PHENOTYPES ASSOCIATED WITH MALIGNANT HYPERThERMIA SUSCEPTIBILITY IN SWINE GENOTyped AS HOMOZYGOUS OR HETEROZYGOUS FOR THE RYANODINE RECEPTOR MUTATION†

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SUMMARY
We have examined the phenotypic expression of several parameters associated with malignant hyperthermia (MH) susceptibility in three groups (homozygous normal, homozygous abnormal and heterozygous) of Yorkshire/Duroc swine genotyped by a mutation in the ryanodine receptor. Subgroups of homozygous normals were classified further by the appearance or absence of muscle rigidity on prolonged in vivo challenge with halothane and suxamethonium. Four swine heterozygous for the proposed MH mutation were indistinguishable from five homozygous normal swine in temperature, heart rate, lactate concentrations, base excess and pH determined during the prolonged halothane and suxamethonium challenge. Resting creatine kinase concentrations, the in vivo barnyard challenge, the in vitro contracture response of skeletal muscle to 3% halothane and the threshold for Ca²⁺-induced Ca²⁺ release were also similar for subgroups of homozygous normals and heterozygotes. Therefore, inheritance of only one allele carrying the defect in the ryanodine receptor does not significantly alter phenotypes associated with MH susceptibility in this strain of swine. As four swine homozygous for the proposed MH defect did not exhibit rigidity and three of these had no other signs of MH on prolonged halothane and suxamethonium challenge, we conclude that the reported mutation in the ryanodine receptor may explain variability within individuals in human MH. (Br. J. Anaesth. 1993; 71: 410–417)

KEY WORDS
Hyperthermia: malignant, porcine, phenotypes, genotypes.

Anaesthesia-induced malignant hyperthermia (MH) is a potentially fatal inherited disorder of skeletal muscle [1–3] manifested typically by muscle rigidity, hyperthermia, hyperkalaemia, tachycardia, signs of lactic acidosis and greatly increased myoplasmic concentrations of Ca²⁺ in the response to halothane [4].

Swine have been used as an animal model of MH, as they may exhibit an inherited MH-like syndrome in response to stress or anaesthetics [5]. In humans, the syndrome varies greatly from individual to individual and even in the same individuals at different times, such that at times an episode may not occur [6, 7]. Similar observations have not been reported in swine. However, age is an important factor for expression of porcine MH [8] and variation in the degree of response between strains of swine has been reviewed [5]. These findings suggest that one or more factors in addition to the MH gene modulate the expression of the syndrome in humans and swine.

There is evidence suggesting that the threshold of Ca²⁺-induced Ca²⁺ release may be reduced in MH-susceptible muscle [9-11]. However, in isolated terminal cisternae preparations from MH muscle, the dose–response profile for halothane-induced Ca²⁺ release is normal [9, 11-14], suggesting that factors other than, or in addition to, a defective Ca²⁺ release channel, or ryanodine receptor, are essential for the exaggerated response of the MH sarcoplasmic reticulum to halothane. More recently, a specific polymorphism (C-to-T nucleotide substitution at position 1843) in the gene encoding the ryanodine receptor (yr1), causing a Cys⁶¹⁵ to Arg⁶¹⁵ amino acid substitution, has been associated with MH susceptibility in swine [15]. Five to 10 % of the swine homozygous for this mutation do not exhibit an MH episode on barnyard challenge with halothane [16, 17]. It has been argued that the barnyard challenge is less sensitive than a prolonged challenge with halothane and suxamethonium and may not detect all MH-susceptible subjects. However, as no other studies verified that the pigs would trigger with a prolonged halothane and suxamethonium challenge, this finding could also be interpreted as a variable

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expression of MH dependent on a modifying factor. Recent, highly detailed, functional studies of ryanodine receptor activity have detected defects in MH susceptible humans that are similar to those in a subpopulation of humans that are phenotypically not MH susceptible as regards their in vivo response to anaesthetics [18]. All of the above observations suggest that abnormal ryanodine receptor function alone is insufficient for expression of the MH syndrome.

The present study is the first to examine a wide variety of phenotypes associated with well-defined MH genotypes, including heterozygotes, in a strain of swine derived from a Yorkshire/Duroc cross. This study also examined whether the proposed MH genotype is sufficient for expression of the MH syndrome in Yorkshire/Duroc swine, or whether individuals from this strain of swine exhibit variability in the response to triggering agents, as has been reported for humans.

MATERIALS AND METHODS

Thirty tissue specimens from swine previously examined for a variety of phenotypes (table I) had been preserved in liquid nitrogen. The genomic DNA in these specimens was analysed for the identified porcine mutation, as described below. We did not have complete phenotyping for all the genotyped swine.

In vivo challenge and clinical laboratory findings

The swine (Yorkshire/Duroc cross) were obtained from Iowa State University. The study was approved by the Hahnemann University Animal Welfare Committee. From 1 to 6 weeks after their arrival at Hahnemann University, the pigs (15–40 kg) were anaesthetized with non-triggering agents (xylazine, ketamine, nitrous oxide), as described previously [11, 22]. In brief, an 8000–12 000 £ fraction isolated from longissimus dorsi removed in vivo challenge and Ca release was determined on a heavy sarcoplasmic reticulum fraction prepared from skeletal muscle. The gels were stained with ethidium bromide.

Threshold of calcium-induced calcium release assay

The threshold of Ca2+-induced Ca2+ release was determined on a heavy sarcoplasmic reticulum fraction isolated from longissimus dorsi removed under non-triggering anaesthesia, as described previously [11, 22]. In brief, an 8000–12 000 £ fraction was isolated by differential centrifugation [10] and the threshold of Ca2+-induced Ca2+ release was determined in the presence of pyrophosphate [23] to increase the sensitivity of the assay. Calcium was added in 10-µmol litre−1 pulses and the uptake and eventually the release of Ca2+ from the vesicles monitored with arsenazo III [10, 11, 22]. (We have recently identified a systematic error in our previously published values for the threshold of Ca2+-induced Ca2+ release. To compare the present study with previous studies from our laboratory, the values...
in the older studies (accepted before January, 1993) must be divided by a factor of two.)

### RESULTS

#### Distribution of phenotypes examined for each genotype

A total of 30 swine were genotyped and the phenotypes examined for each of these are identified individually in table I. Of the 30 swine, 15 were diagnosed initially as MH susceptible by barnyard challenge (rigidity to 6% halothane within 3 min), A–O and H blood typing and creatine kinase (CK) values (table I) and these were all found to be homozygous for the proposed MH mutation. An additional 15 swine were diagnosed initially as not being MH susceptible by the same criteria and, of these, eight were heterozygous for the proposed MH mutation and seven were homozygous normal. The 15 MH-positive swine and 14 of the 15 MH-negative swine were diagnosed for MH susceptibility by the halothane and caffeine contracture tests also, using the North American procedure. All the swine were challenged in vivo with halothane and suxamethonium, but detailed clinical data (temperature, heart rate, lactate concentrations, base excess, pH) were obtained for only five of the seven homozygous normals, four of the eight heterozygotes and 10 of the 15 homozygous MH-positive swine. The 10 homozygous MH-positive swine in which clinical data were gathered were challenged with halothane and suxamethonium and were grouped further into those exhibiting rigidity ($n = 6$) and those in which no rigidity was observed ($n = 4$), in order to examine if other physiological and biochemical changes associated with MH were observed in the absence of rigidity during the halothane and suxamethonium challenge.

#### In vivo challenge with halothane and suxamethonium

Two of the rigid group of MH-susceptible swine exhibited rigidity to halothane alone and all five exhibited rigidity after the first administration of halothane and suxamethonium. The non-rigid MH susceptibles had no signs of rigidity even after five bolus injections of suxamethonium.

Starting temperature has been suggested to influence the response of MH-susceptible swine to triggering anaesthesia [25]. The oesophageal temperatures before administration of halothane (mean (SEM)) for the homozygote controls (36.6 (0.1) °C), heterozygotes (38.1 (1.6) °C), homozygote MH sus-

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### Table I. Phenotypes examined for each swine genotyped for the proposed porcine MH mutation. The groups were subdivided by genotype, including homozygous normal (C/C), heterozygotes (C/T) and MH susceptibles (T/T). n.d. = Not determined. *Reactions of red blood cells with antisera for the H system (Ha or Hc), or with either the A or O blood factors. ** = no reaction [24]. † Either muscle rigidity (+) or no rigidity (–) within 3 min of an in vivo challenge with 6% halothane. ‡ Outcome either MH susceptible (+), or normal (–). Y = Details recorded; N = details not recorded for the prolonged in vivo halothane and suxamethonium challenge; NR = no rigidity; R = muscle rigidity during the challenge.

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Blood type*</th>
<th>Resting CK value (u litre$^{-1}$)</th>
<th>Barnyard challenge†</th>
<th>Contracture test‡</th>
<th>In vivo challenge</th>
<th>Threshold ($\mu$mol Ca$^{2+}$/mg protein)</th>
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<tbody>
<tr>
<td>Homozygous C/C (normal)</td>
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<tr>
<td>1</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>O</td>
<td>36</td>
<td>–</td>
<td>N/NR</td>
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<tr>
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<td>O</td>
<td>36</td>
<td>–</td>
<td>N/NR</td>
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<tr>
<td>14</td>
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<td>46</td>
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<td>N/NR</td>
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<td>Y/R</td>
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<tr>
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<td>Y/R</td>
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<td>+</td>
<td>Y/R</td>
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<tr>
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<td>Y/R</td>
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<td>Y/R</td>
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<td>Y/R</td>
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<tr>
<td>22</td>
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<tr>
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<td>–</td>
<td>1140</td>
<td>+</td>
<td>Y/R</td>
<td>3.0</td>
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<tr>
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<td>+</td>
<td>Y/R</td>
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<tr>
<td>25</td>
<td>a/a</td>
<td>–</td>
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<td>+</td>
<td>Y/R</td>
<td>1.4</td>
</tr>
<tr>
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<td>a/a</td>
<td>–</td>
<td>316</td>
<td>+</td>
<td>N/R</td>
<td>n.d.</td>
</tr>
<tr>
<td>27</td>
<td>a/a</td>
<td>–</td>
<td>157</td>
<td>+</td>
<td>N/R</td>
<td>n.d.</td>
</tr>
<tr>
<td>28</td>
<td>a/a</td>
<td>–</td>
<td>272</td>
<td>+</td>
<td>N/R</td>
<td>1.7</td>
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<tr>
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<td>a/a</td>
<td>–</td>
<td>499</td>
<td>+</td>
<td>N/R</td>
<td>2.1</td>
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<tr>
<td>30</td>
<td>a/a</td>
<td>–</td>
<td>216</td>
<td>+</td>
<td>N/R</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
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3% 2% halothane Sux. 1 mg kg⁻¹

3% 2% halothane Sux. 1 mg kg⁻¹

7.50

FIG. 1. Mean (SEM) changes in (A) pH; (B) base excess (BE); (C) lactate concentration; and (D) heart rate (HR) accompanying prolonged in vivo challenge with halothane and suxamethonium. Blood was obtained at time 0, which was immediately before addition of 3% halothane for values in A–C. Heart rate was also recorded immediately before addition of halothane for time 0. Halothane was reduced to 2% as indicated by the arrow and maintained at 2% for the remainder of the challenge. Suxamethonium (Sux.) 1 mg kg⁻¹ was injected five times, as indicated. Genotypes:

-/- = homozygous normal (C/C) (○) (n = 5);
-/+ = heterozygous (C/T) (●) (n = 4);
+/+ = homozygous MH susceptible (T/T) exhibiting rigidity on prolonged halothane and suxamethonium challenge (△) (n = 6); +/+(-) = homozygous MH susceptible (T/T) not exhibiting rigidity on prolonged halothane and suxamethonium challenge (▽) (n = 4). The four groups were tested at each time point by a one-way ANOVA and Scheffe test.

* Significantly different from control (P < 0.05).
concentrations at the end of the challenge were not significantly different between the groups, as determined by one-way ANOVA (data not shown). The difference in muscle tone between rigid and non-rigid pigs was unmistakable. The heart rate, lactate concentrations and pH values in all but one case supported the subjective determination of rigidity for the MH swine. The lactate value (17 mmol litre⁻¹) for one non-rigid MH pig was greater than that of one of the rigid MH pigs (16 mmol litre⁻¹) and the pH value (6.88) was smaller only than that in the same rigid MH pig (7.29). While slight increases in muscle tone may have occurred in response to this extreme in vivo challenge in the four non-rigid MH swine, these would not be detected or interpreted as clinical signs of MH by an anaesthetist.

In vitro contracture response studies

Other investigators have reported that several strains of swine heterozygous for the MH defect have intermediate [26], or MH-like [27] in vitro contracture responses to halothane. However, neither of these studies directly genotyped the heterozygotes. Therefore, we determined the phenotypic expression of the heterozygotes in pigs genotyped for the mutation in the ryanodine receptor in the Yorkshire/Duroc strain. First, all the homozygous normal and heterozygote swine tested were diagnosed as normal by the North American MH Group procedure (table I). All homozygous MH-susceptible swine were diagnosed as MH susceptible (table I). Based on the maximum response for the three strips tested, the in vitro contracture response to 3% halothane in homozygous controls (0.07 (0.03) g; n = 6) was not significantly different (two-tailed grouped t test) from that of the heterozygotes (0.28 (0.09) g; n = 8) and there was considerable overlap between the groups. These values for controls and heterozygotes are similar to the human control values reported by the North American MH Group [28]. The MH susceptibles had markedly stronger responses to halothane (1.42 (0.13) g; n = 15) than either the heterozygotes or homozygous normals.

Resting CK values

The resting CK values were significantly different (P < 0.05): 20 (4) u litre⁻¹ (n = 6) for the homozygous controls and 43 (8) u litre⁻¹ (n = 8) for the heterozygotes. However, there was considerable overlap in values, making it impossible to differentiate between homozygous normals and heterozygotes by CK values alone (table I). The CK value for MH susceptibles (374 (76) u litre⁻¹; n = 15) were considerably greater than those of the other groups in all cases (table I).

Threshold of Ca²⁺-induced Ca²⁺ release in a heavy sarcoplasmic reticulum fraction

The threshold of Ca²⁺-induced Ca²⁺ release has been reported to be reduced in heavy sarcoplasmic reticulum fractions from MH-susceptible skeletal muscle [9—11]. The observation was supported in the present study for preparations from MH swine, whether or not they exhibited rigidity on prolonged halothane and suxamethonium challenges (table II). The values for the three heterozygotes (table I) were not statistically different from normals (P = 0.43 by two-tailed t test) (table II). To test the association of a reduced threshold of Ca²⁺-induced Ca²⁺ release with outcome of the diagnostic contracture test for MH, the values for the heterozygotes and homozygote normals were pooled to form the MH-negative group and the values from the homozygous MH-positive swine with and without rigidity on prolonged in vivo halothane and suxamethonium challenge were pooled for the MH-positive group in figure 2. Overall, the threshold of Ca²⁺-induced Ca²⁺ release of swine diagnosed as MH-positive was significantly less than that from MH-negative swine (P < 0.05; two-tailed t test) (fig. 2). However, there is considerable variability in this variable, with values occurring within 1 SEM of the mean for MH-negative swine in some MH-positive muscle and

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**Table II. Threshold of Ca²⁺-induced Ca²⁺ release in genotyped swine (mean (SEM)). Genotypes: —/— = homozygous normal (C/C); —/+ = heterozygous (C/G); +/+ (+) = homozygous MH susceptibles (T/T) exhibiting rigidity on prolonged halothane and suxamethonium challenge; +/+ (—) = homozygous MH susceptibles (T/T) not exhibiting rigidity on prolonged halothane and suxamethonium challenge. * Significantly less than —/— (P < 0.05; one-tailed t test)**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Threshold (µmol Ca²⁺/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—/—</td>
<td>6</td>
<td>3.8 (0.6)</td>
</tr>
<tr>
<td>—/+</td>
<td>3</td>
<td>3.1 (0.5)</td>
</tr>
<tr>
<td>+/+ (+)</td>
<td>8</td>
<td>2.5 (0.4)*</td>
</tr>
<tr>
<td>+/+ (—)</td>
<td>4</td>
<td>2.2 (0.4)*</td>
</tr>
</tbody>
</table>

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**Fig. 2.** The threshold for Ca²⁺-induced Ca²⁺ release in swine diagnosed as normal (MH—) (homozygous normals and heterozygotes: mean 3.6 (SEM) 0.4 µmol Ca²⁺/mg protein; n = 9) or susceptible to MH (MH+) (rigid plus non-rigid: mean 2.4 (0.3) µmol Ca²⁺/mg protein; n = 12) by the in vitro contracture test. Three MH-negative swine had threshold values of 2.6 µmol Ca²⁺/mg protein and two had values of 4.2 µmol Ca²⁺/mg protein. Two MH-positive swine had values of 1.7 µmol Ca²⁺/mg protein and two had values of 1.6 µmol Ca²⁺/mg protein.
values occurring within 1 SEM of the mean for MH-positive in some MH-negative muscle.

**DISCUSSION**

The present study extends to the Yorkshire/Duroc cross of swine the association of the C-to-T point mutation in nucleotide 1843 of the gene encoding the ryanodine receptor with MH susceptibility. In the Yorkshire/Duroc swine, inheritance of two mutated alleles appears to be necessary for MH susceptibility, in agreement with a recessive pattern of inheritance. However, even when both alleles are inherited, this mutation is not sufficient to elicit the MH syndrome at all times, despite potentially lethal anaesthesia. Instead, there appears to be an important modulating factor of the MH syndrome that must be present for full expression of the MH syndrome or, if it is absent, the progression of events resulting in the syndrome is interrupted and the syndrome is not observed, as was the case in three of the four non-rigid MH swine. These four swine were definitely MH susceptible, as they had previously exhibited rigidity in the barnyard challenge.

Unlike one other strain of swine [26] in which the heterozygotes exhibited an intermediate in vitro contracture response to halothane and another [27] in which the heterozygotes exhibited a positive contracture response by the European MH Group procedure [29], the Yorkshire/Duroc cross heterozygotes have about the same in vitro contracture response to halothane as homozygous normals. It should be noted that neither of the previous studies [26, 27] truly genotyped the swine, but relied instead on phenotypic information to establish a "genotype" indirectly. The mean of the resting CK concentrations was slightly greater in the Yorkshire/Duroc heterozygotes, but the phenotypes of the heterozygotes and the homozygous normals would be impossible to distinguish clearly using CK values, contracture tests, or both. Similarly, the mean of the values of the threshold of Ca\(^{2+}\)-induced Ca\(^{2+}\) release was normal for heterozygotes. A recent study has reported that heterozygotes form a metabolically distinct phenotype that is more closely related to controls than to homozygote MH susceptibles [30].

In agreement, the prolonged in vivo challenge with halothane and suxamethonium in the Yorkshire/Duroc swine. While this finding would suggest that the threshold of Ca\(^{2+}\)-induced Ca\(^{2+}\) release values for the MH-positive swine not exhibiting rigidity, as a group, were the same as those for the rigid MH-positive swine. While this finding would suggest that the threshold of Ca\(^{2+}\)-induced Ca\(^{2+}\) release was also independent of the MH syndrome modifying factor, the wide range of values in both groups and the wide range of modulation of this variable within individuals with other muscle disorders [35] make this an unreliable predictor of MH susceptibility.

Some strains of swine might express the factor modifying the response to anaesthetics more consistently than others and this may account for the strain-to-strain variation in the expression of the MH syndrome [5]. The Yorkshire/Duroc MH-susceptible swine are somewhat less responsive than some other strains. Our findings in the Yorkshire/
Duroc swine are the first to suggest that the expression of the MH syndrome in homozygous abnormal individuals in this strain can vary in the same manner as observed in humans with MH. One major difference between human MH and porcine MH is the pattern of inheritance. While both exhibit an autosomal inheritance, MH is inherited as a dominant disorder in humans and recessive disorder in swine. A second major difference is that all porcine MH has been suggested to result from a single genetic defect in the ryanodine receptor [15, 16] and this was verified for the strain of swine in the present study. In contrast, an analogous defect in humans is rarely inherited [36] and, if so, it is inherited as a single allele in MH humans. Also, genetic heterogeneity has been observed in humans, suggesting that genes other than the Ca\textsuperscript{2+} release channel can cause MH [37–41].

These findings in the Yorkshire/Duroc strain of swine suggest that inheritance of a single copy of the genetic mutation in the ryanodine receptor in the Yorkshire/Duroc swine does not significantly alter muscle function in the absence of anaesthetics, or the response to MH triggering agents. Inheritance of the porcine mutation as a single allele causative of MH in humans is inconsistent with the present and other studies in swine. Therefore, species-related differences in the Ca\textsuperscript{2+} release channel function or expression must be invoked to explain human MH. Alternatively, the possibility exists that the porcine mutation, when inherited as a single allele, in humans may not cause MH.

Inheritance of two abnormal alleles does result in abnormal muscle function in these swine in both the absence (increased CK values) and presence of anaesthetics. The absence of the MH syndrome in a subpopulation of swine homozygous for the MH defect, even with a prolonged halothane and succinemethionine challenge, suggests that swine may be used to study a modifying factor responsible for the variability of the syndrome in MH-susceptible humans. The outcome of the halothane and caffeine contracture tests appears to reflect the presence of the MH mutation independent of the modifying factor.

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