ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (1): 118-123, 2008
ISSN 1682-296X

Characterization of SNPs of Bovine Prolcatin Gene of Holstein Cattle

[1]Reza Halabian, [1]Morad Pasha Eskandari Nasab, [2]Mohammad Reza Nassiry,
[2]Ali Reza Heravi Mossavi, [3]Seyed Abolfazl Hosseini and [1]Saber Qanbari
[1]Department of Animal Science, Faculty of Agriculture, University of Zanjan, Zanjan, Iran
[2]Center of Excellence in Animal Science, Faculty of Agriculture,
Ferdowsi University of Mashhad, Mashhad, Iran
[3]Department of Biology, Faculty of Science, STT University, Sabzevar, Iran

Abstract: In this study, DNA isolated from blood samples of Iranian Holstein cows (N = 120) and PCR products (156 bp) were analyzed by SSCP technique with MDE gel. Then 4 patterns (Z1, Z2, ...Z4) of provided by SSCP were sequenced and then using POLYPHRED 5.04 software, 4 SNPs were detected in positions 6237, 6263, 6268 and 6297. The genotypes were TC and TG in position 6237, TT in position 6263, AG in position 6268 and AC in position 6297. From of detected SNPs, they that located in positions 6237 and 6268 caused change in the amino acids sequence of protein. These data provide evidence that *bPRL* gene is a good polymorphic source for single nucleotides that can be used for association with milk yield and investigate whether mutations in this sequences might be responsible for quantitative variations in milk yield and composition.

Key words: Bovine prolactin, single strand conformation polymorphism, single nucleotide polymorphism, direct sequencing

INTRODUCTION

Prolactin is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary gland, the lactotrophs (Bole-Feysot *et al*., 1998). The varied effects of prolactin on mammary gland include growth and development of the mammary gland (mammogenesis), synthesis of milk (lactogenesis), maintenance of milk secretion (galactopoiesis), expression milk protein genes, lactose and lipid (Bern and Nicol, 1968; Akers *et al*., 1981; Mitra *et al*., 1993; Dybus, 2002). However, has been shown that prolactin has over 300 separate biological activities (Bole-Feysot *et al*., 1998). Indeed, not only does prolactin subserve multiple roles in reproduction other than lactation, but it also plays multiple homeostatic roles in organism (Grosvenor and Whitworth, 1974; Bern, 1975; Shennan, 1994; Buskila and Shoenfeid, 1996; Goffin *et al*., 1998; Neidhart, 1998).

The detection and genotyping of sequence variations is at the core of all genetic analysis and all approaches to disease gene mapping ultimately lead to variation discovery across a candidate region or gene(s) to identify genetic variants that affect the trait of interest (Nickerson *et al*., 1997).

SNPs are considered to be useful polymorphic and ideal markers for genetic studies of polygenic traits and a worldwide effort to collect SNPs has achieved an accumulation of millions of them in public databases (Tomonari *et al*., 2001; Jalving *et al*., 2004). SNPs are most abundant type of polymorphism in the genome. Therefore, SNPs are becoming the preferred markers for high density mapping (Vignal *et al*., 2002).

In human, agricultural (Bovethuts and Weller, 1994; Rothschild *et al*., 1994) and experimental populations (Mackay and Langley, 1990), polymorphisms at candidate genes have been associated with quantitative genetic variation. Therefore, *bPRL* gene can be an important and excellent candidate for linkage analysis with Quantitative Trait Loci (QTL) affecting milk production traits (Brym *et al*., 2005) and for mapping hSNP (haplotype SNP) and their association with milk performance trait.

The *bPRL* found on the chromosome 23, in the bovine genome (Hallerman *et al*., 1998). The *bPRL* gene is about 10 kb in size and is composed of 5 exons and 4 introns (Camper *et al*., 1984). This encodes the 199 amino acids mature protein (Wallis, 1974). In relation to survey of polymorphisms of *bPRL* gene, several study have been carried out by SSCP and PCR-RFLP methods (Cowan *et al*., 1989; Hart *et al*., 1993; Zhang *et al*., 1994;

Corresponding Author: Reza Halabian, Department of Animal Science, Faculty of Agriculture, University of Zanjan, Zanjan, Iran
Tel: +985712225342

Mitra *et al*., 1993; Chung *et al*., 1996; Chrenek *et al*., 1999; Udina *et al*., 2001). By analysis of four different mRNA clones, four silent nucleotide substitutions were identified (Sasavage *et al*., 1982). On the basis of sequencing of PCR product representing different SSCP pattern, six SNPs were detected (Brym *et al*., 2005). Also by using Milkprotchip, 5 SNPs in *bPRL* gene were genotyped (Kaminski *et al*., 2005).

The aim of the present study was detection and genotyping of SNPs of *bPRL* gene by SSCP method with MDE gel, sequencing of different SSCP patterns and analysis by POLYPHRED software.

MATERIALS AND METHODS

Sampling and DNA extraction: Blood Samples (N = 120) were collected from Iranian Holstein cattles in Astan Ghods farm located at Mashhad. Whole blood (100 μL) was used as source for DNA, which was extracted by a modified Guanidine Isothiocyanate-Silica gel method (Boom *et al*., 1989). The concentration and purity of the DNA obtained were assessed by spectrophotometery and electrophoresis in 1% agarose gels, respectively.

PCR amplification: Polymerase Chain Reaction (PCR) was carried out with Genepak PCR universal kit that was containing 10 μL PCR diluents, 3 μL mixture of primers (5 pm μL^{-1}), 6 μL of ddH_2O and 1 μL of genomic DNA. To detect of SNPs of the *bPRL* gene, a fragment of exon 3 (156 bp) was amplified with primers (PRL-Forward; 5′-CGAGTCCTTATGAGCTTGATTCT-3'and PRL-Reverse; 5'-GCCTTCCAGAAGTCGTTTTT-3). PCR with primers was performed under following condition: initial denaturation (94°C for 5 min), 30 cycles of denaturation (94°C for 30 sec), annealing (59°C for 40 sec), extension (72°C for 20 sec) and final synthesis (72°C for 3 min). The yield and specificity of PCR products were evaluated after electrophoresis in 2% agarose gel with ethidium bromide.

SSCP-MDE analysis: In order to detect mutations, SSCP analysis (Orita *et al*., 1989; Travis, 1995) with MDE® gel solution (Camberx Bio Science Rockland) was performed according to the guidelines described by Camberx Bio Science Rockland. Briefly, 2.0 μL of PCR product was mixed with 18 μL of the denaturing solution (EDTA 0.5 molar, 95% formamide, 0.25% bromophenol blue and 0.25% xylenecyanol), then denatured for 5 min at 95°C, rapidly quenching on ice and, then 4 μL of sample loaded on precast gels (20-25 cm, 0.5 mm) containing 20 mL 2X MDE gel solution, 4.8 mL 10X TBE buffer, 55.2 mL ddH_2O, polymerized by the addition of 500 μL of 10% w/v ammonium persulfate and 50 μL TEMED. The gel was run at a constant power of 6W at room temperature for 8 h. The gels were stained by sliver staining method (Benboza *et al*., 2006).

Sequencing and sequencing analysis: PCR products representing different SSCP were sequenced with an ABI 3730 XL sequencer. All sequences were base-called and assembled using the phred/phrap/consed (Ewing *et al*., 1998; Ewing and Green, 1998; Gordon *et al*., 1998) suit of programs. A related programs polyphred version 5.04 (Nickerson *et al*., 1997) was used to identify SNPs in the assembled sequences. Differences between individual PCR products as well as between PCR products and reference sequences available in GenBank (AF 426315) classified as SNPs.

RESULTS AND DISCUSSION

To detect of SNPs within *bPRL* gene, first we obtained a specific PCR product of expected size (156 bp) with high quality as shown by agarose gel electrophoresis (Fig. 1). This PCR product was consist of whole exon 3 and part of introns 2 and 3.

We used MDE-SSCP method in order to initial screening for polymorphism in 2 herds of Iranian Holstein cattle (N = 120). Four different SSCP patterns were observed: Z1, Z2… Z4 (Fig 2). The frequencies for patterns have been understood in Table 1, that Z1 pattern had higher frequency (0.68) than others.

After direct sequencing of 4 patterns of SSCP and analysis of assembled sequences by polyphred 5.04 revealed 4 SNPs (Table 2). Genotypes of each SNP correspond to SSCP patterns have shown in Table 3. The

Table 1: Frequencies of SSCP patterns in the Iranian Holstein cattle breed

Patterns	Z1	Z2	Z3	Z4
Frequencies	0.68	0.04	0.09	0.19

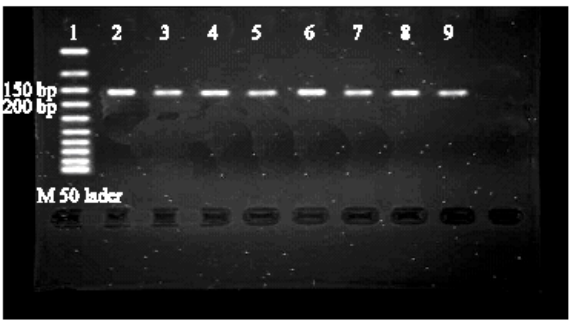

Fig. 1: Electrophoregram in 2% agarose gel. Lane 1 is ladder M 50 and lanes 2-9 are 156 bp segment of the *bPRL* gene consist of whole exon 3 and part of introns 2 and 3

Table 2: Positions and properties of identified SNPs in this study

SNPs	Positions
T changed to G and C (Transversion and Transition)	6237
G changed to T (Transversion)	6263
A changed to G (Transition)	6268
C changed to A (Transversion)	6297

Table 3: Genotypes of identified SNPs within PCR products representing SSCP patterns using resequencing and polyphred 5.04 software

	Position of SNPs			
SSCP patterns	6237	6263	6268	6297
Z1	TC	TT	-	-
Z2	TG	TT	AG	AC
Z3	-	TT	AG	-
Z4	-	TT	-	AC

Table 4: Allelic frequencies of detected SNPs

	Position of SNPs		
Alleles	6237	6268	6297
A	-	0.9333	0.1198
C	0.3375	-	0.8802
G	0.0167	0.0667	-
T	0.6458	-	-

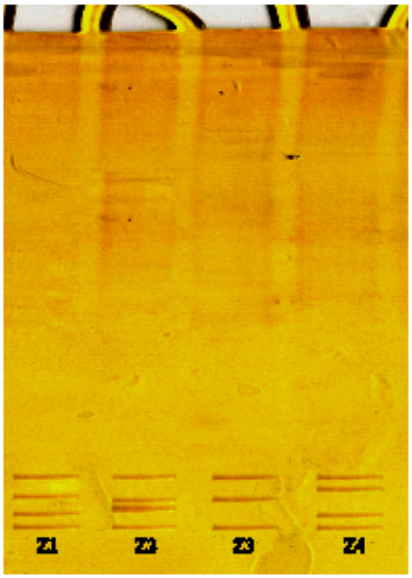

Fig. 2: SSCP analysis of a 156-bp PCR product of the *bPRL* gene with MDE gel. Four SSCP patterns are visible: 1, Z2...Z4

allelic frequencies for 4 SNPs have shown in Table 4, that G allele had lower frequency (0.0167) than others. Also part of results of direct sequencing that presented SNPs in the *bPRL* gene in different SSCP patterns in (Fig. 3).

SNPs in position 6237, 6263 and 6268 are located within exon 3 of *bPRL* (cSNP), that SNPs located in positions 6363 was silent with respect to amino acids sequence of the protein, but SNP located in positions

Table 5: List of charged amino acids correspond to detected SNPs

Position of SNP	Native amino acid	Amino acid after substitution
6237	Thr	Ala (for genotype TC)
6237	Thr	Pro (for genotype TG)
6268	Leu	Pro

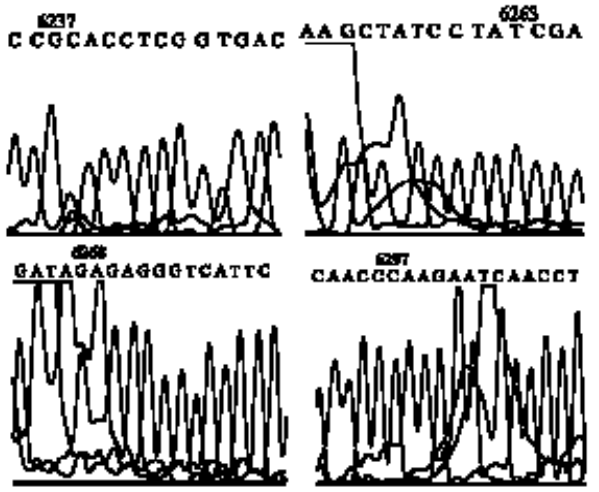

Fig. 3: Result of direct sequencing of SSCP different patterns of a 156 bp fragment of the *bPRL* gene, showing four SNPs at positions: 6237, 6263, 6268 and 6297 that detected by POLYPHRED 5.04 software

6237 and 6268 caused change in the amino acids sequence of protein as shown in Table 5. Also SNP in positions 6297 are located in intron3.

Because of *bPRL* plays a crucial role in mammary gland development, in the initiation and maintenance of lactation and because of milk production is under the indirect influence of it, thus we decide to detection SNPs of *bPRL* gene for next study in regard of their association with milk performance traits.

Since, MDE gel solution is very sensitive and efficient at detecting sequence polymorphisms in PCR amplicon and because MDE gel solution causes DNA separation to occur on the basis of both size and conformation, thus increasing the probability of detecting differences from as low as 15% in standard polyacrylamide gels, to approximately 80%; therefore, we used it in stand of standard polyacrylamide gel (Soto and Sukumar, 1992; Martins-Lopes *et al*., 2001).

With its decreasing costs and rapidly-expanding scale, DNA sequencing seems likely to play a key role in genetic analysis. As studies identify genomic regions associated with biologically or medically important phenotypes, this will inevitably be followed by sequencing of these regions in appropriate individuals.

Also because of resequencing of genes to identify DNA variations play a major role in the post-genomics analysis of animal and human biology, we resequenced different patterns of SSCP and analyzed their with POLYPHRED 5.04 software that automatically detects the presence of heterozygous single nucleotide substitutions by fluorescence based sequencing PCR product. POLYPHRED 5.04 focuses particularly on detecting SNPs through the identification of heterozygous individuals and is therefore well suited to SNP detecting in diploid samples obtained following DNA amplification. It is substantially more accurate than existing approaches and, importantly, provides a useful quantitative measure of its confidence in each potential SNP detected and in each genotype called. The essence of SNP identification is elimination of sequence errors and misaligned bases. therefore, because of quality bases were available, we used polyphred version 5.04. POLYPHRED was shown to be successful in this approach (Nickerson *et al*., 1997).

Within the *bPRL* gene several polymorphisms have been reported but most papers only have described RFLP and SSCP mutations without explaining its nature and locations. Zhang *et al*. (1994) observed three patterns by SSCP analysis of a 857 bp PCR segment of the *bPRL* gene consist of exon 1 and intron 1. Also Brym *et al*. (2005) reported three patterns by SSCP-PCR on exon 4 of the *bPRL* gene. Hart *et al*. (1993) observed a four-allele SSCP in the *bPRL* gene 5' flank. Among of all SNPs identified in earlier studies, SNP located in position 8398 of the *bPRL* (in exon 4) that recognizable by Rsa I endonuclease, has become a popular genetic marker for genetic characterization of cattle populations by mean of PCR-RFLP (Mitra *et al*., 1993; Chrenek *et al*., 1998; Brym *et al*., 2005). Udina *et al*. (2001) and Dybus (2002) analysed a 156 bp fragment of exon 3 of the *bPRL* gene by PCR-RFLP with Rsa I, but did not presented position of SNP. Because of this work with these techniques (MDE-SSCP, direct sequencing and different method of detection and genotyping SNPs by polyphred 5.04 software) was carried out for first time on exon 3 of *bPRL* gene, therefore we could not compared our results with others.

It is noteworthy that SNPs identified in this work can compose intragenic haplotypes (at least two SNPs within a single locus). Because of the limited number of animals genotyped, we were able to show only two haplotypes (Table 3). Intragenic haplotypes can be used as more informative markers in polygenic and association studies. Such linked SNPs may be used in the near future for construction of a bovine haplotype map applied in a new strategy of QTL mapping.

Present finding reveals that *bPRL* gene is a good polymorphic source for single nucleotides, that opens interesting prospects for future selection programs, specially marker-assistant selection for milk production traits.

ACKNOWLEDGMENTS

This research was carried out in Laboratory of Animal Biotechnology of Center of Excellence in Animal Science of Ferdowsi University of Mashhad.

ABBREVIATIONS

MDE : Mutation Detection Enhancement.
SNP : Single Nucleotide Polymorphism.
SSCP : Single Strand Conformation Polymorphism.
bPRL : Bovine prolactin.
cSNP : Coding SNP.

REFERENCES

Akers, R., D. Bauman and A. Capuco *et al*., 1981. Prolactin regulation of milk secretion and biochemical differentiation of mammary epithelial cells in periparturient cow. Endocrinology, 109: 23-30.

Benboza, H., J. Jacquemin, J. Baudoin and G. Mergeai, 2006. Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotechnol. Agron. Soc. Environ., 10 (2): 77-81.

Bern, H. and C. Nicol, 1968. The comparative endocrinology of prolactin. Rec. Prog. Horn. Res., 24: 681-720.

Bern, H., 1975. Prolactin and osmoregulation. Am. Zool., 15: 937-949.

Bole-Feysot, C., V. Goffin, M. Edery, N. Binart and P. Kelly, 1998. Prolactin (PRL) and its receptor: Action, signal transduction pathways and phenotype observed in PRL receptor Knockout mice. Endocrinol. Rev., 19: 225-268.

Boom, R., C. Sol and M. Salimans *et al*., 1989. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol., 28: 495-503.

Bovethuts, H. and J.I. Weller, 1994. Mapping and analysis of dairy cattle quantitative trait loci by maximum likelihood methodology using milk protein genes as genetic markers. Genetics, 136: 267-275.

Brym, P., S. Kaminski and E. Wojcik, 2005. Nucleotide sequence polymorphism within exon 4 of the bovine prolactin gene and its associations with milk performance traits. J. Applied, 45: 179-185.

Buskila, D. and Y. Shoenfeid, 1996. Prolactin, bromocriptine and autoimmune diseased. Isr. J. Med. Sci., 32: 23-27.

Camper, S., D. Luck, Y. Yao, P. Woychik, R. Goodwin, R. Lyons and F. Rottman, 1984. Characterization of the bovine prolactin gene. DNA, 3: 237-249.

Chrenek, P., D. Vasicek, M. Bauerova and J. Bulla, 1998. Simultaneous analysis of bovine growth hormone and prolactin alleles by multiplex PCR and RFLP. Czech J. Anim. Sci., 43: 53-55.

Chrenek, P., J. Huba, M. Oravcova, L. Hetenyi, D. Peskovicova and J. Bulla, 1999. Genotypes of bGH and *bPRL* genes in relationships to milk production. EAAP-50th Annual Meeting. Zurich, 40.

Chung, E., T. Rhin and S. Han, 1996. Association between PCR-RFLP markers of growth hormone and prolactin genes and production traits in dairy cattle. Korean J. Anim. Sci., 38: 321-336.

Cowan, C., M. Dentine, R. Ax and L. Schuler, 1989. Restriction fragment length polymorphism associated with growth hormone and prolactin genes in Holstein bulls: Evidence for new growth hormone allele. Anim. Genet., 20: 157-165.

Dybus, A., 2002. Associations of Growth Hormone (GH) and prolactin (PRL) genes polymorphism with milk production trait in polish black-and-white cattle. Anim. Sci. Pap. Rep., 20: 203-212.

Ewing, B. and P. Green, 1998. Base-calling of automated sequencer trace using phred II. Error probabilities. Genome. Res., 8: 186-194.

Ewing, B., L. Hillier, M. Wendi and P. Green, 1998. Base-calling of automated sequencer traces using phred I. Accuracy assessment. Genome Res., 8: 175-185.

Goffin, V., B. Bouchard, C. Ormandy, E. Welmann, F. Ferray and P. Touraine *et al*., 1998. Prolactin: A hormone at the crossroads of neuroimmunoendocrinology. Ann. N.Y. Acad. Sci., 840: 498-509.

Gordon, D., C. Abajian and P. Green, 1998. Consed: A graphical tool for sequence finishing. Genome. Res., 8: 195-202.

Grosvenor, C. and N. Whitworth, 1974. Evidence for a steady rate of secretion of prolactin following sucking in the rat. J. Dairy Sci., 57: 900-904.

Hallerman, E., J. Theilmann, J. Beckmann, M. Soller and J. Womack, 1998. Mapping of bovine prolactin and rhdopsin genes in hybrid somatic cells. Anim. Genet., 19: 123-131.

Hart, G., J. Bastiaansen, M. Dentine and B. Kirkpatrick, 1993. Detection of a four-allele single strand conformation polymorphism (SSCP) in the bovine prolactin gene 5′ flank. Anim. Genet., 24: 149.

Jalving, R., R. Van't Slot and B.A. Van Oost, 2004. Chicken single nucleotide polymorphism identification and selection for genetic mapping. Poult. Sci., 83: 1925-1931.

Kaminski, S., A. Ahman, A. Ruse, E. Wojcik and T. Nualewski, 2005. MilkProtChip-a microarray of SNPs in candidate genes associated with milk protein biosynthesis-development and validation. J. Applied Genet., 46: 45-58.

Mackay, T.F. and C.H. Langley, 1990. Molecular and phenotypic variation in the *Achaete-scute* region of *Drosophila melangaster*. Nature, 348: 64-66.

Martins-Lopes, P., H. Zhang and R. Koebner, 2001. Detection of single nucleotide mutations in wheat using single strand conformation polymorphism gels. Plant Mol. Rep., 19: 159-162.

Mitra, A., P. Schlee, C. Balakrishnan and F. Pirchner, 1993. Polymorphism at growth hormone and prolactin loci in Indian cattle and buffalo. J. Anim. Breed Genet, 112: 71-74.

Neidhart, M., 1998. Prolactin in autoimmune diseases. Proc. Soc. Exp. Biol., 217: 408-419.

Nickerson, D., V. Tobe and S. Taylor, 1997. Polyphred: Automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. Nucleic. Acids Res., 25: 2745-2751.

Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi and T. Sekiya, 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single strand conformation polymorphism. Proc. Nat. Acad. Sci. USA., 86: 2766-2770.

Rothschild, M.F., C. Jacobson, D.A. Vaske, C.K. Tuggle and T.H. Short, 1994. A major gene for litter size in pigs. 5th World Congress Genet. Applied Livestock Prod., 21: 228-275.

Sasavage, N., J. Nilson, S. Horowitz and F. Rottman, 1982. Nucleotide sequence of bovine prolactin messenger RNA. J. Biol. Chem., 257: 678-681.

Shennan, D., 1994. Regulation of water and solute transport across mammalian plasma cell membranes by prolactin. Dairy Res., 61: 155-166.

Soto, D. and S. Sukumar, 1992. Improved detection of mutations in the P53 gene in human tumors as single-stranded conformation polymorphs and double-stranded heteroduplex DNA. PCR Methods Applied, 2: 96-98.

Tomonari, S., T. Tahira, A. suzuki, A. Higasa, K. Kukita, S. Baba and K. Hayashi, 2001. Precise estimation of allele frequencies of single nucleotide polymorphism by a quantitative SSCP analysis of pooled DNA. Am. J. Hum. Genet., 68: 214-218.

Travis, G., 1995. Step by step SSCP. Laboratory of molecular systematics. Smithsonian Institution Washington, DC. Res. Rep., pp: 3-5.

Udina, I., S. Turkova, M. Kostuchenko, L. Lebedeva and G. Sulimova, 2001. Polymorphism of bovine prolactin gene, microsatellites, PCR-RFLP. Russian J. Genet., 4: 407-411.

Vignal, A., D. Milan, M. Sancristobal and A. Eggen, 2002. A review on SNP and others marker and their use in animal genetics. Genet. Sel. Evol., 34: 275-305.

Wallis, M., 1974. The primary structure of bovine prolactin. FEBS Lett., 44: 205-208.

Zhang, H., S. Denise and R. Ax, 1994. Diallelic single strand conformational polymorphism detected in the bovine prolactin gene. J. Anim. Sci., 72: 256.