NOTE *Virology*

Frequencies of PrP Genotypes in Meat Breeds of Japanese Sheep and Trail of Selective Breeding in Experimental Sheep Flock

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ABSTRACT. The selection of sheep with scrapie-resistant PrP genotypes is one of the control measures for transmissible spongiform encephalopathies in ruminants. In this study, we investigated the frequencies of PrP genotypes in meat breeds in Japan. The nationwide surveillance revealed that nearly half of the Suffolk sheep, a major meat breed in Japan, carried scrapie-susceptible AQ/AQ and AQ/VQ genotypes. In addition, the VQ haplotype, which confers high susceptibility to scrapie within sheep, was also found in Poll Dorset sheep. A trial of selective breeding using sires with scrapie-resistant PrP genotypes AQ/AR and AR/AR could raise the ratio of scrapie-resistant sheep from less than 50% to 80% within 3 years. However, the use of sires with the AR/AR genotype and the selection of ewes would be required to achieve a higher ratio of scrapie-resistant sheep.
KEY WORDS: prion, scrapie, transmissible spongiform encephalopathy.

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Scrapie is a fatal neurodegenerative disease of sheep and goats, and is classified as a transmissible spongiform encephalopathy (TSE). In the sheep prion protein (PrP) gene, nucleotide polymorphisms causing amino acid substitutions have been reported in more than 20 codons [14]. Among these, polymorphisms at codons 136 (valine [V]/alanine [A]) and 171 (glutamine [Q]/arginine [R]/histidine [H]/lysine [K]) are closely associated with susceptibility to scrapie. The VQ haplotype confers a high susceptibility to the disease, and the wild-type AQ haplotype also confers disease susceptibility while the AR haplotype is associated with resistance to scrapie [16]. Due to the clear genetic susceptibility to the disease, the scrapie susceptibility of sheep and flocks can be estimated by analyzing PrP genotypes.

In Japan, there are around 20,000 sheep, more than half of which are being reared in Hokkaido. Although the sheep population is relatively small in Japan, there are sporadic occurrences of scrapie. In a previous study, we analyzed the PrP genotypes of Japanese Suffolk sheep, although their numbers are limited [18]. It is important to estimate the scrapie susceptibility of sheep flocks for the control of scrapie prevalence; however, the recent and precise frequencies of PrP genotypes in Japanese sheep remain unclear.

Breeding programs that aim to increase the population of scrapie-resistant sheep by eliminating the scrapie-susceptible VQ haplotype and increasing the scrapie-resistant AR haplotype are considered as part of the measures to eradicate scrapie. In European countries including the UK, the Netherlands, France, and Germany, breeding programs have been started [9, 11]. However, in Japan, a breeding program has not been implemented yet.

In order to contribute to the control measures for TSE in Japan, we carried out large-scale PrP genotyping of meat breeds of sheep in Japan. In addition, to estimate the efficacy of selective breeding using sires carrying the scrapie-resistant PrP genotype, we have conducted a trial of selective breeding using an experimental sheep flock since 2002. Here we report the frequencies of sheep PrP genotypes in Japan and the transition of the frequencies of PrP genotypes by selective breeding using sires carrying the AR haplotype.

For the investigation of the frequencies of PrP genotypes in Japan, a total of 880 sheep, including 648 Suffolk, 92 Poll Dorset, 20 Southdown, 44 cross-bred Suffolk and Southdown, and 76 cross-bred Poll Dorset and Southdown sheep, were used in this study. The genomic DNA of 195 Suffolk sheep was obtained from the obex samples that were used for active surveillance of scrapie in sheep, and other DNA samples were obtained from the venous blood collected at the farms. These samples were collected between 2003 and 2005. For the evaluation of the efficacy of selective breeding, blood samples were collected from 884 Suffolk sheep in the experimental flock of Shintoku Hokkaido Animal Research Center (HARC). Samples at HARC were collected during 2001–2006. The QIAamp DNA Blood Mini Kit (QIAGEN) and the DNeasy Tissue Kit (QIAGEN) were used for purification of genomic DNA from blood and obex, respectively.

DNA fragments corresponding to the open reading frame of the PrP gene were amplified using PCR with primers sPrP104 and sPrP105 (Table 1). After amplification, the excesses of primers and nucleotides were removed using an S-300HR spin column (GE Healthcare), and the amplified

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Table 1. Nucleotide sequencing of primers and Taqman probes used in this study

Primers for PCR and/or nucleotide sequencing	
sPrP104	5'-CATCATGGTGAAAAGCCACATAGGCAG-3'
sPrP105	5'-ATGAAAACAGGAAGGTTGCCCCTATCC-3'
sPrP109	5'-GGTCAAGGTGGTAGCCACAG-3'
sPrP110	5'-GTCAGTTTCGGTGAAGTTCTC-3'
Primers for SNPs analysis	
356F	5'-CTGCAGCTGGAGCAGTGGTA-3'
450R	5'-GTCCTCATAGTCATTGCCAAAATGTATA-3'
467F	5'-ACATGTACCGTTACCCCAACCA-3'
553R	5'-TGTTGACACAGTCATGCACAAAG-3'
Taqman probes for SNPs analysis[a)]	
136A	5'-FAM-TGCTGGGAAGTGCCA-MGB-3'
136V	5'-VIC-ATGCTGGGAAGTGTCA-MGB-3'
171Q	5'-VIC-CAGTGGATCAGTATAGTAA-MGB-3'
171R	5'-FAM-CAGTGGATCGGTATAGTA-MGB-3

a) Taqman probes possess fluorophore (either FAM or VIC) at 5' and MGB quencher at 3'.

fragments were subjected to direct sequencing with the above primers and additional internal primers, sPrP109 and sPrP110. The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit, and nucleotide sequences were determined using an ABI-3100 Avant-Genetic Analyzer (Applied Biosystems).

Single nucleotide polymorphisms (SNPs) at codons 136 (GCC for alanine and GTC for valine) and 171 (CAG for glutamine and CGG for arginine) were analyzed by allelic discrimination using Taqman assay. The PCR primers used for SNPs analysis at codon 136 were 356F and 450R, and those for codon 171 were 467F and 553R (Table 1). Taqman probes used for SNPs analysis at codon 136 were 136A and 136V, and those for codon 171 were 171Q and 171R (Table 1). SNPs analysis was performed in a 25 μl reaction mixture consisting of 10 *n*g of genomic DNA, 900 nM of each primer for PCR, 250 nM of each Taqman probe, and 1x Taqman Universal PCR Master Mix (Applied Biosystems). Cycling conditions were one cycle of 2 min at 50°C and 10 min at 95°C, followed by 35 cycles of 15 sec at 92°C and 1 min at 60°C. The PCR reaction and the following allelic determination were carried out with an ABI PRISM 7900 HT using SDS 2.1 software (Applied Biosystems).

If samples showed polymorphisms at both codons 136 and 171, the PCR products amplified with primers sPrP104 and sPrP105 were cloned into a pCRII-TOPO vector (Invitrogen) and then sequenced. The PrP haplotype was determined by the co-incidence of the polymorphisms at codons 136 and 171 in the cloned fragments.

In a previous study, six haplotypes, MARQ, TARQ, MVRQ, MAHQ, MARR, and MARH, based on polymorphic codons at 112, 136, 154, and 171, were identified in Japanese Suffolk sheep [18]. In the present study, nucleotide sequencing of the PrP gene in 98 Suffolk sheep revealed four haplotypes, MARQ, TARQ, MVRQ, and MARR. Since polymorphisms at codons 136 and 171 greatly influence scrapie susceptibility, in the following study we focused on determining polymorphisms at codons 136 and 171 using SNPs analysis. Table 2 shows the frequencies of PrP genotypes in Japanese Suffolk sheep. The frequencies of the scrapie-susceptible AQ/AQ and AQ/VQ genotypes were 48.0%, indicating that nearly half of the Japanese Suffolk sheep are susceptible to the disease. In contrast, only 9.6% of the sheep were homozygous for AR. Nationwide surveillance also revealed that PrP genotype frequencies varied with location; Tochigi and Miyagi prefectures showed higher frequencies of AQ/AQ and AQ/VQ genotypes than Hokkaido ($p<0.05$ in χ^2 test).

Table 3 shows the frequencies of PrP genotypes in meat breeds at 14 private farms in Hokkaido. In the Suffolk sheep, the frequency of the AQ/AQ genotype varied from 30.9% to 100%. In four out of ten farms, more than half of the Suffolk sheep carried the AQ/AQ genotype, indicating that scrapie susceptibility varies with the flocks. At farm D, the percentage of sheep bearing the AR/AR genotype (27.4%) was higher than that at other farms. The breeding records confirmed that a single sire was primarily used for breeding at this farm, suggesting that the genotypes of sires used for breeding influenced the scrapie susceptibility of the flocks. We also investigated the PrP genotypes of Poll Dorset sheep at two farms and those of Southdown sheep and cross-breeds at two other farms (Table 3). The Poll Dorset sheep showed a lower frequency of the AQ/AQ genotype than the Suffolk sheep, and had a high percentage of sheep carrying the AR haplotype (72.2% at farm L and 93% at farm K). Furthermore, the percentage of sheep carrying the VQ haplotype among the Poll Dorset sheep (13.1% at farm

Table 2. Frequencies of PrP genotypes in Suffolk sheep from various parts of Japan

Genotype	Prefectures								Japan[a)]	
	Hokkaido		Aomori		Miyagi		Tochigi			
	n=468	%	n=18	%	n=24	%	n=130	%	n=648	%
AQ/AQ	200	42.7	9	50.0	13	54.2	82	63.1	307	47.4
AQ/VQ	0	0	0	0	2	8.3	2	1.5	4	0.6
AQ/AR	209	44.7	8	44.4	9	37.5	41	31.5	272	42.0
AR/AR	57	12.2	1	5.6	0	0	4	3.1	62	9.6
AR/VQ	2	0.4	0	0	0	0	1	0.8	3	0.5

a)In addition to sheep samples collected from the prefectures indicated in the table (n=640), total number includes 8 additional sheep samples from Niigata (n=7) and Ishikawa (n=1) prefectures.

Table 3. Frequencies of sheep PrP genotypes at private farms in Hokkaido

Farms	Breed	n[a)]	Genotype									
			AQ/AQ		AQ/AR		AR/AR		AQ/VQ		AR/VQ	
			n	%	n	%	n	%	n	%	n	%
A	Suffolk	14	8	57.1	5	35.7	1	7.1	0	0	0	0
B	Suffolk	55	17	30.9	30	54.5	8	14.5	0	0	0	0
C	Suffolk	20	10	50.0	9	45.0	1	5.0	0	0	0	0
D	Suffolk	73	24	32.9	29	39.7	20	27.4	0	0	0	0
E	Suffolk	88	37	42.0	39	44.3	12	13.6	0	0	0	0
F	Suffolk	57	22	38.6	26	45.6	8	14.0	0	0	1	1.8
G	Suffolk	32	10	31.3	20	62.5	2	6.3	0	0	0	0
H	Suffolk	12	6	50.0	6	50.0	0	0	0	0	0	0
I	Suffolk	6	6	100	0	0	0	0	0	0	0	0
J	Suffolk	6	2	33.3	1	16.7	3	50.0	0	0	0	0
K	Poll Dorset	61	14	23.0	27	44.3	12	19.7	3	4.9	5	8.2
L	Poll Dorset	29	0	0	11	37.9	13	44.8	2	6.9	3	10.3
M	Southdown	8	4	50.0	3	37.5	1	12.5	0	0	0	0
	PD × SD[b)]	76	10	13.2	45	59.2	17	22.4	3	3.9	1	1.3
N	Southdown	12	3	25.0	3	25.0	6	50.0	0	0	0	0
	S × SD[c)]	44	13	29.5	19	43.2	11	25.0	1	2.3	0	0

a) Number of sheep analyzed.
b) Poll Dorset crossed with Southdown.
c) Suffolk crossed with Southdown.

Table 4. PrP genotype of sires and ewes used for selective breeding at HARC

	PrP genotype	2001	2002	2003	2004	2005
Sire	AQ/AQ	4	0	0	0	0
	AQ/AR	3	3	5	4	6
	AR/AR	0	0	0	2	3
Ewe	AQ/AQ	–	59	68	56	61
	AQ/AR	–	37	43	53	67
	AR/AR	–	8	11	12	18

L and 17.2% at farm K) was higher than that in the Suffolk sheep. The PrP genotype distributions of the Poll Dorset sheep were similar to those reported in other countries [17].

At HARC, a trial of selective breeding using sires with the AQ/AR and AR/AR genotypes has been conducted since 2002. Table 4 shows the PrP genotype of sires and ewes used for the breeding program. More than 100 ewes were used every year, however, only sires have been selected on the basis of PrP genotype since 2002. Table 5 shows the transition of the frequencies of PrP genotypes in lambs born in each season. There were significant differences in the frequencies of PrP genotypes between lambs born before

Table 5. The transition of the frequencies of PrP genotypes in the experimental flock at HARC
a. Genotype frequency

Genotypes[a)]	2002		2003		2004		2005		2006	
	n=186	%	n=220	%	n=214	%	n=220	%	n=154	%
AQ/AQ	97	52.2	86	39.1	60	28.0	44	20.0	37	24.0
AQ/AR	71	38.2	104	47.3	116	54.2	132	60.0	78	50.6
AR/AR	18	9.7	30	13.6	38	17.8	44	20.0	39	25.3

b. Statistical analysis[b)]

	2002	2003	2004	2005	2006
vs 2002	–	7.09 (<0.05)*	24.85 (<0.001)*	46.64 (<0.001)*	32.20 (<0.001)*
vs 2003	–	–	6.14 (<0.05)*	19.54 (<0.001)*	13.17 (<0.01)*
vs 2004	–	–	–	3.96 (0.14)	3.21 (0.20)
vs 2005	–	–	–	–	3.25 (0.20)

a) Genotypes of lambs born in each year were determined by SNPs analysis.
b) Differences in the frequencies of PrP genotypes between years were analyzed by χ^2 test. χ^2-values and *p*-values (in parentheses) are shown. Asterisks indicate significant differences.

(born in 2002) and after selective breeding (born in 2003 and later). In the selective breeding during 2002, which brought lambs early in 2003, we used only three sires with the AQ/AR genotype. However, the frequencies of the PrP genotypes in lambs born during 2003 differed significantly from those in 2002. In 2005, three years after the implementation of selective breeding, the ratio of the AQ/AQ genotype was reduced from 52.2% to 20%, and that of the scrapie-resistant genotypes (AQ/AR and AR/AR) increased from 47.9% to 80%. However, no increase in the ratio of scrapie-resistant genotypes was observed between 2005 and 2006.

The results in this study revealed that the Japanese Suffolk sheep population, the main breed in Japan, is largely comprised of scrapie-susceptible sheep. The frequencies of the scrapie-susceptible genotypes are higher, and those of the resistant genotypes are lower than those reported in European countries [12]. In the UK, where selective breeding was started in accordance with the National Scrapie Plan, more than 90% of Suffolk sheep are reported to have the AR/AR or AQ/AR genotype [10]. Restriction on the use of rams carrying the VQ haplotype is the highest priority in the selective breeding of scrapie-resistant flocks. Although PrP genotype distributions differ with breed, the Suffolk and Poll Dorset sheep used here possessed the VQ haplotype. Selective breeding programs have not been implemented in Japan, however the high percentage of scrapie-susceptible sheep and the presence of the VQ haplotype suggest a requirement for selective breeding to raise the ratio of scrapie-resistant sheep.

Before beginning selective breeding, sires carrying AR/AR in the flock at HARC were rare so that we used sires with the AQ/AR genotype in the first two years of breeding. Although we used sires with the AQ/AR genotype and ewes were used randomly, the breeding program could convert flocks into more scrapie-resistant ones within 2 or 3 years. However, the increase in the ratio of scrapie-resistant sheep appeared to plateau between 2005 and 2006. This suggests that the use of sires with AR/AR and the selection of ewes based on PrP genotype are required to raise the ratio of scrapie-resistant genotypes to a higher level.

It is also important to determine whether the selection of sheep with a specific PrP genotype affects any traits such as meat quality and production. Several studies have reported that some traits, such as litter size, 135 days weight, and daily liveweight gain, could be influenced by PrP genotypes in some breeds [1, 5, 8, 19]. However, it is unlikely that unfavorable associations of scrapie-resistant PrP genotypes with performance parameters, if they really exist, will be a common occurrence in sheep. In fact, no association between PrP genotypes and traits has been observed in the Suffolk sheep produced by the selective breeding at HARC so far (Tokari et al. unpublished results). In Japan, the selective breeding for scrapie-resistance has not been implemented yet; however, because of the small number of sheep populations, it is relatively easy to control sheep PrP genotypes using high-performance sires with scrapie-resistant genotypes. The PrP genotyping carried out in this study will contribute to the selection of sires and ewes used for breeding in the future.

Unusual scrapie cases in sheep with the ARR/ARR and ARQ/ARR genotypes have recently been reported in Germany, the UK, France, and Belgium [6, 7, 10, 13]. This raises questions regarding the increase in sheep populations bearing the ARR haplotype, although the risk of the atypical scrapie to public health remains to be elucidated [3]. The unusual scrapie cases have not been recognized in Japan so far, thus increasing the frequency of scrapie-resistant genotypes is still believed to be effective in reducing and/or eliminating a prevalence of classical scrapie, even though sheep carrying the ARR/ARR genotype are not fully resistant to scrapie [18]. Sheep carrying ARR/ARR genotype were resistant to BSE [3, 4, 20] although not completely [2, 15]. This fact also suggests that selection of the ARR haplotype will contribute to reducing the possibility of human exposure to BSE via sheep. The control of PrP genotype distributions through genotyping, not only for codons 136 and 171, but also for codons 141 and 168 that appear to influence TSE susceptibility especially for atypical scrapie, in addition to continuous surveillance of TSE occurrence, will contribute to the control of TSE in small ruminants.

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