

ELISAおよび免疫拡散法を用いた我が国の羊における
Corynebacterium pseudotuberculosis感染の血清・疫学
的調査

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著者	親松, 静江 菊池, 直哉 趙, 宏坤
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Seroepidemiological Survey of *Corynebacterium pseudotuberculosis* Infection in Sheep in Japan using Enzyme-linked Immunosorbent Assay and Immunodiffusion

Shizue CHIKAMATSU, Hong-Kun ZHAO, Naoya KIKUCHI, and Takashi HIRAMUNE*

Department of Epizootiology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan

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ABSTRACT. Sera from 1186 apparently healthy sheep in Hokkaido were examined by enzyme-linked immunosorbent assay (ELISA) and immunodiffusion (ID) for the presence of antibodies against *Corynebacterium pseudotuberculosis*. ELISA-positives were 466 (39.3%) while ID-positives were 330 (27.8%). Spread of caseous lymphadenitis in sheep in Hokkaido was thus clarified. Although ID was less sensitive than ELISA in detecting the antibodies against *C. pseudotuberculosis* it did not give any non-specific reaction. From the results and in view of the simplicity of the test procedure, ID was found to be of practical diagnostic value. Distribution by age group of anti-*C. pseudotuberculosis* antibodies in 758 sheep in a herd detected by both tests showed that the ratio of positives was low in sheep aged less than 1 year, and the ratio increased significantly in those aged 1 year and continued to increase with age until it reached a plateau at the age of 4–5 years.—**KEY WORDS:** *Corynebacterium pseudotuberculosis*, ELISA, ID, ovine caseous lymphadenitis, serodiagnosis.

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Caseous lymphadenitis in sheep is caused by *Corynebacterium pseudotuberculosis* and is of great economic importance in countries exporting mutton. The disease is characterized by discrete, chronic abscesses containing a caseous pus, particularly in superficial lymph nodes and also in visceral nodes and organs [1]. Sheep breeding industry in Hokkaido, where half the number of sheep of Japan are raised, has been promoted by the recent increase of mutton imports to Japan and home demand for meat. The number of sheep in Hokkaido was 13,100 in 1986, which was 1.7 times the number of 1982. Under these conditions *C. pseudotuberculosis* infection is suspected to be a problem in the sheep of Hokkaido. Only a few reports have been available on *C. pseudotuberculosis* infection in Japan. Nakamura *et al.* [3]

isolated the agent from the abscess of 3 sheep imported from the United States. In 1987 in the authors' laboratory, Zhao *et al.* [5] reported that sera of 13 sheep which were affected with caseous lymphadenitis and from which *C. pseudotuberculosis* was isolated were found to be enzyme-linked immunosorbent assay (ELISA)-positive while sera of 235 sheep which did not have the lesions of caseous lymphadenitis were ELISA-negative, with only 2 exceptions. Thus, ELISA was found to be of diagnostic value.

The present study deals with seroepidemiological survey of caseous lymphadenitis in 1186 apparently healthy sheep in various parts of Hokkaido using ELISA and immunodiffusion (ID), and to estimate serodiagnostic value of ID.

*CORRESPONDENCE TO: HIRAMUNE, T., Department of Epizootiology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan.

MATERIALS AND METHODS

Sera: Sera from 1186 apparently healthy

sheep, different in breed and age, were used for seroepidemiological survey. The sera were collected from February 1985 to September 1987 from various parts of Hokkaido: 95 from 24 herds near Abashiri in eastern Hokkaido; 5 from 3 herds in Eniwa and 237 from a herd in Noboribetsu (total 242 in southern Hokkaido); and 2 from a herd in Tobetsu, 771 from a herd in Takikawa, 54 from 22 herds in Shinshinotsu and 22 from 2 herds in Ebetsu (total 849 in central Hokkaido). The sampling was made from almost all of the sheep raised in these herds.

Sera of 251 sheep in and around Ebetsu were also used. Of these, 248 were previously examined by Zhao *et al.* [5] and 3 were the sera of additional sheep which were pathologically and bacteriologically diagnosed in the authors' laboratory as caseous lymphadenitis. These 251 sera were used especially for evaluating diagnostic value of ID.

ELISA: The method described by Maki *et al.* [2] for diagnosis of ovine *C. pseudotuberculosis* infection was used; the antigen was the culture filtrate (toxin antigen) of *C. pseudotuberculosis* ATCC 19410 and ELISA procedures were similar to those described. The only exception in the method was the removal of non-specifically reactive substances in sera, as previously described by the authors [5]. Briefly, freeze-dried organisms of *Rhodococcus equi* type 2 of Prescott (ATCC 33702) were suspended in phosphate buffer saline (pH 7.2) containing 0.5% Tween 80 and 1% bovine serum albumin (Sigma) at the ratio of 2.5 mg/ml. Each serum (50 μ l) was mixed with 950 μ l of the *R. equi* suspension, incubated at room temperature for 1 hr and centrifuged at 1,300 \times g in 4°C for 30 min. The supernatant was used for ELISA.

Determination of ELISA positive/negative cut off point: The optical density (OD) of the contents of each well was measured using Immuno Reader (NJ 2000; Nihon

Inter Med Co.) at a wave length of 405 nm. OD of 0.474 was taken as the cut off point, as described previously [5], which was the mean of the sera from 235 sheep with no lesions of caseous lymphadenitis plus 3 times of the standard deviation.

Preparation of antigen for ID: The antigen used for ID was prepared according to Shigidi [4]. Briefly, culture filtrate of *C. pseudotuberculosis* (ATCC 19410) was precipitated with ammonium sulfate, dialyzed, concentrated with polyethylene glycol, subjected to gel filtration through Sephadex G-100 and concentrated with polyethylene glycol. The concentrated filtrate was used as the antigen. Titer of the antigen was examined with the anti *C. pseudotuberculosis* (ATCC 19410) goat antiserum previously prepared [5]. The highest antigen dilution showing a clear precipitin line was 1 unit of the antigen; 2 units of the antigen were used in the present study.

Procedure of ID: A micro agar gel diffusion test was carried out using 1% agar (BBL) in saline. Two units of antigen were filled in the central well and undiluted test sera were filled in the peripheral wells. Slides were kept in a moist atmosphere at room temperature and the result was read on the 2nd day.

Statistical analysis: The results were analysed by the χ^2 test.

RESULTS

A serological survey by ELISA and ID was made with the sera collected from 1186 apparently healthy sheep in various parts of Hokkaido. The results are shown in Table 1. In 2 herds raising 771 and 237 sheep, respectively, 43.6% and 38.0% were positive by ELISA and 28.4% and 32.5% were positive by ID. In total, 466/1186 (39.3%) were positive by ELISA and 330/1186 (27.8%) were positive by ID, and the difference was significant ($P < 0.05$). ELISA-

Table 1. Distribution of antibodies against *C. pseudotuberculosis* in sheep in Hokkaido detected by ELISA and ID

Area	City or town	No. of sheep (No. of herds)	No. of ELISA-positive (%)	No. of ID-positive (%)
Southern Hokkaido	Eniwa	5 (3)	1 (20.0)	1 (20.0)
	Noboribetsu	237 (1)	90 (38.0)	77 (32.5)
Central Hokkaido	Tobetsu	2 (1)	0	0
	Takikawa	771 (1)	336 (43.6)	219 (28.4)
	Shinshinotsu	54 (22)	5 (9.3)	5 (9.3)
	Ebetsu	22 (2)	16 (72.8)	16 (72.8)
Eastern Hokkaido	Abashiri	95 (24)	18 (18.9)	12 (12.6)
Total		1186 (54)	466 (39.3)	330 (27.8)

Table 2. Correlation of caseous lymphadenitis in sheep with results of antibody detection by ID

	Caseous lymphadenitis	
	Yes	No
ID-positive	12 ^{a)}	0
ID-negative	4 ^{a)}	235 ^{b)}

a) Isolation of *C. pseudotuberculosis* was positive. b) Bacteriological examination was not done, as those sheep were without caseous lymphadenitis.

positives were greater in number than or equal to ID-positives in every herd examined.

A comparison of ELISA and ID for detecting antibodies against *C. pseudotuberculosis* in sera of sheep revealed that the sera which were ELISA- and ID-positive were 329 (27.7%), those ELISA-positive but ID-negative were 137 (11.6%), that ID-positive but ELISA-negative was only 1 (0.1%), and those ELISA- and ID-negative were 719 (60.6%).

In order to examine diagnostic value of ID, the sera of 251 sheep were examined by ID (Table 2). Of the 251 sheep, 248 were previously examined in the authors' laboratory [5]; 13 had caseous lymphadenitis and 235 did not have the lesions. The remaining 3 were pathologically and bacteriologically diagnosed in the authors' laboratory as

Table 3. Distribution by age group of anti-*C. pseudotuberculosis* antibodies in 758 sheep in a herd

Age (Year)	No. examined	ELISA	ID
<1	266	11 (4.1)	6 (2.3)
1	160	82 (51.3)	49 (30.6)
2	103	53 (51.5)	29 (28.2)
3	86	58 (67.4)	33 (38.4)
4	61	50 (82.0)	41 (67.2)
5	52	48 (92.3)	32 (61.5)
6	27	22 (81.5)	17 (63.0)
8	3	2 (66.7)	2 (66.7)

caseous lymphadenitis, and the sera showed positive reaction in ELISA (the value of OD was 1.698, 1.312 and 0.698 respectively). Of the 16 sheep with the lesions of caseous lymphadenitis 12 (75%) were ID positive while of the 235 sheep with no the lesions of caseous lymphadenitis none were ID-positive.

Distribution by age group of anti-*C. pseudotuberculosis* antibodies was examined by ELISA and ID in September 1987 with a total of 758 sheep raised in a herd in Takikawa of central Hokkaido (Table 3). The ratio of positives was low in sheep aged less than 1 year and the ratio increased significantly in those aged 1 year, irrespective of the test method, and continued to increase with age until it reached a plateau

at the age of 4–5 years.

DISCUSSION

The present serological survey of the sera of 1186 sheep indicated that caseous lymphadenitis may be spreading in Hokkaido.

Based on the authors' present and previous [5] studies, the serodiagnostic value in caseous lymphadenitis of ELISA and ID may be compared as follows. 1) ELISA detected more positives than ID but ELISA gave non-specific reaction occasionally; 16 sheep (13 in the previous study plus 3 in the present study), which had the lesions of caseous lymphadenitis and from which *C. pseudotuberculosis* was isolated, were all ELISA positive, while 235 sheep which did not have the lesions were all ELISA negative, with 2 exceptions [5]. 2) Although ID was less sensitive than ELISA in detecting the antibodies against *C. pseudotuberculosis* it did not give any non-specific reaction; of the 16 sheep, which had the lesions of caseous lymphadenitis and from which *C. pseudotuberculosis* was isolated, 12 (75%) were ID positive, while 235 sheep which did not have the lesions were ID negative with no exceptions. From the above findings and in view of the simplicity of the test procedure, ID may be of practical usefulness in detecting the disease in the field. Four of the 16 sheep had the lesions of caseous lymphadenitis but were ID negative. The amount of the antibodies in these sheep might be insufficient to be detected by ID. Further studies are needed to solve the discrepancy.

Distribution by age group of anti-*C. pseudotuberculosis* antibodies of sheep in a herd was clarified in the present survey. The fact that the ratio of positives was low in sheep aged less than 1 year and the ratio increased most significantly in those aged 1 year, may suggest that *C. pseudotuberculosis* infection occurred in this herd mostly in sheep at 1 year of age.

Although the detection of antibody alone is not sufficient for the diagnosis of the disease, the present results of the serological survey in Japan of ovine *C. pseudotuberculosis* infection, together with our previous bacteriological evidence [5], may be of epidemiological importance to this country.

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要 約

ELISA および免疫拡散法を用いた我が国の羊における *Corynebacterium pseudotuberculosis* 感染の血清・疫学的調査：親松静江・趙 宏坤・菊池直哉・平棟孝志(酪農学園大学獣医学科家畜伝染病学教室)——北海道各地で飼育されている見かけ上健康な羊1,186頭の *Corynebacterium pseudotuberculosis* に対する抗体を ELISA および免疫拡散法(ID)により調査した。ELISA 陽性の羊は466頭(39.3%)、また、ID 陽性のそれは330頭(27.8%)で、本菌感染羊が道内に高率に存在することが示唆された。ID は ELISA よりも抗体検出感度がやや劣るが、非特異反応が認められないこと、および簡便さの点で実用的な診断法と思われた。多数を飼育している1牧場の758頭の羊の年齢と抗体陽性率の関係をみたところ、抗体陽性率は、1歳未満では低率であったが、1歳で有意に急上昇し、以後、4-5歳まで加齢とともに上昇した。