Virology, Note

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

GENETIC RESISTANCE AGAINST BLV INFECTION

Takumi HAYASHI¹, Hirohisa MEKATA², Satoshi SEKIGUCHI¹, Yumi KIRINO³, Shuya MITOMA¹, Kazuyuki HONKAWA⁴, Yoichiro HORII⁴, ⁵ and Junzo NORIMINE¹, ⁵*

¹) Laboratory of Animal Infectious Disease and Prevention, Department of Veterinary Sciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen-Kibanadai-Nishi, Miyazaki, 889-2192, Japan
²) Organization for Promotion of Tenure Track, University of Miyazaki, 1-1 Gakuen-Kibanadai-Nishi, Miyazaki, 889-2192, Japan
³) Zoonosis Education and Research Project, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen-Kibanadai-Nishi, Miyazaki, 889-2192, Japan
⁴) Division of Research and Training for Livestock, Honkawa Ranch, 3898 Takase, Hita, Oita, 877-0056, Japan
⁵) Division of International Cooperation and Education, Center for Animal Disease Control, University of Miyazaki, 1-1 Gakuen-Kibanadai-Nishi, Miyazaki, 889-2192, Japan

*Corresponding author at: 1-1 Gakuen-Kibanadai-Nishi, Miyazaki, 889-2192, Japan.
TEL&FAX: +81-(985)-58-7591
E-mail address: nori@cc.miyazaki-u.ac.jp
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 WORD

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
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 \textit{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 50ng/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., Shiga, Japan) and indicated as virus copy number per 50ng DNA according to manufacturer’s instructions.

To identify the \textit{DRB3*0902} allele, we first used PCR-restriction fragment length polymorphism (RFLP) method [17] in which only the BstYI 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’-TTTAAATTTCGCGCTCACCTCGCCGCT-3’) were used to amplify the \textit{DRB3} exon 2. For the second round, primers HL030 and HL032 (5’-TCGCCGCTGCACAGTGAACCTCTC-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 BstYI, 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
3DRB3*0902 had significantly lower proviral load compared with cattle without
3DRB3*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.
4
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 don’t 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 BstYI. 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.

ACKNOWLEDGMENTS

This study was supported by JSPS KAKENHI Grant Number 26850175 and 25450468, and the Ito Foundation. We are thankful for the cooperation of the Miyazaki prefecture, NOSAI veterinarians, and farmers.
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Fig. 1. Association between the DRB3*0902 allele and proviral load. Proviral load is indicated as the number of copies per 50ng 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).
Table 1. Frequency of *BoLA-DRB3*<sup>0902</sup> allele in animals giving E band in PCR-RFLP

<table>
<thead>
<tr>
<th>Allele</th>
<th>Japanese Black (n=653)</th>
<th>Holstein (n=182)</th>
<th>Total (n=835)</th>
<th>Frequency of each allele (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(allele count=1,306)</td>
<td>(allele count=364)</td>
<td>(allele count=1,670)</td>
<td></td>
</tr>
<tr>
<td>DRB3&lt;sup&gt;0901&lt;/sup&gt;</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0.18 (0.05 - 0.57)&lt;sup&gt;a)&lt;/sup&gt;</td>
</tr>
<tr>
<td>DRB3&lt;sup&gt;0902&lt;/sup&gt;</td>
<td>44</td>
<td>13</td>
<td>57</td>
<td>3.41 (2.62 - 4.43)&lt;sup&gt;a)&lt;/sup&gt;</td>
</tr>
<tr>
<td>DRB3&lt;sup&gt;0903&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00 (0.00 - 0.29)&lt;sup&gt;a)&lt;/sup&gt;</td>
</tr>
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Total (allele count) 45  15  60

<sup>a)</sup> indicates 95% confidence interval for the percentage of each allele. There was no animal with homozygous DRB3<sup>0901</sup> nor DRB<sup>*0902</sup> allele.
Fig. 1

Proviral load (copies/50ng)

W    W/O    W    W/O

Japanese Black  Holstein

P<0.000001  P<0.000001