Strategies for Diagnosis of Diseases in Pigs Using Molecular Markers Review

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Abstract
In certain breeds of pigs were identified the porcine stress syndrome (PSS) that determine the appearance of some carcasses inadequate for processing. Because PSS can be triggered by halothane, the gene responsible for the syndrome is often referred to as the "halothane gene" (Hal). The metabolic and physiological changes that occur in halothane positive pigs (homozygous recessive "nn") are produced by gene located in the Hal locus responsible for synthesis of the Ca^{2+} channel receptor in the sarcoplasmatic reticulum of skeletal muscle fiber, called Ryanodin receptor (RYR1). The Ryanodin receptor locus in pig populations is important not only for economic losses caused by homozygous recessive pigs, but also for the fact that this locus is linked to other quantitatively additive genes which determine muscle hypertrophy. The unconscious promotion of carrier and positive animals in the herd, due to the intention to produce a new generation characterized by muscle hypertrophy, led to the automatic increase of frequency of the mutant allele "n" in the pig population. PCR assay of porcine genetic background can help determine with great precision the frequency of specific alleles in the RYR1 locus, offering the specialists a possibility of reducing the recessive allele frequency through selection. Therefore, the present review underlines the necessity of implementing such testing programs in Romania in order to prevent the risk of dissemination of PSS in pigs.

Keywords: HAL gene, malignant hyperthermia, pork meat quality, PSE, PSS, RYR1.

Introduction

In recent years, increasing the quality of pig meat has become the subject of much research, all aimed at obtaining high quality meat, in order to meet the ever increasing demands of consumers, who aim to eat a nutritious, tasty and healthy meat [1]. Meat quality is a complex characteristic that is difficult to quantify [2]. The traits which contribute to the pig meat quality are different depending on the meat producer, meat processor or consumer, but often they include carcass weight, fat thickness, carcass lean meat percentage, meat marbling, loin eye area, pH, color, water-holding capacity, juiciness and flavor of meat.

The characters which determine the pig meat quality are controlled by many genes located in different loci. Thus, besides other factors, the quality of pork is influenced by the porcine stress syndrome (PSS), a complex genetic disease that is the clinical manifestation of a triangle of conditions; the two other are the malignant hyperthermia (MH) and the pale, soft, exudative muscle (PSE) [3].

Pork quality and porcine stress syndrome

The most significant issues that negatively affect the quality of pig meat is the stress syndrome [4]. The PSS condition was first described by Topel et al., who noted that physically stressed, susceptible

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pigs would collapse in a shock-like state and die [5]. PSS leads to carcasses containing pale, soft, exudative meat, faded, soft texture, but less tender and exudative, with a low water retention capacity and a rapid decrease of pH, combined with a high temperature of the meat which leads to a denatured sarcoplasmatic protein, making the meat unfit for processing.

PSS or malignant hyperthermia (MH) is a hypermetabolic syndrome involving skeletal muscle characterized by hyperthermia, tachycardia, tachypnea, increased oxygen consumption, cyanosis, cardiac dysrhythmias, metabolic acidosis, respiratory acidosis, muscle rigidity, unstable arterial blood pressure, and death [6]. The primary features of MH are a direct consequence of loss of skeletal muscle cell calcium homeostasis with a resulting increased intracellular calcium ion concentration [7]. In pigs the syndrome is autosomal recessive, while in humans inheritance has an autosomal dominant pattern [8]. Although MH was initially recognized as a fatal syndrome in humans, the term describing its occurrence in swine is porcine stress syndrome (PSS) death. MH affects humans, pig breeds, dogs, horses, and probably other animals. In pigs, stress syndromes have been reported for several breeds but the incidence is higher in lean, heavily muscled breeds, such as Pietrain, Poland China, Landrace, Duroc, and Large White [6]. PSS was discovery in Danish Landrace and other pig breeds selected for muscling, a condition in which stressed pigs develop "pale, soft, exudative" flesh (a manifestation of the effects of malignant hyperthermia) rendering their meat less marketable after slaughter. Pig farmers use halothane cones in swine yards to expose piglets to halothane, a fluorinated hydrocarbon anaesthetic. Therefore, testing with halothane can be used as a screening method. Those that die were MH-susceptible, thus saving the farmer the expense of raising a pig whose meat he would not be able to market. This also reduced the use of breeding stocks carrying the genes for PSS [9].

Etiology

This syndrome has been associated with a dysfunction of intracellular calcium homeostasis in skeletal muscle [10-11]. In swine, susceptibility to the syndrome is caused by a single point mutation (Arg^{615} to Cys^{615}) in the sarcoplasmic reticulum (SR) Ca^{2+} release channel (ryanodine receptor - RyR1) [12]. Loss in regulation of muscle cell Ca^{2+} is believed to be the primary etiologic event for induction of MH. It is consistently triggered in genetically susceptible animals by excitement, apprehension, exercise, or environmental stress. Expression of PSS results on account of physical stressors such as exercise, fighting, marketing, vaccination, castration, estrus, mating, parturition and hot weather. It has also been noted that volatile anesthetics such as halothane can bring about the onset of PSS [13]. This is particularly true in pigs, but exercise-induced MH has also been reported in dogs, suggesting the existence of canine stress syndrome. Exposure to volatile anesthetics or depolarizing neuromuscular blocking agents will consistently trigger MH in susceptible animals. Subsequent to the initial challenge or stress, the hypersensitive ryanodine receptor floods the myoplasm of skeletal muscle with Ca^{2+}. Muscle contracture and hypermetabolism develop rapidly as a direct result of this uncontrolled and sustained increase in myoplasmic Ca^{2+}. ATP in skeletal muscle is depleted as the energy requirements for contracture exceed the supply. Increased aerobic and anaerobic metabolism results in excessive CO_2 and lactic acid production, while thermogenesis and peripheral vasoconstriction increase core body temperature. As the MH episode progresses, the combination of increased temperature, acidosis, and ATP depletion leads to rhabdomyolysis. Myoplasmic enzymes and electrolytes are released from the cells, and additional Ca^{2+} enters the myoplasm. Contracture and its subsequent energy requirements are further enhanced and eventually, due to temperature and pH changes, contracture proceeds independently of myoplasmic Ca^{2+} levels. Death occurs due to an increase in serum K^+, which causes cardiac dysrhythmia and arrest [6].

Clinical signs

Signs of MH include muscle stiffness or fasciculations that progress to muscle rigor, increase in end-tidal carbon dioxide and decrease in arterial oxygen saturation, tachycardia and dysrhythmias, and tachypnea. Ventricular tachycardia develops early and continues until serum K^+ reaches cardiotoxic levels. Blanching and erythema followed by blotchy cyanosis are seen in the skin of light-colored animals. Another sign is hyperthermia, when body temperatures can rapidly increase up to 45°C antemortem. In the affected animals muscle mass is pale and soft and

appears exudative or wet. Therefore, pale, soft exudative pork syndrome is linked to MH [6].

All these changes are the result of rapid accumulation of Ca^{2+} ions in the cytoplasm of skeletal muscle that causes spontaneous muscle contractions, accumulation of lactate dehydrogenase and creatinine phosphokinase and also the increase of glycolysis with production of an excess of water, carbon dioxide, heat and excessive oxygen consumption.

Malignant hyperthermia in humans

In humans the disease is a very serious problem. According to data reported by MHAUS (Malignant Hyperthermia Association in the United States) between 1981-1983, in the United States, it occurred with an incidence of 1/100.000 in adults and 1/8.500 in childrens, often leading to fatal reactions. Recent data indicate a different distribution, depending on age and sex of the population. In the United States the laboratory test applied to detect persons at risk of MH is the simulation in vitro of 10-15 g of muscle contraction as a response to treatment with halothane (3%) and high doses of caffeine (2 mM). Diagnostic accuracy obtained by CHCT (Caffeine /Halothane Contracture Test) has 97% sensitivity and 78% specificity [14-15].

Genetic analysis has shown that the candidate gene responsible for the manifestation of the human MH syndrome is the RYR1gene, located on chromosome 19q 13.1 [16]. This gene has 18 mutations, it is expressed as autosomal dominant and is associated with the emergence of MH syndrome present in 45% of cases. It is believed that the effect of this gene is reinforced by other groups of genes present in some additional chromosomal loci: 1, 3, 7 and possibly 17.

Pigs manifesting stress syndrome are an ideal experimental model for studying MH genetics in humans.

Malignant hyperthermia in pigs

At the origin of biochemical and physiological changes to the porcine stress syndrome is a recessive mutation of the gene from "Hal" locus, called Halothane sensitive locus. The researches of Fujii et al. (1991) have shown that metabolic and physiological changes manifested in halothane positive pigs (recessive homozygous „nn") are produced by a gene located in Hal locus which is responsible for the synthesis of the Ca^{2+} channel receptor of sarcoplasmatic reticulum of skeletal muscle fiber, called Ryanodin receptor (RYR1) [9].

This protein belongs to a family of Ca^{2+} channel receptors, which are present in three structural forms: RYR1, 2 and 3, each encoded by different genes. All three protein isoforms are composed of identical subunits with sizes of 565 kDa. They open and close Ca^{2+} channels in the skeletal muscle fiber. It is interesting that in muscle fibers were identified only RYR1 and RYR2 subtypes. In other non-muscular tissues (brain) was located only the RYR3 structural form. Specific skeletal muscle isoform proteins have a tetrameric structure composed of four subunits that have each a hydrophobic region located at the membrane level and a hydrophilic region that forms the cytoplasmic domain. This structure forms a junction, a channel of release that ensures the dissemination of Ca^{2+} ions through the receiver. Opening and closing the receiver is the result of conformational changes in the cytoplasmic domain that binds two types of regulatory proteins: Imunophiline ($FKBP_{12}$) and Calmodulin (CaM) [17]. The research conducted in this area has shown that the immunoprecipitation or depletion of the regulatory protein $FKBP_{12}$ increases Ca^{2+} influx into the cell. In pigs RYR1 locus was identified on chromosome 6^{p11}-^{q21}[18]. Studies of Fujii et al. (1991) showed that the punctiform mutation of nucleotide cytosine (C) into the nucleotide thymine (T), in the normal gene, that occurred through the transition in position 1843 on DNA, led to the change of coresponding codon, causing the synthesis of a RYR1 mutant receptor protein that has included in position 615 instead of arginine, the aminoacid cysteine. The consequence of this fact is calcium channel opening excitation and the inhibition of closure, with dramatic consequences on the skeletal muscle fiber physiology [9].

Knowing the structure of this locus in pig populations is important not only for economic losses represented by recessive homozygous pigs, by producing low-quality meat (PSE) or death, but also because it is linked to „other quantitatively additive genes" that lead to *muscle hypertrophy*. Homozygous recessive pigs (halothane positive-nn) and the heterozygous pigs (Nn) have a very appealing conformation for the specialists who make the selection. The unconscious promotion of these animals (nn and Nn) in the herd, with the intention to produce a new generation, led to the

automatic increase of frequency of the mutant gene "n" in many pig populations. Experiments organized at the Swine Research Center at the University of Illinois measured the effects of recessive homozygous genotype (nn) and the carrier heterozygous genotype (Nn) on piglet growth rate, carcass quality and meat quality. Studies have used DNA HAL-1843 test (developed by Fujii et al.1991) [9] in the offspring of three lines: A-halothane positive (nn), C-halothane negative (NN) and B - descendants that carried the mutant gene (Nn), resulting from the cross of A and C lines, which are used by the Pig Improvement Company, USA, to obtain commercial lines. Evaluation of growth rate was based on average daily gain and specific consumption and carcass quality evaluation was made based on indices of cutting, fat content and carcass lean meat percentage. Meat quality was determined by the pH measured immediately after slaughter, color, juiciness, firmness, marbling and loss of water. Research results showed that the halothane gene has no significant effect on growth rate, although the progeny from line A had consumed 400 g less food per day and achieved a daily average gain smaller with 200 g per day, requiring an additional 14 days to achieve delivery weight. The effect of gene on carcass and meat quality was manifested by significant differences recorded in the percentage of meat in carcass, that can reach up to 2.3%. In the „n" allele carrier animals, pH and juiciness were lower. For characters such as juiciness and tenderness, that give palatability to the meat, there were also found differences between genotypes. Animals with PSE syndrome obtained much lower values for these two characteristics. Subsequent studies have shown that high glycolitic potential and high palatability of the meat are genetically determined by another gene called RN (Rendement Neapole) that has been identified for the first time in Yorkshire breed animals and in their hybrids [19].

Diagnosis and disease management

The RYR1 has already been studied for four decades. A DNA-based molecular genetic test is specific for the HAL (halothane sensitivity) gene, lately known as RYR1 (ryanodine receptor), controlling sensitivity of pigs to stress factors [20]. This DNA-based assay is used to detect mutation in the ryanodine receptor gene and can identify homozygous MH-resistant (NN) and MH-susceptible animals (nn) as well as heterozygous carriers (Nn). It is well documented that T allele at RYR1 locus, corresponding to the recessive allele, is positively associated with increased proportion of lean meat in the carcass and inferior quality of pork (PSE meat) [20-22]. The T allele is due to a C to T transition at nucleotide 1843 of RYR1 locus, associated with MH susceptibility [9]. Based on the discovery of the causal mutation, a DNA-based test using a PCR assay followed by enzymatic restriction with *HhaI* was elaborated in order to enable the direct identification at the molecular level of the mutation within RYR1 gene [18,23-24]. Therefore, knowledge of molecular alteration associated with MH allowed development of accurate DNA-based tests, which are reliable, fast and cost-effective, and can be successfully applied in the wide-scale screening of pig populations. Reducing the prevalence of MH within the swine population requires genetic selection against the trait. With the advent of DNA-based assays, it is possible to cull MH-susceptible animals and carriers. At this time, prevention of MH episodes in individual animals requires that management practices to minimize stress be followed.

Conclusions

Several DNA markers have been used for a range of different traits in swine breeding up to this moment. These markers regarding variation in growth, lean percent, litter size, meat quality, susceptibility to developmental abnormalities, and even disease resistance have been identified and incorporated into breeding programs. Such DNA markers are already being applied at a significant level in the swine industry today [25]. The ability to efficiently combine this information with quantitative genetics is the key to delivering continuing value for the swine industry.

Knowing the precise frequency of specific alleles of this gene and of the three kinds of genotypes (NN, Nn, nn) in pure breeds of pigs has two advantages: **a)** gives the specialists the possibility to reduce the gene frequency through selection; **b)** helps the specialists "to build" deliberately more efficient heterozygous (Nn) commercial animals (Rehal Pietrain breed).

Currently, selection companies have abandoned the halothane test in pigs for the identification of homozygous recessive genotype, and instead use the genotyping PCR-RFLP technique.

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