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Simultaneous Detection of Malignant Hyperthermia and Genetic Predisposition for Improved Litter Size in Pigs by Multiplex PCR-RFLP

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The ryanodine receptor gene (*RYR1*) and the estrogen receptor gene (*ESR*) are the best commercially used markers for predisposition of stress susceptibility (malignant hyperthermia – *MH*) and increased litter size, respectively. A simplified method of simultaneous detection of *MH* and *ESR* genotypes has been developed. The method is based on simultaneous amplification of fragments of two genes by multiplex PCR and subsequent digestion of the products with two restriction enzymes. The PCR and the digestion could be performed in a single tube and all genotypes could be detected by electrophoretic separation on the same agarose gel. Thus, the development of the method can decrease the cost of the sample analysis and increase the speed and efficiency of the analysis. In our study, frequencies of mutated *T* allele of the *RYR1* gene in Large White (LW), White Meaty (WM) and Landrace (L) were 0.11, 0.13, and 0.15, respectively. Frequencies of the preferred *B* allele of the *ESR* gene in the same breeds were 0.35, 0.26, and 0.06, respectively.

Key words: Multiplex PCR-RFLP, malignant hyperthermia, litter size, pig.

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Malignant hyperthermia (*MH*) is a genetic disorder controlled by a recessive gene at a single autosomal locus (GRONERT *et al.* 1988). It can be induced by stress and brings great economic losses in pig industry. FUJII *et al.* (1991) detected a C→T point mutation in the ryanodine receptor gene (*RYR1*) associated with *MH*. Based on the discovery of the causal mutation, DNA tests were elaborated that enable its direct identification on the molecular level (OTSU *et al.* 1992; BRENIG & BREM 1992). *RYR1* is considered as the best commercially used marker of stress susceptibility.

The estrogen receptor (*ESR*) gene has been identified as a major gene for litter size in Meishan and Large White breeds. The preferred *B* allele of the gene (*Pvu II* polymorphism) was associated with an increase of 1.15 and 0.50 pigs per litter for each copy of the allele in first and later parities, respectively, in Meishan crosses (ROTHSCHILD *et al.* 1996). In Large White pigs the effect varied from 0.31 to 0.42 pigs per litter in first and later parities (SHORT *et al.* 1997). *ESR* seems to be the best commercially used marker of increased litter size in pigs.

The aim of the present work was to develop a method of simultaneous detection of malignant hyperthermia (*RYR1* genotypes) and genetic predisposition for increased litter size (*ESR* genotypes) in a single tube by the multiplex PCR-RFLP method.

Material and Methods

We analyzed 155 unrelated sows of Large White (LW), 132 sows of Landrace (L) and 134 sows of White Meaty (WM) breeds from six Slovak breeding farms. In order to verify the reliability of simultaneous detection, the pigs were tested for *RYR1* and *ESR* markers by "classical" methods according to OTSU *et al.* (1991) and SHORT *et al.* (1997), respectively.

Genomic DNA for multiplex PCR-RFLP was isolated from hair roots by silica matrix according

to BAUEROVÁ *et al.* (1999). The multiplex PCR reaction mix (final volume 20 μl) contained 200 μmol/l dNTPs, PCR reaction buffer (20 mmol/l Tris-HCl pH 8.4, 50 mmol/l KCl), 2.0 mmol/l $MgCl_2$, 0.5 μmol/l each of primers RYR-56.1 and RYR-56.2 (BRENIG & BREM, 1992), ESR1 and ESR2 (SHORT *et al.* 1997), 1 U *Taq* DNA polymerase (Life Technologies) and approximately 50 ng of DNA. The PCR was performed in the Primus thermal cycler (MWG-Biotech). After 4 min denaturation at 94°C, the DNA was amplified for 35 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 56°C for 30 s and an extension step at 70°C for 30 s with an additional 8 min extension step at 72°C in the last cycle.

PCR products were digested simultaneously with *Hha* I (New England BioLabs) and *Pvu* II (Advanced Biotechnologies) restriction endonucleases at 37°C overnight. The reactions (final volume 20 μl) contained 1x reaction buffer (Blue buffer, Advanced Biotechnologies), 5 U of each restriction enzyme and 15 μl of PCR product. The digested DNA was electrophoresed on 5% agarose gel (Promega) containing ethidium bromide (0.5 μg/ml).

Frequencies of the *RYR1* and *ESR* genotypes were calculated as a genotype percentage in the population. A calculation of allele frequencies was based on the direct gene count method.

Results and Discussion

Representative results of multiplex PCR-RFLP analysis are shown in Figure 1. The digestion of the multiplex PCR products with *Hha* I and *Pvu* II revealed a variable number of fragments depending on genotypes. Fragments 55 and 65 bp are distinctive for the *B* allele and 120 bp fragment for *A* allele of the *ESR* gene. The presence of 50 and 84 bp fragments is characteristic for the *C* allele and 134 bp fragment for the *T* allele of the *RYR1* gene. The analysis can be carried out simultaneously in a single tube and all genotypes can be detected by electrophoretic separation on the same agarose gel. Thus, simultaneous detection can decrease the cost and increase the speed and efficiency of sample analysis.

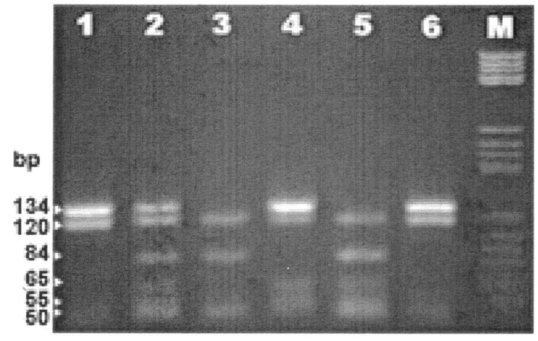

Fig. 1. Representative results of multiplex PCR-RFLP analysis: Lane M – pBR322/Hae III; lane 1 – amplified fragments of the *RYR1* (134 bp) and *ESR* (120 bp) genes; lane 2 – genotype *ABCT* (50, 55, 65, 84, 120 and 134 bp); lane 3 – genotype *AACC* (50, 84 and 120 bp); lane 4 – genotype *ABTT* (55, 65, 120 and 134 bp); lane 5 – genotype *ABCC* (50, 55, 65, 84 and 120 bp); lane 6 – genotype *AATT* (120 and 134 bp).

There is a possibility of replacing the *Hha* I enzyme by izoschisomers *Cfo* I or *Hin*P1 I, however, compatibility of the reaction buffer used must be considered. In any case, detection of 50 and 55 bp bands is not entirely reliable. However, the presence of 84 and 65 bp fragments is sufficient for clear detection of the *C* and *B* alleles, respectively.

The described method was applied in pig testing to verify its reliability. The results obtained by multiplex PCR-RFLP were in 100 % accordance with the ones obtained by "classical" methods. The frequencies of the *RYR1* and *ESR* genotypes and alleles are given in Table 1. Higher frequencies of the *CC* genotype and very low frequencies of the *TT* genotype were found in all tested breeds as a consequence of systematic selection. The frequencies of the mutated *T* allele of the *RYR*1 gene ranged from 0.09 (LW) to 0.16 (L). MATOUŠEK *et al.* (2003) found the frequencies of the *T* allele from 0.03 to 0.05 in LW, KMIEC *et al.* (2000) reported the frequency of 0.22 in L. *ESR* gene analysis showed higher frequencies of *AA* genotypes in WM and L, and *AB* genotypes in LW. The frequencies of the *B* allele obtained with LW (0.33) and L (0.08) pigs could be compared with data published by SHORT *et al.* (1997), who found the frequency of the *B* allele ranging from 0,64 to 0,74. In the Czech Republic, the *B* allele frequency varies from 0.3 to 0.4 in LW and from 0.09 to 0.13 in L (MATOUŠEK *et al.* 2003; VRTKOVÁ & DVOŘÁK,

Table 1

Frequencies of the *RYR1* and *ESR* genotypes and alleles in Large White (LW), White Meaty (WM) and Landrace (L) pigs

Breed	Number of sows	Genotypes (%)				Alleles	
		RYR1	CC	CT	TT	C	T
		ESR	AA	AB	BB	A	B
LW	155	RYR1	81.9	18.1	0	0.91	0.09
		ESR	42.6	48.4	9.0	0.67	0.33
WM	134	RYR1	74.6	25.4	0	0.87	0.13
		ESR	53.0	44.8	2.2	0.75	0.25
L	132	RYR1	70.5	27.3	2.3	0.84	0.16
		ESR	84.1	15.9	0	0.92	0.08

2001). No other investigation of the *ESR* gene in WM breed has been published and therefore no opportunity exists to confirm the present results.

RYR1 and *ESR* markers are utilised in swine breeding programs in many European countries and in the USA. Multiplex PCR-RFLP is an efficient technique for detection of *RYR1* and *ESR* genotypes simultaneously. It is rapid, accurate, gives reproducible results, decreases the cost and increases the speed and efficiency of sample analysis. Therefore, the method could be widely utilized in breeding programs.

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