Survey of Natural Scrapie in Japan: Analysis of RFLP Types of the PrP Gene and Detection of PrP^{Sc} Mainly in Suffolk Sheep

Asako ONODERA, Tetsuya IKEDA, Motohiro HORIUCHI, Naotaka ISHIGURO, Misao ONUMA¹⁾, Norio HIRANO²⁾, Takeshi MIKAMI³⁾, Eiichi HONDA⁴⁾, Katsuya HIRAI⁵⁾, Kazunari KAI⁶⁾, Hiroyuki YUGI⁷⁾, Yasukazu MURAMATSU⁸⁾, and Morikazu SHINAGAWA*

Department of Veterinary Public Health, School of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, ¹⁾Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060, ²⁾Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka 020, ³⁾Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, ⁴⁾Department of Veterinary Microbiology, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu 183, ⁵⁾Department of Veterinary Microbiology, Faculty of Agriculture, Gifu University, Gifu 501-11, ⁶⁾Department of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, ⁷⁾Department of Veterinary Public Health, Faculty of Agriculture, Kagoshima University, Kagoshima 890, and ⁸⁾Department of Veterinary Public Health, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan

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ABSTRACT. We examined the brains, spleens and/or lymph nodes of 197 mainly Suffolk sheep collected from Hokkaido, and the Tohoku, Kanto and Chubu districts to detect PrP^{Sc} and thus to estimate scrapie contamination in Japan. Sixteen sheep in Hokkaido and 2 sheep in other districts that were introduced from Hokkaido were positive for PrP^{Sc}. By comparison of the frequencies of the restriction fragment length polymorphism (RFLP) types of these 18 scrapie sheep with 128 healthy sheep, we confirmed the association of specific RFLP types of the PrP gene with natural scrapie. The frequency of RFLP type I in scrapie sheep was significantly higher than that in healthy sheep and those of other types in scrapie sheep. In contrast, the frequencies of type II and VI in healthy sheep were higher than those in scrapie sheep. Therefore, type I sheep seemed to be susceptible but type II and VI sheep seemed to be resistant to natural scrapie in Suffolk sheep in Japan. Furthermore, we investigated the distribution of the RFLP types of the PrP gene in 161 sheep in Japan to learn about the genetic background of the susceptibility to scrapie. There were variations in the distribution of the RFLP types in each district.—**KEY WORDS:** PrP^{Sc}, RFLP type, scrapie.

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Scrapie is a transmissible neurodegenerative disease of sheep and goats. It is characterized by the presence of scrapie-associated fibril protein (PrP^{Sc}), which is a partially protease-resistant isoform of a host membrane glycoprotein (PrP^C) [19] and is the major component of scrapie-associated fibrils (SAF) [22, 27].

PrP^{Sc} is detected in the brain, spleen and lymph nodes of scrapie-affected animals [27, 29]. Ikegami *et al.* [19] reported that PrP^{Sc} was present in the biopsied lymph nodes of an experimentally infected sheep before the onset of the disease. In the experimental case of mice, PrP^{Sc} was detectable in the spleen and lymph nodes in the early stage of infection [6, 28].

Muramatsu *et al.* [23] reported that natural scrapie-infected sheep could be diagnosed at the preclinical stage through detection of PrP^{Sc} in the peripheral lymph nodes obtained by biopsy. They subsequently observed that some mice inoculated with a spleen homogenate of apparently healthy sheep whose lymph nodes were PrP^{Sc}-positive developed scrapie. These results suggest the possibility that scrapie can be diagnosed at the preclinical stage through detection of PrP^{Sc}.

The incubation period of scrapie is under the control of

alleles of *Sip* in sheep [5, 8] or *Sinc* in mice [18, 33]. Restriction fragment length polymorphisms (RFLP) of the PrP gene in sheep or mice are associated with the *Sip* alleles [10, 15] or *Sinc* alleles, respectively [1, 13, 18, 25, 26, 30, 33]. Hunter *et al.* [15] reported that RFLP types of the PrP gene classified by the restriction enzymes *Eco*RI and *Hind*III, are closely linked to the *Sip* character in Cheviot sheep inoculated with scrapie strain SSBP/1, *i.e.*, haplotypes e1 and h2 are linked to sA, while haplotypes e3 and h1 are linked to pA. The genotype and RFLP type of sheep which have extremely long incubation periods are *Sip* pApA and e3e3, respectively, whereas those of sheep showing short incubation periods are *Sip* sAsA or sApA and e1e1 or e1e3 [11, 14–17, 21].

Muramatsu *et al.* [24] classified the RFLP patterns of the PrP gene into six types (types I–VI) by using two enzymes, *Eco*RI and *Hind*III, in Suffolk sheep mainly raised in the northern Japanese island of Hokkaido. The frequency of RFLP type I found in natural scrapie sheep was much higher than that in healthy sheep, indicating that PrP gene polymorphisms are associated with the susceptibility to scrapie in Suffolk sheep as reported for Cheviot sheep [10, 14, 15, 21]. To further examine the relationship between the RFLP types of the PrP gene and the susceptibility to scrapie, an extensive survey was carried out.

In this study, we investigated the distribution of RFLP

* CORRESPONDENCE TO: SHINAGAWA, M., Obihiro University of Agriculture and Veterinary Medicine, W2-11, Inada-cho, Obihiro, Hokkaido 080, Japan.

types of the PrP gene in Japan to learn about the genetic background for susceptibility to scrapie. We also attempted to detect PrP^{Sc} in several organs to estimate the incidence of latent contamination with scrapie agent among sheep.

MATERIALS AND METHODS

Sheep: The total of 239 sheep examined in this study consisted of 229 Suffolk, 4 mixed breed Suffolk and Corriedale, 3 Corriedale, 2 Blue de Dorset and 1 St. Croix sheep. Eighty-seven of these sheep had been reported in previous studies [19, 23, 24] about the detection of PrP^{Sc} and/or RFLP analysis. The breeds of sheep are listed in Table 1. Sheep tissues from the central nervous system (CNS), spleen, lymph nodes, liver and/or blood, were obtained from 153 healthy sheep collected in slaughterhouses from 1988 to 1993 in various parts of Japan, mainly in Hokkaido and the Chubu district, and were examined for both PrP^{Sc} and RFLP analysis. The remaining samples derived from 86 sheep were collected in our laboratory, including 10 sheep diagnosed as having scrapie.

Detection of PrP^{Sc} by Western blot analysis: To detect PrP^{Sc} from CNS, spleen, and lymph nodes, SDS-PAGE and Western blot analyses were carried out as described previously [19]. Rabbit anti-sheep PrP serum diluted 1:5,000 was used as the primary antibody and horseradish peroxidase-linked anti-rabbit IgG-donkey IgG (Amersham, U.K.) diluted 1:5,000 was used as the secondary antibody. An ECL Western blotting detection system

(Amersham, U.K.) was used to detect the bands of PrP^{Sc} on the nitrocellulose membranes (Schleicher & Schuell, German).

Southern blot analysis: The preparation of high-molecular weight DNA from liver tissues or white blood cells, digestion with the restriction enzymes *Eco*RI (GIBCO BRL, MD) and *Hind*III (Takara, Japan), electrophoresis, blotting and hybridization of the sheep DNA were carried out as described previously [24]. A fragment of the sheep PrP coding region of about 700 bp was used as the probe and was labeled with [α -³²P]dCTP (Amersham, U.K.) by the random priming method [7].

RESULTS

Detection of PrP^{Sc}: Detection of PrP^{Sc} was carried out in 469 tissue samples from a total of 197 sheep collected from 1988 to 1993. Twenty-eight of 32 tissue samples from 14 sheep diagnosed as having scrapie by clinical and/or histopathological examinations, were positive for PrP^{Sc}. In addition to the samples from the scrapie sheep, PrP^{Sc} was detected in the spleen and lymph nodes from 4 apparently healthy sheep (Table 2). Although 13 of 14 sheep diagnosed as having scrapie by clinical and/or histopathological examinations were positive for PrP^{Sc} in the brain, 3 of 4 sheep at the preclinical stage of scrapie were negative. According to the individual information available for the scrapie sheep, all of them were produced in Hokkaido. PrP^{Sc} was not detected in 425 tissue samples from 179 healthy sheep, which were mainly collected in slaughterhouses.

RFLP analysis of the sheep PrP gene: A total of 161

Table 1. Breeds of sheep tested for PrP^{Sc} and RFLP

Breed ^{d)}	No.	Examinations			
		Detection of PrP ^{Sc}			Analysis of RFLP
		CNS ^{a)}	Spleen	Lymph	
S	63(19) ^{c)}	o ^{b)}	o	o	o
S	50(9)	o	o	o	
S	52(14)				o
S	33(5)	o			o
S	27(27)	o	o		
S	7	o			
S	3(1)	o	o		o
S	3(1)	o		o	o
S	1(1)		o	o	o
S	1(1)		o	o	
SxC	3(3)	o	o	o	o
SxC	1(1)			o	o
C	2(2)	o	o		
C	1(1)	o			o
B	1(1)	o	o	o	
B	1(1)	o	o		
SC	1				o
Total	250(87)	194	152	123	161

a) Central nervous system. b) Test was done. c) Numbers in parentheses indicate the sheep reported in the previous studies [19, 23, 24]. d) S, Suffolk. C, Corriedale. SxC, Mixed breed of Suffolk and Corriedale. B, Blue de Dorset. SC, St. Croix.

Table 2. Detection of PrP^{Sc} and RFLP type of scrapie sheep^{g)}

Sex ^{a)}	Age ^{b)} (Month)	Clinical signs	Histological lesions	Detection of PrP ^{Sc}			RFLP type
				CNS	Spleen	Lymph	
F	120	+	+	+	+	ND ^{e)}	IV
M	48	+	+	+	+	+	VI
F	60	+	NT ^{d)}	+	ND	ND	IV
M	10	+	+	+	+	+	V
F	19	+	+	+	+	+	III
M	14	+	+	+	+	+	III
F	29	+	+	+	ND	ND	III
F	76	+	+	+	+	+	I
F	52	+	+	-	+	+	I
F	48	+	+	+	-	+	V
F	48	+	+	+	-	ND	I
F	UK ^{c)}	+	+	+	ND	ND	II
F	35	+	+	+	+	ND	III
F	24	-	+	+	ND	-	I
M	5 ^{f)}	-	NT	-	+	+	I
F	48 ^{f)}	-	-	-	+	+	I
M	8 ^{f)}	-	-	-	+	+	I
M	36 ^{f)}	-	NT	+	+	+	I

a) M, male; F, female. b) Age at the onset of scrapie. c) Unknown. d) Not tested. e) Not determined. f) Age at euthanasia of the apparently healthy sheep. g) The remaining 179 sheep, which were negative for PrP^{Sc}, were omitted from this table.

Fig. 1. RFLP types of the sheep PrP gene. The RFLP types were designated as reported by Muramatsu *et al.* [24].

sheep, including 18 scrapie sheep, were examined for RFLP of the PrP gene with the restriction enzymes *EcoRI* and *HindIII*. We found six RFLP types, which were the same as those identified by Muramatsu *et al.* [24] (Fig. 1). Digestion with *EcoRI* showed 6.8 kb (e1) and 4.4 kb (e3) fragments of the PrP gene, whereas *HindIII* gave 5.0 kb (h1) and 3.4 kb (h2) fragments. In Southern DNA-DNA hybridization with the PrP probe, we were not able to detect the 5.2 kb fragment (e2) that was identified in Suffolk sheep in the United Kingdom [12].

Following the previous study [24], we increased the number of healthy and scrapie sheep examined for the RFLP type of the PrP gene. As the results, the distribution of RFLP types and the association of specific RFLP types with natural scrapie were obtained (Table 3). Although we found types II and VI, which had not yet been identified in natural scrapie in Japan [24], the frequencies of these types were the lowest in scrapie sheep. Type I was the most predominant RFLP pattern in natural scrapie sheep. The healthy sheep with types III and V showed high frequencies compared to those with other types. The frequency of type III in scrapie sheep was nearly the same as that in healthy sheep.

Frequencies of the PrP gene haplotypes: Three haplotypes, e1h1, e1h2 and e3h1, were defined by RFLP patterns in this study (Table 4). These haplotypes corresponded to *prp-a*, *prp-b* and *prp-c* reported by Muramatsu *et al.* [24]. The frequencies of these 3 haplotypes in natural scrapie sheep were compared to those in healthy sheep using chi-square tests of association. The frequency of haplotype e1h2 in scrapie sheep was significantly higher

Table 3. RFLP types of the PrP gene in the sheep

Group of sheep	Number and frequency (%)							Total
	I	II	III	IV	V	VI		
Healthy	No.	12	23	30	10	29	24	128
	%	9.4	18.0	23.4	7.8	22.6	18.7	
Scrapie	No.	8	1	4	2	2	1	18
	%	44.4	5.6	22.2	11.1	11.1	5.6	
Haplotypes		e1h2	e3h1	e1h2	e1h1	e1h2	e3h1	
		e1h2	e3h1	e3h1	e1h1	e1h1	e1h1	

Table 4. Frequencies of the PrP gene haplotypes

Haplotype	Fragment length (kb)		Healthy		Scrapie	
	<i>EcoRI</i>	<i>HindIII</i>	No.	%	No.	%
e1h1	6.8	5.0	73	28.5	7	19.4
e1h2	6.8	3.4	83	32.4	22	61.1
e3h1	4.4	5.0	100	39.1	7	19.4

than those of the other 2 haplotypes ($\chi^2 = 12.500$, $P < 0.05$) and that in healthy sheep ($\chi^2 = 11.487$, $P < 0.05$). The frequencies of the 3 haplotypes in healthy sheep did not show significant differences ($\chi^2 = 4.367$, $P > 0.05$).

Age analysis: As can be seen from Table 2, the ages of 13 sheep at the onset of scrapie were known, the average being 45.0 months (M). The average age of the sheep with each RFLP type was estimated as follows: type I, 50.0 M; type II, unknown; type III, 25.3 M; type IV, 90.0 M; type V, 30.0 M; type VI, 48.0 M. Since most of the sheep appear to be infected soon after birth [9], the age of onset represents the incubation period. The incubation period of type III was much shorter and that of type IV was much longer than the average value.

Distribution of RFLP types in Japan: To study the genetic background for the susceptibility to natural scrapie, we studied the distribution of each RFLP type in Japan (Table 5). Since we were not able to obtain tissue samples from sheep in the Kinki, Chugoku, Shikoku and Kyushu districts, where the number of sheep is very small, these districts were omitted from this table. Among the 161 samples examined for RFLP of the PrP gene, the most predominant RFLP type was type III, while type IV was relatively rare. However, some variation in the distribution of the RFLP types was observed in sheep raised in the different districts. The most dominant RFLP types were type III in Hokkaido (30.0%), type II in the Tohoku district (34.5%) and type V in the Chubu district (38.1%).

DISCUSSION

In this study, we investigated the incidence of scrapie contamination in Japan, the association of specific RFLP types of the PrP gene with natural scrapie in Suffolk sheep and the distribution of RFLP types in Japan to examine the genetic background for susceptibility to scrapie.

Table 5. Distribution of RFLP types in Japan

District ^{a)}	RFLP types							
		I	II	III	IV	V	VI	Total
Hokkaido	No. (%)	15 (18.7)	12 (15.0)	24 (30.0)	6 (7.5)	13 (16.3)	10 (12.5)	80
Tohoku	No. (%)	1 (3.4)	10 (34.5)	8 (27.6)	2 (6.9)	1 (3.4)	7 (24.2)	29
Kanto	No. (%)	2 (20.0)	1 (10.0)	3 (30.0)	2 (20.0)	1 (10.0)	1 (10.0)	10
Chubu	No. (%)	4 (9.5)	4 (9.5)	9 (21.4)	2 (4.8)	16 (38.1)	7 (16.7)	42
Total	No.	22	27	44	12	31	25	161

a) Kinki, Chugoku, Shikoku and Kyushu districts were omitted from this table.

With regard to the 18 scrapie sheep, including the 4 sheep with latent infections, it was noted that most of the sheep with clinical signs and histological lesions were positive for PrP^{Sc} in the brain, spleen and lymph nodes, whereas most of the insidiously scrapie-infected sheep without those changes were negative in the brain but positive in the spleen and lymph nodes. It is suggested that the accumulation of PrP^{Sc} in the brain follows the accumulation in the spleen and lymph nodes, and leads to the onset of the clinical signs and the appearance of scrapie lesions. This presumption is consistent with the previous observations in mice, hamster and sheep [3, 4, 6, 19, 20], regardless of the fact that PrP^{Sc} was not detected in the brain sample of one sheep showing clinical signs and histological lesions. In various regions of the scrapie-infected brain, there are wide variations in the accumulation of PrP^{Sc} [2, 4, 31, 32]. Therefore, the part of the brain examined in this study might have been unfit for the detection of PrP^{Sc}.

Concerning the detection of PrP^{Sc}-harboring sheep in each district, PrP^{Sc} was found in 16 sheep in Hokkaido, 1 sheep in Tohoku and 1 sheep in the Kanto district. Each scrapie sheep found in the Tohoku and Kanto districts had been introduced from Hokkaido. The occurrence of scrapie so far seems to be restricted to sheep produced in Hokkaido. Although the number of sheep obtained from the Chubu district was second to that from Hokkaido, PrP^{Sc} was not detected at all. Thus, the Chubu district seemed to be free from scrapie.

Because of an increase in the number of scrapie sheep examined for the RFLP type of the PrP gene, the frequency of type I in scrapie sheep (44.4%) was lower than in a previous study (70.0%) [24]. However, the frequency of type I (haplotype e1h2) in scrapie sheep was still much higher than that in healthy sheep or those of other types in scrapie sheep. Our results are consistent with the previous finding [24] that Suffolk sheep with type I are relatively susceptible to natural scrapie in Japan. In contrast, the frequency of type II in scrapie sheep was the lowest, but were high in healthy sheep. Therefore, type II

seems to be resistant to natural scrapie in Japan as reported in the United Kingdom [14, 16, 17]. Type III has been reported to be susceptible to scrapie in Cheviot sheep [10, 14, 15]. In Suffolk sheep, type III seems to be associated with a short incubation period as in Cheviot sheep. However, the frequency of type III in scrapie sheep was nearly the same as that in healthy sheep, indicating that type III in Suffolk sheep seems to have intermediate susceptibility to natural scrapie in contrast to that in Cheviot sheep. Type IV, which is homozygous for haplotype e1h1, has an extremely long incubation period, as previously reported for Suffolk sheep by Goldmann *et al.* [11]. Since type V had a short incubation period, haplotype e1h1 may be fully recessive for e1h2. However, the frequencies of types V and VI in healthy sheep were high compared to those in scrapie sheep. This result could have been affected by a large sheep population with type V derived from the Chubu district where scrapie had not yet occurred. Therefore, the association of type V with susceptibility to scrapie was not defined precisely. Type VI seemed to be generally resistant to scrapie. This observation corresponds to evidence that haplotypes e3h1 and e1h1 are associated with a long incubation period [11].

Regarding the distribution of RFLP types in Japan, the frequency of type I associated with high susceptibility to scrapie was the second highest in Hokkaido, where scrapie has occurred sporadically. In the Tohoku district, the frequencies of types II and VI associated with lower susceptibility were higher than those of other types.

We confirmed that the RFLP type I is associated with natural scrapie in Suffolk sheep in Japan. We also reported that thus far scrapie contamination is limited to Hokkaido. Therefore, the spread of scrapie infection may be prevented by restricting the introduction of sheep from Hokkaido.

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