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NOTE

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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

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Table 1. Frequency of *BoLA-DRB3*0902* allele in animals giving E band in PCR-RFLP

	Japanese Black (n=653) (allele count=1,306)	Holstein (n=182) (allele count=364)	Total (n=835) (allele count=1,670)	Frequency of each allele (%) (allele count=1,670)
*DRB3*0901*	1	2	3	0.18 (0.05–0.57)[a)]
*DRB3*0902*	44	13	57	3.41 (2.62–4.43)[a)]
*DRB3*0903*	0	0	0	0.00 (0.00–0.29)[a)]
Total (allele count)	45	15	60	

a) Indicates 95% confidence interval for the percentage of each allele. There was no animal with homozygous *DRB3*0901* nor *DRB*0902* allele.

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 *n*g 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 *Bst*YI 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′-ATCCTCTCTCTGCAGCACATTTCC-3′) and HL031 (5′-TTTAAATTCGCGCTCACCTCGCCGCT-3′) were used to amplify the *DRB3* exon 2. For the second round, primers HL030 and HL032 (5′-TCGCCGCTGCACAGTGAAACTCTC-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 *Bst*YI, 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). The mean of proviral load (± SD) of Japanese Black cattle with *DRB3*0902* was 68.1 (± 319.1) and without was 49,219.9 (± 1,156,950), Holstein cattle with *DRB3*0902* was 13.8 (± 19.3) and without was 2,902.5 (± 3,660.6). Wilcoxon analysis indicated that cattle with *DRB3*0902* had significantly lower proviral load compared with cattle without *DRB3*0902* in both Japanese Black and Holstein cattle ($P<0.000001$). The proviral load of one head of Japanese Black cattle with *DRB3*0901* was 1,205.9. The two heads of Holstein cattle had proviral loads of 2.3 and less than 2.

We have focused on *BoLA-DRB3*0902* for the resistant genotypes and confirmed that cattle with *DRB3*0902* were clearly associated with very low BLV proviral load in the large-scale survey. Considering the fact that cattle with *DRB3*0902* are strongly resistant for developing PL [18], animals with this allele could have an immunogenetic advantage to reduce virus in the host and as a result do not develop PL and lymphosarcoma. Cytokines produced by $CD4^+$T lymphocytes are generally thought to play a role for disease progression during chronic retroviral infection [3]. In BLV infection, it has been reported that the production of IL-2 was associated with asymptomatic animals, while production of IL-10 was increased in PL animals [16]. Ohira *et al.* [15] have also reported that numbers of IFN-*γ* producing $CD4^+$T lymphocytes decreased with disease progression after BLV infection. These cytokines are mainly produced by activated antigen-specific $CD4^+$T lymphocytes and the activation depends on antigenic epitopes presented by MHC class II molecules. Therefore, it is reasonable to assume that certain MHC class II alleles can lead BLV-infected animals to a strong protective immunity but the others may fail to do so. We suggest that animals with *DRB*0902* can mount strong protective immunity against BLV infection and control the virus by eliciting strong BLV-specific $CD4^+$T lymphocytes, while some of the animals without *DRB*0902* may not be able to do so, resulting in high proviral load and disease progression.

To identify animals with *DRB3*0902*, we used a simplified conventional PCR-RFLP method with only *Bst*YI. 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

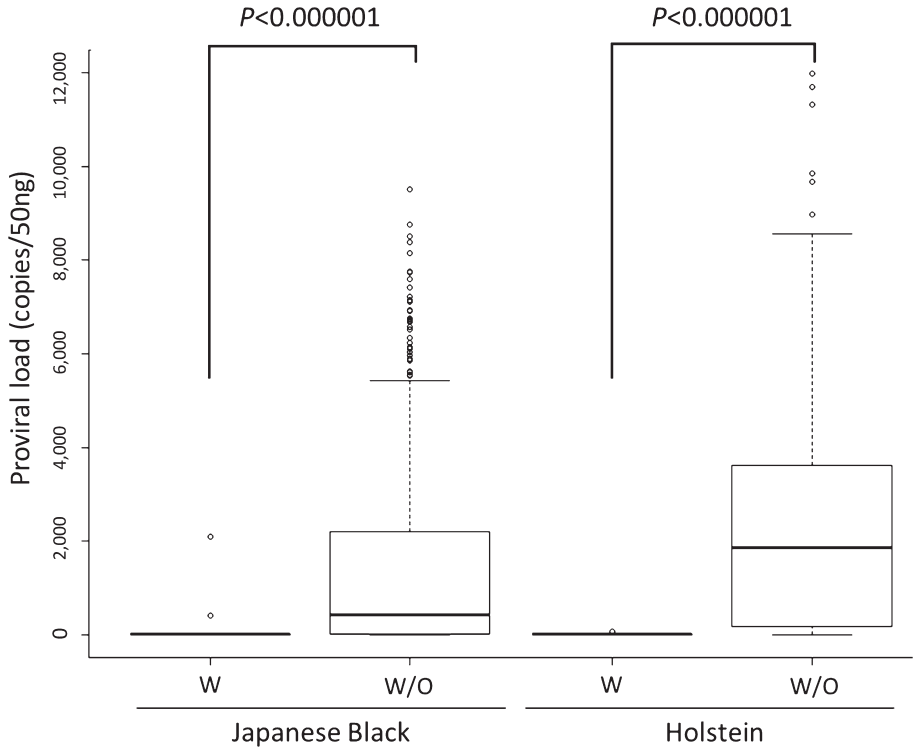

Fig. 1. Association between the *DRB3*0902* allele and proviral load. Proviral load is indicated as the number of copies per 50 *n*g DNA. Holstein cattle with *DRB3*0902*: W; without *DRB3*0902*: W/O (P<0.000001). Japanese Black cattle with *DRB3*0902*: W; without *DRB3*0902*: W/O) (P<0.000001). This indicates that cattle with *DRB3*0902* had significantly lower proviral load compared with cattle without *DRB3*0902* in both Japanese Black and Holstein cattle. Statistical analysis and box plot was performed by R (version 3.2.2; free statistical analysis software).

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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