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A RAPID SCREENING METHOD BY THE POLYMERASE CHAIN REACTION - SINGLE STRAND CONFORMATION POLYMORPHISM USING AUTOMATIC SEQUENCER FOR PORCINE RYANODINE RECEPTOR GENE MUTATION, AND USE FOR MEAT QULITY EVALUATION.

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Introduction

The method to detect the ryanodine receptor gene mutation from C to T at position 1843 is important for porcine breeding and meat quality evaluation, because this mutation is the cause of porcine stress syndrome (PSS) which resembles to human malignant hyperthermia. PSS has been detected by halothane test conventionally (Otsu *et al.*, 1991;Dickson, 1993), however, halothane test is time consuming, and can only detect homozygous mutated animal. In 1991, porcine ryanodine receptor gene was cloned and a missense mutation (C1843 to T) of the gene was discovered as the cause of PSS (Fujii *et al.*, 1991). Several methods to detect this mutation have been established and the studies on the relation between the mutation and the quality of meat have been published (Otsu *et al.*, 1991; Fujii *et al.*, 1991; Rempel *et al.*, 1993; Pommier and Houde, 1993; Horiuchi *et al.*, 1996; Shibata, 1996). The restriction endonulcease fragment length polymorphism (RFLP) of the polymerase chain reaction (PCR) products has been used generally (Otsu *et al.*,1991; Fujii *et al.*, 1991).,but this method is time consuming. The single strand conformation polymorphism (SSCP) method using gel electrophoresis with silver staining has been used (Shibata, 1996;Nakajima *et al.*, 1996), however, it is still complex method. Recently, the SSCP with fluorescence-based automatic sequencer was developed (Makino *et al.*, 1992), and this method is considerably rapid.

In this study, we established the PCR-SSCP method using fluorescence labeled primer on ALFred automatic sequencer. And analyzed 241 animals from 4 farms in Japan by this method. We also analyzed low quality meat.

Materials and Methods

Animals and meat, and DNA preparation Hair roots of swine were collected from four farms of different location in Japan. The hair roots were packed in a plastic bag and mailed. Low quality meats were collected from meat companies. One to two hair roots or a slice of meat were put into a plastic tube with 50 μl of PCR buffer and 1 μl of proteinase K (20mg/ml concentration). After incubation at 50°C for 2 h, the tubes were heated at 95°C for 5 min and cool in ice. The solution was stored at 4°C and used as the template DNA.

PCR method and Restriction enzyme digestion. The PCR was performed by the method of Fujii *et al.* (1991) with the same primers. A 5 μl of PCR product was used for restriction enzyme digestion by the recommended condition with total 10 μl volume at 37 °C for 2 to 18 h. The samples were finally electrophoresed on 15 % of polyacrylamide gel for 1 hour at 150 V, and stained with ethidium bromide.

SSCP method using Automatic sequencer. The PCR were performed using the same primers but Cy-5 labeled forward primer at the same condition. A 0.5 μl of the PCR product was mixed with 20 μl of denaturing solution (95% formamide, 19 mM EDTA and 0.001% bromophenol blue) and was heated at 95°C for 5 min, then was cooled immediately. The 1.5 μl of sample was electrophoresed with ALFred automatic sequencer.

Results and Discussion

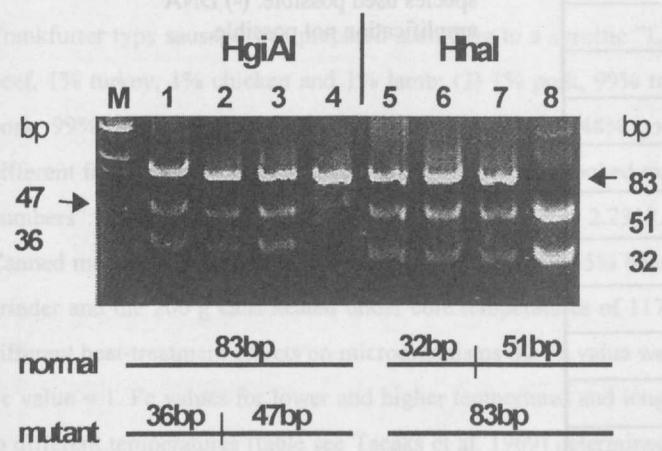

Fig. 1. PCR-RFLP of ryanodine receptor.
Lane 1 and 5, 2 and 6, 3 and 7, 4 and 8 are the same animals

Restriction enzyme analysis (RFLP method) Hair roots of swine were collected and analyzed by PCR-RFLP method. The PCR product was the same size (83bp) as the report of Fujii *et al.* (1991). Hha I digests GCGC sequence, and Hgi AI digested GTGCTC sequence, therefore, the mutation from C to T at position 1843 creates a Hgi AI site and deletes Hha I site. The lane 4 and 8 in Fig 1, show the 83 bp band was not digested by Hgi AI but digested by Hha I. This result shows the sample is normal. The lanes 1,2,3,5,6 and 7 show that the 83 bp band was digested by Hgi AI and Hha I partially, therefore, the samples were heterozygote. To confirm the results, we subcloned and sequenced those samples and detected the mutation of C1843 to T.

PCR-SSCP method We tried to detect the mutation by silver staining PCR-SSCP method. The samples confirmed by sequencing were used. It seemed to be possible to separate the normal (C) and mutated (T) allele, however, the difference of mobility between C and T allele was so small, hence we considered automatic sequencer. PCR was performed with Cy-5 labeled forward primer. The electrophoresis was performed under the several temperature conditions (4°C、10°C、22°C). For standard samples, the C-plasmid which has C/C allele and the T-plasmid which has T/T allele were made. The PCR

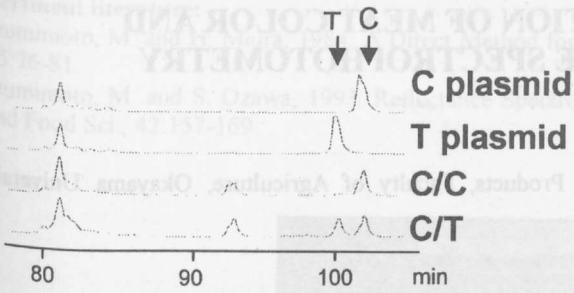

Fig. 2. PCR – SSCP with Automatic Analyzer of Ryanodine Receptor of Swine.

A ryanodine receptor gene was amplified with Cy-5 labeled forward primer and usual reverse primer, and analyzed by ALFred automatic sequencer. C plasmid: the plasmid having C/C sequence was used as the template for C/C standard; T plasmid: the plasmid containing the T/T sequence was used as the template for T/T standard; C/C: normal animal; C/T: heterozygote animal.

product obtained from C-plasmid was clearly separated from the PCR product from T-plasmid under the 4°C and 22°C, however the separation at 4°C was more clear than that at 22°C. Therefore the electrophoresis was performed at 4°C. (Fig.2) The two peaks were detected in heterozygous sample (Fig. 2, C/T) and only the normal peak was detected in normal sample. We can analyze up to 38 samples at one time. We run the samples on the same gel for three times without any treatment in a day, and the result was not different from each other. Therefore, this method seemed to be suitable to analyze the large number of samples in short time to compare with halothane test and other genetic methods.

Screening of the mutation

We examined total 241 samples in 4 farms by this PCR-SSCP method(Table I). There was no homozygous mutated sample found in this study. The average of the heterozygous mutation frequency was 28.6%. The frequency of heterozygote in farm A was 9.6%, and 14.3%, 39.7%, 37.5% for farm B, C, and D respectively. The frequency of the heterozygote in male was 34.5 % and in female was 10 %. Among the farms, only farm A performed the halothane test. The percentage of heterozygote in male swine Duroc and LWD in farm C was so high. However, there is no claim of meat quality of this LWD. The low quality meats were also analyzed by PCR-RFLP (Table 2).

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Table 1. Screening of Ryanodine Receptor Mutation in Swine at 4 Farm

Farm	Strain	Sex	C/C	C/T	Ratio of Heterozygote
Farm A	D	M	5	1	
		F	10	1	
	W	M	1	0	
		F	7	0	9.6
	L	M	4	2	
		F	20	1	
Farm B	D	M	7	0	
	LW	F	22	6	14.3
	L	F	7	0	
Farm C	D	M	11	11	
	LW	F	8	0	39.7
	LWD	U	59	40	
	LWB	U	1	1	
Farm D	D	M	10	6	37.5
Total			172	69	28.6

*D: Duroc; W:Large White; L: Landrace; LW:Landrece-Large White; LWD:Landrace-Large White-Duroc. In farm C. LWD is the child of male Duroc and female LW. The LWB is the child of male Berkshire and female LW. The farm A, C, and D are farrow-to-wean operation farm. The farm B is farrow-to-finish operation farm. T/T allele was not observed at those swine.

Table 2. Analysis of low quality meat by PCR-RFLP

No.	Quality	pH	Color	RYR 1843
1	Poor	5.6	3	C/C
2	Poor	5.7	3.5	C/C
3	Poor	5.4	3.5	C/C
4	Poor	5.4	3	C/C
5	Good	6.0	3	C/C
6	Good	5.7	1.5	C/T
7	Good	6.4	2.5	C/C
8	Good	6.3	4.5	C/C
9	Poor	6.0	1.0	C/T
10	Poor	6.1	2.5	C/T
11	Poor	6.4	5.0	C/T
12	Poor	6.9	3.5	C/C
13	Good	6.6	3.0	C/C
14	Good	5.9	2.0	C/C
15	Good	6.4	4.5	C/C

Color is expressed by Japanese meat color standard.