

Prevalence and Allele Frequency Estimation of Bovine Leukocyte Adhesion Deficiency (BLAD) in Holstein-Friesian Cattle in Japan

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ABSTRACT. Blood samples from 796 Holstein dairy cows in 20 herds from 6 districts in Japan from June 1994 to August 1995 were examined to determine whether they were BLAD-free, BLAD carriers, or BLAD-affected by use of DNA-polymerase chain reaction (PCR) analysis. The usage of semen of confirmed BLAD-carriers for artificial insemination in the Hokkaido district and two selected dairy farms was examined to estimate the gene frequency of BLAD carriers of sires. BLAD-carrier prevalence in 20 herds (796 cows, over 2.5 years old) ranged from 0 to 23.5%, and the mean BLAD-carrier prevalence was 8.1%. The BLAD-carrier prevalence in 10 herds (363 cows) in which the occurrence of BLAD was not detected by the DNA-PCR test ranged from 0 to 12.5% with a mean of 5.4%. The BLAD-carrier prevalence in 10 herds (433 cows) in which the occurrence of BLAD was confirmed by DNA-PCR analysis ranged from 2.6 to 23.5% with a mean of 10.8%, and these values were significantly ($P < 0.05$) higher than those of dairy herds in which the occurrence of BLAD was not detected. The age distribution in BLAD carriers in these cows ranged from 2.5 to 11 years. The mean gene frequencies of BLAD among 796 cows from 20 herds and 433 cows from 10 herds in which the occurrence of BLAD was detected were 0.041 and 0.054, respectively. The proportional usage of semen of BLAD carriers for artificial insemination in the Hokkaido district in 1992 was 12.6%, and its gene frequency was 0.058. On two selected farms in which higher BLAD-carrier rates were detected, the prevalences were 35.5% and 25.8%, and their gene frequencies were 0.177 and 0.129, respectively. The occurrence of BLAD-affected in Holstein dairy cattle was estimated to be 0.16–0.31% at birth in Japan without genetic control. — **KEY WORDS:** BLAD, carrier prevalence, gene frequency.

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Bovine leukocyte adhesion deficiency (BLAD) in Holstein cattle is an autosomal-recessive congenital disease characterized by recurrent bacterial infections, delayed wound healing and stunted growth, and is also associated with persistent marked neutrophilia [2, 8, 12, 17, 19]. There is a deficiency in β_2 integrin of leukocytes from cattle with BLAD, and neutrophil functions are severely impaired [8, 12]. It is likely that the gene encoding defective CD18 on leukocytes is widespread in Holstein dairy cattle. The occurrence of BLAD in Holstein cattle has been reported in various countries, including the United States [5, 8], Germany [17], the Netherlands [9], Denmark [2], England [3] and Japan [11, 19]. It is important to know the prevalence of the gene encoding impaired CD18 for BLAD in Holstein dairy herds for evaluation of a control program for the BLAD-associated gene from Holstein cattle. The prevalence of BLAD carriers and the gene frequency of BLAD in Holstein cattle in Japan are as yet unclarified. In our study, blood samples from 796 Holstein cattle from 20 dairy herds in 5 districts in Japan were tested to determine whether they were BLAD-free, BLAD carriers or BLAD-affected, by use of the DNA-polymerase chain reaction (PCR) test. The objective of the study reported here was to describe the BLAD-carrier prevalence, the age distribution of BLAD carriers, the geographical distribution and the related gene frequency among Holstein dairy cattle in Japan.

MATERIALS AND METHODS

Bulls and utilization of semen: Pedigree analysis of sires of cattle affected with BLAD was carried out using the registration certificates. The number of semen specimens of BLAD carriers used for artificial insemination was examined in total numbers of semen specimens used in 1992 in two selected areas, in which higher occurrence of BLAD had been found as compared to other areas.

Holstein dairy cows: A total of 796 Holstein-Friesian dairy cows aged from 2.5 to 11 years from 20 herds were collected from the Hokkaido, Tohoku, Kanto, Chugoku, and Kyushu districts in Japan from June 6th, 1994 to August 10th, 1995. In our study, 433 dairy cows from 10 herds where the occurrence of cattle affected with BLAD had been detected were used to evaluate the BLAD-carrier prevalence. In comparison, 363 dairy cows from 10 herds, where the semen from bulls with BLAD-carriers had been utilized in mating records were selected.

Blood samples: Ten ml of blood was collected from the tail vein into a tube containing heparin (20 IU/ml), and blood samples were transported to the laboratory at 10°C for the DNA-PCR test.

CD18 genotype analysis by DNA-PCR test: Identification of the normal and defective bovine CD18 alleles by a DNA-PCR test was performed according to the procedure described by Shuster *et al.* [16] with slight modifications. Isolated DNA solution (3 μ l) from leukocytes was amplified

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for 35 cycles (94°C 15 sec, 69°C 20 sec) in a 20 μ l reaction mixture containing 1 \times PCR buffer, 0.2 mM dNTPs, 0.5 unit of amplified polymerase (Perkin-Elmer Cetus), and 4 pmol each of sense primer (5'-TCCGGAGGGCCAAGGGCTA) and antisense primer (5'-GAGTAGGAGAGGTCCATCAGGTAGTACAGG). Reaction tubes and contents were kept on ice until placed directly into the hot thermal cycler block. Ten-microliter aliquots of the amplification product were subjected to restriction endonuclease digestion separately by direct addition of 4 units each of Taq I or Hae III followed by incubation for 1.5 hr at 65°C or 37°C, respectively. The digested products were analyzed by 4% agarose gel electrophoresis and ethidium bromide staining, and were classified as BLAD-free (homozygous for the normal allele), BLAD-carrier (heterozygotes) and BLAD-affected (homozygotes, D128G mutation), respectively.

Analysis: The gene frequencies of BLAD-free cattle, BLAD carriers and BLAD-affected cattle were calculated based on the Hardy-Weinberg law [14] as follows:

$$P = \frac{2(AA) + (Aa)}{2N} \quad , \quad q = \frac{2(aa) + (Aa)}{2N}$$

where p =the gene frequency of BLAD-free normals, q =the gene frequency of BLAD-carriers, N =the total number of cattle tested, AA =the number of BLAD-free cattle, Aa =the number of BLAD carriers, and aa =the number of cattle with BLAD-affected.

The differences in BLAD-carrier prevalence among the 2 groups in which BLAD was detected or not detected in the dairy herds were analyzed statistically by X^2 analysis and Student's t -test. Values of $p < 0.05$ were regarded as significant. The estimation of the occurrence of BLAD-affected in Holstein herds was calculated based on the gene frequencies of BLAD-carriers.

RESULTS

Pedigree analysis of cattle affected with BLAD showed that the bulls were linked genetically to the bull (Osborndale Ivanhoe), which was previously identified as the ancestral carrier of BLAD, and 5 major bulls, A to E, were confirmed to be the sires of BLAD-affected calves, resulting in the occurrence of BLAD-affected cattle in Japan from 1983 to 1995 (Fig. 1).

The prevalence of the gene encoding defective CD18 for BLAD in Japanese Holstein dairy herds was evaluated (Fig. 2). Geographic distribution of BLAD carriers in 18 of 20 Holstein dairy herds showed that the gene encoding defective CD18 for BLAD was widespread in Holstein dairy cattle in Japan.

No homozygote for BLAD was identified in 796 dairy cows in the present study. BLAD-carrier prevalences in 796 dairy cows from 20 herds ranged from 0 to 23.5%, and the mean BLAD-carrier prevalence was 8.1% in Japan.

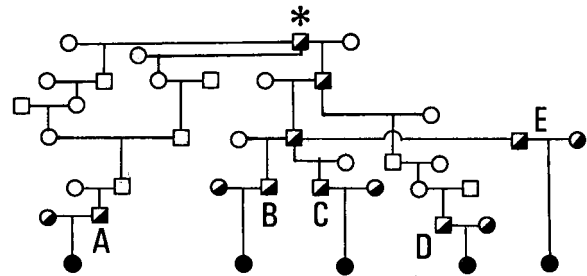


Fig. 1. Pedigrees of cattle affected with BLAD. *Common ancestral sire=Osborndale Ivanhoe. =Male. =Female. Filled symbols indicate homozygous BLAD, and half-filled symbols denote carriers. Open symbols remained to be clarified. A: HB187 (period in use: 2/1990-), B: H330 (6/1983-7/1994), C: H310 (12/1982-10/1987), D: H333 (5/1990-3/1994), E: H311 (5/1983-5/1992).

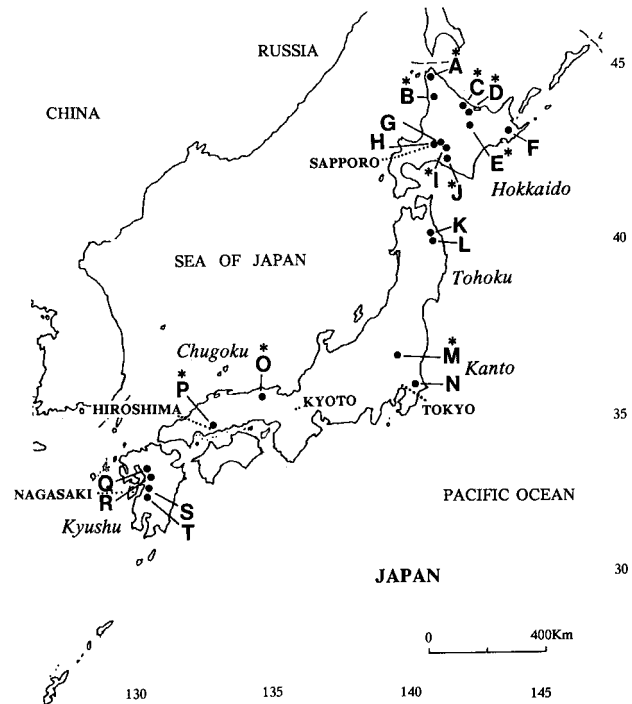


Fig. 2. Geographical distribution of BLAD-carriers in Holstein dairy herds in Japan. A total of 796 dairy cows from 20 herds were tested to determine the presence of BLAD carriers. Two dairy herds K and Q had no BLAD carriers. The asterisks indicate the Holstein herds that the occurrence of BLAD-affected had been detected during 1990-1995.

BLAD-carrier prevalences among 453 Holstein dairy herds in which the occurrence of BLAD was detected by the DNA-PCR test ranged from 2.6 to 23.5% with a mean of 10.8%, and these values were significantly ($P < 0.05$, $X^2 = 5.026$, $df = 1$) higher than those of dairy herds where the occurrence of BLAD was not detected (Table 1). Herd-specific BLAD-carrier prevalences among 363 Holstein dairy cows from 10 herds where the occurrence of BLAD was not detected were

Table 1. The rates of BLAD carriers among 796 Holstein dairy cows from 20 herds in Japan

Herds and Districts	No. of Carriers/ No. of cows tested	Carrier (%)	Gene frequency of BLAD-carriers
Group 1			
<i>Hokkaido</i>			
A Wakkanai	8/59	13.6	
B Enbetsu	9/72	12.5	
C Monbetsu-M	2/27	7.4	
D Monbetsu-N	5/55	9.1	
E Abashiri	5/47	10.6	
I Chitose	1/39	2.6	
J Tomakomai	5/33	15.2	
<i>Kanto</i>			
M Tochigi	2/43	4.7	
<i>Kansai</i>			
O Hyogo-Tanba	8/34	23.5	
<i>Chugoku</i>			
P Hiroshima	2/24	8.3	
Total 10	47/433 ^{a)}	10.8 ± 5.9 ^{b)}	0.054
Group 2			
<i>Hokkaido</i>			
F Shibetsu	5/58	8.6	
G Ebetsu-U1	8/71	11.3	
H Ebetsu-U2	3/36	8.3	
<i>Tohoku</i>			
K Iwate-S	0/15	0	
L Iwate-C	2/16	12.5	
<i>Kanto</i>			
N Chiba	1/48	2.1	
<i>Kyushu</i>			
Q Ooita-A	0/15	0	
R Ooita-B	1/30	3.3	
S Kumamoto-U	2/39	5.1	
T Kumamoto-M	1/35	2.9	
Total 10	23/363	5.4 ± 4.5	0.032

a) $P < 0.05$ (X^2 -test). b) $P < 0.05$ (Student's t -test). Group 1=The occurrence of BLAD was detected in dairy herds by DNA-PCR test. Group 2=The occurrence of BLAD was not detected in dairy herds by DNA-PCR test.

0 to 12.5% with a mean of 5.4%. Approximately 35% of the dairy herds had a prevalence of more than 10% BLAD-carriers. The highest gene frequency of BLAD was 0.118 found in a herd in which the BLAD-carrier prevalence was 23.5% and 3 affected calves had been previously detected, and the occurrence of BLAD was estimated to be 1.52% at birth.

The age distribution of Holstein dairy cows that were BLAD carriers ranged from 2.5 to 11 years old and was slightly different from that of BLAD-free control cows, among which there were few more than 5 years old (Fig. 3).

The proportional usage of BLAD-carrier-positive semen for artificial insemination in the Hokkaido district in 1992 was 12.6%, and on two dairy farms, A and O, in which the BLAD-carrier prevalences detected were 35.5% (22/62) and 25.8% (16/62), respectively (Table 2). The gene frequencies of BLAD carriers among 796 Holstein dairy cows and of semen used in the Hokkaido district were 0.044 and 0.058, respectively. The occurrence of BLAD at birth in Holstein

calves was conservatively estimated to be 0.16% in herds in which BLAD had not been detected previously and 0.31% in herds in which BLAD had been detected in Japan.

DISCUSSION

The pathogenesis of Holstein cattle affected with BLAD has been elucidated and this disorder, formerly known as a bovine granulocytopeny syndrome is now termed BLAD [6, 10, 18]. Cattle affected with BLAD suffer from stunted growth, are susceptible to life-threatening infectious complications such as pneumonia, and subsequently die at a young age. No overt abnormalities except for a few neutrophil extravasations into inflamed tissues of cattle affected with BLAD are observed in patho-anatomical studies. Only limited data concerning the prevalence of BLAD carriers in Holstein cattle throughout the world has been reported, such as the reports in the United States [16] and Denmark [7]. Information concerning the prevalence

of BLAD-carriers in Holstein dairy herds is required to institute an effective control program. In the present

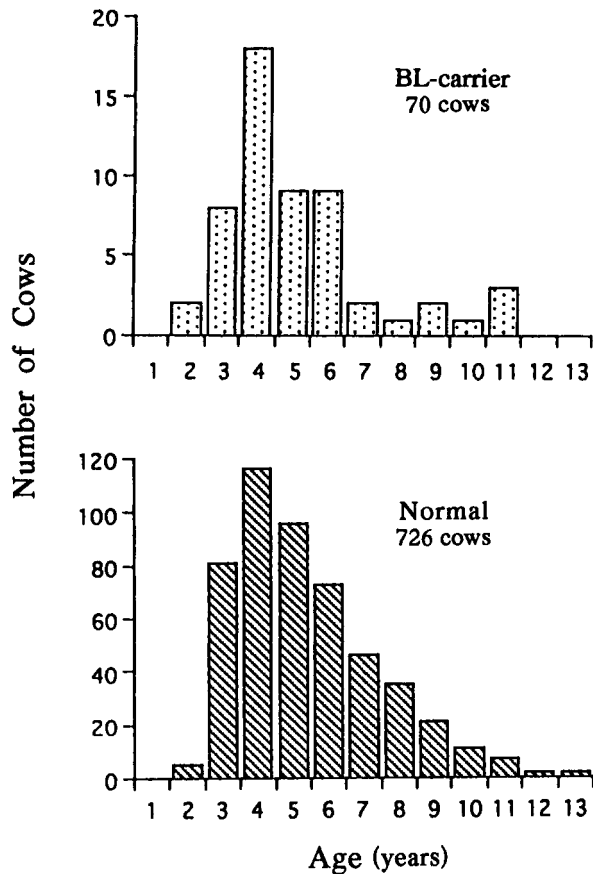


Fig. 3. Age distribution of Holstein BLAD-carrier and BLAD-free dairy cows in Japan. A total of 796 dairy cows were divided into 2 groups, BLAD (BL)-carrier and BLAD-free (Normal).

study, we performed DNA-PCR tests for BLAD in selected Holstein dairy herds in Japan and clarified that the gene encoding defective CD18 expression on leukocytes is widespread in Holstein dairy herds.

About 11% of Japanese Holstein-Friesian breeding bulls were found to be carriers in a preliminary study [13], and semen from BLAD-carrier bulls has been widely used in Japanese dairy herds. In addition, some imported semen was also confirmed to carry BLAD [4]. Pedigree analysis confirmed that BLAD carriers were also present in the background of dams. These findings indicate that the genetic defect of BLAD appears to have had a considerable effect on the dairy industry in Japan for at least 12 years. As this disorder is inherited as an autosomal recessive, the occurrence of BLAD can theoretically be controlled by using non-carrier bulls even though BLAD carriers in dairy herds were highly prevalent. However, semen from BLAD-carrier bulls is still used for artificial insemination, and calves affected with BLAD are still being born in certain areas.

Geographic analysis of BLAD carriers in Holstein dairy herds demonstrated that the gene encoding defective CD18 for BLAD was widely distributed in Holstein dairy cattle throughout Japan. This is consistent with the results on the occurrence of a series of affected heifers showing persistent pneumonia, severe ulcerative periodontitis and marked neutrophilia in Holstein dairy calves in Hokkaido, Japan in 1984 to 1985 [1, 15]. This also confirmed the finding that semen from BLAD-carrier bulls had been employed since 1982 or perhaps even earlier in Hokkaido. The presence of 11-year-old cows that were BLAD carriers demonstrated that BLAD carriers already existed before 1983. This finding is consistent with that of the above-mentioned affected calves showing marked neutrophilia.

A significantly higher prevalence of BLAD carriers was observed in dairy herds (10.8%) in which BLAD had been detected between 1991 and 1995 as compared with 5.4%

Table 2. The prevalences of BLAD carriers and gene frequencies of bulls and semen for artificial insemination used in the Hokkaido district and in 2 selected dairy herds

	Normal	BLAD carriers	Total number	Carrier prevalence (%)	Gene frequency (BLAD carriers)
Bulls ^{a)}	755	91	846	10.8	0.054
Units of semen used in Hokkaido ^{b)}	882198	115802 ^{c)}	998000	11.6	0.058
Units of semen used on farm A ^{b)}	40	22 ³⁾	62	35.5	0.177
Units of semen used on farm O ^{b)}	46	16 ³⁾	62	25.8	0.129

a) Data from the report of Nagahata and Morita, 1995. b) Data of 1992. c) Numbers are comprised of BLAD-carrier bulls used for artificial insemination. Results are shown based on the data of 1992.

for 10 herds in which BLAD had not been detected. Variations in the prevalence and geographic distributions of BLAD-carriers among Holstein dairy herds in Japan are considered to be closely associated with the frequency of utilization of the semen of BLAD carriers, for artificial insemination, and the culling risk of dairy cows that were BLAD carriers in each district and farm. To control of the occurrence of BLAD in Holstein cattle, bulls registered as BLAD carriers have already been assigned the designation *BL and thus should be noted in the selection of semen for practical use.

The BLAD-carrier prevalence was 10.8% for all bulls tested by DNA-PCR analysis among 846 Japanese Holstein bulls, including candidate sires [13]. This value was slightly lower than the value of 14.1% in the United States [16]. The rate for utilization of semen of BLAD carriers had a great influence on the BLAD-carrier prevalence of dairy herds. The highest value for the gene frequency of BLAD carriers in dairy herds was 0.118, found in herd O where the BLAD-carrier rate was 23.5% and in which 3 calves affected with BLAD had been previously detected, and the occurrence of BLAD was estimated to be 1.52% at birth. Semen from BLAD-carrier bulls used for artificial insemination in 2 selected dairy herds where carrier rates of BLAD were highly prevalent, was found to be highly utilized, with rates of 35.5% and 25.8%, respectively. The occurrence of calves affected with BLAD was estimated to be 0.19% in herds in which the occurrence of BLAD had not been detected and 0.31% in those in which BLAD had been detected at birth among Japanese Holstein cattle, based on the frequencies of 0.058 for utilization of semen of BLAD carriers, and 0.032 and 0.054 for 2 groups of Holstein dairy cows. Although the elimination of BLAD-carrier bulls from the Holstein world would be the most efficient method to control this genetic disorder, many BLAD-carrier bulls are still listed commercially for artificial insemination and BLAD is still occurring in Japan. A program for the elimination of the gene encoding defective CD18 in Holstein cattle is being enacted slowly in Japan. Monitoring the prevalence of BLAD-carriers in random selected herds may be helpful in judging the effectiveness of the BLAD-control program.

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