IJAS Iranian Journal of Applied Animal Science

DNA Polymorphisms at Candidate Gene Loci and Their Relation with Milk Production Traits in Murrah Buffalo (*Bubalus bubalis*)

Research Article

D.S. Kale[1*], B.R. Yadav[1] and J. Prasad[2]

[1] Livestock Genome Analysis Laboratory, National Dairy Research Institute, Karnal, 132001, Haryana State, India
[2] Department of Animal Genetics and Breeding, Faculty of Veterinary Science, Allahabad Agricultural Institute Deemed University, Allahabad, 211007, Utter Pradesh, India

Received on: 24 Jan 2013
Revised on: 13 Mar 2013
Accepted on: 16 Mar 2013
Online Published on: Mar 2014

*Correspondence E-mail: deepakkaleccmb@gmail.com

Online version is available on: www.ijas.ir

ABSTRACT

DNA polymorphism within diacylglycerol transferase 2 (DGAT2) / monoacyl glycerol transferases 2 (MOGAT2), leptin and butyrophilin genes were analysed using PCR-SSCP in Murrah buffalo. The single strand conformation polymorphism (SSCP) analysis of amplified gene fragment in exon 5 of MOGAT2, exon 3 of leptin and intron 1 of butyrophilin gene revealed different patterns. A, B and C showed the following frequencies for each candidate gene in 53 (A=0.49, B=0.36 and C=0.15), 65 (A=0.38 and B=0.62) and 55 samples (A=0.6, B=0.31 and C=0.09) from Murrah buffaloes, respectively. The strand conformation polymorphism (SSCP) followed by DNA sequencing revealed one single nucleotide polymorphism (SNP) that was (c.193T>C) in MOGAT2, one single nucleotide polymorphism (SNP) (c.25 T>C) in leptin and one single nucleotide polymorphism (SNP) (c.184C>T>G) in butyrophilin gene confirmed BTI1 SSCP. The statistical analysis using general linear model procedure for association study, indicated that Murrah buffalo monoacyl glycerol transferases 2 (MOGAT2) (c.193T>C) single nucleotide polymorphism (SNP) and (c.25 T>C) in leptin genotypes were not significantly different (P>0.01) in Murrah buffalo for milk production traits milk yield, fat percentage and single nucleotide polymorphism (SNP) percentage. However, the statistical analysis for association study indicated BTI1 SSCP was significantly (P≤0.05) associated with 305-days lactation milk yield. The Murrah buffaloes with BTI1BB genotypes had 683.93 kg and 320.48 kg higher milk yield as compared to BTI1AA and BTI1CC genotypes, respectively. The positive association of butyrophilin single strand conformation polymorphism (SSCP) polymorphism with milk yield will be a useful tool for future selection and breeding strategies and genetic improvement of buffaloes for milk yield.

KEY WORDS butyrophilin, DGAT2 / MOGAT2, leptin, milk yield, Murrah buffalo, PCR-SSCP.

INTRODUCTION

The candidate gene approach is one of the most important approaches to search for genetic markers associated to production traits and in analyzing polymorphism of structural and protein coding genes. Currently, genetic markers research applied to animal breeding and production is focussed mainly on analysing mutations located within candidate genes and their association with economically important production traits (Oikonomou *et al.* 2011). There are several validated DNA polymorphisms in various loci related with milk production traits in dairy cattle (Chamberlain *et al.* 2012). Diacylglycerol transferase1 (DGAT1) was the first identified gene encoding a protein with DGAT activity (Cases *et al.* 1998) in which a missense mutation (Lys^{232}→Ala) has been shown to be significantly associated with variation in milk fat percentage in cattle (Winter *et al.* 2002). DGAT-like activity has also been shown in other enzymes encoded by other genes and led to the detection of diacylglycerol transferase2 (DGAT2), monoacyl glycerol

transferases 1 (MOGAT1) and monoacyl glycerol transferases 2 (MOGAT2) which are the members of the same family (Winter *et al.* 2003). Leptin is a 16-kDa protein secreted predominantly from white adipose tissue and it performs important roles in controlling the body weight, feed intake, immune function and reproduction in dairy cattle (Block *et al.* 2001). Nevertheless, Liefers *et al.* (2005) reported the relationship between the two extreme genotype combinations (polymorphisms located at a 135 bp region of the leptin promoter) showing and increase in milk production on dairy cattle. Butyrophilin has been shown to be a mammary-specific gene and it is normally used as a genetic marker to investigate allelic substitution effects on several quantitative milk production traits (Franke *et al.* 1981). Recently, the association of butyrophilin gene polymorphism with milk yield, protein yield and SNF yield was reported in Korean dairy proven and young bulls (Jang *et al.* 2005).

India is a rich country in buffalo genetic resource having 97 million of buffaloes in 2003 which accounts for 59.5% to total world buffalo's population. Buffalo is the most important farm animal species in Asia, especially in India, where it is extensively used for milk, meat, fuel and fertilizer production (from manure) as well as for draught power (Borghese, 2005). The buffaloes contribute 55.6% to the country's total milk production and Murrah buffalo is the most important dairy breed with superior genetic potential (Borghese, 2005). However, their inherent potential for growth and production has not been yet exploited due to inadequate information about genetic basis and breeding strategies. River buffalo and domestic cattle are closely related because they belong to the same bovidae family, cytogenetically characterized, and they share homology in chromosome banding (Gallagher and Womack, 1992) and gene mapping (Di Meo *et al.* 2005). Therefore, comparative genomic approaches with various DNA marker techniques might prove to be a feasible approach in analysing the buffalo genome. The present work has been carried out to analyse candidate genes; DGAT2 / MOGAT2, eptin and butyrophilin using PCR-SSCP followed by DNA sequencing to find out possible relationships with milk production traits in Murrah buffaloes.

MATERIALS AND METHODS

Sample collection

The study group included a total 173 well maintained Murrah buffaloes at NDRI, Karnal herd whose breeding tracts are the Haryana and Punjab states of India. The SSCP analysis of candidate genes (DGAT2/MOGAT2, leptin and butyrophilin genes) was carried out using 53, 65 and 55 samples for each gene, respectively.

Blood samples (10 mL) were collected by jugular puncture using vacuum tubes containing acid citrate dextrose solution (ACD) as anticoagulant.

DNA isolation and primers

Genomic DNA isolation was carried out from blood using the phenol chloroform extraction protocol (Clamp *et al.* 1993) with some modifications like addition of sodium acetate (3 *M*) to the aqueous phase looking for the final concentration of 0.33 *M* and thorough mixing. The integrity of the DNA was assessed following electrophoresis in a 0.8% agarose gel stained with ethidium bromide. In addition, the OD ratio 260 / 280 nm was measured to check for protein contamination and to calculate the DNA concentration. All stock DNA samples were kept at -80 ˚C for longer storage and the working aliquots were maintained at -20 ˚C. The PCR primers (Table 1) designed for DGAT2 / MOGAT2, leptin and butyrophilin genes analysis in buffaloes were used on the basis of cattle GenBank sequence encompassing polymorphic restriction site with PRIMER3 software (Rozen and Skaletsky, 1998). There is a close evolutionary relationship between cattle and buffaloes that hences the bovine genome is a useful source of markers for the buffalo genome mapping (Gallagher and Womack, 1992). Therefore, the designing of primers based on cattle genome works for some of the buffalo genomic regions.

Polymerase chain reaction (PCR) amplification

The PCR was carried out on about 100 ng of genomic DNA in a 25 µL reaction volume. The reaction mixture consisted of 2.5 µL of 10 × PCR assay buffer containing 1.5 m*M* $MgCl_2$, 200 µ*M* each of dNTPS, 0.75 units *Taq* DNA polymerase and 10 pmol of each primer. Amplification was carried out in a Biometra thermal cycler using the following PCR cycling conditions: 95 ˚C for 5 min and 34 cycles of 45 seconds at 95 ˚C, T^A ˚C and 72 ˚C consecutively, followed by a five minute final extension at 72 ˚C. The PCR amplification was verified by electrophoresis of the PCR products with loading dye (95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol) on 2% (w/v) agarose gel in 0.5 × TBE buffer using a 100 bp ladder as marker for confirmation of the length of the PCR products. The agarose gels were stained with ethidium bromide (0.5 µg/mL). The amplified products (5 µL) were detected on 2% agarose gel using 1 µL of loading dye as a stop dye; electrophoresis was performed and visualized using UV light after ethidium bromide staining.

Strand conformation polymorphism (SSCP) analysis

The DGAT2 / MOGAT2, leptin and butyrophilin gene PCR products were resolved by SSCP analysis (Orita *et al.* 1989).

Table 1 Details of primer sequences used for Murrah buffalo DGAT2 / MOGAT2, leptin and butyrophilin gene PCR-SSCP analysis

Name of gene	Primer	Primer sequence	Annealing temperature / region
DGAT2	DGAT2-1141	FP: 5′-TTGACCACGTGCAGAAGGTA-3′ RP: 5′-CCTGAACCTCTGGTCTCCAA-3′	54 ˚C / intron 4
	DGAT2-1561	FP: 5′-GGTGGACAAAAATGCAACCT-3′ RP: 5′-TTGGCAGGTAGGGATCATGT -3′	61 ˚C / intron 4
	DGAT2-1861	FP: 5′-TACTTCTACCGCCCTGTTGG-3′ RP: 5′-GGGCAAAGCCAATGTACTTC-3′	61 ˚C / intron 4
MOGAT2	MOGAT2	FP: 5′-TTTGGTCTTATGCCCTACCG-3′ RP: 5′-GGACAGGGTGATCTTTTGGA-3′	65 ˚C / exon 5
Leptin	LEPE3	FP: 5′-CAATGACCTGGAGAACCTC-3′ RP: 5′-ACATAGGCTCTCTTCTCCTGT-3′	62 ˚C / exon 3
Butyrophilin	BTI1	FP: 5′-CCTGCTTATTTCCCTAGTCTC-3′ RP: 5′-CCACCCTAAGGTTAGTCAATC-3′	57 ˚C / intron 1
	BTI3	FP: 5′-AACTGGCTATAAAGCCCTAGA-3′ RP: 5′-ACTACACAAGGGAACTGAGGT-3′	57 ˚C / intron 3
	BTI4	FP: 5′-AGATCTCACAGACATTCCAGA-3′ RP: 5′-TGCTGAACCAGAGGTAGAGTA-3′	62 ˚C / intron 4

Various factors were tested for each fragment in order to optimize factors such as the amount of PCR product (4-10 ul), denaturing solution, acrylamide concentration (8 to 14%), percentage cross linking (2%), presence or absence (10%) glycerol, voltage (60 to 400 volts), running time (12 to 30 hours) and temperature (10-15 ˚C and room temperature) (by indicating the final optimized conditions used on this experiment in the results section). PCR products were diluted in denaturing solution (95% formamide, 10 m*M* NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 m*M* EDTA) and heat denatured at 95 ˚C for ten minutes. After denaturation PCR products were immediately transferred to chilled ice pack and kept in -20 ˚C for 10 minutes. The PCR products were resolved on a non-denaturing 20% acry1amide: bis-acrylamide (49:l) gel. The vertical gel electrophoresis was carried out in a Bio-Rad Protean® II Xi Cell electrophoreses unit using 1X TBE buffer at 10-12.5 V/cm for 24 hours at room temperature. Gels were silver-stained (Sambrook and Russell, 2000) and photographed using digital camera for SSCP pattern analysis.

DNA sequencing

The PCR products representing different SSCP patterns were analyzed using florescent sequencing technique from automated DNA sequencing service (Bangalore Genei). The chromatograms were used to verify the nucleotide sequence on the basis of peaks. The nucleotide sequence alignments were carried out using alignment tools from Geneious software, Clustalw and BLAST to reveal single base variations. These allelic variants nucleotide sequences were analysed and submitted in NCBI GenBank. The observed DNA sequence polymorphisms observed were used to genotype Murrah buffalo population.

Statistical analysis

The frequency of polymorphic allelic variants, genotypes and their accordance with Hardy-Weinberg law was assessed by POPGENE 1.31 software.

The association between polymorphic allelic variants of candidate genes and milk production traits were analysed using GLM procedure (SYSTAT). The following model was used:

$$Y_{ijkl} = \mu + g_i + s_j + p_k + h_l + e_{ijklm}$$

Where:

Y_{ijklm}: observation on the m^{th} animal of i^{th} genotype, l^{th} season, k^{th} parity, and h^{th} year.

μ: population mean.

g_i: effect of i^{th} genotype (i=1, 2 and 3).

s_j: effect of j^{th} season.

p_k: effect of k^{th} parity.

h_l: effect of l^{th} year.

e_{ijklm}: random error associated with the measurement on the m^{th} animal of the i^{th} genotype, l^{th} season, k^{th} parity and h^{th} year, assuming $e_{ijklm} \sim NII\ (0, \sigma^2_{\varepsilon})$.

RESULTS AND DISCUSSION

The DGAT2 gene fragments amplified using primers: DGAT2-1141, DGAT2-1561 and DGAT2-1861 (Table 1) did not exhibit SSCP polymorphisms indicating monomorphic nature of intron 4 region of buffalo DGAT2 gene. The SSCP analysis of the amplified gene fragment of exon 5 of the MOGAT2 gene resulted in three different patterns; A, B and C (Figure 1) with the following frequencies (A=0.49, B=0.36 and C=0.15).

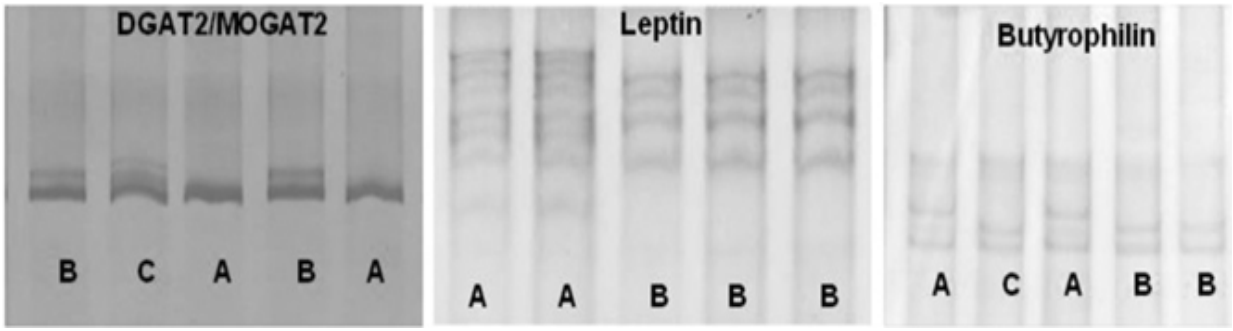

Figure 1 PCR-SSCP within DGAT2 / MOGAT2, leptin and butyrophilin gene in Murrah buffalo

Where:
A, B and C: gene fragment specific SSCP patterns

The SSCP patterns for LEPE3 primer were better resolved in 12% acrylamide-bisacrylamide with other conditions kept similar: voltage (200 volts), temperature (25 ˚C), time (8 h) with 10% glycerol.

The SSCP analysis of amplified exon 3 fragment of the leptin gene using primer LEPE3 resulted in two different patterns: A and B (Figure 1) with the following frequencies in 65 Murrah buffaloes (A=0.38 and B=0.62). The Butyrophilin SSCP patterns were better resolved in 12% acrylamide-bisacrylamide with SSCP conditions: voltage (200volts), temperature (25 ˚C), time (12 h) with 10% glycerol. The frequency of polymorphic SSCP patterns in BTI1 (Figure 1) amplified fragments was found with the following frequencies: A= 0.6, B= 0.31 and C= 0.09 in 55 Murrah buffaloes. The gene sequences arising out of this, i.e. EU239373 and EU239374 were submitted to GenBank. Leptin gene sequence analysis revealed one SNP (T-C substitution) in exon 3 at 25th nucleotide position denoted as (c.25 T>C) and nucleotide sequences arising out of this were submitted to GenBank (EU078405 and EU030441). The Butyrophilin PCR products representing three SSCP patterns (A, B and C) were confirmed after sequencing and nucleotide sequences arising were submitted to GenBank (EU194868, EU1997977 and EU199798).

The ANOVA results indicated non-significant (P>0.01) effects of different Murrah buffalo MOGAT2 genotypes: (c.193T>C) TT and (c.193T>C) TC on 305 days milk yield, fat percentage as well as SNF percentage.

The least squares mean values of the milk production traits studied in Murrah buffaloes differing in their MOGAT2 (c.193T>C) genotypes are given in Table 2. In a bovine MOGAT2 gene polymorphism study Winter *et al.* (2003) found 15 SNPs outside exons and two silent exon SNPs and a standard chi-square test did not reveal a significant association of allele frequencies with breeding values for milk fat content in any of the three analyzed dairy breeds. Similarly, results of ANOVA in leptin gene indicated non significant (P>0.01) effect of allelic variants (c.25T>C) on milk yield, fat percentage and SNF percentage. The least square mean values of milk production traits not differing in their leptin (c.25T>C) genotypes are given in Table 2. In this line, other researchers reported only SNPs in bubaline Leptin gene including the 5' region (Vallinoto *et al.* 2004). The butyrophilin gene after variance analysis indicated that, BTI1 SSCP was significantly (P≤0.05) associated with 305-days lactation milk yield of Murrah buffaloes. The Murrah buffaloes with BTI1BB genotypes had 683.93 kg and 320.48 kg higher milk yield as compared to BTI1AA and BTI1CC genotypes, respectively. The positive association of BTI1 SSCP polymorphism with milk production traits may be useful for improving milk performance in dairy buffaloes.

Table 2 Least square means and standard error (SE) of Murrah buffalo milk production traits in reference to MOGAT2, leptin and butyrophilin allelic variants

Candidate gene	Variant genotype	N	Milk yield±SE	FAT*±SE	SNF*±SE
MOGAT2	c.193T > C TT	26	1775.81^{ns}±241.06	0.297^{ns}±0.005	0.315^{ns}±0.001
	c.193T > C TC	27	1678.21^{ns}±264.85	0.297^{ns}±0.005	0.314^{ns}±0.001
Leptin	c.25T > C CC	40	2052.87^{ns}±290.38	0.297^{ns}±0.004	0.315^{ns}±0.001
	c.25T > C CT	25	2421.37^{ns}±280.95	0.294^{ns}±0.004	0.315^{ns}±0.001
Butyrophilin	BTI1 AA	33	2204.56±223.75	0.297^{ns}±0.004	0.315^{ns}±0.001
	BTI1 BB	17	2888.49^{s}±295.83	0.296^{ns}±0.006	0.315^{ns}±0.002
	BTI1 CC	05	2568.01±415.78	0.309^{ns}±0.008	0.318^{ns}±0.002

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
NS: non significant and SE: standard error.
SNF: solids not fat.

Furthermore, Jang *et al.* (2005) reported in cattle that butyrophilin polymorphism (BTN3) was associated with 305 days production traits (P<0.05).

CONCLUSION

The present study revealed that PCR-SSCP and DNA sequencing are one of the most effective molecular biological techniques to detect DNA sequence variation at candidate gene loci in buffaloes. The possible reasons for non-significant effect of DGAT2 / MOGAT2 and leptin allelic variants might be due to the fact that sample size was small, absence of some genotypes, high standard error and uneven distribution of data. The studies concerning associations between DGAT2 / MOGAT2 and leptin gene polymorphism and production traits of riverine buffaloes are, however, fairly scarce. As these two candidate genes have genetic and functional similarity with identified markers is necessary to characterize them completely in buffalo genome. However, in butyrophilin gene, the variance analysis revealed that the BTI1 SSCP variant BTI1BB genotype was differing significantly with 305-days lactation milk yield of Murrah buffaloes. Therefore, validation of the identified polymorphism with a larger dataset might be a good candidate for marker assisted breeding in order to get better milk production in dairy buffaloes. The observed nucleotide sequence variation in the butyrophilin gene and positive association with production trait can be also a useful information resource for buffalo genetic improvement, conservation, management and breeding decisions.

ACKNOWLEDGEMENT

The financial grant as National Fellowship to Dr. B.R. Yadav from Indian Council of Agricultural Research (ICAR) and assistance in the laboratory by Mr. R.K. Tonk, Mr. Naresh Kumar and Mr. Nankoo Singh is duly acknowledged.

REFERENCES

Block S.S., Butler W.R., Ehrhardt R.A., Bell A.W., Van Amburgh M.E. and Boisclair Y.R. (2001). Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. *J. Endocrinol.* **171,** 339-348.

Borghese A.M.M. (2005). Buffalo Production and Research. FAO Rome, Regional Office for Europe.

Cases S., Smith S.J., Zheng Y.W., Myers H.M., Lear S.R., Sande E., Novak S., Collins C., Welch C.B., Lusis A.J., Erickson S.K. and Farese R.V. (1998). Identification of a gene encoding an acyl-CoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proc. Nat. Acad. Sci.* **95,** 13018-13023.

Chamberlain A.J., Hayes B.J., Savin K., Bolormaa S., McPartlan H.C., Bowman P.J., Van Der Jagt C., MacEachern S. and Goddard M.E. (2012). Validation of single nucleotide polymorphisms associated with milk production traits in dairy cattle. *J. Dairy Sci.* **95(2),** 864-875.

Clamp P.A., Feltes R., Shalvevet D., Beever J.E., Atac E. and Schook L.B. (1993). Linkage relationship between ALPL, EN01, GPI, PGD, TGFB1 on porcine chromosome 6. *Genomics*. **17,** 324-329.

Di Meo G.P., Perucatti A., Uboldi C., Roperto S., Incarnato D., Roperto F., Williams J., Eggen A., Ferretti L. and Iannuzzi L. (2005). Comparative mapping of the fragile histidine triad (FHIT) gene in cattle, river buffalo, sheep and goat by FISH and assignment to BTA22 by RH-mapping: a comparison with HSA3. *Anim. Genet.* **36(4),** 363-364.

Franke W.W., Heid H.W., Grund C., Winter S., Freudenstein C., Schmid E., Jarasch E.D. and Keenan T.W. (1981). Antibodies to the major insoluble milk fat globule membrane associated protein: specific location in apical regions of lactating epithelial cells. *J. Cell. Biol.* **89,** 485-494.

Gallagher D.S.Jr. and Womack J.E. (1992). Chromosome conservation in the Bovidae. *J. Hered.* **83(4),** 287-298.

Jang G.W., Cho K.H., Kim T.H., Oh S.J., Cheong I.C. and Lee K.J. (2005). Association of candidate genes with production traits in Korean dairy proven and young bulls. *Asian-australas J. Anim. Sci.* **18,** 165-169.

Liefers S.C., Veerkamp R.F., Te Pas M.F., Delavaud C., Chilliard Y., Platje M. and Van der Lende T. (2005). Leptin promoter mutations affect leptin levels and performance traits in dairy cows. *Anim. Genet.* **36(2),** 111-118.

Oikonomou G., Michailidis G., Kougioumtzis A., Avdi M. and Banos G. (2011). Effect of polymorphisms at the STAT5A and FGF2 gene loci on reproduction, milk yield and lameness of Holstein cows. *Res. Vet. Sci.* **91,** 235-239.

Orita M., Suzuki Y., Sekiya T. and Hayashi K. (1989). Rapid and sensitive detection of point mutation and DNA polymorphisms using polymerase chain reaction. *Genomics*. **5,** 874-879.

Rozen S. and Skaletsky H.J. (1998). Primer 3. Code. available at: http://www.genome.wi.mit.edu/gnome_software/other/primer3.htm.

Sambrook J. and Russel D.W. (2000). Molecular Cloning: A Laboratory Manual. Cold Spring Harbour, New York.

Vallinoto M., Schneider M.P.C., Silva A., Iannuzzi L. and Brenig B. (2004). Molecular cloning and analysis of the swamp and river buffalo leptin gene. *Anim. Genet.* **35,** 462-463.

Winter A., Eckeveld Van M., Bininda Emonds O.R.P., Habermann F.A. and Fries R. (2003). Genomic organization of the DGAT2 / MOGAT gene family in cattle (*Bos taurus*) and other mammals. *Cytogen. Genom. Res.* **102,** 42-47.

Winter A., Kramer W., Werner F.A., Kollers S., Kata S., Durstewitz G., Buitkamp J., Womack J.E., Thaller G. and Fries R. (2002). Association of a lysine-232 / alanine polymorphism in a bovine gene encoding acyl-CoA: diacylglycerol acyltransferase (DGAT1) with variation at a quantitative trait locus for milk fat content. *Proc. Nat. Acad. Sci.* **20,** 20-27.