



## Stress syndrome: Ryanodine receptor (RYR1) gene in malignant hyperthermia in humans and pigs

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Malignant hyperthermia (MH) - stress syndrome is an inherited myopathy in which skeletal muscle contracture with attendant hypermetabolism and elevation in body temperature are triggered by inhalational anesthetic and skeletal muscle relaxants (4, 17). MH has been described in species other than humans and swine; sporadic cases have occurred in racing dogs, cats, race horses, cattle and giraffes (4). While occurrence in these species is relatively rare, appearance in humans exposed to a combination of potent inhalation anesthetics and depolarising skeletal muscle relaxants presents a hazard to those genetically predisposed to MH (4). The commonly used combination of halothane and succinylcholine can trigger skeletal muscle rigidity, accompanied by hypermetabolism, high fever and cellular ion imbalances in susceptible individuals. If therapy is not immediately initiated, the patient may die within minutes from ventricular fibrillation, within hours from pulmonary edema or coagulopathy, or within days from neurological damage or obstructive renal failure. The human syndrome was characterised in the mid 1950s, following the extensive use of halothane and suxamethonium. In certain families the combination was fatal to genetically predisposed individuals (5). Syndrome appears in about 1 in 15,000 administrations of anesthetics of children and 1 in 50,000 adult anesthetics.

The discovery of early symptoms of a MH episode and prompt termination of anesthetic process followed by and infusion of antidote (Dantrolene) in recent years has lowered the death rate for such episode from over 80% to less than 7%.

Since MH has not posed a serious threat to susceptible individuals in their daily lives or in any way incapacitated most of them, a major goal of MH research has been directed to identification of MH-susceptible individuals prior to administration of anesthetics. For these purposes *in vitro* diagnostic tests for MH susceptibility were developed (6). The *in vitro* caffeine-halothane diagnostic tests, North American (15) or European (8), are based on the use of a fibre from a muscle biopsy, that attached to a force displacement transducer and then exposed to single or incremental doses of caffeine or halothane, or to caffeine in the presence of halothane. Muscle fibres from normal and MH-susceptible individuals differ in their limits of induced tension, or in their sensitivity to halothane. Diagnostic testing has confirmed the autosomal dominance of inheritance of the MH gene.

MH in swine has worldwide economic consequences (1). Pigs are seldom exposed to anesthesia, but animals homozygous for the abnormality respond to stress in the same way that heterozygous humans respond to anesthetics, i.e. with muscle rigidity, hypermetabolism and high fever. The stress-induced death of such animals (porcine stress syndrome or PSS) is one aspect of economic loss due to the syndrome. An equally serious problem is that the same reaction can be activated when a hog experiences acute stress prior to slaughter, resulting in pale, soft, exudative (PSE) pork in large segments of the carcasses of susceptible animals. The incidence of MH (PSS) in swine varies from breed to breed and from country to country. A selected study by Canadians has shown that over 10% of commercial animals were heterozygous carriers for the syndrome, while about 1.5% were homozygous (19). Up to 12% of homozygotes died of PSS and up to 50% of carcasses of homozygotes were devaluated through PSE. The gene responsible for such deleterious consequences may also have a beneficial economic effects in pig breeding. Such effects of the MH gene are associated with leanness and with muscle hypertrophy, appearing to add 2-3% to lean dressed carcass weight (21). In selected breeding stock for characteristics such as large ham conformation, large loin eye area, and excessive leanness, selection is inadvertently being made for the MH gene.

The deleterious effects of the MH gene were first reported in 1953 (4) providing the stimulus for the elimination of it from the breeding stock. These efforts have been frustrated by the continued selection for desirable meat characteristics and secondly, until now, by impossibility to detect heterozygous carriers of the MH gene with the accuracy required to eliminate the gene within the acceptable limits of cost. The ultimate result of this is the incidence of the MH gene stabilises in most lean, heavily muscled breeds of swine.

### Physiological basis of MH

Rises in extracellular fluid  $Ca^{2+}$  play a sufficiently important role in the pathogenesis of MH. The muscle rigidity associated with MH most likely results from abnormalities in  $Ca^{2+}$  regulation in skeletal muscle (4). The primary biochemical abnormalities associated with the syndrome occur in skeletal muscle whose contraction,

relaxation, and energy metabolism are regulated by  $\text{Ca}^{2+}$ . The major regulator of  $\text{Ca}^{2+}$  concentration in muscle is the sarcoplasmic reticulum (14).  $\text{Ca}^{2+}$  is pumped into the sarcoplasmic reticulum by a  $\text{Ca}^{2+}$  ATP-ase ( $\text{Ca}^{2+}$  pump) to start relaxation, stored within the lumen of junctional terminal cisternae, and then released through a  $\text{Ca}^{2+}$  release channel to initiate muscle contraction. Glycolytic and aerobic metabolism proceed only rapidly enough to maintain the energy balance of the cell. The  $\text{Ca}^{2+}$  release channel can be regulated (stimulated) by  $\text{Ca}^{2+}$  itself, ATP,  $\text{Mg}^{2+}$ , and calmoduline. Under normal physiological conditions its open time is short. However, during a MH episode  $\text{Ca}^{2+}$  release channel is sensitive to a lower concentrations of stimulators of opening, releases  $\text{Ca}^{2+}$  at enhanced rates and do not close readily. The abnormal channel floods the cell with  $\text{Ca}^{2+}$  and overpowers the  $\text{Ca}^{2+}$  pump that ordinarily lowers cytoplasmic  $\text{Ca}^{2+}$ . Sustained muscle contraction accounts for rigidity and permanent glycolytic and aerobic metabolism account for the generation of lactic acid,  $\text{CO}_2$  and heat and enhanced oxygen uptake. Damage to cell membranes and imbalances of ion transport can account for the life threatening systemic problems that appear during a MH episode.

The  $\text{Ca}^{2+}$  release channel of the sarcoplasmic reticulum has been characterised in fibres (7, 16) and as a single channel after its incorporation into lipid bilayers (12, 16). The protein was identified and isolated through its high affinity binding to a plant alkaloid, ryanodine, which modulates channel opening. The name ryanodine receptor has been applied to the two isoforms of the  $\text{Ca}^{2+}$  release channel, that seem to be the only cellular sites of ryanodine binding. cDNA encoding human (22) and porcine (9) skeletal muscle isoforms (RYR1) were cloned.

The  $\text{Ca}^{2+}$  release channel is a tetrameric complex made from identical subunits of 565 kD (16). The channel consists of the transmembrane sequences and cytoplasmic subunit bridging the gap between the sarcoplasmic reticulum and the transverse tubule (20). Opening of the  $\text{Ca}^{2+}$  channel in the muscle depends on the presence of  $\text{Ca}^{2+}$  and ATP, and  $\text{Mg}^{2+}$  and calmoduline (16). While  $\text{Ca}^{2+}$  and ATP act synergistically to open the channel,  $\text{Mg}^{2+}$  and calmoduline inhibit channel opening.

The release of  $\text{Ca}^{2+}$  is the end result of a cascade of events which include depolarisation of nerve, muscle and transverse tubular membrane and the opening of  $\text{Ca}^{2+}$  release channel.  $\text{Ca}^{2+}$  pumps, exchangers in the plasma membrane and carriers in the mitochondrial membrane are also regulated by  $\text{Ca}^{2+}$  and contribute to  $\text{Ca}^{2+}$  regulation within muscle cells (16). An abnormality in regulation of  $\text{Ca}^{2+}$  within skeletal muscle could account for all the symptoms of MH caused by continued presence of  $\text{Ca}^{2+}$  within the cell, exchanged glycolytic and aerobic metabolism which deplete ATP, glucose and oxygen causing the excess production of  $\text{CO}_2$ , lactic acid and heat.

Irregularities in regulation of the intracellular concentrations of  $\text{Ca}^{2+}$  that lead to MH might result from mutation in genes regulating the  $\text{Ca}^{2+}$  pump, the  $\text{Ca}^{2+}$  re-

lease channel, or other proteins responsible for the depolarisation of the skeletal muscle fibre.

### Genes and the genetic basis of MH

$\text{Ca}^{2+}$  release channels are encoded by two genes of the sarcoplasmic reticulum: RYR1 encodes the  $\text{Ca}^{2+}$  release channel of both slow- and fast-contracting skeletal muscle, while RYR2 encodes a  $\text{Ca}^{2+}$  release channel that exists in the cardiac muscle and brain (16). RYR1 is located on human chromosome 19 q 13.1 in a linkage group containing human GPI (16), and RYR2 is located on human chromosome 1 (16). A linkage group for the porcine MH (HAL) gene, localised near the centromere of pig chromosome 6 (11) includes GPI and PGD, suggesting that parts of these regions of human chromosome 19 and pig chromosome 6 are homologous. From these it is obvious that RYR1 is a candidate gene for MH in both humans and pigs. MH in humans is, at worst, a subclinical myopathy for heterozygous human carriers of abnormal genes, while in swine it affects homozygous recessive animals.

Linkage between RYR1 and human MH has not been found in all human families studied (13, 16). There is evidence that individuals with central core disease, King-Domborough syndrome, muscular dystrophy, and other myopathies (16) are at risk for anesthetic-induced MH episodes. Abnormalities in cellular  $\text{Ca}^{2+}$  regulation are probably secondary events in such myopathies. If these abnormalities were provoked pharmacologically to the point where excess  $\text{Ca}^{2+}$  remained in the muscle, MH episode could result. It is likely that abnormalities in proteins other than  $\text{Ca}^{2+}$  release channel, leading to poor  $\text{Ca}^{2+}$  regulation within the cell, may eventually be shown to give rise to other form of MH susceptibility in those families in which MH can not be linked to RYR1.

Linkage between RYR1 and MH made it imperative to initiate a search for sequence differences in the RYR1 gene between MH and normal individuals. In a comparison of the RYR1 cDNA sequences of MH (Pietrain) and normal (Yorkshire) pigs, only a single deduced amino acid sequence change was found (9): substitution of T for C at nucleotide 1843 leads to the substitution of Cys for Arg 615 in the deduced amino acid sequence. Association between inheritance of this mutation and MH was shown in some 80 animals from five different breeds. In analysis of linkage in 376 British Landrace swine, including 338 representing informative meioses, the co-segregation of the MH phenotype with the Cys-for-Arg 615 substitution was complete (18).

The appearance of an identical mutation in five lean, heavily muscled pig breeds suggested that the mutation in all breeds appeared in a founder animal. Haplotype and genotype analysis using three markers covering about 150 kb within the RYR1 gene showed the inheritance of the same haplotype in every homozygous MH animal examined and the potential for the inheritance of the chromosome with this haplotype in every heterozygous animal (9). Leanness and the heavy muscling may be manifestation of the gene (21) and

these traits are readily selected by swine breeders. There has been a physiological rationale for the contributions of the gene to leanness and heavy muscling. An abnormal  $Ca^{2+}$  release channel could stimulate spontaneous muscle contraction in these inactive animals. The continued toning of such muscles would result in muscle hypertrophy and because of greater energy utilisation, in the limitation of fat deposition. Alternatively, the MH mutation could be very closely linked to gene(s) responsible for the desirable carcass traits and maintained by disequilibrium in the linkage between the genes.

Searching for the corresponding mutation in 35 human MH families, the equivalent mutation was found in a single family of five members in which the mutation segregated with MH (10). Co-segregation of the mutation with MH in swine combined with the appearance of the corresponding mutation across a species barrier between swine and humans strongly supports the proposal that this mutation is a cause of MH susceptibility in most pigs and at least some human families.

#### Our studies on MH in swine

We have used the DNA-based test (16) for the mutation associated with PSS to determine the prevalence of the PSS mutation in various breeds of swine in Croatia, and studied the influence of the MH genotypes on meat quality. For this trait animals were selected by PCR testing (DNA testing) and by the halothane challenge test, respectively (2, 3). Genomic testing was carried out by the method described elsewhere (12). Briefly, blood samples (1 ml) from pigs of various breeds were collected. Genomic DNA was isolated from 0.5 ml blood sample, which was added to 50 ml of TE buffer pH 8.0 and spun for 10 sec at 1300 g. Then the pellet was washed 3 times with TE, and resuspended in 100 ml of Perkin Elmer Cetus gelatin-free buffer supplemented with 0.5% Tween 20 and 10 mg/ml Proteinase K. The mixture was incubated at 60°C for 30 minutes and reaction terminated by immersion in boiling water for 10 min. This mixture was used to isolate 659 bp fragment by PCR amplification. The reaction was done in Perkin Elmer Cetus PCR buffer containing 1 mM  $MgCl_2$ . Genomic DNA (200 - 400 ng) and 100 ng of each of primers were added to the mixture and program carried at 94°C for 1 min, 53°C for 2 min, and 72°C for 3 min. The forward primer was 5' - TCCAGTTTGCCACAGGTCCATACCA - 3' and the reverse primer was 5' - ATTCACCGGAGTGGAGTCTCTGAG - 3'. The amplified sequence was cut with HgiAI to detect the presence of C/T mutation and restriction fragments were resolved in 3% agarose gel. In halothane challenge test pigs were forced to inhale halothane gas.

From a total of 873 pigs the lowest percentage of heterozygous carrier of the PSS mutation was found in Hampshire swine, being the highest in Hypor (commercial synthetic line) (Table 1). Out of the common breeds Landrace (L) swine were affected much higher than Yorkshire (Y) and the crossbreed between LxY, respectively. Occurrence of homozygotes for PSS was rare,

observed in only 2 out of 195 Landrace, 2 out of 204 Yorkshire, and 4 out of 361 their F1 hybrid.

Genomic testing for detecting pigs that carry a genetic predisposition to the PSS has shown to be a powerful tool which, unlike halothane screening, allows to

TABLE 1

Prevalence of the porcine stress syndrome (PSS) mutation in breeding stock from various breeds of swine in Croatia.

Breed	No. of swine tested	No. and (%) of heterozygous swine	No. and (%) of PSS swine	G <sup>†</sup>
Hypor (Hy)	21	8 (38.1)	1 (4.8)	0.238
Landrace (La)	195	57 (29.2)	2 (1.0)	0.156
Yorkshire (Yo)	204	37 (18.1)	2 (1.0)	0.100
Hampshire (Ha)	19	1 (5.3)	1 (5.2)	0.079
(LaxHy)F1	75	24 (32.0)	1 (1.3)	0.173
(LaxYo)F1	361	54 (15.0)	4 (1.1)	0.086

<sup>†</sup>PSS gene frequency = [(No. of PSS homozygotes x 2) + No. of heterozygotes] / No. of tested swine

TABLE 2

Effectiveness of DNA testing in detection of heterozygous genotype among halothane crossbred pigs<sup>a</sup>.

Number of animals tested	No and (%) of phenotypes from halothane test		No and (%) of genotypes from DNA testing of Hal pig		
	Hal <sup>+</sup>	Hal	NN	Nn	nn
300	31 (10.33)	269 (89.67)	225 (84.12)	42 (15.61)	2 (0.74)

<sup>a</sup>(Large white x German Landrace)F<sub>1</sub> x Belgian Landrace

identify a combination of normal and stress genes in pigs genetic makeup. Table 2 shows the effectiveness of such testing in detection of heterozygous genotype among halothane negative crossbred pigs. As shown, halothane challenge tested pigs may be classified as halothane positive (10.33%) or halothane negative (89.67%). However, genomic testing revealed that among Hal<sup>-</sup> animals 15.61 percentage belonged to heterozygous nonreactors (Nn), and even two animals have shown to be recessive homozygotes (nn). It is likely that the presence of three genotypes related to PSS may influence animal market value.

According to this research, the effect of the presence of the gene inside a pig appears to change according to the weight at the moment of slaughter. Table 3 shows that meat traits from animals of various genotypes at different slaughter weights vary a lot among the groups. The heterozygous pigs having Nn genotype showed the most surprising observations concerning their meat quality characteristics: at a weight of 80 kg, the meat quality of Nn genotypes was comparable to that of NN animals not carrying the mutation in RYR1 gene. Around 105 kg liveweight colour and water-holding capacity of meat was

TABLE 3  
Influence of different genotypes on meat quality.

TRAIT	Meat quality of different genotypes estimated at slaughtered weight of					
	105 kg			130 kg		
	NN	Nn	nn	NN	Nn	nn
Colour	47.2 <sup>a</sup>	46.9	46.8	48.1	46.1	45.9
Water-holding capacity (drop loss)	6.25	6.95	7.3	6.52	7.81	7.75
pH <sub>45</sub>	6.57	6.25	6.24	6.58	7.9	8.1
pH <sub>u</sub>	5.78	5.45	5.48	5.72	6.2	6.9

<sup>a</sup>Absolute numbers without standard deviations

No differences in meat quality were found between animals of 80 kg and 105 kg (80 kg omitted)

pretty much the same in heterozygous (Nn) and homozygous recessive (nn) pigs. However, at 130 kg the meat of Nn resembled closely to that of PSS-sensitive stock for the normal quality parameters such as colour and drop loss.

This study confirmed application of a method for large-scale, rapid, accurate, DNA-based laboratory diagnosis of the mutation associated with susceptibility to PSS. Prevalence of the PSS mutation varied markedly with breed. Of the common breeds of swine, Landrace (Gf 0.156) by affection to PSS followed Hypor-the synthetic line of swine (Gf 0.238). With the development of the PSS test described here, the mutation of RYR1 gene can be used in selection of swine for increased muscularity and leanness. However, it is likely that each breed should be tested for live performance and carcass characteristic, comparing them among normal, heterozygous, and homozygous for PSS mutation to get the economic impact of the beneficial effects.

There has been a common opinion that the gene for PSS should be eliminated only from one parental line, e.g. from the mother, but retained in the other parent because of its association with better carcass yield and leanness. Studies concerning the pigs slaughtered at around 100 kg have suggested that this approach would carry no more than a low risk of perpetuating quality defects in the progeny grown for meat. The data presented have shown that the elimination of both carriers of recessive homozygotes (nn) and heterozygotes (Nn) of mutated RYR1 gene among the progeny grown for meat should be of great importance for the meat quality of pigs at higher market weights. The molecular method described here offers a new tool for quick and inexpensive detection of all carriers of mutated RYR1 gene in pigs.

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**ABSTRACT*****Stress syndrome: Ryanodine receptor (RYR1) gene in malignant hyperthermia in humans and pigs***

*Anesthesia can induce skeletal muscle rigidity, hypermetabolism and high fever in men genetically predisposed to malignant hyperthermia; such episodes can lead to tissue damage and sudden death, if not immediately reversed. In pigs with reciprocal condition stress can induce death or lead to devalued meat products. Muscle contraction is controlled by sarcoplasmic Ca<sup>2+</sup>, and the abnormalities mentioned above can reside in the skeletal muscle Ca<sup>2+</sup> release channel gene RYR1. It has been reported that a single RYR1 mutation causes malignant hyperthermia in all breeds of pigs and in some human families. The substitution of Cys for Arg 615 has been found to be the cause of malignant hyperthermia in all breeds of swine; the appearance of the corresponding mutation, Cys for Arg 614 in a few human families also cosegregates with malignant hyperthermia. However, linkage of malignant hyperthermia to RYR1 gene is not observed in all human families with malignant hyperthermia. The results described in this paper present the prevalence of the porcine stress syndrome mutation in breeds of pigs in Croatia, as well as the influence of malignant hyperthermia genotype on meat quality of pigs.*

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