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PCR GENOTYPING OF THE RYANODINE RECEPTOR GENE RYR1 IN YUGOSLAV MEAT SWINE

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The aim of this study was to investigate presence of a single point mutation, the T-C transition at nt 1843 in the RYR1 gene, in Yugoslav meat breed swine. Recently it has been shown that a single point mutation within the porcine skeletal muscle ryanodine receptor gene accounts for all cases of malignant hyperthermia in five major breeds of swine. The calcium channel, also known as the ryanodine receptor, plays a critical role in the initiation of muscle contraction. The results of the present study on Yugoslav meat breed swine using the PCR/restriction endonuclease test (DNA test) confirm the hypothesis of Fujii and Otsu (1991) that the cause of MH is a mutation at nt 1843 in the RYR1 gene. Mutation in the RYR1 gene is present in Yugoslav meat swine in the heterozygous state. The frequency of the RYR1 (C) allele is 0.90 and RYR1 (T) is 0.1. Development of a definitive diagnostic test for susceptibility to PSS is a major objective of research and cost-effective application in the swine selection programe.

Key words: PCR, ryanodine receptor RYR1, stress syndrome, swine.

INTRODUCTION

Sudden, stress-induced death; pale, soft, exudative meat (PSE); and sensitivity to halothane induced malignant hyperthermia (MH) are manifestations of the porcine stress syndrome (PSS) (Vogeli et. al., 1992). PSS is an economically important genetic defect of pigs. The molecular basis for susceptibility to porcine stress syndrome is a hypersensitive triggering mechanism of the calcium release channel of skeletal muscle sarcoplasmic reticulum. The calcium channel, also known as the ryanodine receptor, plays a critical role in the initiation of muscle contraction. An abnormality in the calcium release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine receptor) may account for malignant hyperthermia, because in skeletal muscle both contraction and metabolism are regulated by the concentration of intracellular $Ca^{2}+$. The skeletal muscle ryanodine (RYR1) gene and the porcine MH (HAL) locus have been localized to pig chromosome 6p11q21 suggesting linkage between porcine MH and RYR1. Moreover, a point mutation associated with malignant hyperthermia in several

breeds of swine has been identified in a putatively important regulatory region of RYR1. Recently Fujii et al. (1991) cloned and sequenced the cDNA of the porcine skeletal ryanodine receptor (RYR1) gene. A replacement of C (cytosine) at nucleotide (nt) 1843 from the MH normal animal by a T (thymine) in the cDNA of the MH-susceptible animal leads to an alteration in amino acid sequence from an arginine at position 615 in the MH normal animal to a cysteine in the MH susceptible animal. These events suggest that this mutation is the causal mutation in porcine and hence that (n) and (T) are synonymous. Of the three phenotypes, only halothane sensitivity can be measured in the living animal through short, controlled exposure to the anesthetic gas halothane (Eikelenboom and Minkema, 1974). Susceptibility to halothane-induced MH has been shown to be controlled by a recessive gene at a single autosomal locus (HAL) with both alleles exhibiting incomplete penetrance. Because sensitivity to halothane induced MH is a recessive trait in pigs, we can identify three genotypes; homozygous resistant animals (N/N), heterozygous carriers (N/n) and recessive homozygous-stress susceptibility (n/n)(Vogeli, et al. 1992). MacLennan and Wayne (1993) suggested possible $Ca^{2}+$ binding sites in segments of the ryanodine receptor. He constructed a 14 trpE fusion protein of the skeletal ryanodine receptor covering about 90% of the receptor. This fusion protein containing residues 4014-4765 (FP13) as a major $Ca^{2}+$ binding fusion protein. The strong $Ca^{2}+$ binding domain of FP13 was then localized in subfragments FP13b containing residues 4246 to 4377 and in FP13c containing residues 4364 to 4529. MacLennan (1993) then made polyclonal antibodies against their sequences and determined whether they affected $Ca^{2}+$ release in planar bilayers. He observed that the anti 13c2 antibody activated the $Ca^{2}+$ release channel by increasing both open probability and opening time without affecting channel conductance. The specific modulatory effect of this antibody directed against a probable $Ca^{2}+$ binding domain in the skeletal muscle ryanodine receptor, strongly suggested that the 13c2 sequence is involved in the $Ca^{2}+$ induced $Ca^{2}+$ release mechanism.

The halothane challenge test for pigs (Eikelenboom and Minkema, 1974) provided diagnosis that is widely used but which has important limited accuracy. This test does not discrimate HAL (N/n) from HAL (N/N) individuals, provides a false positive reaction in up to 3% of HAL (N/n) and HAL (N/N) animals and fails to elicit a response in up to 10% of HAL (n/n) animals (Vogeli, 1988). Some authors have shown that a combination of the halothane test and analysis of inheritance of linked genetic markers allows HAL genotypes to be predicted with an accuracy of 92-97% in various pigs breeds (Imlah, 1982.; Gahne, and Tuneja, 1985).

Development of a definitive diagnostic test for susceptibility to PSS has been a major objective of research in swine selection programmes. Numerous tests have evolved for detection of the functional defect associated with susceptibility to PSS or for associated protein or DNA polymorphisms. However, early tests lacked sensitivity or specificity, or were too expensive for application in swine selection programmes. Discovery of the mutation site in PSS has made available the first definitive and cost effective test for PSS, the DNA test or PCR/restriction endonuclease test. The correlation between inheritance of MH and the C-T

mutation at nt 1843, has been confirmed supporting the hypothesis that this mutation is the causal factor of porcine MH in Yugoslav meat swine, and this paper introduces a laboratory protocol (Vogeli, et al. 1994) for the cost-effective application of a PCR/restriction endonuclease test for susceptibility to PSS.

MATERIALS AND METHODS

Animals. The analysis was carried out on 26 pigs of the Yugoslav meat breed swine, of different age, sex and unknown PSS status.

Blood samples. From each animal, 1 to 2 ml of blood was drawn into sterile tubes containing K_3EDTA. Blood samples were kept at -20^{0}C until DNA preparation. The method of DNA isolation based on that of Higuchi (1989) was used. After thawing 600 μl sample was transfered to a sterile Eppendorf tube, to which 0.5 ml TE (10 mM Tris-HCl pH 7,5; 0.32 M D-saccharose; 5 mM $MgCl_2$, 1% Triton X-100) was added and the resulting mixture spun in an Eppendorf centrifuge for 30 sec. The supernatant was then removed and the pellet resuspended in 1.0 ml of TE by vortexing. This step was repeated three times before the pellet was finally resuspended in 0.5 ml of K buffer (10 mM Tris-HCl pH 8,3; 50 mM KCl, 0.1 mg/ml^{-1} gelatin; 0.45% Nonidet P40 and 0.45% Tween 20) and 50 μl of 20 mg/ml^{-1} proteinase K. The samples were incubated at 54^{0}C for at least 2 h, then boiled for 10 min. to inactivate the proteinase K, before being stored at -20^{0}C.

PCR protocol. We investigated the RYR1 locus: C/C, C/T and T/T genotyping at nt 1843. Genomic DNA samples were thawed and 3 μl, containing 0.2-0.3 μg DNA was used per reaction. Each reaction consisted of 60 μM dNTP's each, 10 mM Tris-HCl pH 8,3, 50 mM KCl and 0.1 mg/ml gelatin to which 0.20 μM of each primer was added. The forward primer was (5'-TCCAGTTTGC CACAG-GTCCTACCA-3') and the reverse primer was (5'-ATTCACCGG AGTGGAGTCCCTGAG-3') (FL17F2/FL17R2), and 2.5 μl of Taq DNA polymerase (Boehringer, Mannheim, Germany) was added in a total reaction volume of 51 μl. Each reaction mixture was overlayed with 51 μl of heavy paraffin oil. The PCR conditions were as follows: an initial cycle consisted of denaturation at 94^{0}C for 5 min., followed by 35 cycles of 40 sec. denaturation at 94^{0}C, 2 min. annealing at 53^{0}C, and extension for 2 min. at 72^{0}C. An extra extension step of 7 min. was added after 35 cycles. PCR products were stored at 4^{0}C until RFLP analysis. Additions to the reaction tubes were made quickly on ice and in the described order, to prevent uncontrolled polymerization.

RFLP analysis Using a restriction enzyme, the PCR product was cut into fragments at the mutation site and at a control site common to the normal and mutated gene. A 16 μl sample of each PCR product was digested for at least 2 h with 4U of isoschizomer BsiHKAI of Asp HI restriction enzyme (BioLabs, Beverly, MA, USA) in the buffer supplied at 60^{0}C in a total volume of 20 μl. Restriction digests were loaded with 4 μl of 40% sucrose, 0,25% bromophenol blue tracking dye, and were run on a 3% agarose gel (type I, Sigma, USA) in Tris-borate/EDTA (TBE) buffer pH 8,0. Samples were electrophoresed at constant voltage (80 V) for approximately 1 h. The bands were readily detected by staining

with ethidium bromide added to the agarose buffer mixture shortly after boiling. The gel was photographed on a UV transilluminator in a tabletop darkroom (UVP inc., USA) by a Polaroid MP4 Land camera.

RESULTS AND DISCUSSION

The PCR/restriction endonuclease test is based on analysis of the region of DNA coding for the abnormal portion of the calcium channel in porcine stress syndrome. Examination of photographs of electrophoretograms of DNA amplified by PCR technology and digested with restriction endonuclease clearly revealed 3 genotypes: normal homozygotes (N/N) or (C/C), homozygotes for the PSS mutation (n/n) or (T/T), and heterozygotes for the PSS mutation (N/n) or (C/T) (Figure 1).

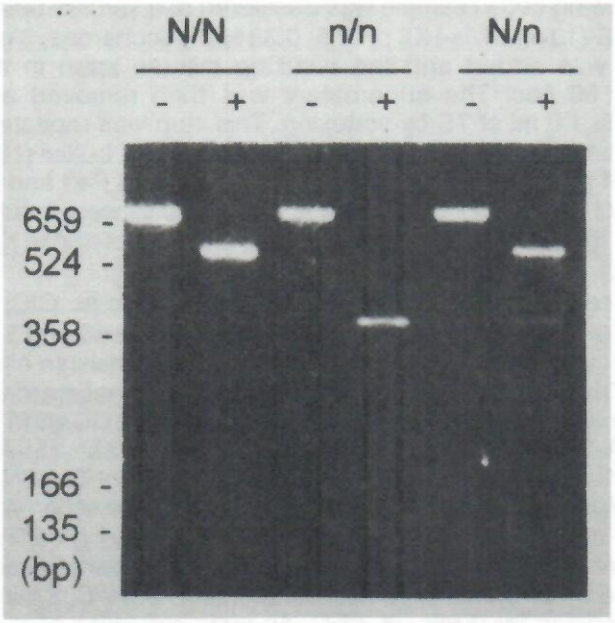

Figure 1. Detection of the C 1843 to T mutation in the RYR1 gene by amplification and subsequent digestion of the amplified product with BsiHKAI. (-) The PCR-amplified product; (+) the same product after digestion. Digestion of the N/N genotype generates 524- and 135-bp fragments from the constant BsiHKAI site, while digestion of the n/n genotype generates 358-, 166-, and 135-bp fragments through a combination of digestion of the constant and variant BsiHKAI sites. Fragments of 524, 358, 166 and 135 bp are generated in an N/n genotype. BsiHKAI is isoschizomer of the AspHI restriction enzyme.

A typical diagnostic electrophoretogram allows diagnosis of the genotype for 20 samples. The RFLP pattern for swine homozygous for the mutation C-T nt 1843 was characterized by a bright band at 358 base pairs (bp) and faint bands at 166 and 135 bp. For normal swine, a bright band was observed at 524 bp, with a faint band at 135 bp. For heterozygote carriers of the mutation, bright bands

were observed at 524 and 358 bp, with lightly fluorescing bands at 166 and 135 bp. If the sample was not cut by the restriction endonuclease a band at 659 bp was visible. Primer polymers appeared at 96 bp (tetramer) or 48 bp (dimer). Primer was observed at 24 bp (Figure 2).

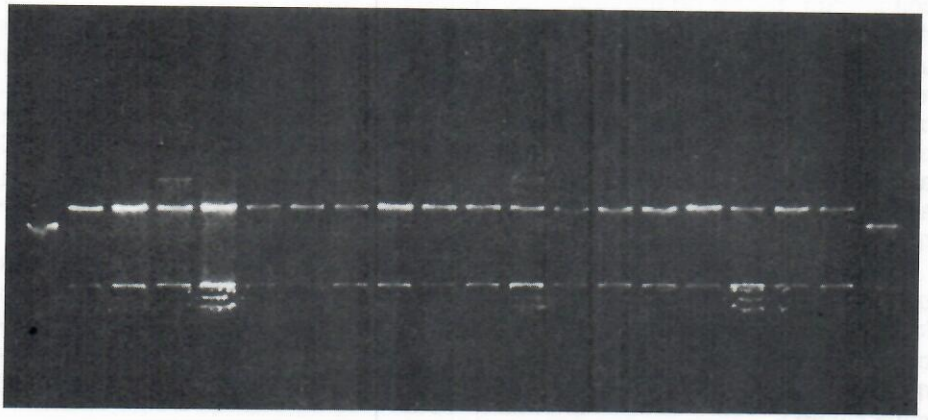

Figure 2. Result of the PCR/restriction endonuclease test for detection of the C 1843 - T mutation in the RYR1 gene by amplification and subsequent digestion of the amplified product with BsiHKAI in Yugoslav meat swine. 1'PCR-amplified product non digested with BsiHKAI (-); 1 (+) PCR amplified product digested with BsiHKAI restriction endonuclease; A - place of sample application; B, C, D - products of PCR digested products; 3, 8, 12, 13, 16 N/n genotypes with generated fragments of 524, 358, 166 and 135 bp; other samples N/N genotypes with generated 524 and 135 bp fragments from the constant BsiHKAI site.

Figure 2. shows the genotype analysis at nucleotide 1843 in the RYR1 gene of Yugoslav meat swine samples. In this electrophoretogram we did not detect any (n/n) animals, only (N/N) and (N/n).

This mutation was found in 5 major breeds of swine: Landrace, Yorkshire, Duroc, Pietrain and Poland China. Approximately 1 of 5 pigs was a heterozygous carrier of the PSS mutation. The prevalence of the PSS mutation varied among breeds, being by the highest in Pietrain swine. Of the common breeds, Landrace swine were affected at approximately twice the extent of other breeds. The prevelance of the PSS mutation among Hampshire, Yorkshire, Duroc, and Large White swine was similar (O'Brien, et al. 1993).

Deleterious and beneficial effects of the PSS mutation are well known in the swine industry. The mutation is associated with increased muscularity and leanness and increased development of PSE pork, with the net economic effects of increased lean carcass yield being at least partially offset by lower pork quality. In addition, the PSS mutation may have deleterious effects on growth rate and fertility, and in swine that are homozygous for the mutation there is a substantial risk of sudden death caused by PSS. Despite deleterious effects of the PSS mutation on pork quality, however, the strong association of PSS with lean muscle mass has resulted in strong selection pressure by swine breeders in North

America and Europe for swine with the PSS mutation. Such a selection program has been facilitated by the absence of penalties on swine breeders for producing swine with inferior quality meat, such as PSE pork. The best strategy for the swine industry to adopt in selection of breeding stock, with respect to their PSS susceptibility is not entirely clear (O'Brien, et al. 1992). Several factors must be considered. Swine homozygous for the PSS mutation have too high a risk of developing PSS and severe PSE to make them useful as market hogs. They are used more as a source of the PSS mutation for breeding programs and for research purposes. However, using swine heterozygous for the PSS mutation as market hogs may by advantageous. They probably benefit in part from the positive effects of the mutation, apparently have a minimal risk of developing PSS, and may have and acceptable prevelance and severity of PSE, if during marketing and slaughter, the environmental and management factors that provoke or aggravate PSE are minimized.

Our study cónfirmed that the problem of PSS in the swine industry could be attributable to a single point mutation at nt 1843 in the skeletal muscle gene for the calcium release channel of the sarcoplasmic reticulum. As in other swine breeds, in Yugoslav meat swine the mutation at nt 1843 in the RYR1 gene is the mutation responsible for PSS. We did not find genetic heterogeneity in Yugoslav meat swine.

The results of the present study confirms the hypothesis of Fujii et al. (1991) and Otsu et al. (1991), that the cause of MH is a mutation only at one locus, the T-C transition at nt 1843 in the RYR1 gene The availability of a diagnostic test for the mutation provides breeders with the opportunity to eliminate the MH gene from their herds, thereby eliminating the major cause of stress-induced death and PSE pork. On the other hand the MH gene is known for its association with leaness and heavy muscling in swine and breeders might find it advantageous to retain the gene and benefit from heterozygous pigs which are stress resistant but lean.

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PCR GENOTIPIZACIJA GENA ZA RIJENODIN RECEPTOR RYR1 KOD JUGOSLOVENSKE MESNATE SVINJE

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SADRŽAJ

Cilj rada bio je ispitivanje prisutnosti pojedinačne tačkaste mutacije C1843-T1843 u genu za RYR1 kod jugoslovenske mesnate rase svinja kao glavnog uzročnika nastanka maligne hipertermije (stres sindroma). Kalcijum kanal, takođe poznat i kao rijenodin receptor, igra značajnu ulogu prilikom mišićne kontrakcije. Rezultati dobijeni u ovom radu uz pomoć PCR / restrikcione endonukleaze testa (DNK test) potvrđuju hipotezu Fujii i Otsu (1991) da je uzrok nastanka maligne hipertermije mutacija na nukleotidu 1843 u genu za RYR1 skeletne muskulature svinja. Utvrđeno je na ispitivanim uzorcima prisustvo mutacije kod jugoslovenske mesnate rase u heterozigotnom stanju (N/n). Frekvencija alela RYR1(C) iznosi 0.90, a alela RYR1(T) 0.1. Razvoj pouzdanog dijagnostičkog testa za utvrđivanje podložnosti svinja na stres sindrom omogućio je ekspanziju istraživačkog rada i njene primene u selekcionom programu.