NOTE *Public Health*

Identification of the first case of atypical scrapie in Japan

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ABSTRACT. A Corriedale ewe was confirmed as the first atypical scrapie case during an active surveillance program for transmissible spongiform encephalopathies in small ruminants in Japan. The animal was homozygous for the $AF_{141}RQ$ haplotype of *PRNP*. The animal showed clinical neurological signs possibly due to listeriosis before culling. Western blot analysis showed an unusual multiple banded pattern with a low-molecular fragment at ~7 kDa. Histopathology revealed suppurative meningoencephalitis caused by listeriosis in the brainstem. Fine granular to globular immunostaining of disease-associated prion proteins was mainly detected in the neuropil of the spinal tract of the trigeminal nerve and in the white matter of the spinocerebellar tract. Based on these results, this case was conclusively diagnosed as atypical scrapie with encephalitic listeriosis.

KEY WORDS: atypical scrapie, coinfection, listeriosis, *PRNP* genotype, surveillance

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Scrapie is a neurodegenerative and fatal disorder that causes abnormal behavior in small ruminants. Scrapie belongs to a group of transmissible spongiform encephalopathies (TSEs), otherwise known as prion diseases. More than 20 strains of scrapie have been classified in inbred mice on the basis of incubation period, lesion profile and distribution patterns of disease-associated prion proteins (PrP^{Sc}), which are misfolded cellular prion proteins [5].

A distinct phenotype of scrapie called atypical or Nor98 scrapie was initially recorded in sheep from Norway in 1998 [4]. Atypical scrapie has been sporadically reported in most European countries [2, 3] as well as in the United States [18] and Canada [19]. The origin of atypical scrapie remains hitherto unclear. Interestingly, atypical scrapie has been reported to occur both in the Falkland Islands [9] and in New Zealand [14], although no case of the classical form of scrapie has been reported in either of these countries. Atypical scrapie generally occurs in older sheep that are more than six years of age, suggestive of a spontaneous/sporadic form of the disease. Additionally, homo- or heterozygous genotypes of AHQ, AHR, ARR and ARQ at codons 136, 154 and 171 in the ovine prion protein gene (*PRNP*) are commonly associated with the resistance to classical scrapie [3, 4, 12, 13, 16]. Scrapie in sheep has occurred sporadically in Japan since 1981 [28], with 64 cases of classical scrapie in sheep confirmed to date. Six cases of classical scrapie in sheep have been identified since June 2003, the beginning of the active TSE surveillance program on fallen sheep, goats and deer older than 12 months, conducted by the Japanese Ministry of Agriculture, Forestry and Fisheries. However, prior to the present case, no case of atypical scrapie had been reported in Japan (Table 1). This short report describes the pathological and biochemical features of the first case of atypical scrapie in Japan.

The ewe investigated was a 133-month-old Corriedale who had been reared for the entirety of its life at the Fukuoka Agriculture and Forestry Research Center in Fukuoka prefecture. The animal displayed gait abnormality and circling 4 months prior to culling, and it appeared anorexic and weak for 3 months prior to culling. The animal was euthanized on March 15, 2016 under anesthesia due to astasia and neurological signs of disease. The brainstem region around the level of the obex, consisting of pons, medulla oblongata and C1 of the spinal cord, was sampled and sent to the National Institute of Animal Health (NIAH) for the detection of PrP^{Sc} by western blotting (WB) and immunohistochemistry (IHC). The WB procedure for detection of proteinase K-resistant PrP^{Sc} was performed by pretreating the sample with 40 μg/m*l* proteinase K for 30 min at 37°C. Sodium sulfate–polyacrylamide gel electrophoresis was then performed using NuPage pre-cast 12% Bis-Tris gels in MES running buffer (Invitrogen, Carlsbad, CA, U.S.A.). The blotted membranes were probed with the monoclonal antibody (mAb) P4 (R-Biopharm, Darmstadt, Germany) according to a previously described method [23]. IHC for PrP^{Sc} was performed using the mAb T1 [27] or F99/97.6.1 (VMRD, Pullman, WA, U.S.A.) as previously described [22–24]. Additionally, formalin-fixed and paraffin-embedded tissue sections were stained with an anti-*Listeria monocytogenes* (Lm) polyclonal antibody [1] and a secondary Alexa Fluor 546-conjugated antibody for dual immunofluorescence. We used brains of sheep affected with atypical scrapie obtained from the Veterinary Laboratories Agency (currently the Animal and Plant Health Agency, Addlestone, Surrey, U.K.) and sheep with classical scrapie from the NIAH archive as positive controls for the WB and IHC tests. A scrapie-negative

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Table 1. Number of samples tested as a result of active TSE surveillance between April 2003 and May 2016 in Japan

	Sheep	Goat	Deer	Total number	Classical scrapie	Atypical scrapie
2003	195	111	3	309	3[a)]	
2004	91	151	6	248		
2005	103	134	9	246	1[a)]	
2006	102	166	7	275		
2007	98	183	5	286		
2008	114	240	16	370		
2009	105	160	6	271		
2010	132	175	12	319		
2011	253	217	15	485	2[a)]	
2012	150	216	12	378		
2013	153	261	19	433		
2014	153	260	18	431		
2015	181	207	7	395		
2016	43	28	2	73		1[b)]
Total	1,873	2,509	137	4,519	6[a)]	1[b)]

a) All cases were confirmed as sheep scrapie,b) Present case.

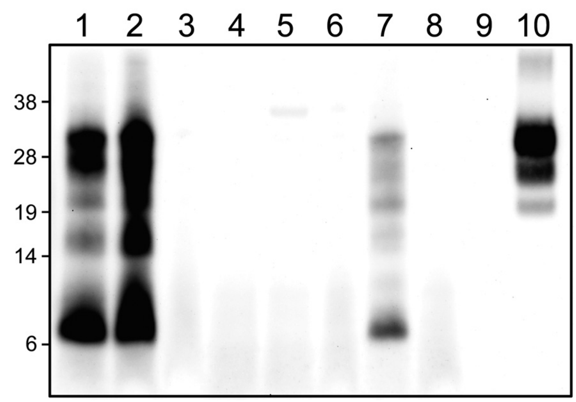

Fig. 1. Western blotting detection with mAb P4 of proteinase K-resistant PrP^{Sc} from the brain of sheep. Lane 1, atypical scrapie positive control (AHQ/AHQ sheep); lane 2, atypical scrapie positive control (AHQ/ARQ sheep); lane 3, offspring #1 with meningoencephalitis; lane 4, offspring #2 with meningoencephalitis; lane 5, flock mate goat #1 with meningoencephalitis; lane 6, flock mate goat #2 without meningoencephalitis; lane 7, present Japanese atypical case (obex); lane 8, negative control (sheep obex); lane 9, empty lane; lane 10, classical scrapie positive control (obex). Lanes 1-8 and lane 10 were loaded with 2 mg and 0.01 mg tissue equivalent, respectively. Molecular markers are shown on the left side (kDa).

brainstem sample was used as a negative control.

WB analysis of the present case (Fig. 1; lane 7) with mAb P4 revealed the same multiple banding pattern as the atypical scrapie positive control (Fig. 1; lanes 1 and 2), exhibiting a characteristic low-molecular fragment at ~7 kDa [6, 14, 26] which is clearly not present in the classical scrapie positive control (Fig. 1; lane 10).

Histopathological examination of the obex region revealed several foci of microabscess, mainly composed of neutrophils with some macrophages, located in the reticular formation and the nucleus of the spinal tract of the trigeminal nerve (Fig. 2A). Mild lymphocytic perivascular cuffing with hemorrhage was present adjacent to these micro-abscesses. A single intraneuronal vacuole, which seemed to be associated with scrapie, was detected in the dorsal motor nucleus of the vagus nerve (DMNV; Fig. 2B). A small number of fine granular to globular PrP^{Sc} deposits were identified in the DMNV using IHC staining (Fig. 2C). These deposits were mainly located in the neuropil of the spinal tract of the trigeminal nerve (Fig. 2D), in the white matter of the spinocerebellar tract and of the olivocerebellar tract, and in the neuropil throughout the brainstem and C1 of the spinal cord. Intraneuronal and other extracellular PrP^{Sc} staining was not detected in any of the examined sections. In addition, granular PrP^{Sc} was observed by dual immunofluorescence and found to be located at the periphery of axons, suggestive of an ad-axonal location in the white matter of the spinocerebellar tract and the olivocerebellar tract (Fig. 2E) [22]. Association of PrP^{Sc} with either astrocytes or microglia [23] was not detected in the brainstem (data not shown), agreeing with other reports of atypical scrapie cases [3, 25]. Short rod-shaped positive staining against Lm was located in the necrotic lesions (Fig. 2F). Immunohistochemical features, such as PrP^{Sc} staining types, neuroanatomical distribution patterns of PrP^{Sc} and magnitude of PrP^{Sc} deposition in the brainstem, were similar to those features of atypical scrapie in sheep that have been reviewed in the literature [3, 20, 29].

The present case was thus conclusively diagnosed as atypical scrapie co-infected with Lm based on both pathological and biochemical features [8]. To the best of our knowledge, this is the first report of atypical scrapie in sheep in Japan. There is no doubt that the ewe had histopathological and immunohistochemical features in the obex region that were characteristic of encephalitic listeriosis. A sheep affected with atypical scrapie may display circling and additional symptoms, but other neurological diseases that cause similar clinical signs, particularly listeriosis, should be considered along with atypical scrapie [15, 17]. In the present case, abnormal behavior observed prior to culling might have been a result of the meningoencephalitis that was caused by Lm. This study indicates the detection of PrP^{Sc} in the brains of aged sheep is necessary to discriminate atypical scrapie from other infectious diseases that may also cause circling.

The presence of a dimorphism at codon 141 (leucine [L] or phenylalanine [F]) has been found only in sheep with the ARQ genotype [21]. The *PRNP* genotype of the present case was $AF_{141}RQ/AF_{141}RQ$, which is known to be highly susceptible to atypical scrapie compared to the wild-type $AL_{141}RQ$ [6, 10, 12, 21]. The *PRNP* genotype rate from 975 sheep sampled between April 2003 and May 2016 was as follows: ARQ/ARQ, 46.5%; ARQ/ARR, 30.9%; and ARR/ARR, 10.1% (Table 2). The $L_{141}F$ substitution ($AF_{141}RQ$) was detected in 76 (7.8%) out of the 975 sheep, including both homozygous and heterozygous genotypes (Table 2). The amino acid sequences of sheep and goat prion proteins are similar to each other, but *PRNP* genotypes of goats are more variable and do not show polymorphisms at codon 136

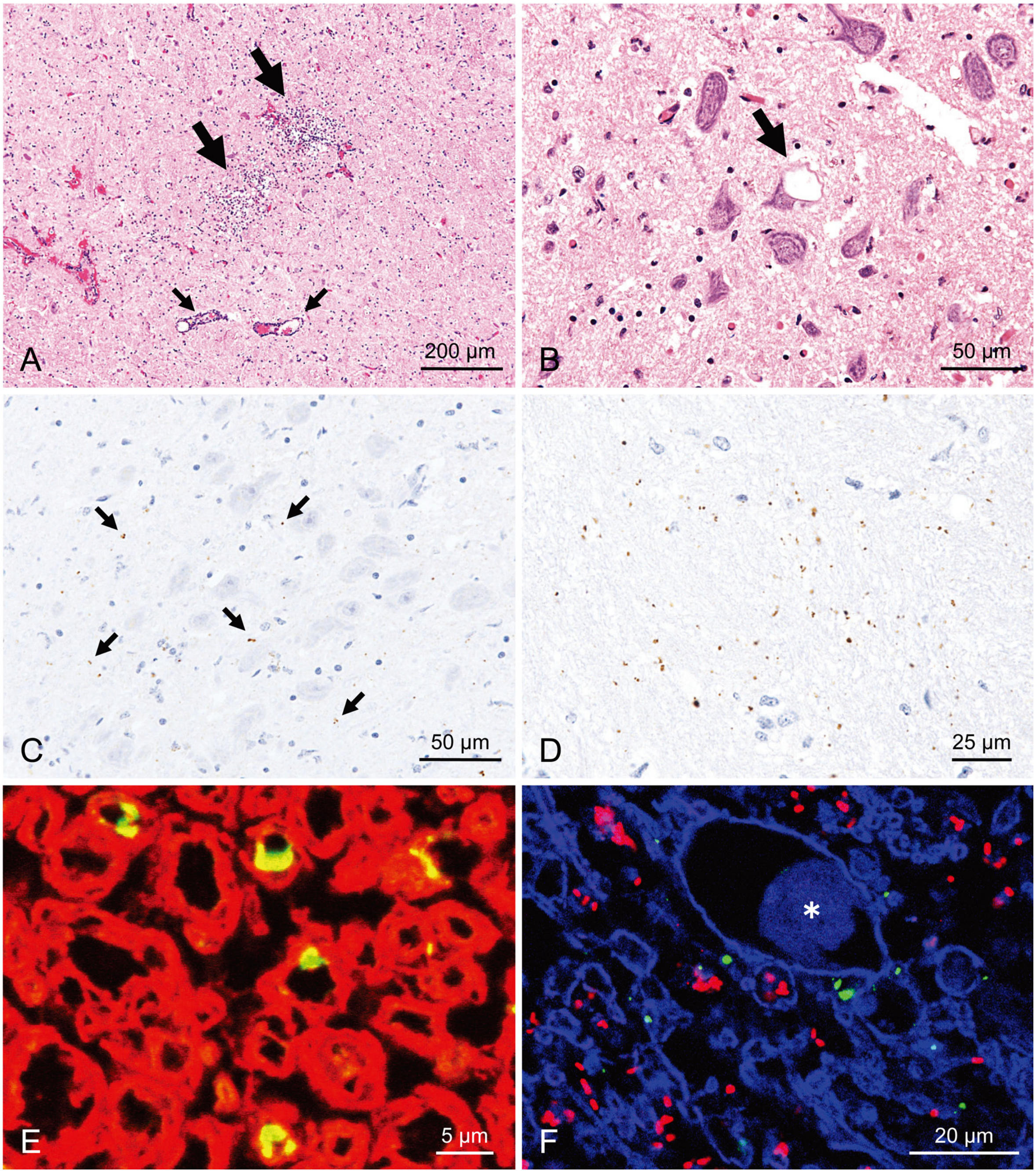

Fig. 2. Histopathological and immunohistochemical features in the medulla oblongata at the obex of the Japanese atypical scrapie sheep. A, Focal microabscesses composed of neutrophils with some macrophages (large arrows) and perivascular mononuclear cell cuffing (small arrow) at the nucleus of the spinal tract of the trigeminal nerve. B, A single intraneuronal vacuole (arrow) in the dorsal motor nucleus of the vagus nerve (DMNV). C, Fine granular PrP^{Sc} immunolabeling with mAb T1 (arrows) in the neuropil of the DMNV. D, Fine granular to globular PrP^{Sc} immunolabeling with mAb T1 in the spinal tract of the trigeminal nerve. E, Granular PrP^{Sc} at the ad-axonal location (yellow) in the white matter of the olivocerebellar tract. PrP^{Sc} (green; Alexa Fluor 488) and myelin sheath (red; Alexa Fluor 546) were labeled with mAb F99/97.6.1 and myelin basic protein (clone 12; Millipore, Billerica, MA, U.S.A.) and imaged by confocal microscopy (LSM 510; Carl Zeiss, Oberkochen, Germany). F, Colocalization of PrP^{Sc} (green; Alexa Fluor 488) and *Listeria monocytogenes* (red; Alexa Fluor 546) in the necrotic lesion of the spinocerebellar tract by dual immunofluorescence with mAb F99/97.6.1 and an antibody to *Listeria* followed with TO-PRO-3 counterstaining (blue). Asterisk (*) indicates axonal swelling.

Table 2. PRNP genotypes of sheep in Japan

Polymorphism at codons 136, 141, 154 and 171	Total	%
ALRQ/ALRQ	390	40
ALRQ/ALRR	301	30.9
ALRR/ALRR	98	10.1
ALRH/ALRQ	9	0.9
ALRH/ALRR	4	0.4
ALHQ/ALRQ	16	1.6
ALHQ/ALRR	14	1.4
ALHQ/ALRH	1	0.1
ALHQ/ALHQ	6	0.6
AFRQ/ALRQ	32	3.3
AFRQ/ALRR	21	2.2
AFRQ/ALHQ	9	0.9
AFRQ/AFRQ	8	0.8
AFRQ/VLRQ	4	0.4
AFRQ/ALRH	2	0.2
VLRQ/ALRQ	29	3
VLRQ/ALRR	20	2.1
VLRQ/ALRH	1	0.1
VLRQ/ALHQ	1	0.1
VLRQ/VLRQ	9	0.9
	975	100

Table 3. PRNP genotypes of goats in Japan

Polymorphisms at codons 136, 141, 154 and 171	Total	%
ALRQ/ALRQ	292	97.7
ALRQ/ALRR	4	1.3
ALRR/ALRR	1	0.3
AFRR/ALRR	1	0.3
ALHQ/ALHQ	1	0.3
	299	100

or 171 [11]. It has been reported that histidine (H) at codon 154 in goats is associated with atypical scrapie [7]. Interestingly, the $L_{141}F$ substitution, which is usually found in sheep, has also been found in a goat (Table 3) [7]. Eight animals, two goats and six Corriedale sheep, including three offspring, were reared in the same flock and culled due to an investigation of TSE. Two offspring sheep ($AF_{141}RQ/AF_{141}RQ$ and $AL_{141}RQ/AF_{141}RQ$) and one goat showed similar clinical signs and were diagnosed with listeriosis. The one remaining offspring ($AF_{141}RQ/AF_{141}RQ$), the two sheep flock mates ($AF_{141}RQ/AF_{141}RQ$ and $AL_{141}HQ/AL_{141}RQ$) and the other goat were all healthy. All offspring and flock mates were negative for atypical and classical scrapie (Fig. 1; lanes 3–6).

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