

Serological Survey to Determine the Occurrence of Blue Tongue Virus, Bovine Leukemia Virus and Herpesvirus Infections in the Japanese Small Ruminant Population from Northern Districts

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Abstract

Ovine sera, collected from the northern prefectures of Hokkaido, Aomori and Iwate in Japan, were examined for the presence of antibodies against Blue tongue virus (BTV), bovine leukemia virus (BLV), bovine *Herpesvirus* type 1, agent of infectious bovine rhinotracheitis (IBR), ovine *Herpesvirus* type 2 (OvHV-2), agent of sheep-associated malignant catarrhal fever (SA-MCF), and bovine *Herpesvirus* type 4 (BoHV-4), using agar immune diffusion, serum neutralisation (SN) and enzyme linked immunosorbent assay (ELISA) tests. No animals were positive to IBR, BoHV-4 or BLV antigens. Antibodies against BTV have been detected in 3 samples (1.11%) in two flocks from Hokkaido. The seroprevalence of OvHV-2 was observed in twelve flocks from the 3 considered prefectures, in 56 sheep and two goats, with 37.66% of samples giving a positive reaction in the serum neutralization test. The infections did not appear to be related to the reduction in sheep productivity. Immune reaction reported in goats could refer to Caprine *Herpesvirus*-2 (CpHV-2). These results indicate that sheep are reservoirs for OvHV-2 in the field in Japan.

Keywords: Blue tongue ; Bovine *herpesvirus* type 4 ; Bovine leukemia virus; Infectious bovine rhinotracheitis; Japan ; Malignant catarrhal fever ; Small ruminants

Introduction

In Japan, bovine farming represents an important economical resource, with production excellences like black Japanese cattle breed, producing Kobe beef, while ovine farming is a relatively minor sector, and population is constituted by approximately 10,000 heads [1]. In some farms, cows are housed close to sheep pens or have access to common pastures. Such close contacts may represent a potential role in the diffusion of pathogenic agents to valuable cattle breeds.

Pathogens affecting cattle welfare and health have been accurately investigated in Japan. However, until now, only scarce information is available on epidemiology of virus pathogens in sheep. Among relevant pathogens, Blue tongue virus (BTV) was reported in cattle from Tochigi prefecture in 1994 [2] and in 2001 [1], and from Hiroshima and Fukushima prefectures in 2005 and 2006 [1]. Studies on bovine leukemia virus (BLV) demonstrated the occurrence of the disease in cattle [3]. Concerning *Herpesvirus* infections, studies undertaken in Japan on bovine *Herpesvirus* type 1 (BoHV-1), agent of infectious bovine rhinotracheitis (IBR), pathogen of worldwide importance affecting primarily cattle, showed the diffusion in cattle population [4,5]. Similarly, studies on ovine *Herpesvirus* type 2 (OvHV-2), known cause of the sheep-associated malignant catarrhal fever (SA-MCF) [6,7], and on bovine *Herpesvirus* type 4 (BoHV-4) demonstrated the occurrence of the diseases in cattle in the country [3,8].

Concerning small ruminants, sheep are naturally sensible to BTV and *Herpesvirus* infections (BoHV-1, OvHV-2 and BoHV-4) [9-11]. In particular, sheep are natural reservoir of OvHV-2. Generally, as other natural reservoir species for the viruses causing MCF, sheep does not exhibit any clinical signs of infection [10]. However, if infected with very high doses of virus, sheep can develop a mild form of MCF [12,13]. Domestic goats harbor their own closely-related strain of MCF virus. It has been termed caprine *Herpesvirus*-2 (CpHV-2) [14]. Although natural BLV infection occurs only in cattle, water buffaloes, and

caprybas, sheep are highly susceptible to infection by inoculation of the virus, with a persistent antibody response, and develop tumours more often and at a younger age than cattle [15]. Therefore, risk of iatrogenic contamination has to be considered.

BTV was reported in sheep from Tochigi prefecture in 1994 (77 serologically positive animals) [2] and in 2001 (9 affected animals) [1]. Only one previous report showed prevalence of OvHV-2 in sheep in Japan [16], which described a seroprevalence of 64.3% in 238 sheep samples originated from 10 farms from Hokkaido. No further investigation has been reported. No previous epidemiological surveys on BLV, BoHV-1 or BoHV-4 have been undertaken in small ruminants in Japan. Furthermore, no clinical cases due to these infections have been reported among sheep flocks.

In order to explore the presence of BTV, BLV, and *Herpesvirus* (BoHV1, OvHV-2 and BoHV-4) infections in sheep, and to obtain a preliminary picture of their epidemiology among the Japanese sheep population, a serological survey was carried out between September 2007 and January 2008. These samples came from the northern prefectures in Japan, Hokkaido, Aomori and Iwate, as they have the most part of small ruminant population, approximately more than 50% in 2007 [1].

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Materials and Methods

The survey was performed on sheep raised both commercially and traditionally, from farms with a limited number of animals up to large flocks of 700 heads. The number of flocks was determined according to the animal population of each prefecture and is representative of the livestock production systems in the country. Ten flocks were sampled in Hokkaido prefecture, considering that about 37% of sheep breeding in



Japan is concentrated in this region [1]. The ten flocks were arbitrarily chosen to include flocks from different regions of Hokkaido prefecture. The sampling was completed with four flocks from Tohoku area, two from Iwate prefecture and two from Aomori prefecture (Figure 1 and Table 1).

A maximum of 20 animals were sampled from each flock selected for sampling, according to the national standard of flock composition (number of rams, ewes and yearlings). In two of the 14 flocks, only 11 and 16 sheep, respectively, were available for sampling, and in one flock five goats were additionally sampled, and therefore, a total of 272 serum samples were collected. 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. The majority of the sampled animals were Suffolk and Suffolk cross-breed. Other breeds were represented by Romanov, Cheviot, Corriedale, Friesland, Black Welsh Mountain and Poll Dorset sheep. Goats were cross-breed Shibayaghi x Tokara x Dane. All the sera were stored at -20°C prior to examination. The collected ovine sera were subsequently transported to laboratories in Italy for further analyses, under the authorization permitted by the Ministry of Health, Rome, Italy.

Serological testing for OvHV-2 and BoHV-4 was carried out using serum neutralisation (SN) test. In a 96-well plate, heat-inactivated serum samples were diluted from an initial dilution of 1:2 to 1:256, in double, and placed in contact with 100 TCID₅₀ of previously titrated *Alcelaphine herpesvirus 1* WC-11 strain or BoHV-4 Movar 33/63 strain. After incubation for 1 h at 37°C under 5% CO₂ to enable viral neutralisation, $5 \times 10^5/\text{ml}$ of bovine turbinate cells (Madin-Darby bovine kidney for BoHV-4) suspended in minimum essential medium (MEM) (Eurobio, France). The medium contains penicillin 100 IU/ml, streptomycin 100 $\mu\text{g}/\text{ml}$, gentamicin 5 $\mu\text{g}/\text{ml}$, nystatin 50 IU/ml and 10% fetal calf serum (FCS) (Sigma, Germany), was added to each well. After 5 days, the cytopathic effect (CPE) in the wells was evaluated and the antibody titre was defined as the highest serum dilution able to inhibit at least 75% of the CPE. The positive and negative reference sera, cells and virus controls (Istituto Zooprofilattico Sperimentale, dell'Abruzzo e del Molise 'G. Caporale', IZSA&M, Teramo, Italy), were included in each plate.

Flock	Prefecture	Flock characteristics				Sampled animals		
		Number of animals	Flock composition	Breeds	Number	Gender		
						Rams	Ewes	
1	Hokkaido	50	5 rams, 35 ewes, 10 yearlings	Cross breed, Suffolk, Cheviot, Romanov, Black, South down and Corriedale	20	0	20	
2	Hokkaido	84	4 rams, 47 ewes, 33 yearlings	Suffolk, some South down and Black	20	0	20	
3	Hokkaido	410	10 rams, 200 ewes, 200 yearlings	Suffolk	20	0	20	
4	Hokkaido	178	4 rams, 80 ewes, 94 yearlings	Suffolk	20	3	17	
5	Hokkaido	600	13 rams, 270 ewes, 30 yearlings, 287 lambs	Poll dorset, cross breeds Suffolk x Cheviot	20	1	19	
6	Hokkaido	221	1 ram, 80 ewes, 40 yearlings, 100 lambs	Cross breeds South down x Poll dorset	20	0	20	
7	Hokkaido	82	2 rams, 29 ewes, 14 yearlings, 37 lambs	Suffolk and Suffolk cross breed	20	0	20	
8	Hokkaido	700	260 ewes, 440 yearlings	Cross breed, Romanov, Poll dorset, Suffolk	20	0	20	
9	Hokkaido	100	2 rams, 40 ewes, 58 yearlings	Cross breed, Suffolk, Romanov, Friesland, Black welsh mountain	20	12	8	
		100 goats		Cross breed Shibayaghi / Tokara / Dane	5			
10	Hokkaido	35	25 ewes, 10 lambs	Corriedale	11	8	3	
11	Iwate	34	2 rams, 24 ewes, 8 yearlings	Cross breed, Suffolk, Romanov, Cheviot, Corriedale	20	1	19	
12	Iwate	52	1 ram, 30 ewes, 21 yearlings	Suffolk	20	1	19	
13	Aomori	34	1ram, 26 ewes, 7 yearlings	Suffolk	20	0	20	
14	Aomori	22	22 ewes	Suffolk	16	0	16	
Total					272	26	241	

Table 1. Details of flocks sampled for serological testing of antibodies against pathogens in sheep from prefectures of northern Japan. Data show the number of samples.

Screening for anti - BLV antibodies was performed using an agar gel immunodiffusion (AGID) (a prescribed test for international trade) in accordance with the Manual of diagnostic tests and vaccines for terrestrial animals of the World Organisation for Animal Health (*Office*

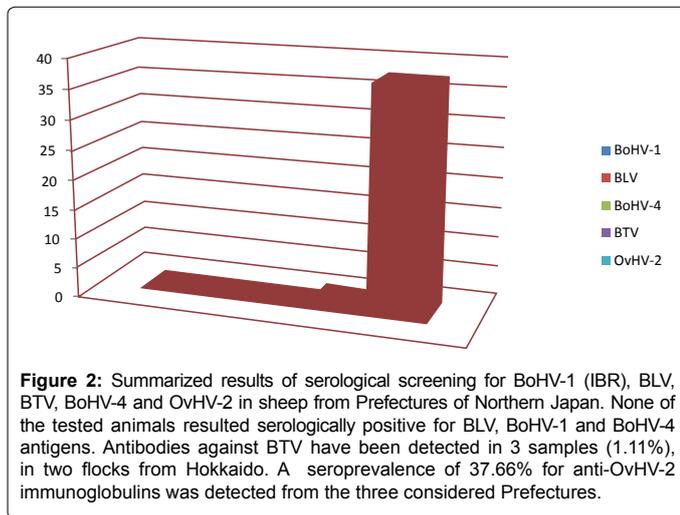


Figure 2: Summarized results of serological screening for BoHV-1 (IBR), BLV, BTV, BoHV-4 and OvHV-2 in sheep from Prefectures of Northern Japan. None of the tested animals resulted serologically positive for BLV, BoHV-1 and BoHV-4 antigens. Antibodies against BTV have been detected in 3 samples (1.11%), in two flocks from Hokkaido. A seroprevalence of 37.66% for anti-OvHV-2 immunoglobulins was detected from the three considered Prefectures.

	Positive	% Positive*	Negative	NE	Toxic	Total
IBR (ELISA)	0	0	272	-	-	272
BLV (AGID)	0	0	272	-	-	272
BTV (ELISA)	3	1.11	272	-	-	272
BoHV-4 (SN)	0	0	208	64	-	272
OvHV-2 (SN)	58	37.66	96	38	80	272

NE: not executed due to insufficient aliquots for testing

*: percentage computed excluding samples resulting toxic or not tested for insufficient serum quantity

Table 2. Results of serological screening for antibodies to IBR, BLV, BTV, BoHV-4 and OvHV-2 in sheep from Prefectures of Northern Japan.

Flock No.	Prefecture	SN titre								Toxic	NE	Total /floc
		Negative	4	8	16	32	64	128	256			
1	Hokkaido	12	1	3	1	0	0	1	0	2	0	20
2	Hokkaido	3	0	2	1	3	1	1	0	6	3	20
3	Hokkaido	4	1	2	0	0	2	1	1	9	0	20
4	Hokkaido	0	0	0	0	0	0	0	0	0	20	20
5	Hokkaido	14	0	0	1	0	1	1	0	3	0	20
6	Hokkaido	11	0	0	0	1	0	1	0	5	2	20
7	Hokkaido	11	0	0	2	0	0	0	0	7	0	20
8	Hokkaido	15	0	0	0	0	0	0	0	4	1	20
9	Hokkaido	6	0	3	1	0	0	0	0	9	1	20
9	Hokkaido*	1	0	0	0	0	1	1	0	2	0	5
10	Hokkaido	1	0	0	0	1	0	0	0	0	9	11
11	Iwate	8	0	0	0	0	1	0	0	11	0	20
12	Iwate	2	0	2	1	1	1	5	2	5	1	20
13	Aomori	7	0	0	0	1	1	4	0	6	1	20
14	Aomori	1	0	0	0	1	1	1	1	11	0	16
Total		96	2	12	7	8	9	16	4	80	38	272
Total seropositives		58 (37.66%**)										

NE: not executed due to insufficient aliquots for testing

*: goats

** : percentage computed excluding samples resulting toxic or not tested for insufficient serum quantity

Table 3. Results of serum neutralization (SN) screening test for antibodies to OvHV-2 virus in sheep from prefectures of northern Japan.

International des Épizooties: OIE) [15]. Antigen and control sera were obtained from the IZSA&M, Teramo, Italy.

Serological testing for antibodies against BoHV-1 glycoprotein B was performed by enzyme linked immunosorbent assay (ELISA), using a commercial kit (IDEXX IBR gB, IDEXX, USA), following the manufacturers' instructions.

Antibodies against BTV were detected using a competitive ELISA kit developed by the National Reference Centre for Exotic Diseases (IZSA&M, Teramo, Italy) [17].

Farmers were interviewed regarding flock management, productivity and losses, referring also to previous years. Concerning flock production, the annual lambing rate was calculated as number of lambs born per ewes exposed to the ram and it was based on a lambing season occurring from February to April, with an exception being made for one farm where the reproductive cycle was related to 3 breeding seasons. In order to evaluate a possible relationship between the prevalence of infection and production parameters such as annual lambing rate, annual lamb mortality rate and annual adult mortality rate, the animals were compared for their screened pathogens infection rate proportions by computing Pearson's correlation coefficients statistics. Differences were considered to be significant at $P < 0.05$.

Results

Results of serological screening for antibodies to BTV, BLV, BoHV-1 (IBR), OvHV-2 and BoHV-4 in sheep from prefectures of northern Japan are summarized in Figure 2 and Table 2. All the 272 sera were submitted to BTV, BLV, and BoHV-1 testing. Not all the samples were applicable to serological tests for OvHV-2 and BoHV-4 antigens (Table 2). Some sera showed cytotoxicity (indicated by cell death, probably caused by the sub-optimal condition of the samples) or they were not tested for insufficient serum quantity, and therefore, all these samples ($n=64$ for BoHV-4 and $n=118$ for OvHV-2) were excluded.

None of the tested animals resulted serologically positive for BLV,

BoHV-1 and BoHV-4 antigens. Antibodies against BTV have been detected in 3 samples (1.11%), in two flocks (number 1 and 10) from Hokkaido, with a positivity rate at flock level of 10% and 9.09%, respectively.

The SN test revealed 58 samples, out of the 154 sera examined, positive for anti- OvHV-2 immunoglobulins (Tables 2 and 3); this corresponds to a seroprevalence of 37.66%. The overall flock seroprevalence was based on a single positive result within a flock with 85.71% of flocks being positive. The average incidence of seropositive animals in individual herds was from 11.11% (flock No.11) up to 85.71% (flock No.12). Titers ranged from 1:4 to 1:256 (Table 3). OvHV-2 infection was detected in 12 flocks out of the 14 sampled flocks. Levels of infection were found in flocks from Hokkaido, Aomori and Iwate prefectures. Hokkaido prefecture, the most involved in sheep production, had an overall seroprevalence of 30.97%. The serological results shows 80% of flocks were seropositive. Relatively higher prevalences were reported from the other two prefectures of Iwate and Aomori, 56.52% and 55.55%, respectively, with all the sampled flocks showing seropositive animals. Four rams and 54 ewes, and two goats were affected. Seroconversions were present in animals of all age categories from one year of age and older than eight years of age. Comparison between different age categories for the percentage of sheep positive for antibodies to OvHV-2 is reported in Table 4. Evaluation of the possible impact of OvHV-2 infection on production in the sampled flocks did not reveal a clear correlation with the reported levels of seropositive animals (Table 5).

Age category (year)	OvHV-2
1	37.5
2	44.44
3	65
4	20
5	54.16
6	21.42
7	30
≥8	33.33
Not known	34.78

Table 4. Comparison between different age categories for the percentage of sheep positive for antibodies to OvHV-2. Percentage computed excluding samples resulting toxic or not tested for insufficient serum quantity.

Flock No.	Prefecture	OvHV-2	Annual lambing rate	Annual lamb mortality rate	Annual culling rate	Mortality rate among adults
1	Hokkaido	33.33	NR	NR	NR	5
2	Hokkaido	72.72	0.72	1.29	0	4.76
3	Hokkaido	63.63	1.62	3.46	14.77	9.2
4	Hokkaido	NE	1.1	12.78	2.97	0
5	Hokkaido	17.64	1.61	20	11.73	8.33
6	Hokkaido	15.38	1.48	17.09	10.33	9.09
7	Hokkaido	15.38	1.58	16.92	6.66	2.22
8	Hokkaido	0	2.44	20.53	NR	NR
9	Hokkaido	40	1.23	0	0	0
10	Hokkaido	50	NR	NR	NR	10
11	Iwate	11.11	1.61	6.89	0	11.76
12	Iwate	85.71	1.38	9.83	24.03	4.8
13	Aomori	46.15	1.54	25.35	17.64	2.94
14	Aomori	80	1.14	21.87	0	9.09

NE: not executed due to insufficient aliquots for testing

NR: not recorded

Table 5. Comparison between different production parameters for the percentage of sheep positive for antibodies to OvHV-2.

Discussion

This survey has demonstrated absence of positiveness for antibodies to BLV, IBR and BoHV-4 in sheep flocks in the northern prefectures of Japan, where the majority of the Japanese sheep are bred. The potential for infection in sheep remains consistent when considering that these infections are present in cattle [3,4,5,8]. In particular IBR is most frequent in Hokkaido, as indicated by reports from 2005 to 2011, with up to 42 outbreaks in 2009 [1]. Nevertheless, the importance of sheep in the epidemiology of IBR remains limited, considering the lower capacity of spreading the virus [18]. Similarly, sheep can be infected by BLV only via iatrogenic accident. However, according to the World Animal Health Organization (OIE), IBR and BLV are included in the list of reportable diseases of importance to international trade [19].

The screening for antibodies against BTV showed a very low prevalence in sheep from Hokkaido. However, the c-ELISA used for testing ensured specificity of the results and avoided non specific reactions due to cross reactivity with the Ibaraki virus, endemic in Japan [2]. This is the first serological evidence of BTV in sheep in the northern regions of Japan, as previous reports referred to central prefecture of Tochigi in the Honshu Island [1,2]. Giving that this seropositivity was observed in sera collected in 2007 and 2008, it might be in relation to the circulation of the virus occurred during the last reported outbreaks in cattle in 2005 and 2006 [1].

From these serological data it is clear that exposure to OvHV-2 is widespread across northern Japan with all the three prefectures having seropositive flocks. The numbers of samples tested from the prefectures varied, but the rate of positivity did not appear to relate directly to the number of samples tested. This survey provides the first serological evidence of the occurrence of the infection in sheep in the Iwate and Aomori prefectures. Interviews to farmers revealed that no previous investigations on the pathogen have been carried out in all of the randomly selected sampling units for this study, thus providing preliminary information on their epidemiology and distribution referring to years 2007 and 2008.

The seroprevalence reported in the present study (37.66%) was lower when compared with the previous study conducted in Japan (64.3%) [16]. In the U.S.A., OvHV-2 was associated with 53-59% of serologically positive sheep [20], in comparison with Germany where there have been reported prevalence of 75% [21], whereas in the

Middle East even above 70% to 95% [22]. Furthermore, generally, in contrast to a lower degree of seropositivity in MCF-susceptible species, including cattle, bison, deer, caribou, elk (*Cervus elaphus*) and moose, which ranges from a few percent to 50% seropositive [20,21], species that may harbour MCF viruses, including sheep and goats, have a high frequency of seropositivity (>90%) [20], indicating their status as inapparent carriers of MCF viruses. The reported lower prevalence might be due to the use of SN test, different from the tests used in other studies. For example, complement fixation test was used in the previous study conducted in Japan [16]. In the U.S.A. and Germany, OvHV-2 antibodies were detected using enzyme-linked immunosorbent assay [20,21], and in Israel was used PCR [22]. Nevertheless, ideally a comparison with other tests would increase information about the reliability of infection rate obtained with the test used but was outside the scope of this study.

Although no diagnostic measures were in place, evaluation of the possible impact of MCF infection on production in the sampled flocks was considered taking into account that sheep can develop a mild form of MCF [12,13], with an eventual reduced productivity. This study did not reveal any clear correlation between production and the reported levels of seropositive animals. This is in line with the general subclinical course of the infection in MCF reservoir host species.

In this study, considering that one sheep flock (Hokkaido 9) was breed with a large number of goats, additional goat samples have been collected for testing MFC virus infection. Two caprine serum samples resulted positives. Reasonably, immune reaction reported in goats could refer to CpHV-2, genetically related, and cross reacting with the other MCF herpes viruses. However, seropositivity could be ascribable to immune reaction against OvHV-2, taking into account the close contact with infected sheep (40% resulted positive in the sheep flock) and the susceptibility of goats to the ovine virus. OvHV-2-specific DNA sequences have been reported in goats [23]. In addition, OvHV-2 sequences could be detected by PCR in 17% of goats surveyed in Indonesia [24]. Discrimination between CpHV-2 and OvHV-2 was not possible by the used serological test.

Considering the potential direct or indirect adverse effects on bovine welfare and production, suitable intervention and control measures should be introduced to avoid diffusion and impact on valuable breeding cattle farming. In some farms, other domestic animals and in particular cows were housed close to sheep pens or had access to common pastures. A sheep flock (Hokkaido 7) originated from a farm mainly focused on dairy cattle breeding, thus being in close contact with a herd of 700 Japanese black cows. Similarly, preventive measures should be considered to protect wild fauna. Cervids, such as sika deer which in Japan is represented by a large population, are even more sensible than cattle to OvHV-2 infection. Many deer die within 48 h of the first clinical signs and affected bison generally die within three days [25].

Primarily, close contacts with sheep flocks should be avoided. However, although most infections occur when the carrier host and susceptible animals are in close contact, transmission of OvHV-2 has been reported when cattle was separated by a distance of at least 70 meters from lambs [10]. Similarly, SA-MCF was also reported in bison herds up to 5 km from a lamb feedlot [10].

An important aspect to be considered is that MCF-associated OvHV-2, shed intermittently in nasal secretions [26], appears to be transmitted by contact or aerosol, mainly from lambs under one year of age, particularly by six to nine months old lambs [27]. Taking into account that the majority of lambs are not infected until after two

months of age, under natural conditions [27], production of virus-free hosts may also be considered. If lambs are removed from contact with infected sheep prior to that age, by early weaning and isolation, they remain uninfected and can be raised virus free [28]. This knowledge is being used by farmers to produce OvHV-2-free sheep in USA and Europe [29]. Similarly, the production of CpHV-2-free goats could be obtained.

The demonstration of OvHV-2 circulation in sheep flocks in the northern prefectures of Japan, based on serological analysis, advanced 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. Knowledge and awareness on the disease should be improved and disseminated to veterinarians and farmers.

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