally reproductive mammals (Malpaux *et al.*, 2001). These effects are exerted through the binding of melatonin receptors located in the brain and control reproductive function (Migaud *et al*., 2005). Melatonin receptors are classified as MTNR1A and MTNR1B subtypes but only the first is involved in the regulation of seasonal reproductive activity in mammals (Weaver *et al.*, 1996 and Dubocovich *et al*., 2003). The MTNR1A receptor gene in sheep is on chromosome 26, in cattle on chromosome 27 and in buffalo on chromosome 1 which is a fusion of Bos Taurus chromosome 1 and 27 (Iannuzzi *et al*., 2003 and Miziara *et al*., 2007). Restriction fragment length polymorphism (RFLP) in the MTNR1A gene was noticed in several pig, sheep and cattle breeds (Messer *et al*., 1997). The RFLP Hpa I polymorphism in the MTNR1A gene is associated with seasonal reproduction in Mediterranean Italian buffaloes (Carcangiu *et al*., 2011). Two RFLP polymorphic sites for Mnl I and Rsa I enzymes have been found in exon II of the MTNR1A gene in sheep (Messer *et al*., 1997). Studies also suggest that these RFLP polymorphic sites, position 612 for sheep (Carcangiu *et al*., 2009) and position 52 for goat, of MTNR1A exon II are associated with a decrease in seasonal reproductive activity (Chu *et al*., 2007; Carcangiu *et al*.2009). Relationship between polymorphism of these two RFLP sites and seasonal reproduction varied within different breeds (sheep or goat) and/or in environmental conditions. Therefore, we were interested to study polymorphic sites for Mnl I, Rsa I and Hpa11 using three different primers of expected size of 268 bp, 824 bp and 856 bp in murrah buffaloes.

Material and Methods:

Genomic DNA Preparation

Blood samples (10 ml) were collected from the murrah buffaloes from dairy farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana and private dairy farms around Ludhiana in EDTA containing centrifuge tubes. Genomic DNA was extracted from the buffy coat using a commercially available kit (Genomic DNA Mini kit, Geneaid) and stored at -20°C until further use. Polymerase chain reaction (PCR) was run using 100-150 ng template DNA and primers described by Messers *et al.*, 1997 and Lai et al, 2013. The primer sequences synthesized by Hysel India Pvt. Limited were as follows:

Primer	primer sequence (5'- 3')	product size (bp)
P1	F1: AGCTCAGCCTACACGATCGC R1: CCAGCAAATGGCAAAGAGGAC	268
P2	F2:TGT GTT TGT GGT GAG CCT GG R2: ATG GAG AGG GTT TGC GTT TA:	824
P3	F3: GCC TGG CAG TTG CAG ACC TG R3: CAT TTT TAA ACG GAG TCC ACC	856

Genotypic analysis

The PCR reaction was carried out in 50µl volume containing approximately 25 µl EmeraldAmp GT PCR Master Mix (2X Premix), 0.25µl each primer, 100-150ng template DNA. PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min, with a final extension at 72°C for 10min on Master cycler. PCR products were separated by electrophoresis on 1% agarose gel in parallel with a 100bp DNA marker.

Restriction Fragment Length polymorphism: PCR products of 10 µl were digested separately with Fast Digest restriction enzymes (Fermentas) i.e. MnlI, RsaI and HpaII at 37°C for 3-5 minutes in a water bath. The digestion reaction was carried out in 30µl volume containing 10µl PCR product, 2µl (10 x) FastDigest Green buffer, 1µl FastDigest enzyme and 17µl nuclease free water. The resulting fragments were separated by electrophoresis on 3% (w/v) agarose gel in parallel with 100bp DNA marker.**Statistical Analysis:** Genotypic frequency was calculated among 73 tested buffaloes.

Results and Discussion:

PCR amplification of MTNR1A gene: Genomic DNA of 66, 30 and 73 buffaloes subjected to PCR using primer pairs 1, 2 and 3 for exon 2 of the MTNR1A gene, respectively. PCR of genomic DNA using primers 1, 2 and 3 resulted in successful amplification of bands of 824 bp, 268 bp and 856 bp corresponding to the main part of the exon II of the MTNR1A melatonin receptor gene.

RFLP analysis of MTNR1A gene:

Primer 1: Digestion of 267 bp PCR product with *MnlI, RsaI and Hpa-II* resulted in an undigested product of 200 bp (Fig 1).

Primer 2: Digestion of 824 bp PCR product with *MnlI* resulted in 218, 150, 75 and 50bp

fragments (Fig 2, Table 1). Fragments of 218, 150 and 75 bp were detected in 66 buffaloes. Polymorphism was observed only at 50 bp restriction site. Allele M contained the restriction site for *MnlI* and presence or absence of restriction site at 50 bp fragments was considered as MM and mm allele, respectively. Genotypic frequency for MM and mm allele among the tested cows was 0.62 and 0.38. Carcangiu *et al*., (2011) cloned PCR product of 824 bp of MTNR1A gene in Mediterranean Italian buffaloes and observed that digestion with HpaI resulted in cleavage site caused by the presence of C and after electrophoresis it produced two bands of 79 and 745 bp. The absence of this cleavage site was caused by the presence of T which leaves the uncut 824 bp fragment. Frequency of C and T alleles was respectively 0.44 and 0.56 in the analyzed population.Digestion of 824 bp PCR product with *RsaI* resulted in 411, 267 and 110 bp fragments (Fig 2). Restriction site at 411 bp was observed in 66 buffaloes, wheres polymorphism was observed at 267 and 110 bp restriction sites. Allele R contained the restriction site for *RsaI* and presence or absence of 267 / 110 bp restriction sites was considered as RR-1/ RR-2 and rr-1 / rr-2, respectively. Genotypic frequencies for RR-1/ RR-2 and rr-1 / rr-2 were 0.95 / 0.03 and 0.05 / 0.97, respectively. The uniform 824 bp PCR product was digested with restriction endonucleases *Mnl*I and *Rsa*I, and checked for the presence of restriction sites in six goat breeds (Chu *et al*., 2007). They did not observe polymorphism at the *Mnl*I cleavage sites and no relationship could be established between the *Mnl*I cleavage sites of *MTNR1A* gene and reproductive seasonality in goats. For polymorphic *Rsa*I cleavage site at base position 53, only genotype *RR* (267/267 bp) was detected in Jining Grey goats, both genotype *RR* and genotype *Rr* (267 /320 bp) were found in all other goat breeds, no genotype *rr* (320 / 320 bp) was detected in all six goat breeds.

Primer 3: Digestion of 856 bp PCR product with *MnlI* resulted in 236, 218, 150, 75 and 50 bp fragments (Fig 3). Restriction sites at 218, 150 bp, 75 bp were detected in 73 buffaloes. Polymorphism was observed at 236 and 50 bp restriction sites. Presence or absence of restriction site at 236 / 50 bp fragments was considered as MM -1 / MM-2 and mm-1 / mm-2 alleles, respectively. Gene frequencies for MM -1 / MM-2 and mm-1 / mm-2 were 0.04 / 0.76 and 0.96 / 0.24, respectively. *RsaI* digestion of 856 bp product resulted in five fragments of 411, 320, 267, 110, 50 bp (Fig 3). Restriction sites at 411, 267 and 110 bp were detected in 73 cows and polymorphism was visible at 320 and 50 bp fragments. Presence and absence of 320 / 50 bp restriction sites was considered as RR-1/ RR-2 and rr-1 / rr-2, respectively. Genotypic frequencies for RR-1/ RR-2 and rr-1 / rr-2 were 0.10 / 0.05 and 0.90 / 0.95, respectively. Lai et al (2013) amplified MTNR-1A gene of different breeds of goat using 856 bp primers, digested the product with Eco31 I and RFLP analysis suggested the presence of three genotypes (CC for 279/ 577 bp, DD for 856 bp and CD for 279/577/856 bp) in all the analysed breeds of goat. Mateescu *et al*., (2009) amplified MTNR-1A gene using 824 bp primers and digestion with *MnlI* and resulted in 236- and 67-bp fragments, Allele

M contained the restriction site for *MnlI* whereas the absence of the restriction site in the *m* allele resulted in a single 303-bp fragment. Similarly, allele *R* contained the restriction site for *RsaI* and resulted in 290- and 5-bp fragments, whereas the absence of the restriction site in the *r* allele resulted in a single 295-bp fragment. Gene frequencies were 0.64 and 0.36 for the *MnlI* polymorphism (alleles *M* and *m*, respectively) and 0.35 and 0.65 for the *RsaI* polymorphism (alleles *R* and *r*, respectively). Among the 116 ewes, genotypic frequencies were 0.43, 0.44, and 0.13 for *MM*, *Mm*, and *mm* genotypes at the *MnlI* polymorphism and 0.13, 0.43, and 0.44 for *RR*, *Rr*, and *rr* genotypes at the *RsaI* polymorphism.

824 bp (n=66)		856 bp (n=73)	
Restriction sites (bp, number of animals)		Restriction sites (bp, number of animals)	
MnII	*RsaI*	*MnII*	*RsaI*
--	411 (66)	--	411 (73)
--	--	--	320 (4)
--	267 (63)	--	267 (73)
--	--	236 (3)	--
218 (66)	--	218 (73)	--
150 (66)	--	150 (73)	--
--	110 (2)	--	110 (73)
75 (66)	--	75 (73)	--
50 (41)	--	50 (56)	50 (8)

Table1. Distribution of total animals according to restriction sites digested with *MnII* and *RsaI* using 824 and 856 bp primers.

Table 2: Distribution of heifers and buffaloes according to restriction sites, digested with *MnII* and *RsaI* using 824 and 856 bp primers.

Restriction sites (bp)	Heifers (n=11)		Cows (n=11)	
	824 bp	856 bp	824 bp	856 bp
MnII				
218	11	11	11	11
150	11	11	11	11
75	11	11	11	11
50	6	8	5	10
RsaI				
411	11	11	11	11
267	9	11	11	11
110	2	11	1	11
50	-	2	-	-

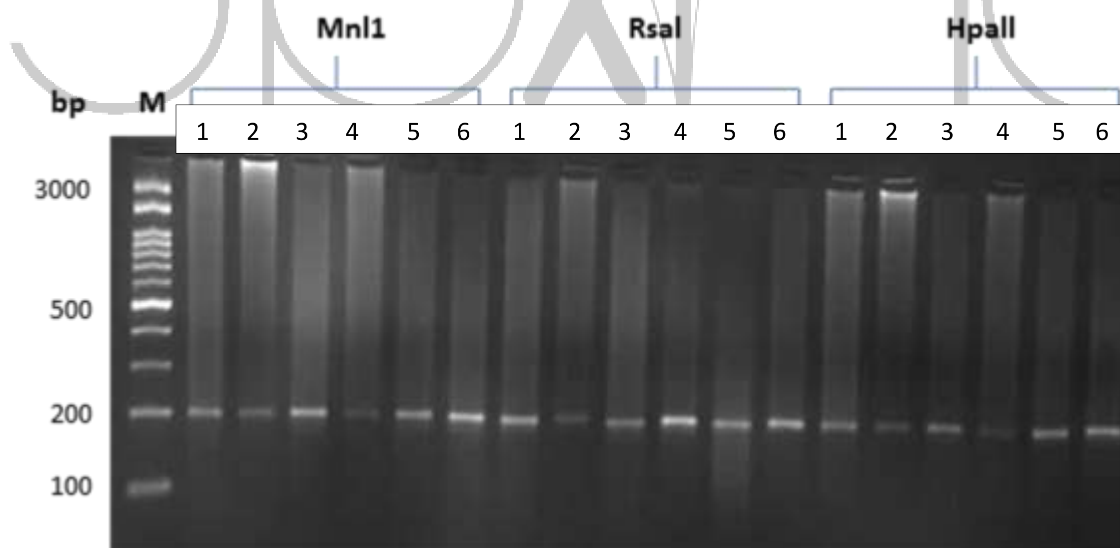

Fig 1. Electrophoresis of digestion of 267 bp PCR product with *MnII*, *RsaI and* HpaI on a 3% agarose gel in Murrah buffalo. M, 100 bp DNA marker and 1-6 indicate samples of different buffaloes.

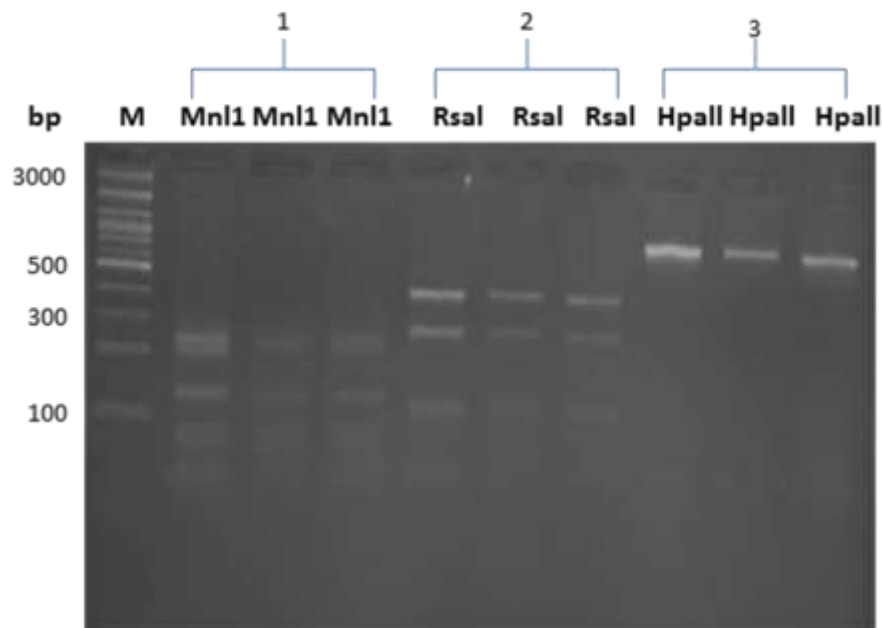

Fig2. Electrophoresis of digestion of 824 bp PCR productwith *MnII*, *RsaI and* HpaI on a 3% agarose gel in Murrah buffalo. M, 100 bp DNA marker and 1, 2, 3 indicate samples of different buffaloes.

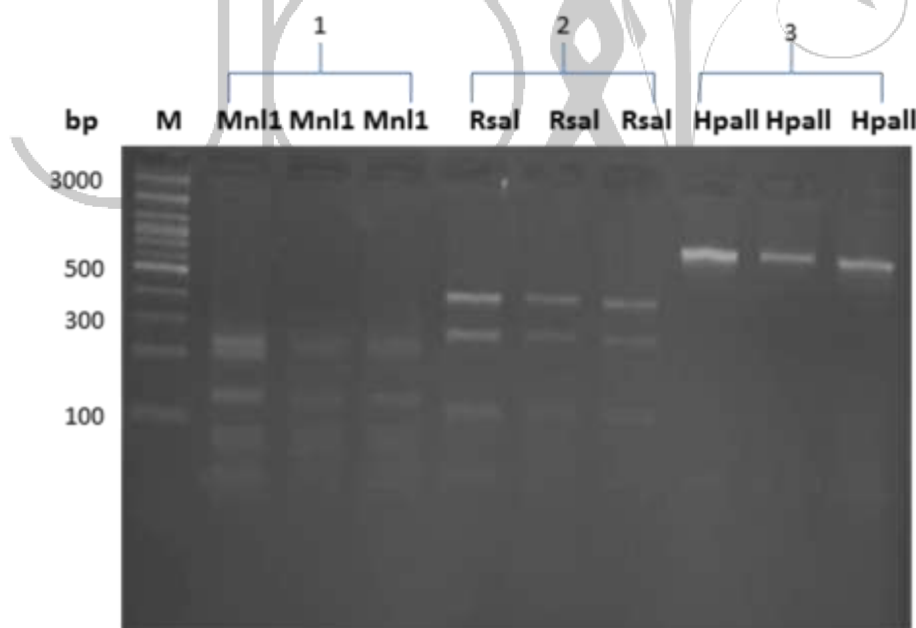

Fig 3. Electrophoresis of digestion of 856 bp PCR product with *MnII*, *RsaI and* HpaI on a 3% agarose gel in Murrah buffalo. M, 100 bp DNA marker and 1 - 3 indicate samples of different buffaloes.

Digestion of 824 bp and 856 bp PCR products with *Hpa-II* resulted in an undigested fragment of 850 bp in all tested buffaloes (Fig 1, 2). Luridiana *et al*., (2012) subjected genomic DNA of Mediterranean Italian buffaloes to PCR using 824 bp primers and its digestion with *Hpa*I confirmed one polymorphic site in position 82 C.T. The cleavage site was caused by the presence of a C nucleotide in position 82; after electrophoresis, this resulted in two bands of 82 bp and 742 bp. With a T nucleotide in position 82, the cleavage site was absent, resulting in an uncut 824-bp fragment. The frequency of the C and T alleles in the population analysed was 0.44 and 0.56, respectively.**Difference of *MTNR1A* genotype distribution in heifers and cows:** Distribution of heifers and cows according to restriction sites is given in Table 2. A difference in genotypic frequency was observed at only 50 bp restriction site digested with *MnlI* between heifers and cows. Genotypic frequency for MM allele using primers 824 / 856 was 0.54 / 0.72 and 0.45 / 0.90 in heifers and cows, respectively. Similarly, difference in frequency was observed at 267 / 110 and 50 bp restriction sites upon digestion of 824 and 856 bp PCR products with *RsaI* among heifers and cows, respectively. Digestion of 824 bp product with *RsaI* resulted in genotypic frequency of 0.82 and 0.18 for RR-1 and RR-2 among heifers, whereas, it was only 0.09 for RR-2 among cows. Digestion of 856 bp product with *RsaI* showed a genotypic frequency of 0.18 for RR-2 only in heifers.

Luridiana *et al*., (2012) were of the view that polymorphism in *MTNR1A* gene may be considered as a genetic marker to identify buffaloes that are able to reproduce out of the breeding season. Our study also indicated that polymorphism observed in *MTNR1A* gene in Murrah buffaloes may have impact on summer anestrous. Observations further revealed a difference in polymorphism of *MTNR1A* gene amid heifers and cows. Relationship of polymorphism of melatonin receptor MT1 gene to age of first mating and calving and between consecutive calvings in Murrah buffaloes needs to be investigated.

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