Convenient genotyping of six myostatin mutations causing double-muscling in cattle using a multiplex oligonucleotide ligation assay

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Summary

We herein describe a procedure that allows for simultaneous genotyping of six loss-of-function mutations in the bovine myostatin gene associated with the double-muscling phenotype. The proposed method relies on a multiplex oligonucleotide ligation assay and detection of the fluorescently labelled products using automatic sequencers.

Keywords: bovine myostatin gene, double-muscling phenotype, multiplex oligonucleotide ligation assay

It has recently been shown that loss-of-function mutations in the myostatin gene cause a double-muscling or muscular hypertrophy (*mh*) phenotype in cattle (Grobet *et al.* 1997; Kambadur *et al.* 1997; McPherron & Lee 1997; Cappucio *et al.* 1998; Grobet *et al.* 1998). So far six such bovine mutations have been described (Fig. 1). (i) An 11 bp deletion (*nt821(del11)*) resulting in a truncation of the bioactive C-terminal domain of the protein found in Belgian Blue, Blonde d'Aquitaine, Limousine, Parthenaise, Asturiana and Rubia Gallega. (ii) A G → A transition at nucleotide position 938 resulting in the substitution of a highly conserved cysteine by a tyrosine (*C313Y*) reported in Gasconne and Piedmontese. (iii) An insertion/deletion at position 419 counting from the initiation codon, replacing 7 bp with an apparently unrelated stretch of 10 bp, revealing a premature stop codon in the N-terminal latency-associated peptide at amino-acid position 140 (*nt419(del7-ins10)*) reported in Maine-Anjou. (iv) A C → T transition at nucleotide position 610 yielding a premature stop codon in the N-terminal latency-associated peptide at amino-acid positions 204 (*Q204X*) observed in Charolais and Limousine. (v) A G → T transversion at nucleotide position 676 also causing a premature stop codon in the

same N-terminal latency-associated peptide at amino-acid position 226 (*E226X*) identified in Maine-Anjou. (vi) A G → T transversion at nucleotide position 874 causing a premature stop codon in the bioactive C-terminal domain (*E291X*) encountered in Marchigiana.

The discovery of this allelic series of loss-of-function mutations disproved the initial hypothesis postulating that double-muscling would be genetically homogeneous, having appeared in Shorthorn first and having then spread from the British Isles to the continent and the rest of the world by migration (Ménissier 1982). It indicates that myostatin has a unique regulatory potential during myogenesis, being a prime target for mutations that dramatically and exclusively affect muscle mass (Lee & McPherron 1999). It is noteworthy that animals have been identified (particularly in Blonde d'Aquitaine) which exhibit the double-muscling phenotype yet do not show an altered myostatin amino-acid sequence nor reduced myostatin mRNA concentrations (Poncelet *et al.* unpublished observations).

The calving difficulties associated with double-muscling have led several breeding organizations to treat this condition as a genetic defect that needs to be eliminated. In specific economic contexts, however, the gains in feed conversion ratio, dressing out percentage and meat quality (increased lean and tenderness) have outweighed the costs of dystocia, leading either to a systematic selection for double-muscling animals or their use in cross breeding (Hanset 1991). Understanding the molecular basis of double-muscling has opened the possibility to select either for or against this phenotype directly at the genotypic level. In this context, a diagnostic test based on allele-specific PCR has previously been described for the *nt821(del11)* and *C313Y* mutations (Fahrenkrug *et al.* 1999). Herein, we describe the development of a diagnostic test aimed at simultaneously detecting one or more of the six previously described myostatin mutations in a DNA sample.

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Fig. 1. Schematic representation of the bovine myostatin gene. The grey boxes correspond to the untranslated leader and trailer sequences (large diameter), and the intronic sequences (small diameter). The blue, pink and green boxes correspond to the sequences coding for the leader peptide, N-terminal latency-associated peptide, and bioactive C-terminal domain of the protein, respectively. The blue arrows represent the two primer pairs used for multiplex amplification of exons II and III, respectively. The corresponding primer sequences are reported in Table 1. The positions of the six bovine loss-of-function mutations are shown as red lines on the myostatin gene. Each mutation is connected via an arrow with a box reporting the details of the corresponding DNA sequence and encoded peptide sequence. In each box the mutation is compared with the wild-type sequence, and differences highlighted in red.

The test is based on the simultaneous amplification of the second and third myostatin exons using primers located respectively in intron I and intron II, and intron II and 3' UTR (Table 1). PCR amplification was performed in 10 µl reactions using 25 ng of template DNA in the presence of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 300 µM of each of the four dNTP, 0.5 µM of each of the six primers and 1 U of AmpliTaq™ Gold polymerase (Perkin-Elmer-Roche, Blanchburg, NJ, USA). This reaction mix was subjected to 30 cycles including: (i) a denaturation step at 95 °C for 1 min (except first cycle: 5 min); (ii) an annealing step at 60 °C for 1 min; and (iii) an extension step at 72 °C for 1 min. At the end of the PCR reaction, the *Taq* DNA polymerase was inactivated at 99 °C for 30 min.

Five microlitres of this PCR reaction were then used as template in a multiplex oligonucleotide ligation reaction (OLA) (Grossman *et al.*

1998). The OLA was performed in the presence of 18 primers corresponding to 6 × 3 primers per loss-of-function mutation (Table 2). A common 3' primer was phosphorylated at its 5' end and extended with three to eight 18-atom spacer arms (Spacer™ Phosphoramidite-CLONTECH, Palo Alto, CA, USA) at its 3' end in order to spread the molecular weight of the OLA products corresponding to the different mutations. This allowed them to be easily separated by gel electrophoresis. The two allele-specific 5' primers were labelled at their 5' end with distinct fluorophores: HEX (PE Applied Biosystems, Foster City, CA, USA) for the wild-type allele and 6-FAM (PE Applied Biosystems) for the double-muscling allele. The OLA reaction was performed in a final volume of 20 µl in the presence of 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl₂, 0.1% Nonidet P40, 10 µM NAD, 1 mM DTT, 20 µM of each primer and 5 U of thermostable *Tsc* DNA ligase (Boehringer

Table 1. Primers for PCR amplification

	UP-primer	DN-primer
Exon II	5'-GTTTCATAGATTGATATGGAGGTGTTTCG-3'	5'-ATAAGCACAGGAAACTGGTAGTTATT-3'
Exon III	5'-GAAATGTGACATAAGCAAAATGATTAG-3'	5'-ATACTCWAGGCCTAYAGCCTGTGGT-3'

Table 2. Primers for the OLA reaction

Mutation	Wild-type	Loss-of-function	Common
<i>Nt419(del7-ins10)</i>	HEX-T G(T/C)T TCT TTA AAT T-3'	FAM-T TCT AAG CAT ACA A-3'	MP-5'-TAG CTC TAA GAT AC-(SP)3
<i>Q204X</i>	HEX-C ACT GGT ATT TGG C-3'	FAM-C ACT GGT ATT TGG T-3'	MP-5'-AGA GCA TTG ATG TG-(SP)4
<i>E226X</i>	HEX-C AAC TTA GGC ATT G-3'	FAM-C AAC TTA GGC ATT T-3'	MP-5'-AAA TCA AAG CTT TA-(SP)5
<i>nt821(del11)</i>	HEX-GTG ATG AAC ACT CC-3'	FAM-TGG GCT TGA TTG TG-3'	MP-5'-ACA GAA TCT CGA TG-(SP)6
<i>C313Y</i>	HEX-TG CTC TGG AGA ATG-3'	FAM-TG CTC TGG AGA ATA-3'	MP-5'-TGA ATT TGT ATT TT-(SP)7
<i>E291X</i>	HEX-AA CTG TGG ATT TTG-3'	FAM-AA CTG TGG ATT TTT-3'	MP-5'-AAG CTT TTG GAT GG-(SP)8

Mannheim, Lowes, UK). This reaction mix was subjected to 40 cycles including: (i) a denaturation step at 94 °C for 30 s (except first cycle, 2 min); and (ii) a ligation step at 39 °C for 3 min. The *Tsc* DNA ligase was then heat inactivated at 99 °C for 10 min.

One microlitre of this OLA reaction was

mixed with loading buffer (3 µl of formamide, 0.5 µl of dextran blue), denatured for 5 min at 95 °C, loaded on a conventional denaturing 6% PAGE sequencing gel, and electrophoresed using an ABI373 automatic sequencer. The resulting gel files were analysed with the Genescan software (PE Applied Biosystems). Figure 2 il-

Fig. 2. Results of the multiplex OLA test for the diagnosis of MSTN loss-of-fucntion mutation performed for seven animals. The position of the OLA products corresponding to the six known MSTN mutations are shown on the left. Yellow bands correspond to the wild-type alleles and blue bands to the corresponding mutations. The deduced genotypes of the seven animals are indicated above the figure.

illustrates the typical patterns obtained from such experiments.

To verify its accuracy, we performed the OLA diagnostic test on genomic DNA from 32 previously described double-muscled individuals representing 10 different continental breeds (Grobet *et al.* 1998), a double-muscled Marchigiana animal (Cappucio *et al.* 1998) and two controls (Holstein-Friesian and Jersey). The genotype of these individuals had previously been determined by sequencing their entire myostatin coding sequence. All results obtained with the OLA test were in perfect agreement with the sequencing data (data not shown).

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