

ARTÍCULO DE INVESTIGACIÓN BÁSICA

Molecular Diagnostics of Porcine Stress Syndrome Susceptibility Associated with the Arg615Cys Mutation Using Real-Time PCR with Fluorescent Hybridization Probes

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ABSTRACT

Objective: The purpose of the present study was to develop a molecular genotyping method test by using a real time PCR hybridization probe and applying it to the analysis of C1843T mutations of the *Sus scrofa* RYR1 gene.

Animals population Three PSS-susceptible and PSS non-susceptible crossbred swine races were used for the experiments: Pietrain X Landrace Belga, Pietrain X Large White and Pietrain X Duroc.

Methods: We have developed a genotyping method by using a hybridization probe and applied it to the analysis of C1843T mutations of the RYR1 gene, associated with PSS susceptibility. Genotyping results obtained by hybridization probe strategies were confirmed by restriction analysis and sequencing. In addition, phenotype/genotype correlation analyses were developed by using the in vitro contracture test and confirmed the in vivo halothane-succinylcholine challenge.

Results: The real-time PCR with fluorescent hybridization probe methodology was designed to identify homozygous PSS-resistant, PSS-susceptible animals as well as heterozygous carriers. All cases genotyped by fluorescent hybridization probes were in agreement with PCR restriction enzyme digestion and sequencing and showed a 100% concordance between the in vivo and in vitro porcine stress syndrome (PSS) susceptibility results.

Conclusions and clinical relevance: The real-time PCR with fluorescent hybridization probe method described here provides a rapid, easily interpretable and reliable tool for genotyping the C1843T (Arg615-Cys) polymorphism of the RYR1 gene. This new methodology may be useful in the wide-scale genotyping of PSS-susceptibility and genetic selection.

Keywords: Malignant Hyperthermia, Caffeine-Halothane Contracture Test, Malignant Hyperthermia Diagnosis, Halothane Test, Succinylcholine Test, Porcine stress syndrome susceptibility, Arg615Cys, C1843T SNP, molecular diagnostic, real-time PCR, fluorescent hybridization probes

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INTRODUCTION

The Porcine Stress Syndrome (PSS) susceptibility is a hypermetabolic syndrome involving skeletal muscle disorder associated with a failure of the calcium homeostasis in muscle fibers, characterized by hyperthermia, muscle rigidity, and autonomic dysfunction_(1,2). Ryanodine receptor 1 (RYR1) and the dihydropyridine receptor (DHPR) are involved in the release of the calcium (Ca²⁺) stored in sarcoplasmic reticulum_(3,4). PSS-susceptibility is inherited as an autosomal recessive dysfunction that has been clearly demonstrated and associated with a single point mutation C1843T (Arg615Cys) in the Ca²⁺ release channel at the sarcoplasmic reticulum RYR1, where the susceptible mutant allele is denominated T and the wild-type - nonsusceptible allele C_(5,6).

The frequency of the PSS gene in swine populations varies according to genetic breeds and country of origin. Porcine stress syndrome has been reported in different swine breeds and the incidence is higher in lean, heavily muscled breeds such as Duroc, Large White, Pietrain, and Landrace. This susceptibility trait was related to the incidence of acute stress and sudden death, and an inferior quality meat generally referred to as pale, soft, exudative, dark, firm and dry, with back muscle necrosis₍₇₎.

Despite the number of laboratory tests developed with the aim to identify PSS-susceptible animals or carriers, none enables rapid diagnosis of PSS in an acute situation and most of these lack the sensitivity and specificity to identify PSS-susceptible animals. The *in vitro* contracture test remains the standard test for the diagnosis of malignant hyperthermia (MH) susceptibility in which live muscle specimens are exposed to halothane and caffeine in the laboratory and the contracture response correlates with elevated myoplasmic Ca²⁺ concentrations_(8,9). In spite of the fact that the sensitivity of the *in vitro* contracture test is 97%, and the specificity is approximately 85%, when this was used for subjects at high risk₍₁₀₎, this methodology had limited application in animals. This was because special laboratory facilities are required and the test must be run within minutes after the specimen is obtained. Genotyping must meet several key criteria, including specificity, sensitivity, fast turnaround, and cost-effectiveness. Real-time PCR using fluorescein-labeled hybridization probes fulfils these criteria and furthermore is a rapid, versatile, and cost-effective method for this type of analysis₍₁₁₎.

The purpose of the present study was to develop a molecular genotyping method test by using a hybridization probe and applying it to the analysis

of C1843T mutations of the RYR1 gene, which is associated with the PSS susceptibility. Hybridization probe methodology was designed to identify homozygous PSS-resistant, PSS-susceptible animals as well as heterozygous carriers. Genotyping results obtained by hybridization probe strategies were confirmed by restriction analysis and sequencing. In addition, phenotype/genotype correlation analyses were developed by using the *in vitro* contracture test and confirmed the *in vivo* halothane-succinylcholine challenge. Our results showed that the simplicity of real-time Polymerase Chain Reaction (PCR) technologies using fluorescence quenching schemes, allowed the diagnosis of the three genotypes of the PSS. This new methodology is a tool that may contribute to genetic selection in order to obtain resistant pigs to Porcine Stress Syndrome. The real-time PCR with fluorescent hybridization probes method described here provides a rapid, easily interpretable and reliable tool for genotyping the C1843T (Arg615Cys) polymorphism of the RYR1 gene.

EXPERIMENTAL PROCEDURES

Three PSS-susceptible and PSS non-susceptible crossbred swine races were used for the experiments: Pietrain X Landrace Belga, Pietrain X Large White and Pietrain X Duroc. Swine were housed in standard lighting and were given water and food *ad libitum* until the beginning of the experiment, when they were moved to a surgery room. All procedures were in accordance to the Guide for the Care and Use of Laboratory Animals (National Institute of Health), and were approved by the Ethics Commission of the Veterinary School at the National University of Colombia.

Muscle samples biopsies, subjected to halothane-caffeine *in vitro* contracture tests were tested according to the protocol of the North American Malignant Hyperthermia Group protocol_(8,12). Genotyping was done using a real-time PCR-based system (LightCycler, Roche, and Mannheim, Germany) with hybridization probes specific for the RYR1 C1843T SNP. Additionally, individual samples representing the C/C, C/T or T/T genotypes analyzed by real-time PCR were also genotyped by DNA sequencing and restriction fragment length polymorphism techniques, proving the accuracy of the real-time PCR method.

Initially, random samples of peripheral blood were obtained from ninety-five swine in a farm located outside the metropolitan area of Bogotá, Colombia. DNA samples plus controls (CC, CT and TT RYR1 genotyping, donated by Dr. R. Omelka, from the De-

partment of Botany and Genetics, Constantine the Philosopher University, Slovak Republic) were processed for screening the RYR1 C/T SNP (C1843T), using polymerase chain reaction and restriction fragment length polymorphism analysis with the enzyme HhaI (Invitrogen), as described elsewhere⁽¹³⁾. For this purpose 5 mL of peripheral venous blood was gathered from each swine by standard venepuncture and each blood sample was collected in sterile tubes containing K3EDTA solution. The animals were genetically tested by standard methods using genomic DNA isolated from blood leucocytes (ChargeSwitch® gDNA Blood Kit, Invitrogen, USA), according to the manufacturer's recommendations. The purity and concentration of the extracted DNA were determined by a UV-Vis spectrophotometer. Twenty-one swine genotyping with the restriction enzyme HhaI for the RYR1 C1843T SNP (CC n=9, TC n=7, and TT n=5), were randomly selected for the later development of the study.

Sequences for PCR primers and hybridization probes used for SNP detection can be found in Table 1. The DNA sequencing templates were PCR amplified from the *Sus scrofa* strain Yorkshire RYR1 gene (Accession M91456.1 [GenBank]) and primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers amplified a 329 bp fragment encompassing the polymorphism C1843T.

The detection probe attached to the polymorphic site and the nucleotides flanking it were specific for the T-allele (figure 1). Hybridization probes for genotyping RYR1 gene whose mutation C1843T (Arg615Cys) is associated with PSS -susceptibility, were designed and obtained from Roche Molecular

Table 1

Target Genes	Primer Sequence	Product size (bp)	GenBank accession #
RYR1 (<i>Sus scrofa</i>)	Forward 5' - AGACCTTCTCTTTGACCTTGAT-3' Reverse 5' - CCAGACCTGGTGACATAGTTGA-3'	329	M91456.1
Hybridization probe			
Donor probe	5' - CGTGTGCTCCAACCAA-FL		
Acceptor probe	5' - LC Red 640-CTCATTACTGAGAAGCTTGCTGCCTGGC-PH		

The primer and Hhybridization probe sequences are shown. Donor probe is labeled with fluorescein at the 3', whilst the adjacent acceptor probe is labeled with the specific fluorophore Red 640 (LC Red 640) at the 5' end region

Biochemical®. Acceptor probe were labeled at the 5'-end with LC Red 640 and phosphorylated on the 3'-end by block extension. The donor probe was labeled on the 3'-end with fluorescein and was designed to bind at a distance of three bases from the acceptor probe.

The PCR reaction was carried out using the LightCycler real-time PCR machine 2.0 (Roche Diagnostics, Germany) and the software version 4.0. The reaction was performed in a volume of 20 µl with 2.5 mM MgCl₂, 0.25 µM of both primers, 0.25 µM of both probes and 2 µl of 10 × LightCycler® FastStart DNA Master HybProbe buffer (Roche Diagnostics), and about 50 ng of template DNA. The PCR protocol consisted of initial denaturation at 95 °C for 10 min, followed by 37 cycles of denaturation (95 °C for 5 s, 20 °C/s), annealing (60 °C for 15 s, 20 °C/s) and elongation (72 °C for 20 s, 20 °C/s). This was followed by melting curve analysis consisting



Figure 1: A representative fragment of the *Sus scrofa* strain Yorkshire RYR1 gene, showing the PCR primers and the fluorophore-labeled hybridization probes for SNP C1843T. Probes designed to detect the SNP C1843T were complementary to the antisense (reverse) strands of the RYR1. The SNP creates an A-T match between the antisense strand and the donor probe the allele 1843T. Complete matching of donor probe to the antisense strand results in a higher T_m of the mutant allele's hybrid. The mismatch destabilizes the wild type hybrid such that a decrease in the probe T_m occurs. P indicates the addition of a phosphate group.

of 1 cycle at 95 °C for 30 s (20 °C/s), 40 °C for 20 s (20 °C/s) and a temperature rise to 85 °C at a slope of 0.2 °C/s with continuous measurement of fluorescence. The genotyping results were interpreted according to derivative melting curves [$-d(F2 / F1) / dt$] plotted against temperature.

To confirm the genotyping results obtained by real-time PCR using hybridization probes strategies, restriction analysis and sequencing were used (figure 2, 3). All PCR products were purified using Marligen's Gel Extraction Systems Kit (Marligen Biosciences Inc.), according to the manufacturer's recommendations. Sequencing was performed in both directions by DYEnamic™ ET Dye Terminator Kit (MegaBACE™) on a MegaBace DNA Analysis Systems (Amersham Biosciences). Sequencing results were analyzed using nucleotide-nucleotide BLAST 2 SEQUENCES software ([GenBank]) to locate and identify base changes. In addition, analyses of DNA RYR1 amplicons were subjected to restriction enzyme digestion during 4 hours, with the enzyme HhaI (Invitrogen), according to the manufacturer's recommendations. The digested DNA fragments were visualized on a 1.5% agarose gel, with a 100-bp DNA ladder (Invitrogen) as a molecular weight marker, and stained with ethidium bromide (10 mg/

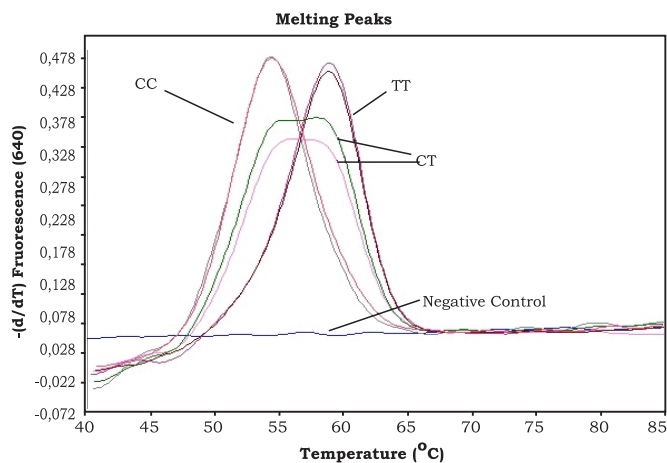
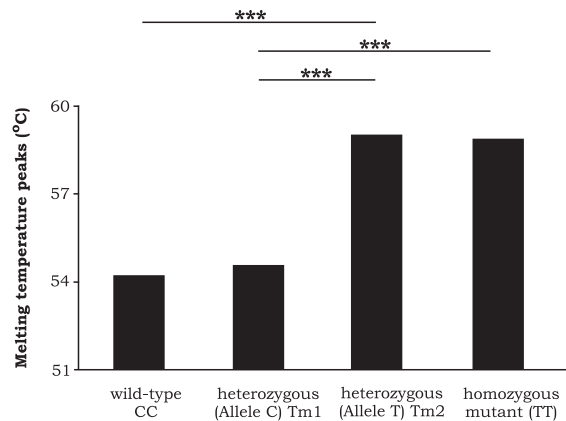


Figure 2: Derivative melting curve ($-dF/dT$) showing the RYR1 single base genotyping (C/T). Samples are wild type with a C-T mismatch to the hybridization probe and a melting temperature of 54.2°C; the mutation with a perfect match (T) to the hybridization probe and a melting temperature of 58.97°C, and two heterozygous samples with both wild type and mutant alleles. Melting analysis of a no template control was also performed. The melting peaks indicate that the RYR1 1843C wild type allele sequence has a lower T_m than the mutant 1843T allele sequence due to homology between the probe and mutant allele.

mL). Images of the gels were taken using Molecular imaging Systems (Bio-Rad). In all assays, negative and positive sample controls with known SNP C/T genotypes were used.

To determine the associations between genetically tested swine for the RYR1 C1843T SNP and the stress susceptibility phenotype, six PSS-susceptible and PSS-nonsusceptible swine (C/C, n=2; C/T, n=2; T/T, n=2; weight, 30 +/- 10 kg) were investigated. In vivo studies with 3% halothane and in vitro contracture test from the gracilis muscle biopsy exposed to halothane-caffeine were undertaken⁽¹²⁾. Parallel to the in vivo study, viable muscle specimens were taken and exposed to in vitro contracture tests following the protocol described by Marilyn Green Larach and the North American Malignant Hyperthermia Group⁽⁸⁾.



RYR1 genotypes				
Genotype	wild-type CC	heterozygous (Allele C) Tm1	heterozygous (Allele T) Tm2	homozygous mutant (TT)
$\bar{X} = T_m$ (°C)	54,20±0,10 n=9	54,53±0,20 n=5	58,97±0,48 n=5	58,82±0,35 n=7

Figure 3: Melting Temperatures for Fluorescein Probe/Allele. The homozygous mutant genotype (TT) formed a perfect match T:A and were annealed to the probe at the fluorescence acquisition temperature of 58.82 °C. The fluorescein probe formed a T:G mismatch with the wild-type sequence (CC), lowering the T_m of the probe by 4.62°C from the completely complementary duplex. Heterozygous (CT) genotype had a melting peak at 58.97 °C (perfect match T:A) and other acquisition temperature of 54.53 °C (T:G mismatch). Data were expressed as means ± SEM (CC: n= 9; CT: n= 5; TT: n= 7), and their statistical comparison was made by ANOVA followed by the unpaired Student's t-test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

A schematic representation of the adjacent fluorescent hybridization probes used for genotyping the RYR1 C1843T SNP is shown in figure 1. Table 1 lists the sequences for PCR primers and hybridization probes used for SNP detection. These probes were designed so that during hybridization the donor probe formed a mismatch with the wild-type allele (C:T). The data collected during the melting phase were used to genotype each sample and melting peaks were obtained by plotting the fluorescence (F) versus temperature data as $2dF/dT$.

Genotype analysis for the C1843T SNP is shown in figure 2, where all genotypes are clearly distinguishable. The peak assignment is as given in figure 3. The G:T mismatch formed between the wild-type allele with the fluorescein probe, created a ΔT_m of 4.62°C from the completely Watson-Crick paired duplex (homozygous mutant allele T). A distinct peak at $54.2 \pm 0.10^\circ\text{C}$ is indicative of allele C1843, whereas both the homozygous mutant allele (TT) and the allele with the 1843T mutation from the heterozygous samples, were annealed to the probe at the fluorescence acquisition temperature of $58.82 \pm 0.35^\circ\text{C}$ (see figure 3).

Swine single point mutation C1843T (Arg615-Cys) genotyping performed in the LightCycler instrument was compared with conventional PCR restriction fragment length analysis and sequence. PCR restriction fragment length analysis was performed on all samples for the C1843T mutation with the enzyme HhaI (figure 4). Forward and reverse primers were designed to produce a 329-bp-long amplicon (see table 1 and figure 4A). The RYR1 C1843T mutation destroyed a HhaI restriction site that upon restriction digestion of the wild-type genomic DNA, yielded fragments of 239 and 90 bp (figure 4B). All cases genotyped by fluorescent hybridization probes agreed with PCR restriction fragment length analysis. In addition, results of genotyping with hybridization probes and restriction analysis were compared with PCR amplicon sequences and aligned to the *Sus scrofa* strain Yorkshire RYR1 gene to verify melting curve analyses (figure 5). Single base change at the targeted SNP site corresponded with results of melting curve analyses (Figure 2). Direct DNA sequencing analysis confirmed the different RYR1 genotypes (figure 5) and revealed a C-to-T substitution that results in an arginine-to-cysteine amino acid replacement at codon 615 (Arg615Cys). Variations from wild-type to mutant alleles resulted in 4.62°C T_m change (Figure 3).

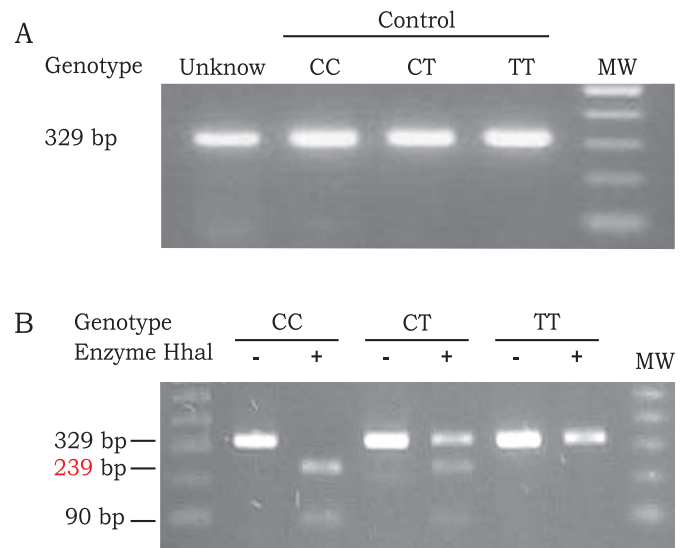


Figure 4: Restriction analysis for the swine RYR1 C1843T SNP, determined by digestion with HhaI on 1.5% agarose gel electrophoresis stained with ethidium bromide. Allele specific PCR for the C1843T variant showing band patterns after screening: undigested 329-bp PCR product; wild-type (CC) genotype restriction fragments of 239 and 90 bp; heterozygous (CT) genotype restriction fragments of 329, 239 and 90 bp; and homozygous mutant (TT) genotype restriction fragment of 329 bp. MW, molecular weight marker 100 bp.

In the current study, the correlation between RYR1 C1843T genotype determination above by different molecular approaches and the porcine stress syndrome susceptibility has been investigated. Phenotype/genotype correlation analyses were developed by using the in vitro contracture test and confirmed by the in vivo halothane-succinylcholine challenge (Figure 6A andB), as was described elsewhere⁽¹²⁾. All swine diagnosed as PSS-susceptible by in vitro contracture test, were T allele homozygous (TT) and heterozygous (CT) for the RYR1 mutation, and all PSS non-susceptible swine were homozygous for the C1843 allele (Figure 6). All results were in agreement with contracture test parameters seen with an increase in twitch height as Allen described elsewhere⁽¹²⁾. Furthermore, all swine diagnosed as PSS-susceptible by the in vivo halothane-succinylcholine challenge were homozygous for the 1843T allele whereas the wild-type (CC) and heterozygous swine were PSS non-susceptible (data not shown). These results confirm once again that autosomal recessive Porcine Stress Syndrome susceptibility disease is caused by the C1843T mutation in the gene encoding the skeletal muscle calcium release channel RYR1.

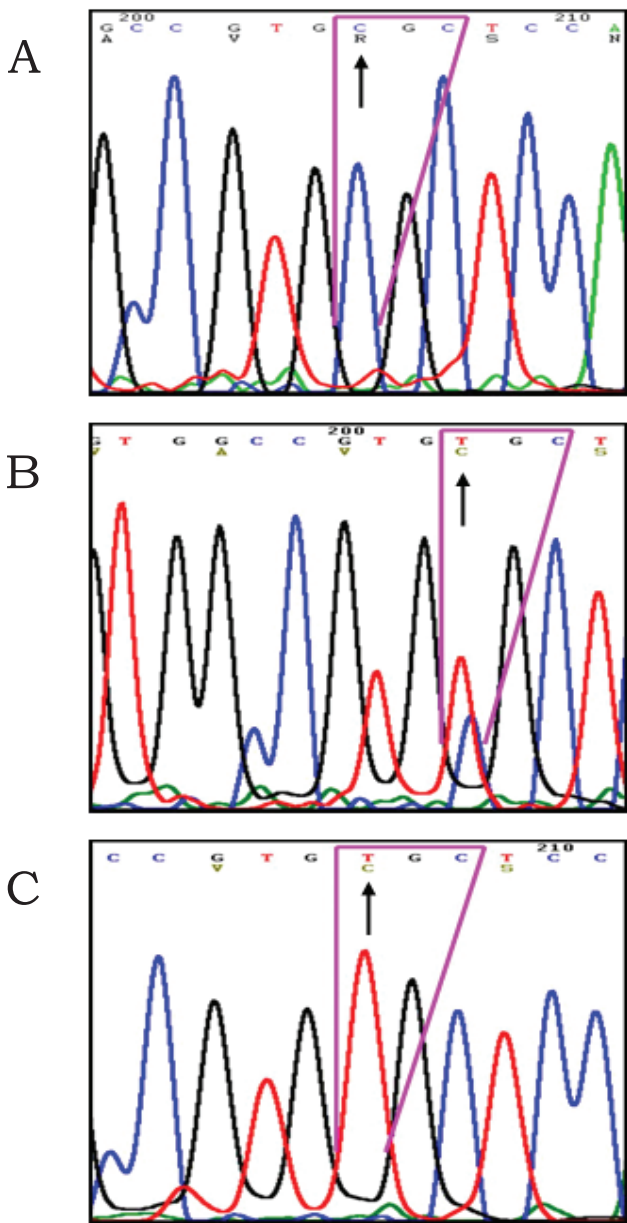


Figure 5: Results of direct sequencing of a 329-bp fragment of the RYR1 gene, showing the polymorphic site at position: A) wild-type genotype (CC), B) heterozygous pattern (CT) and C) homozygous mutant (TT) (indicated by arrows).

DISCUSSION

Porcine stress syndrome (PSS) susceptibility, has been associated with a single point mutation C1843T (Arg615Cys) in the Ca²⁺ release channel at the sarcoplasmic reticulum ryanodine receptor 1 (RYR1). The RYR1 gene is the best commercially available marker used for predisposition of stress susceptibility in several swine breeds. Changes in

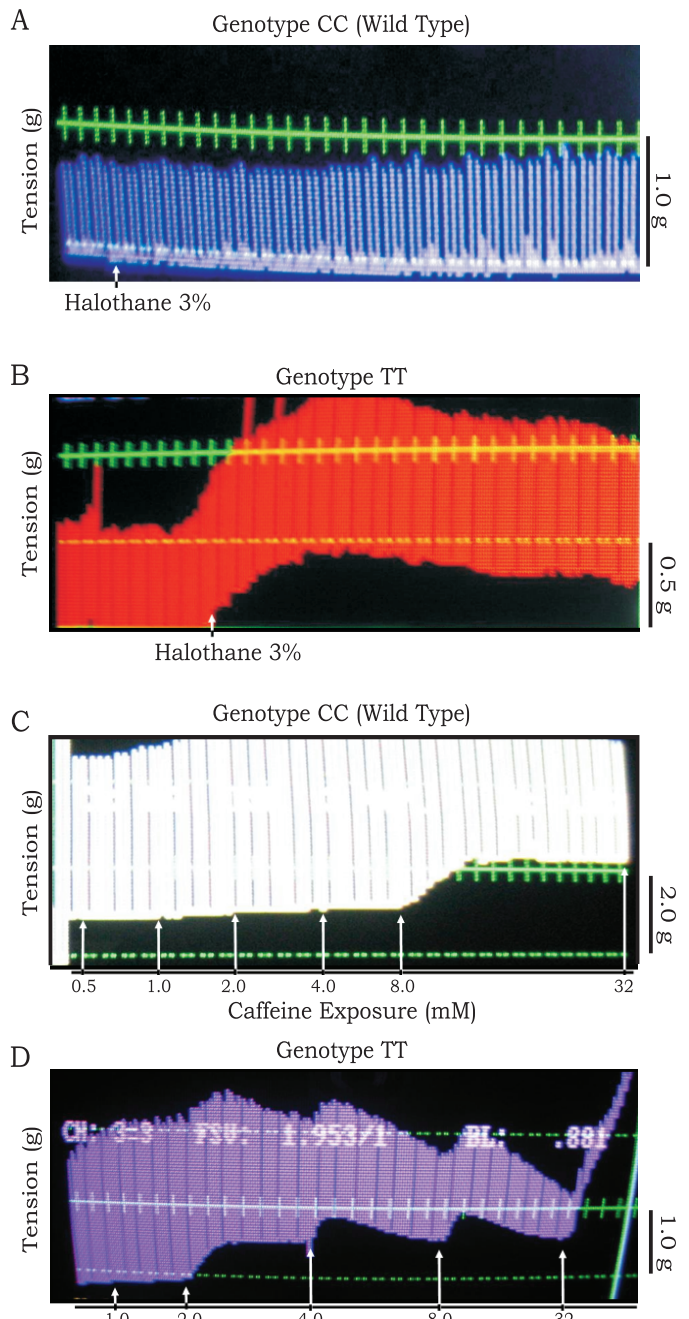


Figure 6: The Caffeine-Halothane Contracture Test in PSS-nonsusceptible and PSS-susceptible from the swine gracilis muscle biopsy. (A) Normal (negative) and (B) abnormal (positive) response after exposure to 3% halothane. A positive halothane contracture test is defined as a contracture greater than 0.2 – 0.7 g after exposure to 3% halothane bubbled through the tissue bath for 10 min. (C) Normal (negative) and (D) abnormal (positive) response to caffeine. Caffeine exposure is to 0.5, 1.0, 2.0, 4.0, 8.0, and 32 mM for 4 min in each concentration. A contracture greater than 0.3 g to 2 mM caffeine or less indicates susceptibility. 32 mM caffeine is added to the bath to determine maximal response.

the intracellular free calcium concentration are reflected in a variety of cellular responses, such as muscle contraction, neuronal excitability, gene expression and metabolism variation. The functional effect of the RYR1 Arg615Cys mutation (C1843T) linked to neuromuscular disorders lead to a dysregulation of intracellular calcium homeostasis. The mechanisms leading to the PSS-susceptibility crisis involve a high concentration of free calcium in the myoplasm released by a faulty membrane system in calcium storing organelles including sarcoplasmic reticulum⁽¹⁴⁾. In the present study, we used molecular and physiological (in vitro and in vivo) approaches to determinate the PSS susceptibility.

Current methods for genotyping the C1843T Porcine stress syndrome (PSS) susceptibility mutations include allele-specific oligonucleotide hybridization and PCR restriction fragment length analysis, and these methods require multiple manual steps and are time consuming^(5,15). An alternative is the use of fluorescent hybridization probes and rapid-cycle PCR, a technique that provides homogeneous amplification and genotyping in approximately 45 minutes. We have developed a genotyping method by using a hybridization probe and applied it to the analysis of C1843T mutations of the RYR1 gene, associated with PSS susceptibility.

Methods available for PSS- susceptibility genotyping are based on sequencing and restriction fragment analysis, which requires several technical steps. Although different PCR-based fluorescent techniques are being used for the diagnosis of PSS-susceptibility none of them genotype by allele / hybridization probe T_m ⁽¹⁶⁾. In addition, the technology of fluorescence resonance energy transfer avoids additional steps, and the genotyping can be done directly on extracted genomic DNA from swine. Real time PCR using hybridization probe systems based on fluorescence resonance energy transfer (FRET) have been established as effective tools that allow the discrimination between closely related sequences on the basis of a melting curve analysis that confer very sensitive and highly specific detection. This technology is useful for diagnostic purposes such as genotyping (SNP analysis) and determination of genetic predisposition to disease⁽¹¹⁾. In addition, Polymerase Chain Reaction amplification and genotyping by fluorescent probe melting temperature, which reduces the cost and invasiveness of sample collection, is a rapid, versatile, and cost-effective method for this type of analysis. Derivative melting curves for the

three genotypes of the RYR1 C1843T (Arg615Cys) polymorphism are shown in Figure 2.

The thermal stability of a DNA duplex relies on GC content, duplex length, and Watson-Crick base pairing. Fluorescence from hybridization probes is the direct result of the hybridization of two independent probes and this result in very high specificity. Changes from Watson-Crick pairing destabilize a duplex by varying degrees depending on the length of the mismatched duplex, the specific mismatch, the position of the mismatch, and neighboring base pairs. The probe melting temperature is sequence dependent, providing a simple method to genotype mutations, including single base mutations⁽¹¹⁾. In this study, the single base mismatch G:T (C1843T) in the probe region decreases the melting temperature as little as 4.62 °C in comparison with the completely Watson-Crick paired duplex. The average melting temperature was 54.2 ± 0.10 °C for the Wild-type C allele and 58.82 ± 0.35 °C for the mutant T allele. The donor fluorescein probe formed an G:T mismatch with the wild-type allele lowering the T_m of the probe by 4.62 °C from the Watson-Crick paired duplex, assuring a good separation of the C1843 and 1843T genotypes. The donor probe attached to the polymorphic site was specific for the T-allele. All cases genotyped by fluorescent hybridization probes were in agreement with PCR restriction enzyme digestion and sequencing in a random set of samples representing all genotypes. When we validated the method with positive DNA control generously donated by Dr. R. Omelka (Department of Botany and Genetics, Constantine the Philosopher University, Slovak Republic), the results matched completely.

Finally, we have demonstrated a 100% concordance between the in vivo and in vitro porcine stress syndrome (PSS) susceptibility results and the ones obtained by means of molecular genotyping such as real-time PCR using fluorescein-labeled hybridization probe, sequencing and restriction analysis. The simplicity of real-time PCR technologies using a fluorescence quenching schemes, allowed diagnosis of the three genotypes of the PSS, and this methodology is a tool that may contribute to the genetic selection and to obtain pigs resistant to PSS. In summary, this assay shows that real-time with fluorescent hybridization probes provide a rapid and sensitive analysis for genotyping the RYR1 C1843T (Arg615Cys) polymorphism and may be useful in the wide-scale genotyping of PSS-susceptibility.

This new methodology is a tool that may contribute to the genetic selection and to obtain pigs resistant to PSS and may be useful in the wide-scale genotyping of PSS-susceptibility.

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