Cattle with the BoLA class II DRB3*0902 allele have significantly lower bovine leukemia proviral loads

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ABSTRACT. The bovine MHC (BoLA) class II DRB3 alleles are associated with polyclonal expansion of lymphocytes caused by bovine leukemia virus (BLV) infection in cattle. To examine whether the DRB3*0902 allele, one of the resistance-associated alleles, is associated with the proviral load, we measured BLV proviral load of BLV-infected cattle and clarified their DRB3 alleles. Fifty-seven animals with DRB3*0902 were identified out of 835 BLV-infected cattle and had significantly lower proviral load ($P<0.000001$) compared with the rest of the infected animals, in both Japanese Black and Holstein cattle. This result strongly indicates that the BoLA class II DRA/DRB3*0902 molecule plays an important immunological role in suppressing viral replication, resulting in resistance to the disease progression.

KEY WORDS: BLV, BoLA class II, DRB3*0902, polymorphism, proviral load

Bovine leukemia virus (BLV) is the etiological agent of enzootic bovine leukosis (EBL) and belongs to the family Retroviridae. While most of the BLV-infected cattle remain asymptomatic for life, about 30% of infected cattle develop a persistent B-cell lymphocytosis (PL) and 1–5% develop lymphosarcoma [1, 2]. In Japan, the number of cattle with the lymphosarcoma is increasing every year [13] and progressively causing more economic loss for farmers. Although this is a nationwide problem in Japan, no control measures have been officially implemented. In order to establish an effective control measure, we previously investigated how BLV transmission routes and BLV proviral load were associated with spread of the disease and demonstrated that cattle with higher BLV proviral load have higher risk of either horizontal or vertical transmission [9, 10]. To reduce and control BLV infection, segregating infected and uninfected cattle is the most effective measure if culling infected cattle is not an option [8]. It is now known that a small population of BLV-infected animal maintains low BLV proviral load and does not transmit the virus to the neighboring animals, though uninfected cattle neighboring to infected cattle have a significantly higher risk for BLV infection [5, 8]. In farms with tie-stall housing or stanchion, it is feasible to place these cattle with extremely low proviral load between infected and uninfected cattle as shields to prevent the virus transmission. Although the mechanisms are still unknown, there is accumulated evidence that the bovine MHC (bovine leukocyte antigen: BoLA) class II DRB3 gene is strongly associated with resistance to developing PL and a reduction in proviral load [4, 6, 11, 12, 14, 18, 19]. Therefore, animals with the resistant DRB3 allele could be useful for such a strategy for controlling BLV infection. In this study, we have focused on the DRB3*0902 allele for the resistant genotype, and analyzed the relationship between the DRB3*0902 allele and the BLV proviral load in both Japanese Black and Holstein cattle.

Whole blood samples were collected from Japanese Black and Holstein cattle in Miyazaki and Oita prefectures in Japan and examined for antibodies against BLV gp51 using the BLV enzyme-linked immunosorbent assay (ELISA) kit (JNC, Tokyo, Japan). After the ELISA test, genomic DNAs were extracted from blood samples collected from BLV-seropositive cattle (653 heads...
of Japanese Black and 182 heads of Holstein cattle) and examined for their proviral loads and the DRB3*0902 allele. Genomic DNA extraction from the blood samples was performed using a Wizard® Genomic DNA Purification Kit (Promega, WI, U.S.A.), according to manufacturer’s instruction. Extracted DNA was quantified using NanoDrop 8000 (Thermo Fisher Scientific, MA, U.S.A.) and adjusted to 50 ng/µl in water. Quantitative real-time PCR was performed using an Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Forster City, CA, U.S.A.). BLV proviral loads were measured using the Cycleave PCR bovine leukemia virus detection kit (TaKaRa Bio Inc., Otsu, Japan) and indicated as virus copy number per 50 ng DNA according to manufacturer’s instructions.

To identify the DRB3*0902 allele, we first used PCR-restriction fragment length polymorphism (RFLP) method [17] in which only the BsrYI enzyme was used to digest PCR products and detect e-pattern bands (112, 87 and 85 bp), hereafter referred to as “E band”. For the first round, primers HL030 (5′-ATCCTCTCTGTGACGACATTTCC-3′) and HL031 (5′-TTTAAATTCGCCCTCCTCGACGAGATTTCCT-3′) were used to amplify the DRB3 exon 2. For the second round, primers HL030 and HL032 (5′-TCGGCCGTGCACTGAACTCCT-3′) were used as a hemi-nested PCR and the amplicon was used for RFLP analysis [17]. Ten µl of the amplicon for each sample were digested with BsrYI, and the restriction fragment pattern was obtained by electrophoresis in 3% Metaphor agarose gel (Lonza, Basel, Switzerland). Because this pattern may include other alleles, such as DRB3*0901 and DRB3*0903 alleles, each amplicon was extracted from agarose gel by using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), cloned into Mighty TA-cloning vector (TaKaRa Bio Inc.) and sequenced. Sequencing was performed in both direction using M13 forward and reverse primers with BigDye®Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems) and the data were analyzed using an Applied Biosystems 3730 DNA Analyzer. The Wilcoxon test in R (version 3.2.2) was used to analyze association between the proviral load and the DRB3*0902 allele and the P value less than 0.01 (P<0.01) was considered to be statistically significant.

Sixty animals had the E band among 835 BLV-seropositive animals examined. Among the 60 animals, 45 were Japanese Black cattle and 15 were Holstein cattle. The sequence analysis revealed that the 57 animals, 44 heads of Japanese Black cattle and 13 heads of Holstein cattle had DRB3*0902. Three animals, 1 head of Japanese Black cattle and 2 heads of Holstein cattle, were confirmed to have DRB3*0901 (Table 1). We have not identified any animal with DRB3*0903 in this study.

The animals were divided into 4 groups (Japanese Black cattle with and without DRB3*0902, Holstein cattle with and without DRB3*0902) and the median of proviral load in each group was calculated. The median of proviral load of Japanese Black cattle with DRB3*0902 was 20 and without was 417.5, Holstein cattle with DRB3*0902 was 5.8 and without was 1,853 (Fig. 1).

To identify the DRB3*0902 allele, we used a simplified conventional PCR-RFLP method with only BsrYI. The sequence analysis reveal that this method could not only effectively identify the DRB3*0902 allele (57/60) but also include the DRB3*0901 allele (3/60) as shown in Table 1. Because it is not clear whether animals with DRB3*0901 allele manifest the resistance [7], additional modification to the simplified method is needed to accurately distinguish the DRB3*0902 allele from the others.

To control BLV infection, measuring proviral loads is particularly important because higher proviral loads increase the risk of...
BLV infection [5, 10]. In this regard, animals with \( DRB^*0902 \), even if infected, are not likely to be a source of BLV transmission and thus can be used as shields placed between BLV-infected and non-infected animals when the farmer doesn’t have enough space to separate them. In fact, we have successfully used this strategy to reduce the BLV infection rate for one heavily infected farm in Miyazaki (data not shown).

Taken together, animals with \( DRB3^*0902 \) and infected with BLV have low proviral load and thus a low risk of spreading BLV infection. Animals with the heterozygous \( DRB3^*0902 \) could be found in the field at about 6.8% (>7% if included uninfected cattle: data not shown) and be useful to protect uninfected animals from infected animals. Finally, studies on immunological mechanisms involving the resistance are anticipated.

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REFERENCES

1. Burny, A., Cleuter, Y., Kettmann, R., Mammerickx, M., Marbaix, G., Portetelle, D., van den Broeke, A., Willems, L. and Thomas, R. 1988. Bovine leukaemia: facts and hypotheses derived from the study of an infectious cancer. *Vet. Microbiol.* 17: 197–218. [Medline] [CrossRef]

2. Burny, A., Bruck, C., Cleuter, Y., Couez, D., Deschamps, J., Gregoire, D., Ghysdael, J., Kettmann, R., Mammerickx, M., Marbaix, G., et al. 1985. Bovine leukaemia virus and enzootic bovine leukosis. *Onderstepoort J. Vet. Res.* 52: 133–144. [Medline]

3. Clerici, M., Balotta, C., Meroni, L., Ferrario, E., Riva, C., Trabattoni, D., Ridolfi, A., Villa, M., Shearer, G. M., Moroni, M. and Galli, M. 1996. Type 1 cytokine production and low prevalence of viral isolation correlate with long-term nonprogression in HIV infection. *AIDS Res. Hum. Retroviruses* 12: 1053–1061. [Medline] [CrossRef]

4. Forletti, A., Juliarena, M. A., Ceriani, C., Amadio, A. F., Esteban, E. and Gutiérrez, S. E. 2013. Identification of cattle carrying alleles associated with resistance and susceptibility to the Bovine Leukemia Virus progression by real-time PCR. *Res. Vet. Sci.* 95: 991–995. [Medline] [CrossRef]

5. Juliarena, M. A., Barrios, C. N., Ceriani, M. C. and Esteban, E. N. 2016. Hot topic: Bovine leukemia virus (BLV)-infected cows with low proviral load are not a source of infection for BLV-free cattle. *J. Dairy Sci.* 99: 4586–4589. [Medline] [CrossRef]

6. Juliarena, M. A., Poli, M., Ceriani, C., Sala, L., Rodríguez, E., Gutierrez, S., Dolcini, G., Odeon, A. and Esteban, E. N. 2009. Antibody response against three widespread bovine viruses is not impaired in Holstein cattle carrying bovine leukocyte antigen DRB3.2 alleles associated with bovine leukemia virus resistance. *J. Dairy Sci.* 92: 375–381. [Medline] [CrossRef]

7. Juliarena, M. A., Poli, M., Sala, L., Ceriani, C., Gutierrez, S., Dolcini, G., Rodriguez, E. M., Mariño, B., Rodriguez-Dubra, C. and Esteban, E. N. 2008. Association of BLV infection profiles with alleles of the BoLA-DRB3.2 gene. *Anim. Genet.* 39: 432–438. [Medline] [CrossRef]
8. Kobayashi, S., Tsutsui, T., Yamamoto, T., Hayama, Y., Muroga, N., Konishi, M., Kameyama, K. and Murakami, K. 2015. The role of neighboring infected cattle in bovine leukemia virus transmission risk. *J. Vet. Med. Sci.* **77**: 861–863. [Medline] [CrossRef]

9. Mekata, H., Sekiguchi, S., Konnai, S., Kirino, Y., Horii, Y. and Norimine, J. 2015. Horizontal transmission and phylogenetic analysis of bovine leukemia virus in two districts of Miyazaki, Japan. *J. Vet. Med. Sci.* **77**: 1115–1120. [Medline] [CrossRef]

10. Mekata, H., Sekiguchi, S., Konnai, S., Kirino, Y., Honkawa, K., Nonaka, N., Horii, Y. and Norimine, J. 2015. Evaluation of the natural perinatal transmission of bovine leukemia virus. *Vet. Rec.* **176**: 254. [Medline] [CrossRef]

11. Mirsky, M. L., Olinstead, C., Da, Y. and Lewin, H. A. 1998. Reduced bovine leukemia virus proviral load in genetically resistant cattle. *Anim. Genet.* **29**: 245–252. [Medline] [CrossRef]

12. Miyasaka, T., Takeshima, S. N., Imba, M., Matsumoto, Y., Kobayashi, N., Matsuhashi, T., Sentsui, H. and Aida, Y. 2013. Identification of bovine leukocyte antigen class II haplotypes associated with variations in bovine leukemia virus proviral load in Japanese Black cattle. *Tissue Antigens* **81**: 72–82. [Medline] [CrossRef]

13. Murakami, K., Kobayashi, S., Konishi, M., Kameyama, K. and Tsutsui, T. 2013. Nationwide survey of bovine leukemia virus infection among dairy and beef breeding cattle in Japan from 2009–2011. *J. Vet. Med. Sci.* **75**: 1123–1126. [Medline] [CrossRef]

14. Nikbakht Brujeni, G., Ghorbanpour, R. and Esmailnejad, A. 2016. Association of BoLA-DRB3.2 Alleles with BLV Infection Profiles (Persistent Lymphocytosis/Lymphosarcoma) and Lymphocyte Subsets in Iranian Holstein Cattle. *Biochem. Genet.* **54**: 194–207. [Medline] [CrossRef]

15. Ohira, K., Nakahara, A., Konnai, S., Okagawa, T., Nishimori, A., Maekawa, N., Ikebuchi, R., Kohara, J., Murata, S. and Ohtani, K. 2016. Bovine leukemia virus reduces anti-viral cytokine activities and NK cytotoxicity by inducing TGF-β secretion from regulatory T cells. *Immun. Inflamm. Dis.* **4**: 52–63. [Medline] [CrossRef]

16. Pyeon, D., O’Reilly, K. L. and Splitter, G. A. 1996. Increased interleukin-10 mRNA expression in tumor-bearing or persistently lymphocytotic animals infected with bovine leukemia virus. *J. Virol.* **70**: 5706–5710. [Medline]

17. van Eijk, M. J. T., Stewart-Haynes, J. A. and Lewin, H. A. 1992. Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP. *Anim. Genet.* **23**: 483–496. [Medline] [CrossRef]

18. Xu, A., van Eijk, M. J. T., Park, C. and Lewin, H. A. 1993. Polymorphism in BoLA-DRB3 exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. *J. Immunol.* **151**: 6977–6985. [Medline]

19. Zanotti, M., Poli, G., Ponti, W., Polli, M., Rocchi, M., Bolzani, E., Longeri, M., Russo, S., Lewin, H. A. and van Eijk, M. J. 1996. Association of BoLA class II haplotypes with subclinical progression of bovine leukemia virus infection in Holstein-Friesian cattle. *Anim. Genet.* **27**: 337–341. [Medline]