Screening of \textit{RYR1} genotypes in swine population by a rapid and sensitive method

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Abstract

Genetic polymorphism in the gene controlling the calcium release channel (ryanodine receptor, \textit{RYR1}) of sarcoplasmic reticulum in skeletal muscle have been shown to affect meat quality in pigs, producing porcine stress syndrome (PSS) and malignant hyperthermia (MH). The MH condition produces pale, soft and exudative (PSE) meat resulting in reduced commercial value of pork. Therefore, monitoring swine population for \textit{RYR1} genetic variants is important in the subsequent process of breeding and improving meat quality in pigs. The aim of the present study was to establish and validate a rapid and sensitive molecular genotyping assay to detect C1843T mutations of the \textit{Sus scrofa} \textit{RYR1} gene using quantitative PCR (qPCR) followed by high-resolution melting (HRM) analysis. The qPCR-HRM assay allowed the rapid and sensitive identification of \textit{RYR1} genotypes for discrimination of wild type and mutant alleles, so the carriers can be detected. The method was validated by analyzing a total of 195 DNA samples that were previously genotyped by PCR-RFLP method. The results demonstrate that qPCR-HRM method is a fast, simple and reliable assay for genotyping the C1843T (Arg615Cys) polymorphism of the \textit{RYR1} gene. The high frequency of \textit{RYR1} mutant allele suggested that implementing a routine testing system is necessary to gradually eradicate the MH condition.

Key words: C1843T SNP, high-resolution melting, malignant hyperthermia, molecular diagnostic, porcine stress syndrome, ryanodine receptor

Introduction

Ryanodine receptor (\textit{RYR1}) is the calcium release channel from the sarcoplasmic reticulum in skeletal muscle whose contraction, relaxation and energy metabolism are regulated by the concentration of intracellular Ca\textsuperscript{2+} (D.H. MACLENNAN & al. [1]). An abnormality in the ryanodine receptor facilitates opening and inhibits closing of Ca\textsuperscript{2+} channel resulting from an altered low-affinity Ca\textsuperscript{2+} binding site in the channel pore and thus causes malignant hyperthermia (MH). Currently, this condition has worldwide economic consequences and represents an economical major concern in the swine industry (D.H. MACLENNAN & al. [1]; M. FILL & al. [2]; Y. KIM & al. [3]).

MH is a hypermetabolic syndrome involving the skeletal muscle characterized by muscle rigidity, hyperthermia, tachycardia, tachypnea, increased oxygen consumption, cyanosis, cardiac dysrhythmias, metabolic acidosis, respiratory acidosis, unstable arterial blood pressure, and death (E. SUSAN & al. [4]). The primary features of MH are a direct
consequence of loss of skeletal muscle cell calcium homeostasis with a resulting increased intracellular calcium ion concentration (P. J. HALSALL & P.M. HOPKINS [5]).

Fujii et al. (1991) reported that a point mutation in pig RYR1 gene is associated with porcine stress syndrome (PSS), and is a major gene known to have a direct linkage with pork quality (J. FUJII & al. [6]). The PSS-susceptibility is inherited as an autosomal recessive dysfunction (J.E. ROJAS & al. [7]) and was first described by Topel et al. (1968), who noted that physically stressed, susceptible pigs would collapse in a shock-like state and die (D.G. TOPEL & al. [8]). PSS leads to carcasses containing pale, soft, exudative (PSE) meat, with a low water retention capacity and a rapid decrease of pH, combined with a high temperature of the meat which leads to a denatured sarcoplasmatic protein, making the meat unfit for processing. Expression of PSS results on account of physical stressors such as excessive movement, fighting, marketing, vaccination, castration, oestrus, mating, parturition and hot weather (G. MITCHELL & J.J.A HEFFRON [9]). It has also been noted that volatile anaesthetics such as halothane bring about the onset of PSS (A.J. WEBB & C.H. C. JORDAN [10]). Pigs that are homozygous for the recessive RYR1 n allele are subject to sudden death from stress. In addition, those surviving and those heterozygous for the condition have many meat quality problems. This gene has multiple denominations, being called the stress gene, halothane gene and PSS gene (A.TÄNAVOTS & al. [11]).

The porcine RYR1 locus was localized on chromosome 6p11-q21 where a substitution at position 1843 (C1843T) lead to the substitution of Arg for Cyst at position 615 in amino acid sequence (Arg615Cys), where the susceptible mutant allele is denominated T and the wild-type – non-susceptible allele C (J. FUJII & al. [6]; I. HARBITZ & al. [12]; A. HOUDE & al. [13]; P. VOGELI & al. [14]).

In pigs, MH have been reported for several breeds, however, the incidence is higher in lean, heavily muscled breeds, such as Pietrain, Poland China, Landrace, Duroc, and Large White (E. SUSAN & al. [4]). This incidence is due to a dual effect of the gene: negative and, in the same time, beneficial. The beneficial effects of the MH gene are associated with leanness and muscle hypertrophy (M.A. MANEA & al. [15]). It is this association that has allowed the gene frequency to be increased initially through selection for increased muscularity.

Knowing the structure of this locus in pig populations is extremely important for economic losses represented by recessive homozygous pigs. Homozygous recessive pigs, nn (T/T) and the heterozygous pigs, Nn (C/T) have a very appealing conformation for the specialists who make the selection. The unconscious promotion of these animals (nn and Nn) in the herd, with the intention to produce a new generation, led to the automatic increase of the mutant allele n frequency in pig populations.

In the recent years, several genetic tests were described to identify mutations and type single-nucleotide polymorphisms (SNPs) including the RYR1. The majority of these techniques require processing, separation steps or allele-specific primers or probes, which make them less favourable for high-throughput assays (H. GRIEVINK & K. M STOWELL [16]). The new approach reported is based on a High-resolution melting (HRM) assay that was introduced as a homogeneous closed-tube system that allows post-PCR analysis of genetic mutations or variance without the need of separation steps. This method is based on the properties of dissociation (melting) of double-stranded DNA, which is subsequently examined by melting curve analysis. Different melting profiles are obtained from the transition of double-stranded DNA to single-stranded DNA due to a gradual increase in temperature after the PCR (G.H. REED, & C.T. WITTWER [17]).

Melting curve analysis has advantages over other mutation detection methods that derive information from the amplification process itself (M. HOFFMANN & al. [18]). HRM is considered the simplest method for genotyping and mutation detection because it is...
done in the same tube and immediately after the PCR (J. MONTGOMERY & al. [19]).

We consider that the HRM is a reliable assay for fast, high-throughput post-PCR analysis of genetic mutations or variance in nucleic acid sequences that can facilitate marker assisted selection. Different sequence variants can be identified based on differences in melting curves using the LightCycler Nano Software. Heterozygous samples are distinguished from homozygous samples by an altered shape in the melting curve. These differences are best visualized using difference plots because slight differences in curve shape and melting temperature (Tm) become obvious (H. GRIEVEINK & K. M STOWELL [16]).

The aim of the current study was to develop and test the feasibility of an inexpensive and high-throughput HRM assay using the LightCycler Nano Real-Time PCR System in order to allow genotyping of C1843T (Arg615Cys) polymorphism of the \textit{RYR1} gene in swine. Genomic DNA samples of known \textit{RYR1} genotypes were used to validate the HRM assays.

Materials and Methods

Samples and DNA extraction

In order to develop a HRM method for screening of the \textit{RYR1} C/T SNP (C1843T), we analyzed a total of 195 animals belonging to four distinct breeds (Landrace, Duroc, Pietrain and Large White) from commercial farms in which a low frequency of halothane-susceptible animals was known to exist.

Blood samples for DNA genotyping were collected in 2 mL vacutainers containing K$_2$EDTA. Genomic DNA was isolated from blood samples (300 µl) using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA), according to manufacturers’ instructions. DNA concentration was evaluated spectrophotometrically, with NanoDrop-2000 (Thermo Fisher Scientific Inc., USA), and visually by standard agarose gel electrophoresis [1% agarose (w/v) in TBE] (J.M.S. BARTLETT & D. STIRLING [20]).

PCR and HRM conditions

Initially, the 195 DNA samples were processed for screening the \textit{RYR1} C/T SNP (C1843T), using PCR-RFLP analysis with the enzyme \textit{HhaI} (Fermentas, Thermo Fisher Scientific Inc., USA), as described elsewhere (B. BRENIG & G. BREM [21]; A.TÄNAVOTS & al. [11]). The digested DNA fragments were visualized on a 3.5% agarose gel, with a standard DNA ladder (pUC19/Sau3A) as a molecular weight marker, and stained with ethidium bromide (10 mg/mL). Images of the gels were taken using Vilber Lourmat Print II Systems.

Primers \textit{RYR1-F} (5’- GTG CTG GAT GTC CTG TGT TCC CT-3’) and \textit{RYR1-R} (5’- CTG GTG ACA TAG TTG ATG AGG TTT G-3’) for \textit{RYR1} gene used for both normal PCR and qPCR - HRM assay amplified a 134 bp fragment (B. BRENIG & G. BREM [21]).

Starting from our normal PCR conditions using C1000 Thermal Cycler (Bio-Rad Laboratories, Inc.), we first determined the optimal conditions to perform the reaction in the LightCycler® 480 High Resolution Melting master mix containing a saturating DNA dye.

Real-Time PCR was then performed on a LightCycler Nano Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany), in 8 tube strips, in 18 µl volume containing 20 ng of genomic DNA as template, 10 pmoles of each primer, 2.5 mM of MgCl$_2$ and 1x LightCycler 480 High Resolution Melting Master (Roche Diagnostics GmbH, Mannheim, Germany) consisting of FastStart Taq DNA Polymerase, dNTP mix, reaction buffer and high resolution melting dye. Cycling conditions were: one cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds, 60°C for 25 seconds and 72°C for 15 seconds. After amplification, the PCR amplicons were denatured at 95°C for 1 minute and cooled down to 40°C for 1 minute to allow heteroduplex formation. The final HRM
step was performed from 65°C to 95°C with 0.05°C increase/second. Each assay performed included reference samples of the 2 genotypes of RYR1 (NN and Nn) and a negative control containing distilled water instead of template DNA. One negative control (i.e. DNA from the normal animal) was used to normalize melting profiles of the other samples against this pre-defined horizontal baseline.

**Data analysis**

Data were analyzed using automated quantification, Tm calling (melt temperature analysis) and HRM (high resolution melt analysis and genotyping) provided by the LightCycler Nano Software 1.0. For sample analysis, melting curves were normalized, temperature – adjusted and, finally, a difference plot was generated. Samples analyzed by HRM were automatically grouped according to their melting profiles. The melting curves were normalized by calculation of two normalization regions before and after the major fluorescence decrease representing the melting of the amplicons. This algorithm allows the direct comparison of the samples that have different starting fluorescence levels (M. BALIC & al. [22]). Also, afterwards, agarose gels were run to confirm that clean products of the expected length had been obtained.

**Results and Discussion**

From a total of 195 animals used in the present study belonging to four distinct breeds (Landrace, Duroc, Pietrain and Large White), 7.00% had the allele n associated with susceptibility to porcine stress syndrome, while 93.00% had the allele N normal or resistant to PSS. Genotypic frequencies observed were 86.00% homozygous normal or resistant individuals (NN), 14.00% of individuals heterozygous (Nn), while for homozygous susceptible (nn) we did not find any animal. The obtained results suggest that in Romanian swine population it still a potential impact of susceptible allele (n) of RYR1 gene (Table 1).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Breed</th>
<th>No.</th>
<th>Genotypic frequency</th>
<th>Allelic frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NN (C/C)</td>
<td>Nn (C/T)</td>
</tr>
<tr>
<td>RYR1</td>
<td>Landrace</td>
<td>69</td>
<td>0.826 (57)</td>
<td>0.174 (12)</td>
</tr>
<tr>
<td></td>
<td>Duroc</td>
<td>48</td>
<td>0.812 (39)</td>
<td>0.188 (9)</td>
</tr>
<tr>
<td></td>
<td>Pietrain</td>
<td>35</td>
<td>0.942 (33)</td>
<td>0.058 (2)</td>
</tr>
<tr>
<td></td>
<td>Large White</td>
<td>43</td>
<td>0.860 (37)</td>
<td>0.140 (6)</td>
</tr>
</tbody>
</table>

For establishment and optimization of the HRM assay, DNA samples with known genotype were firstly tested by PCR-RFLP technique by using the HhaI restriction enzyme. The genotyping analysis for the C1843T SNP obtained through PCR-RFLP assay is shown in Figure 1, where genotypes found in investigated animals (NN and Nn) were clearly distinguishable.

Figure 1. The PCR-RFLP analysis for the swine RYR1 C1843T SNP, determined by digestion with HhaI on 3.5% agarose gel electrophoresis stained with ethidium bromide. Lane 1 standard markers pUC19/Sau3A; Lanes 2-4, 6, 8-10 DNA from homozygous normal individuals NN (C/C); Lanes 5 and 7 DNA from heterozygous individuals Nn (C/T)

Amplicon melting analyses in the presence of the LightCycler 480 HRM dye binding
the dsDNA heteroduplexes were used to detect RYR1 polymorphisms employing the LightCycler Nano Real-Time PCR System. The analyzed C1843T SNP located in RYR1 gene affect the melting behaviour of PCR products and therefore generates different melting curves. Amplicons were 134 bp in length and allow discrimination and identification of RYR1 genotypes by high - resolution melting analysis.

We started our study with PCR mix optimization. The concentration of Mg$^{2+}$ supplied in the PCR buffers is normally not enough to ensure efficient amplification of template. An increase in the concentration of Mg$^{2+}$ to 2-3 mM significantly enhances the amplification. Increased Mg$^{2+}$ concentration enhance nonspecific amplification and therefore has to be optimized for each primer set (K.T. WOJDACZ & al. [23]). In the current study 2.5 mM MgCl$_2$ was found to be optimal (Figure 2).

![Figure 2: Effect of increasing MgCl2 on melting behavior. The four different curves show the dependence of amplicon melting on MgCl2 concentration.](image)

The fluorescence-based data were normalized with the following pre- and post-melt normalization regions: 84.12–84.61°C and 88.23–88.76°C. Figure 3 shows the normalized, temperature-shifted melting curves produced by the HRM analysis, which followed the real-time PCR amplification of 134 bp amplicons from genomic DNA using the LightCycler 480 HRM dye. HRM analysis allows clear discrimination between the normal homozygous and heterozygous genomic DNA samples, based on shape of the melting curves and difference in $T_m$. All samples were of known genotypes and were grouped correctly by the LightCycler Nano Software. The $T_m$ values were: 86.45 – 86.58°C for heterozygotes variants (C/T) and 86.84 – 86.96°C for homozygotes variants (C/C). The $T_m$ difference between the heterozygous (C/T) and homozygous (C/C) variants was 0.26°C and proved to be sufficient for discrimination between the two variants.
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Figure 3. Normalized HRM plots related to the RYR1 amplicons. The two genotypes are clearly distinguished in this assay. The C/C homozygote melts at the highest temperature (blue) while the C/T homozygote melts at 0.26°C lower (red).

The assay was validated by screening 166 homozygous wild-type and 29 heterozygous genomic DNA samples of known genotypes for the RYR1 C/T SNP (C1843T) causing the Arg615Cys substitution in amino acid sequence.

The difference plots in the HRM assay for the 134 bp amplicons shown in Figure 4 clearly separate normal homozygous genomic DNA samples (C/C) from heterozygous ones (C/T). Heterozygotes were easily distinguished from normal homozygotes based on the shape of the melting curves. Discrimination of different genetic variants is based on differences in Tm and amplicon melting data should be analyzed both with and without temperature shifting.

Figure 4. The difference plots related to the RYR1 amplicons, using one of the normal samples (C/C) as the baseline. Difference plot analysis allowed differentiation between wild-type (blue) and heterozygous (red) samples.
In HRM analysis, differences in $T_m$ and normalized curve shape are used together to discriminate between different genotypes. A good reaction optimization and an appropriate assay design are crucial points that can increase the amplitude of the profile difference and make sequence discrimination easier. However, the primers used for HRM must generate short amplicons. According to the manufacturer's recommendation the best results can be obtained with amplicons up to 300 bp (www.roche-applied-science.com) because reducing amplicon size increases the difference in signal at a given temperature between two sequences that differ at only one nucleotide position. After primers design, reaction optimization can involve primer and DNA template concentrations and also the use of a thermal gradient in order to determine the best annealing temperature that yields 100% reaction efficiency and produces a single product. Thus, careful sample preparation and assay design are crucial for robust and reproducible results.

In the present study, only $RYR1\, NN$ (C/C) and $Nn$ (C/T) variants occurring in the investigated population were genotyped. Our result showed that HRM is a close tube, homogenous genotyping assay, that can be used successfully in genotyping of $RYR1$ and can replace PCR-RFLP analysis, which is more labour intensive and expensive.

HRM analysis characterizes samples by their dissociation behaviour, which is based on sequence length, GC content and DNA sequence complementarities, and can be used to detect single base sequence variations (M. LIEW & al. [24]). When HRM genotyping accuracy was compared to PCR-RFLP assay no discordant results were observed. The commonly used PCR–RFLP technique was laborious and time-consuming comparing with qPCR-HRM assay, where the results were obtained faster and with less labour. The qPCR-HRM assay setup was fast, since it took less than 2 hours to prepare the PCR mix, to perform PCR reaction, HRM analysis and genotypic data acquisition. We were able to obtain results within 6 h for the whole procedure, including the genomic DNA extraction and spectrophotometric measurements.

Our findings are in accordance with those of other researchers which have previously stated that high-resolution melting analysis is a convenient technique, with no processing or separation steps required (C.T. WITTWER & al. [25]). In addition to genotyping, high-resolution melting analysis is an accurate mutation scanning tool (G.H. REED & al. [26]).

Conclusions

A more recent development in fluorescent analysis of PCR amplicons was investigated, named high-resolution melting analysis, and this assay was applied to the analysis of $RYR1$ gene in pigs. In summary, our results showed that the HRM technique was a rapid, reliable and cost-effective assay that allowed us genotyping the C1843T SNP located in $RYR1$ swine gene, based on post-PCR melting curve analysis under high-resolution conditions.

The detection of $RYR1$ polymorphism using HRM technique can be widely used by breeding companies and producers in order to reduce the frequency of susceptible allele ($n$) in Romanian swine population trough proper selection. This could lead to an improvement of the pork meat quality, with beneficial effects in overall profitability of farms.

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