Bovine leukemia virus proviral load is more strongly associated with bovine major histocompatibility complex class II DRB3 polymorphism than with DQA1 polymorphism in Holstein cow in Japan

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Abstract
Bovine leukemia virus (BLV) causes enzootic bovine leukosis and is closely related to the human T-lymphotropic virus. Bovine major histocompatibility complex (BoLA) are used extensively as markers of disease and immunological traits in cattle. For BLV diagnosis, proviral load is a major diagnosis index for the determination of disease progression and transmission risk. Therefore, we investigated the frequency of BoLA-DRB3 alleles, BoLA-DQA1 alleles, and haplotypes of BoLA class II isolated from the heads of 910 BLV-infected cows out of 1290 cows assessed from BLV-positive farms, in a nationwide survey from 2011 to 2014 in Japan. Our aim was to identify BoLA class II polymorphisms associated with the BLV proviral load in the Holstein cow. The study examined 569 cows with a high proviral load and 341 cows with a low proviral load. Using the highest odds ratio (OR) as a comparison index, we confirmed that BoLA-DRB3 was the best marker for determining which cow spread the BLV (OR 13.9 for BoLA-DRB3, OR 11.5 for BoLA-DQA1, and OR 6.2 for BoLA class II haplotype). In addition, DRB3*002:01, *009:02, *012:01, *014:01, and *015:01 were determined as BLV provirus associated alleles. BoLA-DRB3*002:01, *009:02, and *014:01 were determined as resistant alleles (OR > 1), and BoLA-DRB3*012:01 and *015:01 were determined as susceptible alleles (OR < 1). In this study, we showed that BoLA-DRB3 was a good marker for determining which cow spread BLV, and we found not only one resistant allele (BoLA-DRB3*009:02), but also two other disease-resistant alleles and two disease-susceptible alleles. This designation of major alleles as markers of susceptibility or resistance can allow the determination of the susceptibility or resistance of most cows to disease. Overall, the results of this study may be useful in eliminating BLV from farms without having to separate cows into several cowsheds.

Keywords: BoLA-DRB3, BoLA-DQA1, Bovine leukemia virus, Proviral load, Japanese Holstein
cause earlier EBL onset, high accident rates, and low rates of conception and low milk production [6, 10–15]. Therefore, techniques for identifying high-risk cows are urgently needed to mitigate economic losses. It has been posited that cows classified as high-risk show a proviral load of over 14,000 copies/10^5 cells and 18,000 copies/10^5 cells in blood samples secreting BLV provirus into nasal and saliva, respectively [16]. It has been suggested that these cows may cause a high-risk for BLV transmission via coming into direct contact with healthy cows. In addition, it appears that proviral load correlates not only with BLV infection, but also with BLV disease progression [17–19]. Thus, BLV proviral load is an important index for estimating the stage of BLV infection.

Studies on BLV-associated host factors identified polymorphisms within the bovine major histocompatibility complex (MHC) (BoLA) [20–29]. BoLA is a highly polymorphic and tightly linked gene cluster [30]. Functionally, the BoLA class II gene is classified into two groups, DR and DQ. The DR molecule was constructed from a single DRA locus and a single DRB3 locus, and DQ molecules were constructed from at least two DQA loci and two DQB loci [31]. To date, 136 DRB3, 65 DQA, and 87 DQB alleles have been registered on the IPD-MHC database (http://www.ebi.ac.uk/ipd/mhc/bola). Recently BLV proviral load quantification methods have been developed [17, 32] and several studies have successfully identified SNPs or BoLA-DRB3 alleles that are associated with increasing or suppressing the BLV provirus load [27–29, 33–36]. However, the results of association studies that compared the frequencies of BoLA alleles in low proviral load cows with those in high proviral load cows were strongly affected by the allele frequencies in normal cows, and there is little information on how the allele frequencies were stable year-on-year in a countries.

For BLV diagnosis, proviral load is one of the major diagnosis indices for determining disease progression and transmission risk. Therefore, in this study, we investigated BoLA-DRB3 and BoLA-DQA1 allele frequencies in Japan over 4 years, performed an association study using the specific BoLA class II allele to determine BLV provirus load in Holstein cows, and determined proviral load-associated polymorphisms using cow which collected among 4 years.

We collected blood samples from 1290 cow heads over 6 months old from BLV-positive farms in a nationwide survey in Japan from 2011 to 2014, isolated genomic DNA and sera from peripheral blood. Cows determined as BLV-positive by anti-BLV gp51 antibody ELISA kit using sera (JNC Corporation, Kanagawa Japan) (Table 1) and the BLV proviral load measured by the BLV-CoCoMo-qPCR-2 method [32] using genomic DNA. First, we confirmed the allele frequencies of BoLA-DRB3 gene in each 4 years and confirmed the allele frequency is stable in these 4 years in Holstein in Japan (Fig. 1). Next, our previous report showed that cows with a detected proviral load of over 14,000 copies/10^5 cells (as determined by the BLV-CoCoMo-qPCR-2 method) secreted BLV provirus into nasal secretions [16]. Thus, these cows may be high-risk transmitters. Therefore, we here categorized the 910 BLV-infected cows into two groups, as follows: (i) cows with proviral load over 10,000 copies/10^5 cells—high-risk BLV spreader cows, and (ii) cows with proviral load under 10,000 copies/10^5 cells—low-risk BLV spreader cows (Table 1). The 910 cow heads tested were separated into 341 heads of “low-risk spreaders” and 569 heads of “high-risk spreaders.”

Next, these 910 cows were subjected to BoLA-DRB3 genotyping using a PCR-sequence-based typing (PCR-SBT) method [37]. From 910 BLV-positive cows, a total of 1820 BoLA-DRB3 alleles were detected, which were classified into 23 types of known BoLA-DRB3 alleles (Fig. 2). BoLA-DRB3 allele frequencies of these two groups, i.e., 682 alleles originating from low-risk spreaders and 1138 alleles originating from high-risk spreaders, were calculated, and estimated p values and odds ratios (ORs) for each BoLA-DRB3 allele in the two spreader groups were compared (Fig. 2).

### Table 1 Samples collected from Japanese BLV-positive farms (over 60% positive rate in the test prior to collection for this study) and distribution of proviral load in BLV-positive cow

|                | 2011 | 2012 | 2013 | 2014 | Total |
|----------------|------|------|------|------|-------|
| Number of animals |      |      |      |      |       |
| Collected       | 322  | 390  | 290  | 288  | 1290  |
| BLV-positive    | 222  | 275  | 199  | 214  | 910   |
| High risk spreader* | 137  | 160  | 133  | 139  | 569   |
| Low risk spreader** | 85   | 115  | 66   | 75   | 341   |
| **Distribution of age in BLV positive cow (month)** | | | | | |
| Average of age  | 57.1 | 58.0 | 56.0 | 55.3 | 56.7  |
| Maximum age     | 148  | 191  | 142  | 134  | 191   |
| Minimum age     | 12   | 11   | 15   | 17   | 11    |
| Standard deviation of age | 24.2 | 28.6 | 24.7 | 22.2 | 25.3  |
| **Distribution of proviral load in BLV positive cow (copies per 10^5 cells)** | | | | | |
| Average         | 33564| 27,927| 42,850| 42,014| 35,878|
| Maximum         | 137,905| 135,662| 154,306| 135,950| 154,306|
| Minimum         | 0    | 0    | 0    | 0    | 0     |
| Standard deviation | 32,786| 28,945| 38,382| 39,536| 35,285|

* High risk spreader: proviral load > 10,000/10^5 cells
** Low risk spreader: proviral load ≤ 10,000/10^5 cells
which significantly low frequency in low risk spreader than high risk spreader (OR > 1), we determined that the allele was resistant allele. Moreover, in the case that the allele which significantly high frequency in high risk spreader than low risk spreader (OR > 1), the allele was determined as susceptible allele. From these 23 BoLA-DRB3 alleles, DRB3*002:01, DRB3*009:02, DRB3*012:01, DRB3*014:01:01, and DRB3*015:01 were determined as BLV provirus-associated alleles. BoLA-DRB3*002:01, DRB3*009:02, and DRB3*014:01:01 were determined to be alleles associated with BLV resistance (OR > 1), whereas BoLA-DRB3*012:01 and DRB3*015:01 were determined to be alleles associated with BLV susceptibility (OR < 1).

There are DR and DQ genes embedded in the BoLA class II region, and these genes were closely linked to each other [30]. Indeed, we previously identified 39 DRB3-DQA1 haplotypes in 507 Japanese Black cows [38]. Therefore, to determine the effect of other class II genes, we genotyped the second polymorphic class II genes, such as the DQA1 gene. The 910 Japanese Holstein cow heads were subjected to genotyping of the BoLA-DQA1 gene using a PCR-SBT method [39] and 899 cows were succeeded to genotyping for BoLA-DQA1 alleles. These 899 cows were divided into low-risk (N = 336) and high-risk spreaders (N = 563), based on whether their proviral load was under or over 10,000 copies/10^5 cells, respectively. In total, 1798 BoLA-DQA1 alleles were detected, and these alleles were assigned as one of 14 kinds of known BoLA-DQA1 alleles (Fig. 3). BoLA-DQA1 allele frequencies of these two groups (672 alleles originating from low-risk spreaders and 1126 alleles originating from high-risk spreaders), were analyzed using Fisher's exact test. Three kinds of BoLA-DQA1 allele—DQA1*002:04, DQA1*012:01:01, and DQA1*014:02—were significantly associated with the high proviral load (Fig. 3). DQA1*002:04 and DQA1*014:02 showed ORs > 1 (12.7 and 2.47, respectively), as these two alleles were disease resistant. Conversely, the OR of DQA1*012:01 was 0.34 and the allele indicated disease susceptibility.

Notably, DRB3 and DQA1 were highly linked [38]: for example, DRB3*009:02 was linked with DQA1*002:04
and DRB3*014:01:01 was linked with DQA1*014:02 in Japanese Holstein cows [35]. Therefore, we identified that DRB3*009:02-DQA1*002:04 and DRB3*014:01:01-DQA1*014:02 haplotypes were indicated disease resistance. Table 2 shows that animals with the resistant haplotype were detected at a significantly higher level in the low proviral load group compared with the high proviral load group. However, the OR was lower when the DRB3-DQA1 haplotype was used as a marker (OR = 11.46) than when the DRB3 allele alone was used (OR = 13.88).

In this study, we used three markers, BoLA-DRB3, BoLA-DQA1, and BoLA class II haplotypes, to determine the risk of BLV spread through cows in the farm environment. Using the biggest OR as a comparison index, we confirmed that BoLA-DRB3 was the best marker for determining which cow spread the BLV (OR = 13.9 for BoLA-DRB3, OR = 11.5 for BoLA-DQA1, and OR = 6.2 for BoLA class II haplotype).

The most strongly associated allele was BoLA-DRB3*009:02, which was determined to be a BLV-resistant allele in our study, and was also detected in several studies, such as those by Julliarena et al. [36], Miyasaka et al [35], Forletti et al [40], Lutzelscheab et al. [21], Carignano et al [34], and Hayashi et al [33]. Moreover, we explored the other resistant alleles, BoLA-DRB3*002:01 and DRB3*014:01:01, and susceptible alleles, DRB3*012:01 and DRB3*015:01. The effects of these alleles were weaker than that of BoLA-DRB3*009:02, but they were more frequently detected in the farm [41]. Therefore, obtaining information about these common alleles is more important than obtaining information about rare alleles. It is true that the PVL we determined is only about single time point, the PVL may be changing in future. However, in our limited data in lab, the PVL tends to be stable at least 6 months. Needs more research to confirm how long the PVL shows stable. As the BLV PVL is the most variable quantitative index for assessing the risk of BLV transmission [42], the information about
Table 2 Association between cows with BLV-resistant DRB3-DQA1 haplotypes and cows without BLV-resistant DRB3-DQA1 haplotypes (p value = 9.029 × 10^{-15}, odds ratio = 6.168086)

| Number of DRB3-DQA1 haplotype | Category of proviral load |
|--------------------------------|---------------------------|
|                                | Low (≤ 10,000 copies/10^6 cells) | High (> 10,000 copies/10^6 cells) |
| BLV resistant*                 | 544                         | 1192                        |
| Non BLV resistant              | 62                          | 22                          |

* BLV resistant haplotype: BoLA-DRB3*009:02-DQA1*002:04 or BoLA-DRB3*014:01-DQA1*014:02

disease susceptible and resistant alleles may be useful to eliminate BLV from the farm without separating cows into several sheds.

Abbreviations
BLV: bovine leukemia virus; BoLA: bovine leukocyte antigen; EBL: enzootic bovine leukeosis; HTLV: human T-lymphotropic virus; MHC: major histocompatibility complex; OR: odds ratio; PCR-SBT: PCR-sequence based typing; PVL: proviral load.

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Authors’ contributions
Study conception and design: YA and ST. Data acquisition, analysis, and interpretation: ST and AO. Contribution of reagents/materials/analysis tools: YA. Drafting and revising the manuscript: ST and YA. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional file.

Ethics approval and consent to participate
All animals were handled by veterinarians from the veterinary officers of the prefectural Livestock Hygiene Service Centers, and RIKEN, Japan in strict accordance with good animal practice following the guidelines of RIKEN. The study was approved by the RIKEN Animal Experiments Committee (approval number H29-2-104).

Consent for publication
Signed informed consents were obtained from the study subjects.

Competing interests
The authors declare that they have no competing interests.

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