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DETECTION OF MUTATION T-C 1843 IN THE RYR 1 GENE, POLYMORPHIC PROTEIN AND ENZYME SYSTEMS ASSOCIATED WITH MALIGNANT HYPERTHERMIA IN YUGOSLAV AUTOCHTONAL PIG BREEDS

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The aim of this study was to investigate the presence of a single point mutation, the T-C transition, at nucleotide (nt) 1843 in the RYR1 gene in the Yugoslav autochtonal pig breeds: Bikovačka mangulica and Moravka. Recently it has been shown that a single point mutation within the porcine skeletal muscle ryanodine receptor 1 gene accounts for all cases of malignant hyperthermia in many breeds of swine. The results of the present study on Yugoslav autochtonal pig breeds using the PCR/restriction endonuclease test (DNA test) confirm the hypothesis of Fujii et al. (1991) that the cause of MH might be mutation at nt 1843 in the RYR1 gene. The frequency of the RYR1 (C) allele is 0.95 and RYR1 (T) is 0.05 Polymorphism of GPI, PGD and PGM was examined using agarose gel electrophoresis at pH 7.2. The GPI system was controlled by two allelic genes A and B with frequencies fo 0.5 and 0.5. The PGD system was controlled by two allelic genes A and B with frequencies of 0.95 and 0.05. The PGM system was also controlled by two allelic genes with frequencies A = 0.4 and B = 0.7. Using two-dimensional agarose at pH 5.4 in the first dimension and horizontal PAGE (pH 9.0) with 4, 8, 12% separation gel was examined the polymorphism of three different α-protease inhibitors, PI1, PO1A, PI2, and three other proteins, PO2, Hpx and Tf. The systems of α-protease inhibitors showed the following polymorphism; the PI1 system was controled by two allelic genes with frequencies A = 0.7, B = 0.3. Five allelic genes were found in the PO1A system, namely S, F, I, D and R, with frequencies of 0.1, 0.45, 0.2, 0.1, 0.15, respectively in seven phenotypes. The PI 1 system was controlled by two allelic genes with frequencies S = 0.7 and I = 0.3. The PO2 system in the examined population did not exhibit polymorphism, only the PO2 F type being observed. The hemopexin system was controlled by three alellic genes (1, 3, 5) with frequencies of 0.4,0.35, 0.35, respectively in five genotypes. The Tf system was controlled by two alellicgenes with frequencies of B allele 0.8 and A 0.2. Swine resistant status is associated with 90% PGD (AA) genotype, 100% PO2 (FF) genotype, 60% P12 (IS) and 60% Tf (BB) genotype. The GPI, PGM, PI1, PO1A

and Hpx systems did not show any significant associations between stress resistant status and high frequencies of certain genotypes.

Key words: DNA test, RYR1, 2D PAGE, MH status, Yugoslav autochtonal pig breeds.

INTRODUCTION

Defining a genetic abnormality at the level of nucleotide sequence is a formidable task. In the past decade, advances in the use of linkage analysis have greatly facilitated the identification of many loci. Using an approach called positional cloning, the location of a genetic locus responsible for a given hereditary syndrome can be identified without prior knowledge of the biochemical or physiological abnormalities underlying the disease process. An abnormality in the intracellular Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum / ryanodine receptor 1 (RYR1) (Fleischer et al., 1985) may account for malignant hypothermia because in skeletal muscle both contraction and metabolism are regulated by the concentration of intracellular Ca^{2+} (Mickelson et al. 1992). Recently, Fujii et al. (1991) cloned and sequenced the cDNA of the porcine skeletal ryanodine receptor (RYR1) gene. A replacement of C (cytosine) at nt 1843 in the normal animal by a T (thymine) in the cDNA in the malignant hyperthermia (MH) susceptible animal leads to an alteration in amino acid sequence from an arginine at position 615 in the normal animal to a cysteine in the MH-susceptible animal. The T mutation, or T allele, was shown to be associated with MH in five different breeds of Canadian pigs, leading Fujii et al. (1991) to the hypothesis that this is indeed the causal mutation in porcine MH, and hence that n and T are synonymous. Other methods for the halothane (HAL) gene detection are based on its linkage to other genes, such as erythrocyte enzyme loci glucosephosphate isomerase (GPI) in selectionist groups known as PHI, 6-phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM), serum protein locus α-1-B glycoprotein (A1BG) or post albumin-2 (PO2) (Gahne & Juneja, 1985; Hojny, et al. 1985; Vogeli, 1989), and serum protein locus α-protease inhibitors (PI1, PI2) (Juneja, et al. 1983). This linkage group is localized on chromosome 6 in swine (Davies, et al. 1988; Harbitz, et. al. 1990). In this study, we have compared results from the DNA-based test for mutation in the RYR1 gene with results from the GPI, PGD, PGM, A1BG, PI1, PI2, Hpx, Tf haplotype analysis used to diagnose MH in two autochtonal pig breeds, Bikovačka mangulica and Moravka. The study confirms the correlation between inheritance of MH and the C-T mutation at nt 1843, supporting the hypothesis that this mutation is may be the causal factor of porcine MH.

MATERIALS AND METHODS

Animals, and blood samples: The analysis was carried on 30 pigs (15 pigs of the Bikovačka mangulica breed, and 15 pigs of the Moravka breed) of various ages, different sex and unknown porcine stress syndrome status (MH status).

Blooe was drawn into tubes containing K_3EDTA or trisodium citrate. The plasma and washed red cells were stored at -20^{0}C until analysis of DNA preparations.

Biochemical polymorphisms: All animals were typed for the plasma proteins PI1, PI2, P2 (A1BG), PO1A, Hpx and Tf, using the two-dimensional electrophoresis method described by Juneja et al. (1983) with some modifications (Vogeli, et al. 1994; Sarač, et al. 1996). GPI, PGD and PGM genotyping was performed according to Gahne and Juneja (1985) with some modifications by Vogeli et al. (1994)

PCR/restriction endonuclease test:

DNA isolation. The methodof DNA isolation based on Higuchi (1989) was used. After thawing the samples,600 μl was transferred to a sterile Eppendorf tube, which contained 0.5 ml TE buffer (Sarač et al. 1996), and the resulting mixture was spun in an Eppendorf centrifuge for 30 sec. The supernatant was then removed and the pellet resuspended in 1.0 ml of TE buffer by vortexing. This step was repeated three times before the pellet was finally resuspended in 0.5 ml of K buffer 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 genotypes 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, in 10 μM Tris-HC1 pH 8.3, with 50 μM KCl and 0.1 mg/ml gelatin to which 0.20 μM of each primer was added. The forward primer was (5'- TCCAGTTGCCACAG-GTCCCTACCA-3') and the reverse primer (5'- ATTCAC-CGGAGTGGAGTCCCTGAG-3) (FL17F2/FL17R2) 2.5 μl of Taq DNA polymerase (Boehringer, Mannheim, Germany) was added in a total reaction volume of 51 ml. 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 the 35 cycles. PCR products were stored at 4^{0}C until RFLP analysis.

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 AspHI restriction enzyme (BioLabs, Beverly, MA, USA) in the buffer supplied at 60^{0}C in total volume of 20 μl. Restriction digests were loaded with 4 μl of 40% sucrose, 0.25% bromophenol blue tracking due, and were run on a 3% agarose gel (type I, Sigma) in Tris-borate/EDTA (TBE) buffer pH 8.0. Samples were electrophoresed at constant voltage (80V) 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 Polaroid MP4 Land camera.

RESULTS AND DISCUSSION

The PCR/restriction endonuclease test (DNA-test or MHS-test) is based on analysis of the region of DNA coding for the abnormal portion of the intracellular calcium release channel im porcine stress syndrome. Examination 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 porcine stres syndrom (PSS) mutation (n/n) or (T/T) and heterozygotes (N/N) or (C/C) (Figure 1.)

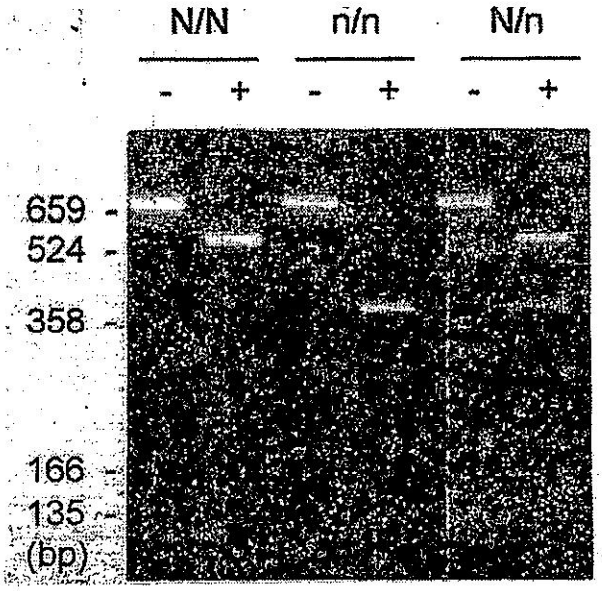

Figure 1. Detection of the C 1843 to T mutation in the RYR1 gene by amplification and subsequent digestion of the amplified product with BsiHKAI. (-) the PCR-amplified product; (+) the same product after digestion, digestion of the N/N genotype generates 524-and 135-bp fragments from the constant BsiHKAI 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 restriction fragment lenght polymorphism 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).

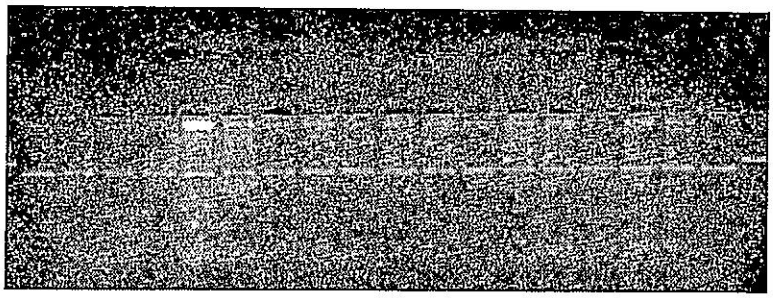

Figure 2. Result of the PCR/restriction endonuclease test for detection of the C-1843-T mutation in the RYR1 gene by amplification and subsequent digestion of the amplified product with BsiHKAI in autochtonal pig breeds Bikovačka mangulica 1-5, and Moravka 6-10, L1 PCR-amplified product non digested with BsiHKAI (-); 1 (+) PCR amplified product digested with BsiHKAI restriction enzyme; A-place of sample application; B, C, D - products of PCR digested products.

Figure 2 shows the genotype analysis at nucleotide 1843 in the RYR1 gene of the autochtonal pig breeds; Bikovačka mangulica and Moravka. In this electrophoretogram we did not detect (n/n) animals, but 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 swine was a heterozygous carrier of the MH mutation. The prevalence of the MH mutation varied among breeds, being by far the highest in Pietrain swine. Of the common breeds, Landrace swine were affected at about twice the extent of other breeds. The prevelance of the MH mutation among Hampshire, Yorkshire, and Duroc breeds was similar (O'Brien, 1993).

Table 1. Distribution of the allele frequencies and genotypes (%) for RYR1 and some polymorphic enzyme and protein systems in two autochtonalpig breeds. (n=30)

System	Allele frequencies	Genotypes of system
GPI ⇨	(A)=0.5 (B)=0.5 [s^2=0.0125; S=0.111]	40% (AA), 40% (AB), 20% (BB)
PGD ⇨	(A)=0.95 (B)=0.05 $\check{S}s^2$=0.00237; S=0.0487]	90% (AA), 10% (AB), /
PGM ⇨	(A)=0.3 (B)=0.7 $\check{S}s^2$=0.0105; S=0.0487]	10% (AA), 40% (AB), 50% (BB)
RYR1 ⇨	(C)=0.95 (T)=0.05 $\check{S}s^2$=0.00237; S=0.0487]	90% (CC), 10% (CT)
PI1 ⇨	(S)=0.7 (F)=0.3 $\check{S}s^2$=0.0105; S=0.1024]	50% (SS), 40% (FS), 10% (FF)
PO2 ⇨	(S)=0.0 (F)=1.0 $\check{S}s^2$=0.00; S=0]	/ / 100% (FF)
PI2 ⇨	(S)=0.7 (I)=0.3 $\check{S}s^2$=0.0105; S=0.1024]	40% (SS), 60% (IS)
PO1A ⇨	(S)=0.1 (F)=0.43 (I)=0.2 (D)=0.1 (R)=0.15	20% (FF), 10% (FI), 20% (DF), 20% (FR), 10% (RS), 10% (II), 10% (IS)
Hpx ⇨	(1)=0.4 (3)=0.25 (5)=0.35	20% (11), 10% (13), 30% (15), 20% (55), 20% (33)
Tf ⇨	(A)=0.2 (B)=0.8 $\check{S}s^2$=0.008, S=0.0894]	60% (BB), 40% (AB)

GPI-glucosephosphate isomerase, PGD-6-phosphogluconate dehydrogenase, PGM-phosphoglucomutase, RYR1-ryanodine receptor 1 skeletal muscle, PI1, PI2, PO1A-systems of α- protease inhibitors, PO2 (A1BG)-postalbumin 2 (α-1-B-glycoprotein), Hpx-hemopexin, Tf-transfennin; s^2 variance, S-standard deviation.

Deleterious and beneficial effects of the MH mutation are well known in the swine industry. The mutation is associated with increased muscularity and leanness and increased development of pale soft exudative (PSE) pork, with the net economic effects of increased lean carcass yield being at least partially offset by decreased pork quality. In addition, the MH 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 porcine stress syndrome. Polymorphisms in functionally important proteins are of particular interest since they could provide information about diseases related to the functional role of the protein in question. The study of polymorphisms is of particular interest in common diseases that appear to have significant genetic factors in their etiology since the genes contributing to disease susceptibility would be expected to be frequent. The polymorphism of GPI, PGD and PGM was examined using agarose gel electrophoresis at pH 7.2. The GPI system was controlled by two allelic genes A and Bwith frequencies of 0.5. The PGD system was controlled by two allelic genes A and B with frequencies of 0.95 and 0.05. The PGM system was also controlled by two allelic genes with frequencies A=0.3 and B=0.7. Using two-dimensional agarose gel at pH 5.4- in the first dimension and horizontal PAGE (pH 9.0) with 4, 8, 12% separation gel the polymorphism of three different α-protease inhibitors (PI1, PO1A, PI2) and three other proteins PO2, Hpx and Tf was examined. The α-protease inhibitors showed the following polymorphism; the PI1 system was controlled by two allelic genes with frequencies A=0.7, B=0.3. Five allelic genes were established in the PO1A system, namely S, F, I, D and R with frequencies of 0.1, 0.45, 0.2, 0.1, 0.15, respectively in seven genotypes. The PI2 system was controlled by two allelic genes with frequencies S=0.7 and I=0.3. In the examined population the PO2 system did not exhibit polymorphism, as only thePO2 F type was found. The hemopexin system was controlled by three allelic genes (1, 3, 5) with frequencies of 0.4, 0.35, 0.35, respectively in five genotypes. The Tf system was controlled by two allelic genes with high frequencies of the B allele (0.8) and A allele (0.2). The frequencies of the RYR1 C allele was 0.95 and the RYR1 T allele 0,05.

In Swedish Landrace pigs, GPI (B) is associated with 93% of the Hal n/n genotype, while PHI (A) is associated with 7% of the Hal n/n genotype.In Danish Landrace pigs, GPI (B) is associated with 100% of the Hal n haplotypes (Nielsen et al. 1984). In Swiss Landrace pigs GPI (B) was also associated with the Hal n haplotypes (Vogeli and Schworer, 1982). Moreover, in the Swedish Yorkshire breed, GPI (B), PO2 (S) and PGD(A) accounted for 99%, 87% and 95% of Hal n haplotypes, respectively. However, in contrast to several other breeds PHI (A) and not PHI (B) appears to be predominantly associated with Hal n/n genotype in the Duroc breed.In tab. 1 we show which protein and enzyme genotypes of Yugoslav autochtonal pig breeds are associated with MH resistant status. A good

exampleis the PGD system, where stress resistant status is associated with 90% (AA) genotype. For the PO2 system, stress resistant animals have 100% (FF) genotype. The PI2 system showed that stress resistance status is associated with 60% PI2(IS) genotype and the transferrin system with 60% (BB) genotype. The GPI, PGM, PI1,PO1A and Hpx systems did not show any significant association between stress resistant (MH-resistant) status and high frequencies of some genotypes.

As in other swine breeds, in the Yugoslav autochtonal pig breeds, Bikovačka mangulica and Moravka, mutation at nt 1843 in the RYR1 gene is mutation responsibility for PSS malignant hyperthermia. We did not find genetic heterogeneity for MH in Yugoslav autochtonal pigs. In these breeds polymorphisms of enzyme and protein systems associated with MH showed many different characteristics from other breeds.

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DETEKCIJA MUTACIJE T-C 1843 U GENU ZA RIJENODIN RECEPOR 1, POLIMORFNIH PROTEINSKIH I ENZIMSKIH SISTEMA UDRUŽENIH SA MALIGNOM HIPERTERNIJOM KOD JUGOSLOVENSKIH AUTOHTONIH RASA SVINJA

M.SARAČ, M. GAGRČIN I S. JOVANOVIĆ

SADRŽAJ

Cilj rada bio je ispitivanje prisustva pojedinačne tačkaste mutacije, tranzicije T-C na nukleotidu 1843 u genu za rijenodin receptor (RYR1) u jugoslovenskim autohtonim rasama svinja, bikovačka mangulica i moravka. Rezultati sadašnjih studija na jugoslovenskim autohtonim rasama svinja, upotrebom PCR/restrikcione endonukleaze testa (DNK test) potvrđuju hipotezu Fujii i Otsu (1991) da je kauzalni uzrok maligne hipertermije mutacija na nukleotidu 1843 u genu za RYR1. Frekvencija (C) alela je 0.95, a RYR1 (T) alela 0.05. Upotrebom agaroze gel elektroforeze pH 7.3 ispitivan je polimorfizam GPI, PGD i PGM. GPI sistem kontrolišu dva alelna gena A i B sa frekvencijama 0.5 i 0.5. PGD sistem kontrolisan je takođe sa dva alelna gena A i B sa frekvencijama 0.95 i 0.05. PGM sistem takođe kontrolišu dva alela gena sa frekvencijama A 0.4 i B 0.7. Upotrebom dvodimenzionalne agaroza gel elektroforeze pH 5.4 za prvu dimenziju i horizontalne poliakrilamid separacione 4,8, 12% gel elektroforeze ispitivan je polimorfizam tri različita a- proteze inhibitor sistema PI1, PO1A, PI2 i tri proteina PO2, Hpx i Tf. Sistem a-proteaze inhibitora pokazivao je sledeći polimorfizam. PI sistem kontrolisan je sa dva alelna gena sa frekvencijama A 0.7 i B 0.3. Pet alelnih gena pronašli smo u PO1A sistemu, S, F, I, D, R sa frekvencijama0.1, 0.45, 0.2, 0.1, 0.15 raspoređenih u sedam genotipova. PI1 sistem kontrolišu dva alelna gena sa frekvencijama S 0.7, i I 0.3. PO2 sistem u ispitivanoj populaciji nije pokazivala polimorfizam. Sistem hemopeksina kontrolišu tri alelnih gena 1, 3, 5, sa frekvencijama 0.4, 0.35, 0,35 u pet genotipova. Sistem transferina kontrolišu dva alelna gena sa frekvencijama A 0.2 i B alela 0.8. Status rezistencije na stress sindrom - malignu hipertermiju reguliše 90% PGD (AA) genotipa, 100% PO2 (FF) genotipa i 60% PI2 (IS genotipa. GPI, PGM, PI1, PO1A, and Hpx sistem nisu pokazali značajnu frekvenciju genotipova koji bi bili udruženi sa statusom rezistencije na malignu hipertermiju.