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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.

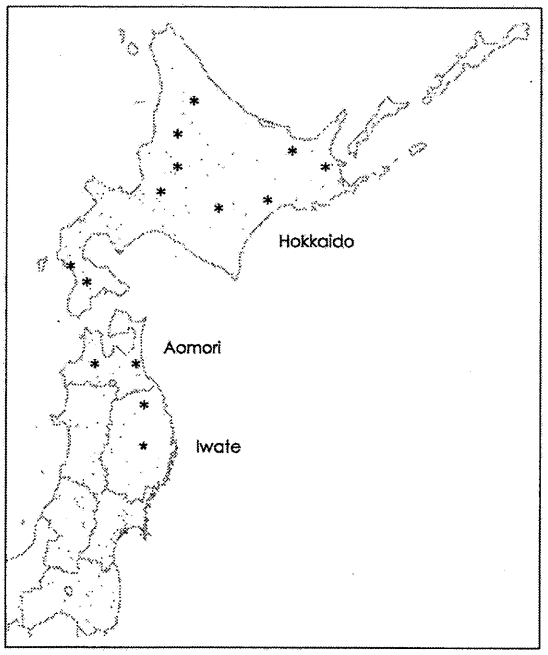
Materials and methods

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			

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

Diagnostic system controls						
	Plate 1		Plate 2		Plate 3	
Negative control OD values	0.042	0.03	0.034	0.031	0.043	0.04
Positive control OD values	1.358	1.465	1.629	1.662	1.388	1.352
OD negative mean	0.036		0.033		0.042	
OD positive mean	1.4115		1.6455		1.37	
DELTA P-N	1.376		1.613		1.329	
Minimum values OD positive mean >= 0.350; OD X positive/negative ratio >= 3.5						

OD optical density at 450 nm
S/P sample to positive ratio (S/P = S–negative control [NC]/positive control [PC] mean-NC)
S/P reference values 110-120, cut-off: 120

positive result was revealed by either assay. No evidence of the presence of either the viral or proviral form of the virus was therefore detected in any of the 237 samples submitted for analysis, including samples H/1/10 and H/11/6 (sample H/4/8 was not tested).

Visna-maedi infection was detected in three flocks out of the 14 sampled. Prevalence of infection was found to vary between prefectures (Table V). Positive sera originated from two flocks in the Hokkaido Prefecture and from one flock in the Iwate Prefecture. None of the sera collected from the Prefecture of Aomori were found to be positive. The percentage of positive sheep was 1.6% and 2.5% in the Hokkaido and Iwate Prefectures, respectively. The average incidence of seropositive animals in individual herds was 5% in the three sampling groups from affected flocks.

Table V
Comparison between different prefectures of northern Japan of percentages of sheep positive for antibodies to visna-maedi virus

Prefecture	No. of flocks sampled	Positive (%)	No of samples	Positive (%)
Hokkaido	10	20	191	1.6
Iwate	2	50	40	2.5
Aomori	2	0	36	0

The seropositive sheep were all females, but of different breeds, namely: Cheviot, Suffolk and Romanov (Tables II and VI). With the exception of the Romanov ewe which was in poor condition, the other two animals were apparently healthy. In the present study, no diagnostic measures were taken. Proportions of infected sheep were found to increase with age (Table VI). Two positive animals were five-year-old Suffolk ewes and eight-year-old Cheviot ewes, respectively. The Romanov ewe was an adult sheep but the exact age was not

Table VI
Comparison between different age categories of the percentage of sheep positive for antibodies to visna-maedi virus

Age category	No. of animals	Positive to visna-maedi virus	Percentage
1 year	16		0
2	27		0
3	32		0
4	57		0
5	34	1 ewe Suffolk (H/4/8)	2.94
6	26		0
7	33		0
8	10	1 ewe Cheviot (I/11/6)	10
9	3		0
10	6		0
12	1		0
Not known	22	1 ewe Romanov (H/1/10)	
Total	267	3	

recorded. No seropositive animals were found in the age groups from one to four years of age or from animals over eight years of age.

The annual lambing rate was based on a lambing season extending from February to April, with the exception for one farm were the reproductive cycle was related to three breeding seasons: a first in the autumn (from 1 September to 15 October), the second during the late autumn (from 15 October to 15 December) and the third in spring, for empty ewes after the autumn season or for yearlings, associated with 9 days progesterone sponge application and the administration of pregnant mare's serum gonadotropin (PMSG) 24 h before sponge removal (from mid-May to mid-June). The average annual wool yield included uncleaned material from shearing between April and May. The annual milk yield covered a period of 150 lactation days and was reported from only one farm that is unique in Japan for ovine dairy production. On the other farms, lambs were allowed to milk with their mothers, therefore milk yield recording was not applicable.

An evaluation of the possible impact of visna-maedi infection on production in the sampled flocks did not reveal any correlation with the reported levels of seropositive animals (Table VII). In particular, in regard to flock reproductive performance, annual lambing rates from two infected flocks ranged from 1.1 to 1.61 (mean 1.35) and, in flocks with no evidence of the infection, from 0.72 to 1.62 (mean 1.36) and up to 2.44 on only one farm. The mean proportion of multiple offspring (twin and triplets), was slightly lower in flocks affected by visna-maedi (46.25%) in comparison to disease-free flocks (53.64%). Furthermore, the rates of ewes that did not conceive after mating were 0% and 5.62% in two affected flocks, and ranged from 0% to 30.15% in the others. Similarly, variations in average live body weight and annual wool yield were not related to the presence of animals that tested positive to visna-maedi.

Table VII
Average production and reduction of survivability of flocks
Data are referred to means from two years (2006 and 2007) prior to sampling

Flock	Annual lambing rate (lambs/ ewes)	Infertility rate of ewes	Annual mortality rate of lambs	Average no. of lambs/ ewes that survived	Annual culling rate	Mortality rate among adults	Live weight mean	Annual milk yield	Annual wool yield
1	NR	NR	NR	NR	NR	5%	NR	NA	NR
2	0.72	NR	1.29%	0.71	0%	4.76%	Ewes 50 kg	NA	3 kg
3	1.62	NR	3.46%	1.57	14.77%	9.2%	Ewes 55 kg	NA	3.5 kg
4	1.1	5.62%	12.78%	0.96	2.97%	0%	Ewes 60 kg	NA	NR
5	1.61	NR	20%	1.29	11.73%	8.33%	Ewes 75 kg Rams 110 kg	NA	3 kg
6	1.48	5.06%	17.09%	1.22	10.33%	9.09%	Ewes 60 kg Rams 80 kg	NA	2 kg
7	1.58	0%	16.92%	1.31	6.66%	2.22%	Ewes 60 kg	NA	NR
8	2.44	1.02%	20.53%	1.94	NR	NR	NR	150 l	4 kg
9	1.23	30.15%	0%	1.23	0%	0%	Ewes 50 kg	NA	2 kg
10	NR	NR	NR	NR	NR	10%	NR	NA	NR
11	1.61	0%	6.89%	1.5	0%	11.76%	Ewes 65 kg	NA	3.5 kg
12	1.38	9.09%	9.83%	1.25	24.03%	4.8%	Ewes 65 kg Rams 100 kg	NA	3.5 kg
13	1.54	4.34%	25.35%	1.15	17.64%	2.94%	Ewes 65 kg	NA	6.5 kg
14	1.14	3.12%	21.87%	0.89	0%	9.09%	Ewes 50 kg	NA	2.5 kg

NR not recorded
NA not applicable

Discussion

Antibodies against the virus were detected in two of the three northern prefectures of Japan where serum samples were collected (Table II). These results confirm the results of previous studies on the presence of visna-maedi in sheep in Hokkaido which was first reported by Yonemichi *et al.* (20) and again by Okada and Yonemichi (12), who provided a follow-up study for visna-maedi infections in Hokkaido and reported the first case in Iwate Prefecture.

Our present survey demonstrated that visna-maedi infection is present in the northern prefectures of Japan. However, the very low prevalence reported suggests the possibility of eradicating the disease, as the pathology caused by this virus is known to adversely affect animal production.

Serology results for infection showed excellent complementarity between AGID and ELISA tests. For a single case in Hokkaido 4/8, the intensity of the precipitation line and the OD value also corresponded. Nevertheless, the combined use of the two serological tests helped to facilitate the interpretation of results. According to the *Manual of standards for diagnostic tests and vaccines* of the OIE, both tests are prescribed for regulatory purposes and are indicated as reference tests for visna-maedi. The sensitivity of the commercial ELISA used in our work is reported to be equivalent or even higher than AGID (3, 8), thus being of particular interest, especially in cases of weak-positive reactions obtained by immune precipitation.

Attempts to detect the viral genome, or its proviral form, by PCR were not successful, and consequently did not provide an opportunity for the genomic evaluation of visna-maedi viral strains circulating in the Japanese ovine population, or a comparison with virus strains from other parts of the world. However, this does not indicate the absence of the infection and the detection of seroconversion in animals remains a reliable proof of virus circulation. Direct detection of viral nucleic acids by PCR is possible, but fluctuates, usually with very low levels of circulating virus which makes this difficult. Upon infection of a cell, lentiviruses integrate into the host genome, generating a proviral form, so that the cell is permanently and irreversibly infected. This proviral stage is easier to detect by PCR and is a more sensitive indicator of infection. PCR for visna-maedi virus detection is therefore traditionally performed on whole blood samples, enriched peripheral blood mononuclear cell (PBMC) preparations, or tissues, where integrated provirus may be found, rather than on serum, where insufficient cellular genomic DNA from leukocytes is usually present. The PCR was performed in accordance with the method described by Zhang *et al.* (21) for quantitative analysis of the proviral DNA load in peripheral blood monocytes and alveolar macrophages, and it is likely that the viral load in the serum of these visna-maedi infected animals was insufficient for detection with the PCR method used. Studies on CAEV in Japan have revealed positive results with PCR testing of peripheral blood leukocytes, carpal joint fluid and homogenised synovial membrane added to foetal lamb lung (FLL) cell culture (10, 11). Finally, it cannot be excluded that there is a difference in the genome sequence of the virus strains in these animals, so that one or both of the primers did not anneal properly.

The positive rate (1.12%) in our study revealed a very low pathogenic pressure and was lower than the level of infection previously reported (8.86%) (12). In previous studies undertaken in Hokkaido, Suffolk, Corriedale, and Cheviot sheep showed the presence of visna-maedi virus-like particles (20). Furthermore, animals identified as being serologically positive were Suffolk and Corriedale. The Cheviot and Landrace sheep tested were found to be negative (12). In our study, Romanov, Cheviot and Suffolk ewes were affected. Taking into account the fact that the susceptibility to visna-maedi infection varies across sheep breeds, with coarse-wool breeds (such as Texel, Border Leicester, Finnish Landrace and Cheviot) apparently more susceptible than the fine-wool sheep (such as Columbia, Rambouillet, Corriedale and Suffolk) (18), the low percentage reported in the present study might be related to the breeds of animals tested. The

majority of the sampled animals were Suffolk and Suffolk cross-breeds.

In our study, visna-maedi infections could not be linked to losses in sheep production. The very low levels of infection were insufficient to enable the determination of any appreciable negative impact of infection on overall sheep flock performance. In the different flocks, reproduction records revealed levels of reduced fertility, lambing rate, number of offspring and lamb survivability. In addition to stillbirths, lamb mortalities were reported to occur generally during the first week, followed by a second peak when lambs were separated from their mothers at three months of age. The causes of losses were not elucidated. Similarly, the reduction of survivability was observed in the different flocks considered, but this appeared not to be influenced by visna-maedi infections. Culling did not generally follow a specific management policy. A 1%-10% mortality rate among adults, often reported as being due to lambing complications, was also the result of various other causes such as predators, road accidents or stress. This mortality rate does not represent a relevant finding to the extent that it occurs normally in sheep flocks. Milk yield could not be considered among production parameters since only one farm, unique in Japan, managed dairy products. Nevertheless, with an annual production of 17 metric tons of milk with 6% fat and 10% proteins, based on early weaning at 21 days, this represents a good example of the potential for sheep production systems in the country.

An increased number of adult sheep in poor condition is the most common feature of virus spread in the flock. This is directly related to an increased number of ewes being culled and also a reduction in conception rates and lower proportions of twin and triplet lambs. Ewes in poor condition produce smaller lambs which are less likely to survive. Lower volumes of colostrum and milk are produced, resulting in increased lamb mortality and reduced lamb growth rates. Briefly, the potential economic impact can be estimated to 10%-20% adult mortality after the development of clinical signs and a 30% loss of the potential lamb crop. The estimation of losses due to visna-maedi in previous studies undertaken in other countries indicate that losses gradually increase over several years and reach 15%-30% annually in some flocks (13).

A comparison of the proportions of seropositivity to visna-maedi between flocks with different survival rates (i.e. annual percentage of culled sheep) in 2006 and 2007 (prior to sampling), did not show any relation with the infection rate. Occurrence of visna-maedi infection was reported in flocks with a reduced survival rate of 0%-2.97%. Annual culling rates in the other flocks ranged from 0% to 24.03% (mean 9.46). Similarly, flocks were also compared according to the mortality rate of lambs and adults during the two years under consideration, which did not reveal any positive correlation with the infection rate. Annual lamb mortality rates in infected flocks ranged from 6.89% to 12.78%, resulting in an average of surviving lambs per ewe of 1.23. In other flocks, the reported lamb mortality varied from 0% to 25.35% (mean 13.63), with an average rate of surviving lambs of 1.13. Visna-maedi-positive sheep belonged to flocks with an adult mortality rate of 0% to 11.76% (mean 5.58%). The other flocks showed a mean rate of 6.04%, with values from 0% to 9.09%. Older animals were more often affected (five to eight years old). This corresponds to the findings of surveys conducted in other countries (9). Mortality or culling probably explains the absence of seropositive sheep over the age of eight years. Nevertheless, previous studies performed in Hokkaido reported a majority of visna-maedi infections in young animals. Seropositive status was observed in two animals aged 1.5 years, three aged 2 years, and only one case aged 5 years out of the 12 sheep tested with intranasal tumours (20). In another study performed on a sample group of 79 sheep aged between 1 and 7 years, seropositive animals were 2-3 years old (12).

In countries where infection exists at very low levels of prevalence, like Japan, flock owners may neglect visna-maedi, ignoring the potential problem, and may consider the chance of their flock becoming infected as quite remote. Furthermore, in Japan, field

practitioner veterinarians have never diagnosed a case of visna-maedi, probably because the disease is still not very common, the signs can resemble a number of other diseases and veterinary involvement in sheep flocks is at an all-time low. Nevertheless, the control of the infection remains important in order to reduce animal trade barriers.

As neither antiviral treatment nor vaccination is available (6), diagnostic tests are the backbone of most of the schemes implemented to prevent the spread of visna-maedi. Serological identification and the subsequent elimination of infected animals is the starting point of control schemes in order to gain visna-maedi-free accredited status. Given that the pathogen is fragile in the external environment and transmission is mainly due to direct contact between infected animals, control programmes rely on the prevention of the introduction of the disease based on serological monitoring of newly introduced animals. In our study, evidence of the introduction of infected animals was revealed. In one flock (number 4) only one of the 20 animals tested was positive. This animal was introduced into the flock one month before sampling, without having been subjected to prior serological testing. Two other ewes were introduced at the same time, but were seronegative. Therefore, the infection can be prevented by following sound biosecurity practices, including the quarantine of animals of unknown health status, and feeding newborn animals colostrum and milk known to be free of the virus. Such stringent rules will help to prevent the flock from becoming infected.

Considering that visna-maedi can infect goats and CAEV can infect sheep, although documented cases of natural cross-species transmission are rare (14, 16), and that recombination has recently been demonstrated between visna-maedi and CAEV (15), in addition to the fact that CAE was recently reported in Japan (10, 11), any eradication programmes should address both visna-maedi and CAE infections simultaneously.

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NOTE *Bacteriology*

Prevalence of Hemoplasma Infection among Cattle in the Western Part of Japan

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ABSTRACT. We have examined for hemoplasma infection among cattle in the Hiroshima and Miyazaki prefectures by using a sensitive real-time PCR, with SYBR Green I and with melting curve analysis, which allow to distinguish the two bovine hemoplasma species, *Mycoplasma wenyonii* and '*Candidatus* M. haemobos'. We found 69.4% of 36 cattle in Hiroshima and 93.8% of 32 cattle in Miyazaki infected with either of these two hemoplasma species. High morbidity in western part of Japan may reflect the activity of arthropod vectors for hemoplasma transmission. We also demonstrated neonatal calves less than three months old affected with hemoplasmas without grazing in summer, suggesting a possibility of vertical transmission.

KEY WORDS: hemoplasma, mycoplasma, vertical transmission.

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Only two hemoplasma species, *Mycoplasma wenyonii* and '*Candidatus* M. haemobos' (synonym for '*C.* M. haemobovis') are currently recognized in cattle [6, 9]. Although *M. wenyonii* has been shown to exhibit worldwide geographical distribution, '*C.* M. haemobos' has been solely reported from Switzerland, China, Germany and Japan [2, 3, 8, 9]. Both hemoplasma species are causative of infectious anemia in cattle to some extent and have been demonstrated in Hokkaido and Tohoku regions of this country. Incidences of *M. wenyonii* and '*C.* M. haemobos' infections have been reported as 21.8 and 16.7%, respectively, in Hokkaido, and 40.3 and 56.8%, respectively, in the Miyagi prefecture [7, 9]. Infection has spread by transmission routes that we do not fully understand. Despite the relatively high incidence in these areas, little is known about the prevalence of bovine hemoplasma infections in western parts of Japan. Therefore, we examined for hemoplasma prevalence among cattle in the Hiroshima and Miyazaki prefectures by using a sensitive real-time PCR to detect the 16S rRNA gene [4], with SYBR Green I and with melting curve analysis, which allow to distinguish these two hemoplasma species.

EDTA-anticoagulated or heparinized blood samples from 68 cattle in different herds of the Hiroshima and Miyazaki prefectures were randomly collected between 2008 and 2011. Information on clinical diagnoses and age of all the cattle included in this study was obtained from the relevant veterinarians or owners. The ages of cattle were ranging from one month to 15 years old. Cattle included less than yearling in both the prefectures. Total DNA was extracted from 200 μl whole blood samples by using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Negative controls consisting of 200 μl phosphate-buffered saline solution were included with each batch. Extracted DNA samples were stored at –20°C prior to examination.

To detect the both hemoplasma species in real-time PCR, specific primers for the 16S rRNA gene were used as described previously [7]. Forward primer, 5'-ATATTCCTACGGGAAGCAGC-3', equivalent to nucleotide numbers 328 to 347 of *M. wenyonii* and reverse primer, 5'-ACCGCAGCTGCTGGCACATA-3', equivalent to nucleotide numbers 503 to 522 of *M. wenyonii* amplified a 195 and 173 bp for *M. wenyonii* and '*C.* M. haemobos', respectively. Nucleotide sequences and sizes bracketed by the primers are peculiar to each hemoplasma. The 22-bp gap in the PCR product from '*C.* M. haemobos' attributes to a genetic marker to distinguish it from *M. wenyonii* in the real-time PCR [7].

Real-time PCR was performed in a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with SYBR Premix Ex *Taq* (Code #RR041A, TaKaRa Bio., Shiga, Japan). The reaction mixture contained 1 μl of each primer (10 pmol/μl), 12.5 μl of 2X premix reaction buffer and water to volume of 23 μl. Finally, 2 μl of DNA samples as templates were added to this mixture. Amplification was achieved with 40 cycles of denaturation at 95°C for 5 sec, renaturation at 57°C for 20 sec, and elongation at 72°C for 15 sec, after the initial denaturation at 94°C for 30 sec. Positive cattle were found affected with each hemoplasma irrespective of the age.

After real-time PCR, melting experiment was performed from 60 to 95°C at 0.2°C/sec with smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature (*Tm*) as described previously [1]. Melting curve analysis of the amplified products allowed differentiation of these two

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hemoplasma species, since nucleotide sequences and sizes bracketed by the primers are distinct between the species. Thus, the variations in the *Tm* may serve as a differential marker for hemoplasma species. The input amount of DNA, the copy number of the target as well as presence of co-infections with several targets did not influence the *Tm*. No melting peak was evident on negative cattle.

In the present study, we found overall 69.4% (25/36) and 93.8% (30/32) cattle infected with hemoplasmas in Hiroshima and Miyazaki, respectively (Fig. 1). This incidence was much higher than that in Hokkaido and Miyagi, suggesting factors in geographical latitude affected in infections. The geographical difference in morbidity may attribute to activity of arthropod vectors for hemoplasma transmission. '*C.* M. haemobos' infection was predominant in both the prefectures. Thirty-six cattle in Hiroshima included 18 stores from yearling to two years old and 18 adult dairy cows. Despite missing exact age, no relationship to infections was evident among age and bleed of cattle in Hiroshima. In Miyazaki, although extremely high incidence of infections was demonstrated over all, relatively low incidence was found in elder cattle. More surprising result is that all the calves less than three months born in winter were unexpectedly infected with '*C.* M. haemobos' (Table 1). It raises a question of whether vectors for the hemoplasma transmission are unnecessary, since ticks have been suspected as a vector for feline hemoplasma infection [10]. Given the absence of vectors in winter, it will turn out that vertical transmission is most likely route of neonatal infection, since cattle may become infection while grazing in summer.

The hemoplasma-infected cattle in the present study did not exhibit clinical signs such as anemia attributable to hemoplasmosis, though hemoplasma infections in cattle were first recognized in Swiss dairy cows with hemolytic anemia [3]. In our study, no significant association was found between the infection status and anemic syndromes. One possibility is that there might be distinct strains in virulence or anemia might be caused by opportunistic infections. As none of the PCR-positive cattle caused clinical manifestations of severe anemia or all cattle had been presented for reasons unrelated to hemoplasmosis, laboratory parameters were not subjected to in-depth investigation. Although our results indicate wide distribution of *M. wenyonii* and '*C.* M. haemobos' among cattle population in this country without developing anemic syndromes, infected animals probably remain chronic carriers after clinical signs have resolved. Thus, the persistent infections with hemoplasmas may contribute to the progression of retroviral, neoplastic, or immune-mediated diseases [5]. Collectively, our findings suggest possibility of vertical transmission because of neonatal infections and monitoring of hemoplasma infection may help to maintain hygienic conditions for cattle production.

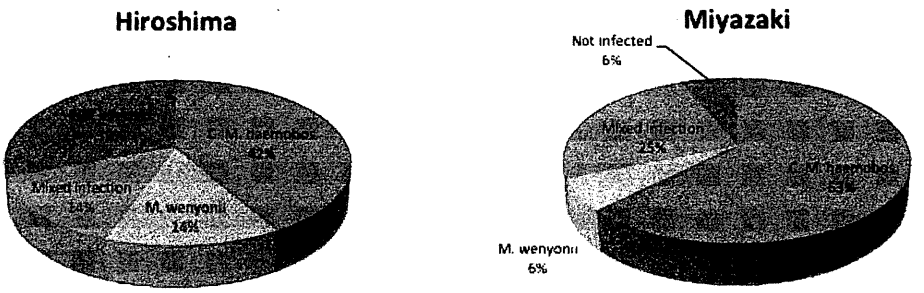

Fig. 1. Hemoplasma infections among cattle in Hiroshima and Miyazaki prefectures. Percentages were shown in round figures.

Table 1. Incidence of hemoplasma infections in cattle of the Miyazaki Prefecturre

Age	No. of cattle examined	*M. wenyonii*	'*C.* M. haemobos'
Less than 3 months	4	0	4
Yearling	23**	7*	21*
2 years old	1	0	1
7 years old	1	0	0
11 years old	1	1*	1*
14 years old	1	1	0
15 years old	1	1*	1*
Total	32	10 (31.3%)	28 (87.5%)

* Numbers include mixed infections. ** One of the yearlings was not infected with either of the hemoplasma species.

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