N.R. 1996. *Mycoplasma fermentans* simplifies our view of the catalytic core of ribonuclease P RNA. *RNA* **2**: 452–462.
14. Sykes, J.E., Lindsay, L. L., Maggi, R.G. and Breitschwerdt, E.B. 2010. Human coinfection with Bartonella henselae and two hemotropic mycoplasma variants resembling *Mycoplasma ovis*. *J. Clin. Microbiol.* **48**: 3782–3785.
15. Tanner, M.A. and Cech, T.R. 1995. An important RNA tertiary interaction of group I and group II introns is implicated in Gram-positive RNase P RNAs. *RNA* **1**: 349–350.
16. Tasker, S., Helps, C.R., Day, M.J., Harbour, D.A., Shaw, S.E., Harrus, S., Baneth, G., Lobetti, R.G., Malik, R., Beaufils, J.P., Belford, C.R. and Gruffydd-Jones, T.J. 2003. Phylogenetic analysis of hemoplasma species: an international study. *J. Clin. Microbiol.* **41**: 3877–3880.
17. Tasker, S., Peters, I.R., Mumford, A.D., Day, M.J., Gruffydd-Jones, T.J., Day, S., Pretorius, A.-M., Birtles, R.J., Helps, C.R. and Neimark, H. 2010. Investigation of human haemotropic mycoplasma infections using a novel generic haemoplasma qPCR assay on blood samples and blood smears. *J. Med. Microbiol.* **59**: 1285–1292.
18. Willi, B., Filoni, C., Catão-Dias, J.L., Cattori, V., Meli, M.L., Vargas, A., Martínez, F., Roelke, M.E., Ryser-Degiorgis, M.-P., Leutenegger, C.M., Lutz, H. and Hofmann-Lehmann, R. 2007. Worldwide occurrence of feline hemoplasma infections in wild felid species. *J. Clin. Microbiol.* **45**: 1159–1166.
19. Zuker, M. and Stiegler, P. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary. *Nucleic Acids Res.* **9**: 133–148.

NOTE *Virology*

Epidemiological Survey of Border Disease Virus among Sheep from Northern Districts of Japan

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ABSTRACT. The first epidemiological survey of *Border disease virus* (BDV) was undertaken in small ruminants in Japan. Ovine sera, collected from the northern prefectures of Hokkaido, Aomori and Iwate, were examined for the presence of antibodies against BDV using the neutralization peroxidase-linked antibody test. Twenty-nine (17.6%) of one hundred and sixty-five samples were seropositive for BDV. Results were specific, excluding cross-reactions with *bovine viral diarrhea virus* (BVDV). Only one sample (0.6%) was positive for BVDV, and was negative for BDV. Despite serological evidence of virus circulation, there have been no clinical cases of border disease in sheep in Japan. Although no diagnostic measures were performed, the infection did not appear to be associated with a reduction in ewe fertility nor with lamb mortality.

KEY WORDS: Border disease virus, epidemiology, Japan, *Pestivirus*, sheep.

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Border disease virus (BDV) is a recognized species in the genus *Pestivirus* of the family *Flaviviridae* [7]. Border disease (BD) affects mainly sheep and goats, and has substantial loss-related economic implications worldwide. In addition to cattle, goats, sheep and pigs, ruminant pestiviruses have been isolated from many other wild ruminant species, and serologic surveys have demonstrated infection with pestiviruses in more than 40 species worldwide [3, 24].

Sheep and goat farming is a relatively minor sector in Japan. Land dedicated to small ruminant farming is limited because the terrain mainly consists of mountains unsuitable for pasture use. The diversity of farms is consequently particularly reduced, with few available hectares, and economic problems associated with keeping animals. Furthermore, the winter climate conditions necessitate food supplementation, resulting in increased costs. The small ruminant population consists mainly of goats (21,000) and sheep (11,000) (data from Japan Livestock Industry Association 2000). In 1994, sheep numbered 24,000 and goats 30,000. This marked decrease probably arose because of a lack of adequate support for the optimization of animal farming and the development of production technologies. Ovine and caprine breeds are little studied, either from the animal husbandry or veterinary health aspect. Currently, the production of wool, milk and milk products is practically nonexistent. Only isolated and specific, but promising, studies are being undertaken at an embryonic stage.

At present in Japan, only Caprine Arthritis Encephalitis virus, Scrapie and Visna Maedi have been examined in small ruminant populations, having recently been the subject of epidemiological studies [10, 12, 13, 16, Giangaspero *et al.*, unpublished]. No previous epidemiological surveys on BDV have been undertaken in small ruminants in Japan. Furthermore, no clinical cases of BD have been reported among sheep flocks. The other genetically related *Pestivirus* species, namely bovine viral diarrhea virus (BVDV) type 1, BVDV type 2, and classical swine fever, have been previously reported in the country [8, 15, 19].

In order to verify the presence of BDV infection and to obtain a preliminary indication of its epidemiology, a serological survey of antibodies to BDV was carried out. This was to determine the prevalence of the virus in the northern prefectures in Japan, i.e., Hokkaido, Aomori and Iwate, where the largest concentrations of sheep, a total of 4,775 sheep (43%), are bred. The survey was performed on sheep raised both commercially and traditionally. Farmers were interviewed regarding flock management, productivity and losses, referring also to previous years, to define possible factors influencing the epidemiology of BDV infection, and to explore the potential impact of disease on sheep productivity.

Fourteen sheep flocks from Hokkaido, Aomori and Iwate were sampled from September 2007 to January 2008. The

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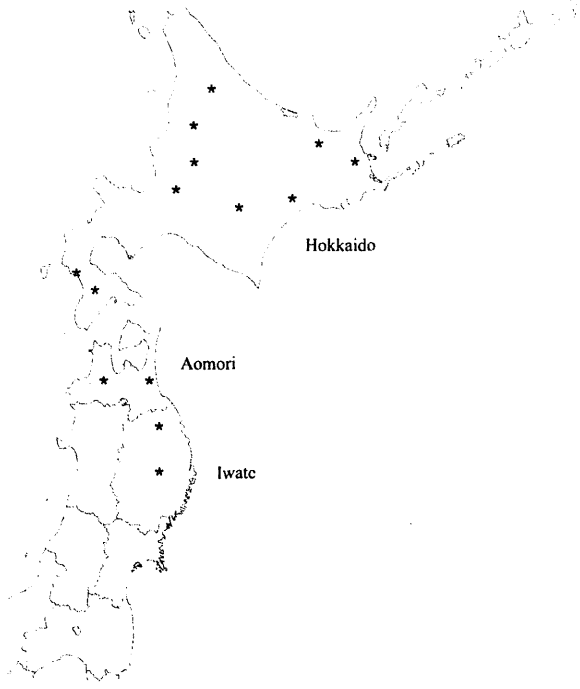

Fig. 1. Northern prefectures of Japan. Gray line: prefecture boundaries; light gray line: municipal boundaries; * sampling locations in Hokkaido, Aomori and Iwate prefectures.

number of flocks was identified according to the animal population of each prefecture and was representative of the livestock production systems in the country as a whole. Ten flocks were sampled in the Hokkaido prefecture, considering that about 37% of sheep breeding in Japan is concentrated in this region. The 10 flocks were arbitrarily chosen to include flocks from different parts of Hokkaido prefecture. The samples also included two flocks from Aomori prefecture and two from Iwate prefecture in the Tohoku region (Fig. 1).

Twenty sheep from each flock were selected for sampling, according to the national standard of flock composition (number of rams, ewes and yearlings). All age categories, from 1 to 12 years of age, were sampled. The majority of the sampled animals were Suffolk and Suffolk cross-breed. Lambs were not sampled to avoid interpretation difficulties arising from the potential presence of maternal antibodies. In 2 of the 14 flocks, only 11 and 16 sheep, respectively, were available for sampling, and therefore, a total of 267 serum samples were collected. All the sera were stored at –20°C prior to examination. The collected ovine sera were subsequently transported to laboratories in Italy and the United Kingdom for further analyses. The sera were imported legally into these countries, under the authorization of the Department of Public Veterinary Health, Nutrition and Food Safety, Ministry of Health, Rome, Italy, and license AHZ/963/93/4 issued under the terms of the Importation of Animal Pathogens Order by the Department for the Environment, Food and Rural Affairs, London, U.K.

Taking into account the genetic relatedness between BDV and BVDV, and the potential for cross-reactivity, detection of BDV antibodies was accompanied by anti-BVDV antibody screening. The presence of BDV antibodies was determined using the neutralization peroxidase-linked antibody (NPLA) test as described by Hyera *et al.* [11]. The sera underwent a serum neutralization test, followed by immunoperoxidase staining. Sera were double-diluted to a 1/40 and tested using BDV isolate S137/4 [23] and bovine turbinate cells. Cell lines were determined to be negative for pestivirus contamination. Peroxidase staining was performed using a mixture of monoclonal antibodies [WB103, WB105 & WB112 as provided by Animal Health and Veterinary Laboratories Agency (AHVLA), Surrey, U.K.] [5, 6, 17]. Testing for neutralizing antibodies against BVDV was performed by serum neutralization assay as described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organisation for Animal Health – Office International des Épizooties [25]. The BVDV CP strain NADL obtained from the American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.) [1] was grown on cell substrate bovine turbinate cells (Central Veterinary Laboratory, Surrey, U.K.).

Concerning flock production, the annual lambing rate was calculated as number of lambs born per ewes exposed to a ram, and was based on the lambing season occurring from February to April, with an exception being made for one farm where the reproductive cycle was related to three breeding seasons. Average annual wool yield referred to uncleaned material from shearing from April to May.

Percentages of sheep positive for antibodies to BDV among different age categories were compared using Fisher's exact probability test. Pearson's correlation coefficients were calculated for a possible relationship between the prevalence of BDV infection and production parameters such as annual lambing rate, annual lamb mortality rate, annual adult mortality rate, and annual wool yield. Differences were considered to be significant at $P<0.05$.

Out of the 267 serum samples, 16 had insufficient aliquots for testing, 18 were contaminated with fungi or bacteria, and 68 showed cytotoxic reactions, and therefore, all these samples (n=102) were excluded. As a result, a total of 165 samples were examined in the NPLA test, and 29 samples (17.6%) were observed to be positive for anti-BDV immunoglobulins (Table 1). Titers ranged from 20 to >40. Toxicity in the samples was indicated by cell death, probably caused by the sub-optimal condition of the samples.

BDV infection was detected in nine out of the 14 sampled flocks. Infection was found in flocks from Hokkaido and Iwate prefectures, but not from Aomori prefecture. The prevalence of BDV infection was found to vary between prefectures (Table 2). Sera showed positivity in 8 of the 10 flocks from Hokkaido and in 1 of the 2 flocks from Iwate prefecture. One flock from Hokkaido could not be evaluated because of cytotoxicity and insufficient serum quantity.

Table 1. Neutralization peroxidase-linked antibody (NPLA) test. Results of serological screening for antibodies to Border disease virus in sheep from prefectures of northern Japan

Flock No.	Prefecture	NPLA titer					Contaminated	Toxic	NE	Total /folck
		Negative	20	30	40	>40				
1	Hokkaido	19	0	1	0	0	0	0	0	20
2	Hokkaido	18	1	0	0	0	1	0	0	20
3	Hokkaido	15	0	1	0	2	1	0	1	20
4	Hokkaido	0	0	0	0	0	0	6	14	20
5	Hokkaido	12	3	2	0	3	0	0	0	20
6	Hokkaido	13	1	2	0	0	1	3	0	20
7	Hokkaido	13	1	3	0	0	3	0	0	20
8	Hokkaido	8	2	0	0	0	3	6	1	20
9	Hokkaido	14	0	0	0	0	0	6	0	20
10	Hokkaido	5	2	0	2	0	0	2	0	11
11	Iwate	6	0	0	0	0	2	12	0	20
12	Iwate	4	2	1	0	0	6	7	0	20
13	Aomori	8	0	0	0	0	1	11	0	20
14	Aomori	1	0	0	0	0	0	15	0	16
Total		136	12	10	2	5	18	68	16	267

NE: not executed because of insufficient aliquots for testing.

Table 2. Comparison between different prefectures of northern Japan for the percentage of sheep positive for antibodies to Border disease virus

Prefecture	Sampled flocks	% Positive	Samples	Samples not considered*	% Positive
Hokkaido	10	88.9	191	48	18.2
Iwate	2	50.0	40	27	23.1
Aomori	2	0	36	27	0
Total	14	69.2	267	102	17.6

* Samples showing toxicity (n=68), contamination (n=18) or not tested because of insufficient serum quantity (n=16).

None of the sera collected from Aomori prefecture were found to be positive. The percentage of sheep with positive sera was 18.2% in Hokkaido prefecture and 23.1% in Iwate prefecture. The mean prevalence of seropositive animals in individual herds ranged from 5.0 to 42.9% in the nine sampling groups from seropositive flocks.

Results were specific, and showed no cross-reactions with BVDV. Only one serum sample (0.6%) was positive for BVDV, and was negative for BDV. The sample was from a sheep in a flock from Hokkaido, showed a serum-neutralization titer of 1:256 against BVDV.

Comparison between different age categories showed that the highest percentage of sheep positive for antibodies to BDV was in animals 3 years old (45.5%), with another peak (33.3%) in animals 6 years of age (Table 3), but there was no statistically significant difference in the prevalence among different age categories.

Evaluation of the possible impact of BDV infection on production in the sampled flocks did not reveal any correlation with BDV seropositivity.

This survey has demonstrated that infection with BDV is present in sheep in the northern prefectures of Japan, and provides the first serological evidence of the occurrence of the disease in the country. Antibodies against BDV were found in two prefectures (Hokkaido and Iwate), out of three tested, where 43% of Japanese sheep are bred. This first report is based on specific serological results. Taking into account genetic relatedness among species in the genus *Pestivirus*, BVDV was investigated to exclude cross-reactivity. Only one serum sample was positive for BVDV, and was negative for BDV. Interestingly, the sample came from a sheep flock on a farm mainly focused on dairy cattle breeding, thus being in close contact with a herd of 700 Japanese Black cows, a possible source of the BVDV.

Despite serological evidence of virus circulation, no clinical cases of BD have been reported among sheep flocks in Japan. Furthermore, although no diagnostic measures were performed, the infection was not related to a reduction in sheep production, as determined by fertility, lambing rate and mortality in lambs.

The relatively high number of newly deposited sequences of BDV isolates from domestic and wild animals, and the recent evidence of novel "atypical" pestivirus sequences, in particular recent reports of atypical strains from clinical cases of BD from domestic and wild small ruminants [2, 4, 20–22], indicate that laboratory diagnosis of BD has to be expanded and it should not be limited in domestic ruminants but also include wild ruminants. Recent reports

Table 3. Comparison between different age categories for the percentage of sheep positive for antibodies to Border disease virus

Age category (year)	No. animals	No. excluded	No. tested	No. BDV positive	BDV positive: breed	%*
1	16	10	6	1	1 ewe: Corriedale	16.7
2	27	13	14	1	1 ewe: Cross breed	7.1
3	32	21	11	5	3 ewe: Corriedale; Suffolk 2 rams: Corriedale	45.5
4	57	14	43	8	7 ewes: Cross breeds Suffolk × Cheviot; Suffolk 1 ram: Cross breeds Suffolk × Cheviot	18.6
5	34	16	18	2	2 ewes: Suffolk; Cross breeds South down × Poll Dorset	11.1
6	26	9	17	6	6 ewes: Suffolk; cross breeds Suffolk × Cheviot; cross breed	35.3
7	33	11	22	3	3 ewes: Cross breeds Suffolk × Cheviot; Cross breeds South down × Poll Dorset	13.6
≥8	20	8	12	2	2 ewes: Suffolk; cross breeds South down × Poll Dorset	16.7
Not known	22	0	22	1	1 ewe: Suffolk	4.5
Total	267	102	165	29	29	17.6

* Percentage excluding samples with toxicity, contamination or not tested because of insufficient serum quantity.

demonstrated confirmed cases with isolates of ruminant pestivirus and disease with a significant population impact on free-range animals [14, 18, 22]. An outbreak of a previously unreported disease, associated with a new pestivirus belonging to the BDV virus cluster, was reported in Southern chamois (*Rupicapra pyrenaica*) in the Catalan Pyrenees (northeast Spain) in 2001 and 2002, and was supposedly responsible for a population decrease of 40–45% in the most affected areas [14]. In Japan, evidence for pestivirus infection in free-living Japanese serows (*Capricornis crispus*) has also been reported [9]. These recent cases indicate the potential for a disease to emerge in wild animal populations, with a possible severe impact on farmed populations. Furthermore, the role of wild animals as a reservoir for spreading the disease in domestic populations could increase the risk of mutation, resulting in increased virulence.

The demonstration of BDV circulation in sheep flocks in the prefectures of Hokkaido and Iwate, based on serological analysis, advances the knowledge on pathogens affecting domestic sheep in Japan. These interesting findings deserve further evaluations in order to examine the full extent of the problem in small ruminant populations, taking into account that BDV infection has a potential negative impact on reproduction. The complexity of the topic suggests the need for lager, more detailed epidemiological studies in the animal population and, considering the genetic heterogeneity of pestiviruses, molecular studies should be undertaken to extend the serological findings. Furthermore, investigations should include wild ruminants, because of concern for the protection of the valuable ruminant wild fauna present in Japan.

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REFERENCES

1. Colett, M. S., Larson, R., Gold, C., Strick, D., Anderson, D. K. and Purchio, A. F. 1988. Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. *Virology* **165**: 191–199.
2. De Mia, G. M., Greiser-Wilke, I., Feliziani, F., Giammarioli, M. and De Giuseppe, A. 2005. Genetic characterization of a caprine pestivirus as the first member of a putative novel pestivirus subgroup. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **52**: 206–210.
3. Doyle, L. G. and Heuschele, W. P. 1983. B.V.D.v. infection in captive exotic ruminants. *J. Am. Vet. Med. Assoc.* **183**: 1257.
4. Dubois, E., Russo, P., Prigent, M. and Thiéry, R. 2008. Genetic characterization of ovine pestiviruses isolated in France, between 1985 and 2006. *Vet. Microbiol.* **130**: 69–79.
5. Edwards, S., Sands, J. J. and Harkness, J. W. 1988. The application of monoclonal antibody panels to characterize pestivirus isolates from ruminants in Great Britain. *Arch. Virol.* **102**: 197–206.
6. Edwards, S., Moennig, V. and Wensvoort, G. 1991. The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. *Vet. Microbiol.* **29**: 101–108.
7. Fauquet, C. M., Mayo, M. A., Maniiloff, J., Desselberger, U. and Ball, L. A. 2005. Virus Taxonomy. Classification and Nomenclature of Viruses, Elsevier, Academic Press, San Diego.
8. Harasawa, R. and Giangaspero, M. 1999. Genetic variation in the 5' end and NS5B regions of classical swine fever virus genome among Japanese isolates. *Microbiol. Immunol.* **43**: 373–379.
9. Harasawa, R., Aoyama, F., Goryo, M., Okada, K., Nishimura, T., Matsubara, K., Tsujimoto, T. and Giangaspero, M. 2006. Evidence for pestivirus infection in free-living Japanese serows, *Capricornis crispus*. *Microbiol. Immunol.* **50**: 817–821.
10. Horiuchi, M., Nemoto, T., Ishiguro, N., Furuoka, H., Mohri, S. and Shinagawa, M. 2002. Biological and Biochemical Characterization of Sheep Scrapie in Japan. *J. Clin. Microbiol.* **40**: 3421–3426.
11. Hyera, J. M. K., Liess, B. and Frey, H-R. 1987. A direct neutralizing peroxidase-linked antibody assay for detection and titration of antibodies to bovine viral diarrhoea virus. *J. Vet. Med. B* **34**: 227–239.
12. Konishi, M., Tsuduku, S., Haritani, M., Murakami, K., Tsuboi, T., Kobayashi, C., Yoshikawa, K., Kimura, K. M. and Sentsui, H. 2004. An epidemic of caprine arthritis encephalitis in Japan: isolation of the virus. *J. Vet. Med. Sci.* **66**: 911–917.
13. Konishi, M., Haritani, M., Kimura, K., Tsuboi, T., Sentsui, H. and Murakami, K. 2007. Epidemiological survey and pathological studies on Caprine arthritis encephalitis (CAE) in Japan. *Bull. Natl. Inst. Anim. Hlth.* **113**: 23–30.
14. Marco, I., Rosell, R., Cabezon, O., Mentaberre, G., Casas, E., Velarde, R., Lopez-Olvera, J. R., Hurtado, A. and Lavin, S. 2008. Epidemiological study of border disease virus infection in Southern chamois (*Rupicapra pyrenaica*) after an outbreak of disease in the Pyrenees (NE Spain). *Vet. Microbiol.* **127**: 29–38.
15. Nagai, M., Sato, M., Nagano, H., Pang, H., Kong, X., Murakami, T., Ozawa, T. And Akashi, H. 1998. Nucleotide sequence homology to bovine viral diarrhea virus 2 (BVDV 2) in the 5' untranslated region of BVDVs from cattle with mucosal disease or persistent infection in Japan. *Vet. Microbiol.* **60**: 271–276.
16. Okada, K. and Yonemichi, H. 1982. Slow virus infections and nasal carcinoma in sheep. *Iwate Vet.* **8**: 77–91.
17. Paton, D. J., Sands, J. J. and Edwards, S. 1994. Border disease virus: delineation by monoclonal antibodies. *Arch. Virol.* **135**: 241–252.
18. Pioz, M., Loison, A., Gibert, P., Dubray, D., Menaut, P., Le Tallec, B., Artois, M. and Gilot-Fromont, E. 2007. Transmission of a pestivirus infection in a population of Pyrenean chamois. *Vet. Microbiol.* **119**: 19–30.
19. Sakoda, Y., Ozawa, S., Damrongwatanapokin, S., Sato, M., Ishikawa, K. and Fukusho, A. 1999. Genetic heterogeneity of porcine and ruminant pestiviruses mainly isolated in Japan. *Vet. Microbiol.* **65**: 75–86.
20. Thabti, F., Letellier, C., Hammami, S., Pepin, M., Ribiere, M., Mesplede, A., Kerkhofs, P. and Russo, P. 2005. Detection of a novel border disease virus subgroup in Tunisian sheep. *Arch. Virol.* **150**: 215–229.
21. Valdazo-Gonzalez, B., Alvarez-Martinez, M. and Greiser-Wilke, I. 2006. Genetic typing and prevalence of Border disease virus (BDV) in small ruminant flocks in Spain. *Vet. Microbiol.* **117**: 141–153.
22. Valdazo-Gonzalez, B., Alvarez-Martinez, M. and Sandvik, T. 2007. Genetic and antigenic typing of border disease virus isolates in sheep from the Iberian Peninsula. *Vet. J.* **174**: 316–324.
23. Vilček, S., Nettleton, P. F., Paton, D. J. and Belak, S. 1997. Molecular characterization of ovine pestiviruses. *J. Gen. Virol.* **78**: 725–735.
24. Weber, K. H. 1982. Uber das Vorkommen des BVD-MDv. bei zerviden in Rheinland-Pfal. *Dtsch. Tierärztl. Wochenschr.* **89**: 1–3 (in German).
25. World Organisation for Animal Health (OIE). 2008. Bovine viral diarrhoea, Chapter 2.4.8. p. 704. *In*: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE, Paris.

NOTE *Bacteriology*

Two Genetic Clusters in Swine Hemoplasmas Revealed by Analyses of the 16S rRNA and RNase P RNA Genes

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ABSTRACT. Only two hemoplasma species, *Eperythrozoon parvum* and *Mycoplasma suis*, have been recognized in pigs. Here we demonstrate the genetic variations among six hemoplasma strains detected from pigs, by analyzing the 16S rRNA and RNase P RNA (*rnpB*) genes, and propose a novel hemoplasma taxon that has not been described previously. Phylogenetic trees based on the nucleotide sequence of the 16S rRNA gene indicated that these six hemoplasmas were divided into two clusters representing *M. suis* and a novel taxon. We further examined the primary and secondary structures of the nucleotide sequences of the *rnpB* gene of the novel taxon, and found it distinct from that of *M. suis*. In conclusion, we unveiled a genetic cluster distinct from *M. suis*, suggesting a new swine hemoplasma species or *E. parvum*. Our findings also suggest that this novel cluster should be included in the genus *Mycoplasma*.

KEY WORDS: Eperythrozoon, hemoplasma, mycoplasma.

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Hemoplasma is a trivial name for the group of hemotrophic prokaryotes that lack a cell wall, but have never been cultured *in vitro* [10]. This group is composed of former *Eperythrozoon* and *Hemobartonella* species as well as newly identified hemotrophic mycoplasmas. Currently only two hemoplasma species, *Eperythrozoon parvum* and *Mycoplasma suis* previously known as *E. suis*, are recognized in pigs (*Sus scrofa domestica*) [11, 12, 18]. Hemoplasmas parasitize the cell surface of swine erythrocyes, where it causes membrane deformation and damage [10, 25]. Damaged erythrocytes are subsequently removed from the blood circulation, resulting in hemolytic anemia and icterus in pig [10].

The clinical signs of *M. suis* infection are variable, but in the acute form, sows usually develop anemia, icterus and anorexia for a few days [6], and it causes severe anemia in newborn and weaned piglets [5]. Chronic carrier states have been associated with reproductive failures in sows and decreased weight gains in piglet [26]. Mycoplasmas have host specificity to some extent, but human infections with *M. suis* have been reported in China, suggesting a zoonotic pathogen [23, 24]. Pathogenicity of *E. parvum* has not well been documented, but it also causes severe anaemia accompanied with fever on splenectomised pig [1].

In the present study, we examined the genetic variations among swine hemoplasmas detected from a commercial farm in Japan. Heparin-anticoagulated blood samples collected from 12 pigs in Morioka (latitude 39.7N and longitude 141.1E), Japan in December 2010, were stored at –80°C prior to analysis. Total DNA was extracted from 200 μl of the Heparin-anticoagulated whole blood samples by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, eluting into 200 μl of buffer AE, and stored at –20°C until examination in the PCR assay.

For the preliminary screening of hemoplasma infections by real-time PCR, specific PCR primers (forward primer: 5'-ATATTCCTACGGGAAGCAGC-3' and reverse primer: 5'-ACCGCAGCTGCTGGCACATA-3') for the 16S rRNA gene of hemoplasmas were used as described previously [22]. After real-time PCR, melting experiment was performed as described previously [4]. Six (50%) out of the 12 blood samples tested by real-time PCR were found to be positive for hemplasma infection. No mixed infection with both the hemoplasma species was found in this screening test. The six samples designated Morioka1, Morioka4, Morioka5, Morioka6, Morioka8, and Morioka9 were divided into two clusters, A and B, according to the melting temperature (*Tm*) levels. *Tm* (mean ± SE) of the cluster A consisting of Morioka5, Morioka6, Morioka8 showed lower temperature at 82.40 ± 0.08°C, and the cluster B including Morioka1, Morioka4 and Morioka9 showed higher temperature at 83.33 ± 0.22°C.

These six positive samples were further subjected to endpoint PCR to amplify entire region of the 16S rRNA gene by using universal primers as described previously [22]. DNA extracted from PCR products was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Six nucleotide sequences of the almost entire region of the 16S rRNA gene have been deposited to the DDBJ, EMBL, GSDB and NCBI nucleotide sequence databases under the accession numbers AB610845 to AB610850.

A phylogenetic tree constructed with the neighbor-joining method [16] from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model [7]

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divided the six swine hemoplasmas into two clusters, A and B, as same as the *Tm* values in the real-time PCR experiments (Fig. 1). Nucleotide sequences of Morioka5, Morioka6 and Morioka8 in cluster A were identical and closely related to *M. suis* (more than 99% homology) in the phylogenetic tree of the 16S rRNA gene sequences. On the contrary, the three strains, Morioka1, Morioka4 and Morioka9, in cluster B were distinct from the former sequences (less than 95% homology), suggesting a different taxon in the hemoplasma taxonomy according to the cutoff value of the 16S rRNA gene sequence identity for species definition [2].

We have further examined the nucleotide sequences of the RNase P RNA (*rnpB*) gene of the cluster B hemoplasmas, a distinct taxon revealed by 16S rRNA sequences, to compare with other hemoplasma species [14]. The *rnpB* gene has been shown to be suitable for phylogenetic discrimination of closely related taxonomic groups when examined by the 16S rRNA sequence comparison [20]. The *rnpB* gene of only the strains Morika1, Morika4, and Morioka9 in cluster B was successfully amplified by end-pint PCR with forward primer [5'-TATTTAAAGTAGAG-GAAAGTC-3' equivalent to nucleotide numbers 10 to 30 of *M. suis* (EF523602)] and reverse primer [5'-GAGGAGTT-TACCGCGTTT-3' equivalent to nucleotide numbers 206 to 223 of *M. suis* (EF523602)]. Reaction was the same as used for the amplification of the 16S rRNA gene except for the annealing temperature at 56°C instead of 58°C. PCR product was then subjected to direct sequencing as described previously and the nucleotide sequences of the *rnpB* gene of these strains, which were identical, have been deposited to the DNA database under the accession numbers AB641639 to AB641641. A phylogenetic tree was constructed with the *rnpB* gene sequences of these three strains with other hemoplasma sequences from DNA databases (Fig. 2). The *rnpB* gene sequence of the three strains in cluster B showed less than 90% homology to *M. suis*, and it included deletion of three serial nucleotides in the gene, supporting the notion

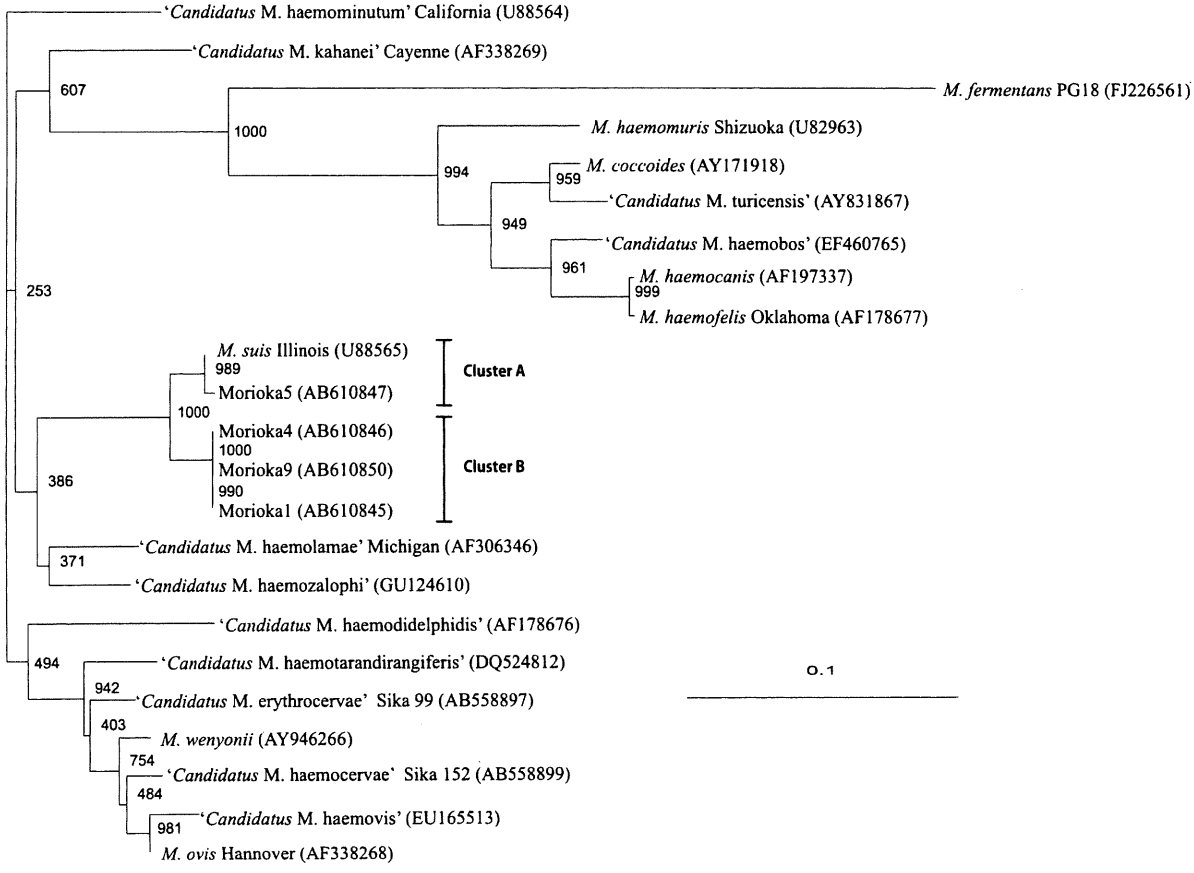

Fig. 1. A phylogenetic tree based on the 16S rRNA gene comparison among mycoplasmas including 18 hemoplasma species (accession numbers are given in a parenthesis) and putative taxa created by clusters A and B. Genetic distances were compared with CLUTAL W [21]. Morioka5, representative of morioka6 and Morioka8 in cluster A was identified as *M. suis*. Morioka4, Morioka9 and Morioka1 in cluster B were distinct from *M. suis*. Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications. Scale bar indicates the estimated evolutionary distance.

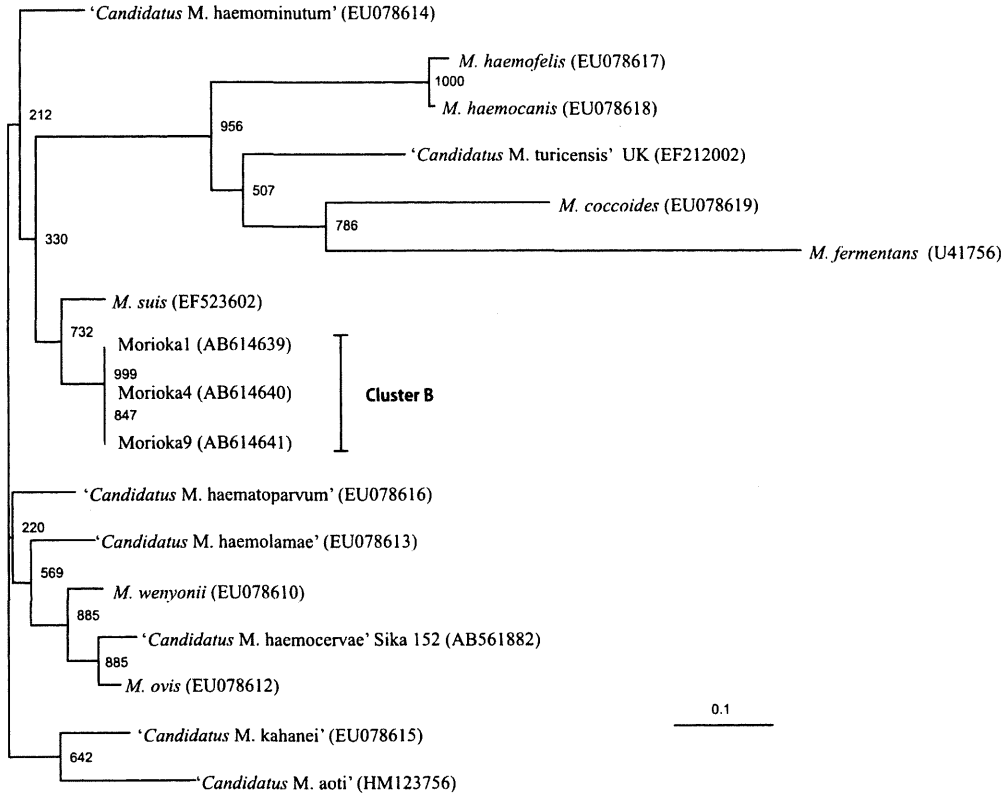

Fig. 2. A phylogenetic tree generated by partial *rnpB* gene sequences among mycoplasmas including 13 hemoplasma species (accession numbers are given in a parenthesis) and a putative taxon as cluster B. Genetic distances were compared with CLUTAL W [21]. Hemoplasma strains Morioka1, Morioka4 and Morioka9 in cluster B were detected from a commercial swine farm in the Iwate prefecture. Numbers in the relevant branches refer to the values of bootstrap probability of 1,000 replications. Scale bar indicates the estimated evolutionary distance.

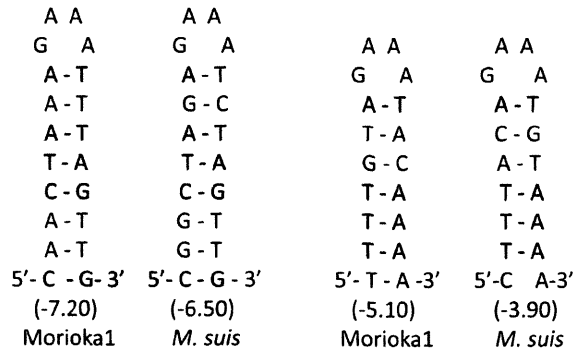

Fig. 3. Comparison of the representative secondary structures containing the GAAA tetraloop modules in the *rnpB* gene. *M. suis* sequences in the left and right structures correspond to the nucleotide numbers 93–112 and 189–206, respectively, of the accession number EF523602. Homologous sequences between *M. suis* and Morioka1 are shown on a gray background. Minimum free energy (Kcal/mol) is given in a parenthesis.

that the cluster B was distinct from *M. suis*.

As an additional means of assessing genetic relatedness between *M. suis* and cluster B, we compared the secondary structures of *rnpB* predicted according to the algorithm of Zuker and Stiegler [27]. Two copies of the GAAA tetraloop module were allocated in the *rnpB* gene in the cluster B as well as *M. suis*. Transitional substitutions between pyrimidines and purines were evident in the stem regions of the GAAA tetraloop modules (Fig. 3).

The GAAA tetraloop has high affinity for the motif called tetraloop receptor and exerts an important function in ribozyme activity [8, 9]. Nucleotide base-pairings at the stem region were variable between these two hemoplasma taxa, but were capable to form a stable secondary structure to minimize free energy. The folding energy of the each GAAA tetraloop module showed substantial negative free energy [3]. Palindromic nucleotide substitutions in base-pairings correspond to radical evolutionary changes, which can generate new genotypes or species. Several secondary structure modules in *rnpB* molecules have been predicted from the phylogenetic comparison, which play the interac-

tion with a defined structural motif. Analysis of the secondary structures may provide a clear picture for species identification. Point mutations occur continuously and at random through the prokaryotic genome at every multiplication phase. Although point mutations occur in both the translated and untranslated regions at the same rate, incidence of some nucleotide substitutions, observed in the controlling regions of prokaryotic genomes, are biased by the selection of lethal mutation. These lethal mutations are not obvious in critical regions of the prokaryotic *rnpB* gene that is responsible for processing the 5' end of tRNA by cleaving a precursor and leading to tRNA maturation. The secondary structure differences delineated the differences between the two species more clearly than the nucleotide sequence alignments, which only showed a small number of differences and some of these are common to both species. Therefore, comparison of the secondary structures may be more meaningful than the phylogenetic analysis of the *rnpB* nucleotide sequences.

There have only been two hemoplasma species, *M. suis* and *E. parvum*, recognized in swine [18], that have been distinguished by morphology and pathogenecity [17, 19]. In the present study, we demonstrated two genetic clusters among swine hemoplasmas. One of them was classified *as M. suis* according to nucleotide sequence homology of the 16S rRNA gene. The nucleotide sequence of the 16S rRNA gene of *M. suis* was first determined on the Illinois strain which had been maintained by serial passage in pigs, and deposited in DNA databases under the accession number U88565 [15]. However, *E. parvum* has not been maintained *in vivo* so far, and this has long hampered to identification of this particular species. The accumulated data in the present study suggested the cluster B strains, distinct from *M. suis* by phylogenic analysis, were thus a new hemoplasma species or most likely to be *E. parvum*, which is the only hemoplasma species remaining in the genus *Eperythrozoon* [13]. All the other recognized species in this genus have already been transferred to the genus *Mycoplasma* [11, 12]. Our results indicated that the novel cluster was genetically close to the *Mycoplasma* species, suggesting that this taxon should be included in the genus *Mycoplasma*. In conclusion, the present study reports the existence of two genetically distinct clusters among swine hemoplasmas, representing *M. suis* and a provisional *M. parvum*.

REFERENCES

1. Barnett, S. F. 1963. *Eperythrozoon parvum* in pig in Kenya. *Bull. Epiz. Dis. Afr.* **11**: 185–195.
2. Drancourt, M. and Raoult, D. 2005. Sequence-based identification of new bacteria: a proposition for creation of an orphan bacterium repository. *J. Clin. Microbiol.* **43**: 4311–4315.
3. Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, M., Caruthers, M. H., Nielson, T. and Turner, D. H. 1986. Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. U.S.A.* **83**: 9373–9377.
4. Harasawa, R., Mizusawa, H., Fujii, M., Yamamoto, J., Mukai, H., Uemori, T., Asada, K. and Kato, I. 2005. Rapid detection and differentiation of the major mycoplasma contaminants in cell cultures using real-time PCR with SYBR Green I and melting curve analysis. *Microbiol. Immunol.* **49**: 859–863.
5. Henderson J. P., O'Hagan, J., Hawe, S. M. and Pratt, M. C. 1997. Anaemia and low viability in piglets infected with *Eperythrozoon suis*. *Vet. Rec.* **140**: 144–146.
6. Henry, S. C. 1979. Clinical observations on eperythrozoonosis. *J. Am. Vet. Med. Assoc.* **174**: 601–603.
7. Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111–120.
8. Krasilnikov, A. S., Yang, X., Pan, T. and Mondragon, A. 2003. Crystal structure of the specificity domain of ribonuclease P. *Nature* **421**: 760–764.
9. Massire, C., Jaeger, L. and Westhof, E. 1997. Phylogenetic evidence for a new tertiary interaction in bacterial RNase P RNAs. *RNA* **3**: 553–556.
10. Messick, J. B. 2004. Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. *Vet. Clin. Pathol.* **33**: 2–13.
11. Neimark, H., Johansson, K. E., Rikihisa, Y. and Tully, J. G. 2001. Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with the descriptions of '*Candidatus* Mycoplasma haemofelis', '*Candidatus* Mycoplasma haemomuris', '*Candidatus* Mycoplasma haemosuis' and '*Candidatus* Mycoplasma wenyonii'. *Int. J. Syst. Evol. Microbiol.* **51**: 891–899.
12. Neimark, H., Johansson, K. E., Rikihisa, Y. and Tully, J. G. 2002. Revision of haemotrophic mycoplasma species names. *Int. J. Syst. Evol. Microbiol.* **52**: 683.
13. Neimark, H., Peters, W., Robinson, B. L. and Stewart, L. B. 2005. Phylogenetic analysis and description of *Eperythrozoon coccoides*, proposal to transfer to the genus *Mycoplasma* as *Mycoplasma coccoides* comb. Nov. and request for an opinion. *Int. J. Syst. Evol. Microbiol.* **55**: 1385–1391.
14. Peters, I. R., Helps, C. R., McAuliffe, Neimark, H., Lapin, M. R., Gruffydd-Jones, T. J., Day, M. J., Hoelzle, L. E., Willi, B., Meli, M., Hofmann-Lehmann, R. and Tasker, S. 2008. RNase P RNA gene (*rnpB*) phylogeny of hemoplasmas and other *Mycoplasma* species. *J. Clin. Microbiol.* **46**: 1873–1877.
15. Rikihisa, Y., Kawahara, M., Wen, B., Kociba, G., Fuerst, P., Kawamori, F., Suto, C., Shibata, S. and Futohashi, M. 1997. Western immunoblot analysis of *Haemobartonella muris*, and comparson of 16S rRNA gene sequences of *H. muris*, *H. felis*, and *Eperythrozoon suis*. *J. Clin. Microbiol.* **35**: 823–829.
16. Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
17. Seamer, J. 1960. Studies with Eperythrozoon parvum Splitter, 1950. *Parasitology* **50**: 67–80.
18. Splitter, E. J. 1950. *Eperythrozoon suis* n. sp. and *Eperythrozoon parvum* n. sp., two new blood parasites of swine. *Science* **111**: 513–514.
19. Splitter, E. J. 1953. *Eperythrozoon parvum*, a filtrable blood parasite of swine. *Nature* **172**: 40.
20. Täpp, J., Thollesson, M. and Hermann, B. 2003. Phylogenetic relationships and genotyping of the genus *Streptococcus* by sequence determination of the RNase P RNA gene, *rnpB*. *Int. J. Syst. Evol. Microbiol.* **53**: 1861–1871.
21. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids*

Res. **22**: 4673–4680.
22. Watanabe, Y., Fujihara, M., Obara, H., Matsubara, K., Yamauchi, K. and Harasawa, R. 2010. Novel hemoplasma species detected in free-ranging Sika deer (*Cervus nippon*). *J. Vet. Med. Sci.* **72**: 1527–1530.
23. Yao, C., Liang, A., Yuan, C., Xia, J., Bo, L., Zheng, W., Yang, Z. and Hua, X. 2010. Overexpresssion of complement receptor type 1 (CR1, CD35) on erythrocytes in patients with hemoplasma infection. *Microbiol. Immunol.* **54**: 460–465.
24. Yuan, C. L., Liang, A. B., Yao, C. B., Yang, Z. B., Zhu, J. G., Cui, L., Yu, F., Zhu, N. Y., Yang, X. W. and Hua, X. G. 2009. Prevalence of *Mycoplasma suis* (*Eperythrozoon suis*) infection in swine and swine-farm workers in Shanghai, China. *Am. J. Vet. Res.* **70**: 890–894.
25. Zachary, J. F. and Basgall, E. J. 1985. Erythrocyte membrane alterations associated with the attachment and replication of *Eperythrozoon suis*: a light and electron microscopic study. *Vet. Pathol.* **22**: 164–170.
26. Zinn, G. M., Jesse, G. W. and Dobson, A. W. 1983. Effect of eperythrozoonosis on sow productivity. *J. Am. Vet. Med. Assoc.* **182**: 369–371.
27. Zuker, M. and Stiegler, P. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary. *Nucleic Acids Res.* **9**: 133–148.

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Epidemiological survey for visna-maedi among sheep in northern prefectures of Japan

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Summary

Ovine sera collected from the northern Prefectures of Hokkaido, Iwate and Aomori in Japan, were examined for the presence of antibodies against visna-maedi virus using the agar gel immunodiffusion test and enzyme-linked immunosorbent assays. Three animals (1.12%), out of 267 samples tested, were found to be seropositive to the visna-maedi antigens in both tests. Levels of infection were found in flocks from Hokkaido and Iwate Prefectures, but not in the Aomori Prefecture. Nucleic acid detection by polymerase chain reaction on serum samples did not give positive results. Although no diagnostic measures were in place, the infection could not be related to losses in sheep production or to reduced survival rates. The very limited visna-maedi distribution indicates a highly favourable condition for the application of eradication strategies in this area.

Keywords

Japan, Sheep, Virus, Visna-maedi.

Indagine epidemiologica per visna-maedi in pecore in prefetture settentrionali del Giappone

Riassunto

Sieri ovini prelevati nelle Prefetture settentrionali di Hokkaido, Iwate e Aomori in Giappone, sono stati esaminati per la presenza di anticorpi contro il virus visna-maedi usando i test di immuno-diffusione su gel di agar e enzyme-linked immunosorbent assays. Tre animali (1,12%), su 267 campioni testati, sono risultati sieropositivi per l'antigene visna-maedi in entrambi i test. Livelli d'infezione sono stati trovati in greggi provenienti dalle Prefetture di Hokkaido e Iwate, ma non nella Prefettura di Aomori. La messa in evidenza di acido nucleico attraverso la reazione a catena della polimerasi sui campioni di siero non ha dato esito positivo. Sebbene non siano state applicate misure diagnostiche, l'infezione non ha potuto essere messa in relazione a perdite di produzione ovina o a ridotti tassi di sopravvivenza. La distribuzione molto

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limitata di visna-maedi indica una condizione particolarmente favorevole per l'applicazione di strategie di eradicazione in questa area.

Parole chiave
Giappone, Ovini, Virus, Visna-maedi.

Introduction

Visna-maedi is a chronic and persistent infectious disease of sheep and rarely goats that is caused by the visna-maedi virus, family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Lentivirus*, which is genetically related to caprine arthritis encephalitis virus (CAEV) (7). Most infections are asymptomatic but once clinical signs appear, usually after a long incubation period, the disease most often progresses slowly, but irreversibly, and invariably has a lethal outcome. In certain regions, serological conversion may reach 25% in young animals and up to 85% in adults (1, 2, 5). However, the level of clinically diseased animals does not generally exceed 20% in affected flocks (4, 19). Visna-maedi is economically significant due to premature culling of animals, and export restrictions, as this disease is notifiable to the World Organisation of Animal Health (*Office International des Épizooties*: OIE). This infection, also known as ovine progressive pneumonia, *zwoegerziekte*, *Graaff-Reinet disease* and *Montana progressive pneumonia*, first described in 1954 in Iceland (17), occurs in different parts of the world, in most sheep-producing countries. Only Australia, New Zealand and Iceland are considered free of the disease. At present in Japan, among ovine lentiviruses, only CAEV has been examined, having recently been the object of extensive epidemiological studies (10, 11). Clinical suspicion of the disease was reported in Shiba goats from a farm in the Nagano Prefecture in 2002. Japan had been assumed to be free of the disease, since there were no records or related diagnostic methods available.

Although visna-maedi has not been sufficiently investigated in Japan, virus-like particles associated with intranasal tumour of the ethmoid olfactory mucosa in sheep were first observed by electron microscopy in samples obtained from sheep originating from the Takikawa city area in Hokkaido in 1978 (20). Furthermore, in 1982, 79 serum samples were collected from sheep, between 1 and 7 years of age, originating from the Prefecture of Hokkaido and tested using the enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies (12). Seven of these sera were found to be positive. No eradication measures have been taken and no further investigations have been reported. According to OIE's World Animal Health Information System, Japan is currently free from visna-maedi and is the object of general surveillance only.

In order to verify the presence of the infection and to obtain a preliminary picture of its epidemiology 30 years after the first report, a serological survey of antibodies to the visna-maedi virus was conducted. This was to determine the prevalence of the virus in the northern prefectures in Japan, namely: Hokkaido, Aomori and Iwate, where most Japanese sheep, a total of 4 775 sheep (43%), are bred. The survey was performed on sheep raised both commercially and traditionally. Farmers were interviewed regarding flock management, productivity and losses, incidents that may have occurred in previous years, to define possible factors that influence the epidemiology of the infection and to explore the potential impact of disease on sheep productivity.

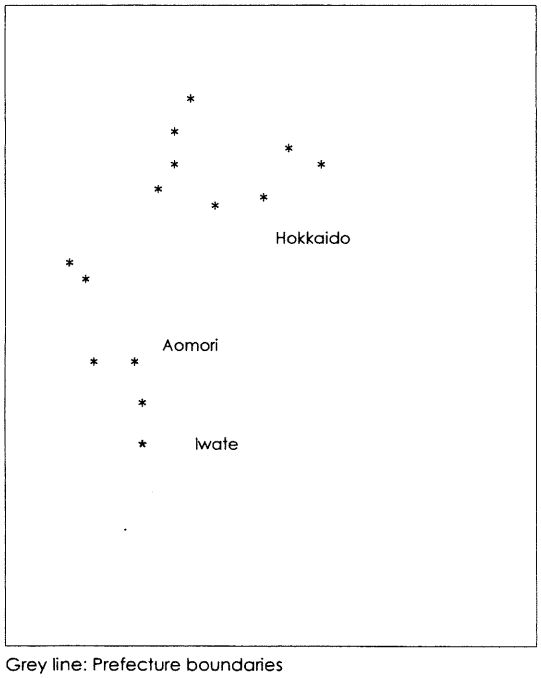
Materials and methods

Sample collections

Fourteen sheep flocks from the three northern prefectures in Japan (Hokkaido, Iwate and Aomori), were sampled from September 2007 to January 2008 (Fig. 1). The number of flocks was chosen according to the animal population of each prefecture (Table I) and is representative of the livestock production systems in the country. Ten flocks were sampled in the Hokkaido Prefecture, considering that about 37% of sheep breeding in Japan is concentrated in this region. The sampling was completed with four flocks from

the Tohoku area, two from Iwate Prefecture and two from Aomori Prefecture (Table II).

Twenty sheep from each flock were selected for sampling, according to the national standard of flock composition (number of rams, ewes and yearlings). All age categories, from one year to 12 years of age, were sampled. Lambs were not sampled to avoid interpretation difficulties due to the potential presence of maternal antibodies. A total of 267 serum samples were collected. All the sera were stored at –20°C prior to examination. The ovine sera collected were subsequently transported to laboratories in Italy and the United Kingdom for further analysis. The sera were legally imported into these countries under the authorisation permitted by the Department of Public Veterinary Health, Nutrition and Food Safety, Ministry of Health in Rome, and license AHZ/963/93/4 issued under the terms of the Importation of Animal Pathogens Order by the Department for Environment, Food and Rural Affairs in London, respectively.



Grey line: Prefecture boundaries

* Sampling locations in Hokkaido, Aomori and Iwate Prefectures

Figure 1
Northern prefectures of Japan

Agar gel immunodiffusion test

A commercial agar gel immunodiffusion (AGID) test kit for the detection of visna-maedi antibodies was used (Maeditect 1000, Veterinary Laboratories Agency, Weybridge). Briefly, strain WLC-1 and ovine reference sera precipitating the visna-maedi virus envelope glycoprotein 135, were used to perform the test. Reading was performed up to 14 days of incubation. Doubtful reactions were retested with 1:2 diluted antigen. Sera that formed lines of non-identity peripheral to the major glycoprotein line were retested. Curved lines and lines of identity with the control serum lines were considered positive, excluding non-specific reactions.

Enzyme-linked immunosorbent assay

The ELISA was performed using the Pourquier® ELISA visna-maedi/CAEV serum screening kit (Institute Pourquier, Montpellier), with coated 96-well flat-bottom microplates. This was based on the use of an immunogenic peptide of the transmembrane protein and of the recombinant protein P28 which enters into the composition of the viral capsid. Test sera were diluted 1/20 with phosphate buffered saline (PBS)-Tween 20 and 200 µl of this dilution were placed into a coated ELISA microplate well. A duplicate 200 µl 1/20 dilution series of positive and negative ovine reference antisera were placed in each test plate. Plates were incubated at 37°C for 1 h on a rotary shaker. After subsequent washing, 100 µl of peroxidise-labelled rabbit anti-ruminant immunoglobulin G diluted 1/100 in PBS-blocking reagent were added to each well and incubated at 37°C for 1 h on a rotary shaker. The reaction was revealed with 100 µl of tetramethylbenzidine per well after washing, then stopped after 20 min with 100 µl of 0.5 M sulphuric acid per well. Optical density (OD) reading was performed using a spectrophotometer at 450 nm (Microplate Reader 680, Bio-Rad, Hercules, California). Validation criteria for the diagnostic system were a minimal mean OD 450 value of 0.350 of the positive control serum, and a mean OD 450 value positive negative control ratio ≥3.5. Sera were considered positive when their sample to positive (S/P) ratio (S/P = 100 × [OD 450 value of

Table I
Sheep population in the different prefectures of Japan in 2000
(Japan Livestock Industry Association)
A total of 11 121 sheep are reported from 947 farms
Sheep breeding is absent in the Prefectures of Okinawa, Kochi and Tottori

Prefecture	Farms	Animals	Percentage	Prefecture	Farms	Animals	Percentage
Hokkaido	334	4.135	37.18	Aichi	9	110	0.98
Aomori	28	165	1.48	Mie	1	20	0.17
Iwate	34	475	4.27	Shiga	0	140	1.25
Miyagi	37	267	2.4	Kyoto	10	58	0.52
Akita	25	214	1.92	Osaka	5	65	0.58
Yamagata	11	278	2.49	Hyogo	4	108	0.97
Fukushima	85	519	4.66	Nara	7	51	0.45
Ibaraki	14	188	1.69	Wakayama	10	35	0.31
Tochigi	7	227	2.04	Shimane	7	50	0.44
Gunma	23	812	7.3	Okayama	11	85	0.76
Saitama	15	121	1.08	Hiroshima	8	181	1.62
Chiba	7	252	2.26	Yamaguchi	8	62	0.55
Tokyo	8	89	0.8	Tokushima	1	27	0.24
Kanagawa	30	249	2.23	Kagawa	1	31	0.27
Niigata	4	48	0.43	Ehime	9	14	0.12
Toyama	4	60	0.53	Fukuoka	9	77	0.69
Ishikawa	3	23	0.2	Saga	0	49	0.44
Fukui	7	30	0.26	Nagasaki	6	87	0.78
Yamanashi	21	130	1.16	Kumamoto	12	397	3.56
Nagano	62	422	3.79	Oita	13	69	0.62
Gifu	23	258	2.31	Miyazaki	2	52	0.46
Shizuoka	30	356	3.2	Kagoshima	2	26	0.23

the sample – OD450 value of the negative control]/[mean OD 450 value of the positive control – OD450 value of the negative control]) was higher than or equal to 120%. Doubtful results were recorded when the S/P ranged between 110% and 120%. Samples with a S/P≤110% were considered negative.

Virus genome and integrated 'provirus' detection

Attempts to isolate the visna-maedi virus genome and integrated 'provirus' detection in the ovine serum samples were performed using a polymerase chain reaction (PCR) based on the method described by Zhang *et al.* (21). Due to insufficient serum aliquots, out of the 267 sera collected, 237 samples were submitted for the analysis.

Nucleic acid extraction

DNA was extracted from 200 µl of serum, using the Qiagen blood mini kit (Catalogue No. 51306), according to the manufacturer's instructions (Qiagen handbook, 'Blood or body fluids, spin protocol', November 2007 edition). The DNA was eluted in a final volume of 200 µl elution buffer. RNA was extracted from 140 µl of serum, using the Qiagen QIAamp® viral RNA mini kit (Catalogue No. 52906), in accordance with the instructions of the manufacturer. The RNA was eluted in a final volume of 60 µl elution buffer. The quality and quantity of nucleic acids were evaluated spectrophotometrically and were stored at –20°C until tested.

Table II
Flocks submitted to serological screening for antibodies to visna-maedi in sheep from prefectures of northern Japan

Flock	Flock characteristics			Animals sampled			Visna-maedi seropositive animals (AGID, ELISA)
	No. of animals	Flock composition	Breeds	No.	Gender		
					Rams	Ewes	
1	50	5 males, 35 ewes, 10 yearling	Cross-breed, Suffolk, Cheviot, Romanov, Black, South Down and Corriedale	20	0	20	1 ewe Romanov (H/1/10)
2	84	4 rams, 47 ewes, 33 yearlings	Suffolk, some South Down and Black	20	0	20	
3	410	10 rams, 200 ewes 200 yearlings	Suffolk	20	0	20	
4	178	4 rams, 80 ewes, 94 yearlings	Suffolk	20	3	17	1 ewe Suffolk (H/4/8)
5	600	13 rams , 270 ewes, 30 yearlings, 287 lambs	Poll Dorset, cross-breeds Suffolk × Cheviot	20	1	19	
6	221	1 ram, 80 ewes, 40 yearlings, 100 lambs	South Down × Poll Dorset cross-breeds	20	0	20	
7	82	2 rams, 29 ewes, 14 yearlings, 37 lambs	Suffolk × Suffolk cross-breed	20	0	20	
8	700	260 ewes, 440 yearlings	Cross-breed, Romanov, Poll Dorset, Suffolk	20	0	20	
9	100	2 rams, 40 ewes, 58 yearlings	Cross-breed, Suffolk, Romanov, Friesland, Black Welsh Mountain	20	12	8	
10	35	25 ewes, 10 lambs	Corriedale	11	8	3	
11	34	2 rams, 24 ewes, 8 yearlings	Cross-breed, Suffolk, Romanov, Cheviot, Corriedale	20	1	19	1 ewe Cheviot (I/11/6)
12	52	1 ram, 30 ewes, 21 yearlings	Suffolk	20	1	19	
13	34	1ram, 26 ewes, 7 yearlings	Suffolk	20	0	20	
14	22	22 ewes	Suffolk	16	0	16	
Total				267	26	241	3 ewes

Flock numbers 1 to 10 represent Hokkaido
Numbers 11 and 12 represent Iwate
Numbers 13 and 14 represent Aomori Prefectures, respectively

AGID agar gel immunodiffusion test
ELISA enzyme-linked immunosorbent assay

Oligonucleotides

Two primers amplifying a 217 base-pair region of the *pol* gene of the visna-maedi virus were selected, namely: 4231_F (5′-ata gta aat ggc atc aag atg c-3′) and 4448_R (5′-tcc cga att tgt ttc tac cc-3′), based on the POL4 and POL5 sequences published by Zhang *et al.* (21).

cDNA synthesis

Prior to reverse transcription, 10 µl RNA solution was denatured with 1 µl primer 4448_R (50 pmol/ µl) for 5 min at 65°C, then chilled rapidly. The reaction mixture was incubated at 42°C for 60 min after the addition of 8 µl first-strand buffer (Invitrogen, Carlsbad, New Mexico), 2 µl dNTP mix (10 mM each) (Promega, Madison, Wisconsin), 80 U RNasin (Promega) and 400 U Superscript II reverse transcriptase (Invitrogen). The total volume of the reaction was 40 µl. Following this reaction, the enzyme was inactivated by heating at 70°C for 15 min.

Polymerase chain reaction

The amplification of the viral sequence was performed in micro-tubes, using 5 µl of cDNA or 30 µl of DNA as template. The reaction mixture consisted of 5 µl 10X buffer (Promega), 6 µl 25 mM $MgCl_2$ (Promega), 1 µl dNTP mix (10 mM each) (Promega), 1 µl each of primers 4231_F and 4448_R (50 pmol/ µl each), and 2.5 U taq polymerase in buffer A (Promega), with water added to a total of 50 µl. The reaction cycle was carried out 35 times with denaturation at 94°C for 45 sec, annealing at 51°C for 30 sec and extension at 72°C for 60 sec followed by a final elongation step at 72°C for 300 sec in a thermocycler with a heated lid. PCR products were detected by agarose gel electrophoreses.

Results

Using the AGID test, three samples out of the 267 sera examined were positive for anti-visna-maedi virus gp135 immunoglobulins (Table III); this corresponds to a prevalence rate of 1.12%. All sera were seen to be positive after 24 h of incubation; none of the other tested sera showed a positive reaction at all during observation over the following two weeks. Two sera (samples H/1/10 and I/11/6) formed strong lines of identity peripheral to the major glycoprotein line. Serum sample H/4/8 showed a weaker identity line.

Corresponding results were obtained using the ELISA (Table IV). High OD values (>3.5) were recorded with serum samples H/1/10 and I/11/6 that showed strong precipitating lines of identity in the AGID test. Serum sample H/4/8 was also positive, but with a lower OD value (3.137), corresponding to the weaker identity line observed by AGID. All the other tested sera were negative by ELISA, with no doubtful reactions.

Attempts to detect the visna-maedi viral genome and integrated 'provirus' by PCR in the ovine serum samples were performed. No

Table III
Agar gel immunodiffusion test: results of serological screening for antibodies to visna-maedi virus in sheep from Prefectures of northern Japan

Flock/ sample	Result	Flock/ sample	Result	Flock/ sample	Result	Flock/ sample	Result	Flock/ sample	Result
Hokkaido 1		Hokkaido 2		Hokkaido 3		Hokkaido 4		Hokkaido 5	
1	Negative	1	Negative	1	Negative	1	Negative	1	Negative
2	Negative	2	Negative	2	Negative	2	Negative	2	Negative
3	Negative	3	Negative	3	Negative	3	Negative	3	Negative
4	Negative	4	Negative	4	Negative	4	Negative	4	Negative
5	Negative	5	Negative	5	Negative	5	Negative	5	Negative
6	Negative	6	Negative	6	Negative	6	Negative	6	Negative
7	Negative	7	Negative	7	Negative	7	Negative	7	Negative
8	Negative	8	Negative	8	Negative	8	Positive	8	Negative
9	Negative	9	Negative	9	Negative	9	Negative	9	Negative
10	Positive	10	Negative	10	Negative	10	Negative	10	Negative
11	Negative	11	Negative	11	Negative	11	Negative	11	Negative
12	Negative	12	Negative	12	Negative	12	Negative	12	Negative
13	Negative	13	Negative	13	Negative	13	Negative	13	Negative
14	Negative	14	Negative	14	Negative	14	Negative	14	Negative
15	Negative	15	Negative	15	Negative	15	Negative	15	Negative
16	Negative	16	Negative	16	Negative	16	Negative	16	Negative
17	Negative	17	Negative	17	Negative	17	Negative	17	Negative
18	Negative	18	Negative	18	Negative	18	Negative	18	Negative
19	Negative	19	Negative	19	Negative	19	Negative	19	Negative
20	Negative	20	Negative	20	Negative	20	Negative	20	Negative

Table III (contd)
Agar gel immunodiffusion test: results of serological screening for antibodies to visna-maedi virus in sheep from Prefectures of northern Japan

Flock/ sample	Result	Flock/ sample	Result	Flock/ sample	Result	Flock/ sample	Result	Flock/ sample	Result
Hokkaido 6		Hokkaido 7		Hokkaido 8		Hokkaido 9		Hokkaido 10	
1	Negative	1	Negative	1	Negative	1	Negative	1	Negative
2	Negative	2	Negative	2	Negative	2	Negative	2	Negative
3	Negative	3	Negative	3	Negative	3	Negative	3	Negative
4	Negative	4	Negative	4	Negative	4	Negative	4	Negative
5	Negative	5	Negative	5	Negative	5	Negative	5	Negative
6	Negative	6	Negative	6	Negative	6	Negative	6	Negative
7	Negative	7	Negative	7	Negative	7	Negative	7	Negative
8	Negative	8	Negative	8	Negative	8	Negative	8	Negative
9	Negative	9	Negative	9	Negative	9	Negative	9	Negative
10	Negative	10	Negative	10	Negative	10	Negative	10	Negative
11	Negative	11	Negative	11	Negative	11	Negative	11	Negative
12	Negative	12	Negative	12	Negative	12	Negative		
13	Negative	13	Negative	13	Negative	13	Negative		
14	Negative	14	Negative	14	Negative	14	Negative		
15	Negative	15	Negative	15	Negative	15	Negative		
16	Negative	16	Negative	16	Negative	16	Negative		
17	Negative	17	Negative	17	Negative	17	Negative		
18	Negative	18	Negative	18	Negative	18	Negative		
19	Negative	19	Negative	19	Negative	19	Negative		
20	Negative	20	Negative	20	Negative	20	Negative		
Iwate 11		Iwate 12		Aomori 13		Aomori 14			
1	Negative	1	Negative	1	Negative	1	Negative		
2	Negative	2	Negative	2	Negative	2	Negative		
3	Negative	3	Negative	3	Negative	3	Negative		
4	Negative	4	Negative	4	Negative	4	Negative		
5	Negative	5	Negative	5	Negative	5	Negative		
6	Positive	6	Negative	6	Negative	6	Negative		
7	Negative	7	Negative	7	Negative	7	Negative		
8	Negative	8	Negative	8	Negative	8	Negative		
9	Negative	9	Negative	9	Negative	9	Negative		
10	Negative	10	Negative	10	Negative	10	Negative		
11	Negative	11	Negative	11	Negative	11	Negative		
12	Negative	12	Negative	12	Negative	12	Negative		
13	Negative	13	Negative	13	Negative	13	Negative		
14	Negative	14	Negative	14	Negative	14	Negative		
15	Negative	15	Negative	15	Negative	15	Negative		
16	Negative	16	Negative	16	Negative	16	Negative		
17	Negative	17	Negative	17	Negative				
18	Negative	18	Negative	18	Negative				
19	Negative	19	Negative	19	Negative				
20	Negative	20	Negative	20	Negative				

Table IV
Enzyme-linked immunosorbent assay results for the detection of antibodies against visna-maedi among 267 sheep sera from northern prefectures of Japan

Flock/ sample	OD value	S/P ratio	Flock/ sample	OD value	S/P ratio	Flock/ sample	OD value	S/P ratio	Flock/ sample	OD value	S/P ratio
Hokkaido 1			Hokkaido 2			Hokkaido 3			Hokkaido 4		
1	0.161	9.09	1	0.492	33.15	1	0.092	4.07	1	0.159	8.94
2	0.148	8.14	2	0.074	2.76	2	0.085	3.56	2	0.133	7.05
3	0.094	4.22	3	0.065	2.11	3	0.053	1.24	3	0.07	2.47
4	0.155	8.65	4	0.213	12.87	4	0.23	14.10	4	0.077	2.98
5	0.305	19.56	5	0.117	5.89	5	0.204	12.21	5	0.645	44.27
6	0.055	1.38	6	0.078	3.05	6	0.253	15.78	6	0.108	5.23
7	0.042	0.44	7	0.135	7.20	7	0.206	12.36	7	0.098	4.51
8	0.118	5.96	8	0.13	6.83	8	0.31	19.92	8	3.137	225.45
9	0.082	3.34	9	0.096	4.36	9	0.106	5.09	9	0.106	5.09
10	>3.5	>250	10	0.071	2.54	10	0.078	3.05	10	0.123	6.32
11	0.339	22.03	11	0.266	16.72	11	0.169	9.67	11	0.083	3.42
12	0.099	4.58	12	0.116	5.82	12	0.101	4.73	12	0.083	3.42
13	0.099	4.58	13	0.069	2.40	13	0.325	21.01	13	0.117	5.89
14	0.072	2.62	14	0.065	2.11	14	0.14	7.56	14	0.094	4.22
15	0.114	5.67	15	0.048	0.87	15	0.082	3.34	15	0.075	2.84
16	0.253	15.78	16	0.082	3.34	16	0.061	1.82	16	0.073	2.69
17	0.197	11.70	17	0.08	3.20	17	0.077	2.98	17	0.135	7.20
18	0.095	4.29	18	0.076	2.91	18	0.068	2.33	18	0.197	11.70
19	0.064	2.04	19	0.071	2.54	19	0.112	5.53	19	0.068	2.33
20	0.064	2.04	20	0.061	1.82	20	0.151	8.36	20	0.076	2.91
Hokkaido 5			Hokkaido 6			Hokkaido 7			Hokkaido 8		
1	0.109	5.31	1	0.076	2.70	1	0.097	4.00	1	0.214	11.25
2	0.085	3.56	2	0.09	3.56	2	0.556	32.46	2	0.155	7.59
3	0.383	25.23	3	0.086	3.32	3	0.43	24.64	3	0.145	6.97
4	0.083	3.42	4	0.226	12.00	4	0.155	7.59	4	0.107	4.62
5	0.176	10.18	5	0.761	45.16	5	0.131	6.11	5	0.131	6.11
6	0.114	5.67	6	0.111	4.87	6	0.122	5.55	6	0.094	3.81
7	0.2	11.92	7	0.072	2.45	7	0.138	6.54	7	0.099	4.12
8	0.168	9.60	8	0.085	3.25	8	0.112	4.93	8	0.4	22.78
9	0.196	11.63	9	0.075	2.63	9	0.154	7.53	9	0.137	6.48
10	0.074	2.76	10	0.196	10.14	10	0.394	22.41	10	0.164	8.15
11	0.284	18.03	11	0.1	4.18	11	0.117	5.24	11	0.107	4.62
12	0.118	5.96	12	0.612	35.93	12	0.107	4.62	12	0.142	6.79
13	0.788	46.84	13	0.163	8.09	13	0.171	8.59	13	0.099	4.12
14	0.35	19.68	14	0.167	8.34	14	0.195	10.07	14	0.824	49.07
15	1.229	74.18	15	0.132	6.17	15	0.185	9.45	15	0.067	2.14
16	0.207	10.82	16	0.08	2.94	16	0.213	11.19	16	0.133	6.23
17	0.192	9.89	17	0.18	9.14	17	0.087	3.38	17	0.076	2.70
18	0.198	10.26	18	0.071	2.39	18	0.089	3.50	18	0.084	3.19
19	0.077	2.76	19	0.145	6.97	19	0.134	6.29	19	0.088	3.44
20	0.106	4.56	20	0.11	4.80	20	0.135	6.35	20	0.203	10.57

Table IV (contd)
Enzyme-linked immunosorbent assay results for the detection of antibodies against visna-maedi among 267 sheep sera from northern prefectures of Japan

Flock/ sample	OD value	S/P ratio	Flock/ sample	OD value	S/P ratio	Flock/ sample	OD value	S/P ratio
Hokkaido 9			Hokkaido 10			Iwate 11		
1	0.244	13.11	1	0.116	5.18	1	0.097	4.18
2	0.059	1.64	2	0.094	3.81	2	0.096	4.10
3	0.132	6.17	3	0.084	3.19	3	0.19	11.18
4	0.107	4.62	4	0.121	5.49	4	0.783	55.81
5	0.112	4.93	5	0.069	2.07	5	1.223	88.93
6	0.205	10.69	6	0.301	19.53	6	>3.5	>250
7	0.167	8.34	7	0.107	4.93	7	0.097	4.18
8	0.062	1.83	8	0.077	2.67	8	0.132	6.81
9	0.112	4.93	9	0.187	10.95	9	0.089	3.58
10	0.07	2.32	10	0.095	4.03	10	0.139	7.34
11	0.102	4.31	11	0.074	2.45	11	0.063	1.62
12	0.072	2.45				12	0.086	3.35
13	0.163	8.09				13	0.094	3.95
14	0.345	19.37				14	0.233	14.41
15	0.089	3.50				15	0.101	4.48
16	0.109	4.74				16	0.075	2.52
17	0.106	4.56				17	0.103	4.63
18	0.104	4.43				18	0.117	5.68
19	0.176	8.90				19	0.091	3.73
20	0.126	5.80				20	0.111	5.23
Iwate 12			Aomori 13			Aomori 14		
1	0.094	3.95	1	0.091	3.73	1	0.109	5.08
2	0.1	4.40	2	0.112	5.31	2	0.162	9.07
3	0.091	3.73	3	0.06	1.39	3	0.106	4.86
4	0.132	6.81	4	0.081	2.97	4	0.087	3.42
5	0.127	6.44	5	0.115	5.53	5	0.139	7.34
6	0.076	2.60	6	0.071	2.22	6	0.139	7.34
7	0.074	2.45	7	0.061	1.47	7	0.057	1.17
8	0.134	6.96	8	0.151	8.24	8	0.193	11.40
9	0.192	11.33	9	0.07	2.15	9	0.083	3.12
10	0.172	9.82	10	0.083	3.12	10	0.149	8.09
11	0.151	8.24	11	0.079	2.82	11	0.092	3.80
12	0.106	4.86	12	0.253	15.92	12	0.144	7.72
13	0.104	4.70	13	0.146	7.87	13	0.124	6.21
14	0.062	1.54	14	0.108	5.01	14	0.088	3.50
15	0.094	3.95	15	0.073	2.37	15	0.42	28.49
16	0.169	9.60	16	0.417	28.26	16	0.173	9.90
17	0.084	3.20	17	0.128	6.51			
18	0.375	25.10	18	0.093	3.88			
19	0.107	4.93	19	0.139	7.34			
20	0.08	2.90	20	0.177	10.20			