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DIAGNOSTICATION OF MALIGNANT HYPERTHERMIA IN SWINE

DIAGNOSTICAREA HIPERTERMIEI MALIGNE LA SUINE

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Malignant hyperthermia (MH) causes neurological, liver, and kidney damage and death in humans and major economic losses in the swine industry. A single point mutation in the gene for the skeletal muscle ryanodine receptor (Ryrl) was found to be correlated with MH in major breeds of swine. This mutation generates the production of an abnormal protein which alters the structure and function of the Ca^{2+} *release channel of skeletal muscle sarcoplasmic reticulum (ryanodine receptor). Because the mutation destroys the recognition site for the restriction endonuclease HhaI, it was possible to develop a PCR-RFLP method for MH diagnostication in all swine breeds. Our objective was to identify the normal homozygous, heterozygous carriers and affected homozygous swine for MH trait, using this method. Results suggest that the genetic test will be useful in identifying heterozygous pigs for the MH trait.*

Key words: swine, malignant hyperthermia, PCR-RFLP, diagnostication.

Introduction

Malignant hyperthermia (MH) is an inherited myopathy in which skeletal muscle shrinkage with attendant hypermetabolism and elevation in body temperature, which is triggered by inhalation anaesthetic and skeletal muscle relaxants (O'Brien, 1987) in humans. The syndrome can cause neurological, liver and kidney damage, and is frequently fatal.

MH also occurs in domestic animals such as swine and therefore has worldwide economic consequences (David H. *et al*, 1992). Homozygous animals for the abnormality respond to stress in the same way like heterozygous humans respond to anaesthetics: muscle rigidity, hypermetabolism, and high fever. The stress-induced death of such animals (Porcine Stress Syndrome or PSS) is only one aspect of economic loss due to the syndrome. An equally serious problem is that the same reaction can be triggered when an acute stress is induced before slaughter determining pale, soft, exudative pork in large segments of the carcasses of susceptible animals.

The incidence of MH in swine varies from breed to breed, with a higher incidence in specialized lean breeds of swine. 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. In selecting breeding stocks for such characteristics as large ham conformation, and excessive leanness, selection is inadvertently being marked for the MH gene.

The disease is caused by a single point mutation (C→T) in the gene for the skeletal muscle ryanodine receptor (Ryrl). This mutation generates the production of an abnormal protein (Cys→Arg), which alters the structure and function of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum (Fuji *et al*. 1991). In MH pigs, the Ca^{2+} release channel (Ryr1) releases Ca^{2+} at enhanced rates, and does not close readily. The abnormal channel floods the cell with Ca^{2+} and overpowers the Ca^{2+} pump that ordinarily lowers cytoplasmic Ca^{2+}. Sustained muscle contraction accounts for rigidity, and sustained glycolytic and aerobic metabolism account for the generation of lactic acid, CO_2, and heat and enhanced oxygen uptake (David *et al*. 1992).

Materials and Methods

We used blood samples from 40 pigs (S.C. Romsuintest Periş), preserved in EDTA anticoagulant. The isolation of genomic DNA from fresh blood was performed with Wizard Genomic DNA Extraction Kit (Promega).

PCR was done using a GeneAmp 9700 PCR System (AppliedBiosystems). The reactions were carried out in 25-μl final volume containing PCR Buffer, $MgCl_2$, 200μM dNTP, 0.5μM of each primer (F-CTGGGACATCATCCTTCTGG; R-GGGTTCTAAGCTCTGGGGTC), 0.5 units of AmpliTaq Gold DNA Polymerase, diluted DNA and nuclease-free water. PCRs were performed in 0.2 ml tubes using 40 cycles with denaturation at 95ºC (30s), annealing at 58°C (30s) and extension at 72°C (60s). The first denaturation step was of 10 minutes at 95ºC and the last extension was of 10 minutes at 72°C.

PCR products were detected by electrophoresis in 2% agarose gel stained with ethidium bromide and then digested with restriction endonuclease *HhaI* at 37°C for 3 hours. Restricted products were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide.

For sequencing, we performed the PCR under the same conditions above. The amplified fragments were sequenced by ABI Prism 310 Genetic Analyzer, using the ABI Prism ® BigDye Terminator Cycle Sequencing Reaction Kit after purification with the Wizard System Kit (Promega). The sequences were processed using DNA Sequencing Analysis 5.1 Software (AppliedBiosytems) and the nucleotide sequences were aligned with the BioEdit program and refined manually.

Results and Discussions

Our goal was to develop an easy and efficient method, which can be used to correctly identify the normal, carrier and affected swine for MH and to sequence the fragment of the gene that contains the mutation.

For MH diagnostication we used PCR-RFLP method. The set of primers was designed to amplify only a 274bp fragment from the ryanodine receptor gene containing or not the single point mutation (C→T). This mutation modifies the recognition site for *HhaI* restriction endonuclease (GCG↓C → GTGC). The PCR conditions were selected in such a way that the two primers could amplify the DNA from normal, carrier and MH affected swines.

Successful amplification and digestion with *HhaI* yielded one, two or three alleles with an expected size of 123, 149 and (or) 272bp. The number and the size of alleles depends on whether the animal tested is a heterozygote (carrier) or homozygote (normal or MH affected). For a normal pig (NN) we should obtain two bands at 123 and 149bp since the enzyme cuts the PCR products in two fragments. For homozygous affected pigs (nn) only one band of 272bp was obtained because the mutation modified the restriction site and the enzyme did not cut. Three bands of 123, 149 and 272bp are obtained for the heterozygous carriers (Nn) because one allele is normal and the other one contains the single point mutation (Figure 1).

In our study we identified homozygous normal (NN) and heterozygous (Nn) pigs but we didn't find any homozygous affected (nn) pigs**.**

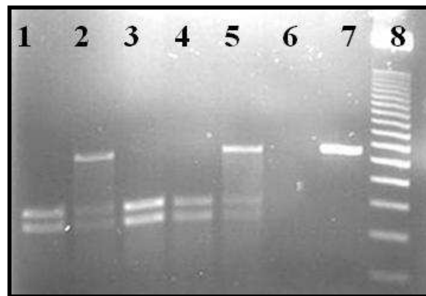

Figure 1: Electrophoresis pattern of ryanodine locus after digestion with *HhaI* enzyme. Line 1, 3, 4 - two fragments of 123 and 149bp indicate normal homozygous pigs (NN); Line 2,5 - three fragments of 123, 149 and 272bp indicate heterozygous pigs (Nn); Line 6 - negative control; Line 7 - uncut PCR product; Line 8 - molecular size marker-50 bp DNA Step Ladder.

To confirm our findings we sequenced the 274bp fragment of the ryanodine receptor gene. Figures 2 and 3 illustrate the profiles of the region from the PCR products that may contain the point mutation and BioEdit fragment alignment of a region of Ryanodin receptor gene and our PCR products, in the case of a homozygous normal (NN) pig.

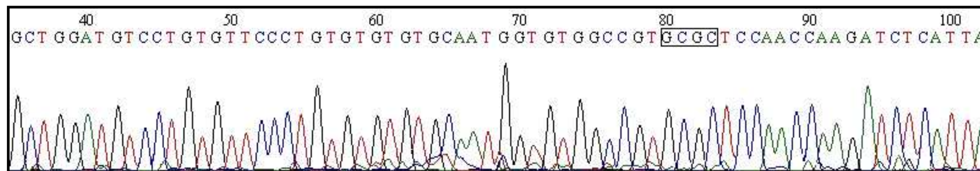

Figure 2: The sequence of the region from the PCR product that may contain the single point mutation inside the recognition site for *HhaI* for a homozygous normal pig (NN).

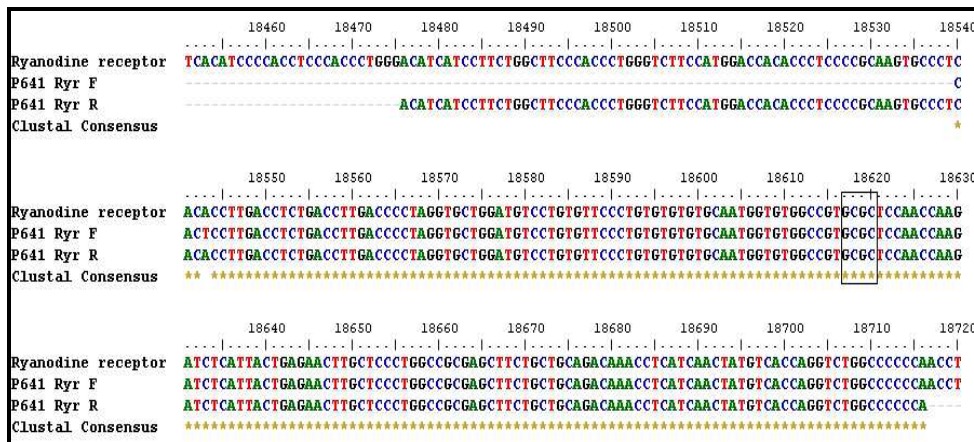

Figure 3: BioEdit fragment alignment of a fragment from ryanodine receptor gene and our PCR product for a homozygous normal pig (NN).

The profile of the region of the PCR products that contains the point mutation is shown in figure 4. The BioEdit fragment alignment of the region of ryanodine receptor gene and our PCR products from heterozygous (Nn) pigs is presented in figure 5. To confirm this heterozygosis we were obliged to clone the two different amplification products (data not shown).

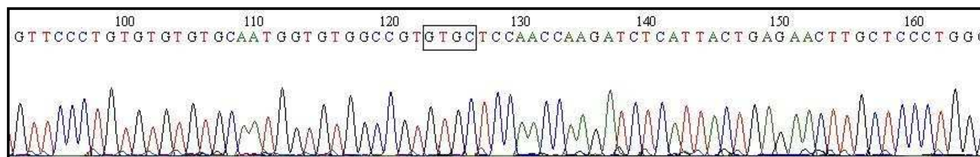

Figure 4: The sequence of the region from the PCR product that may contain the single point mutation inside the recognition site for *HhaI* for a heterozygous carrier pig (Nn).

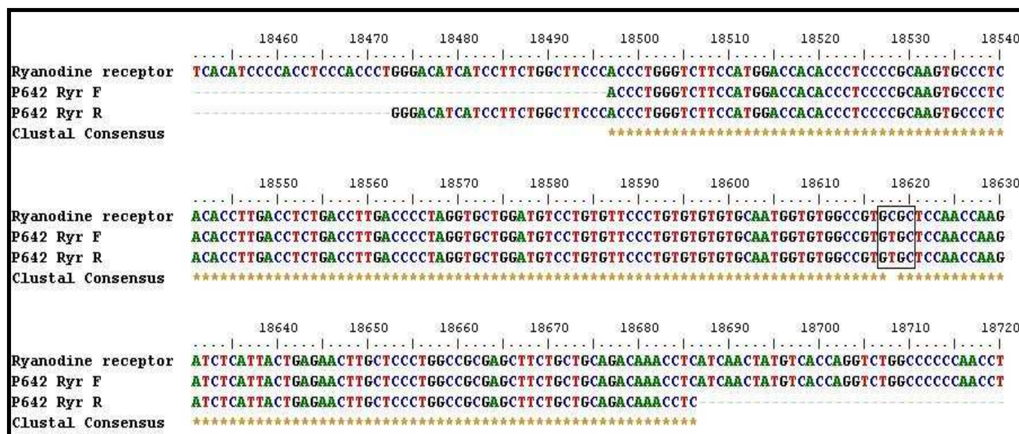

Figure 5: BioEdit fragment alignment of a fragment from ryanodine receptor gene and our PCR product for a heterozygous carrier pig (Nn).

Conclusions

The major focus of this study has been to identify the normal or affected homozygous and heterozygous carriers for MH trait in Romanian swine breeds and to implement a useful diagnosis methodology in order to assist veterinarians and breeders in disease control. Knowledge of molecular alteration associated with MH and with a specific Ryrl genotype has allowed us to develop a simple, accurate, and noninvasive test for the altered Ryrl gene that will make it possible to eliminate this gene from livestock.

Since the heterozygous carries for MH trait don't show any clinical symptoms of the disease it is very difficult to identify these animals without a genetic test. Our results showed that, using this technique, it is easy to identify the heterozygous carriers for MH trait and to exclude these animals for forward breeding programs.

The method presented above is reliable, fast and cost-effective, and can be successfully applied in the wide-scale screening of different pig populations.

Bibliography

1. **David H. M., M. S. Phillips** (1992) - *Malignant hyperthermia.* Science 256: 789-794.

2. **Fill M., Coronado R., Mickelson J.R., Vilven J., Ma J., Jacobson M. A., Louis C. F.** (1990) - *Abnormal ryanodine receptor channels in malignant hyperthermia*. Biophys. J. 50, 471-475.

3. **Fujii J., Otsu K., Zorzato S. de Leon S., Khanna V.K., Weiler J.E., O'Brien P.J., Maclennan D.H.** (1991) - *Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia*. Science 253:448-451.

4. **O'Brien P.J.** (1987) - *Etiopathogenetic defect of malignant hyperthermia: hypersensitive calcium-release channel of skeletal muscle sarcoplasmic reticulum.* Veterinary Research Communications 11: 527-559.

5. **Stanisław K., A. Ruść, K. Wojtasik** (2002) – *Simultaneous identification of ryanodine receptor 1 (RYR1) and estrogen receptor (ESR) genotypes with the multiplex PCR-RFLP method in Polish Large White and Polish Landrace pigs*. J. Appl. Genet. 43(3): 331-335.

6. **Wappler F.** (2001) – M*alignant hyperthermia.* European Journal of Anaesthesiology 18, 632-652.