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An improved method for characterization of the mutation associated to porcine stress syndrome by PCR amplification followed by restriction analysis

Um método melhorado para caracterização da mutação associada à síndrome do estresse suíno por amplificação por PCR seguido de análise de restrição

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- NOTE -

ABSTRACT

A mutation in the gene coding for the ryanodine receptor 1 (RYR1), also known as halothane (hal) gene or swine stress gene, is associated to the porcine stress syndrome (PSS). Detection of the mutation is normally accomplished by PCR amplification of an 81bp fragment of the hal gene, followed by digestion with the HhaI restriction endonuclease. Wild-type allele (N) is cut in two fragments, whereas the mutant allele (n) is not digested by the restriction enzyme. Electrophoresis of the digested DNA on agarose gel and ethidium bromide staining allows the reading of the result. The correct interpretation is difficult due to the small size of the DNA fragments. In this study we designed a new set of primers for amplification of a 144bp fragment that facilitates the reading of the result. In addition, we optimized the PCR reaction to allow amplification from a single hair bulb, added directly into the PCR mix without previous treatment. This improved method was used to genotype 165 sows and boars used in a breeding program. Forty-nine percent of the animals had the NN genotype, whereas 50% were Nn and only 1% was nn.

Key words: *PCR-REA, plucked hair, swine stress gene, PSE pork.*

RESUMO

Uma mutação no gene que codifica o receptor ryanodine 1 (RYR1), também conhecido como gene do halotano (hal) ou gene do estresse suíno, está associada à Síndrome do Estresse Suíno (PSS). A mutação é geralmente detectada por PCR,, a partir da amplificação de um fragmento de 81pb do gene hal, seguida por digestão com a endonuclease de restrição HhaI. O alelo normal (N) é cortado em dois fragmentos, enquanto que o alelo mutado (n) não é digerido pela enzima de restrição. A eletroforese do DNA digerido em gel de agarose corado com brometo de etídio permite a leitura do resultado. A interpretação correta é difícil devido ao pequeno tamanho dos fragmentos. Neste estudo, foi projetado um novo par de iniciadores para a amplificação de um fragmento de 144pb, o que facilita a leitura do resultado. Adicionalmente, foi otimizada a reação de PCR para permitir a amplificação a partir de um único bulbo capilar, acrescentado diretamente na mistura de PCR, sem tratamento prévio. Esse método foi usado para genotipar 165 reprodutores utilizados em granjas produtoras de matrizes. Quarenta e nove porcento dos animais apresentaram genótipo NN, 50% Nn e apenas 1% nn.

Palavras-chave: *PCR-REA, pelo, gene do estresse suíno, carne PSE.*

Testing of the ryanodine receptor 1 gene (*RYR1*) whose mutation C11843T is associated with stress susceptibility and a decrease of some parameters of meat quality (FUJII et al., 1991) is important in swine breeding programs. The RYR1 gene is located on chromosome 6 (p1.1-q2.1) and the receptors are Ca^{2+} release channels of skeletal muscle sarcoplasmic reticulum (OTSU et al., 1991). Porcine stress syndrome (PSS) is an inherited myopathology in which skeletal muscle contraction, hypermetabolism and an elevation in body temperature are triggered by inhaled anesthetics and environmental stress (SANTORO & FAUCITANO, 1996). In recessive homozygotes (*nn*) the *RYR1* gene leads to PSS and the *post-mortem* manifestation of pale, soft and exudative pork (PSE). In

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heterozygosis (*Nn*) the *hal* gene produces lower carcass quality (BAND et al., 2005a; BASTOS et al., 2001; POMMIER & HOUDE, 1993), but possibly higher carcass weight (BAND et al., 2005b; LEACH et al., 1996). The polymorphism at nucleotide 1843 of the *hal* gene has been characterized by PCR amplification associated to restriction endonuclease assay (PCR-REA) using blood or muscle biopsy as the source of genomic DNA (HOUDE et al., 1993; KAMINSKI et al., 2002).

In a previous study, BASTOS et al. (2000) described a PCR-REA test using plucked hair as source of DNA, which is a noninvasive and convenient method to screen farm animals. In this study we made significant improvements to the method. Using a new set of primers and modifications in the PCR conditions, higher amplification efficiency was obtained even from a single hair bulb used directly in the PCR reaction without previous treatment. The larger amplified fragment made the reading of the result much easier.

Pluck hair was collected from 165 pigs from commercial farms. The samples were placed in an envelope and shipped to the laboratory through the standard postal service. Upon arrival, samples were stored at 4ºC until use. For performing the PCR amplification, a single hair was picked with tweezers and the bulb was placed in a 0.2ml PCR tube. Scissors used to cut the hair were wiped with 70% ethanol between samples to avoid cross-contamination. DNA extraction was originally carried out by the protocol described by BASTOS et al. (2000). Briefly, the hair bulb was incubated for 5min in 0.5µl of 0.1M NaOH, after which 4.5µl of 0.02M Tris-HCl, pH 7.4, was added. The PCR reaction mixture (20µl) was then added to the tube containing the extract. In an attempt to simplify the procedure, the hair bulb was added directly to the PCR reaction mixture (25µl) without prior treatment. It was observed that the reactions employing DNA extracted with sodium hydroxide showed a weaker band than those performed with the untreated hair bulbs, upon electrophoresis of the PCR reaction on a 2% agarose gel stained with ethidium bromide. The use of untreated hair bulbs has the advantage of reducing one step in the amplification process, thus minimizing the risk of contamination.

The primers described by FUJII et al. (1991) result in the amplification of an 81bp fragment. Digestion of the amplified fragment results in two bands of 49bp and 32bp if the animal has the *NN* genotype; no digestion (81 bp fragment) if the animal has the *nn* genotype; and three bands of 81bp, 49bp and 32bp if the animal is *Nn*. Visualization of such small bands is difficult on agarose gel. Primer dimers can sometimes make the reading of the result hard to be accomplished with accuracy. The use of primers described by O'BRIEN et al. (1993) would alleviate this problem as the PCR product is 659 bp long, however the efficiency of amplification of such large fragment by PCR is lower and requires DNA extracted from blood or muscle tissue samples. Therefore we sought new primer sequences that would allow amplification of a larger DNA fragment with high efficiency. After retrieving the DNA sequence corresponding to the *hal* gene from GenBank (accession number M91456), the sequence was analyzed with the aid of the VectorNTI 10.0 software (Invitrogen). A new forward primer was designed (5'- CCACACCCTCCCCGCAAGTGC - 3'), which together with the original reverse primer (5' – GCCAGGGAGCAAGTTCTCAGTAAT – 3') allows the amplification of a 144bp fragment.

PCR reactions were performed in a final volume of 25µl containing 200µM dNTP, 1x reaction buffer, 0.4pmol of each primer and 2.5 units of *Taq* DNA polymerase (CENBIOT-ENZIMAS, UFRGS). Different magnesium concentrations were tested, ranging from 1.5 to 3mM $MgCl_2$. In addition the use of additives such as 10% glycerol, 5% DMSO and 45µg ml^{-1} BSA were evaluated in order to optimize the amplification reaction. The use of High-fidelity DNA polymerase (Invitrogen) was also tested. For the amplification, a Mastercycler Gradient (Eppendorf) was used with the following settings: 9 min at 95°C followed by 30 cycles of 60 sec at 95°C, 60 sec at 56°C and 60 sec at 72°C and then a final extension of 7 min at 72°C. These settings were used for testing the different magnesium concentrations and additives. A hot-start PCR was also evaluated by adding the Taq DNA polymerase and the $MgCl_2$ after the initial denaturing step at 95°C for 9min. A volume of 10µl of the PCR reaction was submitted to a 2% agarose gel electrophoresis. After confirming the amplification of the expected DNA fragment, the remaining 15µl were digested with 5U *Hha*I (Invitrogen) overnight at 37°C, and then submitted to a 4% agarose gel electrophoresis.

Different concentrations of magnesium and the addition of 10% glycerol, 5% DMSO, 45µg/ml BSA, or the use of High-fidelity DNA polymerase fail to improve the result. Therefore, the standard 1.5mM $MgCl_2$ concentration was used throughout the study. A significant improvement was observed with the hot-start strategy, resulting in a strong single band of the expected size (data not shown). A good amplification is essential for an accurate determination of the genotype.

Digestion of the PCR products with the *Hha*I enzyme generated three distinct patterns (Figure 1). The normal genotype (*NN*) resulted in two fragments

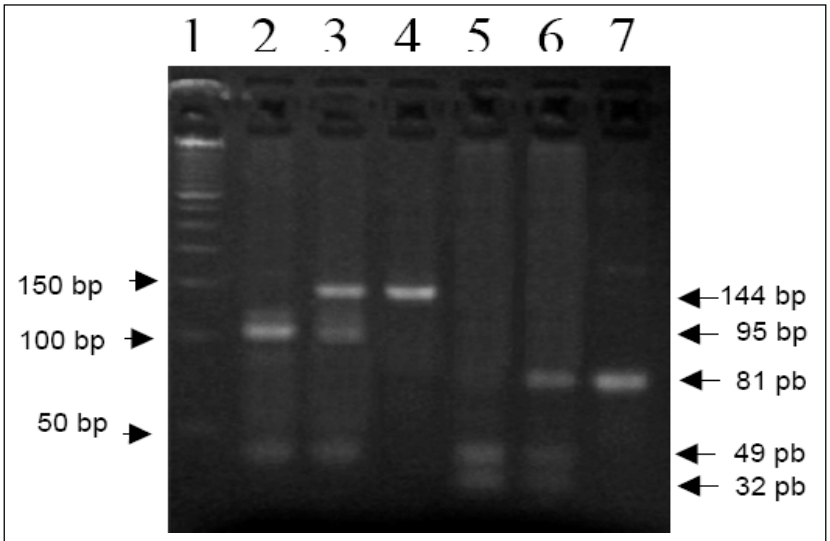

Figure 1 - Agarose gel electrophoresis of the *hal* gene amplified by PCR and digested with the *Hha*I enzyme. Lane 1, Molecular weight marker Ladder 50 bp (Invitrogen); lane 2 – 4, results obtained with the new set of primers - normal homozygote (*NN*); heterozygote (Nn); and recessive homozygotes (*nn*), respectively; lanes 5 – 7, results obtained with the old set of primers - normal homozygote (*NN*); heterozygote (Nn); and recessive homozygotes (*nn*), respectively.

of 95 and 49bp, respectively. The homozygote mutated genotype (*nn*) was not cut by the restriction enzyme, and the heterozygote (*Nn*) produced three fragments of 144, 95 and 49bp, respectively. The relatively larger size of these fragments compared to the original PCR (81, 49 and 32bp) facilitates the visualization of the bands. Samples tested by the method previously described and this improved method resulted in the same genotype.

Of 165 animals tested, 49% were *NN*, 50% were *Nn*, and 1% was *nn*. This high frequency of the *n* allele is the result of the different breeds used in the breeding program of these commercial farms. Considering the economical losses associated to mortality and lower carcass quality, the presence of the *n* allele in the herd should be avoided. The genotyping of breeding animals by the PCR-REA method described here is convenient, as it uses plucked hair as source of DNA, and easy to be performed.

In summary, we have described an improved method for genotyping the swine stress gene. A new set of primers that allows amplification of a larger DNA fragment, associated to a hot-start PCR reaction, resulted in an efficient amplification from a single hair bulb added directly to the PCR mix. These changes represent a significant improvement over previously described methods which needed DNA extracted from blood or muscle tissue samples and whose results were difficult to interpret.

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