Real-time PCR genotyping and frequency of the myostatin F94L mutation in beef cattle breeds

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This research developed two real-time PCR assays, employing high-resolution melt and allele-specific analysis to accurately genotype the F94L mutation in cattle. This mutation (g.433C>A) in the growth differentiation factor 8 or myostatin gene has recently been shown to be functionally associated with increased muscle mass and carcass yield in cattle. The F94L mutation is not, like other myostatin mutations, associated with reduced fertility and dystocia. It is therefore a candidate for introgression into other breeds to improve retail beef yield and the development of a simple and accurate test to genotype this specific mutation is warranted. Variations in the efficiency of enzyme cleavage compromised the accuracy of genotyping by published methods, potentially resulting in an overestimation of the frequency of the mutant allele. The frequency of the F94L mutation was determined by real-time PCR in 1140 animals from 15 breeds of cattle in Australia. The mutation was present in Simmental (0.8%), Piedmontese (2%), Droughtmaster (4%) and Limousin (94.2%) but not found in Salers, Angus, Poll Hereford, Hereford, Gelbvieh, Charolais, Jersey, Brahman, Holstein, Shorthorn or Maine Anjou. The low prevalence of F94L in all beef breeds except Limousin indicates the significant potential for this mutation to improve retail yield in Australian beef cattle.

Keywords: beef cattle, F94L, marker-assisted selection, myostatin, real-time PCR

Implications

In Australian beef cattle, the F94L mutation is found in high frequency in Limousins and in very low frequency in other breeds indicating a significant potential to utilize this mutation for improving retail beef yield in commercial beef herds. The two assays developed in this study provide simple, reliable and robust methodologies for genotyping the F94L mutation in cattle. The availability of an inexpensive assay to genotype this mutation will facilitate marker-assisted selection to improve retail beef yield through the introgression of these alleles into other beef breeds.

Introduction

Myostatin (MSTN), or growth differentiation factor 8 (GDF8) has been identified as playing an important role in negatively regulating skeletal muscle mass and is highly conserved among vertebrate species (Kambadur et al., 1997; McPherron et al., 1997). The gene has attracted considerable attention over the last 10 years due to its potential for genetic manipulation in farm animals.

A large number of mutations (one single nucleotide polymorphism (SNP) every 100 bp) have been identified in both coding and non-coding regions of the gene (McPherron and Lee, 1997; Grobet et al., 1998; Dunner et al., 2003; Bellinge et al., 2005). Many of these, including the F94L mutation identified in exon 1 (g433C>A) - a transversion resulting in a phenylalanine-to-leucine substitution at amino acid position 94), were not predicted to interfere with the myostatic function of the encoded protein (McPherron and Lee, 1997; Grobet et al., 1998). Recent studies, however, have demonstrated significant phenotypic effects associated with the F94L mutation (Alexander et al., 2007; Sellick et al., 2007; Esmailizadeh et al., 2008). This mutation is therefore potentially useful for genetic selection and introgression into other breeds to increase retail beef yield.

Development of a rapid and cost-effective allele-detection methodology would facilitate the selection of animals based on F94L genotype. Previous investigators (Sellick et al., 2007; Esmailizadeh et al., 2008) have genotyped the F94L mutation by the laborious PCR-restriction fragment length polymorphism (RFLP) method, since the sequence context of the SNP prevented genotyping by indirect colorimetric primer extension using SNPware 96 SNP genotyping kit (Orchid Biosciences, Princeton, NJ, USA). Whilst SNP chip and SNP-plex
platforms are the most cost-effective methodologies for genotyping large numbers of SNPs, alternative methods are required for detection of individual SNP. High resolution melting (HRM) analysis shows great promise for rapid genotyping of individual polymorphic loci. It is applicable to both high and low throughput, is a closed tube assay, which minimizes handling and contamination and therefore an ideal tool for a routine testing laboratory. The objective of this research is to facilitate marker-assisted selection through the development of an inexpensive and simple test for the F94L mutation in cattle and to investigate the frequency of this mutation in Australian cattle breeds.

Material and methods

Samples
Blood, semen or hair follicle samples were collected from 1140 cattle representing Angus (56), Brahman (50), Charolais (50), Droughtmaster (113), Gelbvieh (40), Hereford (70), Holstein (50), Jersey (50), Limousin (279), Maine Anjou (50), Piedmontese (35), Poll Hereford (60), Salers (50), Shorthorn (124) and Simmental (63) breeds. These samples were a subset of those submitted for parentage testing through a commercial testing laboratory. To minimize bias from inbreeding, only parent samples were included in situations where offspring were also submitted. In addition, for any one breed, samples were selected from at least four different herds from diverse geographic locations. Different sample types necessitated the use of different extraction methods. High quality genomic DNA was extracted from semen using a phenol-chloroform method (Sambrook et al., 1989) and from blood and hair roots using the method described by Aljanabi and Martinez, (1997). DNA was also extracted from hair roots using a rapid, low cost method (Healy et al., 1995) suitable for routine microsatellite genotyping but producing low quality DNA.

PCR-HRM

PCR amplification reactions were performed on all DNA extracts in 12 μl reactions consisting of 2 μl (approximately 30 to 100 ng) template DNA, 0.25 μM each of the forward and reverse primers, 0.2 mM dNTPs, 2.5 mM MgCl₂, 1.5 μM SYTO® 9 fluorescent nucleic acid stain, 0.6 U Immolase™ DNA polymerase (Bioline, Randolph, Massachusetts, USA) and 1.2 μl of 10× ImmoBuffer. Primers to amplify an 87-base pair product that included the SNP g.433C > A were as follows:

Forward: (5’-3’)  
CCT CCA CTC CTG GAA CTG ATT
Reverse: (5’-3’)  
GCG GTG GTA GTC ATC GTC TT

PCR was performed using a RotorGene 6000 (Corbett Research, Sydney, Australia) and cycling conditions consisted of 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 20 s. Data acquisition was at 72°C on the 6-carboxyfluorescein channel (green) at a gain of two. Post amplification melt curve data were acquired on the same channel, ramping between 77°C and 90°C rising by 0.1°C each step and optimizing gain before melt on all tubes. Control samples for each extraction method representing the AA, CC and AC genotypes as well as no-template controls were included in all runs.

Allele specific real-time PCR

Allele-specific PCR was performed on DNA extracts using the RotorGene 6000 (Corbett Research) in 12 μl reactions consisting of 2 μl template DNA, 6 μl SensiMixPlus SYBR (Quantace, Australia Pty Ltd, Alexandria, NSW, Australia), 0.2 μM Forward primer, 0.2 μM Reverse-T primer and 0.045 μM Reverse-G primer per reaction. Primer sequences were as follows:

Forward: (5’-3’)  
AAA CAG CTC CTA ACA TCA GC
Reverse-T: (5’-3’)  
GGC ATC TCT CGT GAC ATT T
Reverse-G: (5’-3’)  
GCG CCG CGC GCA TCT CTC TTG ACA TTG

PCR cycling conditions were 95°C for 15 s followed by 40 cycles of 60°C for 60 s, with data acquisition occurring at 60°C on the 6-carboxyfluorescein channel (green) at a gain of two. Following amplification melt curve data were acquired using a ramping rate of 0.5°C/60 s from 72°C to 90°C and melt curves were analyzed using the RotorGene software with the digital filter set as ‘light’. No-template and genotype controls for each extraction method were included in all runs.

PCR-RFLP and sequencing

For confirmation of real-time PCR results, DNA samples from 200 Limousin sires (including 170 AA, 3 CC and 27 AC genotypes) and 25 Holstein animals (all CC) were also genotyped using the PCR-RFLP protocol outlined previously (Sellick et al., 2007). All 225 animals were selected independently of any F94L result: Limousin sires were selected to provide F94L genotypes to the breed society and Holstein animals were selected to provide F94L genotypes from a dairy breed. In addition, 100 samples were selected for sequencing based on RFLP, HRM and allele-specific (AS) results. All AC phenotypes observed across the breeds (33) and any samples with discordant results across methodologies were sequenced along with a selection of suspected AA and CC phenotypes, based on HRM and AS or RFLP results (30 AA and 37 AC). Sequencing was used to determine the nucleotide sequences implied by HRM, allele-specific and RFLP methodologies. Sequencing reactions were carried out using the same primers as for PCR-RFLP genotyping, and using the Big Dye Termination Technology (Applied Biosystems, Scoresby, Victoria, Australia) following the manufacturer’s protocol.

Statistical analysis

To compare the proportions of alleles correctly detected between RFLP and HRM methodologies, two-tailed P-values were calculated using paired data and both McNemar’s test uncorrected for continuity and Fisher’s exact test. Assuming allelic independence, separate analyses were performed for A and C alleles using the Pairs etc. module (version 2.19) in the WinPepi statistical package (version 9.6; Abramson, 2004).
The prevalence of mutation for Australian Pure Limousins born in the 1980s was compared to those born after 1995 using a $\chi^2$ test and assuming independence of samples.

**Results**

Extracted DNA from 1140 samples was amplified using real-time PCR and post-amplification analysis employed HRM technology. The HRM curves were analyzed using the RotorGene software and were able to clearly differentiate between the AA, AC and CC genotypes based on changes in the shape of the melting curve (Figure 1). The homozygotes had $T_m$ values that differed by $\sim 0.5^\circ C$. Different extraction methods resulted in melt curve shifts to the left or right but did not alter the $T_m$ difference between AA and CC homozygotes. Small melt curve shifts were also observed in samples that had not reached an amplification plateau due either to a low quantity or quality of input DNA or the presence of inhibitors. These variables could confuse the discrimination of homozygotes where samples from different extraction methods or with large variations in DNA concentration are run concurrently. The position of the heterozygote melt curve was similarly shifted under varying conditions but melt curve shape was unaffected, therefore allowing for easy discrimination of heterozygote genotypes.

HRM genotyping accuracy was compared with RFLP and sequencing results. Discordant results between HRM and RFLP methodologies were observed in seven samples. All cases involved an RFLP AA result while HRM and sequencing results were AC. Thus the proportion of A alleles ($n = 93$) correctly identified by both HRM and RFLP methods was 100% while of the 107 C alleles, HRM identified 100% and RFLP identified 93.5% (McNemar’s test $P = 0.008$; Fisher’s exact test $P = 0.016$). The PCR-HRM method proved simple and robust for discrimination of F94L genotypes provided the same extraction procedure was used for all samples being analyzed concurrently. Alternatively, where sample types such as semen necessitated different extraction procedures to be run together, discrimination of homozygous genotypes was readily achieved by including control samples for each extraction method in each run.

Two samples displayed aberrant HRM curves and sequencing revealed an additional non-functional mutation within these amplicons (Figure 2).

Allele-specific real-time PCR was employed as an alternative testing strategy to allow accurate genotyping of

![Figure 1](image1.png)

**Figure 1** Normalized high resolution melting curves of F94L SNP genotypes from the RotorGene 6000 demonstrating clear discrimination of AA, CC and AC genotypes based on melt curve shape and position. $T_m$ values for homozygous AA and CC genotypes differed by $\sim 0.5^\circ C$.

![Figure 2](image2.png)

**Figure 2** Aberrant high resolution melting profiles observed in Brahman and Droughtmaster samples showing intermediate melting curve profiles and sequence data indicating the single base changes responsible for producing the different melting profiles.
samples that demonstrated aberrant HRM profiles (Figure 3). As with HRM, different extraction methods and large variations in DNA concentration resulted in small changes in $T_m$ values but did not affect robustness of homozygous allele scoring, with a $T_m$ difference of $\sim 2.2^\circ C$ between AA and CC homozygotes.

Genotyping results using allele-specific PCR were compared with HRM and sequencing results. Six samples were inaccurately genotyped using the allele-specific PCR methodology. All incorrect genotyping involved poor quality DNA samples (as determined by A260/A280 ratios) and inaccuracies were all due to poor amplification of the A allele in heterozygotes. In all cases, the correct genotypes could be obtained by titrating the template DNA. In comparison to HRM analysis, allele-specific genotyping of heterozygotes was more sensitive to DNA quality but provided a valuable backup assay for differentiating homozygotes and assessing aberrant HRM curves.

Samples from unrelated animals representing different cattle breeds in Australia were tested for the presence of the F94L mutation. The overall frequency of the A allele in Limousins was 94.2%. When analyzed for origin, the frequency in French Pure Limousins ($n=136$) was 98.9%. The prevalence of the mutation in Australian Pure Limousins born in the 1980s ($n=18$) was 63.9% compared to 92.5% for the 107 animals born after 1995 ($P<0.001$). Of the 15 other breeds tested, the mutation was only found in very low frequencies in Simmental (0.8%), Piedmontese (2%) and Droughtmaster (4%).

**Discussion**

The F94L mutation has been shown by independent studies to increase beef yield with no effect on birth weight, growth rate, meat tenderness, pH and cooking loss of the *M. longissimus dorsi*. Muscle mass was increased up to 12% to 15% in homozygous calves and 5.9% to 7.3% in heterozygous calves, with a reduction in carcass fat (Alexander *et al.*, 2007; Sellick *et al.*, 2007; Esmailizadeh *et al.*, 2008). By contrast, other myostatin mutations that cause the extreme double-muscled phenotypes result in around 20% increase in skeletal muscle mass (Swatland and Kieffer, 1974) but are also associated with poor fertility, reduced stress tolerance, low calf viability, high incidence of dystocia (Arthur *et al.*, 1988 and 1989) and reduced flavour ratings (Bellinge *et al.*, 2005). The benefits of these mutations have been maximized through the development of simple and cost-effective assays that allow selection of heterozygous dams (Fahrenkrug *et al.*, 1999), which do not suffer a significant increase in dystocia and produce heterozygous calves with increase retail product yield up to 8% (Casas *et al.*, 1999). These studies indicate that the F94L mutation is at least as effective for increasing retail beef yield as the more severe double muscling mutations, without any associated reproductive disadvantages. The development of an inexpensive test for genotyping F94L would facilitate its utility as a genetic selection tool.

HRM analysis employs saturating fluorescent DNA dyes to characterize samples by their dissociation behaviour, which is based on sequence length, GC content and DNA sequence complimentarity, and can be used to detect single base sequence variations (Liew *et al.*, 2004). Coupled with real-time PCR, amplification reactions can be monitored for abnormalities that may lead to erroneous results, providing a simple, closed-tube, homogenous genotyping assay (Kristensen and Dobrovic, 2008). DNA melting behaviour is also known to be affected by salts in the reaction mix and melt curve shifts in both the HRM and allele-specific assays reported here were observed when DNA from different extraction methods were analyzed together. This highlights the necessity for appropriate extraction controls to be included with each real-time PCR run. With appropriate extraction controls, however, the PCR-HRM assay was more reliable than the previously used PCR-RFLP method for genotyping the F94L mutation. Discordant results observed with the PCR-RFLP methodology were presumably due to failure of enzyme digestion in selected samples, which has been previously reported (Dennis and Healy, 2001) and is possibly due to unidentified inhibitors in the sample affecting the efficacy of ampiclon. In a commercial testing laboratory setting, the PCR-HRM assay setup is rapid and results are obtained within 3 h. The assay is robust with different DNA extraction procedures, including quick procedures that are inexpensive but result in low quality DNA, and the relatively rare occurrence of aberrant melt curves can be resolved by running high quality DNA extracts from those samples through an additional allele specific assay.

The F94L SNP has been previously shown to have a high frequency in Limousin animals (83%) and has also been identified in Charolais, Blonde d’Aquitaine and Angus cattle (Dunner *et al.*, 2003; Sellick *et al.*, 2007). By comparison, this study did not identify the mutation in Angus or Charolais...
cattle and has demonstrated a higher frequency in Limousin. The significant increase observed in the frequency of the F94L mutation in Limousins over time most likely reflects the effects of selection for increased muscle mass and accounts for frequency differences reported in the literature.

The two assays developed in this study provide simple, reliable and robust methodologies for genotyping the F94L mutation in cattle. The availability of an inexpensive assay for this mutation will facilitate marker assisted selection to improve retail beef yield through the introgression of these alleles into other beef breeds. We have also shown that, in Australian cattle, the F94L mutation is found almost exclusively in the Limousin breed, with very low frequency in other beef breeds, indicating a significant potential to utilize this mutation for improving retail beef yield in commercial beef herds.

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References