博士論文

Studies on association of bovine major histocompatibility complex (BoLA)-*DRB3* polymorphism with bovine leukemia virus infection outcome

(BoLA)-*DRB3* 遺伝子の多型性と

牛白血病ウイルス感染症の相関性に関する研究)

羅　傑文

Lo Chieh-Wen

CONTENTS

Abbreviations

BH	Benjamini–Hochberg
BLV	Bovine leukemia virus
BoLA	Bovine leukocyte antigen
CAT 1	Cationic amino acid transporter 1
CI	Confidence interval
CTL	Cytotoxic T cell
CYP 21	Steroid 21-hydroxylase
EBL	Enzootic bovine leukosis
ELISA	Enzyme-Linked Immunosorbent Assay
Env	Envelope glycoprotein
FPPS	Farnesyl pyrophosphate synthetase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HPVL	High-provirus load
HSP 70	Heat shock protein 70
HTLV	Human T-lymphotropic viruses

IFN-γ	Interferon γ
IL	Interleukin
IPD	Immuno-Polymorphism Database
LPVL	Low-provirus load
LTRs	Long terminal repeats
MHC	Major histocompatibility complex
MMR	DNA mismatch repair
OLA	Ovine leukocyte antigen
OR	Odds ratio
PL	Persistent lymphocytosis
PRMT5	Protein arginine-N-methyltransferase 5
PVL	Provirus load
SBT	Sequenced-based typing
STLV	Simian T-lymphotropic viruses
Th1	T helper type 1
Th2	T helper type 2
TNF	Tumor necrosis factor

Chapter 1.

General introduction

1.1 Bovine leukemia virus (BLV)

1.1.1 *Identification history and taxonomy*

Enzootic bovine leukosis (EBL) is the most common neoplastic disease in cattle. The lymphoproliferative disease was first reported in 1969 by Miller, who identified the viral particle produced from lymphocytes of cows with persistent lymphocytosis (PL) by electron microscopy (Miller *et al.* 1969). The causative agent of EBL is bovine leukemia virus (BLV), an oncogenic retrovirus member of the genus *Deltaretrovirus* of the family Retroviridae and is closely related to Human T-lymphotropic virus types I and II (HTLV-I and -II), and the Simian T-lymphotropic viruse (STLV) (Salemi *et al.* 2000).

1.1.2 *Epidemiology*

BLV has spread worldwide via the trade in breeding animals except in the Western Europe where the BLV eradication program has been performed since the second half of the twentieth century (Nuotio *et al.* 2003, Maresca *et al.* 2015). However, EBL still exists in eastern European nation (Sandev *et al.* 2015).

In North America, the BLV positive rate in the United States is 94.2% at herds level and 46.5% at animals level (LaDronka *et al.* 2018). In South America, relatively high levels of BLV prevalence have been observed, and BLV-induced leukosis is present in the majority of countries. In Peru and Paraguay, 42.3% and over 50 % of individuals are BLV positive. In Argentina, the infection rate at herds and individuals are as high as 90.9% and 77.9%, respectively (Polat *et al.* 2016).

In Asia, the nationwide BLV infection rate is varied in different countries. In China, it was reported as 10% (Ma *et al.* 2021). Epidemiological study in Japan revealed 40.9% of dairy and 28.7% of beef cattle are BLV positive at individual level and 78% in dairy and 69% in beef cattle at herd level (Murakami *et al.* 2013). BLV infection levels

in Philippines ranged from 4.8% to 9.7% (Polat *et al.* 2015), 37% in Myanmar (Moe *et al.* 2020) and 81.8% in Taiwan (Hsieh *et al.* 2019).

In Africa, the BLV prevalence rate for cattle in Kenya was 7.6% (Kathambi *et al.* 2019) and 21.5% to 28.0% in Egypt (Hamada *et al.* 2020)(Metwally *et al.* 2020).

As summarized by Polat *et al.* (Polat *et al.* 2017), the nationwide BLV eradication and control programs were conducted in Australia and New Zealand in 1983 and 1996, respectively. Around 99.7% of Australian dairy herds were free from EBL in 2013, while 2008 for New Zealand.

1.1.3 *Structure of viral genome and viral particle*

The genome of BLV is consisted of 8714 nucleotides (Sagata *et al.* 1985) including *gag*, *pro*, *pol*, *env* structural protein and enzyme coding genes and a pX region, flanked by two identical long terminal repeats (LTRs) (Figure 1). The structural protein and enzyme coding genes are indispensable for the production of infectious virions (Jewell *et al.* 2000). The protein product of *gag* gene is Pr45 Gag, the precursor of three mature proteins (Hamard-Peron *et al.* 2011): the nucleocapsid protein, p12, and the matrix protein, p15 which are involved in viral genome packaging (Wang *et al.* 2003), and the capsid protein, p24, which is the major target of the host immune response (Mager *et al.* 1994). The envelope gene encodes two proteins: the extracellular glycoprotein, gp51, which is responsible for receptor recognition and virion attachment; and the transmembrane glycoprotein, gp30 which induces the fusion of viral and cellular membranes necessary for virus to penetrate into host cell cytoplasm (Zhao *et al.* 2007). The pX region, which is located between *env* and the 3´ LTR, encodes the regulatory proteins Tax and Rex, and the accessory proteins R3 and G4 (Aida *et al.* 2013). The regulatory proteins are important for regulation of viral transcription, transformation of BLV-induced leukemogenesis (Panei *et al.* 2013). In addition to protein, BLV also encodes 10 microRNAs and other long non-coding RNAs, which are shown to associate with BLV pathogenesis (Safari *et al.* 2020).

1.1.4 *BLV life cycle and transmission*

BLV infection begins with the interaction of viral envelope glycoprotein (Env) and the host cellular receptor cationic amino acid transporter **(**CAT) 1 (Bai *et al.* 2019) (Figure 2). After the Env mediated membrane fusion, the viral genome is released into cytosol from the virion (Eckert *et al.* 2001). The viral RNA genome then be reverse

transcribed into cDNA and be transported into nucleus (Bukrinsky 2004). The cDNA copies of viral genome then integrate into host genome, which is called as provirus, and lead to persistent infection (Gillet *et al.* 2013). There are two mechanisms for the subsequent viral replication. One of the mechanisms is that provirus produces viral proteins and genome and assembles as new viral particle and infects other cells (i.e. the infectious cycle). Alternatively, as the provirus is integrated into host genome, clonal expansion of the infected cells (mitotic cycle) is another mechanism for provirus replication (Gillet *et al.* 2013).

Due to the instability feature of free viral particles, BLV is transmitted primarily through the infected lymphocytes via both horizontal and vertical routes (Figure 3). BLV can be transmitted within and between herds through physical contact (Johnson *et al.* 1985). In addition, iatrogenic factors (Figure 3A) such as exposure to nasal secretion and saliva contaminated with infected lymphocytes (Yuan *et al.* 2015), using contaminated dehorning devices (DiGiacomo *et al.* 1985), fixing of tattoos and bull rings, reuse of needles and gloves, and reuse of plastic sleeves for rectal palpation play a significant role in BLV transmission (Hopkins *et al.* 1997). Besides, viral transmission by blood sucking insects was also documented (Ohshima *et al.* 1981). Figure 3B shows the potential routs for BLV vertical transmission i.e. *in utero* infection (Meas *et al.* 2002). Recently, PVL and the infectivity of cells within milk from BLV infected cows were visualized suggesting the transmission risk from colostrum and milk consuming (Watanuki *et al.* 2019).

1.1.5 *Pathogenicity*

The natural hosts of BLV are domestic cattle and water buffaloes. BLV can experimentally infect a variety of animal including rabbits, rats, chickens, pigs, goats, and sheep. However, the pathogenesis was only occurred in sheep and cattle (Aida *et al.* 2013). Approximately 70% of infected cattle remain asymptomatic; 30% of infected individuals develop PL characterized by proliferation of non-malignant polyclonal B-cell; and approximately 5% of infected animals develop B-cell leukemia/lymphoma in various lymph nodes after a long latency period (Gillet *et al.* 2007).

The mechanism of BLV-induced onset is still elusive. The provirus load (PVL) is thought as an index for disease progression (Jimba *et al.* 2012). Sometimes, the provirus integration near cancer driver sites potentially promotes tumorigenesis (Rosewick *et al.* 2017). In addition, the BLV provirus encodes accessory genes Tax and G4, which has been shown the potential promoting cell transformation (Willems *et al.* 1994, Panei *et al.* 2013). Tax activates

viral transcription by acting on the LTR promoter via the CREB/ATF signaling pathway (Nguyên *et al.* 2007). Furthermore, Tax cooperates with Ha-Ras oncogene and induces immortalization of primary rat embryo fibroblasts (Willems *et al.* 1990). The amino acid 245-265 region of Tax plays a role in Tax-mediated transactivation regulation (Tajima *et al.* 2000). The interaction of BLV G4 with farnesyl pyrophosphate synthetase (FPPS), a protein involved in the mevalonate/squalene pathway and in synthesis of FPP, a substrate required for prenylation of Ras is essential for G4 oncogenic potential (Lefèbvre *et al.* 2002). The viral non-coding RNA has been proved playing a role in tumorigenesis, as deletion of non-coding RNA reduces cell proliferation and lacks of pathogenesis (Safari *et al.* 2020).

In addition to PVL and virus related factors, the reduced cell turnover rate which caused by BLV infection is thought as a potential mechanism in lymphocyte accumulation and disease onset (Debacq *et al.* 2003). As the virus alone is not sufficient to induce disease onset, host factors mutation and genetic polymorphism in individuals might also play a role in disease progression. It has been observed that approximately 60% of the EBL cattle carry p53 mutant which relates with the decreased capacities for DNA binding, transactivation and growth suppression (Zhuang *et al.* 1997, Tajima *et al.* 1998). Genetic polymorphism association study found that tumor necrosis factor *(TNF)-α -824G* allele, is related with high lymphoma onset risk and is associated with low transcription activity of the bovine *TNF-α* gene (Konnai *et al.* 2006). The polymorphism of bovine leukocyte antigen (BoLA) class I (Lewin *et al.* 1986) and class II *DQA1* and *DRB3* have been shown the critical role in BLV PVL regulation (Juliarena *et al.* 2008, Miyasaka *et al.* 2013). Among them, BoLA class II is superior to BoLA class I (Xu *et al.* 1993); and *BoLA-DRB3* has been identified as having a stronger association with the BLV phenotype than the *DQA1* gene and could be a prominent target for cattle breeding selection to reduce PVL in cattle (Takeshima *et al.* 2019). The expression level of DNA mismatch repair (MMR) genes were related with lymphoma development (Bai *et al.* 2020). Besides, protein arginine-N-methyltransferase 5 (PRMT5) regulates BLV gene expression and is related with disease progression (Assi *et al.* 2020). Figure 4 shows the host factors that participate in BLV induced-disease progression. In the initial step, BLV Tax protein facilitates lymphocyte immortalization of BLV-infected cattle and leads the cattle transitions from asymptomatic status into lymphocyte polyclonal proliferation status called as PL. To progress to lymphoma stage, several host factors are playing a regulatory role e.g. p53, TNF-α, PRMT5, MMR and BoLA that contribute in the lymphocyte malignant transformation and monoclonal proliferation.

1.1.6 *Current treatment and prevention strategies*

The early success in Europe of BLV control was based on eliminating cattle harboring high blood lymphocyte level (Bendixen 1963); however, this strategy is economically impractical for farms with high prevalence rates. Good management practice such as single-use needles and examination gloves, proper sterilization for serological tools are thought beneficial in reducing virus transmission. In addition, using BLV negative cattle for breeding and avoiding providing milk from BLV positive dam to calves or providing only after proper sterilization are ways to decrease chance of vertical transmission.

Vaccines are powerful approach to protect animals from virus infection. Inactivated virus based vaccine has shown the potential inducing a strong neutralizing humoral response and partially protecting cattle from low but not high dose BLV challenge (Fukuyama *et al.* 1993). Subunit vaccines against BLV capsid protein (p24) and BLV ENV (gp51) have been developed; however, only gp51 subunit vaccine showed protective effect in BLV-infected sheep model (Onuma *et al.* 1984). DNA vaccine targeting Tax elicited a cytotoxic response and decreased PVL (Usui *et al.* 2003); however, it could not further prohibit BLV-induced lymphoma development in sheep model (Van den Broeke *et al.* 2010). Peptide vaccines have lower safety issue compared to other vaccine types. A peptide targeting 9-mer cytotoxic T cell (CTL) epitope of gp51 induced a cell-mediated cytotoxicity but did not protect most vaccinated sheep against infection (Mateo *et al.* 2001). A carbonate apatite capsulated peptide vaccine targeting Env and Gag has shown the antigen specific cell-mediated immune response in BLV susceptibility cattle (Aida *et al.* 2015). But the protection efficiency of this vaccine needs further investigation. The low performance of peptide vaccines is possibly due to the lack of stereochemical structure and limited epitope presentation. Therefore, vaccine development against BLV infection is still urgently needed.

Alternative BLV control strategy is host genetic selection. As the cattle with low PVL has been shown no risk for BLV horizontal transmission (Juliarena *et al.* 2016) and extremely low risk for vertical transmission , it is encouraged including those cattle in farm; in contrast, cattle with high PVL are higher risk for viral transmission, so identifying the most infectious cattle and removing them from farm via segregating or culling may contribute to controlling BLV transmission. A field trial showed that after 2 to 2.5 years of cattle management based on PVL, the overall herd prevalence decreased from 62.0% to 20.7% (Ruggiero *et al.* 2019). Therefore, breeding selection to select BLV resistant cattle and remove susceptible cattle is an applicable strategy to control BLV in farm.

1.2 Major histocompatibility complex (MHC)

1.2.1 *General roles*

MHC is a large genetic region in vertebrates containing over 100 genes with 40% involved at various degrees in immunity (Danchin *et al.* 2004). The MHC class I and II are expressed on cell surface, responsible for peptide antigen presentation for T-cell recognition and initiating adaptive immune response. MHC class I are expressed on all nucleated cells of vertebrates and presents antigen for $CD8^+$ T cell, the cytotoxic T cell, and activates the cell-mediated immune response. The class I molecules bind with intracellular derived antigens, for example, self-antigen, and therefore are particularly important for the susceptibility to organs transplantation rejection and autoimmune diseases (Rock *et al.* 2016). Class II molecules bind with extracellular derived antigen, for example, antigen derived from pathogens. MHC class II are expressed by so-called 'professional antigen-presenting cells' (APCs), which include dendritic cells, macrophages and B cells and presents antigen for $CD4^+$ T cell, the helper T cell, and facilitates the T cell becoming either Th1 or Th2 cell (Bottomly 1988). Th1 cell expresses abundant interferon γ (IFN-γ) and interleukin 2 (IL-2). These cytokines are involved in inducing cell-mediated cytotoxicity such as CTLs proliferation and macrophage activation (Mosmann *et al.* 1986). Th2 cell secretes cytokines IL-4 and IL-5, which could activate B cells producing neutralizing antibody (Murray 1998). MHC class III includes genes coding for the complement system and some inflammation related members such as TNF family (Deakin *et al.* 2006).

1.2.2 *Protein structure*

MHC class I and class II share a similar protein structure. MHC class I is composed of an α- chain containing a transmembrane domain, and a β-chain which derived from microglobulin. MHC class II is consisted of an α-chain and a β-chain, and both chains contain a transmembrane domain (Figure 5). The peptide binding groove of MHC molecules is built from the α-chain of Class I and both α-chain and a β-chain of class II (Figure 5). In MHC class I, the size of bound peptide is usually restricted as 9–10 residues because of the closed structure of binding groove at both ends (Matsumura *et al.* 1992, Trolle *et al.* 2016). In contrast, MHC class II has an open binding groove structure and thus could accommodate peptides of 13–25 residues in length (Chicz *et al.* 1992). The bound peptide species of MHC molecules are governed by the properties of peptide binding pockets of MHC binging groove, i.e. the geometry, charge distribution, and hydrophobicity (Wieczorek *et al.* 2017).

1.2.3 *Genome structure*

The MHC is a gene condensed, highly polymorphic region. In human, the MHC system is in chromosome 6 called as human leukocyte antigen (HLA). A total of 253 loci have been identified from HLA region (Stewart *et al.* 2004). The size of HLA region is approximately 4 Mb (~0.13% of full genome), but contains ~0.5% of the known protein coding genes (Shiina *et al.* 2009).

There are 19 HLA class I gene loci, where 3 are highly polymorphic classical (HLA-A, -B and -C), 3 non-classical (HLA-E, -F and -G) and 13 non-coding genes or pseudogenes. The non-classical HLA class I genes have limited polymorphism compared with classical HLA class I gene. There are 27 loci identified within the class II region (Shiina *et al.* 2009). This region contains the classical class II alpha and beta chain genes, HLA-DP, -DQ and -DR that are expressed on the surface of APCs to present peptides to T-helper cells. The class III region, located between the class I and II regions, contains 75 loci (Shiina *et al.* 2009). Most of the protein coding genes are related with immune function or inflammation.

1.2.4 *Bovine MHC*

The MHC system in cattle is BoLA which has been mapped to the bovine chromosome 23 (Fries *et al.* 1986). BoLA-class I ranges from 770 Kb to 1650 Kb, including *BoLA-A* and *BoLA-B* that are within 200 Kb of each other (Bensaïd *et al.* 1991). The class III region is constituted by genes related to inflammation and immunological functions, such as the complement factors BF and C4, TNF α (*TNFA*), steroid 21-hydroxylase (*CYP 21*), and heat shock protein 70 (*HSP 70*) (Aida *et al.* 2015) (Figure 6). The class II region of BoLA is split into two regions: class IIa and class IIb which are separated by at least 15cM (Andersson *et al.* 1988). The class IIa includes DR locus and DQ locus. *DRA* gene is monomorphic. By contrast, there are three genes that encode for the β chain of the DR (DRB) molecule of which *DRB1* is a pseudogene and *DRB2* is poorly expressed, whereas the *DRB3* locus is the most polymorphic and strongly expressed gene from this group. The DQ cluster comprises five DQA (DQA 1–5) and five DQB (DQB 1–5) genes, which have arisen from gene duplication (Silvina Elena Gutiérrez 2017). The class IIb region includes the *DMA*, *DMB*, *LMP2*, *LMP7*, and *TAP* genes, which are involved in antigen processing and transportation (Behl *et al.* 2012). However, the function of other genes within this region, such as *DYA*, *DYB*, *DIB*, *DOB* and *DNA* is currently unclear (Behl *et al.* 2012). BoLA class II region lies near the centromere of Chromosome

23, whereas the class I region is near the telemetric site (Aida *et al.* 2015). The class III genes locate in the telomeric side to the class IIa region.

1.2.5 *BoLA-DRB3 and related diseases*

BoLA-DRB3 is the highly polymorphic gene with 365 alleles registered in Immuno-Polymorphism Database (IPD)-MHC database so far (accessed on 4 April 2021). Furthermore, it has the highest immune response ability among BoLA-class II molecules (Aida 1995). Therefore, *BoLA-DRB3* polymorphism is associated with various diseases and could potentially be a marker for diseases assessment or be further applied in cattle breeding selection. It has been shown polymorphism of *BoLA-DRB3* is related with the susceptibility of mastitis (Yoshida *et al.* 2009), tick infection (Boophilus microplus) (Martinez *et al.* 2006), foot and mouth disease (FMD) (Othman *et al.* 2018), bovine herpesvirus 1 (BoHV-1) (Morales *et al.* 2020) but not the Bovine tuberculosis (bTB) (Eirin *et al.* 2020). In BLV research, several reports found that *BoLA-DRB3* polymorphism is associated with BLV induced-lymphocytosis and PVL (Panei *et al.* 2009, Miyasaka *et al.* 2013, Nikbakht Brujeni *et al.* 2016, Takeshima *et al.* 2019). However, little is known whether polymorphism at *BoLA-DRB3* relates with BLV-induced lymphoma. In this study, we investigate the association of *BoLA-DRB3* polymorphism with BLV-induced lymphoma in Holstein cows and Japanese Black cattle.

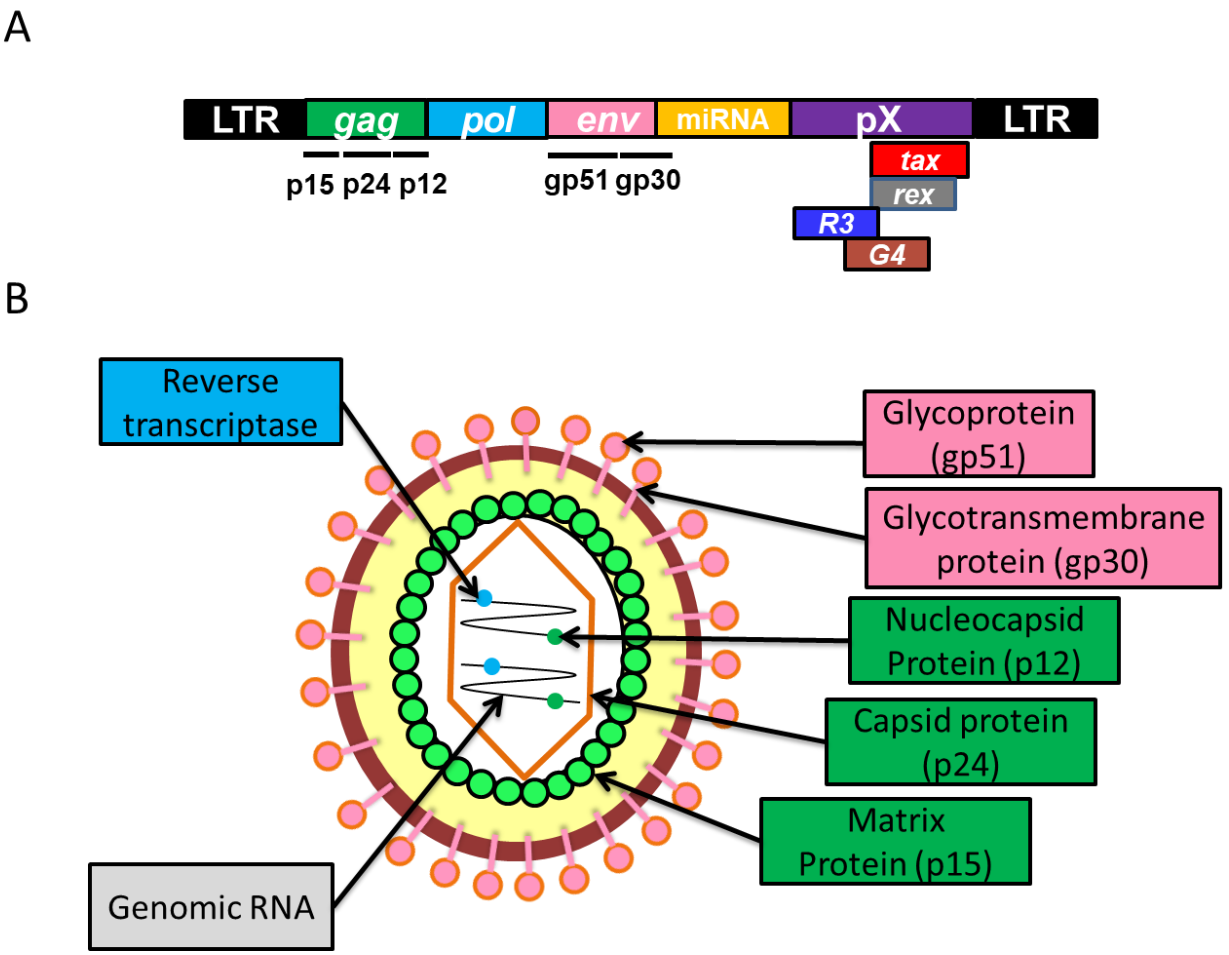


Figure 1 Schematic presentation of genome organization (A) and viral article structure (B) of bovine leukemia virus

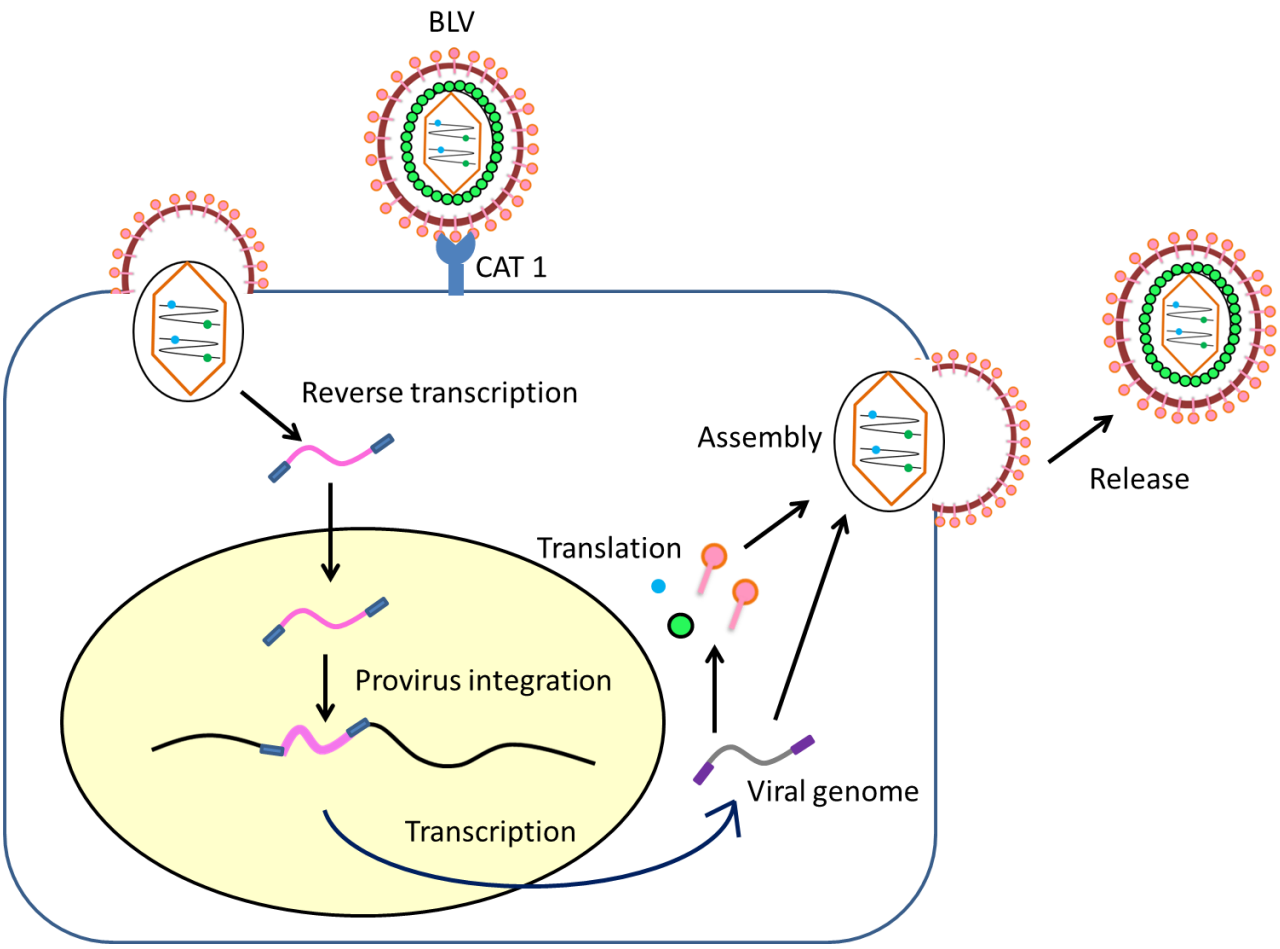
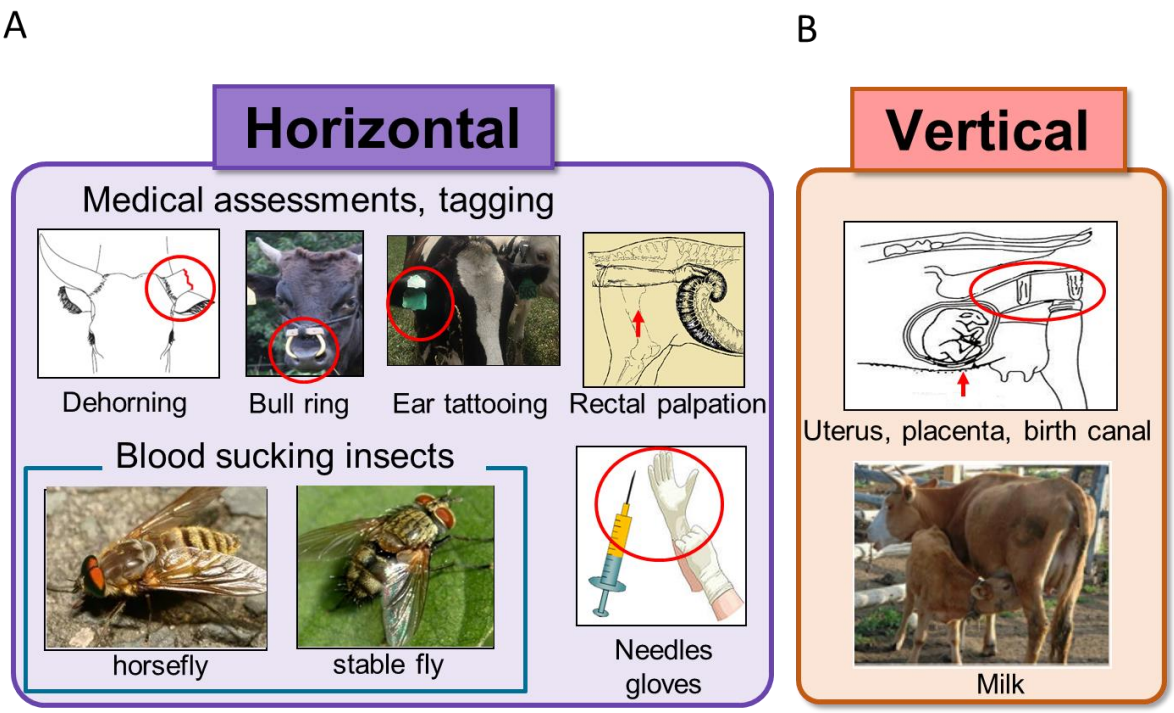


Figure 2 The lifecycle of bovine leukemia virus



(Adapted from Aida and Bai)

Figure 3 Transmission routes of BLV infection.
Horizontal transmission (A) and vertical transmission (B).

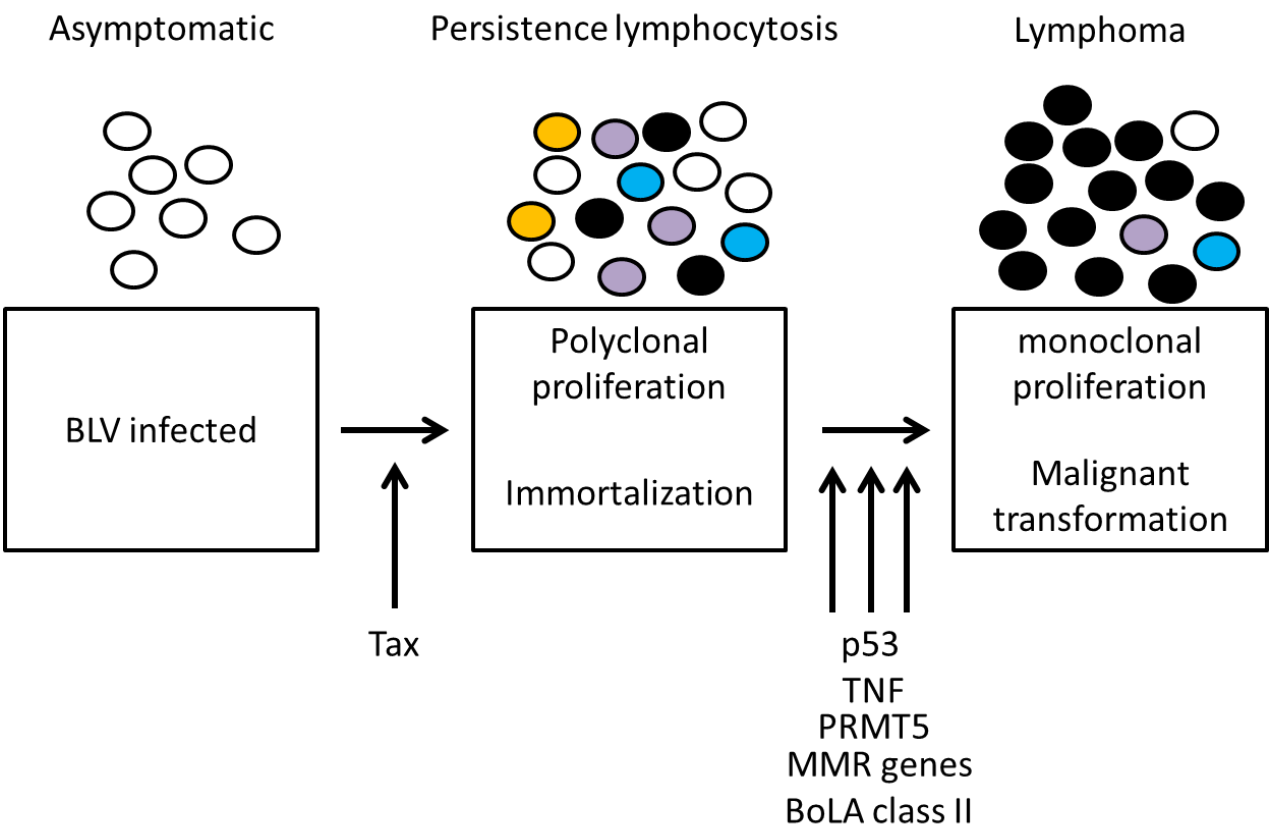


Figure 4 Factors participating in BLV-induced disease progression.

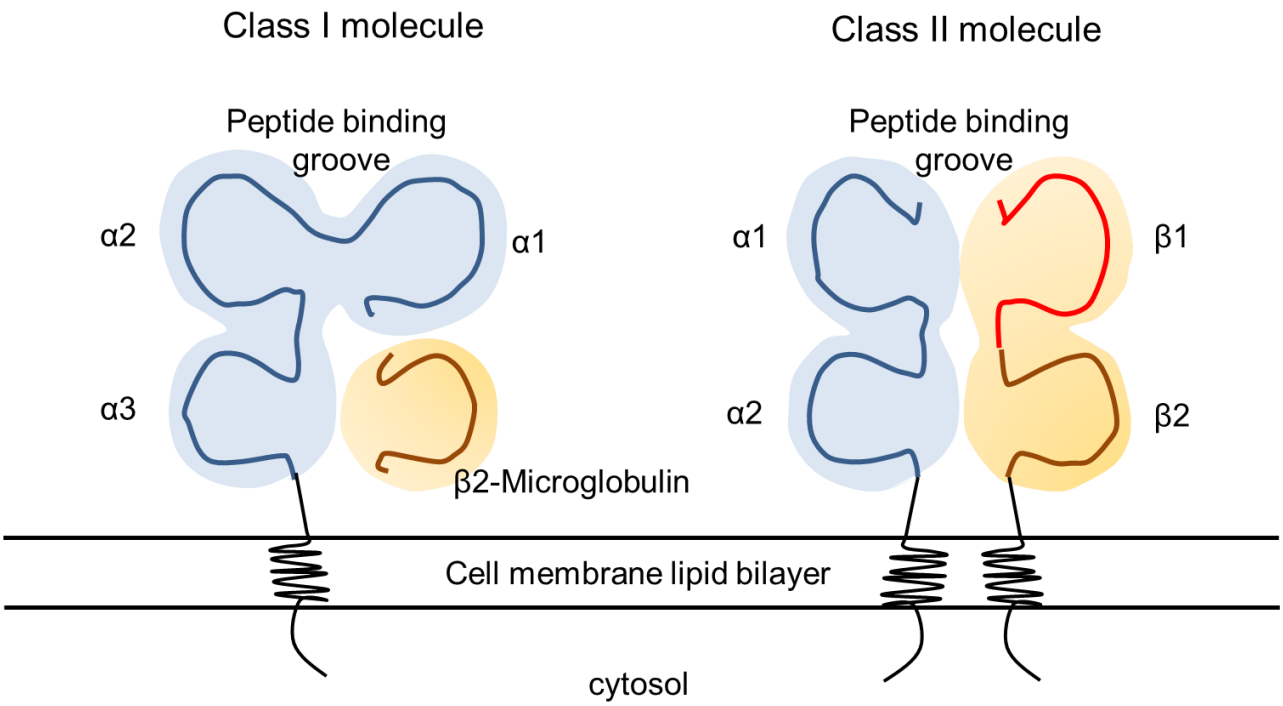


Figure 5 Structure of major histocompatibility complex.
Class I molecule (A) and class II molecule (B).

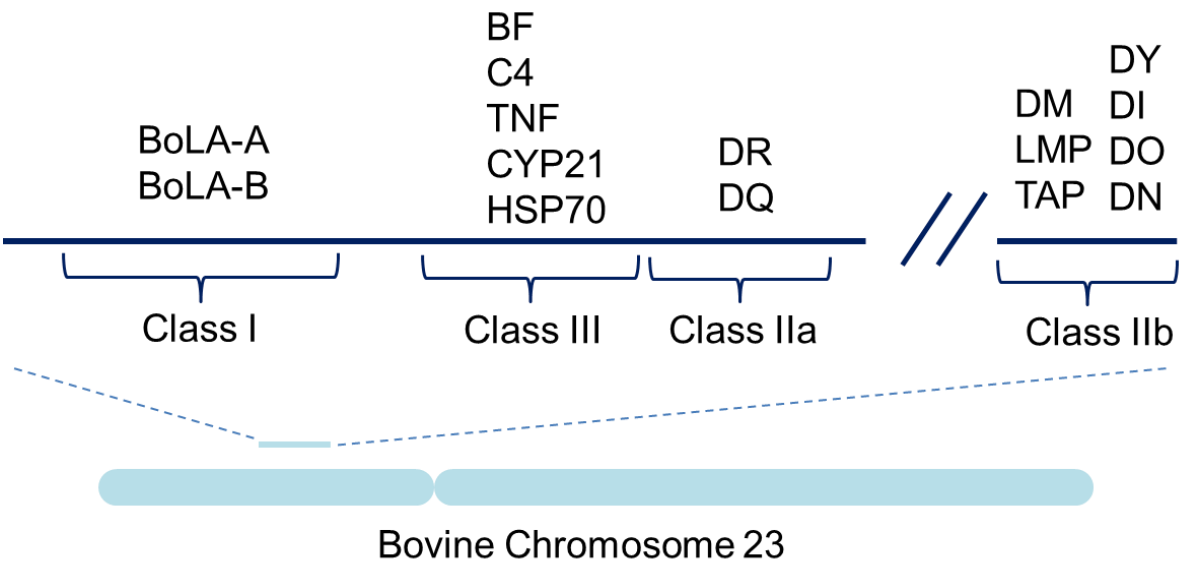


Figure 6 Gene map of bovine major histocompatibility complex

Chapter 2.

BoLA-DRB3 Polymorphism is Associated with Differential Susceptibility to Bovine Leukemia Virus-Induced Lymphoma and Proviral Load

2.1 Abstract

Bovine leukemia virus (BLV) is the causative agent of enzootic bovine leucosis. However, less than 5% of BLV-infected cattle will develop lymphoma, suggesting that, in addition to viral infection, host genetic polymorphisms might play a role in disease susceptibility. Bovine leukocyte antigen (BoLA)-*DRB3* is a highly polymorphic gene associated with BLV proviral load (PVL) susceptibility. Due to the fact that PVL is positively associated with disease progression, it is believed that controlling PVL can prevent lymphoma development. Thus, many studies have focused on the relationship between PVL and *BoLA-DRB3*. Despite this, there is little information regarding the relationship between lymphoma and *BoLA-DRB3*. Furthermore, whether or not PVL-associated *BoLA-DRB3* is linked to lymphoma-associated *BoLA-DRB3* has not been clarified. Here, we investigated whether or not lymphoma-associated *BoLA-DRB3* is correlated with PVL-associated *BoLA-DRB3*. We demonstrate that two *BoLA-DRB3* alleles were specifically associated with lymphoma resistance (**010:01* and **011:01*), but no lymphoma-specific susceptibility alleles were found; furthermore, two other alleles, **002:01* and **012:01*, were associated with PVL resistance and susceptibility, respectively. In contrast, lymphoma and PVL shared two resistance-associated (*DRB3*014:01:01* and **009:02*) *BoLA-DRB3* alleles. Interestingly, we found that PVL associated alleles, but not lymphoma associated alleles, are related with the anti-BLV gp51 antibody production level in cows. Overall, this study is the first to demonstrate that the *BoLA-DRB3* polymorphism confers differential susceptibility to BLV-induced lymphoma and PVL.

2.2 Introduction

Viral load in chronic infections with viruses, such as hepatitis B virus (HBV), hepatitis C virus (HCV), HTLV-1, and human immunodeficiency virus type 1 (HIV-1), has been reported to determine the likelihood of pathogenesis and disease progression (Hisada *et al.* 2005, Chen *et al.* 2009, Iwanaga *et al.* 2010, Shoko *et al.* 2019**)**. For retroviruses, whose genome integrates with the host genome, PVL is an important risk factor of virus-associated disease prediction **(**Furtado *et al.* 2012, Hong *et al.* 2018**)**. BLV is closely related to HTLV-1 and is the causative agent of EBL, a disease that is characterized by long-term symptoms, including PL, which may culminate in B-cell lymphoma (Gillet *et al.* 2007, Aida *et al.* 2013**)**. Several studies indicate that BLV PVL is associated with BLV-related disease progression (Jimba *et al.* 2012, Somura *et al.* 2014, Ohno *et al.* 2015, Kobayashi *et al.* 2019**)**. However, only 5% of infected cattle progress to develop lymphoma, suggesting that in addition to viral infection, host genetic polymorphisms might play a role in disease susceptibility.

MHC, a highly polymorphic gene set, plays a crucial role in antigen presentation and immune responsiveness (Takeshima *et al.* 2006, Panei *et al.* 2009, Garrick *et al.* 2015**)**, and thus, it is associated with numerous infectious diseases. In cattle, the MHC system is known as BoLA. Several studies have identified genetic variations in *BoLA-DRB3*, a functionally important locus and the most highly polymorphic BoLA class II locus in cattle. To date, 330 *DRB3* alleles have been registered in the IPD-MHC database (https://www.ebi.ac.uk/ipd/mhc/group/BoLA/). The *BoLA-DRB3* polymorphism influences susceptibility to BLV-induced lymphoma (Juliarena *et al.* 2008, Nikbakht Brujeni *et al.* 2016, Takeshima *et al.* 2019**)**, and to PVL (Konnai *et al.* 2003, Miyasaka *et al.* 2013, Nieto Farias *et al.* 2017**)**. As PVL is positively related to lymphoma development, it is possible that lymphoma-associated *BoLA-DRB3* is consistent with PVL-associated *BoLA-DRB3*. However, the consistency of above association has not been studied yet.

Indeed, lymphoma development and viral replication depend on different cellular mechanisms, potentially leading to the differential susceptibility of lymphoma and PVL to *BoLA-DRB3*. It has been reported that proviral integration and BLV proteins are required for initial cell transformation (Aida *et al.* 2013, Rosewick *et al.* 2017**)**. However, the host immune system can remove transformed cells by lymphocyte activation via MHC molecules **(**Thibodeau *et al.* 2012). Because MHC class II alleles affect antigen presentation and MHC expression levels in cancer cells (Oldford *et al.* 2004, Marty *et al.* 2017**)**, it is reasonable to hypothesize that MHC class II alleles would bind to peptides

derived from viral or tumor antigens, and that the resulting complex would be recognized by $CD4^+$ T cells. Consequently, some *BoLA-DRB3* alleles might specifically bind with the processed viral antigen, while others might specifically recognize the tumor antigens. Thus, it is likely that different *BoLA-DRB3* alleles are specifically associated with BLV-induced lymphoma and PVL. Consistent with this, PVL does not always correlate with lymphoma development, as many infected cows with a high PVL do not develop lymphoma. On the contrary, attenuated BLV-infected sheep were found to exhibit significantly lower PVL, but still developed lymphoma **(**Florins *et al.* 2007**)**, suggesting that lymphoma and PVL may induce different susceptibilities depending on different *BoLA-DRB3* polymorphisms. In this study, using asymptomatic and lymphoma Holstein cows randomly collected in a nationwide survey in Japan, we demonstrated that *BoLA-DRB3* polymorphism is associated with differential susceptibility to BLV-induced lymphoma and PVL.

2.3 Materials and Methods

2.3.1 *Sample collection and diagnosis*

Blood samples from 611 BLV-infected but clinically normal Holstein cows (asymptomatic cows; information summarized in Tables 1 and 2) and 221 BLV-infected Holstein cows with lymphoma (lymphoma cows; information summarized in Table 3) were randomly collected in a nationwide BLV Proviral Load Determination survey across Japan (32 prefectures out of 47), and the genomic DNA and plasma from peripheral blood were isolated that was done by previous laboratory members. The subclinical stage of BLV infection was diagnosed according to the lymphocyte count (cells/L) and the age of each cow (8,500 = normal and 13,000 = lymphocytosis for cows aged 2–3 years; 5,500 = normal and 7,500 = lymphocytosis for cows aged 6 years). Asymptomatic cows were defined as BLV-infected but clinically and hematologically normal cattle; PL cows were defined as BLV-infected but clinically normal cattle showing with an increase in the number of apparently normal B lymphocytes. Subsequently, lymphoma was diagnosed by both gross and histological observation and by detecting atypical mononuclear cells in the slaughterhouse. In this study, PL cases were excluded and used only samples from asymptomatic cows and lymphoma cows.

2.3.2 *BLV proviral load determination*

BLV infection was estimated by BLV-CoCoMo-qPCR-2 that was partially done by previous laboratory members (RIKEN Genesis, Kanagawa, Japan), as previously described (Jimba *et al.* 2010, Jimba *et al.* 2012, Panei *et al.* 2013, Takeshima *et al.* 2015, Yuan *et al.* 2015). Briefly, the BLV-LTR region was amplified in a reaction mixture containing THUNDERBIRD Probe qPCR Mix (Toyobo, Tokyo, Japan), CoCoMo FRW primer, CoCoMo REV primer, FAM-BLV probe, and 150 ng of template DNA. In addition, the *BoLA-DRA* region was amplified as internal control. The proviral load was calculated using following equation: (number of BLV-LTR copies /number of *BoLA-DRA* copies) X 10^5 cells.

2.3.3 *BoLA-DRB3 genotyping*

BoLA-DRB3 alleles were determined using the PCR-sequenced-based typing (SBT) method, as previously described (Takeshima *et al.* 2011). Briefly, *BoLA-DRB3* exon 2 was amplified by single-step PCR using the DRB3 forward (5'-CGCTCCTGTGAYCAGATCTATCC-3') and DRB3 reverse (5'-CACCCCCGCGCTCACC-3') primer set. The PCR products were purified by ExoSAP-IT PCR product purification kit (USB Corp., Cleveland, OH) and then sequenced using the ABI PRISM BigDye1.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The sequence data were then analyzed using Assign 400ATF ver. 1.0.2.41 software (Gonexio Genomics, Fremantle, Australia) to determine the *BoLA-DRB3* genotype.

2.3.4 *Detection of anti-BLV gp51 antibody by enzyme-linked immunosorbent assay (ELISA)*

The anti-BLV gp51 antibody was measured with an anti-BLV antibody ELISA Kit (JNC, Tokyo, Japan), according to the manufacturer's instructions. Two-fold serial dilutions of plasma samples starting at 1:16 were tested by the ELISA Kit. The OD value in each DRB3 group was compared at each dilution.

2.3.5 *Association study and statistical analysis*

An association study based on Fisher's exact test was performed by comparing the allele and genotype frequencies between asymptomatic and lymphoma cows or low PVL and high PVL cows. The results were penalized with the Benjamini-Hochberg (BH) procedure to correct for the false positive rate. Each allele or genotype

was ranked based on their *p*-value starting from the smallest one. The BH value was calculated based on the equation *p*-value rank / total allele (genotype) number × 0.05). The alleles or genotypes with *p*-value < BH value and odds ratio (OR) < 1 were categorized as resistance alleles. In contrast, those with *p*-value < BH value and OR > 1 were defined as susceptibility alleles or genotypes. The association of cow mean age and birth location with lymphoma or PVL was evaluated by the Mann–Whitney U test and Tukey's multiple comparison test, respectively. When we confirmed the association between age and lymphoma or PVL, we performed logistic regression analyses to adjust for age. To evaluate a multiplicative interaction between *BoLA-DRB3* alleles, we introduced the interaction term in a logistic regression model as conditional analysis (Cordell 2009). We assessed the significance level of the association study by applying a Bonferroni correction according to the number of assessed alleles (adjusted $p < 0.05$). All calculations were performed using R software (version 3.5.0, R Foundation for Statistical Computing, Vienna, Austria).

2.4 Results

2.4.1 *PVL is not fully correlated with lymphoma development*

The PVL of 250 asymptomatic cows (Table 1) ranged from 5–120,482 copies/10^5 cells (mean: 9,401 copies/10^5 cells), while in 221 lymphoma cows (Table 3), the PVL ranged from 28–1,960,674 copies/10^5 cells (mean 99,522 copies/10^5 cells; Figure 7). This difference suggested that animals with a high BLV PVL were at a higher risk of developing lymphoma. Our previous report indicated that cows with a PVL of greater than 14,000 copies/10^5 cells secreted BLV into nasal mucus *(*Yuan *et al. 2015)*, and BLV provirus was detected in milk samples from cows when the PVL in blood samples was higher than 10,000 copies/10^5 cells *(*Watanuki *et al. 2019)*. These results suggest that a PVL around 10,000 copies/10^5 cells in blood might be an indicator of efficiently BLV spreading within whole body and thus is a relatively high number. Therefore, a BLV PVL of 10,000 copies/10^5 cells was set as a threshold to distinguish between high-PVL (HPVL) and low-PVL (LPVL) cows (Figure 7), which is also in line with our previous study *(*Takeshima *et al. 2019)*. Consistently, in lymphoma and asymptomatic cows, the mean PVL was found to be above and below this threshold, respectively (Figure 7). However, 62 HPVL cows remained asymptomatic, whereas 37 LPVL cows developed lymphoma (Table 4), indicating that lymphoma development is

not fully correlated with the PVL. This could be because BLV-induced lymphoma and BLV PVLs are associated with different *BoLA-DRB3* alleles.

2.4.2 *Association study of BoLA-DRB3 with lymphoma*

Next, to explore the association between *BoLA-DRB3* and lymphoma, all 250 asymptomatic and 221 lymphoma cows were typed for *BoLA-DRB3* alleles (Table 5). The alleles with frequencies > 1% are shown in Figure 8. An association study based on Fisher's exact test found that *DRB3*009:02* (OR = 0.23), *DRB3*010:01* (OR = 0.48), *DRB3*011:01* (OR = 0.56), and *DRB3*014:01:01* (OR = 0.57) were classified as lymphoma resistance alleles, whereas *DRB3*012:01* (OR = 2.71) and *DRB3*015:01* (OR = 1.67) were identified as lymphoma susceptibility alleles (Table 6).

To exclude the effect from other potential factors that might associate with lymphoma development such as cow origin and age, we then applied multivariable logistic regression to adjust for the effect of these potential factors. Our calculation indicates that age showed a significant association with disease susceptibility ($p = 6.56 \times 10^{-6}$). However, no significant difference was observed between location and lymphoma susceptibility ($p = 0.182$). Therefore, we performed the logistic regression analysis adjusted by age in the only association study of lymphoma. After studying the association between each *BoLA-DRB3* allele and lymphoma susceptibility, we conducted a stepwise conditional analysis with respect to the top-associated *BoLA-DRB3* alleles (Table 7). In Table 8, a conditional analysis of *DRB3*011:01* revealed an independent association with *DRB3*009:02* and *DRB3*010:01*. A subsequent conditional analysis regarding *DRB3*009:02* and *DRB3*011:01* revealed an independent association with *DRB3*010:01* and *DRB3*014:01:01*. Next, the subsequent conditional analysis of *DRB3*009:02*, *DRB3*010:01*, and *DRB3*011:01* revealed an independent association with *DRB3*014:01:01*. After conditioning *DRB3*009:02*, *DRB3*010:01*, *DRB3*011:01*, and *DRB3*014:01:01*, no significant association locus was observed. We then conducted a multivariate regression analysis incorporating the four associated *BoLA-DRB3* alleles (*DRB3*009:02*, *DRB3*010:01*, *DRB3*011:01*, and *DRB3*014:01:01*). We identified that all were independently associated with lymphoma resistance (Table 8).

For the genotype association study, genotypes with frequencies > 1% are shown in Figure 9 (complete genotype frequencies are summarized in Table 9). However, no genotypes reached statistical significance in terms of their association with lymphoma development after BH correction to adjust the false discovery rate (Table 10).

2.4.3 *Association study of BoLA-DRB3 with PVL*

Subsequently, to determine the association between *BoLA-DRB3* and BLV PVL, we selected an additional 361 asymptomatic cows, in addition to the original 250 asymptomatic cows (used in Figures 7–9). A total of 611 asymptomatic cows were then divided into the HPVL group (n = 294; Table 2) and LPVL group (n = 317; Table 2). The frequencies of *BoLA-DRB3* alleles from LPVL cows and HPVL cows were calculated by Fisher's exact test, and p-values and ORs were estimated for each allele (Table 11). The analysis of allele frequencies (Figure 10) and association (Table 12) established *DRB3*002:01* (OR = 0.15), **009:02* (OR = 0.07), and **014:01:01* (OR = 0.61) as BLV PVL resistance alleles, consistent with previous findings (Takeshima *et al.* 2019). In addition, *DRB3*012:01* (OR = 3.84) was identified as a susceptibility allele.

Next, to exclude the bias that might occur in Fisher's exact test, we assessed the association between PVL and other potential factors, including age and the cows' birth location. However, no association between age (p = 0.170)/location (p = 0.991) and PVL was observed. After studying the association between each *BoLA-DRB3* allele and PVL, we conducted stepwise conditional analysis, with respect to the top-associated *BoLA-DRB3* alleles (Table 13). In Table 14, a conditional analysis of *DRB3*009:02* revealed an independent association with *DRB3*002:01*, *DRB3*012:01*, and *DRB3*014:01:01*. A subsequent conditional analysis regarding *DRB3*009:02* and *DRB3*014:01:01* revealed an independent association with *DRB3*002:01* and *DRB3*012:01*. Next, a conditional analysis of *DRB3*009:02*, *DRB3*012:01*, and *DRB3*014:01:01* revealed an independent association with *DRB3*002:01*. After conditioning *DRB3*002:01*, *DRB3*009:02*, *DRB3*012:01*, and *DRB3*014:01:01*, no significant association locus was observed. We then conducted a multivariate regression analysis, incorporating the four associated *BoLA-DRB3* alleles (*DRB3*002:01*, *DRB3*009:02*, *DRB3*012:01*, and *DRB3*014:01:01*). We identified that *DRB3*002:01*, *DRB3*009:02*, and *DRB3*014:01:01* are resistance alleles and *DRB3*012:01* is a susceptibility allele independently associated with PVL (Table 15).

For genotype association study (Table 15), the genotypes with frequency > 1% are shown in Figure 11. The Fisher's exact test of genotype association (Table 16) indicated that *DRB3*009:02/*015:01* (OR = 0) was determined as the resistance genotype. In contrast, *DRB3*011:01/*012:01* (OR = 6.83), was determined as the susceptibility genotype.

2.4.4 *Differential susceptibility of BoLA-DRB3 polymorphisms to lymphoma and PVL*

We compared the effect of *BoLA-DRB3* on cow susceptibility to lymphoma and PVL, based on the multivariable logistic regression analysis in Tables 8 and 14. Several different types of *BoLA-DRB3* alleles were found to be associated with BLV-induced lymphoma and BLV PVL (Figure 12). There were two lymphoma resistance alleles, *DRB3*010:01*, and *DRB3*011:01*, but no susceptibility alleles were identified. In addition, one allele associated with PVL resistance, *DRB3*002:01*, and one PVL susceptibility allele, *DRB3*012:01*, were found. Two resistance alleles, *DRB3*009:02* and *DRB3*014:01:01*, were commonly identified in lymphoma and BLV PVL.

2.4.5 *BoLA-DRB3 polymorphisms are associated with anti-BLV antibody production levels*

Finally, we tried to link the potential biological functions with *BoLA-DRB3* polymorphisms. Previously, it has been demonstrated that PVL resistance and susceptibility alleles are associated with anti-BLV antibody production levels (Forletti *et al.* 2020). Here, we hypothesized that only PVL-associated alleles, but not lymphoma-associated alleles, would be related to viral antigen-induced immune responses. To test this, we compared the anti-BLV antibody (anti-gp51) production level between the cows with the PVL susceptibility allele (*DRB3*012:01*), PVL resistance allele (*DRB3*002:01*), PVL/lymphoma resistance allele (*DRB3*009:02*), and lymphoma-specific resistance allele (*DRB3*011:01*), as shown in the summary in Figure 6. Two-fold serially-diluted plasma samples were tested by ELISA and the OD value in each *BoLA-DRB3* group was compared at the dilution of 1:2048. Cows with the PVL resistance allele (PVL resistance group and PVL/lymphoma resistance group) had significantly lower anti-gp51 production levels compared to those in cows carrying the PVL susceptibility allele (p = 0.006 and p = 0.012 respectively; Figure 13). However, cows with the lymphoma specific resistance allele did not have explicitly and significantly different levels of anti-gp51, compared to those in animals with the PVL susceptibility allele (p = 0.474), suggesting that the lymphoma specific associated allele has a lesser effect on anti-gp51 production than the PVL associated allele.

2.5 Discussion

In the present study, by using both Fisher's exact test and multivariable logistic regression analysis, we showed for the first time that the susceptibility to BLV-induced lymphoma and PVL is affected by different *BoLA-DRB3*

polymorphisms. For example, two *BoLA-DRB3* alleles, *DRB3*010:01*, and *DRB3*011:01*, were found to be associated with resistance to lymphoma but not to PVL. In addition, *DRB3*002:01* was specifically associated with PVL resistance. In contrast, we found that *DRB3*009:02* was common between lymphoma and PVL resistance, in line with the reciprocal association between PVL and lymphoma development. Thus, we might conclude that host polymorphisms at the *BoLA-DRB3* locus are an important factor in both PVL and lymphoma development; interestingly, the PVL-associated *BoLA-DRB3* allele did not show a major correlation with the lymphoma-associated *BoLA-DRB3* allele. The potential reason for the differential susceptibility to PVL and Lymphoma might be the differential immune response, which depends on *BoLA-DRB3* polymorphisms, as we found that the level of anti-BLV antibody is related to the PVL-associated allele but not the lymphoma-associated allele, suggesting that lymphoma specific-associated alleles have a lesser effect on anti-BLV antibody production than PVL-associated alleles.

Some discrepancies were observed between the association based on Fisher's exact test and multivariable logistic regression analysis. For example, *DRB3*015:01* was indicated as a lymphoma susceptibility allele by Fisher's exact test, but not by multivariable logistic regression analysis. The inconsistencies can also be found in *DRB3*012:01* and *DRB3*014:01:01*. This is a common problem between these two statistical methods, as Fisher's exact test includes all factors, such as environmental and genetically factors, that together influence *BoLA-DRB3* polymorphisms. In contrast, multivariable logistic regression can adjust for the effect of other associated factors, such as age, for the lymphoma association study. Therefore, further experiments for allele functional confirmation are needed.

It is not clear how most BLV-infected cattle do not develop bovine leukosis. The following findings in our study may help solve this issue: (i) In asymptomatic cows, two of the major *BoLA-DRB3* alleles, *DRB3*011:01* (22%) and *DRB3*010:01* (12%), were significantly associated with lymphoma resistance, but were unrelated to PVL. This may explain why some HPVL cows remained asymptomatic. (ii) Susceptibility alleles specifically associated with lymphoma were absent in Japanese Holstein cows, suggesting that malignant transformation requires other factors, besides *BoLA-DRB3* polymorphism. For instance, the deregulation of lymphocyte homeostasis is known to lead to leukemia (Debacq *et al.* 2003). Provirus integration close to cancer-driver sites and transcriptionally active regions may affect host gene expression (Kettmann *et al.* 1982, Gillet *et al.* 2013, Rosewick *et al.* 2017). The viral accessory proteins Tax and G4 also play a crucial role in cell transformation (Willems *et al.* 1994). Besides, p53 mutation

(Dequiedt *et al.* 1995, Tajima *et al.* 1998) and tumor necrosis factor-α polymorphisms (Konnai *et al.* 2006) are also related to lymphoma development. In addition, the expression level of MMR genes and PRMT5 are correlated with diseases progression (Assi *et al.* 2020, Bai *et al.* 2020).

The major function of MHC class II molecules is to present antigens for T cells to activate the adaptive immune response. It is possible that PVL-specific *BoLA-DRB3* and lymphoma-specific *BoLA-DRB3* recognize different antigens and thus the subsequent immune response targeting the virus or tumor cells, respectively. To link *BoLA-DRB3* polymorphisms with their biological functions, we tested the anti-BLV gp51 antibody level in cows with the PVL-associated *BoLA-DRB3* allele and lymphoma-associated *BoLA-DRB3* allele. Interestingly, significantly different anti-gp51 levels were found between cows with PVL resistance and PVL susceptibility alleles. However, no significant difference was found in cows carrying lymphoma-specific resistance alleles compared to those in cows carrying PVL susceptibility alleles. This result is in line with the hypothesis that proteins encoded by PVL-associated and lymphoma-associated *BoLA-DRB3* alleles recognize different antigens and thus trigger different subsequent immune responses. Similar to that in a previous study, we found that cows with PVL resistance alleles exhibit significantly lower anti-gp51 levels than cows with PVL susceptibility alleles (Forletti *et al.* 2020). This is probably because we measured anti-gp51 levels in steady state virus infections, and thus, the antibody concentration might change and correlate with the viral titer in cows. As a result, in PVL resistance cows, which are associated with low viral expression levels, a low anti-BLV antibody level would be detected. In addition to humoral immunity, whether *BoLA-DRB3* polymorphisms are associated with effects on CTLs needs further study.

In the PVL association study, *DRB3*015:01* has been reported as a PVL susceptibility allele in our and other studies (Takeshima *et al.* 2019, Forletti *et al.* 2020). However, we found only one PVL susceptibility allele, *DRB3*012:01*, in the current investigation. This difference might be due to the sample collection bias and also the statistical analysis method. For lymphoma association study, it has been reported that the *BoLA-DRB3*018:02*, *DRB3*032:02*, and *DRB3*009:01* alleles are associated with the susceptibility to BLV-induced lymphoma, whereas *DRB3*001:01* and *DRB3*011:01* are involved in lymphoma resistance in Iranian Holstein cows (Nikbakht Brujeni *et al.* 2016). In the present study, *DRB3*011:01* was confirmed to be a resistance allele. The other identified alleles were different from those previously reported, suggesting that regional genetic variations may exist. Indeed, ethnicity-related differences in the frequency of human MHC alleles have been observed (Bugawan *et al.* 1999, Velickovic *et al.* 2002). Furthermore, allelic diversity in the *BoLA* locus between cattle breeds has been previously

demonstrated (Takeshima *et al.* 2002, Takeshima *et al.* 2003, Takeshima *et al.* 2008, Miyasaka *et al.* 2011). This variability is strongly influenced by selective pressures such as exposure to infectious diseases and breed origin. Therefore, the association between the *BoLA-DRB3* locus and the resistance or susceptibility to BLV-induced lymphoma, as well as the regulation of PVL, should be further explored in different countries and in distinct cow breeds.

In conclusion, we have demonstrated for the first time that BLV-induced lymphoma and PVL are associated with different *BoLA-DRB3* alleles in Holstein cows in Japan. Although BLV infects cattle worldwide, effective treatments and vaccines are not available. Consequently, breed selection based on *BoLA-DRB3* polymorphism is a promising strategy to reduce the burden of BLV-induced lymphoma. Contrarily, the sporadic inconsistency between PVL and terminal diseases might be a common phenomenon due to host genetic polymorphisms during different infectious viral diseases. Indeed, partial inconsistency between PVL and the related symptoms was also observed in HTLV-1-infected patients **(**Pineda *et al.* 2019**).** As BLV is closely related to HTLV-1, the consistency between the susceptibility of host genetic polymorphisms with PVL and HTLV-1-related symptoms is worth confirming.

Table 1. Sample information of asymptomatic cows in *BoLA-DRB3* and BLV-induced lymphoma association study.

no.	Sample ID	*BoLA-DRB3* (a):(b)	[1]PVL	[2]WBC	[3]Lymphocyte	Age (Month)	Birth area
1	H86	*(*009:02):(*027:03)*	48	79	45	47	Hyogo
2	H87	*(*009:02):(*010:01)*	62	64	39	19	Chiba
3	H88	*(*007:01):(*014:01:01)*	67	49	16	120	Chiba
4	H89	*(*011:01):(*015:01)*	68		[4]n.t.	83	Saitama
5	H91	*(*001:01):(*007:01)*	70	106	48	50	Saitama
6	H94	*(*001:01):(*014:01:01)*	98		n.t.	64	Saitama
7	H95	*(*009:02):(*010:01)*	99	99	51	48	Aichi
8	H98	*(*001:01):(*016:01)*	114		n.t.	45	Iwate
9	H99	*(*001:01):(*011:01)*	115	83	36	77	Chiba
10	H102	*(*010:01):(*011:01)*	121	121	43	45	Okinawa
11	H103	*(*006:01):(*011:01)*	123	100	59	34	Chiba
12	H104	*(*002:01):(*016:01)*	124	84	66	55	Hyogo
13	H105	*(*001:01):(*011:01)*	126	53	31	119	Niigata
14	H106	*(*009:02):(*010:01)*	128	85	57	96	Chiba
15	H107	*(*011:01):(*011:01)*	131		n.t.	50	Kanagawa
16	H109	*(*009:02):(*015:01)*	134	101	26	48	Okinawa
17	H110	*(*001:01):(*027:03)*	134	52	24	122	Yamagata
18	H111	*(*014:01:01):(*027:07)*	134	71	38	91	Osaka
19	H112	*(*007:01):(*014:01:01)*	136		n.t.	107	Saitama
20	H113	*(*007:01):(*011:01)*	142	76	24	122	Saitama
21	H117	*(*015:01):(*016:01)*	147	139	39	20	Chiba
22	H118	*(*014:01:01):(*015:01)*	152	59	17	82	Chiba
23	H119	*(*009:01):(*009:02)*	154	64	20	64	Shiga
24	H120	*(*006:01):(*027:03)*	156	118	48	27	Shiga
25	H121	*(*009:02):(*015:01)*	156	93	47	35	Oita
26	H122	*(*011:01):(*027:03)*	157	77	39	48	Chiba
27	H123	*(*001:01):(*009:02)*	163	61	38	100	Niigata
28	H125	*(*001:01):(*011:01)*	167	94	58	59	Iwate
29	H126	*(*012:01):(*014:01:01)*	170	78	36	86	Chiba
30	H127	*(*015:01):(*007:04)*	172	83	61	55	Kanagawa
31	H128	*(*001:01):(*009:02)*	180	62	26	62	Okinawa
32	H129	*(*001:01):(*014:01:01)*	186		n.t.	53	Chiba
33	H130	*(*010:01):(*016:01)*	188		n.t.	52	Tokyo
34	H131	*(*011:01):(*011:01)*	189	64	32	53	Chiba
35	H132	*(*011:01):(*011:01)*	190	92	37	49	Gifu

36	H133	*(*011:01):(*012:01)*	222		n.t.	58	Saitama
37	H134	*(*009:02):(*011:01)*	222	94	33	72	Chiba
38	H135	*(*011:01):(*015:01)*	226	124	26	84	Shizuoka
39	H138	*(*011:01):(*027:03)*	244	72	39	57	Hyogo
40	H140	*(*011:01):(*015:01)*	250		n.t.	62	Chiba
41	H141	*(*001:01):(*011:01)*	266	79	28	44	Chiba
42	H142	*(*011:01):(*011:01)*	266		n.t.	53	Chiba
43	H143	*(*001:01):(*027:03)*	268	63	31	69	Chiba
44	H144	*(*011:01):(*014:01:01)*	270	69	37	43	Aichi
45	H145	*(*001:01):(*007:01)*	275	72	19	24	Kanagawa
46	H146	*(*015:01):(*015:01)*	278	65	49	57	Hyogo
47	H147	*(*001:01):(*011:01)*	290		n.t.	59	Chiba
48	H148	*(*001:01):(*009:02)*	303		n.t.	16	Chiba
49	H149	*(*015:01):(*015:01)*	311	92	63	24	Hyogo
50	H150	*(*010:01):(*012:01)*	313	110	29	60	Chiba
51	H151	*(*011:01):(*011:01)*	323	93	37	49	Osaka
52	H152	*(*001:01):(*027:03)*	338	44	18	65	Chiba
53	H155	*(*001:01):(*014:01:01)*	356	90	39	74	Chiba
54	H156	*(*009:02):(*010:01)*	359	66	28	119	Shizuoka
55	H157	*(*014:01:01):(*015:01)*	360	123	43	35	Chiba
56	H159	*(*001:01):(*011:01)*	374		n.t.	63	Hida
57	H161	*(*010:01):(*011:01)*	409		n.t.		Asahikawa
58	H162	*(*007:01):(*015:01)*	420	62	23	79	Chiba
59	H166	*(*001:01):(*011:01)*	453	94	65	41	Aichi
60	H168	*(*001:01):(*011:01)*	476	103	41	82	Shizuoka
61	H169	*(*011:01):(*014:01:01)*	485		n.t.	35	Matsumoto
62	H170	*(*011:01):(*014:01:01)*	490	96	20	94	Chiba
63	H174	*(*011:01):(*014:01:01)*	539	86	52	46	Sumoto
64	H175	*(*015:01):(*016:01)*	545	83	43	47	Himeji
65	H176	*(*015:01):(*017:01)*	552	90	38	71	Chiba
66	H177	*(*014:01:01):(*015:01)*	561	66	24	64	Osaka
67	H178	*(*011:01):(*027:03)*	576	82	37	50	Nara
68	H179	*(*002:01):(*011:01)*	598	54	31	60	Shizuoka
69	H180	*(*014:01:01):(*015:01)*	598	103	59	29	Niigata
70	H181	*(*011:01):(*014:01:01)*	627	83	34	102	Saitama
71	H182	*(*009:02):(*015:01)*	638	65	37	93	Ishikawa
72	H184	*(*012:01):(*014:01:01)*	676	96	38	46	Chiba
73	H185	*(*002:01):(*015:01)*	691	55	21	114	Osaka
74	H186	*(*011:01):(*027:03)*	693	143	86	15	Chiba

75	H187	*(*001:01):(*015:01)*	725	114	63	64	Gifu
76	H188	*(*002:01):(*014:01:01)*	759	64	41	111	Shizuoka
77	H190	*(*001:01):(*006:01)*	788	62	33	39	Nagasaki
78	H191	*(*012:01):(*015:01)*	805	59	23	120	Shiga
79	H192	*(*001:01):(*016:01)*	851	64	35	47	Himeji
80	H193	*(*001:01):(*014:01:01)*	852		n.t.	43	Nagano
81	H194	*(*011:01):(*015:01)*	870	90	29	85	Saitama
82	H195	*(*001:01):(*014:01:01)*	888	124	49	18	Chiba
83	H196	*(*011:01):(*011:01)*	903		n.t.	23	Chiba
84	H198	*(*014:01:01):(*027:03)*	943	127	37	28	Chiba
85	H199	*(*015:01):(*027:03)*	957		n.t.	42	Hida
86	H201	*(*010:01):(*014:01:01)*	1004		n.t.	40	Chiba
87	H202	*(*001:01):(*011:01)*	1005	96	22	59	Saitama
88	H203	*(*014:01:01):(*015:01)*	1044	71	35	89	Shiga
89	H204	*(*011:01):(*014:01:01)*	1049	59	28	78	Niigata
90	H205	*(*010:01):(*011:01)*	1054	100	45	46	Saitama
91	H206	*(*001:01):(*011:01)*	1100		n.t.		Hokkaido
92	H208	*(*011:01):(*014:01:01)*	1160	75	18	59	Chiba
93	H210	*(*001:01):(*015:01)*	1216	106	28	44	Chiba
94	H211	*(*001:01):(*002:01)*	1230	83	54	57	Ishikawa
95	H212	*(*010:01):(*012:01)*	1253		n.t.	22	Chiba
96	H213	*(*001:01):(*015:01)*	1293	113	31	43	Chiba
97	H214	*(*009:02):(*027:03)*	1328	59	23	70	Saitama
98	H217	*(*001:01):(*010:01)*	1350	106	39	47	Saitama
99	H218	*(*002:01):(*002:01)*	1441	84	24	28	Kanagawa
100	H219	*(*003:01):(*015:01)*	1451	80	40	26	Oita
101	H220	*(*012:01):(*027:03)*	1505	65	41	40	Hyogo
102	H222	*(*011:01):(*011:01)*	1534	136	49	38	Saitama
103	H223	*(*001:01):(*001:01)*	1571	57	25	143	Osaka
104	H224	*(*011:01):(*027:03)*	1604	67	28	40	Saitama
105	H225	*(*011:01):(*015:01)*	1669	74	47	51	Chiba
106	H226	*(*012:01):(*014:01:01)*	1756	73	26	77	Osaka
107	H227	*(*001:01):(*015:01)*	1767		n.t.	33	Tokyo
108	H228	*(*014:01:01):(*015:01)*	1806	99	69	39	Sumoto
109	H229	*(*014:01:01):(*027:03)*	1827	70	30	48	Kanagawa
110	H231	*(*010:01):(*010:01)*	1844	231	76	31	Sumoto
111	H232	*(*011:01):(*015:01)*	1878		n.t.	61	Kanagawa
112	H234	*(*010:01):(*010:01)*	1990		n.t.	113	Chiba
113	H235	*(*002:01):(*011:01)*	2023	85	43	30	Kanagawa

114	H236	*(*001:01):(*011:01)*	2066	76	34	50	Okinawa
115	H237	*(*001:01):(*011:01)*	2100	51	31	63	Nagasaki
116	H241	*(*001:01):(*015:01)*	2277	84	44	34	Okinawa
117	H242	*(*011:01):(*016:01)*	2306	93	63	19	Gifu
118	H243	*(*011:01):(*011:01)*	2309		n.t.	39	Chiba
119	H245	*(*014:01:01):(*015:01)*	2410	86	24	74	Osaka
120	H246	*(*010:01):(*011:01)*	2418	94	58	39	Nagasaki
121	H247	*(*010:01):(*015:01)*	2537	141	72	32	Shizuoka
122	H248	*(*010:01):(*014:01:01)*	2710	106	33	46	Sumoto
123	H252	*(*001:01):(*002:01)*	2893		n.t.	61	Iwate
124	H254	*(*001:01):(*015:01)*	3244	107	77	26	Ishikawa
125	H255	*(*012:01):(*016:01)*	3246	81	39	49	Himeji
126	H256	*(*011:01):(*015:01)*	3394	76	56	41	Kanagawa
127	H257	*(*011:01):(*015:01)*	3453	141	39	46	Chiba
128	H258	*(*011:01):(*027:03)*	3483	89	30	42	Saitama
129	H259	*(*010:01):(*027:03)*	3524	72	45	70	Hyogo
130	H261	*(*001:01):(*011:01)*	4028	77	28	98	Saitama
131	H262	*(*010:01):(*012:01)*	4111	112	66	16	Shizuoka
132	H263	*(*001:01):(*010:01)*	4201	78	26	100	Saitama
133	H265	*(*005:03):(*010:01)*	4462	88	35	50	Saitama
134	H266	*(*010:01):(*010:01)*	4531	140	30	44	Hyogo
135	H267	*(*001:01):(*011:01)*	4548	105	79	27	Hyogo
136	H268	*(*001:01):(*012:01)*	4583	49	21	90	Osaka
137	H269	*(*010:01):(*011:01)*	4918	122	70	106	Chiba
138	H270	*(*001:01):(*002:01)*	5032	36	20	81	Okinawa
139	H271	*(*011:01):(*015:01)*	5056		n.t.	58	Kanagawa
140	H273	*(*015:01):(*015:01)*	5212	144	48	22	Chiba
141	H274	*(*001:01):(*011:01)*	5216		n.t.	39	Iwate
142	H275	*(*001:01):(*010:01)*	5238	101	41	42	Akita
143	H277	*(*010:01):(*015:01)*	5442	90	26	39	Chiba
144	H278	*(*001:01):(*009:01)*	5695	71	32	60	Shizuoka
145	H279	*(*010:01):(*016:01)*	5711	78	37	48	Saitama
146	H280	*(*011:01):(*015:01)*	5835	148	54	20	Chiba
147	H281	*(*007:01):(*011:01)*	5838	75	44	19	Kanagawa
148	H282	*(*002:01):(*010:01)*	5842	100	52	42	Gifu
149	H283	*(*010:01):(*010:01)*	5859	97	39	63	Shiga
150	H284	*(*010:01):(*015:01)*	6103	70	45	16	Shizuoka
151	H285	*(*014:01:01):(*015:01)*	6157	59	30	45	Himeji
152	H286	*(*001:01):(*010:01)*	6229	74	44	67	Kanagawa

153	H287	*(*001:01):(*015:01)*	6288	181	136	52	Kanagawa
154	H289	*(*001:01):(*010:01)*	6468	141	57	20	Chiba
155	H290	*(*010:01):(*011:01)*	6644	91	30	41	Osaka
156	H291	*(*010:01):(*011:01)*	6646	110	56	37	Gifu
157	H293	*(*001:01):(*001:01)*	6831		n.t.	65	Hida
158	H294	*(*001:01):(*001:01)*	6910	112	38	33	Chiba
159	H295	*(*015:01):(*064:01)*	6979	134	86	69	Kanagawa
160	H296	*(*011:01):(*027:03)*	7074		n.t.	37	Iwate
161	H297	*(*001:01):(*015:01)*	7288	83	26	102	Saitama
162	H298	*(*010:01):(*014:01:01)*	7588		n.t.	47	Matsumoto
163	H299	*(*001:01):(*027:03)*	7621		n.t.	71	Iwate
164	H300	*(*010:01):(*015:01)*	7675	118	51	35	Niigata
165	H301	*(*014:01:01):(*015:01)*	7793	95	43	41	Saitama
166	H302	*(*001:01):(*010:01)*	7811	58	22	53	Chiba
167	H303	*(*011:01):(*015:01)*	7830		n.t.	40	Hida
168	H306	*(*010:01):(*027:03)*	8216	65	32	27	Shizuoka
169	H308	*(*015:01):(*015:01)*	8757	87	57	31	Himeji
170	H309	*(*010:01):(*011:01)*	9063	97	62	32	Aichi
171	H310	*(*012:01):(*015:01)*	9076	77	25	64	Chiba
172	H314	*(*011:01):(*014:01:01)*	9583	100	61	43	Aichi
173	H315	*(*014:01:01):(*015:01)*	9685	103	65	35	Iwate
174	H317	*(*011:01):(*027:07)*	9939	114	53	48	Iwate
175	H318	*(*010:01):(*012:01)*	10105	101	24	48	Chiba
176	H323	*(*010:01):(*011:01)*	10949	125	78	28	Aichi
177	H335	*(*011:01):(*027:03)*	12077		n.t.		Kurayoshi
178	H338	*(*010:01):(*027:03)*	12222	134	53	43	Saitama
179	H349	*(*010:01):(*015:01)*	13084	98	51	52	Kagoshima
180	H350	*(*010:01):(*011:01)*	13158		n.t.		Asahikawa
181	H363	*(*010:01):(*010:01)*	14481	141	30	20	Chiba
182	H365	*(*007:01):(*009:02)*	14634	112	35	59	Saitama
183	H368	*(*007:01):(*011:01)*	15000	102	50	71	Saitama
184	H374	*(*012:01):(*018:01)*	15744		n.t.	25	Chiba
185	H395	*(*011:01):(*014:01:01)*	19115		n.t.		Asahikawa
186	H400	*(*011:01):(*015:01)*	19450		n.t.		Asahikawa
187	H406	*(*001:01):(*016:01)*	20000	109	32	23	Chiba
188	H407	*(*011:01):(*015:01)*	20213		n.t.		Kurayoshi
189	H408	*(*015:01):(*015:01)*	20276	95	29	38	Chiba
190	H409	*(*015:01):(*015:01)*	20509		n.t.		Kurayoshi
191	H415	*(*010:01):(*015:01)*	21602	113	31	46	Chiba

192	H420	*(*010:01):(*016:01)*	22130		n.t.		Asahikawa
193	H422	*(*011:01):(*011:01)*	23485	117	36	59	Hokkaido
194	H423	*(*027:03):(*027:03)*	23491		n.t.		Asahikawa
195	H431	*(*010:02):(*011:01)*	24603	136	48	44	Chiba
196	H436	*(*001:01):(*011:01)*	25000	107	66	24	Hokkaido
197	H442	*(*011:01):(*012:01)*	25635	103	57	46	Chiba
198	H447	*(*011:01):(*018:01)*	26159	113	18	83	Saitama
199	H451	*(*001:01):(*011:01)*	27349	95	50	47	Saitama
200	H456	*(*014:01:01):(*020:01:02)*	27982		n.t.		Kurayoshi
201	H462	*(*001:01):(*018:01)*	28708	113	55	46	Nagano
202	H465	*(*011:01):(*015:01)*	29011	96	43	46	Kanagawa
203	H476	*(*012:01):(*015:01)*	31354	104	52	64	Chiba
204	H484	*(*007:01):(*011:01)*	34088	105	28	23	Chiba
205	H495	*(*001:01):(*015:01)*	37122		n.t.		Kurayoshi
206	H498	*(*012:01):(*027:03)*	37829		n.t.		Kurayoshi
207	H500	*(*001:01):(*011:01)*	38028		n.t.		Kurayoshi
208	H532	*(*014:01:01):(*016:01)*	44729		n.t.		Asahikawa
209	H536	*(*011:01):(*014:01:01)*	45169	106	48	80	Saitama
210	H575	*(*015:01):(*015:01)*	59047		n.t.		Kurayoshi
211	H595	*(*015:01):(*027:03)*	68869		n.t.		Kurayoshi
212	H610	*(*016:01):(*027:03)*	120482		n.t.		Asahikawa
213	H479	*(*011:01):(*14011)*	49	77		73	Chiba
214	H480	*(*010:01):(*010:01)*	28225	132	68	73	Chiba
215	H482	*(*002:01):(*011:01)*	56306	82	28	73	Niigata
216	H485	*(*011:01):(*015:01)*	13424	73	54	74	Tottori
217	H487	*(*009:02):(*010:01)*	13	59	28	75	Akita
218	H490	*(*011:01):(*012:01)*	37117	91	55	75	Kanagawa
219	H491	*(*010:01):(*016:01)*	107	54	35	76	Tokyo
220	H496	*(*011:01):(*015:01)*	49555	186	134	77	Saitama
221	H498	*(*011:01):(*015:01)*	58641	121	76	78	Chiba
222	H501	*(*011:01):(*027:03)*	62396	163	113	79	Chiba
223	H506	*(*001:01):(*011:01)*	5	95	37	82	Chiba
224	H509	*(*007:01):(*010:01)*	26214	80	25	82	Chiba
225	H510	*(*007:01):(*011:01)*	71385	120	82	82	Saitama
226	H511	*(*009:02):(*010:01)*	25	72	40	83	Tokyo
227	H516	*(*010:01):(*011:01)*	16484	57	41	83	Kanagawa
228	H519	*(*011:01):(*015:01)*	426	56	36	84	Shizuoka
229	H520	*(*14011):(*015:01)*	451	56	39	84	Tottori
230	H523	*(*010:01):(*14011)*	19661	140	44	85	Chiba
231	H527	*(*012:01):(*015:01)*	19387	201	52	87	Ishikawa

232	H534	*(*002:01):(*010:01)*	518	64	31	91	Kanagawa
233	H535	*(*011:01):(*027:03)*	928	151	61	91	Chiba
234	H539	*(*010:01):(*027:03)*	9100	69	31	94	Osaka
235	H540	*(*010:01):(*011:01)*	16037	52	31	94	Kanagawa
236	H544	*(*011:01):(*015:01)*	57659	129	85	94	Saitama
237	H546	*(*009:02):(*012:01)*	19	104	47	95	Iwate
238	H549	*(*001:01):(*007:01)*	21254	74	40	96	Osaka
239	H551	*(*001:01):(*011:01)*	76263	65	46	96	Kanagawa
240	H552	*(*012:01):(*027:03)*	24061	53	42	97	Tottori
241	H562	*(*009:02):(*015:01)*	44	79	27	103	Chiba
242	H566	*(*010:01):(*015:01)*	23772	36	32	107	Tokyo
243	H569	*(*011:01):(*015:01)*	37934	45	27	109	Kanagawa
244	H574	*(*011:01):(*015:01)*	19524	100	43	114	Chiba
245	H575	*(*001:01):(*010:01)*	14132	76	35	116	Hokkaido
246	H576	*(*14011):(*015:01)*	32279	81	42	117	Yamagata
247	H586	*(*001:01):(*012:01)*	37537	88	50	142	Osaka
248	H588	*(*001:01):(*011:01)*	1199	72	25	150	Osaka
249	H589	*(*011:01):(*015:01)*	38416	106	65	150	Chiba
250	H590	*(*001:01):(*14011)*	13	46	17	167	Osaka

[1]PVL. Proviral load. (expressed as the number of copies of provirus per 10^5 peripheral blood mononuclear cells)
[2]WBC. White blood cell (cells/μL)
[3]Lymphocyte (cells/μL)
[4]n.t. Not tested

Table 2. Sample information of LPVL and HPVL cows in PVL association study.

Number	Sample ID	[1]Status	*BoLA-DRB3* (a):(b)	[2]PVL
1	H316	Low	*(*011:01):(*015:01)*	1
2	H317	Low	*(*011:01):(*011:01)*	1
3	H318	Low	*(*001:01):(*002:01)*	2
4	H319	Low	*(*007:01):(*015:01)*	2
5	H533	Low	*(*009:02):(*015:01)*	2
6	H320	Low	*(*010:01):(*014:01:01)*	2
7	H321	Low	*(*001:01):(*001:01)*	3
8	H461	Low	*(*011:01):(*015:01)*	3
9	H322	Low	*(*010:01):(*010:01)*	4
10	H323	Low	*(*001:01):(*011:01)*	5
11	H324	Low	*(*011:01):(*011:01)*	5
12	H462	Low	*(*002:01):(*011:01)*	5
13	H325	Low	*(*009:02):(*012:01)*	5
14	H326	Low	*(*001:01):(*011:01)*	6
15	H327	Low	*(*011:01):(*014:01:01)*	6
16	H328	Low	*(*001:01):(*011:01)*	6
17	H329	Low	*(*001:01):(*015:01)*	7
18	H534	Low	*(*001:01):(*015:01)*	7
19	H330	Low	*(*011:01):(*012:01)*	8
20	H331	Low	*(*001:01):(*027:03)*	8
21	H332	Low	*(*015:01):(*015:01)*	8
22	H463	Low	*(*001:01):(*002:01)*	8
23	H333	Low	*(*010:01):(*010:01)*	9
24	H334	Low	*(*003:01):(*027:03)*	9
25	H464	Low	*(*009:02):(*015:01)*	10
26	H335	Low	*(*020:01:02):(*027:03)*	10
27	H336	Low	*(*002:01):(*011:01)*	11
28	H337	Low	*(*001:01):(*002:01)*	11
29	H338	Low	*(*007:01):(*012:01)*	11
30	H339	Low	*(*014:01:01):(*015:01)*	11
31	H340	Low	*(*002:01):(*002:01)*	11
32	H341	Low	*(*011:01):(*016:01)*	11
33	H535	Low	*(*001:01):(*027:03)*	12
34	H342	Low	*(*001:01):(*015:01)*	12
35	H465	Low	*(*001:01):(*015:01)*	13
36	H466	Low	*(*012:01):(*014:01:01)*	13
37	H343	Low	*(*015:01):(*016:01)*	13

38	H344	Low	*(*009:02):(*010:01)*	13
39	H345	Low	*(*001:01):(*014:01:01)*	13
40	H467	Low	*(*001:01):(*015:01)*	14
41	H346	Low	*(*011:01):(*027:03)*	14
42	H468	Low	*(*009:02):(*012:01)*	15
43	H347	Low	*(*011:01):(*027:03)*	15
44	H536	Low	*(*009:02):(*015:01)*	16
45	H537	Low	*(*011:01):(*002:01)*	16
46	H469	Low	*(*011:01):(*012:01)*	17
47	H348	Low	*(*014:01:01):(*015:01)*	17
48	H470	Low	*(*002:01):(*014:01:01)*	17
49	H471	Low	*(*007:01):(*009:02)*	19
50	H538	Low	*(*009:02):(*012:01)*	19
51	H349	Low	*(*011:01):(*015:01)*	19
52	H350	Low	*(*001:01):(*011:01)*	20
53	H539	Low	*(*001:01):(*011:01)*	20
54	H351	Low	*(*012:01):(*014:01:01)*	21
55	H352	Low	*(*001:01):(*014:01:01)*	21
56	H353	Low	*(*001:01):(*011:01)*	21
57	H354	Low	*(*001:01):(*015:01)*	22
58	H472	Low	*(*009:02):(*027:03)*	22
59	H540	Low	*(*011:01):(*015:01)*	23
60	H541	Low	*(*001:01):(*014:01:01)*	23
61	H355	Low	*(*009:02):(*010:01)*	24
62	H356	Low	*(*014:01:01):(*015:01)*	24
63	H357	Low	*(*001:01):(*009:02)*	24
64	H358	Low	*(*009:02):(*010:01)*	25
65	H473	Low	*(*009:02):(*015:01)*	26
66	H474	Low	*(*001:01):(*011:01)*	26
67	H475	Low	*(*001:01):(*010:01)*	26
68	H476	Low	*(*014:01:01):(*027:03)*	27
69	H359	Low	*(*009:02):(*015:01)*	30
70	H360	Low	*(*001:01):(*011:01)*	30
71	H361	Low	*(*001:01):(*009:02)*	31
72	H362	Low	*(*009:02):(*014:01:01)*	32
73	H542	Low	*(*009:02):(*012:01)*	34
74	H363	Low	*(*001:01):(*002:01)*	34
75	H477	Low	*(*009:02):(*011:01)*	34
76	H478	Low	*(*011:01):(*027:03)*	37
77	H364	Low	*(*006:01):(*010:01)*	38
78	H479	Low	*(*009:02):(*009:02)*	40

79	H480	Low	*(*009:02):(*015:01)*	40
80	H481	Low	*(*009:02):(*015:01)*	44
81	H1	Low	*(*009:02):(*027:03)*	48
82	H365	Low	*(*011:01):(*014:01:01)*	49
83	H482	Low	*(*001:01):(*027:03)*	50
84	H483	Low	*(*011:01):(*015:01)*	57
85	H366	Low	*(*009:02):(*015:01)*	58
86	H484	Low	*(*009:02):(*015:01)*	59
87	H2	Low	*(*009:02):(*010:01)*	62
88	H3	Low	*(*007:01):(*014:01:01)*	67
89	H4	Low	*(*011:01):(*015:01)*	68
90	H485	Low	*(*014:01:01):(*015:01)*	69
91	H5	Low	*(*001:01):(*007:01)*	70
92	H367	Low	*(*011:01):(*011:01)*	72
93	H368	Low	*(*001:01):(*015:01)*	73
94	H6	Low	*(*001:01):(*014:01:01)*	98
95	H7	Low	*(*009:02):(*010:01)*	99
96	H369	Low	*(*010:01):(*016:01)*	107
97	H370	Low	*(*011:01):(*011:01)*	113
98	H8	Low	*(*001:01):(*016:01)*	114
99	H9	Low	*(*001:01):(*011:01)*	115
100	H371	Low	*(*001:01):(*011:01)*	115
101	H372	Low	*(*009:02):(*015:01)*	120
102	H10	Low	*(*010:01):(*011:01)*	121
103	H11	Low	*(*006:01):(*011:01)*	123
104	H12	Low	*(*002:01):(*016:01)*	124
105	H13	Low	*(*001:01):(*011:01)*	126
106	H14	Low	*(*009:02):(*010:01)*	128
107	H15	Low	*(*011:01):(*011:01)*	131
108	H543	Low	*(*011:01):(*015:01)*	132
109	H16	Low	*(*009:02):(*015:01)*	134
110	H17	Low	*(*001:01):(*027:03)*	134
111	H18	Low	*(*014:01:01):(*027:07)*	134
112	H19	Low	*(*007:01):(*014:01:01)*	136
113	H20	Low	*(*007:01):(*011:01)*	142
114	H544	Low	*(*001:01):(*012:01)*	145
115	H373	Low	*(*001:01):(*011:01)*	146
116	H374	Low	*(*011:01):(*027:03)*	146
117	H21	Low	*(*015:01):(*016:01)*	147
118	H22	Low	*(*014:01:01):(*015:01)*	152
119	H23	Low	*(*009:01):(*009:02)*	154

120	H24	Low	*(*006:01):(*027:03)*	156
121	H25	Low	*(*009:02):(*015:01)*	156
122	H26	Low	*(*011:01):(*027:03)*	157
123	H27	Low	*(*001:01):(*009:02)*	163
124	H486	Low	*(*011:01):(*011:01)*	167
125	H28	Low	*(*001:01):(*011:01)*	167
126	H29	Low	*(*012:01):(*014:01:01)*	170
127	H30	Low	*(*015:01):(*007:04)*	172
128	H31	Low	*(*001:01):(*009:02)*	180
129	H32	Low	*(*001:01):(*014:01:01)*	186
130	H33	Low	*(*010:01):(*016:01)*	188
131	H34	Low	*(*011:01):(*011:01)*	189
132	H35	Low	*(*011:01):(*011:01)*	190
133	H36	Low	*(*011:01):(*012:01)*	222
134	H37	Low	*(*009:02):(*011:01)*	222
135	H38	Low	*(*011:01):(*015:01)*	226
136	H375	Low	*(*012:01):(*027:03)*	229
137	H376	Low	*(*009:02):(*012:01)*	237
138	H39	Low	*(*011:01):(*027:03)*	244
139	H377	Low	*(*002:01):(*002:01)*	249
140	H40	Low	*(*011:01):(*015:01)*	250
141	H41	Low	*(*001:01):(*011:01)*	266
142	H42	Low	*(*011:01):(*011:01)*	266
143	H43	Low	*(*001:01):(*027:03)*	268
144	H44	Low	*(*011:01):(*014:01:01)*	270
145	H45	Low	*(*001:01):(*007:01)*	275
146	H46	Low	*(*015:01):(*015:01)*	278
147	H47	Low	*(*001:01):(*011:01)*	290
148	H48	Low	*(*001:01):(*009:02)*	303
149	H49	Low	*(*015:01):(*015:01)*	311
150	H50	Low	*(*010:01):(*012:01)*	313
151	H51	Low	*(*011:01):(*011:01)*	323
152	H52	Low	*(*001:01):(*027:03)*	338
153	H545	Low	*(*011:01):(*014:01:01)*	344
154	H378	Low	*(*015:01):(*015:01)*	353
155	H53	Low	*(*001:01):(*014:01:01)*	356
156	H54	Low	*(*009:02):(*010:01)*	359
157	H55	Low	*(*014:01:01):(*015:01)*	360
158	H379	Low	*(*001:01):(*011:01)*	370
159	H56	Low	*(*001:01):(*011:01)*	374
160	H380	Low	*(*009:02):(*018:01)*	380

161	H57	Low	*(*010:01):(*011:01)*	409
162	H58	Low	*(*007:01):(*015:01)*	420
163	H546	Low	*(*011:01):(*011:01)*	423
164	H381	Low	*(*011:01):(*015:01)*	426
165	H382	Low	*(*014:01:01):(*015:01)*	451
166	H59	Low	*(*001:01):(*011:01)*	453
167	H383	Low	*(*002:01):(*027:03)*	457
168	H60	Low	*(*001:01):(*011:01)*	476
169	H61	Low	*(*011:01):(*014:01:01)*	485
170	H62	Low	*(*011:01):(*014:01:01)*	490
171	H547	Low	*(*014:01:01):(*015:01)*	494
172	H384	Low	*(*014:01:01):(*015:01)*	517
173	H548	Low	*(*002:01):(*010:01)*	518
174	H63	Low	*(*011:01):(*014:01:01)*	539
175	H64	Low	*(*015:01):(*016:01)*	545
176	H65	Low	*(*015:01):(*017:01)*	552
177	H66	Low	*(*014:01:01):(*015:01)*	561
178	H67	Low	*(*011:01):(*027:03)*	576
179	H68	Low	*(*002:01):(*011:01)*	598
180	H69	Low	*(*014:01:01):(*015:01)*	598
181	H70	Low	*(*011:01):(*014:01:01)*	627
182	H71	Low	*(*009:02):(*015:01)*	638
183	H385	Low	*(*011:01):(*015:01)*	668
184	H72	Low	*(*012:01):(*014:01:01)*	676
185	H73	Low	*(*002:01):(*015:01)*	691
186	H74	Low	*(*011:01):(*027:03)*	693
187	H75	Low	*(*001:01):(*015:01)*	725
188	H76	Low	*(*002:01):(*014:01:01)*	759
189	H386	Low	*(*001:01):(*012:01)*	781
190	H77	Low	*(*001:01):(*006:01)*	788
191	H78	Low	*(*012:01):(*015:01)*	805
192	H79	Low	*(*001:01):(*016:01)*	851
193	H80	Low	*(*001:01):(*014:01:01)*	852
194	H81	Low	*(*011:01):(*015:01)*	870
195	H82	Low	*(*001:01):(*014:01:01)*	888
196	H83	Low	*(*011:01):(*011:01)*	903
197	H387	Low	*(*011:01):(*027:03)*	928
198	H84	Low	*(*014:01:01):(*027:03)*	943
199	H85	Low	*(*015:01):(*027:03)*	957
200	H388	Low	*(*002:01):(*015:01)*	998
201	H86	Low	*(*010:01):(*014:01:01)*	1004

202	H87	Low	*(*001:01):(*011:01)*	1005
203	H88	Low	*(*014:01:01):(*015:01)*	1044
204	H89	Low	*(*011:01):(*014:01:01)*	1049
205	H90	Low	*(*010:01):(*011:01)*	1054
206	H91	Low	*(*001:01):(*011:01)*	1100
207	H549	Low	*(*015:01):(*027:03)*	1129
208	H92	Low	*(*011:01):(*014:01:01)*	1160
209	H550	Low	*(*001:01):(*011:01)*	1199
210	H93	Low	*(*001:01):(*015:01)*	1216
211	H94	Low	*(*001:01):(*002:01)*	1230
212	H95	Low	*(*010:01):(*012:01)*	1253
213	H96	Low	*(*001:01):(*015:01)*	1293
214	H97	Low	*(*009:02):(*027:03)*	1328
215	H389	Low	*(*011:01):(*017:01)*	1336
216	H390	Low	*(*011:01):(*011:01)*	1342
217	H98	Low	*(*001:01):(*010:01)*	1350
218	H99	Low	*(*002:01):(*002:01)*	1441
219	H100	Low	*(*003:01):(*015:01)*	1451
220	H101	Low	*(*012:01):(*027:03)*	1505
221	H391	Low	*(*011:01):(*014:01:01)*	1514
222	H102	Low	*(*011:01):(*011:01)*	1534
223	H103	Low	*(*001:01):(*001:01)*	1571
224	H104	Low	*(*011:01):(*027:03)*	1604
225	H105	Low	*(*011:01):(*015:01)*	1669
226	H106	Low	*(*012:01):(*014:01:01)*	1756
227	H107	Low	*(*001:01):(*015:01)*	1767
228	H108	Low	*(*014:01:01):(*015:01)*	1806
229	H109	Low	*(*014:01:01):(*027:03)*	1827
230	H392	Low	*(*007:01):(*011:01)*	1836
231	H110	Low	*(*010:01):(*010:01)*	1844
232	H111	Low	*(*011:01):(*015:01)*	1878
233	H393	Low	*(*001:01):(*027:03)*	1885
234	H112	Low	*(*010:01):(*010:01)*	1990
235	H113	Low	*(*002:01):(*011:01)*	2023
236	H114	Low	*(*001:01):(*011:01)*	2066
237	H115	Low	*(*001:01):(*011:01)*	2100
238	H551	Low	*(*001:01):(*002:01)*	2142
239	H552	Low	*(*011:01):(*011:01)*	2227
240	H553	Low	*(*011:01):(*011:01)*	2238
241	H116	Low	*(*001:01):(*015:01)*	2277
242	H117	Low	*(*011:01):(*016:01)*	2306

243	H118	Low	*(*011:01):(*011:01)*	2309
244	H554	Low	*(*001:01):(*002:01)*	2325
245	H119	Low	*(*014:01:01):(*015:01)*	2410
246	H120	Low	*(*010:01):(*011:01)*	2418
247	H121	Low	*(*010:01):(*015:01)*	2537
248	H122	Low	*(*010:01):(*014:01:01)*	2710
249	H394	Low	*(*010:01):(*015:01)*	2775
250	H555	Low	*(*001:01):(*012:01)*	2818
251	H556	Low	*(*010:01):(*010:01)*	2863
252	H123	Low	*(*001:01):(*002:01)*	2893
253	H557	Low	*(*001:01):(*002:01)*	2942
254	H124	Low	*(*001:01):(*015:01)*	3244
255	H125	Low	*(*012:01):(*016:01)*	3246
256	H126	Low	*(*011:01):(*015:01)*	3394
257	H127	Low	*(*011:01):(*015:01)*	3453
258	H128	Low	*(*011:01):(*027:03)*	3483
259	H129	Low	*(*010:01):(*027:03)*	3524
260	H558	Low	*(*015:01):(*027:03)*	3785
261	H130	Low	*(*001:01):(*011:01)*	4028
262	H131	Low	*(*010:01):(*012:01)*	4111
263	H132	Low	*(*001:01):(*010:01)*	4201
264	H395	Low	*(*010:01):(*015:01)*	4390
265	H133	Low	*(*005:03):(*010:01)*	4462
266	H134	Low	*(*010:01):(*010:01)*	4531
267	H135	Low	*(*001:01):(*011:01)*	4548
268	H136	Low	*(*001:01):(*012:01)*	4583
269	H137	Low	*(*010:01):(*011:01)*	4918
270	H138	Low	*(*001:01):(*002:01)*	5032
271	H139	Low	*(*011:01):(*015:01)*	5056
272	H396	Low	*(*010:01):(*015:01)*	5181
273	H140	Low	*(*015:01):(*015:01)*	5212
274	H141	Low	*(*001:01):(*011:01)*	5216
275	H142	Low	*(*001:01):(*010:01)*	5238
276	H397	Low	*(*011:01):(*014:01:01)*	5261
277	H143	Low	*(*010:01):(*015:01)*	5442
278	H144	Low	*(*001:01):(*009:01)*	5695
279	H145	Low	*(*010:01):(*016:01)*	5711
280	H146	Low	*(*011:01):(*015:01)*	5835
281	H147	Low	*(*007:01):(*011:01)*	5838
282	H148	Low	*(*002:01):(*010:01)*	5842
283	H149	Low	*(*010:01):(*010:01)*	5859

284	H150	Low	*(*010:01):(*015:01)*	6103
285	H151	Low	*(*014:01:01):(*015:01)*	6157
286	H152	Low	*(*001:01):(*010:01)*	6229
287	H153	Low	*(*001:01):(*015:01)*	6288
288	H398	Low	*(*012:01):(*014:01:01)*	6376
289	H154	Low	*(*001:01):(*010:01)*	6468
290	H155	Low	*(*010:01):(*011:01)*	6644
291	H156	Low	*(*010:01):(*011:01)*	6646
292	H399	Low	*(*001:01):(*012:01)*	6776
293	H157	Low	*(*001:01):(*001:01)*	6831
294	H158	Low	*(*001:01):(*001:01)*	6910
295	H159	Low	*(*015:01):(*064:01)*	6979
296	H160	Low	*(*011:01):(*027:03)*	7074
297	H161	Low	*(*001:01):(*015:01)*	7288
298	H162	Low	*(*010:01):(*014:01:01)*	7588
299	H163	Low	*(*001:01):(*027:03)*	7621
300	H164	Low	*(*010:01):(*015:01)*	7675
301	H165	Low	*(*014:01:01):(*015:01)*	7793
302	H166	Low	*(*001:01):(*010:01)*	7811
303	H167	Low	*(*011:01):(*015:01)*	7830
304	H487	Low	*(*010:01):(*011:01)*	8187
305	H400	Low	*(*015:01):(*020:01:02)*	8193
306	H168	Low	*(*010:01):(*027:03)*	8216
307	H559	Low	*(*014:01:01):(*015:01)*	8568
308	H169	Low	*(*015:01):(*015:01)*	8757
309	H170	Low	*(*010:01):(*011:01)*	9063
310	H171	Low	*(*012:01):(*015:01)*	9076
311	H560	Low	*(*010:01):(*027:03)*	9100
312	H561	Low	*(*011:01):(*014:01:01)*	9142
313	H562	Low	*(*001:01):(*027:03)*	9471
314	H172	Low	*(*011:01):(*014:01:01)*	9583
315	H173	Low	*(*014:01:01):(*015:01)*	9685
316	H401	Low	*(*010:01):(*015:01)*	9902
317	H174	Low	*(*011:01):(*027:07)*	9939
318	H175	High	*(*010:01):(*012:01)*	10105
319	H563	High	*(*011:01):(*015:01)*	10290
320	H315	High	*(*001:01):(*015:01)*	10351
321	H314	High	*(*010:01):(*015:01)*	10495
322	H275	High	*(*001:01):(*011:01)*	10857
323	H176	High	*(*010:01):(*011:01)*	10949
324	H313	High	*(*015:01):(*015:01)*	11364

325	H312	High	*(*011:01):(*012:01)*	11459
326	H402	High	*(*010:01):(*011:01)*	11520
327	H403	High	*(*015:01):(*015:01)*	11642
328	H488	High	*(*014:01:01):(*015:01)*	11662
329	H404	High	*(*014:01:01):(*015:01)*	11728
330	H489	High	*(*011:01):(*014:01:01)*	11802
331	H311	High	*(*001:01):(*001:01)*	11944
332	H564	High	*(*001:01):(*027:03)*	11977
333	H225	High	*(*014:01:01):(*027:03)*	12013
334	H565	High	*(*011:01):(*015:01)*	12053
335	H177	High	*(*011:01):(*027:03)*	12077
336	H250	High	*(*010:01):(*011:01)*	12086
337	H405	High	*(*001:01):(*001:01)*	12140
338	H178	High	*(*010:01):(*027:03)*	12222
339	H264	High	*(*011:01):(*015:01)*	12245
340	H490	High	*(*001:01):(*015:01)*	12284
341	H274	High	*(*015:01):(*015:01)*	12358
342	H566	High	*(*015:01):(*027:03)*	12484
343	H406	High	*(*012:01):(*015:01)*	12494
344	H310	High	*(*001:01):(*014:01:01)*	12544
345	H221	High	*(*001:01):(*011:01)*	12615
346	H249	High	*(*010:01):(*011:01)*	12621
347	H263	High	*(*001:01):(*001:01)*	12762
348	H491	High	*(*001:01):(*001:01)*	12950
349	H179	High	*(*010:01):(*015:01)*	13084
350	H180	High	*(*010:01):(*011:01)*	13158
351	H407	High	*(*011:01):(*015:01)*	13424
352	H492	High	*(*015:01):(*015:01)*	13510
353	H262	High	*(*001:01):(*011:01)*	13685
354	H493	High	*(*011:01):(*015:01)*	13762
355	H248	High	*(*012:01):(*012:01)*	13868
356	H408	High	*(*010:01):(*012:01)*	13885
357	H309	High	*(*002:01):(*007:01)*	14020
358	H409	High	*(*011:01):(*011:01)*	14070
359	H247	High	*(*001:01):(*010:01)*	14132
360	H246	High	*(*010:01):(*011:01)*	14151
361	H308	High	*(*012:01):(*015:01)*	14301
362	H307	High	*(*011:01):(*015:01)*	14474
363	H181	High	*(*010:01):(*010:01)*	14481
364	H245	High	*(*010:01):(*012:01)*	14545
365	H182	High	*(*007:01):(*009:02)*	14634

366	H567	High	*(*011:01):(*012:01)*	14760
367	H261	High	*(*005:03):(*016:01)*	14851
368	H183	High	*(*007:01):(*011:01)*	15000
369	H568	High	*(*010:01):(*011:01)*	15088
370	H215	High	*(*001:01):(*010:01)*	15338
371	H494	High	*(*011:01):(*012:01)*	15391
372	H569	High	*(*015:01):(*016:01)*	15503
373	H244	High	*(*010:01):(*011:01)*	15644
374	H184	High	*(*012:01):(*018:01)*	15744
375	H273	High	*(*011:01):(*027:03)*	15924
376	H495	High	*(*001:01):(*010:01)*	15975
377	H410	High	*(*010:01):(*011:01)*	16037
378	H411	High	*(*014:01:01):(*027:03)*	16131
379	H496	High	*(*014:01:01):(*015:01)*	16201
380	H497	High	*(*010:01):(*011:01)*	16484
381	H306	High	*(*015:01):(*015:01)*	16735
382	H570	High	*(*011:01):(*015:01)*	17059
383	H412	High	*(*001:01):(*015:01)*	17132
384	H571	High	*(*001:01):(*011:01)*	17313
385	H572	High	*(*001:01):(*001:01)*	17515
386	H305	High	*(*011:01):(*012:01)*	17625
387	H498	High	*(*012:01):(*014:01:01)*	17627
388	H304	High	*(*014:01:01):(*015:01)*	17757
389	H573	High	*(*001:01):(*015:01)*	17771
390	H574	High	*(*010:01):(*015:01)*	18336
391	H220	High	*(*001:01):(*010:01)*	18485
392	H413	High	*(*001:01):(*012:01)*	18838
393	H414	High	*(*001:01):(*012:01)*	18950
394	H243	High	*(*012:01):(*027:03)*	19058
395	H185	High	*(*011:01):(*014:01:01)*	19115
396	H260	High	*(*005:02):(*010:01)*	19223
397	H575	High	*(*015:01):(*015:01)*	19261
398	H213	High	*(*001:01):(*015:01)*	19271
399	H499	High	*(*012:01):(*015:01)*	19387
400	H186	High	*(*011:01):(*015:01)*	19450
401	H302	High	*(*011:01):(*015:01)*	19524
402	H303	High	*(*012:01):(*027:03)*	19524
403	H500	High	*(*001:01):(*033:01)*	19646
404	H501	High	*(*010:01):(*014:01:01)*	19661
405	H415	High	*(*001:01):(*011:01)*	19783
406	H187	High	*(*001:01):(*016:01)*	20000

407	H188	High	*(*011:01):(*015:01)*	20213
408	H189	High	*(*015:01):(*015:01)*	20276
409	H190	High	*(*015:01):(*015:01)*	20509
410	H272	High	*(*010:01):(*011:01)*	20609
411	H576	High	*(*001:01):(*027:03)*	20898
412	H577	High	*(*001:01):(*027:03)*	21129
413	H578	High	*(*001:01):(*007:01)*	21254
414	H242	High	*(*010:01):(*010:01)*	21284
415	H191	High	*(*010:01):(*015:01)*	21602
416	H416	High	*(*010:01):(*027:03)*	21657
417	H502	High	*(*001:01):(*012:01)*	21689
418	H579	High	*(*011:01):(*012:01)*	21975
419	H580	High	*(*001:01):(*012:01)*	22104
420	H192	High	*(*010:01):(*016:01)*	22130
421	H503	High	*(*001:01):(*011:01)*	23284
422	H193	High	*(*011:01):(*011:01)*	23485
423	H194	High	*(*027:03):(*027:03)*	23491
424	H259	High	*(*012:01):(*016:01)*	23611
425	H417	High	*(*010:01):(*015:01)*	23772
426	H301	High	*(*012:01):(*015:01)*	23787
427	H504	High	*(*001:01):(*027:03)*	23890
428	H418	High	*(*012:01):(*027:03)*	24061
429	H505	High	*(*010:01):(*011:01)*	24135
430	H300	High	*(*001:01):(*015:01)*	24249
431	H195	High	*(*010:02):(*011:01)*	24603
432	H258	High	*(*015:01):(*015:01)*	24659
433	H271	High	*(*001:01):(*015:01)*	24662
434	H257	High	*(*011:01):(*015:01)*	24851
435	H419	High	*(*001:01):(*001:01)*	24937
436	H196	High	*(*001:01):(*011:01)*	25000
437	H581	High	*(*011:01):(*011:01)*	25041
438	H420	High	*(*011:01):(*015:01)*	25309
439	H218	High	*(*011:01):(*027:03)*	25473
440	H582	High	*(*001:01):(*015:01)*	25517
441	H506	High	*(*011:01):(*012:01)*	25590
442	H197	High	*(*011:01):(*012:01)*	25635
443	H299	High	*(*010:01):(*010:01)*	25839
444	H298	High	*(*011:01):(*011:01)*	25977
445	H297	High	*(*010:01):(*011:01)*	26038
446	H296	High	*(*011:01):(*015:01)*	26111
447	H198	High	*(*011:01):(*018:01)*	26159

448	H241	High	*(*007:01):(*010:01)*	26214
449	H583	High	*(*010:01):(*012:01)*	26311
450	H507	High	*(*011:01):(*012:01)*	26501
451	H199	High	*(*001:01):(*011:01)*	27349
452	H295	High	*(*001:01):(*015:01)*	27705
453	H240	High	*(*001:01):(*015:01)*	27843
454	H584	High	*(*012:01):(*011:01)*	27891
455	H421	High	*(*011:01):(*015:01)*	27953
456	H200	High	*(*014:01:01):(*020:01:02)*	27982
457	H294	High	*(*011:01):(*027:03)*	28112
458	H293	High	*(*010:01):(*010:01)*	28225
459	H422	High	*(*001:01):(*001:01)*	28615
460	H585	High	*(*010:01):(*011:01)*	28617
461	H292	High	*(*001:01):(*001:01)*	28665
462	H201	High	*(*001:01):(*018:01)*	28708
463	H586	High	*(*012:01):(*015:01)*	28874
464	H423	High	*(*011:01):(*015:01)*	28958
465	H202	High	*(*011:01):(*015:01)*	29011
466	H587	High	*(*001:01):(*011:01)*	29053
467	H256	High	*(*001:01):(*011:01)*	29571
468	H508	High	*(*011:01):(*012:01)*	29667
469	H424	High	*(*001:01):(*011:01)*	29998
470	H255	High	*(*002:01):(*008:01)*	30165
471	H254	High	*(*011:01):(*011:01)*	30641
472	H239	High	*(*010:01):(*015:01)*	30668
473	H425	High	*(*001:01):(*001:01)*	30984
474	H588	High	*(*001:01):(*012:01)*	31038
475	H589	High	*(*011:01):(*011:01)*	31330
476	H203	High	*(*012:01):(*015:01)*	31354
477	H253	High	*(*014:01:01):(*015:01)*	31362
478	H216	High	*(*010:01):(*027:03)*	31778
479	H238	High	*(*001:01):(*027:03)*	31808
480	H590	High	*(*014:01:01):(*015:01)*	32279
481	H426	High	*(*011:01):(*012:01)*	32540
482	H509	High	*(*001:01):(*015:01)*	33968
483	H427	High	*(*011:01):(*015:01)*	33971
484	H204	High	*(*007:01):(*011:01)*	34088
485	H510	High	*(*001:01):(*014:01:01)*	34878
486	H511	High	*(*001:01):(*011:01)*	35041
487	H428	High	*(*009:02):(*014:01:01)*	35311
488	H237	High	*(*010:01):(*011:01)*	35325

489	H291	High	*(*001:01):(*015:01)*	35351
490	H512	High	*(*011:01):(*012:01)*	35814
491	H513	High	*(*011:01):(*015:01)*	35870
492	H290	High	*(*015:01):(*015:01)*	36538
493	H429	High	*(*011:01):(*011:01)*	36646
494	H430	High	*(*011:01):(*012:01)*	37117
495	H205	High	*(*001:01):(*015:01)*	37122
496	H431	High	*(*012:01):(*015:01)*	37348
497	H591	High	*(*001:01):(*012:01)*	37537
498	H206	High	*(*012:01):(*027:03)*	37829
499	H514	High	*(*011:01):(*015:01)*	37934
500	H207	High	*(*001:01):(*011:01)*	38028
501	H432	High	*(*011:01):(*015:01)*	38176
502	H289	High	*(*011:01):(*015:01)*	38371
503	H288	High	*(*011:01):(*015:01)*	38416
504	H433	High	*(*011:01):(*011:01)*	38812
505	H592	High	*(*001:01):(*018:01)*	38898
506	H287	High	*(*012:01):(*012:01)*	39314
507	H434	High	*(*010:01):(*011:01)*	39915
508	H593	High	*(*012:01):(*015:01)*	40402
509	H435	High	*(*012:01):(*015:01)*	40563
510	H236	High	*(*010:01):(*011:01)*	41145
511	H515	High	*(*001:01):(*001:01)*	41316
512	H235	High	*(*015:01):(*027:03)*	41824
513	H594	High	*(*015:01):(*015:01)*	41854
514	H516	High	*(*015:01):(*027:03)*	42060
515	H595	High	*(*010:01):(*011:01)*	42082
516	H286	High	*(*001:01):(*010:01)*	42402
517	H596	High	*(*010:01):(*018:01)*	42493
518	H285	High	*(*001:01):(*010:01)*	42723
519	H597	High	*(*010:01):(*016:01)*	42751
520	H234	High	*(*010:01):(*011:01)*	43083
521	H598	High	*(*011:01):(*001:01)*	43099
522	H599	High	*(*001:01):(*012:01)*	43237
523	H600	High	*(*011:01):(*015:01)*	43289
524	H436	High	*(*011:01):(*015:01)*	43352
525	H284	High	*(*007:01):(*012:01)*	43601
526	H437	High	*(*001:01):(*023:01)*	44000
527	H601	High	*(*011:01):(*012:01)*	44069
528	H283	High	*(*001:01):(*012:01)*	44098
529	H602	High	*(*011:01):(*012:01)*	44118

530	H438	High	*(*012:01):(*014:01:01)*	44130
531	H517	High	*(*001:01):(*011:01)*	44479
532	H208	High	*(*014:01:01):(*016:01)*	44729
533	H270	High	*(*001:01):(*015:01)*	44758
534	H233	High	*(*001:01):(*011:01)*	44843
535	H603	High	*(*011:01):(*015:01)*	44851
536	H209	High	*(*011:01):(*014:01:01)*	45169
537	H604	High	*(*011:01):(*012:01)*	45604
538	H232	High	*(*010:01):(*011:01)*	46099
539	H269	High	*(*001:01):(*012:01)*	46208
540	H605	High	*(*010:01):(*011:01)*	46611
541	H439	High	*(*001:01):(*010:01)*	46879
542	H231	High	*(*010:01):(*015:01)*	47524
543	H223	High	*(*012:01):(*012:01)*	47727
544	H518	High	*(*011:01):(*012:01)*	48028
545	H519	High	*(*001:01):(*001:01)*	48799
546	H282	High	*(*015:01):(*015:01)*	49349
547	H268	High	*(*011:01):(*015:01)*	49555
548	H606	High	*(*011:01):(*014:01:01)*	49755
549	H230	High	*(*011:01):(*011:01)*	49949
550	H607	High	*(*011:01):(*015:01)*	51711
551	H229	High	*(*011:01):(*012:01)*	51915
552	H281	High	*(*007:01):(*012:01)*	52158
553	H440	High	*(*011:01):(*012:01)*	52265
554	H228	High	*(*012:01):(*015:01)*	52401
555	H520	High	*(*010:01):(*011:01)*	52431
556	H441	High	*(*010:01):(*015:01)*	52530
557	H521	High	*(*012:01):(*012:01)*	52650
558	H280	High	*(*007:01):(*011:01)*	52941
559	H608	High	*(*011:01):(*014:01:01)*	53898
560	H522	High	*(*015:01):(*015:01)*	53901
561	H523	High	*(*001:01):(*010:01)*	54374
562	H442	High	*(*015:01):(*015:01)*	55007
563	H524	High	*(*012:01):(*015:01)*	55030
564	H252	High	*(*001:01):(*015:01)*	55104
565	H251	High	*(*012:01):(*015:01)*	55109
566	H609	High	*(*001:01):(*011:01)*	55505
567	H525	High	*(*002:01):(*011:01)*	56306
568	H217	High	*(*011:01):(*014:01:01)*	56306
569	H443	High	*(*010:01):(*011:01)*	57202
570	H267	High	*(*011:01):(*015:01)*	57659

571	H444	High	*(*001:01):(*027:03)*	57908
572	H445	High	*(*010:01):(*011:01)*	58407
573	H526	High	*(*001:01):(*015:01)*	58599
574	H279	High	*(*011:01):(*015:01)*	58641
575	H210	High	*(*015:01):(*015:01)*	59047
576	H446	High	*(*015:01):(*015:01)*	59059
577	H447	High	*(*001:01):(*011:01)*	59093
578	H278	High	*(*007:01):(*012:01)*	59136
579	H448	High	*(*010:01):(*015:01)*	59311
580	H277	High	*(*012:01):(*007:04)*	59974
581	H527	High	*(*001:01):(*015:01)*	61446
582	H227	High	*(*011:01):(*027:03)*	62396
583	H449	High	*(*011:01):(*027:03)*	62718
584	H214	High	*(*011:01):(*015:01)*	62837
585	H450	High	*(*016:01):(*027:03)*	63383
586	H451	High	*(*001:01):(*014:01:01)*	63758
587	H452	High	*(*010:01):(*010:01)*	63771
588	H528	High	*(*015:01):(*015:01)*	64386
589	H276	High	*(*007:01):(*011:01)*	64950
590	H529	High	*(*011:01):(*015:01)*	65416
591	H453	High	*(*010:01):(*015:01)*	65924
592	H610	High	*(*001:01):(*015:01)*	66675
593	H454	High	*(*015:01):(*018:01)*	67126
594	H611	High	*(*011:01):(*014:01:01)*	67716
595	H211	High	*(*015:01):(*027:03)*	68869
596	H455	High	*(*010:01):(*018:01)*	69072
597	H456	High	*(*015:01):(*015:01)*	69602
598	H219	High	*(*001:01):(*001:01)*	70085
599	H266	High	*(*007:01):(*011:01)*	71385
600	H530	High	*(*011:01):(*011:01)*	72611
601	H531	High	*(*001:01):(*011:01)*	72873
602	H457	High	*(*001:01):(*011:01)*	76263
603	H458	High	*(*011:01):(*015:01)*	77409
604	H265	High	*(*001:01):(*015:01)*	78942
605	H224	High	*(*001:01):(*015:01)*	81124
606	H532	High	*(*001:01):(*011:01)*	87212
607	H222	High	*(*001:01):(*001:01)*	96990
608	H459	High	*(*015:01):(*016:01)*	99331
609	H460	High	*(*011:01):(*011:01)*	99747
610	H212	High	*(*016:01):(*027:03)*	120482
611	H226	High	*(*015:01):(*015:01)*	166773

Asymptomatic cows used in PVL association study. Total 611 asymptomatic cows were divided into two groups based on the BLV PVL: (i) HPVL, n=294 (ii) LPVL, n=317

[1]Status: Low or High proviral load

[2]PVL. Proviral load. (expressed as the number of copies of provirus per 105 peripheral blood mononuclear cells)

Table 3. Sample information of lymphoma cows in *BoLA-DRB3* and BLV-induced lymphoma association study.

ID	*BoLA- DRB3* (a):(b)	[1]PVL	Age (month)	Birth area	Tumor location															lymph node			cavity	
					Heart	Lung	Liver	Kidney	Spleen	Diaphragm	Mediastinum	Rumen	Reticulum	Omasum	Abomasum	Intestines	Bladder	Uterus	Head	Iliac	Body	Thoracic	Abdominal
L1	*(*009:02):(*012:01)*	28	120	Iwate																		●	
L2	*(*011:01):(*027:03)*	38	48	Hyogo				●								●							
L3	*(*001:01):(*006:01)*	274	89	Tochigi				●															
L4	*(*014:01:01):(*015:01)*	417	84	Hyogo											●								
L5	*(*010:01):(*012:01)*	423	85	Tottori		●		●			●	●			●				●	●			
L6	*(*001:01):(*027:03)*	1095	56	Hokkaido			●																
L7	*(*011:01):(*015:01)*	1486	96	Gunma	[2]n.t.																		
L8	*(*009:02):(*011:01)*	1864	49	Hokkaido											●								
L9	*(*001:01):(*012:01)*	2246	78	Saitama	n.t.																		
L10	*(*010:01):(*015:01)*	2404	49	Gunma	n.t.																		
L11	*(*015:01):(*015:01)*	2479	20	Kanagawa	●																		
L12	*(*001:01):(*027:03)*	2530	108	Hyogo											●					●			
L13	*(*002:01):(*014:01:01)*	2990	24	Hyogo															●				
L14	*(*011:01):(*027:03)*	3150	60	Hyogo												●							
L15	*(*011:01):(*015:01)*	3776	51	Hokkaido	●				●	●		●	●	●	●	●					●		
L16	*(*002:01):(*014:01:01)*	4216	104	Tochigi											●								
L17	*(*010:01):(*014:01:01)*	4289	65	Hokkaido			●																
L18	*(*005:03):(*016:01)*	4348	12	Hokkaido			●																
L19	*(*002:01):(*015:01)*	4830	144	Hyogo												●				●			

L20	*(*011:01):(*015:01)*	5010	61	Hokkaido		●																	
L21	*(*012:01):(*012:01)*	5250	70	Fukushima				●															
L22	*(*001:01):(*001:01)*	5616	53	Tochigi																●			
L23	*(*014:01:01):(*027:03)*	5714	81	Tochigi												●							
L24	*(*001:01):(*010:01)*	5741	76	Hokkaido	n.t.																		
L25	*(*015:01):(*020:01:02)*	5891	104	Iwate												●							
L26	*(*011:01):(*015:01)*	5976	n.t.	Gunma						●													
L27	*(*001:01):(*015:01)*	6260	60	Hyogo				●															
L28	*(*015:01):(*027:03)*	6475	97	Hokkaido					●					●		●					●		
L29	*(*002:01):(*015:01)*	7434	82	Tochigi	●																		
L30	*(*001:01):(*010:01)*	7580	60	Hyogo																●			
L31	*(*006:01):(*007:01)*	9040	85	Shiga												●							
L32	*(*001:01):(*001:01)*	9564	46	Hokkaido										●									
L33	*(*014:01:01):(*015:01)*	9725	134	Hokkaido	●	●										●					●		
L34	*(*012:01):(*016:01)*	9730	12	Hyogo				●															
L35	*(*010:01):(*011:01)*	9767	63	Hokkaido																●			
L36	*(*015:01):(*027:03)*	9813	75	Hokkaido	n.t.																		
L37	*(*011:01):(*012:01)*	9892	25	Tochigi							●												
L38	*(*014:01:01):(*018:01)*	10087	86	Hokkaido		●	●			●					●						●		
L39	*(*002:01):(*015:01)*	10261	89	Tochigi	●																		
L40	*(*027:03):(*027:03)*	10355	132	Fukushima																●			
L41	*(*015:01):(*015:01)*	10369	122	Hokkaido	●																		
L42	*(*011:01):(*011:01)*	10526	112	Hokkaido	●				●		●			●							●		

L43	*(*012:01):(*012:01)*	10776	72	Ibaraki				●															
L44	*(*014:01:01):(*2808)*	10837	56	Tochigi	●																		
L45	*(*002:01):(*009:02)*	10909	n.t.	Gunma																●			
L46	*(*001:01):(*012:01)*	11382	50	Aomori	●																		
L47	*(*001:01):(*007:01)*	11800	24	Hyogo											●								
L48	*(*015:01):(*015:01)*	12624	90	Hokkaido							●												
L49	*(*015:01):(*015:01)*	13036	68	Tochigi											●								
L50	*(*012:01):(*015:01)*	13289	50	Hokkaido			●		●												●		
L51	*(*011:01):(*015:01)*	15573	60	Hokkaido																●			
L52	*(*015:01):(*027:03)*	15700	48	Hyogo								●											
L53	*(*011:01):(*027:07)*	15800	60	Hyogo												●							
L54	*(*011:01):(*016:01)*	16170	92	Tottori	●	●	●	●	●										●				
L55	*(*014:01:01):(*015:01)*	16372	91	Hokkaido					●				●	●	●								
L56	*(*018:01):(*011:04)*	17200	48	Hyogo																●			
L57	*(*011:01):(*011:01)*	17545	110	Hokkaido	●								●	●	●	●							
L58	*(*010:01):(*027:07)*	18500	36	Hyogo												●							
L59	*(*015:01):(*015:01)*	18893	57	Gunma	●																		
L60	*(*011:01):(*015:01)*	18976	49	Gunma	n.t.																		
L61	*(*011:01):(*012:01)*	19220	94	Hokkaido	n.t.																		
L62	*(*011:01):(*011:01)*	19440	99	Tochigi										●									
L63	*(*015:01):(*027:01)*	19576	73	Iwate																●			
L64	*(*001:01):(*001:01)*	19776	82	Tochigi						●													
L65	*(*015:01):(*015:01)*	20463	80	Hokkaido	●	●		●	●			●			●	●					●		

L66	*(*016:01):(*031:03)*	20544	75	Hokkaido	●	●			●						●	●		●			●		
L67	*(*001:01):(*027:03)*	20672	135	Hokkaido					●												●		
L68	*(*011:01):(*015:01)*	20922	79	Hokkaido											●								
L69	*(*010:01):(*010:01)*	21566	52	Tochigi	●																		
L70	*(*001:01):(*007:01)*	22068	44	Saitama	n.t.																		
L71	*(*001:01):(*018:01)*	22500	48	Hyogo				●															
L72	*(*011:01):(*015:01)*	23033	115	Hokkaido	●			●															
L73	*(*001:01):(*015:01)*	23200	63	Aichi														●					
L74	*(*015:01):(*015:01)*	23731	110	Iwate								●	●	●	●								
L75	*(*011:01):(*016:01)*	24600	48	Hyogo											●								
L76	*(*012:01):(*015:01)*	24859	71	Tochigi															●				
L77	*(*011:01):(*027:03)*	27399	53	Aichi				●															
L78	*(*001:01):(*001:01)*	28000	100	Hokkaido																		●	
L79	*(*012:01):(*015:01)*	28565	46	Hokkaido	n.t.																		
L80	*(*014:01:01):(*014:01:01)*	30848	64	Hokkaido	n.t.																		
L81	*(*011:01):(*027:03)*	32902	113	Saitama	n.t.																		
L82	*(*010:01):(*011:01)*	33022	57	Hokkaido												●					●		
L83	*(*011:01):(*014:01:01)*	34375	76	Gunma																●			
L84	*(*001:01):(*001:01)*	34624	66	Hokkaido					●									●	●		●		
L85	*(*001:01):(*027:07)*	34624	108	Hokkaido	●								●	●	●						●		
L86	*(*001:01):(*001:01)*	38328	67	Kohchi	●																		
L87	*(*010:01):(*011:01)*	38588	57	Hokkaido	●			●	●	●	●					●		●			●	●	
L88	*(*012:01):(*012:01)*	38671	103	Hokkaido	●																		
L89	*(*015:01):(*027:03)*	38874	75	Fukushima	●																		

L90	*(*012:01):(*012:01)*	41050	79	Hokkaido	●																		
L91	*(*001:01):(*011:01)*	41549	95	Hokkaido	●																	●	
L92	*(*012:01):(*015:01)*	42384	98	Hokkaido	n.t.																		
L93	*(*011:01):(*015:01)*	42794	53	Gunma	●	●		●		●	●								●		●		
L94	*(*015:01):(*016:01)*	47540	50	Tottori	●	●	●	●		●		●	●	●	●	●					●		
L95	*(*010:01):(*027:03)*	49514	19	Hokkaido	●	●			●							●						●	
L96	*(*015:01):(*015:01)*	50993	92	Hokkaido	●				●													●	
L97	*(*011:01):(*015:01)*	52000	60	Hyogo												●				●			
L98	*(*015:01):(*015:01)*	53036	18	Hokkaido				●							●							●	
L99	*(*012:01):(*015:01)*	53179	41	Hokkaido	●			●	●		●					●						●	
L100	*(*010:01):(*011:01)*	53741	161	Hokkaido	●																		
L101	*(*001:01):(*012:01)*	53957	104	Hokkaido	●						●											●	
L102	*(*012:01):(*015:01)*	54865	54	Hokkaido	●						●		●		●							●	
L103	*(*011:01):(*011:01)*	56516	72	Saitama	n.t.																		
L104	*(*012:01):(*012:01)*	57900	48	Hyogo															●		●		
L105	*(*012:01):(*015:01)*	59804	70	Hokkaido	●	●		●								●			●			●	
L106	*(*015:01):(*018:01)*	60510	52	Gunma	n.t.																		
L107	*(*015:01):(*027:03)*	63732	86	Tottori	●										●						●		
L108	*(*011:01):(*015:01)*	65690	99	Hokkaido	●			●							●								
L109	*(*011:01):(*011:01)*	66757	87	Hokkaido	n.t.																		
L110	*(*015:01):(*015:01)*	66819	76	Hokkaido	●		●								●	●						●	●
L111	*(*001:01):(*015:01)*	69372	84	Hokkaido				●															
L112	*(*010:01):(*015:01)*	69477	76	Tottori	●			●	●			●	●	●	●	●							
L113	*(*001:01):(*010:01)*	69714	71	Tochigi			●																

L114	*(*011:01):(*027:03)*	70139	65	Hokkaido			●	●													●		●
L115	*(*015:01):(*046:01:01)*	70566	n.t.	Aomori												●							
L116	*(*014:01:01):(*015:01)*	71786	n.t.	Gunma																●			
L117	*(*017:01):(*018:01)*	72751	43	Fukushima																●			
L118	*(*012:01):(*015:01)*	73962	73	Hokkaido	n.t.																		
L119	*(*001:01):(*001:01)*	73980	98	Hokkaido	●						●		●								●		
L120	*(*011:01):(*014:01:01)*	74595	80	Tochigi	●																		
L121	*(*015:01):(*015:01)*	76131	57	Gunma	●		●				●	●								●		●	●
L122	*(*005:03):(*016:01)*	77471	110	Hokkaido		●		●	●						●	●					●		
L123	*(*012:01):(*011:04)*	79316	138	Aichi																●			
L124	*(*001:01):(*011:01)*	79346	88	Yamagata				●															
L125	*(*001:01):(*011:01)*	80117	77	Kumamoto															●				
L126	*(*011:01):(*018:01)*	81633	81	Hokkaido					●												●		
L127	*(*015:01):(*015:01)*	82609	54	Hokkaido	●	●	●	●	●				●	●				●	●		●		
L128	*(*015:01):(*015:01)*	82609	55	Hokkaido																●			
L129	*(*015:01):(*017:01)*	82609	55	Hokkaido																●			
L130	*(*012:01):(*015:01)*	82796	100	Ibaraki															●				
L131	*(*001:01):(*015:01)*	84091	66	Hokkaido	●		●	●	●		●		●			●			●		●		●
L132	*(*001:01):(*015:01)*	84906	71	Gunma				●															
L133	*(*010:01):(*016:01)*	86372	57	Hokkaido	●																		
L134	*(*011:01):(*018:01)*	86996	62	Niigata	●																		
L135	*(*015:01):(*015:01)*	87580	63	Tochigi																●			
L136	*(*012:01):(*015:01)*	88618	102	Hokkaido				●															

L137	*(*001:01):(*010:01)*	90000	93	Iwate											●								
L138	*(*016:01):(*016:01)*	90964	90	Tochigi	●																		
L139	*(*015:01):(*027:03)*	91775	78	Tochigi					●														
L140	*(*015:01):(*015:01)*	92222	55	Aichi							●												
L141	*(*010:01):(*011:01)*	92500	n.t.	Ibaraki	●																		
L142	*(*015:01):(*015:01)*	93007	120	Hokkaido						●													
L143	*(*001:01):(*015:01)*	93040	60	Hokkaido	●																		
L144	*(*001:01):(*011:01)*	95354	n.t.	Chiba																●			
L145	*(*001:01):(*001:01)*	96700	60	Aichi														●					
L146	*(*015:01):(*027:03)*	97484	72	Tochigi														●					
L147	*(*001:01):(*027:03)*	98799	63	Hokkaido	n.t.																		
L148	*(*011:01):(*015:01)*	100590	99	Hokkaido	●			●								●					●		
L149	*(*010:01):(*011:01)*	100671	69	Tochigi							●												
L150	*(*010:01):(*014:01:01)*	100946	88	Aichi			●																
L151	*(*012:01):(*012:01)*	101765	61	Yamagata	●																		
L152	*(*001:01):(*015:01)*	103268	75	Aichi																●			
L153	*(*016:01):(*020:01:02)*	104004	76	Miyazaki											●								
L154	*(*016:01):(*016:01)*	108027	72	Fukushima																●			
L155	*(*012:01):(*012:01)*	108920	129	Tochigi																●			
L156	*(*027:03):(*027:03)*	111940	n.t.	Aichi																●			
L157	*(*001:01):(*018:01)*	113462	40	Aichi																●			
L158	*(*001:01):(*015:01)*	114000	60	Hyogo												●							
L159	*(*012:01):(*015:01)*	114463	114	Hokkaido											●								

L160	*(*001:01):(*016:01)*	118033	n.t.	Gunma																●			
L161	*(*001:01):(*012:01)*	118571	n.t.	Gifu												●							
L162	*(*001:01):(*014:01:01)*	120270	42	Niigata	n.t.																		
L163	*(*009:01):(*010:01)*	120606	77	Tochigi	●																		
L164	*(*014:01:01):(*015:01)*	120643	n.t.	Tochigi	n.t.																		
L165	*(*014:01:01):(*014:01:01)*	121858	70	Tochigi												●							
L166	*(*012:01):(*027:03)*	125444	43	Ibaraki																●			
L167	*(*001:01):(*027:03)*	131311	39	Hokkaido	●						●		●	●	●	●	●	●			●		
L168	*(*001:01):(*027:03)*	137056	58	Hokkaido											●								
L169	*(*001:01):(*015:01)*	138554	95	Aichi																●			
L170	*(*001:03):(*015:01)*	140375	46	Tottori	●	●	●	●	●	●								●					
L171	*(*014:01:01):(*015:01)*	140741	78	Hokkaido	●																		
L172	*(*015:01):(*027:03)*	143216	72	Gunma														●					
L173	*(*001:01):(*027:03)*	144277	91	Hokkaido															●				
L174	*(*010:01):(*010:01)*	144309	61	Ibaraki																●			
L175	*(*012:01):(*015:01)*	144444	n.t.	Tochigi						●													
L176	*(*011:01):(*011:01)*	148120	n.t.	Gunma						●													
L177	*(*001:01):(*020:01:02)*	148598	67	Hokkaido			●																
L178	*(*001:01):(*001:01)*	151707	75	Nagano												●							
L179	*(*010:01):(*014:01:01)*	154000	94	Hokkaido	●						●										●		
L180	*(*011:01):(*012:01)*	157529	n.t.	Aichi																●			
L181	*(*011:01):(*014:01:01)*	161789	n.t.	Fukushima						●													
L182	*(*001:01):(*001:01)*	162136	96	Chiba																●			

L183	*(*012:01):(*015:01)*	163514	64	Hokkaido	●			●	●				●			●		●			●		●
L184	*(*001:01):(*001:01)*	163768	103	Iwate											●								
L185	*(*028:01):(*031:01)*	163905	112	Aichi	●																		
L186	*(*011:01):(*011:01)*	164935	67	Tochigi														●					
L187	*(*015:01):(*015:01)*	175789	n.t.	Ibaraki	n.t.																		
L188	*(*001:01):(*016:01)*	177524	67	Ibaraki								●	●	●	●								
L189	*(*011:01):(*015:01)*	179518	63	Tochigi	n.t.																		
L190	*(*001:01):(*015:01)*	180465	65	Iwate																●			
L191	*(*012:01):(*012:01)*	183151	98	Hokkaido	●																		
L192	*(*011:01):(*012:01)*	205344	18	Hokkaido	●	●			●		●					●			●		●		
L193	*(*011:01):(*012:01)*	205344	18	Hokkaido	n.t.																		
L194	*(*012:01):(*014:01:01)*	208909	81	Aichi						●													
L195	*(*015:01):(*015:01)*	211211	81	Tochigi											●								
L196	*(*010:01):(*012:01)*	216000	64	Hokkaido												●							
L197	*(*015:01):(*015:01)*	217341	n.t.	Gunma																●			
L198	*(*002:01):(*011:01)*	219355	80	Gunma				●															
L199	*(*010:01):(*015:01)*	219844	93	Tochigi	●																		
L200	*(*002:01):(*015:01)*	227273	81	Ibaraki						●													
L201	*(*001:01):(*001:01)*	227564	n.t.	Gunma	n.t.																		
L202	*(*001:01):(*011:01)*	247596	n.t.	Ibaraki	n.t.																		
L203	*(*006:01):(*015:01)*	254167	51	Shizuoka				●															
L204	*(*012:01):(*015:01)*	263333	68	Ibaraki						●													
L205	*(*001:01):(*012:01)*	266515	92	Hokkaido	●																		
L206	*(*011:01):(*012:01)*	272121	n.t.	Tochigi																●			

L207	*(*027:03):(*027:03)*	273810	99	Hokkaido										●									
L208	*(*011:01):(*015:01)*	281172	n.t.	Tochigi	●																		
L209	*(*010:01):(*027:08)*	323394	n.t.	Gunma												●							
L210	*(*002:01):(*002:01)*	326351	80	Aichi								●	●	●	●								
L211	*(*002:01):(*002:01)*	328000	71	Iwate			●																
L212	*(*015:01):(*016:01)*	341162	42	Miyazaki											●								
L213	*(*001:01):(*001:01)*	350542	70	Hokkaido	●																		
L214	*(*015:01):(*027:03)*	357367	70	Hokkaido	●																		
L215	*(*010:01):(*011:01)*	370551	65	Tottori	●	●	●	●	●						●	●	●	●					
L216	*(*005:02):(*015:01)*	403341	184	Tochigi																		●	
L217	*(*001:01):(*001:01)*	407194	87	Shizuoka																	●		
L218	*(*015:01):(*027:03)*	442971	103	Tochigi			●																
L219	*(*012:01):(*014:01:01)*	501534	59	Shizuoka			●																
L220	*(*012:01):(*015:01)*	573691	72	Hokkaido												●							
L221	*(*001:01):(*011:01)*	######	74	Hokkaido	●																		

[1]PVL. Proviral load. (expressed as the number of copies of provirus per 105 peripheral blood mononuclear cells)
[2]n.t.. Not tested

Table 4. Summary of PVL distribution in asymptomatic cows and lymphoma cows.

Status	Asymptomatic (*n* = 250)	Lymphoma (*n* = 221)
Low proviral load [1]	188	37
High proviral load [2]	62	184

[1] A PVL of $< 10^4$ copies/10^5 cells was considered Low proviral load; [2] a PVL of $> 10^4$ copies/10^5 cells was considered High proviral load.

Table 5. Association study of *BoLA-DRB3* alleles between asymptomatic cows and lymphoma cows.

BoLA-DRB3 allele	Number of allele		[1]OR	*p*-value	*p-value* rank (l)	[2]BH value (l/allele number)*0.05	[3]Susceptibility
	Asymptomatic	Lymphoma					
**001:01*	70	70	1.156	0.463			[4]-
**002:01*	13	13	1.135	1.000			-
**003:01*	1	0	0.000	1.000			-
**005:02*	0	1	-	0.469			-
**005:03*	1	2	2.268	0.603			-
**006:01*	3	3	1.132	1.000			-
**007:01*	13	3	0.256	0.024	6	0.009	(R)
**007:04*	1	0	0.000	1.000			-
**009:01*	2	1	0.565	1.000			-
**009:02*	19	4	0.231	0.005	5	0.008	R
**010:01*	64	29	0.478	0.001	2	0.003	R
**010:02*	1	0	0.000	1.000			-
**011:01*	111	61	0.561	0.001	2	0.003	R
**011:03*	0	1	-	1.000			-
**011:04*	0	2	-	0.220			-
**012:01*	22	49	2.709	0.000	1	0.002	S
**014:01:01*	44	23	0.569	0.042			(R)
**015:01*	82	109	1.669	0.002	4	0.006	S
**016:01*	14	17	1.389	0.578			-
**017:01*	1	2	2.268	0.603			-
**018:01*	3	8	3.054	0.127			-
**020:01:02*	1	3	3.410	0.346			-
**027:01*	0	1	-	0.469			-
**027:03*	31	31	1.141	0.693			-
**027:07*	2	3	1.702	0.670			-
**027:08*	0	1	-	0.469			-
**028:01*	0	1	-	0.469			-
**028:08*	0	1	-	0.469			-
**031:01*	0	1	-	0.469			-
**031:03*	0	1	-	0.469			-
**046:01:01*	0	1	-	0.469			-
**064:01*	1	0	0.000	1.000			-

BoLA-DRB3 allele frequency of asmptomatic and lymphoma cows. Total 942 *BoLA-DRB3* alleles were detected and classified into 32 known types in the BLV-infected population. The frequencies of 500 *BoLA-DRB3* alleles (of 22 different types) from asymptomatic cows, and 442 alleles (of 28 different types) from lymphoma cows were calculated and p values and odds ratios (ORs) were estimated for each *BoLA-DRB3* allele by Fisher's exact test. Benjamini–Hochberg procedure was used to adjust the false positive rate and determine the significance.

[1] OR: Odds ratio

[2] BH value: The value derived from Benjamini–Hochberg procedure adjusted p-value. I, the rank of p-value.

[3] The allele with p-values < BH value were determined as susceptibility, S, when OR > 1 and resistance, R, when OR < 1. R and S which with ( ) indicate the resistance and susceptibility allele that only significant before correction of multiple testing (BH procedure).

[4] -: OR=∞

Table 6. Fisher's exact test based association analysis of *BoLA-DRB3* alleles in asymptomatic and lymphoma cows.

BoLA-DRB3 Allele	Asymptomatic (250 Cattle)	Lymphoma (221 Cattle)	OR	*p*-Value	*p*-Value Rank (I)	BH Value (I/Allele Number)*0.05	Susceptibility
**001:01*	70	70	1.156	0.463			-
**002:01*	13	13	1.135	1.000			-
**006:01*	3	3	1.132	1.000			-
**007:01*	13	3	0.256	0.024	6	0.009	(R)
**009:02*	19	4	0.231	0.005	5	0.008	R
**010:01*	64	29	0.478	0.001	2	0.003	R
**011:01*	111	61	0.561	0.001	2	0.003	R
**012:01*	22	49	2.709	0.000	1	0.002	S
**014:01:01*	44	23	0.569	0.042			(R)
**015:01*	82	109	1.669	0.002	4	0.006	S
**016:01*	14	17	1.389	0.578			-
**018:01*	3	8	3.054	0.127			-
**027:03*	31	31	1.141	0.693			-

The Benjamini–Hochberg (BH) procedure was performed to adjust the false positive rate. Alleles with a *p*-value < BH value were defined as susceptibility, S, with an odds ratio (OR) > 1 and as resistance, R, with an OR < 1. BH value = (*p*-value rank/total allele number) × 0.05. R and S which with ( ) indicate the resistance and susceptibility allele that only significant before correction of multiple testing (BH procedure).

Table 7. Results of the stepwise conditional analysis of *BoLA-DRB3* alleles in asymptomatic and lymphoma cows.

			Conditional association							
	Univariate[1]		**011:01*		**011:01, *009:02*		**011:01, *009:02, *010:01*		**011:01, *009:02, *010:01, *014:01:01*	
Allele	OR (95% CI)	*p-value*	OR (95% CI)	*p-value*	OR (95% CI)	*p-value*	OR (95% CI)	*p-value*	OR (95% CI)	*p-value*
**001:01*	1.08 (0.75–1.55)	0.692	0.96 (0.66–1.38)	0.81	0.88 (0.6–1.29)	0.513	0.78 (0.53–1.15)	0.215	0.66 (0.44–0.98)	0.041
**002:01*	0.92 (0.43–1.95)	0.818	0.83 (0.39–1.78)	0.631	0.75 (0.35–1.62)	0.465	0.69 (0.32–1.5)	0.348	0.65 (0.3–1.42)	0.284
**006:01*	1.45 (0.28–7.52)	0.658	1.28 (0.24–6.82)	0.769	1.16 (0.22–6.24)	0.86	0.97 (0.18–5.22)	0.969	0.8 (0.15–4.37)	0.797
**007:01*	0.23 (0.06–0.85)	0.027	0.22 (0.06–0.81)	0.023	0.21 (0.06–0.79)	0.021	0.19 (0.05–0.71)	0.014	0.19 (0.05–0.7)	0.013
**009:02*	0.1 (0.02–0.43)	0.002	0.08 (0.02–0.35)	8.54×10^{-4}	—	—	—	—	—	—
**010:01*	0.52 (0.32–0.84)	0.008	0.45 (0.28–0.74)	0.002	0.46 (0.28–0.76)	0.002	—	—	—	—
**011:01*	0.53 (0.36–0.77)	9.91×10^{-4}	—	—	—	—	—	—	—	—
**012:01*	2.36 (1.39–4.02)	0.001	2.1 (1.23–3.59)	0.007	2.05 (1.18–3.54)	0.01	1.89 (1.09–3.29)	0.024	1.74 (0.99–3.06)	0.053
**014:01:01*	0.53 (0.3–0.93)	0.026	0.46 (0.26–0.82)	0.009	0.4 (0.22–0.72)	0.002	0.36 (0.2–0.66)	7.82×10^{-4}	—	—
**015:01*	1.72 (1.23–2.42)	0.002	1.56 (1.1–2.2)	0.012	1.46 (1.02–2.07)	0.036	1.28 (0.89–1.84)	0.191	1.15 (0.79–1.67)	0.468
**016:01*	1.87 (0.85–4.12)	0.119	1.64 (0.74–3.61)	0.224	1.49 (0.67–3.29)	0.327	1.41 (0.63–3.15)	0.397	1.2 (0.54–2.68)	0.659

**018:01*	3.98 (1.02–15.47)	0.046	3.76 (0.97–14.54)	0.055	3.45 (0.89–13.37)	0.073	2.93 (0.75–11.42)	0.121	2.68 (0.69–10.41)	0.154
**027:03*	1.37 (0.77–2.42)	0.28	1.3 (0.73–2.32)	0.38	1.26 (0.7–2.29)	0.438	1.16 (0.64–2.11)	0.626	1.04 (0.56–1.91)	0.907

[1] calculated by logistic regression analysis after adjustment of age.

Table 8. Logistic regression analysis-based association study of *BoLA-DRB3* alleles in asymptomatic and lymphoma cows after adjustments for age.

BoLA-DRB3 Allele	Univariate				Multivariate			
	p-Value	OR	L95	U95	*p*-Value	OR	L95 [1]	U95 [2]
**009:02*	0.002	0.10	0.02	0.43	4.27×10^{-4}	0.07	0.01	0.30
**010:01*	0.008	0.52	0.32	0.84	7.38×10^{-4}	0.43	0.026	0.70
**011:01*	9.91×10^{-4}	0.53	0.36	0.77	5.77×10^{-6}	0.40	0.27	0.59
**014:01:01*	0.026	0.53	0.30	0.93	7.82×10^{-4}	0.36	0.20	0.66

[1] L95, lower 95% confidence interval. [2] U95, upper 95% confidence interval.

Table 9. Association study of *BoLA-DRB3* genotypes between Asymptomatic cows and lymphoma cows.

BoLA-DRB3 genotype	Number of genotype		[1]OR	*p*-value	*p-value* rank (I)	[2]BH value (I/genotype number)*0.05	[3]Susceptibility
	Asymptomatic	Lymphoma					
**001:01/*001:01*	3	14	5.568	0.005	3		(S)
**001:01/*002:01*	3	0	0.000	0.251			[4]-
**001:01/*006:01*	1	1	1.132	1.000			-
**001:01/*007:01*	3	2	0.562	0.689			-
**001:01/*009:01*	1	0	0.000	1.000			-
**001:01/*009:02*	3	0	0.000	0.251			-
**001:01/*010:01*	7	4	0.558	0.393			-
**001:01/*011:01*	21	6	0.263	0.002	1	0.0005	(R)
**001:01/*012:01*	2	5	1.906	0.483			-
**001:01/*014:01:01*	6	1	0.158	0.072			-
**001:01/*015:01*	9	10	1.269	0.645			-
**001:01/*016:01*	3	2	0.752	1.000			-
**001:01/*018:01*	1	2	2.274	0.603			-
**001:01/*020:01:02*	0	1	-	0.469			-
**001:01/*027:03*	4	7	2.012	0.362			-
**001:01/*027:07*	0	1	-	0.469			-
**002:01/*002:01*	1	2	2.274	0.603			-
**002:01/*009:02*	0	2	-	0.220			-
**002:01/*010:01*	2	1	0.374	0.626			-
**002:01/*011:01*	3	1	0.280	0.377			-
**002:01/*014:01:01*	1	1	1.132	1.000			-
**002:01/*015:01*	1	4	4.590	0.191			-
**002:01/*016:01*	1	0	0.000	1.000			-
**003:01/*015:01*	1	0	0.000	1.000			-
**005:02/*015:01*	0	1	-	0.469			-
**005:03/*010:01*	1	0	0.000	1.000			-
**005:03/*016:01*	0	2	-	0.220			-
**006:01/*007:01*	0	1	-	0.469			-
**006:01/*011:01*	1	0	0.000	0.220			-
**006:01/*015:01*	0	1	-	1.000			-
**006:01/*027:03*	1	0	0.000	0.220			-
**007:01/*009:02*	1	0	0.000	0.220			-
**007:01/*010:01*	1	0	0.000	0.501			
**007:01/*011:01*	5	0	0.000	0.057			-
**007:01/*014:01:01*	2	0	0.000	0.501			-

**007:01/*015:01*	1	0	0.000	0.490			-
**009:01/*009:02*	1	0	0.000	0.490			-
**009:01/*010:01*	0	1	-	0.469			-
**009:02/*010:01*	6	0	0.000	0.008			(R)
**009:02/*011:01*	1	1	1.132	1.000			-
**009:02/*012:01*	1	1	0.564	1.000			-
**009:02/*015:01*	4	0	0.000	0.064			-
**009:02/*027:03*	2	0	0.000	0.501			-
**010:01/*010:01*	6	2	0.317	0.183			-
**010:01/*011:01*	12	7	0.551	0.264			-
**010:01/*012:01*	4	2	0.562	0.689			-
**010:01/*014:01:01*	4	3	0.674	0.728			-
**010:01/*015:01*	7	3	0.416	0.231			-
**010:01/*016:01*	4	1	0.223	0.221			-
**010:01/*027:03*	4	1	0.223	0.221			-
**010:01/*027:07*	0	1	-	0.469			-
**010:01/*027:08*	0	1	-	0.469			-
**010:02/*011:01*	1	0	0.000	1.000			-
**011:01/*011:01*	9	7	0.876	1.000			-
**011:01/*012:01*	3	5	1.424	0.740			-
**011:01/*014:01:01*	11	3	0.273	0.037			(R)
**011:01/*015:01*	22	14	0.496	0.039			(R)
**011:01/*016:01*	1	2	2.274	0.603			-
**011:01/*018:01*	1	2	2.274	0.603			-
**011:01/*027:03*	10	5	0.459	0.215			-
**011:01/*027:07*	1	1	1.132	1.000			-
**011:03/*015:01*	0	1	-	0.469			-
**012:01/*011:04*	0	1	-	0.469			-
**012:01/*012:01*	0	8	-	0.002	1	0.0005	(S)
**012:01/*014:01:01*	3	2	0.752	1.000			-
**012:01/*015:01*	4	15	3.568	0.012			(S)
**012:01/*016:01*	1	1	1.132	1.000			-
**012:01/*018:01*	1	0	0.000	1.000			-
**012:01/*027:03*	3	1	0.280	0.377			-
**014:01:01/*014:01:01*	0	2	-	0.220			-
**014:01:01/*015:01*	12	6	0.470	0.169			-
**014:01:01/*016:01*	1	0	0.000	1.000			-
**014:01:01/*018:01*	0	1	-	0.469			-
**014:01:01/*020:01:02*	1	0	0.000	1.000			-
**014:01:01/*027:03*	2	1	0.564	1.000			-
**014:01:01/*027:07*	1	0	0.000	1.000			-

**014:01:01/*028:08*	0	1	-	0.469	-
**015:01/*007:04*	1	0	0.000	1.000	-
**015:01/*015:01*	7	18	3.078	0.013	(S)
**015:01/*016:01*	2	3	1.706	0.669	-
**015:01/*017:01*	1	1	1.132	1.000	-
**015:01/*018:01*	0	1	-	0.469	-
**015:01/*020:01:02*	0	1	-	0.469	-
**015:01/*027:01*	0	1	-	0.469	-
**015:01/*027:03*	2	10	5.877	0.016	(S)
**015:01/*046:01:01*	0	1	-	0.469	-
**015:01/*064:01*	1	0	0.000	1.000	-
**016:01/*016:01*	0	2	-	0.220	-
**016:01/*020:01:02*	0	1	-	0.469	-
**016:01/*027:03*	1	0	0.000	1.000	-
**016:01/*031:03*	0	1	-	0.469	-
**017:01/*018:01*	0	1	-	0.469	-
**018:01/*011:04*	0	1	-	0.469	-
**027:03/*027:03*	1	3	3.427	0.345	-
**028:01/*031:01*	0	1	-	0.469	-

BoLA-DRB3 genotype frequency of 250 asmptomatic and 221 lymphoma cows. A total of 95 different genotypes were detected in the BLV-infected population, and 68 and 70 different genotypes were defined at the *BoLA-DRB3* locus in the 250 asymptomatic cows and the 221 lymphoma cows, respectively. p values and odds ratios (ORs) were estimated for each *BoLA-DRB3* allele by Fisher's exact test. Benjamini–Hochberg procedure was used to adjust the false positive rate and determine the significance.

[1] OR: Odds ratio

[2] BH value: The value derived from Benjamini–Hochberg procedure adjusted p-value. I, the rank of p-value.

[3] The genotype with p-values < BH value were determined as susceptibility, S, when OR > 1 and resistance, R, when OR < 1. R and S which with ( ) indicate the resistance and susceptibility genotype that only significant before correction of multiple testing (BH procedure).

[4] -: OR=∞

Table 10. Fisher's exact test based association analysis of *BoLA-DRB3* genotypes in asymptomatic and lymphoma cows.

BoLA-DRB3 Genotype	Asymptomatic (212 Cattle)	Lymphoma (221 Cattle)	OR	*p*-Value	*p*-Value Rank (I)	BH Value (I/Genotype Number)*0.05	Susceptibility
**001:01/*001:01*	3	14	5.568	0.005			(S)
**001:01/*007:01*	4	2	0.562	0.689			-
**001:01/*010:01*	8	4	0.558	0.393			-
**001:01/*011:01*	24	6	0.263	0.002	1	0.0005	(R)
**001:01/*012:01*	3	5	1.906	0.483			-
**001:01/*014:01:01*	7	1	0.158	0.393			-
**001:01/*015:01*	9	10	1.269	0.645			-
**001:01/* 016:01*	3	2	0.752	1.000			-
**001:01/*018:01*	1	2	2.274	0.603			-
**001:01/*027:03*	4	7	2.012	0.362			-
**002:01/*015:01*	1	4	4.590	0.191			-
**005:03/*016:01*	0	2	-	0.220			-
**007:01/*011:01*	6	0	0	0.057			-
**009:02/*010:01*	8	0	-	0.008			(S)
**010:01/*010:01*	7	2	0.317	0.183			-
**010:01/*011:01*	14	7	0.551	0.264			-
**010:01/*012:01*	4	2	0.562	0.689			-
**010:01/*014:01:01*	5	3	0.674	0.728			-
**010:01/*015:01*	8	3	0.416	0.231			-
**011:01/*011:01*	9	7	0.876	1.000			-
**011:01/*012:01*	4	5	1.424	0.740			-
**011:01/*014:01:01*	12	3	0.273	0.037			(R)
**011:01/*015:01*	30	14	0.496	0.039			(R)
**011:01/*027:03*	12	5	0.459	0.215			-
**012:01/*012:01*	0	8	-	0.002	1	0.0005	(S)
**012:01/*014:01:01*	3	2	0.752	1.000			-
**012:01/*015:01*	5	15	3.568	0.011			(S)
**014:01:01/*015:01*	4	1	0.280	0.377			-
**015:01/*015:01*	7	18	3.078	0.013			(S)
**015:01/*016:01*	2	3	1.706	0.669			-
**015:01/*027:03*	2	10	5.877	0.016			(S)

The Benjamini–Hochberg (BH) procedure was performed, to adjust the false positive rate. Genotypes with a *p*-value < BH value were defined as susceptibility, S, with an odds ratio (OR) > 1 and as resistance, R, with an OR < 1. BH value = (*p*-value rank / total allele number) × 0.05. R and S which with ( ) indicate the resistance and susceptibility genotype that only significant before correction of multiple testing (BH procedure).

Table 11. Association study of *BoLA-DRB3* alleles between LPVL and HPVL cows.

BoLA-DRB3 allele	Number of allele		[1]OR	*p*-value	*p-value* rank (I)	[2]BH value (I/allele number)*0.05	[3]Susceptibility
	LPVL	HPVL					
**001:01*	100	99	1.8681	0.6046			[4]-
**002:01*	29	3	0.1458	< 0.0001	1	0.0019	R
**003:01*	2	0	0	0.2313			-
**005:02*	0	1	-	0.4812			-
**005:03*	1	1	1.4016	1			-
**006:01*	4	0	0	0.1256			-
**007:01*	11	12	1.5744	0.8322			-
**007:04*	1	1	1.4016	1			-
**008:01*	0	1	-	0.4812			-
**009:01*	2	0	0	0.2313			-
**009:02*	41	2	0.0685	< 0.0001	1	0.0019	R
**010:01*	62	66	1.7989	0.4262			-
**010:02*	0	1	-	0.4812			-
**011:01*	133	139	2.2967	0.1933			-
**012:01*	28	64	3.8383	< 0.0001	1	0.0019	S
**014:01:01*	59	24	0.6068	0.0004	4	0.0077	R
**015:01*	102	125	2.5463	0.0224	5	0.0096	(S)
**016:01*	12	10	1.1962	0.8314			-
**017:01*	2	0	0	0.2313			-
**018:01*	1	7	9.968	0.0319			(S)
**020:01:02*	2	1	0.7008	1			-
**023:01*	0	1	-	0.4812			-
**027:03*	39	29	1.1249	0.369			-
**027:07*	2	0	0	0.2313			-
**033:01*	0	1	-	0.4812			-
**064:01*	1	0	0	1			-

BoLA-DRB3 allele frequency of LPVL and HPVL cows. Total of 1222 *BoLA-DRB3* alleles were detected and classified into 26 known types from 611 asympomatic cows. The frequency of 634 alleles (21 different types) from LPVL cows and 588 alleles (20 different types) from HPVL cows, was calculated, and p values and ORs were estimated by Fisher's exact test. Benjamini–Hochberg procedure was used to adjust the false positive rate and determine the significance.

[1] OR: Odds ratio

[2] BH value: The value derived from Benjamini–Hochberg procedure adjusted p-value. I, the rank of p-value.

[3] The allele with p-values < BH value were determined as susceptibility, S, when OR > 1 and resistance, R, when OR < 1. R and S which with ( ) indicate the resistance and susceptibility allele that only significant before correction of multiple testing (BH procedure).

[4] -: OR=∞

Table 12. Fisher's exact test based association analysis of *BoLA-DRB3* alleles in low PVL and high PVL cows.

BoLA-DRB3 Allele	Low PVL (317 Cattle)	High PVL (294 Cattle)	OR	*p*-Value	*p*-Value Rank (I)	BH Value (I/Genotype Number)*0.05	Susceptibility
**001:01*	100	99	1.8681	0.6046			-
**002:01*	29	3	0.1458	< 0.0001	1	0.0019	R
**007:01*	11	12	1.5744	0.8322			-
**009:02*	41	2	0.0685	< 0.0001	1	0.0019	R
**010:01*	62	66	1.7989	0.4262			-
**011:01*	133	139	2.2967	0.1933			-
**012:01*	28	64	3.8383	< 0.0001	1	0.0019	S
**014:01:01*	59	24	0.6068	0.0004	4	0.0077	R
**015:01*	102	125	2.5463	0.0224	5	0.0096	(S)
**016:01*	12	10	1.1962	0.8314			-
**018:01*	1	7	9.968	0.0319			(S)
**027:03*	39	29	1.1249	0.369			-

Benjamini–Hochberg (BH) procedure was performed to adjust the false positive rate. Alleles with *p*-value < BH value were defined as susceptibility, S, with odds ratio (OR) > 1, and as resistance, R, with OR < 1. BH value = (*p*-value rank / total allele number) × 0.05. R and S which with ( ) indicate the resistance and susceptibility allele that only significant before correction of multiple testing (BH procedure).

Table 13. Results of the stepwise conditional analysis of *BoLA-DRB3* alleles in LPVL and HPVL cows.

			Conditional association							
	Univariate[1]		**009:02*		**009:02, *014:01:01*		**009:02, *014:01:01, *012:01*		**009:02, *014:01:01, *012:01, *002:01*	
Allele	OR (95% CI)	*p-value*	OR (95% CI)	*p-value*	OR (95% CI)	*p-value*	OR (95% CI)	*p-value*	OR (95% CI)	*p-value*
**001:01*	1.08 (0.8–1.46)	0.617	1.04 (0.76–1.42)	0.825	0.92 (0.66–1.27)	0.596	1.01 (0.73–1.41)	0.934	0.99 (0.71–1.39)	0.971
**002:01*	0.13 (0.04–0.41)	6.28×10^{-4}	0.12 (0.04–0.38)	4.11×10^{-4}	0.1 (0.03–0.35)	2.20×10^{-4}	0.12 (0.04–0.4)	5.19×10^{-4}	—	—
**007:01*	1.18 (0.51–2.73)	0.692	1.37 (0.57–3.27)	0.483	1.29 (0.53–3.12)	0.577	1.26 (0.51–3.11)	0.621	1.26 (0.5–3.15)	0.628
**009:02*	0.05 (0.01–0.2)	3.29×10^{-5}	—	—	—	—	—	—	—	—
**010:01*	1.15 (0.81–1.64)	0.431	1.17 (0.81–1.68)	0.41	1.06 (0.73–1.53)	0.775	1.17 (0.8–1.7)	0.41	1.08 (0.74–1.58)	0.684
**011:01*	1.17 (0.89–1.54)	0.259	1.01 (0.76–1.34)	0.951	0.92 (0.69–1.23)	0.57	1.02 (0.76–1.37)	0.908	0.93 (0.69–1.26)	0.631
**012:01*	2.65 (1.66–4.22)	4.00×10^{-5}	2.83 (1.73–4.63)	3.31×10^{-5}	2.77 (1.68–4.56)	6.53×10^{-5}	—	—	—	—
**014:01:01*	0.39 (0.23–0.64)	2.40×10^{-4}	0.32 (0.19–0.55)	3.07×10^{-4}	—	—	—	—	—	—
**015:01*	1.39 (1.05–1.85)	0.024	1.36 (1.01–1.84)	0.044	1.33 (0.98–1.81)	0.064	1.5 (1.1–2.05)	0.011	1.37 (1–1.87)	0.053
**016:01*	0.89 (0.38–2.1)	0.799	0.56 (0.22–1.46)	0.238	0.49 (0.19–1.26)	0.137	0.5 (0.19–1.32)	0.164	0.49 (0.18–1.29)	0.147
**018:01*	7.71 (0.94–63.03)	0.057	16.33 (1.28–207.64)	0.031	14.78 (1.12–194.97)	0.041	16.47 (1.19–227.94)	0.037	15.26 (1.07–216.84)	0.044
**027:03*	0.79 (0.48–1.3)	0.347	0.61 (0.35–1.06)	0.081	0.56 (0.32–0.98)	0.042	0.6 (0.34–1.05)	0.075	0.55 (0.31–0.97)	0.037

[1] calculated by logistic regression analysis after adjustment of age.

Table 14. Logistic regression analysis based association study of *BoLA-DRB3* alleles in low PVL and high PVL cows after adjustment of age.

BoLA-DRB3 Allele	Univariate				Multivariate			
	p-Value	OR	L95	U95	*p*-Value	OR	L95	U95
**002:01*	6.28×10^{-4}	0.13	0.04	0.41	5.19×10^{-4}	0.12	0.04	0.40
**009:02*	3.29×10^{-5}	0.05	0.01	0.20	1.42×10^{-5}	0.04	0.01	0.17
**012:01*	4.00×10^{-5}	2.65	1.66	4.22	3.20×10^{-4}	2.51	1.52	4.15
**014:01:01*	2.40×10^{-4}	0.39	0.23	0.64	2.10×10^{-5}	0.31	0.18	0.53

L95, lower 95% confidence interval. U95, upper 95% confidence interval.

Table 15. Association study of *BoLA-DRB3* genotypes between LPVL and HPVL cows.

BoLA-DRB3 genotype	Number of genotype		[1]OR	*p*-value	*p-value* rank (I)	[2]BH value (I/genotype number)*0.05	[3]Susceptibility
	LPVL	HPVL					
**001:01/*001:01*	4	13	3.6201	0.0245			[4]-
**001:01/*002:01*	10	0	0	0.0019	3	0.0016	-
**001:01/*006:01*	1	0	0	1			-
**001:01/*007:01*	2	1	0.5375	1			-
**001:01/*009:01*	1	0	0	1			-
**001:01/*009:02*	5	0	0	0.0624			-
**001:01/*010:01*	7	8	1.2388	0.7958			-
**001:01/*011:01*	27	20	0.784	0.4511			-
**001:01/*012:01*	5	9	1.9705	0.2824			-
**001:01/*014:01:01*	8	3	0.3982	0.226			-
**001:01/*015:01*	15	20	1.4696	0.299			-
**001:01/*016:01*	2	1	0.5375	1			-
**001:01/*018:01*	0	2	-	0.2313			-
**001:01/*023:01*	0	1	-	0.4812			-
**001:01/*027:03*	9	6	0.713	0.6067			-
**001:01/*033:01*	0	1	-	0.4812			-
**002:01/*002:01*	3	0	0	0.2504			-
**002:01/*007:01*	0	1	-	0.4812			-
**002:01/*008:01*	0	1	-	0.4812			-
**002:01/*010:01*	2	0	0	0.5003			-
**002:01/*011:01*	4	1	0.2671	0.3747			-
**002:01/*014:01:01*	2	0	0	0.5003			-
**002:01/*015:01*	2	0	0	0.5003			-
**002:01/*016:01*	1	0	0	1			-
**002:01/*027:03*	1	0	0	1			-
**003:01/*015:01*	1	0	0	1			-
**003:01/*027:03*	1	0	0	1			-
**005:02/*010:01*	0	1	-	0.4812			-
**005:03/*010:01*	1	0	0	1			-
**005:03/*016:01*	0	1	-	0.4812			-
**006:01/*010:01*	1	0	0	1			-
**006:01/*011:01*	1	0	0	1			-
**006:01/*027:03*	1	0	0	1			-
**007:01/*009:02*	1	1	1.0785	1			-
**007:01/*010:01*	0	1	-	0.4812			-
**007:01/*011:01*	3	5	1.8108	0.4908			-

*007:01/*012:01	1	3	3.2577	0.3559			-
*007:01/*014:01:01	2	0	0	0.5003			-
*007:01/*015:01	2	0	0	0.5003			-
*009:01/*009:02	1	0	0	1			-
*009:02/*009:02	1	0	0	1			-
*009:02/*010:01	7	0	0	0.0156			-
*009:02/*011:01	2	0	0	0.5003			-
*009:02/*012:01	5	0	0	0.0624			-
*009:02/*014:01:01	1	1	1.0785	1			-
*009:02/*015:01	13	0	0	0.0002	1	0.0005	R
*009:02/*018:01	1	0	0	1			-
*009:02/*027:03	3	0	0	0.2504			-
*010:01/*010:01	7	5	0.7662	0.7741			-
*010:01/*011:01	9	24	3.042	0.0039			-
*010:01/*012:01	3	4	1.4437	0.716			-
*010:01/*014:01:01	4	1	0.2671	0.3747			-
*010:01/*015:01	8	10	1.36	0.6341			-
*010:01/*016:01	3	2	0.7169	1			-
*010:01/*018:01	0	2	-	0.2313			-
*010:01/*027:03	3	3	1.079	1			-
*010:02/*011:01	0	1	-	0.4812			-
*011:01/*011:01	0	1	-	0.4812			-
*011:01/*002:01	1	0	0	1			-
*011:01/*011:01	17	11	0.6859	0.4393			-
*011:01/*012:01	3	18	6.8261	0.0005	2	0.0011	S
*011:01/*014:01:01	14	7	0.5279	0.1883			-
*011:01/*015:01	19	32	1.9156	0.0396			-
*011:01/*016:01	2	0	0	0.5003			-
*011:01/*017:01	1	0	0	1			-
*011:01/*018:01	0	1	-	0.4812			-
*011:01/*027:03	12	6	0.5295	0.237			-
*011:01/*027:07	1	0	0	1			-
*012:01/*007:04	0	1	-	0.4812			-
*012:01/*011:01	0	1	-	0.4812			-
*012:01/*012:01	0	4	-	0.053			-
*012:01/*014:01:01	6	2	0.355	0.2886			-
*012:01/*015:01	2	12	6.7021	0.0053			-
*012:01/*016:01	1	1	1.0785	1			-
*012:01/*018:01	0	1	-	0.4812			-
*012:01/*027:03	2	4	2.1724	0.4351			-
*014:01:01/*015:01	18	6	0.3461	0.0223			-

**014:01:01/*016:01*	0	1	-	0.4812	-
**014:01:01/*020:01:02*	0	1	-	0.4812	-
**014:01:01/*027:03*	3	2	0.7169	1	-
**014:01:01/*027:07*	1	0	0	1	-
**015:01/*007:04*	1	0	0	1	-
**015:01/*015:01*	6	19	3.5812	0.0067	-
**015:01/*016:01*	3	2	0.7169	1	-
**015:01/*017:01*	1	0	0	1	-
**015:01/*018:01*	0	1	-	0.4812	-
**015:01/*020:01:02*	1	0	0	1	-
**015:01/*027:03*	3	4	1.4437	0.716	-
**015:01/*064:01*	1	0	0	1	-
**016:01/*027:03*	0	2	-	0.2313	-
**020:01:02/*027:03*	1	0	0	1	-
**027:03/*027:03*	0	1	-	0.4812	-

BoLA-DRB3 genotypes frequency of LPVL and HPVL cows. Total of 92 different genotypes were detected at the *BoLA-DRB3* locus in the BLV-infected population, 56 in the HPVL cows and 71 in the LPVL cows. p values and odds ratios (ORs) were estimated for each *BoLA-DRB3* genotype by Fisher's exact test. Benjamini–Hochberg procedure was used to adjust the false positive rate and determine the significance.

[1] OR: Odds ratio

[2] BH value: The value derived from Benjamini–Hochberg procedure adjusted p-value. I, the rank of p-value.

[3] The allele with p-values < BH value were determined as susceptibility, S, when OR > 1 and resistance, R, when OR < 1. R and S which with ( ) indicate the resistance and susceptibility allele that only significant before correction of multiple testing (BH procedure).

[4] -: OR=∞

Table 16. Fisher's exact test based association analysis of *BoLA-DRB3* genotypes in low PVL and high PVL cows.

BoLA-DRB3 Genotype	Low PVL (317 Cattle)	High PVL (294 Cattle)	OR	*p*-Value	*p*-Value Rank (I)	BH Value (I/Genotype Number)*0.05	Susceptibility
**001:01/*001:01*	4	13	3.6201	0.0245			(S)
**001:01/*002:01*	10	0	0.0000	0.0019	3	0.0016	(R)
**001:01/*009:02*	5	0	0.0000	0.0624			-
**001:01/*010:01*	7	8	1.2388	0.7958			-
**001:01/*011:01*	27	20	0.7840	0.4511			-
**001:01/*012:01*	5	9	1.9705	0.2824			-
**001:01/*014:01:01*	8	3	0.3982	0.2260			-
**001:01/*015:01*	15	20	1.4696	0.2990			-
**001:01/*027:03*	9	6	0.7130	0.6067			-
**002:01/*011:01*	4	1	0.2671	0.3747			-
**007:01/*011:01*	3	5	1.8108	0.4908			-
**007:01/*012:01*	1	3	3.2577	0.3559			-
**009:02/*010:01*	7	0	0.0000	0.0156			(R)
**009:02/*012:01*	5	0	0.0000	0.0624			-
**009:02/*015:01*	13	0	0.0000	0.0002	1	0.0005	R
**010:01/*010:01*	7	5	0.7662	0.7741			-
**010:01/*011:01*	9	24	3.0420	0.0039			(S)
**010:01/*012:01*	3	4	1.4437	0.7160			-
**010:01/*014:01:01*	4	1	0.2671	0.3747			-
**010:01/*015:01*	8	10	1.3600	0.6341			-
**010:01/*016:01*	3	2	0.7169	1.0000			-
**010:01/*027:03*	3	3	1.0790	1.0000			-
**011:01/*011:01*	17	11	0.6859	0.4393			-
**011:01/*012:01*	3	18	6.8261	0.0005	2	0.0011	S
**011:01/*014:01:01*	14	7	0.5279	0.1883			-
**011:01/*015:01*	19	32	1.9156	0.0396			(S)
**011:01/*027:03*	12	6	0.5295	0.2370			-
**012:01/*012:01*	0	4	-	0.0530			-
**012:01/*014:01:01*	6	2	0.3550	0.2886			-
**012:01/*015:01*	2	12	6.7021	0.0053			(S)
**012:01/*027:03*	2	4	2.1724	0.4351			-

BoLA-DRB3 Genotype	Low PVL (317 Cattle)	High PVL (294 Cattle)	OR	*p*-Value	*p*-Value Rank (I)	BH Value (I/Genotype Number)*0.05	Susceptibility
**014:01:01/*015:01*	18	6	0.3461	0.0223			(R)
**014:01:01/*027:03*	3	2	0.7169	1.0000			-
**015:01/*015:01*	6	19	3.5812	0.0067			(S)
**015:01/*016:01*	3	2	0.7169	1.0000			-
**015:01/*027:03*	3	4	1.4437	0.7160			-

The Benjamini–Hochberg (BH) procedure was performed to adjust the false positive rate. Alleles with a *p*-value < BH value were defined as susceptibility, S, with an odds ratio (OR) > 1 and as resistance, R, with an OR < 1. BH value = (*p*-value rank / total allele number) × 0.05. R and S which with ( ) indicate the resistance and susceptibility genotype that only significant before correction of multiple testing (BH procedure).

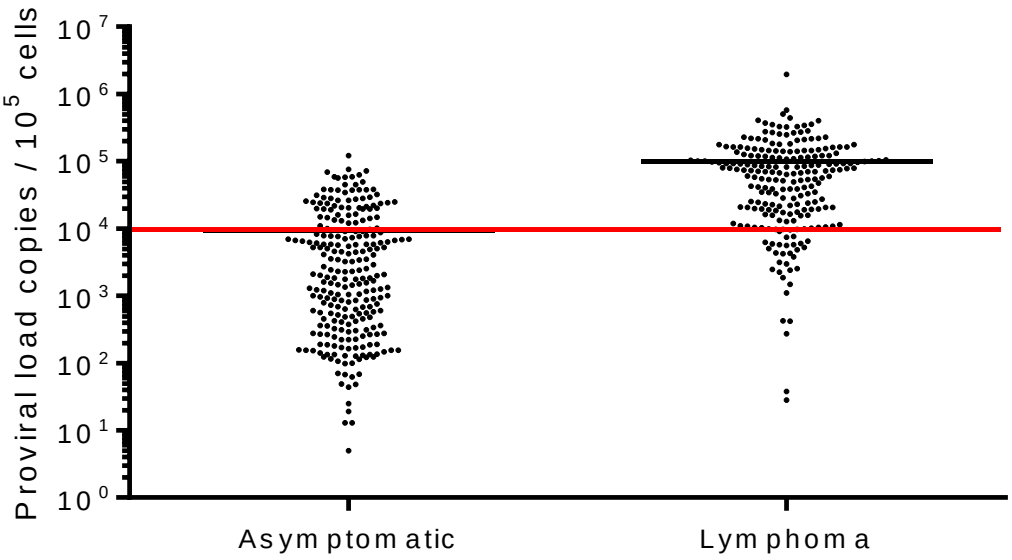


Figure 7. Proviral load (PVL) estimation in Bovine leukemia virus (BLV)-infected but clinically and hematologically normal cows (asymptomatic cows) and BLV-infected cows with lymphoma (lymphoma cows). Blood samples were obtained from 250 asymptomatic (Table 1) and 221 lymphoma (Table 3) cows in a nationwide survey in Japan. BLV infection was analyzed using BLV-CoCoMo-qPCR-2. The red line represents a BLV PVL of 10,000 copies/10^5 cell, which was set as the threshold between high- and low-PVL cows.

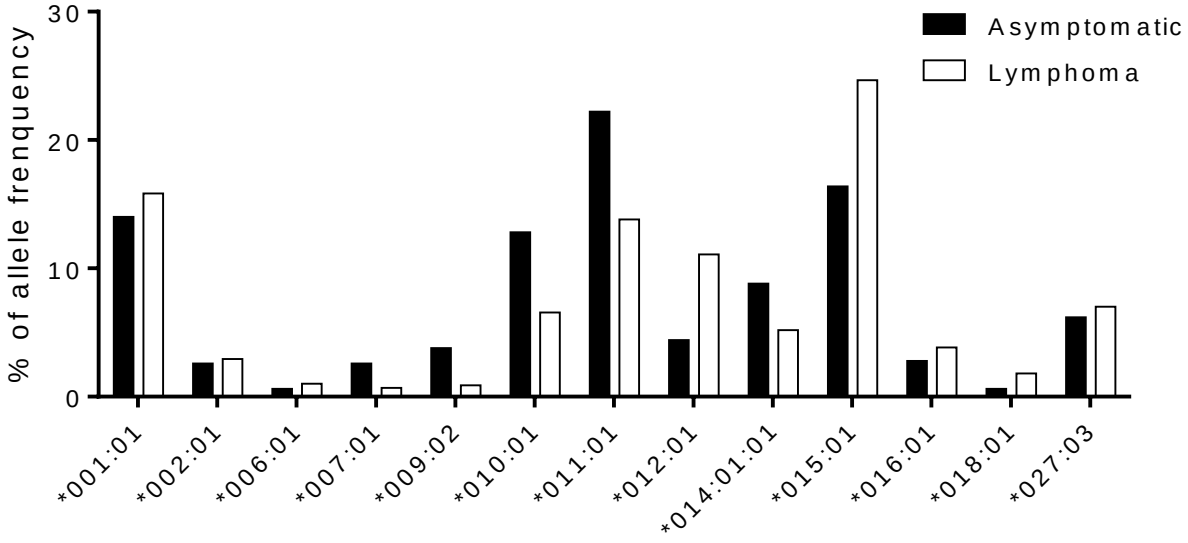


Figure 8. Comparison of *BoLA-DRB3* allele frequencies between asymptomatic and lymphoma cows. Allele frequency in 250 asymptomatic (■) and 221 lymphoma (□) cows were calculated for each *BoLA-DRB3* allele (Table 5); 13 out of 32 alleles with frequency > 1% are shown. The X-axis shows the allele name and the Y-axis shows allele frequency (%) for each *BoLA-DRB3* allele.

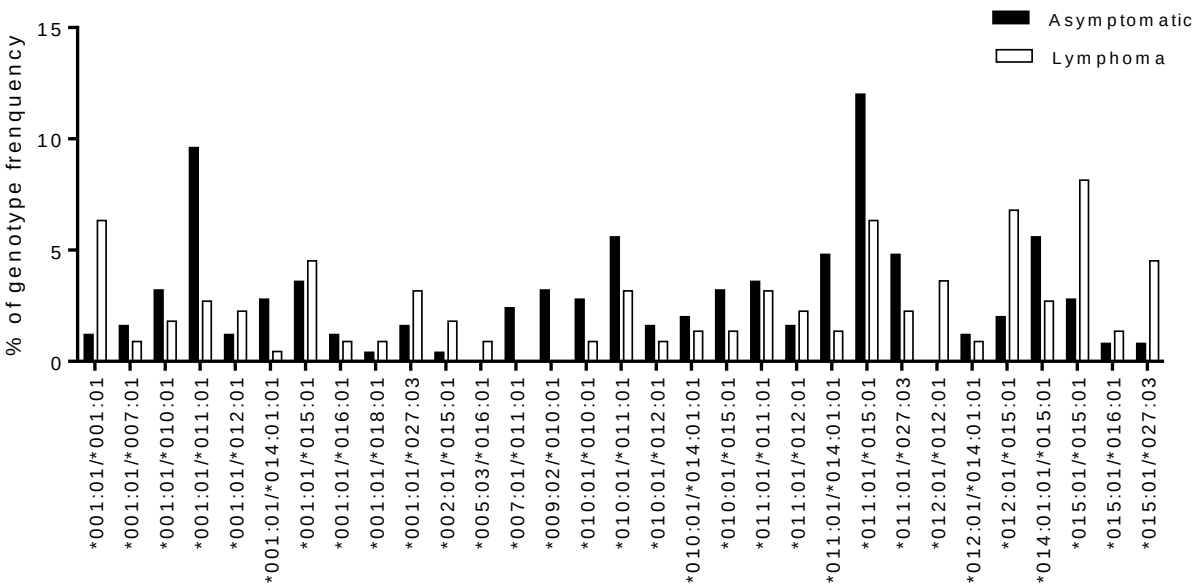


Figure 9. Comparison of *BoLA-DRB3* genotype frequency between asymptomatic and lymphoma cows. Genotype frequency of 250 asymptomatic (■) and 221 lymphoma (□) cows was calculated for each *BoLA-DRB3* genotype (Table 7); a total of 31 out of 94 genotypes with frequency > 1% are shown. The X-axis and Y-axis show the genotype name and frequency (%) for each *BoLA-DRB3* genotype, respectively.

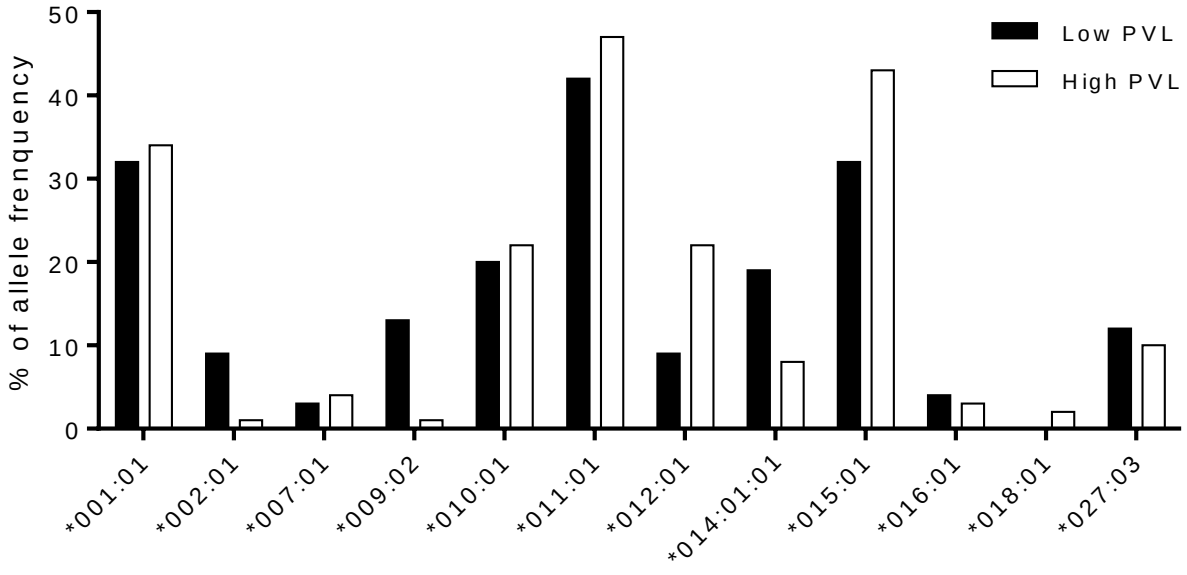


Figure 10. Comparison of *BoLA-DRB3* allele frequencies between LPVL and HPVL cows. *BoLA-DRB3* allele frequencies in LPVL and HPVL cows. The 611 asymptomatic cows comprised 317 LPVL and 294 HPVL individuals. The allele frequencies were calculated in LPVL (■) and HPVL cows (□) for each *BoLA-DRB3* allele (Table 2). Total of 12 out of 26 alleles with frequency > 1% are shown.

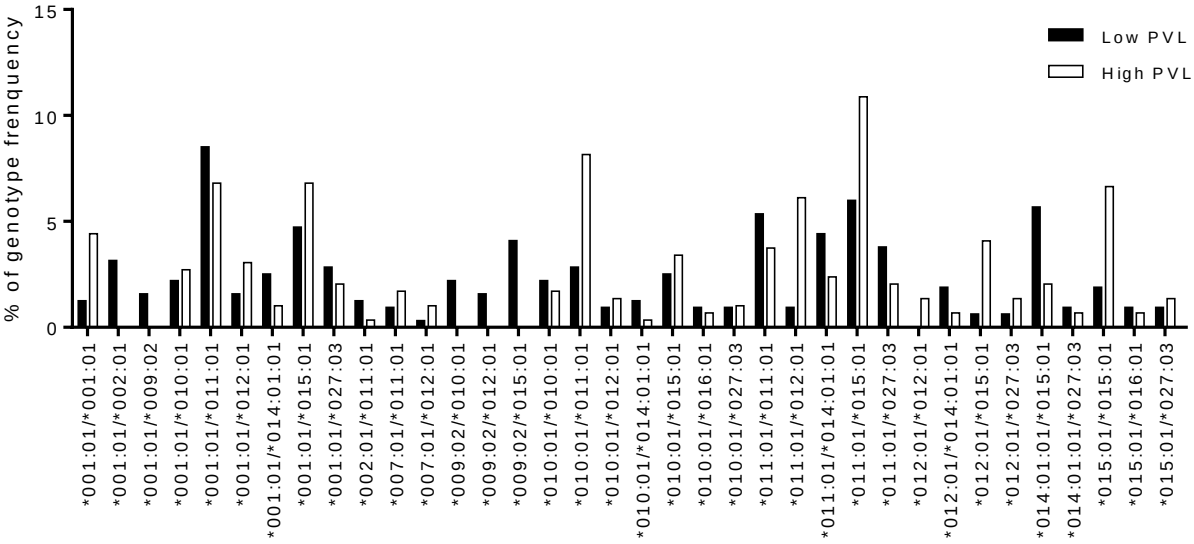


Figure 11. Comparison of *BoLA-DRB3* genotype frequencies between HPVL and LPVL cows. The genotype frequencies of 317 LPVL (■) and 294 HPVL cows (□) were calculated for each *BoLA-DRB3* genotype (Table 15); a total of 36 out of 92 genotypes with frequencies > 1% are shown. The X-axis and Y-axis show the genotype name and frequency (%) for each *BoLA-DRB3* genotype, respectively.

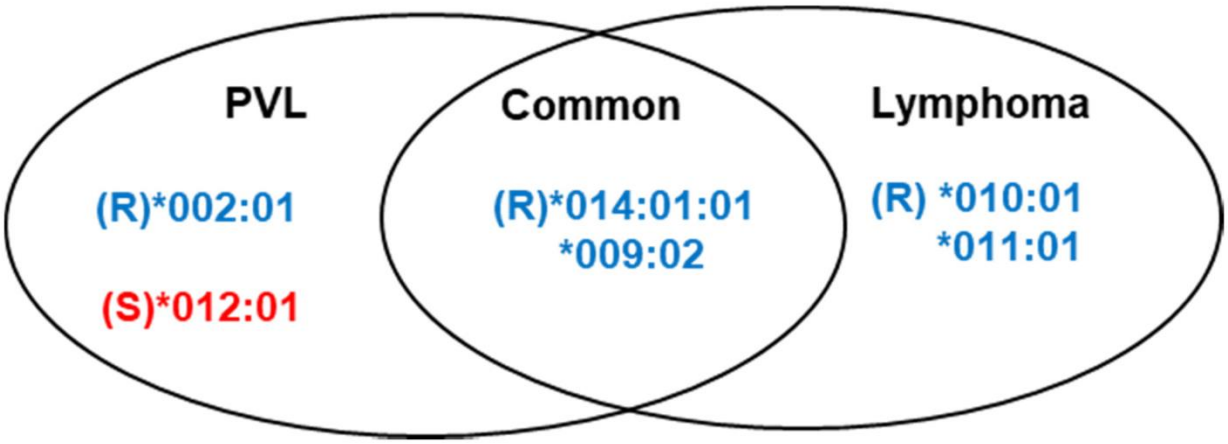


Figure 12. Summary of the differences in *BoLA-DRB3* allele-associated proviral load (PVL) and lymphoma susceptibility, based on the logistic regression association study results. R, resistance; S, susceptibility.

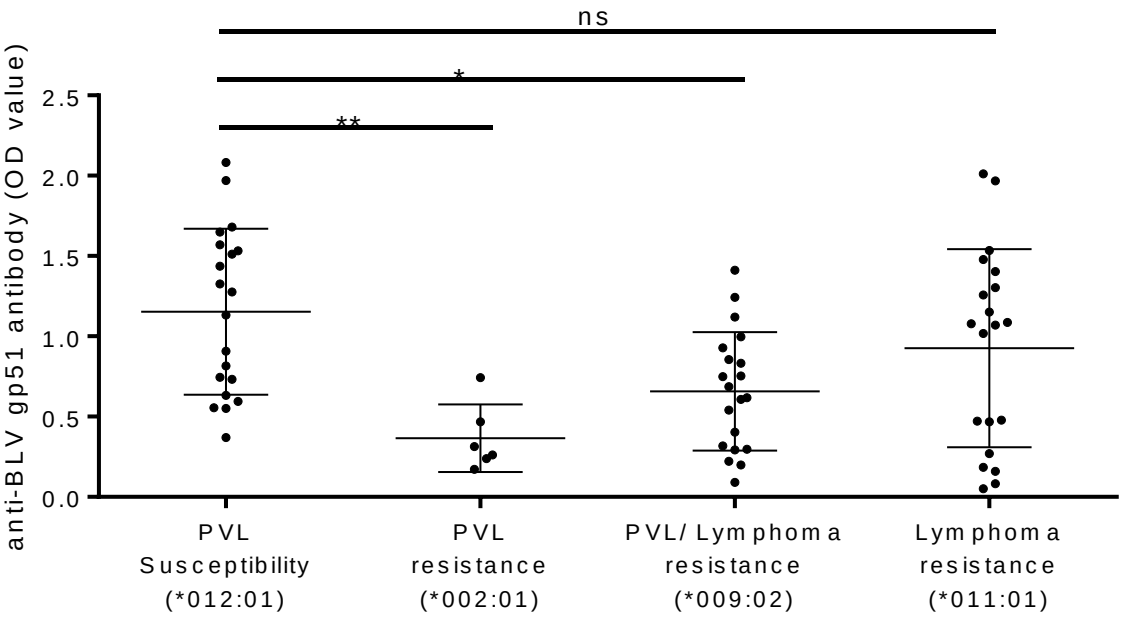


Figure 13. Differential anti-BLV antibody production level in cows with proviral load (PVL) susceptibility, PVL resistance, PVL/lymphoma resistance, and lymphoma-resistance *BoLA-DRB3* allele. The PVL susceptibility group consisted of cows with the PVL susceptibility allele *DRB3*012:01* (n = 20). The PVL resistance group consisted of cows with the PVL resistance allele *DRB3*002:01* (n = 6). The PVL/lymphoma resistance group consisted of cows with the PVL resistance allele *DRB3*009:02* (n = 20). The lymphoma resistance group consisted of the lymphoma resistance allele **011:01* (n = 20). The anti-BLV gp51 antibody was measured in plasma at a 2^{11} dilution level. Optical Density (OD) value data represent the mean ± SD. Statistical comparisons were performed by one-way ANOVA. *, $p < 0.05$. **, $p < 0.01$. ns, not significant

Chapter 3.

Association of Bovine Leukemia Virus-Induced Lymphoma with BoLA-DRB3 Polymorphisms at DNA, Amino Acid, and Binding Pocket Property Level

3.1 Abstract

Bovine leukemia virus (BLV) causes enzootic bovine leucosis, a malignant B-cell lymphoma in cattle. The DNA sequence polymorphisms of bovine leukocyte antigen (BoLA)-*DRB3* have exhibited a correlation with BLV-induced lymphoma in Holstein cows. However, the association may vary between different cattle breeds. Furthermore, little is known about the relationship between BLV-induced lymphoma and DRB3 at the amino acid and structural diversity levels. Here, we comprehensively analyzed the correlation between BLV-induced lymphoma and DRB3 at DNA, amino acid, and binding pocket property levels, using 106 BLV-infected asymptomatic and 227 BLV-induced lymphoma Japanese Black cattle samples. *DRB3*011:01* was identified as a resistance allele, whereas *DRB3*005:02* and *DRB3*016:01* were susceptibility alleles. Amino acid association studies showed that positions 9, 11, 13, 26, 30, 47, 57, 70, 71, 74, 78, and 86 were associated with lymphoma susceptibility. Structure and electrostatic charge modeling further indicated that binding pocket 9 of resistance DRB3 was positively charged. In contrast, alleles susceptible to lymphoma were neutrally charged. Altogether, this is the first association study of *BoLA-DRB3* polymorphisms with BLV-induced lymphoma in Japanese Black cattle. In addition, our results further contribute to understanding the mechanisms regarding how *BoLA-DRB3* polymorphisms mediate susceptibility to BLV-induced lymphoma.

3.2 Introduction

EBL is a lymphoproliferative disease characterized by B-cell lymphoma and is the most common neoplasm disease in cattle (Underwood *et al.* 2015). BLV infection is the causative agent of EBL (Aida *et al.* 2013). As EBL is a lethal disease leading to severe financial burden in the cattle industry, it is recognized by the World Organization for Animal Health as a disease of importance for international trade (OIE 2012). Recent surveys in most countries worldwide except Europe have reported a continuous increase in BLV infection in cattle (Bartlett *et al.*). Consequently, it is difficult to develop new strategies to decrease the disease prevalence rate, as the mechanisms of BLV-induced lymphoma are unknown.

BLV belongs to family *Retroviridae* and genus *Deltaretrovirus* and is closely related to the HTLV (Gillet *et al.* 2007). Like other retroviruses, the DNA copies of BLV RNA genome integrate into the host genome as a provirus and induce lifelong infection. In the majority of cases, approximately 70% of BLV-infected cattle are asymptomatic carriers. Approximately 30% of infected individuals progress to persistent lymphocytosis, characterized by polyclonal expression of the non-neoplastic $CD5^+$ B lymphocyte population. Only 1%–5% of infections develop B-cell lymphoma after a prolonged latency period (Barez *et al.* 2015). The low mortality rate of BLV infection implies that the virus itself may not be sufficient to induce disease onset and that host genetic polymorphisms in individuals potentially play a key role in disease susceptibility.

MHC is a highly polymorphic gene set responsible for peptide antigen presentation and immune responsiveness and is, therefore, associated with disease susceptibility (Takeshima *et al.* 2006). BoLA is the MHC system in cattle (Aida *et al.* 2015). Specifically, *BoLA-DRB3* is the highly polymorphic *BoLA class II* locus, with 365 alleles registered in the IPD-MHC database (https://www.ebi.ac.uk/ipd/mhc/group/BoLA/) (April 4, 2021) and is associated with many infectious diseases in cattle (Yoshida *et al.* 2009, Lei *et al.* 2012, Morales *et al.* 2020). The associations of *BoLA-DRB3* polymorphisms with BLV pro-PVL and related symptoms are well documented (Udina *et al.* 2003, Miyasaka *et al.* 2013, Takeshima *et al.* 2019). In fact, *BoLA-DRB3* polymorphisms have been shown to affect BLV PVL regulation in a cattle experimental infection model (Jimba *et al.* 2012, Forletti *et al.* 2020), thereby strengthening the importance of *BoLA-DRB3* in BLV transmission and disease progression. Indeed, *BoLA-DRB3* is a promising target for breeding selection to decrease BLV transmission and related pathogenesis (Juliarena *et al.* 2016, Lützelschwab *et al.* 2016). However, we recently found that BLV PVL and lymphoma are associated with

differential *BoLA-DRB3* polymorphisms in Holstein cows (Chapter 2 of this thesis) (Lo *et al.* 2020), and thus, further research regarding the effect of *BoLA-DRB3* polymorphisms on lymphoma development is warranted. In addition, the associations between *BoLA-DRB3* polymorphisms and BLV-related diseases vary in different breeds and locations of cows. However, information regarding the association between *BoLA-DRB3* polymorphisms and BLV-induced lymphoma is currently only available in Holstein cows, and therefore the relationship requires elucidation in different breeds of cattle, for example, Japanese Black cattle.

A functional BoLA class II DR molecule consists of an α chain and a β chain, that are encoded from a single polymorphic gene, *BoLA-DRA*, and a highly polymorphic gene, *BoLA-DRB3*, respectively. Therefore, the antigen peptide-binding preference of BoLA class II DR molecule is mainly determined by the BoLA-DRβ polymorphisms. The polymorphisms of *BoLA-DRB3* occur in the exon 2 region, encoding the BoLA-DRβ β1 domain, which is the peptide-binding cleft, containing five peptide-binding pockets, 1, 4, 6, 7, and 9, which constitute the structure and govern the binding strength of peptides with BoLA-DRβ (Shen *et al.* 2013). Consequently, the amino acid composition and the chemical/physical property variations of these five binding pockets may largely affect disease susceptibility. It is known that the binding affinity of peptide antigen with MHC class II is one of the factors that determine subsequent T helper cell-mediated immune responses, i.e., T helper type 1 (Th1) and T helper type 2 (Th2) responses (Murray 1998). Th1 is known for cell-mediated immunity, secreting IFN-γ, and efficacy against viruses (Maloy *et al.* 2000, Boasso 2013). Furthermore, a recent study indicated that a toll-like receptor 7 agonist could activate bovine Th1 and thus promoted anti-BLV infection *in vivo* (Sajiki *et al.* 2021). Th2 is associated with humoral immunity, which is known to be less effective against BLV infection (Ohishi *et al.* 1992). Indeed, experimental infection in an ovine model demonstrated that amino acids 70 and 71 in the binding pocket 4 of ovine leukocyte antigen (OLA)-DRβ were critical for BLV-induced lymphoma susceptibility or resistance by affecting the efficiency of Th1 activation (Nagaoka *et al.* 1999, Konnai *et al.* 2003). This result highlights the importance of amino acid and structure analysis of BoLA-DRβ in understanding how BoLA-DRβ polymorphisms affect lymphoma susceptibility. In addition, it is known that differences in the binding pockets property of MHC molecules affect the peptide binding preference. For example, HLA-DQ2 molecules with positively charged binding pockets compared with that with negatively charged binding pockets has better ability at binding with proline-glutamate rich peptide (Jones *et al.* 2006). Therefore, the study of BoLA-DRβ polymorphisms at amino acid and structural levels could contribute to understanding the binding pocket properties of each BoLA-DRβ molecule and potentially

provide information for future peptide vaccine development, e.g., the charge of peptide should be taken into account. Although few reports have found an association between the *BoLA-DRB3* allele and BLV-induced lymphoma (Nikbakht Brujeni *et al.* 2016, Lo *et al.* 2020), little is known about the relationship between BLV-induced lymphoma and BoLA-DRβ at the amino acid and structure levels. Here, we present the first association study involving BLV-induced lymphoma and *BoLA-DRB3* in Japanese Black cattle. In addition, the relationship was comprehensively investigated at the *BoLA-DRB3* DNA sequence, DRβ amino acid, and DRβ binding pocket structural property levels.

3.3 Materials and Methods

3.3.1 *Sample collection and diagnosis*

Sample collection was done by Professor Aida and previous laboratory members. Blood samples from 106 BLV-infected but clinically normal purebred Japanese Black cattle (asymptomatic cattle) and 227 BLV-infected purebred Japanese Black cattle with lymphoma (lymphoma cattle) which were selected from a nationwide survey (12 out of 48 prefectures) across Japan were used in this study, and the genomic DNA and plasma from the peripheral blood were isolated. Asymptomatic cattle samples were collected from farms and the infection was confirmed by anti-BLV gp51 ELIAS. Lymphoma cattle samples were collected from slaughterhouses and the infection were confirmed by anti-BLV gp51 ELISA and some of the sample tested together with southern blotting and PCR of BLV proviral genomes. As lymphoma onset in cattle occurs, on average, seven years after BLV infection (Tsutsui *et al.* 2016), all asymptomatic cattle were > 9 years old to reduce the likelihood of collecting potential lymphoma cattle. The age difference between lymphoma and asymptomatic cattle was therefore compensated for further association study. Asymptomatic cattle sample were collected from farms and the subclinical stage of BLV infection was diagnosed according to the lymphocyte count (cells/μL) and the age of each cow (≤5500 = normal, between 5500 to 7500 = suspected lymphocytosis and ≥7500 = lymphocytosis). Asymptomatic cattle were defined as BLV-infected but clinically and hematologically normal; PL cattle were defined as BLV-infected but clinically normal cattle with an increase in the number of apparently normal B lymphocytes. In this study, only samples from asymptomatic cattle were used for further analysis. This study was approved by the Animal Ethical Committee, and the Animal Care and Use RIKEN Animal Experiments Committee (approval number H29-2-104). Cattle lymphoma status was diagnosed

using both gross observation of neoplastic tissues in lymph nodes and histological observation in heart, lung, liver, kidney, spleen, intestines and lymph node in the body. In addition, atypical mononuclear cells in blood sample together with genomic southern blotting for testing disease progression were used for confirmation of some cattle samples (Tajima *et al.* 1998).

3.3.2 *BLV infection determination using ELISA*

The anti-BLV gp51 antibody was measured using an anti-BLV antibody ELISA Kit (JNC, Tokyo, Japan) that was done by previous laboratory members, according to the manufacturer's instructions.

3.3.3 *BoLA-DRB3 genotyping*

BoLA-DRB3 alleles were determined using the PCR-SBT method that was done by previous laboratory members, as previously described (Takeshima *et al.* 2011). Briefly, *BoLA-DRB3* exon 2 was amplified via single-step PCR using the *DRB3* forward (5 ´ -CGCTCCTGTGAYCAGATCTATCC-3 ´) and reverse (5 ´ -CACCCCCGCGCTCACC-3 ´) primer set. The PCR products were purified using the ExoSAP-IT PCR product purification kit (USB Corp., Cleveland, OH, USA) and then sequenced using the ABI PRISM BigDye1.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The sequence data were then analyzed using Assign 400ATF ver. 1.0.2.41 software (Gonexio Genomics, Fremantle, Australia) to determine the *BoLA-DRB3* genotype.

3.3.4 *Characterization of amino acid properties*

Amino acid charge property of the BoLA-DRβ binding pocket was characterized according to international ImMunoGeneTics information system (Pommié *et al.* 2004).

3.3.5 *3-Dimensional (3D) protein structure modeling of BoLA-DRB3 molecules*

All *BoLA-DRB3* sequences were downloaded from the IPD-MHC database (https://www.ebi.ac.uk/ipd/mhc/group/BoLA/) (April 4, 2021). The amino acid sequence alignment was performed using Mega X software (Kumar *et al.* 2018). Graphical 3D structures and electrostatic surface potential of BoLA-

DRB3 molecules were determined with PyMOL 2.4 (Schrodinger LLC, New York, NY, USA). The structural models of all *BoLA-DRB3* molecules were constructed based on the crystal structure of HLA-DRB1: Protein Data Bank (PDB) ID: 1dlh (Stern *et al.* 1994).

3.3.6 *Association study and statistical analysis*

An association study based on Fisher's exact test was performed by comparing the allele, genotype, or amino acid frequencies between asymptomatic and lymphoma cows. The results were penalized with the Bonferroni correction procedure to correct for false positive rate. The alleles or genotypes with odds ratios (OR) < 1 were categorized as resistance alleles or genotypes. In contrast, those with OR > 1 were defined as susceptibility alleles or genotypes. All calculations were performed using Prism 6 (GraphPad, San Diego, CA, USA).

3.4 Results

3.4.1 *BoLA-DRB3 genotyping in asymptomatic and lymphoma cattle*

Lymphoma cattle blood samples were collected from 227 BLV-infected, disease onset Japanese Black cattle. Asymptomatic cattle blood sample were collected from 106 BLV-infected Japanese Black cattle. The genotyping was performed in all cattle samples using a PCR-SBT method of *BoLA-DRB3* at exon 2. In total, 21 known alleles were identified in asymptomatic cattle and 24 known alleles were found in lymphoma cattle (Table 17).

3.4.2 *Association study of BoLA-DRB3 with BLV-induced lymphoma*

An association analysis based on Fisher's exact test found that *DRB3*005:02* (OR = 11.260) and *DRB3*016:01* (OR = 2.953) were lymphoma susceptibility alleles, whereas *DRB3*011:01* (OR = 0.186) was a lymphoma resistance allele (Table 17). In addition, *DRB3*005:03* (OR = 5.106) showed a tendency to lymphoma susceptibility, whereas *DRB3*002:01* (OR = 0.419), *DRB3*009:02* (OR = 0.130), and *DRB3*015:01* (OR = 0.511) showed a tendency to lymphoma resistance, although they did not meet the significance threshold after the stringent adjustment for multiple testing (Bonferroni correction).

It was found that the effect of the resistance allele was dominant over the susceptibility allele in PVL association studies (Miyasaka *et al.* 2013). To address whether this observation was true for lymphoma association, we then

performed an association study involving the *BoLA-DRB3* genotype with BLV-induced lymphoma (Table 18). Interestingly, only the *DRB3*016:01/*016:01* homozygote (OR = 7.020) was found as a susceptibility genotype, but no resistant genotypes were found after Bonferroni correction. This may be due to the high divergent level of *BoLA-DRB3* genotypes, but lacked major resistance genotypes. The susceptibility tendency was found in *DRB3*005:02/*016:01* (OR = 13.405) and *DRB3*005:03/*016:01* (OR = 10.283), whereas resistance tendency was found in *DRB3*002:01/*010:01* (OR = 0.180), *DRB3*002:01/*011:01* (OR = 0.041), *DRB3*011:01/*015:01* (OR = 0.050), and *DRB3*011:01/*016:01* (OR = 0.254).

3.4.3 *Association study of BoLA-DRβ with BLV-induced lymphoma at the amino acid level*

The potential mechanisms of allele differential susceptibility in lymphoma are due to variations in antigen recognition sites for their encoding BoLA-DRβ molecules. The sequence of *BoLA-DRB3* at exon 2 allowed us to determine the amino acid variations of 17 antigen recognition sites (positions 9, 11, 13, 26, 28, 30, 37, 47, 57, 61, 67, 70, 71, 74, 78, 85, and 86), which are located within the five peptide-binding pocket regions of BoLA-DRβ *(Bondinas et al. 2007, Takeshima et al. 2009)*. Through amino acid alignment from all BoLA-DRB3 types identified in this study, we found four (9, 47, 78, and 85) out of 17 positions with biallelic polymorphisms, and the remaining 13 positions accommodate more than two amino acid variants (Figure 14A). An association study between antigen recognition sites in peptide-binding pockets with BLV-induced lymphoma indicated that Q^{9}, G^{13}, L^{26}, H^{30}, F^{47}, S^{57}, R^{70}, R^{71}, E^{74}, V^{78}, and V^{86} were resistance amino acids (OR ranging from 0.201–0.554); in contrast, E^{9}, T^{11}, K^{13}, Y^{30}, Y^{47}, D^{57},E^{70}, K^{71}, A^{74}, Y^{78}, and G^{86} were associated with lymphoma susceptibility (OR ranging from 1.832–3.665) (Figure 14B, only statistically significant amino acids are shown).

The MHC bound peptide is determined by the properties of MHC-binding pockets, which are constituted by multiple antigen recognition sites. Therefore, it is important to note the association at the amino acid motif level. We further analyzed the effect of amino acid motifs, which included antigen recognition sites of the BoLA-DRβ chain. We observed that amino acid residues (Figure 14B), encoded by *BoLA-DRB3* in the potential resistance allele group (14 alleles, OR < 1, indicated in blue) or susceptibility allele group (11 alleles, OR > 1, indicated in red), were aligned (Figure 15A). Interestingly, the motifs associated with BLV-induced lymphoma were found in all BoLA-DRβ pockets-1, 4, 6, 7, and 9 (Figure 15B, only significant motifs are shown). In pocket 4, amino acid motifs 13, 26, 71, 74, and 78 were found to associate with lymphoma susceptibility. The motif $K^{13}F^{26}$ and $K^{71}A^{74}Y^{78}$ were

susceptible to lymphoma (OR = 2.84 and 2.95, respectively), whereas $G^{13}L^{26}$ and $R^{71}E^{74}V^{78}$ represented resistance motifs (OR = 0.273 and 0.171, respectively). In addition, the combination of amino acid 86, found in pocket 1, with amino acid 70 or motif 71, 78 of pocket 4 was associated with lymphoma susceptibility (E^{70} G^{86}, OR = 2.36; $Y^{71}G^{78}$ G^{86}, OR = 3.52). In contrast, $R^{70}V^{86}$ and $R^{70}V^{71}V^{78}$ represented the resistance amino acid combinations (OR = 0.329 and 0.202, respectively). In pocket 6, amino acid motifs 11 and 30 showed susceptibility to lymphoma. $T^{11}Y^{30}$ was found to be a susceptibility motif (OR = 2.842) and $H^{11}H^{30}$ represented a resistance motif (OR = 0.273). For pocket 7, the relationship was found in motifs 47 and 71. $Y^{47}K^{71}$ was identified as a susceptibility motif (OR = 3.696), whereas $F^{47}R^{71}$ was a resistance motif (OR = 0.226). In pocket 9, a motif at positions 9 and 57 was identified. $E^{9}D^{57}$ was found to be susceptible to lymphoma (OR = 2.601), whereas $Q^{9}S^{57}$ was resistant to lymphoma (OR = 0.186).

3.4.4 *3D structure and electrostatic charge analysis of BoLA-DRβ binding pocket*

The electrostatic charge of the MHC binding pocket is reportedly associated with disease susceptibility (Zhang *et al.* 2012, Huang *et al.* 2015). The underlying mechanism is influenced by the interaction of the MHC binding pocket with specific bound peptides and the subsequent immunoreaction (Garstka *et al.* 2015). We investigated the charged potential of resistance and susceptibility BoLA-DRβ to determine whether electrostatic charged BoLA-DRβ binding pockets related to BLV-induced lymphoma (Figure 16A). Interestingly, a major charge difference was identified at binding pocket 9 between resistance and susceptibility BoLA-DRβ. A positive electrostatic charge was found in resistance type BoLA-DRβ, BoLA-DRB3*010:11 molecule. However, susceptibility BoLA-DRβ molecules, BoLA-DRB3*005:02 and BoLA-DRB3*016:01, were neutrally charged in binding pocket 9. These results are in line with the amino acid properties within binding pocket 9. Negatively charged amino acids, E^{9} and D^{57}, were found in susceptibility BoLA-DRβ, potentially altering the positively charged environment, resulting in a neutral charged environment (Figure 16B).

3.5 Discussion

Herein, we report on the first association study between *BoLA-DRB3* (BoLA-DR*β*) polymorphisms with BLV-induced lymphoma in Japanese Black cattle at the DNA, amino acid, and electrostatically charged binding pocket levels. Here, *DRB3*011:01* was identified as a resistance allele, whereas *DRB3*005:02* and *DRB3*016:01* were identified as susceptibility alleles. Interestingly, we demonstrated that antigen recognition sites of BoLA-DRβ at

positions 9, 11, 13, 26, 30, 47, 57, 70, 71, 74, 78, and 86, as well as the amino acid motifs that covered all peptide-binding pockets, were related to lymphoma susceptibility. Electrostatic charge potential analysis found that pocket 9 of the resistance *DRB3*011:01* allele encoding BoLA-DRβ was positively charged; in contrast, a neutral charge was observed in both *DRB3*005:02* and *DRB3*016:01*. Our results not only identified *BoLA-DRB3* polymorphism susceptibility in Japanese Black cattle but contribute to understanding the mechanisms underlying how *BoLA-DRB3* polymorphisms affect lymphoma susceptibility, thereby providing useful information for future vaccine development.

The association of *BoLA-DRB3* polymorphisms to BLV PVL varies in different cattle breeds or regions. Our findings show that, in Japanese Black cattle, *DRB3*011:01* represents a resistance allele with frequency 6.3%, whereas *DRB3*005:02* and *DRB3*016:01* represent susceptibility alleles with frequency 3.6% and 36.3%, respectively, among all allele populations in relation to BLV lymphoma. Previously, we found that *DRB3*010:01* and *DRB3*011:01* were lymphoma resistance alleles, but no susceptibility allele was found in Holstein cows in Japan (concluded in Chapter 2 of this thesis) (Lo *et al.* 2020). In Iranian Holstein cows, *BoLA-DRB3*018:02*, *DRB3*032:02*, and *DRB3*009:01* alleles are associated with susceptibility to BLV-induced lymphoma, whereas *DRB3*001:01* and *DRB3*011:01* are involved in lymphoma resistance (Nikbakht Brujeni *et al.* 2016). *DRB3*011:01* was identified as a resistance allele in all three independent studies; however, other resistance/susceptibility alleles are varied in different cattle breeds or regions. The discrepancies may be due to the allele distribution diversities in different breeds/regions of cattle. For example, the Holstein resistance allele, *DRB3*001:01*, also showed the resistance tendency in Japanese Black cattle but did not reach a significant level, as only few cattle carried this allele in the Japanese cattle population. Furthermore, we did not find any Japanese Black cattle harboring the Holstein susceptibility allele *BoLA-DRB3*018:02*, *DRB3*032:02*, and *DRB3*009:01* in the current study. Therefore, association studies should address *BoLA-DRB3* polymorphisms and BLV-induced lymphoma in different regions or breeds of cattle.

The BoLA-DRβ molecule presents a peptide antigen for T cell recognition and initiates the immune response. There are five binding pockets of BoLA-DRβ, namely, pockets 1, 4, 6, 7, and 9, responsible for the interaction with the amino acids of antigen peptides at the corresponding positions (Liu *et al.* 2017). These binding pockets shape the species of bound peptide as well as the susceptibility to specific diseases (Ettinger *et al.* 2006, Garstka *et al.* 2015, Liu *et al.* 2017). We previously found that amino acids 70 and 71 at pocket 4 of OLA DRB1 related with BLV-

induced lymphoma (Nagaoka *et al.* 1999). Animals with the resistance motif at pocket 4 strongly expressed IFN-γ, which is known as a marker of Th1 response, suggesting the role of binding pocket polymorphisms in the determination of immune responsiveness and BLV-related disease susceptibility (Konnai *et al.* 2003). In the current study, in addition to amino acids 70 and 71 at pocket 4, we found that amino acids 9, 11, 13, 26, 30, 47, 57, 70, 71, 74, 78, and 86, which covered all binding pockets 1, 4, 6, 7, and 9, were associated with BLV-induced lymphoma susceptibility. Furthermore, through motif association study, we confirmed that two amino acid motifs: first, 13 and 26, and second, 71, 74, and 78, at pocket 4 were related with lymphoma susceptibility. The correlation was also identified in the amino acid combination of 70 and 86 and 71, 78, and 86 at binding pockets 1 and 4, respectively. At pocket 6, motif 11 and 30; pocket 7, motif 47 and 71; and pocket 9, motif 9 and 57 were found to be associated with BLV-induced lymphoma. As the susceptibility motifs were found in different binding pockets, it is likely that there is more than a single conserved type of BoLA-DRβ that could affect BLV-induced lymphoma susceptibility. Besides, the motif patterns which susceptible/resistant to lymphoma development we identified, could contribute to the prediction of susceptibility of some rare alleles, which could not be evaluated in allele association studies owing to their low frequencies. However, whether cattle with these specific motifs could bind with different peptides and induce differential immune responses still requires further investigation.

The electrostatic protein charge affects protein-protein interactions (Zhou *et al.* 2018). In the case of MHC, the charge of the MHC binding pocket affects peptide binding preference and therefore influences disease susceptibility. For example, positively charged binding pockets of the HLA-DQ2 molecule promote its ability to accommodate peptides with negatively charged anchor residues compared with other HLA-DQ molecules without positively charged binding pockets (Jones *et al.* 2006). This is a key factor in allowing the HLA-DQ2 molecule to present gluten-derived peptides that are rich in prolines and glutamates (Jones *et al.* 2006). Similarly, *HLA-DRB1* polymorphisms lead to differential electrostatic charges of binding pocket 9 and are thus related to the susceptibility to primary sclerosing cholangitis (Donaldson 2011). In this study, we found two lymphoma susceptibility molecules, DRB3*005:02 and DRB3*016:01, which were neutrally charged in binding pocket 9; whereas the resistance BoLA-DRβ molecule, DRB3*011:01, carries a positive charge. This electrostatic charge variation may allow the recognition of different peptide antigens and thus exert a differential immune reaction against BLV-induced lymphoma. Although we found that there were amino acid motifs associated with BLV-induced lymphoma in all binding pockets (1, 4, 6, 7, and 9), pocket 9 showed a major difference in electrostatic potential between resistance

and susceptibility groups. This result implies that there might be other property differences in addition to that in electrostatic potential, such as in hydrophobicity of binding pockets, that affect BLV-induced lymphoma susceptibility. Interestingly, we found that the BoLA-DRB3*010:01 molecule, which is a lymphoma resistance type identified in Holstein cows, also carries a positive charge in pocket 9, in line with the current study (data not shown). However, *BoLA-DRB3*010:01* was not categorized as a resistance allele in this study, suggesting the possibility of other host factors affecting the susceptibility to BLV-induced lymphoma.

The development of BVL-induced lymphoma is a complex result caused by both viral and host factors in addition to *BoLA-DRB3*. For example, in the viral factors, BLV provirus integration close to cancer-driver sites or transcriptionally active regions influences host gene expression (Gillet *et al.* 2013, Rosewick *et al.* 2017). The viral accessory proteins, Tax and G4, are reported as causative agents for cell transformation (Willems *et al.* 1994, Zyrianova *et al.* 2020). For host factors, p53 mutation and the polymorphisms of tumor necrosis factor-α are related to lymphoma development (Dequiedt *et al.* 1995, Tajima *et al.* 1998, Konnai *et al.* 2006). Besides, the deregulation of lymphocyte homeostasis, which is characterized by the downregulation of cell turnover rate, is known to lead to leukemia (Debacq *et al.* 2003, Gillet *et al.* 2007). Recently, we found that the expression levels of DNA mismatch repair genes *MSH2* and *EXO1* were associated with BLV-induced lymphoma, implying that the accumulation of DNA mutations is one of the mechanisms causing disease onset (Bai *et al.* 2020). Furthermore, an arginine-N-methyltransferase, PRMT5, has shown positive correlation with BLV infection with a high pro-viral load and lymphoma stage. Downregulation of PRMT5 expression impaired BLV gene expression and pathogenicity (Assi *et al.* 2020). The factors mentioned above potentially working together with *BoLA-DRB3* polymorphisms contribute to BLV-induced lymphoma development.

Taken together, in addition to the BLV-induced lymphoma association study regarding *BoLA-DRB3* alleles, amino acid and structure level analyses augment the understanding of how BoLA-DRB3 polymorphisms affect lymphoma susceptibility. These results are not only helpful for cattle breeding selection but for future vaccine development against BLV-induced lymphoma.

Table 17. Association of the *BoLA-DRB3* allele with Bovine leukemia virus (BLV)-induced lymphoma.

Allele	Asymptomatic (n. = 212) [1]	Lymphoma (n. = 454)	OR [2]	95% CI [3]	*p*-value	Susceptibility [4]
*001:01	9	8	0.405	0.154–1.064	0.0677	-
*002:01	18	17	0.419	0.212–0.831	0.0147	(R)[5]
*004:01	0	1	1.406	0.057–34.653	1	-
*005:01	1	1	0.466	0.029–7.483	0.5356	-
*005:02	1	23	11.260	1.510–83.945	0.0014	S
*005:03	3	31	5.106	1.543–16.894	0.0021	(S)
*005:04	2	1	0.232	0.021–2.571	0.2391	-
*005:08	0	3	3.295	0.169–64.072	0.5552	-
*006:01	1	2	0.934	0.084–10.354	1	-
*007:01	7	5	0.326	0.102–1.040	0.0606	-
*008:01	1	4	1.876	0.208–16.884	1	-
*009:02	7	2	0.130	0.027–0.629	0.0059	(R)
*010:01	27	45	0.754	0.454–1.253	0.2855	-
*011:01	29	13	0.186	0.095–0.366	<0.00001	R
*012:01	8	20	1.175	0.509–2.713	0.8369	-
*013:01	1	1	0.466	0.029–7.483	0.5356	-
*013:02	13	20	0.705	0.344–1.447	0.3426	-
*014:01:01	7	11	0.727	0.278–1.903	0.6086	-
*015:01	29	34	0.511	0.302–0.864	0.0024	(R)
*016:01	44	198	2.953	2.019–4.319	<0.00001	S
*027:03	2	0	0.093	0.004–1.938	0.1010	-
*020:01:02	1	4	1.876	0.208–16.884	1	-
*034:01	1	3	1.404	0.145–13.573	1	-
*038:01	0	3	3.295	0.169–64.072	0.5552	-
*044:01	0	4	4.245	0.228–79.214	0.3126	-

* indicates the allele;
[1] n.: total allele number (= cattle number multiplied by 2);
[2] OR: Odds ratio;
[3] 95% CI: 95% confidence intervals;
[4] Susceptibility: R = resistance; S = susceptibility after Bonferroni correction;
[5] ( ): significant only before Bonferroni correction.

Table 18. Association of *BoLA-DRB3* genotype with BLV-induced lymphoma

Genotype [1]	Asymptomatic (n. = 106) [2]	Lymphoma (n. = 227)	OR [3]	95% CI [4]	*p*-value	Susceptibility [5]
*001:01/*016:01	2	3	0.696	0.115–4.231	0.6551	-
*002:01/*005:03	1	3	1.406	0.145–13.681	1	-
*002:01/*010:01	5	2	0.180	0.034–0.941	0.0356	(R)[6]
*002:01/*011:01	5	0	0.041	0.002–0.741	0.0031	(R)
*002:01/*016:01	1	7	3.341	0.406–27.507	0.4438	-
*005:02/*016:01	0	13	13.405	0.789–227.680	0.0115	(S)
*005:03/*012:01	0	4	4.289	0.229–80.387	0.311	-
*005:03/*016:01	0	10	10.283	0.597–177.152	0.0341	(S)
*010:01/*010:01	2	3	0.696	0.117–4.312	0.6551	-
*010:01/*011:01	3	3	0.460	0.091–2.317	0.3873	-
*010:01/*015:01	3	4	0.616	0.135–2.802	0.6837	-
*010:01/*016:01	7	19	1.292	0.526–3.175	0.6654	-
*011:01/*015:01	4	0	0.050	0.003–0.939	0.0099	(R)
*011:01/*016:01	7	4	0.254	0.073–0.886	0.0415	(R)
*012:01/*015:01	2	3	0.696	0.115–4.231	0.6551	-
*012:01/*016:01	0	5	5.265	0.289–96.103	0.1822	-
*013:02/*016:01	3	10	1.582	0.426–5.872	0.7621	-
*015:01/*016:01	10	15	0.679	0.295–1.567	0.3769	-
*016:01/*016:01	4	49	7.020	2.462–20.017	<0.00001	S
*016:01/*020:01:02	1	3	1.406	0.145–13.681	1	-

* indicates the genotype;
[1] Only genotypes with frequency >1 are shown;
[2] no.: total genotype number;
[3] OR, Odds ratio;
[4]95% CI: 95% confidence intervals;
[5] Susceptibility, R = resistance, S = susceptibility after Bonferroni correction; 6 ( ), significant only before Bonferroni correction.

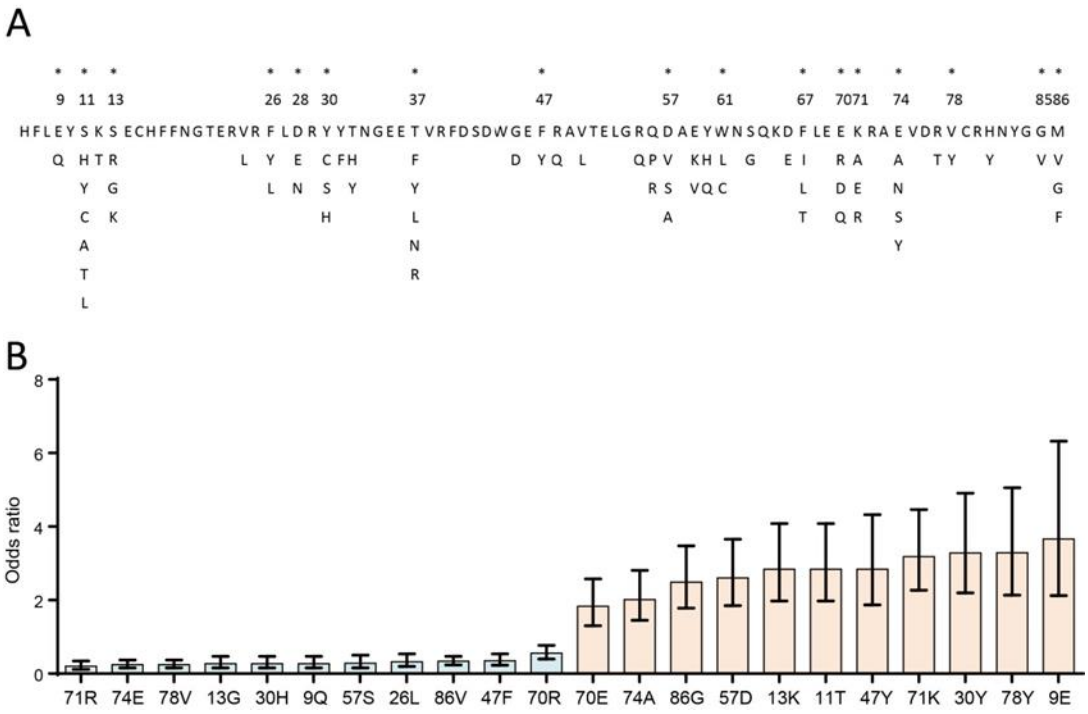


Figure 14. Association of antigen recognition sites of peptide-binding pockets in BoLA-DRβ with lymphoma. (**A**) Amino acid diversities of BoLA-DRβ based on the *BoLA-DRB3* allele, which was identified in this study. The asterisks indicate the antigen recognition sites of BoLA-DRβ, located in the antigen interacting position. (**B**) Odds ratio of BoLA-DRβ binding pocket amino acids and lymphoma. Resistance amino acid: Odds ratio < 1, indicated in blue; Susceptibility amino acids: Odds ratio > 1, indicated in red. The association study was performed using Fisher's exact test followed by Bonferroni correction. Error bars indicate the 95% confidence intervals. Only Bonferroni-corrected significant amino acids are shown.

A

BoLA-DRB3	9	11	13	24	26	30	32	45	47	55	57	59	70	71	74	77	78	86	OR
*027:03	E	Y	R	V	F	C	Y	G	F	Q	D	E	E	R	E	T	V	G	
*009:02	E	S	S	V	F	S	H	G	Y	Q	D	E	E	R	E	T	V	G	
*011:01	Q	H	G	V	L	H	H	G	F	Q	S	E	R	R	E	T	V	V	
*005:04	E	Y	S	L	Y	Y	Y	G	Y	R	D	K	R	K	N	T	Y	F	
*007:01	E	C	R	V	F	C	H	G	F	R	V	V	E	R	E	T	V	V	
*001:01	E	S	S	V	F	Y	T	G	F	R	D	E	E	K	E	R	V	M	
*002:01	E	S	S	V	F	Y	H	G	Y	R	D	E	R	A	A	T	Y	V	< 1
*005:01	E	H	S	L	Y	Y	Y	G	Y	R	D	K	R	K	N	T	Y	V	
*013:01	E	L	S	V	F	Y	Y	G	Y	Q	D	K	R	K	N	T	Y	V	
*015:01	E	S	S	V	Y	Y	H	G	Y	R	V	K	R	E	Y	T	Y	V	
*013:02	E	L	S	V	F	S	Y	D	Y	Q	D	E	R	K	N	R	Y	V	
*014:01:01	Q	H	G	V	L	H	H	G	F	Q	A	E	Q	K	E	T	V	V	
*010:01	E	S	S	V	F	Y	Y	D	Y	Q	V	E	R	A	A	T	Y	G	
*006:01	E	C	R	V	L	C	H	G	F	R	V	E	R	K	E	R	V	G	
*012:01	E	T	K	V	F	Y	H	G	Y	Q	D	E	R	A	A	T	Y	G	
*034:01	E	C	S	V	F	S	Y	G	Y	Q	V	E	Q	K	N	T	Y	V	
*008:01	E	A	S	V	F	Y	Y	G	F	R	S	E	D	E	S	R	Y	V	
*020:01:02	E	C	R	V	L	Y	Y	G	F	Q	S	E	Q	R	A	T	Y	V	
*016:01	E	T	K	V	F	Y	T	G	Y	R	D	E	E	K	A	R	Y	G	
*005:03	E	S	S	L	Y	Y	Y	G	Y	R	D	K	R	K	N	T	Y	F	> 1
*005:02	E	H	S	L	Y	Y	Y	G	Y	R	D	K	R	K	N	T	Y	G	
*004:01	E	S	S	V	F	Y	T	G	F	R	D	E	R	E	A	R	Y	V	
*005:08	E	H	S	V	Y	Y	H	G	Y	R	D	E	R	K	N	T	Y	F	
*038:01	E	S	S	V	Y	Y	T	G	Y	Q	D	E	R	K	N	T	Y	V	
*044:01	E	Y	S	V	F	Y	T	G	F	R	D	K	R	A	A	T	Y	G	

B

Pockets	Amino acid position	Motif	Susceptibility	Asymptomatic	Lymphoma	OR	*p*-value
4	13, 26	$K^{13}F^{26}$	S	52	218	2.842	<0.00001
		$G^{13}L^{26}$	R	36	24	0.273	<0.00001
	71, 74, 78	$K^{71}A^{74}Y^{78}$	S	44	198	2.953	<0.00001
		$R^{71}E^{74}V^{78}$	R	45	20	0.171	<0.00001
1+4	70, 86	$E^{70}G^{86}$	S	53	200	2.362	<0.00001
		$R^{70}V^{86}$	R	91	90	0.329	<0.00001
	71, 78, 86	$K^{71}Y^{78}G^{86}$	S	45	221	3.520	<0.00001
		$R^{71}V^{78}V^{86}$	R	36	18	0.202	<0.00001
6	11, 30	$T^{11}Y^{30}$	S	52	218	2.842	<0.00001
		$H^{11}H^{30}$	R	36	24	0.273	<0.00001
7	47, 71	$Y^{47}K^{71}$	S	66	284	3.696	<0.00001
		$F^{47}R^{71}$	R	39	22	0.226	<0.00001
9	9, 57	$E^{9}D^{57}$	S	109	333	2.601	<0.00001
		$Q^{9}S^{57}$	R	29	13	0.186	<0.00001

Figure 15. Association of amino acid motifs, which are constituted by antigen recognition sites of peptide-binding pockets in BoLA-DRβ, with lymphoma. (**A**) Conservation of lymphoma Resistance/Susceptibility amino acid distribution encoded by the Resistance/Susceptibility *BoLA-DRB3* allele. * indicates each allele. The order is ranked using Odds ratios (OR) determined in Table 1. *BoLA-DRB3* with OR < 1 are considered potential resistance alleles, whereas those with OR > 1 are considered susceptibility alleles. Resistance or susceptibility amino acids are indicated in blue and red, respectively. (**B**) Association of BLV-induced lymphoma with the combination of amino acid residues. Amino acid combinations and their localizations (pockets) are shown. Resistance amino acid combination: OR < 1; Susceptibility amino acid combination: OR > 1. The association study was performed using Fisher's exact test.

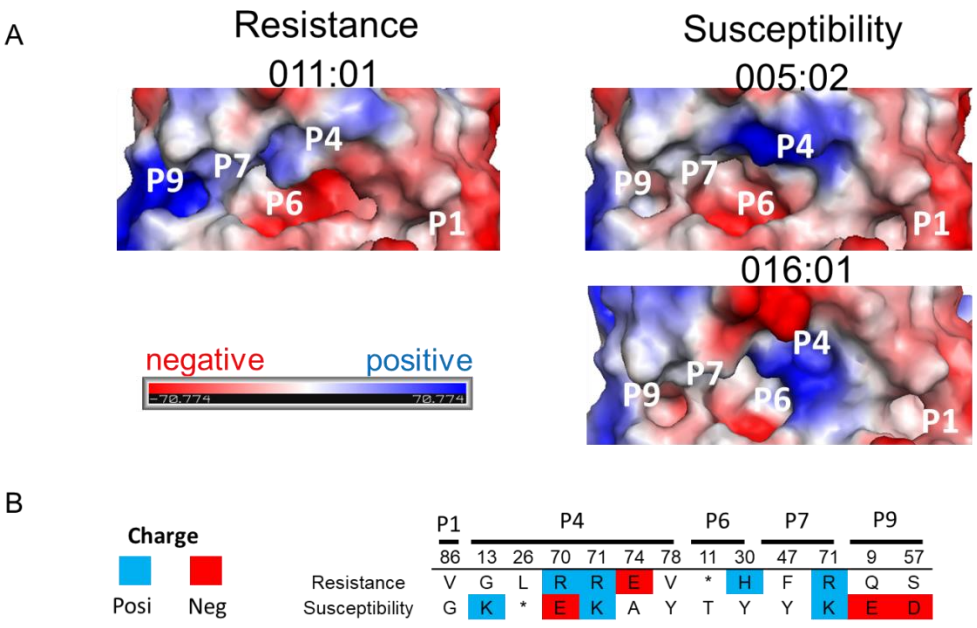


Figure 16. Electrostatic charge differences in binding pockets between lymphoma resistance and susceptibility BoLA-DRβ molecules. (**A**) Electrostatic potential of lymphoma resistance BoLA-DRβ molecule, DRB3*011:01, and susceptibility molecules, DRB3*005:02 and DRB3*016:01, is shown. Binding pockets 1, 4, 6, 7, and 9 are indicated. Negative charge is indicated in red, and positive charge is indicated in blue. (**B**) Lymphoma resistance/susceptibility amino acid charges. Positively charged amino acids are indicated with a blue background and negatively charged amino acids are indicated with a red background.

Chapter 4.

BoLA heterozygote advantage against the outcome of bovine leukemia virus infection

4.1 Abstract

Bovine leukemia virus (BLV) causes enzootic bovine leucosis. Host genetic heterozygosity at the major histocompatibility complex can enhance the ability to combat infectious diseases. However, heterozygote advantage is loci specific and depends on disease type. Bovine leukocyte antigen (BoLA)-*DRB3* polymorphisms are related with BLV-infection outcome; however, whether *BoLA-DRB3* heterozygotes have an advantage against BLV-induced lymphoma and proviral load (PVL) remains unclear. By analyzing 1567 BLV-infected individuals, we found that *BoLA-DRB3* heterozygous status was significantly associated with lymphoma resistance irrespective of cattle breeds ($p < 0.0001$). Similarly, decreased PVL was observed in *BoLA-DRB3* heterozygotes ($p = 0.0407$ for Holstein cows; $p = 0.0889$ for Japanese Black cattle). This report provides first evidence of *BoLA-DRB3* heterozygote advantage against BLV infection outcome.

4.2 Introduction

BLV infects cattle worldwide and is the causative agent of EBL (Gillet *et al.* 2007). It belongs to the *Deltaretrovirus* genus of the Retroviridae family, which also includes the HTLV- I and II (Aida *et al.* 2013). Approximately 70% BLV-infected cattle show no clinical symptoms, whereas 30% infected cattle develop persistent lymphocytosis, which is typified by the polyclonal expansion of non-neoplastic B lymphocyte cells, 1–5% of which lead to $CD5^+$-B cell leukemia/lymphoma after a long latency period (Aida *et al.* 2013, Barez *et al.* 2015). BLV also causes considerable financial loses in the industry because infected cattle lead to a decreased milk production rate (Norby *et al.* 2016). In addition, similar to HTLV-1, DNA copies of the BLV genome are integrated into the host genome, forming proviruses, which lead to lifelong infection (Lairmore 2014). The PVL is therefore positively

associated with both disease progression and viral transmission (Jimba *et al.* 2012, Panei *et al.* 2013, Ruggiero *et al.* 2019).

The (MHC is a highly polymorphic gene set and plays a central role in antigen recognition of pathogens (Takeshima *et al.* 2006, Blackwell *et al.* 2009). Heterozygotes of the MHC loci may exhibit enhanced resistance to infectious diseases compared with homozygotes by presenting a broader array of pathogen-derived peptides to T cells and thus generating a more diverse T-cell repertoire (Penn *et al.* 2002, Pierini *et al.* 2018). This concept is called heterozygote advantage. Numerous studies have shown that heterozygous individuals at the MHC loci exhibit an advantage against viruses including HIV-1, HTLV-1, and HCV (Jeffery *et al.* 2000, Hraber *et al.* 2007, Arora *et al.* 2020). In cattle, heterozygotes of the *BoLA-DQA2* locus and not of the *DRB3* locus exhibit a correlation with mastitis resistance (Takeshima *et al.* 2008), suggesting that the heterozygote advantage to disease susceptibility potentially depends on specific gene loci. Several studies have shown that the genetic variations in *BoLA-DRB3* are associated with the susceptibility to BLV-induced lymphoma and PVL (Juliarena *et al.* 2008, Miyasaka *et al.* 2013, Nikbakht Brujeni *et al.* 2016, Takeshima *et al.* 2019, Forletti *et al.* 2020, Lo *et al.* 2020). However, whether heterozygosity at *BoLA-DRB3* has a superior effect on anti-BLV-induced lymphoma and PVL still needs to be studied further. In this study, we sought to investigate whether heterozygote at *BoLA-DRB3* has advantage against BLV infection outcome.

4.3 Materials and Methods

4.3.1 *Sample collection and diagnosis*

Blood samples from 1567 BLV-infected cattle were randomly collected in a nationwide survey across Japan, and the genomic DNA and plasma from peripheral blood were isolated (Miyasaka *et al.* 2013, Ohno *et al.* 2015, Takeshima *et al.* 2019, Lo *et al.* 2020). Among them, 822 samples were from Holstein cows and 735 were from Japanese Black cattle (Table 19). Asymptomatic cattle samples were collected from farms and the infection was confirmed by anti-BLV gp51 ELIAS. Lymphoma cattle samples were collected from slaughterhouses and the infection were confirmed by anti-BLV gp51 ELIAS and some of the sample tested together with southern blotting and PCR of BLV pro-viral genomes. Asymptomatic cattle sample were collected from farms and the subclinical stage of BLV infection was diagnosed according to the lymphocyte count (cells/μL) and the age of each cow (≤5500

= normal, between 5500 to 7500 = suspected lymphocytosis and ≥7500 = lymphocytosis). Asymptomatic cattle were defined as BLV-infected but clinically and hematologically normal; PL cattle were defined as BLV-infected but clinically normal cattle with an increase in the number of apparently normal B lymphocytes. In this study, only samples from asymptomatic cattle were used for further analysis. In the asymptomatic group, individuals with PVLs above and below 10^4 copies/10^5 cells were categorized as high and low PVL groups, respectively (Takeshima *et al.* 2019, Lo *et al.* 2020). This study was approved by the Animal Ethical Committee, and the Animal Care and Use RIKEN Animal Experiments Committee (approval number H29-2-104). Cattle lymphoma status was diagnosed using both gross observation of neoplastic tissues in lymph nodes and histological observation in heart, lung, liver, kidney, spleen, intestines and lymph node in the body. In addition, atypical mononuclear cells in blood sample together with genomic southern blotting for testing disease progression were used for confirmation of some cattle samples (Tajima *et al.* 1998).

4.3.2 *BLV proviral load determination*

BLV infection was estimated by BLV-CoCoMo-qPCR-2 (RIKEN Genesis, Kanagawa, Japan), as previously described (Jimba *et al.* 2010, Jimba *et al.* 2012, Panei *et al.* 2013, Takeshima *et al.* 2015, Yuan *et al.* 2015). Briefly, the BLV-LTR region was amplified in a reaction mixture containing THUNDERBIRD Probe qPCR Mix (Toyobo, Tokyo, Japan), CoCoMo FRW primer, CoCoMo REV primer, FAM-BLV probe, and 150 ng of template DNA. In addition, the *BoLA-DRA* region was amplified as internal control. The proviral load was calculated using following equation: (number of BLV-LTR copies /number of *BoLA-DRA* copies) X 10^5 cells.

4.3.3 *BoLA-DRB3 genotyping*

BoLA-DRB3 alleles were determined using the PCR-SBT method, as previously described (Takeshima *et al.* 2011). Briefly, *BoLA-DRB3* exon 2 was amplified by single-step PCR using the DRB3 forward (5'-CGCTCCTGTGAYCAGATCTATCC-3') and DRB3 reverse (5'-CACCCCCGCGCTCACC-3') primer set. The PCR products were purified by ExoSAP-IT PCR product purification kit (USB Corp., Cleveland, OH) and then sequenced using the ABI PRISM BigDye1.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The sequence data were then analyzed using Assign 400ATF ver. 1.0.2.41 software (Gonexio Genomics, Fremantle, Australia) to determine the *BoLA-DRB3* genotype.

4.3.4 *Association study and statistical analysis*

An association study based on Fisher's exact test was performed by comparing the heterozygote frequencies between asymptomatic and lymphoma cows or low and high PVL cows. Odds ratios were used to indicates the risk potential: OR<1, cows with heterozygote has lower risk for BLV infection outcome; OR>1, cows with heterozygote has higher risk for BLV infection outcome. All calculations were performed using Prism 6 (GraphPad, San Diego, CA, USA).

4.4 Results

4.4.1 *Association of BoLA-DRB3 heterozygosity with lymphoma in total cattle population*

We first tested whether zygosity of *BoLA-DRB3* was associated with BLV-induced lymphoma in cattle. As shown in Figure 1, DNA samples from 448 BLV-infected lymphoma animals (221 from Holstein cows and 227 from Japanese Black cattle) and 1119 BLV-infected asymptomatic animals (611 from Holstein cows and 508 from Japanese Black cattle) were used for *BoLA-DRB3* genotyping, followed by association studies (Table 19). Forty-four known *BoLA-DRB3* alleles were identified (the IPD-MHC database (https://www.ebi.ac. uk/ipd/mhc/group/BoLA/)). Hardy-Weinberg equilibrium was used to inspect the genotyping errors by analyzing the healthy cattle sample (Arlequin ver. 3.5.2.2) (Excoffier *et al.* 2007). Both Holstein cows and Japanese Black cattle used in this study were followed Hardy-Weinberg equilibrium suggesting typing errors are less likely occurred: Holstein (Observed Heterozygosity = 0.84943, Expected Heterozygosity = 0.86300, p = 0.06419), Japanese Black (Observed Heterozygosity = 0.88189, Expected Heterozygosity = 0.87355, p = 0.23863). The association study indicated that *BoLA-DRB3* heterozygous cattle in comparison to homozygous cattle had a significantly lower frequency in the lymphoma group (OR = 0.41, 95% CI = 0.31-0.53, p < 0.0001) (Table 19).

4.4.2 *Association of BoLA-DRB3 heterozygosity with PVL in total cattle population*

Next, we tested the effect of zygosity of *BoLA-DRB3* on BLV PVL. Among 1119 asymptomatic animals, individuals were classified into low PVL and high PVL groups based on the threshold of 10^4 copies/10^5 cells, as described previously (Takeshima *et al.* 2019, Lo *et al.* 2020). As shown in Figure 17, 478 BLV-infected individuals belonged to the low PVL group (317 Holstein cows and 161 Japanese Black cattle) and 536 BLV-infected

individuals carried a high PVL (294 Holstein cows and 242 Japanese Black cattle). The association between *BoLA-DRB3* zygosity and BLV PVL indicated that the heterozygous state of *BoLA-DRB3* was significantly associated with low PVL (OR = 0.62, 95% CI = 0.43-0.90, $p = 0.0108$) (Table 19).

4.4.3 *Association of BoLA-DRB3 heterozygosity with lymphoma in Holstein cows*

The association between *BoLA-DRB3* polymorphisms and BLV-related diseases varies among different cattle breeds; thus, we tested whether *BoLA-DRB3* heterozygote advantage is retained in different breeds of cattle. For the lymphoma association study in Holstein cows, 211 lymphoma and 611 asymptomatic BLV-infected individuals were used for analysis (Figure 17). The results indicated that *BoLA-DRB3* heterozygous cows had a significantly lower frequency for lymphoma development compared with homozygous cows (OR = 0.48, 95% CI = 0.33-0.70, $p < 0.0001$) (Table 19). To further investigate whether the heterozygote advantage was caused by the influence of a specific genotype or was an overall effect, we performed an association study of the *BoLA-DRB3* genotype with lymphoma (Figure 18A). Interestingly, we found that except *DRB3*010:01* and *DRB3*011:01* homozygous genotypes, which were identified as lymphoma resistance alleles previously, all the other homozygous genotypes exhibited a higher frequency in the lymphoma group (*DRB3*001:01*, *DRB3*012:01*, *DRB3*015:01*, and *DRB3*027:03*). In contrast, most heterozygous genotypes had a higher frequency in the asymptomatic group. However, no genotypes associated with BLV-induced lymphoma were observed, suggesting that heterozygote advantage is a collective effect from all genotypes.

4.4.4 *Association of BoLA-DRB3 heterozygosity with PVL in Holstein cows*

For the PVL association study in Holstein cows, 317 low PVL and 294 high PVL individuals were used for analysis (Figure 17). Similarly, the results showed that *BoLA-DRB3* heterozygous cows had significantly lower PVL compared with homozygous cows (OR = 0.62, 95% CI = 0.39-0.97, $p = 0.0407$) (Table 19). Genotype association of PVL indicated that *DRB3*009:02* and *DRB3*015:01* heterozygosity is associated with PVL resistance, and *DRB3*009:02* was identified as a PVL resistance allele previously; however, *DRB3*011:01* and *DRB3*012:01* heterozygotes were associated with PVL susceptibility (Figure 18B), and *DRB3*012:01* was identified as a PVL susceptibility allele previously. The result obtained from Holstein cows implied that the allele effect is dominant over heterozygote advantage. However, overall, heterozygous cows tend to a have lower PVL.

4.4.5 *Association of BoLA-DRB3 heterozygosity with lymphoma in Japanese Black cattle*

Finally, we confirmed the heterozygote advantage in BLV-induced lymphoma and PVL in Japanese Black cattle. For lymphoma association, 227 lymphoma and 508 asymptomatic BLV-infected cattle were analyzed (Figure 17). Consistently, *BoLA-DRB3* heterozygous cattle had significantly lower frequency for lymphoma development compared with homozygous cattle (OR = 0.33, 95% CI = 0.23-0.50, $p < 0.0001$) (Table 19). To test whether specific *BoLA-DRB3* genotypes contribute to the heterozygote advantage in lymphoma development, we performed an association study of the *BoLA-DRB3* genotype with lymphoma (Figure 19A). Surprisingly, only homozygotes and no heterozygotes of *DRB3*016:01*, which is the lymphoma susceptibility allele identified in our previous study (section 3 of this thesis) (Lo *et al.* 2021), were significantly enriched in the BLV-induced lymphoma group, suggesting homozygotes are more susceptible to lymphoma development; however, the observed overall heterozygote advantage is at least partially caused by the effect of susceptibility allele *DRB3*016:01*.

4.4.6 *Association of BoLA-DRB3 heterozygosity with PVL in Japanese Black cattle*

For PVL association, 161 high PVL and 242 low PVL BLV-infected asymptomatic cattle were analyzed (Figure 17). The result indicated that *BoLA-DRB3* heterozygous cattle had a tendency to carry low PVLs (OR = 0.56, 95% CI = 0.29-1.08, $p = 0.0889$) (Table 19). Genotype association of PVL did not show any genotype that was significantly associated with PVL susceptibility (Figure 19B) in Japanese Black cattle.

4.5 Discussion

In this study, for the first time, we show evidence of *BoLA-DRB3* heterozygote advantage against BLV-induced lymphoma and BLV PVL in cattle. Interestingly, we found that there are potentially different mechanisms underlying the heterozygote advantage in Holstein cows and Japanese Black cattle. Heterozygote advantage is caused by two mechanisms: first, *BoLA-DRB3* heterozygotes recognize a more diverse antigen repertoire and thus exhibit better anti-lymphoma and anti-PVL ability. In agreement with this, in the lymphoma association study in Holstein cows, although no genotypes were found to be associated with BLV-induced lymphoma, *BoLA-DRB3* homozygous individuals had a higher chance of developing lymphoma (*DRB3*001:01*, *DRB3*012:01*, *DRB3*015:01*, and *DRB3*027:03*) except two *BoLA-DRB3* homozygotes (*DRB3*010:01* and *DRB3*011:01*) which

both allele were identified as lymphoma resistant in our previous study (Figure 2A); in contrast, in general, heterozygote individuals had a lower chance of developing lymphoma. Similarly, the HLA allele divergence at specific loci is critical for HIV load in patients in addition to the allele effect (Arora *et al.* 2020). The second potential mechanism of heterozygote advantage is due to the fact that resistance allele is dominant over the susceptibility allele (i.e. genotype that carries both resistance and susceptibility allele belongs to resistance genotype) (Miyasaka *et al.* 2013). Therefore, heterozygote may increase the chance counteracting the effect of susceptibility allele. In line with this, in the lymphoma association study in Japanese Black cattle, we found that homozygotes but no heterozygotes of *BoLA-DRB3*016:01* were significantly associated with disease onset. Our result could also be supported by previous studies in non-Hodgkin lymphoma that show that low MHC diversity provided an advantage for the tumor to escape the immune response, thus leading to a higher chance of disease onset (Drénou *et al.* 2004, Wang *et al.* 2010).

We showed that *BoLA-DRB3* heterozygote advantage is more effective in BLV-induced lymphoma than in terms of PVL in both the tested cattle breeds. Indeed, MHC is known to target cancer directly or by reducing the causative pathogens and indirectly prohibiting cancer development (Garcia-Lora *et al.* 2003, Ghasemi *et al.* 2020). Thus, it is reasonable that the effect of MHC heterozygosity is higher against cancer than against the causative viruses.

MHC class I play a crucial role in tumor immunity (Marty *et al.* 2017). In line with this, the association of BoLA with BLV-induced subclinical progression was firstly found in BoLA class I (Lewin *et al.* 1986); however, BoLA class II were later identified as playing a stronger role in BLV infection outcome (Xu *et al.* 1993). Here, we augment the knowledge that heterozygote at BoLA class II *DRB3* relates with BLV-induced lymphoma and PVL. In fact, MHC class II have recently shown its role in tumor immunity in addition to MHC class I (Marty Pyke *et al.* 2018). Interestingly, tumor mutations which poorly bound to MHC class II are positively selected during tumorigenesis, even more than mutations poorly bound to MHC class I (Marty Pyke *et al.* 2018). This emphasizes the importance of MHC class II-mediated anti-tumor immunity. However, whether heterozygotes of BoLA class I have advantage against infection outcome needs further investigation.

It has been observed that the cattle geographic and genetic background affect the association of *BoLA-DRB3* with BLV. Indeed, the frequencies of resistance and susceptibility *BoLA-DRB3* alleles are varied in different breed of cattle. For example, in Holstein cows *DRB3*009:01*, *DRB3*018:02* and *DRB3*032:02* were found as lymphoma susceptible alleles (Nikbakht Brujeni *et al.* 2016); however, all these alleles are absent in Japanese Black cattle.

Although the existence of genetic differences in different breed of cattle, we found that heterozygote at *BoLA-DRB3* has advantage against BLV infection outcome irrespective to cattle breed.

Table 19. Heterozygosity advantage against BLV induced lymphoma and proviral load

	Symptoms		Homozygote (No. of cows)[1]	Heterozygote (No. of cows)	OR[2]	95 % CI[3]	Z-value[4]	p-value
Total	Lymphoma	Asymptomatic	151	968	0.41	0.31-0.53	6.54	< 0.0001
		lymphoma	124	324				
	PVL[5]	Low PVL	52	426	0.62	0.43-0.90	2.54	0.0108
		High PVL	88	448				
Holstein	Lymphoma	Asymptomatic	91	520	0.48	0.33-0.70	3.86	< 0.0001
		lymphoma	59	162				
	PVL	Low PVL	38	279	0.62	0.39-0.97	2.08	0.0407
		High PVL	53	241				
Japanese Black	Lymphoma	Asymptomatic	60	448	0.33	0.23-0.50	5.46	< 0.0001
		lymphoma	65	162				
	PVL	Low PVL	14	147	0.56	0.29-1.08	1.72	0.0889
		High PVL	35	207				

No.[1]: number
OR[2]: odds ratio
95% CI[3]: 95% confidence interval of OR
Z-value[4]: standard normal deviate
PVL[5]: proviral load

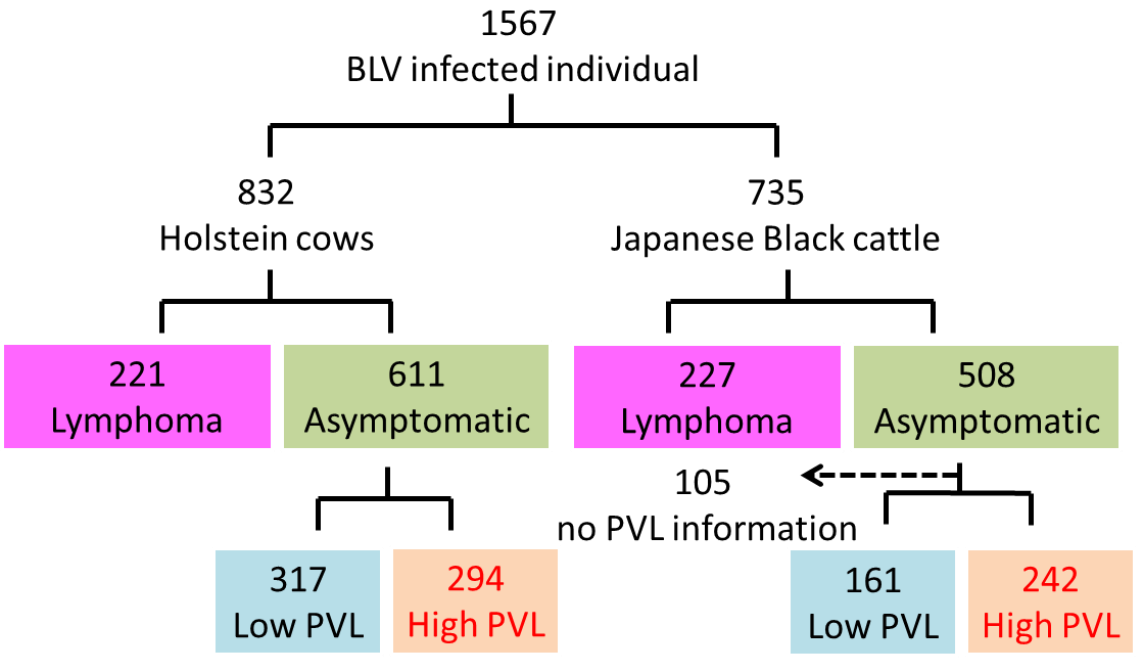


Figure 17 Sample characterization performed for the PVL and lymphoma association study. DNA sample from 1567 BLV-infected animals were used for analysis in this study. Among them, 832 were Holstein cows and 735 were Japanese Black cattle. BLV infection and proviral load (PVL) were determined by anti-BLV gp51 ELISA (JNC, Tokyo, Japan) and/or BLV-CoCoMo-qPCR-2 methods (RIKEN Genesis, Kanagawa, Japan). The subclinical stage of cattle was diagnosed according to the lymphocyte count (for asymptomatic cattle determination), by both gross and histological observations, and by detecting the atypical mononuclear cells (for lymphoma cattle determination), as described previously. In the asymptomatic group, individuals with PVL above or below 10^4 copies/10^5 cells were categorized into high or low PVL groups, respectively.

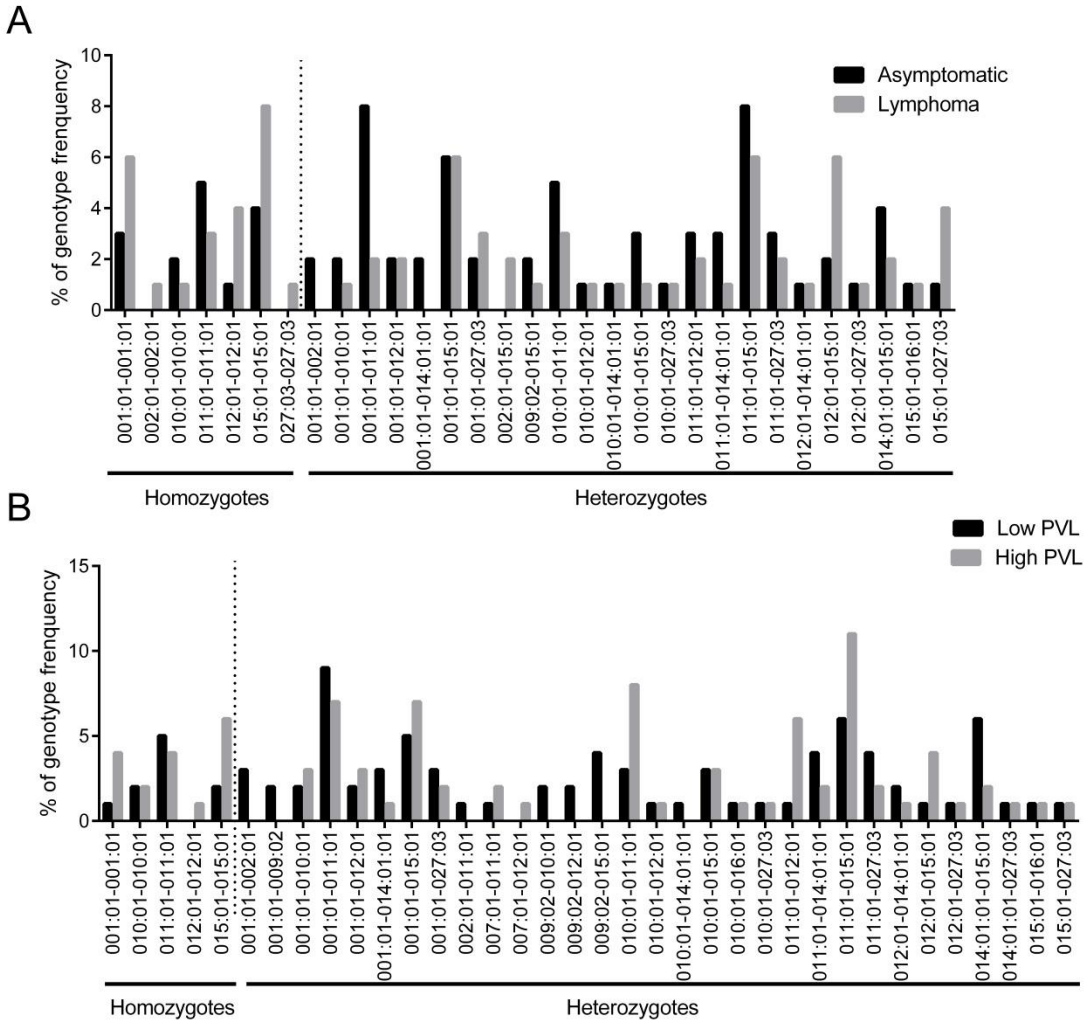


Figure 18 *BoLA-DRB3* genotype distributions in Holstein cows with low/high PVL or in healthy/lymphoma conditions. *BoLA-DRB3* genotyping was performed by sequence-based typing, and then, the genotype was analyzed by Assign 400ATF ver. 1.0.2.41 software (Gonexio Genomics, Fremantle, Australia). Association studies of *BoLA-DRB3* zygosity to PVL or lymphoma were based on Fisher's exact test (GraphPad, San Diego, California, USA). Association of *BoLA-DRB3* genotype to PVL or lymphoma was analyzed by Fisher's exact test, and the resulting *p*-value was then adjusted through Bonferroni correction. The significance was set at a level of adjusted *p*-value = 0.05. Asterisks indicate the significant genotype. R: resistance genotype; S: susceptibility genotype. Only genotype frequencies >1% are shown in the figure. (A) Frequency of *BoLA-DRB3* genotype distribution in Holstein cows with low or high PVL. *BoLA-DRB3* loci were genotyped from DNA samples of 611 BLV infected asymptomatic Holstein cows. A PVL of 10^4 copies/10^5 cells was set as the threshold to classify high PVL and low PVL groups. (B) Frequency of the distribution of the *BoLA-DRB3* genotype in Holstein cows in asymptomatic or lymphoma conditions. The *BoLA-DRB3* loci were genotyped from DNA samples of 611 BLV-infected asymptomatic and 211 BLV-infected lymphoma Holstein cows.

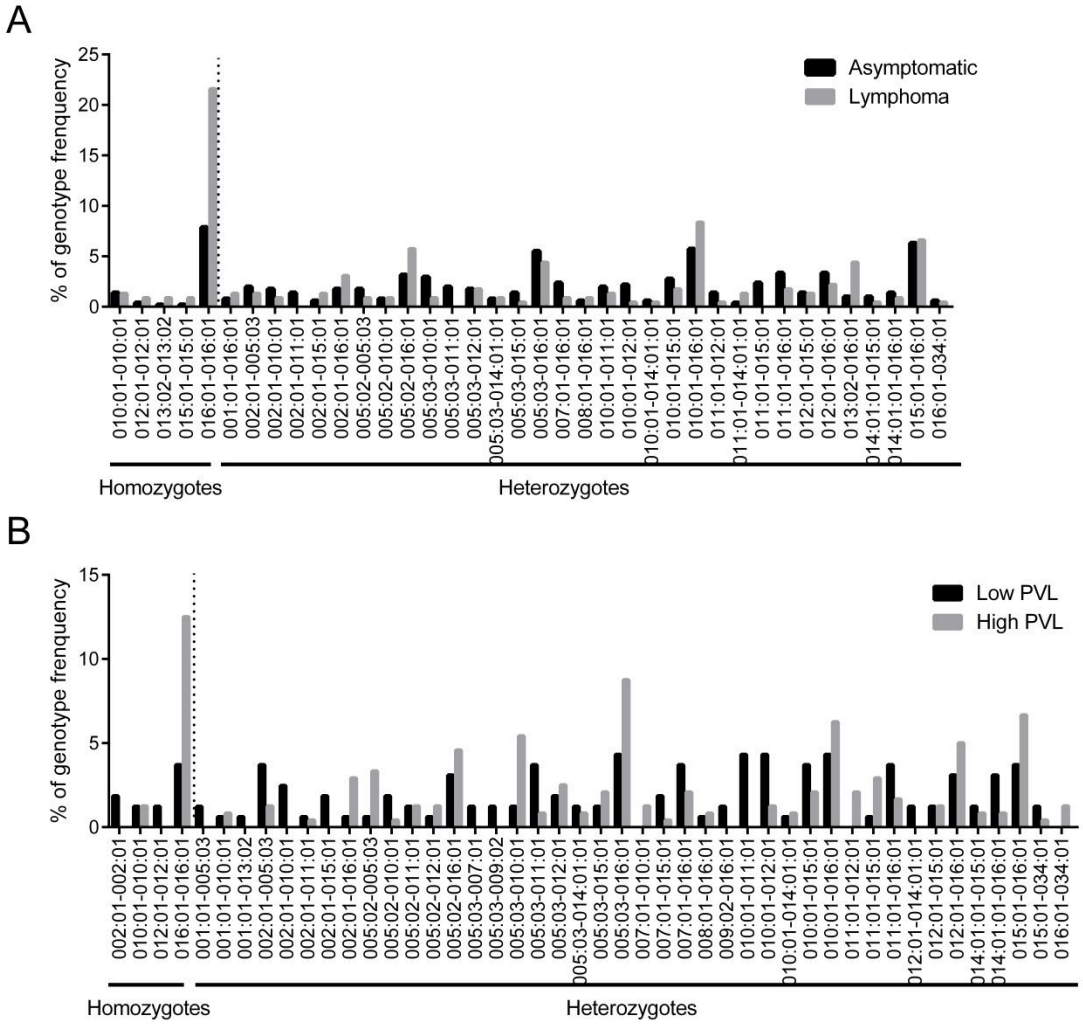


Figure 19 *BoLA-DRB3* genotype distributions in Japanese Black cattle with low/high PVL or in healthy/lymphoma conditions. *BoLA-DRB3* genotyping was performed by sequence-based typing, and then, the genotype was analyzed by Assign 400ATF ver. 1.0.2.41 software (Gonexio Genomics, Fremantle, Australia). Association studies of *BoLA-DRB3* zygosity to PVL or lymphoma were based on Fisher's exact test (GraphPad, San Diego, California, USA). Association of *BoLA-DRB3* genotype to PVL or lymphoma was analyzed by Fisher's exact test, and the resulting *p*-value was then adjusted through Bonferroni correction. The significance was set at a level of adjusted *p*-value = 0.05. Asterisks indicate the significant genotype. S: susceptibility genotype. Only genotype frequencies of >1% are shown in the figure. (A) Frequency of *BoLA-DRB3* genotype distribution in Japanese Black cattle with low or high PVL. *BoLA-DRB3* loci were genotyped from DNA samples of 403 BLV infected asymptomatic Japanese Black cattle. A PVL of 10^4 copies/10^5 cells was set as the threshold to classify high PVL and low PVL groups. (B) Frequency of *BoLA-DRB3* genotype distribution in Japanese Black cattle in asymptomatic or lymphoma condition. *BoLA-DRB3* loci were genotyped from DNA samples of 463 BLV-infected asymptomatic and 211 BLV-infected lymphoma Japanese Black cattle. Only genotype frequencies of >1% are shown in the figure. Association of genotypes to PVL/lymphoma was analyzed by Fisher's exact test followed by Bonferroni correction. Asterisks indicate the significant genotype. S: susceptibility genotype.

Chapter 5.

General conclusions

In this research, three major conclusions have been made. First, BLV-induced lymphoma and PVL are associated with differential *BoLA-DRB3* polymorphism. Second, DRB3 binding pocket property is associated with BLV-induced lymphoma found in Japanese Black cattle. Third, *BoLA-DRB3* heterozygotes have advantage against BLV infection outcome as shown as follows:

1. In Holstein cows, **010:01* and **011:01* were specifically associated with lymphoma resistance; **002:01* and **012:01*, were specifically associated with PVL resistance and susceptibility, respectively. In contrast, lymphoma and PVL shared two resistance-associated (*DRB3*014:01:01* and **009:02*) *BoLA-DRB3* alleles. Interestingly, I found that lymphoma associated alleles are not related with the anti-BLV gp51 antibody production level in cows.
2. In Japanese Black cattle, *DRB3*011:01* was identified as a resistance allele, whereas *DRB3*005:02* and *DRB3*016:01* were susceptibility alleles. Amino acid association studies showed that positions 9, 11, 13, 26, 30, 47, 57, 70, 71, 74, 78, and 86 were associated with lymphoma susceptibility. Structure and electrostatic charge modeling further indicated that binding pocket 9 of resistance DRB3 was positively charged. In contrast, alleles susceptible to lymphoma were neutrally charged.
3. Irrespective of cattle breeds, *BoLA-DRB3* heterozygous status was associated with PVL and lymphoma resistance.

Above results found that *BoLA-DRB3* plays an independent role in lymphomagenesis and PVL clearance. The amino acid and peptide binding pocket charge of DRB3 is crucial for lymphoma susceptibility. In addition, *BoLA-DRB3* heterozygotes alleviate BLV infection outcome. These results contribute for future vaccine development as well as cattle breeding selection against BLV-induced lymphoma.

Acknowledgements

I would like to express my sincere appreciation to my supervisor Prof. Yoko Aida of RIKEN and The University of Tokyo, for her invaluable advice, continuous support, patience and guidance on my research and life during the years in Japan. In addition, I am grateful for that Prof. Aida encourages me working on diverse projects, including the chance of handing SARS-CoV-2 and joining several meeting that broadens my interests and knowledge in science.

I am thankful to Profs. Yasunobu Matsumoto, Hideki Endo, Hiromi Nakanishi and Takeshi Haga of The University of Tokyo, for being my thesis committee member and for reviewing my doctoral thesis.

I genuinely appreciate all the veterinarians and collaborators, Drs. Etsuko Saitou of Hyogo Prefectural Awaji Meat Inspection Center, Katsunori Okazaki of Health Sciences University of Hokkaido, Tetsuya Mizutani of Tokyo University of Agriculture and Technology, Kosuke Okada of Iwate University, Tatsuo Fujita of Livestock Research Institute of Oita Prefectural Agriculture Forestry and Fisheries Research Center and Hidetoshi Inoko of GenoDive Pharma Inc., who kindly help me to collect the sample for my research.

I am grateful to Prof. Shin-nosuke Takeshima of Jumonji University, for teaching me the skill in NGS experiment and data analysis. I am thankful to Dr. Susumu Saito of Jumonji University, for mentoring me and doing experiment with me together. I would like to appreciate Dr. Koya Fukunaga of RIKEN, for his guidance in bioinformatics analysis.

I appreciate the University of Tokyo for offering me the fellowship and appreciate the kindness of Dr. Satoshi Wada of RIKEN, for providing me the research allowance that supports my life in Japan.

I would like to thank all the laboratory members in the Viral Infectious Disease Unit of RIKEN, Ryosuke Matsuura, Meripet Polat, Ayumi Nakatsuchi, Maruko Soya, Samy Metwally, Rania Hamada, Wlaa Assi, Hao Chang, Liushiqi Borjigin, Lanlan Bai, Hirotaka Sato, Lowela Siarot, Yoko Hashimoto and Kyoko Matsumoto for their experimental guidance, support and accompaniment.

Finally, I greatly appreciate my father Fu-Chi Lo, mother Li-Chuan Shen and brother Mao-Wei Lo for having my back and for their warmest encouragement

Reference

Aida, Y. (1995). Characterization and expression of bovine MHC class II genes. Bulletin de la Societe Franco-Japonaise Des Sciences Veterinaires **6**: 17-24.

Aida, Y., J. Kim, N. Okimoto, J. Yamagishi, S. Tada, Y. Ito and S.-n. Takeshima (2015). A novel bovine leukemia virus peptide vaccine targeting susceptible cattle-Production by 3-D modelling and nanotechnology. Retrovirology **12**(1): P48.

Aida, Y., H. Murakami, M. Takahashi and S.-N. Takeshima (2013). Mechanisms of pathogenesis induced by bovine leukemia virus as a model for human T-cell leukemia virus. Frontiers in microbiology **4**: 328-328.

Aida, Y., S.-n. Takeshima, C. L. Baldwin and A. K. KKaushik (2015). Bovine Immunogenetics. The genetics of cattle. D. J. Garrick and A. Ruvinsky, C. A. B. International.

Andersson, L., A. Lundén, S. Sigurdardottir, C. J. Davies and L. Rask (1988). Linkage relationships in the bovine MHC region. High recombination frequency between class II subregions. Immunogenetics **27**(4): 273-280.

Arora, J., F. Pierini, P. J. McLaren, M. Carrington, J. Fellay and T. L. Lenz (2020). HLA Heterozygote Advantage against HIV-1 Is Driven by Quantitative and Qualitative Differences in HLA Allele-Specific Peptide Presentation. Molecular biology and evolution **37**(3): 639-650.

Assi, W., T. Hirose, S. Wada, R. Matsuura, S.-N. Takeshima and Y. Aida (2020). PRMT5 Is Required for Bovine Leukemia Virus Infection In Vivo and Regulates BLV Gene Expression, Syncytium Formation, and Glycosylation In Vitro. Viruses **12**(6): 650.

Bai, L., T. Hirose, W. Assi, S. Wada, S.-n. Takeshima and Y. Aida (2020). Bovine Leukemia Virus Infection Affects Host Gene Expression Associated with DNA Mismatch Repair. Pathogens **9**(11).

Bai, L., H. Sato, Y. Kubo, S. Wada and Y. Aida (2019). CAT1/SLC7A1 acts as a cellular receptor for bovine leukemia virus infection. FASEB journal : official publication of the Federation of American Societies for Experimental Biology **33**(12): 14516-14527.

Barez, P.-Y., A. de Brogniez, A. Carpentier, H. Gazon, N. Gillet, G. Gutiérrez, M. Hamaidia, J.-R. Jacques, S. Perike, S. Neelature Sriramareddy, N. Renotte, B. Staumont, M. Reichert, K. Trono and L. Willems (2015). Recent Advances in BLV Research. Viruses **7**(11): 6080-6088.

Bartlett, P. C., V. J. Ruggiero, H. A.-O. Hutchinson, C. J. Droscha, B. A.-O. Norby, K. A.-O. Sporer and T. A.-O. Taxis Current Developments in the Epidemiology and Control of Enzootic Bovine Leukosis as Caused by Bovine Leukemia Virus. LID - 10.3390/pathogens9121058 [doi] LID - 1058. (2076-0817 (Print)).

Behl, J. D., N. K. Verma, N. Tyagi, P. Mishra, R. Behl and B. K. Joshi (2012). The Major Histocompatibility Complex in Bovines: A Review. ISRN Veterinary Science **2012**: 872710.

Bendixen, H. J. (1963). PREVENTIVE MEASURES IN CATTLE LEUKEMIA: LEUKOSIS ENZOOTICA BOVIS*. Annals of the New York Academy of Sciences **108**(3): 1241-1267.

Bensaïd, A., J. R. Young, A. Kaushal and A. J. Teale (1991). Pulsed-field gel electrophoresis and its application in the physical analysis of the bovine major histocompatibility complex. Gene mapping techniques and applications. L. H. A. M. D. G. Schook L.B. New York, France, Marcel Dekker**:** 127-158, 121 micro-fiche numéro VT910156.

Blackwell, J. M., S. E. Jamieson and D. Burgner (2009). HLA and Infectious Diseases. Clinical Microbiology Reviews **22**(2): 370.

Boasso, A. (2013). Type I Interferon at the Interface of Antiviral Immunity and Immune Regulation: The Curious Case of HIV-1. Scientifica **2013**: 580968.

Bondinas, G. P., A. K. Moustakas and G. K. Papadopoulos (2007). The spectrum of HLA-DQ and HLA-DR alleles, 2006: a listing correlating sequence and structure with function. Immunogenetics **59**(7): 539-553.

Bottomly, K. (1988). A functional dichotomy in CD4+ T lymphocytes. Immunology Today **9**(9): 268-274.

Bugawan, T. L., S. J. Mack, M. Stoneking, M. Saha, H. P. Beck and H. A. Erlich (1999). HLA class I allele distributions in six Pacific/Asian populations: evidence of selection at the HLA-A locus. Tissue Antigens **53**(4): 311-319.

Bukrinsky, M. (2004). A hard way to the nucleus. Molecular medicine (Cambridge, Mass.) **10**(1-6): 1-5.

Chen, C.-J., H.-I. Yang, U. H. Iloeje and R.-H. B. V. S. G. The (2009). Hepatitis B virus DNA levels and outcomes in chronic hepatitis B. Hepatology **49**(S5): S72-S84.

Chicz, R. M., R. G. Urban, L. W.S., J. C. Gorga, L. J. Stern, D. A. Vignali and J. L. Strominger (1992). Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. (0028-0836 (Print)).

Cordell, H. J. (2009). Detecting gene-gene interactions that underlie human diseases. Nat Rev Genet **10**(6): 392-404.

Danchin, E., V. Vitiello, A. Vienne, O. Richard, P. Gouret, M. F. McDermott and P. Pontarotti (2004). The major histocompatibility complex origin. Immunological Reviews **198**(1): 216-232.

Deakin, J. E., A. T. Papenfuss, K. Belov, J. G. R. Cross, P. Coggill, S. Palmer, S. Sims, T. P. Speed, S. Beck and J. A. M. Graves (2006). Evolution and comparative analysis of the MHC Class III inflammatory region. BMC Genomics **7**(1): 281.

Debacq, C., B. Asquith, M. Reichert, A. Burny, R. Kettmann and L. Willems (2003). Reduced cell turnover in bovine leukemia virus-infected, persistently lymphocytotic cattle. Journal of virology **77**(24): 13073-13083.

Dequiedt, F., R. Kettmann, A. Burny and L. Willems (1995). Mutations in the p53 Tumor-Suppressor Gene Are Frequently Associated with Bovine Leukemia Virus-Induced Leukemogenesis in Cattle but Not in Sheep. Virology **209**(2): 676-683.

DiGiacomo, R. F., R. L. Darlington and J. F. Evermann (1985). Natural transmission of bovine leukemia virus in dairy calves by dehorning. Canadian journal of comparative medicine : Revue canadienne de medecine comparee **49**(3): 340-342.

Donaldson, P. T. (2011). Electrostatic modifications of the human leukocyte antigen DR P9 peptide-binding pocket in primary sclerosing cholangitis: Back to the future with human leukocyte antigen DRβ. Hepatology **53**(6): 1798-1800.

Drénou, B., M. Tilanus, G. Semana, M. Alizadeh, B. Birebent, J.-M. Grosset, P. Dias, D. Van Wichen, Y. Arts, D. De Santis, R. Fauchet and L. Amiot (2004). Loss of heterozygosity, a frequent but a non-exclusive mechanism responsible for HLA dysregulation in non-Hodgkin's lymphomas. British Journal of Haematology **127**(1): 40-49.

Eckert, D. M. and P. S. Kim (2001). Mechanisms of Viral Membrane Fusion and Its Inhibition. Annual Review of Biochemistry **70**(1): 777-810.

Eirin, M., H. Carignano, E. Shimizu, M. A. Pando, M. Zumárraga, G. Magnano, A. Macías, S. Garbaccio, P. Huertas, C. Morsella, X. Ferrara Muñiz, A. Cataldi, F. Paolicchi and M. Poli (2020). BoLA-DRB3 exon2 polymorphisms among tuberculous cattle: Nucleotide and functional variability and their association with bovine tuberculosis pathology. (1532-2661 (Electronic)).

Ettinger, R. A., G. K. Papadopoulos, A. K. Moustakas, G. T. Nepom and W. W. Kwok (2006). Allelic Variation in Key Peptide-Binding Pockets Discriminates between Closely Related Diabetes-Protective and Diabetes-Susceptible HLA-DQB1*06 Alleles. The Journal of Immunology **176**(3): 1988.

Excoffier, L., G. Laval and S. Schneider (2007). Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary bioinformatics online **1**: 47-50.

Florins, A., N. Gillet, M. Boxus, P. Kerkhofs, R. Kettmann and L. Willems (2007). Even attenuated bovine leukemia virus proviruses can be pathogenic in sheep. Journal of virology **81**(18): 10195-10200.

Forletti, A., C. M. Lützelschwab, R. Cepeda, E. N. Esteban and S. E. Gutiérrez (2020). Early events following bovine leukaemia virus infection in calves with different alleles of the major histocompatibility complex DRB3 gene. Veterinary Research **51**(1): 4.

Fries, R., R. Hediger and G. Stranzinger (1986). Tentative chromosomal localization of the bovine major histocompatibility complex by in situ hybridization. (0268-9146 (Print)).

Fukuyama, S., K. Kodama, T. Hirahara, N. Nakajima, K. Takamura, O. Sasaki and J. Imanishi (1993). Protection against bovine leukemia virus infection by use of inactivated vaccines in cattle. (0916-7250 (Print)).

Furtado, M. d. S. B. S., R. G. Andrade, L. C. F. Romanelli, M. A. Ribeiro, J. G. Ribas, E. B. Torres, E. F. Barbosa-Stancioli, A. B. d. F. C. Proietti and M. L. Martins (2012). Monitoring the HTLV-1 proviral load in the peripheral blood of asymptomatic carriers and patients with HTLV-associated myelopathy/tropical spastic paraparesis from a Brazilian cohort: ROC curve analysis to establish the threshold for risk disease. Journal of Medical Virology **84**(4): 664-671.

Garcia-Lora, A., I. Algarra and F. Garrido (2003). MHC class I antigens, immune surveillance, and tumor immune escape. Journal of Cellular Physiology **195**(3): 346-355.

Garrick, D. J., A. Ruvinsky and C. A. B. International (2015). The genetics of cattle.

Garstka, M. A., A. Fish, P. H. N. Celie, R. P. Joosten, G. M. C. Janssen, I. Berlin, R. Hoppes, M. Stadnik, L. Janssen, H. Ovaa, P. A. van Veelen, A. Perrakis and J. Neefjes (2015). The first step of peptide selection in antigen presentation by MHC class I molecules. Proceedings of the National Academy of Sciences **112**(5): 1505.

Ghasemi, F., T. M. Tessier, S. F. Gameiro, A. H. Maciver, M. J. Cecchini and J. S. Mymryk (2020). High MHC-II expression in Epstein–Barr virus-associated gastric cancers suggests that tumor cells serve an important role in antigen presentation. Scientific Reports **10**(1): 14786.

Gillet, N., A. Florins, M. Boxus, C. Burteau, A. Nigro, F. Vandermeers, H. Balon, A.-B. Bouzar, J. Defoiche, A. Burny, M. Reichert, R. Kettmann and L. Willems (2007). Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. Retrovirology **4**: 18-18.

Gillet, N. A., G. Gutiérrez, S. M. Rodriguez, A. de Brogniez, N. Renotte, I. Alvarez, K. Trono and L. Willems (2013). Massive Depletion of Bovine Leukemia Virus Proviral Clones Located in Genomic Transcriptionally Active Sites during Primary Infection. PLOS Pathogens **9**(10): e1003687.

Hamada, R., S. Metwally, M. Polat, L. Borjigin, A. O. Ali, A. A. A. Abdel-Hady, A. E. A. Mohamed, S. Wada and Y. Aida (2020). Detection and Molecular Characterization of Bovine Leukemia Virus in Egyptian Dairy Cattle. Frontiers in Veterinary Science **7**: 608.

Hamard-Peron, E. and D. Muriaux (2011). Retroviral matrix and lipids, the intimate interaction. Retrovirology **8**(1): 15.

Hisada, M., N. Chatterjee, Z. Kalaylioglu, R. J. Battjes and J. J. Goedert (2005). Hepatitis C virus load and survival among injection drug users in the United States. Hepatology **42**(6): 1446-1452.

Hong, F., J. L. Jacobs, E. Aga, A. R. Cillo, E. Fyne, D. L. Koontz, L. Zheng and J. W. Mellors (2018). Associations between HIV-1 DNA copy number, proviral transcriptional activity, and plasma viremia in individuals off or on suppressive antiretroviral therapy. Virology **521**: 51-57.

Hopkins, S. G. and R. F. DiGiacomo (1997). Natural Transmission of Bovine Leukemia Virus in Dairy and Beef Cattle. Veterinary Clinics of North America: Food Animal Practice **13**(1): 107-128.

Hraber, P., C. Kuiken and K. Yusim (2007). Evidence for human leukocyte antigen heterozygote advantage against hepatitis C virus infection. Hepatology **46**(6): 1713-1721.

Hsieh, J.-C., C.-Y. Li, W.-L. Hsu and S.-T. Chuang (2019). Molecular Epidemiological and Serological Studies of Bovine Leukemia Virus in Taiwan Dairy Cattle. Frontiers in Veterinary Science **6**: 427.

Huang, R., J. Yin, Y. Chen, F. Deng, J. Chen, X. Gao, Z. Liu, X. Yu and J. Zheng (2015). The amino acid variation within the binding pocket 7 and 9 of HLA-DRB1 molecules are associated with primary Sjögren's syndrome. Journal of Autoimmunity **57**: 53-59.

Iwanaga, M., T. Watanabe, A. Utsunomiya, A. Okayama, K. Uchimaru, K.-R. Koh, M. Ogata, H. Kikuchi, Y. Sagara, K. Uozumi, M. Mochizuki, K. Tsukasaki, Y. Saburi, M. Yamamura, J. Tanaka, Y. Moriuchi, S. Hino, S. Kamihira, K. Yamaguchi and A. T. L. D. i. for the Joint Study on Predisposing Factors of (2010). Human T-cell leukemia virus type I (HTLV-1) proviral load and disease progression in asymptomatic HTLV-1 carriers: a nationwide prospective study in Japan. Blood **116**(8): 1211-1219.

Jeffery, K. J. M., A. A. Siddiqui, M. Bunce, A. L. Lloyd, A. M. Vine, A. D. Witkover, S. Izumo, K. Usuku, K. I. Welsh, M. Osame and C. R. M. Bangham (2000). The Influence of HLA Class I Alleles and Heterozygosity on the Outcome of Human T Cell Lymphotropic Virus Type I Infection. The Journal of Immunology **165**(12): 7278.

Jewell, N. A. and L. M. Mansky (2000). In the beginning: genome recognition, RNA encapsidation and the initiation of complex retrovirus assembly. Journal of General Virology **81**(8): 1889-1899.

Jimba, M., S.-n. Takeshima, K. Matoba, D. Endoh and Y. Aida (2010). BLV-CoCoMo-qPCR: Quantitation of bovine leukemia virus proviral load using the CoCoMo algorithm. Retrovirology **7**: 91-91.

Jimba, M., S.-n. Takeshima, H. Murakami, J. Kohara, N. Kobayashi, T. Matsuhashi, T. Ohmori, T. Nunoya and Y. Aida (2012). BLV-CoCoMo-qPCR: a useful tool for evaluating bovine leukemia virus infection status. BMC Veterinary Research **8**(1): 167.

Johnson, R., C. D. Gibson and J. B. Kaneene (1985). Bovine leukemia virus: A herd-based control strategy. Preventive Veterinary Medicine **3**(4): 339-349.

Jones, E. Y., L. Fugger, J. L. Strominger and C. Siebold (2006). MHC class II proteins and disease: a structural perspective. Nature Reviews Immunology **6**(4): 271-282.

Juliarena, M., M. Poli, L. Sala, C. Ceriani, S. Gutierrez, G. L. Dolcini, E. Rodriguez, B. Mariño, C. Rodríguez-Dubra and E. N. Esteban (2008). Association of BLV infection profiles with alleles of the BoLA-DRB3.2 gene. Animal genetics **39**: 432-438.

Juliarena, M. A., C. N. Barrios, M. C. Ceriani and E. N. Esteban (2016). Hot topic: Bovine leukemia virus (BLV)-infected cows with low proviral load are not a source of infection for BLV-free cattle. Journal of Dairy Science **99**(6): 4586-4589.

Kathambi, E. K., G. K. Gitau, G. M. Muchemi, J. Van Leeuwen and S. Kairu-Wanyoike (2019). Seroprevalence of bovine leukemia virus infection in contrasting farming systems in Kenya. Ethiopian Veterinary Journal **23**(1): 12.

Kettmann, R., J. Deschamps, Y. Cleuter, D. Couez, A. Burny and G. Marbaix (1982). Leukemogenesis by bovine leukemia virus: proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3'

proximate cellular sequences. Proceedings of the National Academy of Sciences of the United States of America **79**(8): 2465-2469.
Kobayashi, T., Y. Inagaki, N. Ohnuki, R. Sato, S. Murakami and K. Imakawa (2019). Increasing Bovine leukemia virus (BLV) proviral load is a risk factor for progression of Enzootic bovine leucosis: A prospective study in Japan. Preventive Veterinary Medicine.
Konnai, S., S.-n. Takeshima, S. Tajima, S. A. Yin, K. Okada, M. Onuma and Y. Aida (2003). The Influence of Ovine MHC Class II DRB1 Alleles on Immune Response in Bovine Leukemia Virus Infection. Microbiology and Immunology **47**(3): 223-232.
Konnai, S., T. Usui, M. Ikeda, J. Kohara, T.-i. Hirata, K. Okada, K. Ohashi and M. Onuma (2006). Tumor necrosis factor-alpha genetic polymorphism may contribute to progression of bovine leukemia virus-infection. Microbes and Infection **8**(8): 2163-2171.
Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular Biology and Evolution **35**(6): 1547-1549.
Lützelschwab, C. M., A. Forletti, R. Cepeda, E. N. Esteban, O. Confalonieri and S. E. Gutiérrez (2016). Co-infection with Mycobacterium bovis does not alter the response to bovine leukemia virus in BoLA DRB3*0902, genetically resistant cattle. Research in Veterinary Science **109**: 10-16.
LaDronka, R. M., S. Ainsworth, M. J. Wilkins, B. Norby, T. M. Byrem and P. C. Bartlett (2018). Prevalence of Bovine Leukemia Virus Antibodies in US Dairy Cattle. Veterinary medicine international **2018**: 5831278-5831278.
Lairmore, M. D. (2014). Animal Models of Bovine Leukemia Virus and Human T-Lymphotrophic Virus Type-1: Insights in Transmission and Pathogenesis. Annual Review of Animal Biosciences **2**(1): 189-208.
Lefèbvre, L., A. Vanderplasschen, V. Ciminale, H. Heremans, O. Dangoisse, J.-C. Jauniaux, J.-F. Toussaint, V. Zelnik, A. Burny, R. Kettmann and L. Willems (2002). Oncoviral bovine leukemia virus G4 and human T-cell leukemia virus type 1 p13(II) accessory proteins interact with farnesyl pyrophosphate synthetase. Journal of virology **76**(3): 1400-1414.
Lei, W., Q. Liang, L. Jing, C. Wang, X. Wu and H. He (2012). BoLA-DRB3 gene polymorphism and FMD resistance or susceptibility in Wanbei cattle. Molecular Biology Reports **39**(9): 9203-9209.
Lewin, H. A. and D. Bernoco (1986). Evidence for BoLA-linked resistance and susceptibility to subclinical progression of bovine leukaemia virus infection. (0268-9146 (Print)).
Liu, Y.-F., C.-Y. Lin and H.-M. Hong (2017). In silico Design, Synthesis and Potency of an Epitope-based Vaccine Against Foot-and-mouth Disease Virus. International Journal of Pharmacology **13**: 122-133.
Lo, C.-W., L. Borjigin, S. Saito, K. Fukunaga, E. Saitou, K. Okazaki, T. Mizutani, S. Wada, S.-N. Takeshima and Y. Aida (2020). BoLA-DRB3 Polymorphism is Associated with Differential Susceptibility to Bovine Leukemia Virus-Induced Lymphoma and Proviral Load. Viruses **12**(3): 352.
Lo, C.-W., S.-n. Takeshima, K. Okada, E. Saitou, T. Fujita, Y. Matsumoto, S. Wada, H. Inoko and Y. Aida (2021). Association of Bovine Leukemia Virus-Induced Lymphoma with BoLA-DRB3 Polymorphisms at DNA, Amino Acid, and Binding Pocket Property Levels. Pathogens **10**(4).
Ma, B.-Y., Q.-L. Gong, C.-Y. Sheng, Y. Liu, G.-Y. Ge, D.-L. Li, N.-C. Diao, K. Shi, J.-M. Li, Z.-B. Sun, Y. Zong, X. Leng and R. Du (2021). Prevalence of bovine leukemia in 1983–2019 in China: A systematic review and meta-analysis. Microbial Pathogenesis **150**: 104681.
Mager, A., R. Masengo, M. Mammerickx and J.-J. Letesson (1994). T cell proliferative response to bovine leukaemia virus (BLV): identification of T cell epitopes on the major core protein (p24) in BLV-infected cattle with normal haematological values. Journal of General Virology **75**(9): 2223-2231.
Maloy, K. J., C. Burkhart, T. M. Junt, B. Odermatt, A. Oxenius, L. Piali, R. M. Zinkernagel and H. Hengartner (2000). CD4(+) T cell subsets during virus infection. Protective capacity depends on effector cytokine secretion and on migratory capability. The Journal of experimental medicine **191**(12): 2159-2170.
Maresca, C., S. Costarelli, A. Dettori, A. Felici, C. Iscaro and F. Feliziani (2015). Enzootic bovine leukosis: Report of eradication and surveillance measures in Italy over an 8-year period (2005–2012). Preventive Veterinary Medicine **119**(3): 222-226.
Martinez, M. L., M. A. Machado, C. S. Nascimento, M. V. G. B. Silva, R. L. Teodoro, J. Furlong, M. C. A. Prata, A. L. Campos, M. F. M. Guimarães, A. L. S. Azevedo, M. F. A. Pires and R. S. Verneque (2006). Association of BoLA-DRB3.2 alleles with tick (Boophilus microplus) resistance in cattle. (1676-5680 (Electronic)).
Marty Pyke, R., W. K. Thompson, R. M. Salem, J. Font-Burgada, M. Zanetti and H. Carter (2018). Evolutionary Pressure against MHC Class II Binding Cancer Mutations. Cell **175**(2): 416-428.e413.

Marty, R., S. Kaabinejadian, D. Rossell, M. J. Slifker, J. van de Haar, H. B. Engin, N. de Prisco, T. Ideker, W. H. Hildebrand, J. Font-Burgada and H. Carter (2017). MHC-I Genotype Restricts the Oncogenic Mutational Landscape. Cell **171**(6): 1272-1283.e1215.

Mateo, L., J. Gardner and A. Suhrbier (2001). Delayed emergence of bovine leukemia virus after vaccination with a protective cytotoxic T cell-based vaccine. (0889-2229 (Print)).

Matsumura, M., D. H. Fremont, P. A. Peterson and I. A. Wilson (1992). Emerging principles for the recognition of peptide antigens by MHC class I molecules. (0036-8075 (Print)).

Meas, S., T. Usui, K. Ohashi, C. Sugimoto and M. Onuma (2002). Vertical transmission of bovine leukemia virus and bovine immunodeficiency virus in dairy cattle herds. Veterinary Microbiology **84**(3): 275-282.

Metwally, S., R. Hamada, A. O. Ali, H. Y. A. H. Mahmoud, N. M. Baker, A. E. A. Mohamed, S. Wada, Y. Matsumoto and Y. Aida (2020). Detection and molecular characterization of bovine leukemia virus in beef cattle presented for slaughter in Egypt. The Journal of veterinary medical science **82**(11): 1676-1684.

Miller, J. M., L. D. Miller, C. Olson and K. G. Gillette (1969). Virus-Like Particles in Phytohemagglutinin-Stimulated Lymphocyte Cultures With Reference to Bovine Lymphosarcoma2. JNCI: Journal of the National Cancer Institute **43**(6): 1297-1305.

Miyasaka, T., S. n. Takeshima, M. Jimba, Y. Matsumoto, N. Kobayashi, T. Matsuhashi, H. Sentsui and Y. Aida (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**(2): 72-82.

Miyasaka, T., S. N. Takeshima, Y. Matsumoto, N. Kobayashi, T. Matsuhashi, Y. Miyazaki, Y. Tanabe, K. Ishibashi, H. Sentsui and Y. Aida (2011). The diversity of bovine MHC class II DRB3 and DQA1 alleles in different herds of Japanese Black and Holstein cattle in Japan. Gene **472**(1-2): 42-49.

Moe, K. K., M. Polat, L. Borjigin, R. Matsuura, S. T. Hein, H. H. Moe and Y. Aida (2020). New evidence of bovine leukemia virus circulating in Myanmar cattle through epidemiological and molecular characterization. PloS one **15**(2): e0229126-e0229126.

Morales, J. P. A., A. López-Herrera and J. E. Zuluaga (2020). Association of BoLA DRB3 gene polymorphisms with BoHV-1 infection and zootechnical traits. Open veterinary journal **10**(3): 331-339.

Mosmann, T. R., H. Cherwinski, B. M.W., G. M.A. and R. L. Coffman (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. (0022-1767 (Print)).

Murakami, K., S. Kobayashi, M. Konishi, K.-i. Kameyama and T. Tsutsui (2013). Nationwide Survey of Bovine Leukemia Virus Infection among Dairy and Beef Breeding Cattle in Japan from 2009–2011. Journal of Veterinary Medical Science **75**(8): 1123-1126.

Murray, J. S. (1998). How the MHC selects Th1/Th2 immunity. Immunology Today **19**(4): 157-162.

Nagaoka, Y., H. Kabeya, M. Onuma, N. Kasai, K. Okada and Y. Aida (1999). Ovine MHC Class II DRB1 Alleles Associated with Resistance or Susceptibility to Development of Bovine Leukemia Virus-induced Ovine Lymphoma. Cancer Research **59**(4): 975.

Nguyên, T. L.-A., S. de Walque, E. Veithen, A. Dekoninck, V. Martinelli, Y. de Launoit, A. Burny, R. Harrod and C. Van Lint (2007). Transcriptional Regulation of the Bovine Leukemia Virus Promoter by the Cyclic AMP-response Element Modulator τ Isoform *. Journal of Biological Chemistry **282**(29): 20854-20867.

Nieto Farias, M., M. Caffaro, L. Pamela, J. Passucci, M. Poli, C. Ceriani and G. L. Dolcini (2017). A novel association of BoLA DRB3 alleles in BLV infected cattle with different proviral loads. Brazilian Journal of Veterinary Research and Animal Science **54**: 215.

Nikbakht Brujeni, G., R. Ghorbanpour and A. Esmailnejad (2016). Association of BoLA-DRB3.2 Alleles with BLV Infection Profiles (Persistent Lymphocytosis/Lymphosarcoma) and Lymphocyte Subsets in Iranian Holstein Cattle. Biochemical Genetics **54**(2): 194-207.

Norby, B., P. C. Bartlett, T. M. Byrem and R. J. Erskine (2016). Effect of infection with bovine leukemia virus on milk production in Michigan dairy cows. Journal of Dairy Science **99**(3): 2043-2052.

Nuotio, L., H. Rusanen, L. Sihvonen and E. Neuvonen (2003). Eradication of enzootic bovine leukosis from Finland. Preventive Veterinary Medicine **59**(1): 43-49.

Ohishi, K., H. Suzuki, Y. Yasutomi, M. Onuma, K. Okada, S. Numakunai, K.-i. Ohshima, Y. Ikawa and M. Sugimoto (1992). Augmentation of Bovine Leukemia Virus (BLV)-Specific Lymphocyte Proliferation Responses in Ruminants by Inoculation with BLV env-Recombinant Vaccinia Virus: Their Role in the Suppression of BLV Replication. Microbiology and Immunology **36**(12): 1317-1323.

Ohno, A., S.-n. Takeshima, Y. Matsumoto and Y. Aida (2015). Risk factors associated with increased bovine leukemia virus proviral load in infected cattle in Japan from 2012 to 2014. Virus Research **210**: 283-290.

Ohshima, K.-i., K. Okada, S. Numakunai, Y. Yoneyama, S. Sato and K. Takahashi (1981). Evidence on Horizontal Transmission of Bovine Leukemia Virus due to Blood-sucking Tabanid Flies. The Japanese Journal of Veterinary Science **43**(1): 79-81.

OIE. (2012). "OIE Enzootic Bovine Leukosis. In: OIE Terrestrial Manual World Organisation for Animal Health."

Oldford, S. A., J. D. Robb, P. H. Watson and S. Drover (2004). HLA-DRB alleles are differentially expressed by tumor cells in breast carcinoma. International Journal of Cancer **112**(3): 399-406.

Onuma, M., T. Hodatsu, Y. S., M. Higashihara, S. Masu, T. Mikami and H. Izawa (1984). Protection by vaccination against bovine leukemia virus infection in sheep. (0002-9645 (Print)).

Othman, O. E., M. G. Khodary, A. H. El-Deeb and H. A. Hussein (2018). Five BoLA-DRB3 genotypes detected in Egyptian buffalo infected with Foot and Mouth disease virus serotype O. (2090-5920 (Electronic)).

Panei, C. J., M. S. Serena, G. E. Metz, M. E. Bravi, E. T. González and M. G. Echeverría (2013). Analysis of the pX region of bovine leukemia virus in different clinical stages of Enzootic Bovine Leukemia in Argentine Holstein cattle. Virus Research **171**(1): 97-102.

Panei, C. J., K. Suzuki, M. G. Echeverría, M. S. Serena, G. E. Metz and E. T. González (2009). Association of BoLA-DRB3.2 alleles with resistance and susceptibility to persistent lymphocytosis in BLV infected cattle in Argentina. International Journal of Dairy Science **4**(3): 123-128.

Panei, C. J., S.-n. Takeshima, T. Omori, T. Nunoya, W. C. Davis, H. Ishizaki, K. Matoba and Y. Aida (2013). Estimation of bovine leukemia virus (BLV) proviral load harbored by lymphocyte subpopulations in BLV-infected cattle at the subclinical stage of enzootic bovine leucosis using BLV-CoCoMo-qPCR. BMC Veterinary Research **9**(1): 95.

Penn, D. J., K. Damjanovich and W. K. Potts (2002). MHC heterozygosity confers a selective advantage against multiple-strain infections. Proceedings of the National Academy of Sciences **99**(17): 11260.

Pierini, F. and T. L. Lenz (2018). Divergent Allele Advantage at Human MHC Genes: Signatures of Past and Ongoing Selection. Molecular Biology and Evolution **35**(9): 2145-2158.

Pineda, M. V., M. B. Bouzas, M. Remesar, A. Fridman, C. Remondegui, L. Mammana, N. Altamirano, P. Paradiso, P. Costantini, L. Tadey, P. Aulicino and A. Mangano (2019). Relevance of HTLV-1 proviral load in asymptomatic and symptomatic patients living in endemic and non-endemic areas of Argentina. PloS one **14**(11): e0225596-e0225596.

Polat, M., A. Ohno, S.-n. Takeshima, J. Kim, M. Kikuya, Y. Matsumoto, C. N. Mingala, M. Onuma and Y. Aida (2015). Detection and molecular characterization of bovine leukemia virus in Philippine cattle. Archives of Virology **160**(1): 285-296.

Polat, M., S.-N. Takeshima and Y. Aida (2017). Epidemiology and genetic diversity of bovine leukemia virus. Virology journal **14**(1): 209-209.

Polat, M., S.-N. Takeshima, K. Hosomichi, J. Kim, T. Miyasaka, K. Yamada, M. Arainga, T. Murakami, Y. Matsumoto, V. de la Barra Diaz, C. J. Panei, E. T. González, M. Kanemaki, M. Onuma, G. Giovambattista and Y. Aida (2016). A new genotype of bovine leukemia virus in South America identified by NGS-based whole genome sequencing and molecular evolutionary genetic analysis. Retrovirology **13**: 4-4.

Pommié, C., S. Levadoux, R. Sabatier, G. Lefranc and M.-P. Lefranc (2004). IMGT standardized criteria for statistical analysis of immunoglobulin V-REGION amino acid properties. Journal of Molecular Recognition **17**(1): 17-32.

Rock, K. L., E. Reits and J. Neefjes (2016). Present Yourself! By MHC Class I and MHC Class II Molecules. Trends in immunology **37**(11): 724-737.

Rosewick, N., K. Durkin, M. Artesi, A. Marçais, V. Hahaut, P. Griebel, N. Arsic, V. Avettand-Fenoel, A. Burny, C. Charlier, O. Hermine, M. Georges and A. Van den Broeke (2017). Cis-perturbation of cancer drivers by the HTLV-1/BLV proviruses is an early determinant of leukemogenesis. Nature Communications **8**(1): 15264.

Ruggiero, V. J., B. Norby, O. J. Benitez, H. Hutchinson, K. R. B. Sporer, C. Droscha, C. L. Swenson and P. C. Bartlett (2019). Controlling bovine leukemia virus in dairy herds by identifying and removing cows with the highest proviral load and lymphocyte counts. Journal of Dairy Science **102**(10): 9165-9175.

Safari, R., J.-R. Jacques, Y. Brostaux and L. Willems (2020). Ablation of non-coding RNAs affects bovine leukemia virus B lymphocyte proliferation and abrogates oncogenesis. PLOS Pathogens **16**(5): e1008502.

Sagata, N., T. Yasunaga, J. Tsuzuku-Kawamura, K. Ohishi, Y. Ogawa and Y. Ikawa (1985). Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. Proceedings of the National Academy of Sciences of the United States of America **82**(3): 677-681.

Sajiki, Y., S. Konnai, T. Okagawa, N. Maekawa, H. Nakamura, Y. Kato, Y. Suzuki, S. Murata and K. Ohashi (2021). A TLR7 agonist activates bovine Th1 response and exerts antiviral activity against bovine leukemia virus. Developmental & Comparative Immunology **114**: 103847.

Salemi, M., J. Desmyter and A. M. Vandamme (2000). Tempo and Mode of Human and Simian T-Lymphotropic Virus (HTLV/STLV) Evolution Revealed by Analyses of Full-Genome Sequences. Molecular Biology and Evolution **17**(3): 374-386.

Sandev, N., D. Ilieva, N. Rusenova and V. Marasheva (2015). Prevalence of Enzootic Bovine Leukosis in Bulgaria. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Veterinary Medicine; Vol 72, No 1 (2015): BULLETIN OF UNIVERSITY OF AGRICULTURAL SCIENCES AND VETERINARY MEDICINE CLUJ-NAPOCA. VETERINARY MEDICINE.

Shen, W.-J., S. Zhang and H.-S. Wong (2013). An effective and effecient peptide binding prediction approach for a broad set of HLA-DR molecules based on ordered weighted averaging of binding pocket profiles. Proteome Science **11**(1): S15.

Shiina, T., K. Hosomichi, H. Inoko and J. K. Kulski (2009). The HLA genomic loci map: expression, interaction, diversity and disease. Journal of Human Genetics **54**(1): 15-39.

Shoko, C. and D. Chikobvu (2019). A superiority of viral load over CD4 cell count when predicting mortality in HIV patients on therapy. BMC Infectious Diseases **19**(1): 169.

Silvina Elena Gutiérrez, E. N. E., Claudia María Lützelschwab, Marcela Alicia Juliarena (2017). Trends and Advances in Veterinary Genetics.

Somura, Y., E. Sugiyama, H. Fujikawa and K. Murakami (2014). Comparison of the copy numbers of bovine leukemia virus in the lymph nodes of cattle with enzootic bovine leukosis and cattle with latent infection. Archives of Virology **159**(10): 2693-2697.

Stern, L. J., J. H. Brown, T. S. Jardetzky, J. C. Gorga, R. G. Urban, J. L. Strominger and D. C. Wiley (1994). Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature **368**(6468): 215-221.

Stewart, C. A., R. Horton, R. J. N. Allcock, J. L. Ashurst, A. M. Atrazhev, P. Coggill, I. Dunham, S. Forbes, K. Halls, J. M. M. Howson, S. J. Humphray, S. Hunt, A. J. Mungall, K. Osoegawa, S. Palmer, A. N. Roberts, J. Rogers, S. Sims, Y. Wang, L. G. Wilming, J. F. Elliott, P. J. de Jong, S. Sawcer, J. A. Todd, J. Trowsdale and S. Beck (2004). Complete MHC haplotype sequencing for common disease gene mapping. Genome research **14**(6): 1176-1187.

Tajima, S. and Y. Aida (2000). The region between amino acids 245 and 265 of the bovine leukemia virus (BLV) tax protein restricts transactivation not only via the BLV enhancer but also via other retrovirus enhancers. Journal of virology **74**(23): 10939-10949.

Tajima, S., Y. Ikawa and Y. Aida (1998). Complete Bovine Leukemia Virus (BLV) Provirus Is Conserved in BLV-Infected Cattle throughout the Course of B-Cell Lymphosarcoma Development. Journal of Virology **72**(9): 7569.

Tajima, S., W. Z. Zhuang, M. V. Kato, K. Okada, Y. Ikawa and Y. Aida (1998). Function and Conformation of Wild-Type p53 Protein Are Influenced by Mutations in Bovine Leukemia Virus-Induced B-Cell Lymphosarcoma. Virology **243**(1): 235-246.

Takeshima, S.-N. and Y. Aida (2006). Structure, function and disease susceptibility of the bovine major histocompatibility complex. Animal Science Journal **77**(2): 138-150.

Takeshima, S.-n., A. Ohno and Y. Aida (2019). 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. Retrovirology **16**(1): 14.

Takeshima, S.-n., Y. Sarai, N. Saitou and Y. Aida (2009). MHC class II DR classification based on antigen-binding groove natural selection. Biochemical and Biophysical Research Communications **385**(2): 137-142.

Takeshima, S., S. Chen, M. Miki, M. Kado and Y. Aida (2008). Distribution and origin of bovine major histocompatibility complex class II DQA1 genes in Japan. Tissue Antigens **72**(3): 195-205.

Takeshima, S., Y. Matsumoto, J. Chen, T. Yoshida, H. Mukoyama and Y. Aida (2008). Evidence for cattle major histocompatibility complex (BoLA) class II DQA1 gene heterozygote advantage against clinical mastitis caused by Streptococci and Escherichia species. Tissue Antigens **72**(6): 525-531.

Takeshima, S., Y. Nakai, M. Ohta and Y. Aida (2002). Short Communication: Characterization of DRB3 Alleles in the MHC of Japanese Shorthorn Cattle by Polymerase Chain Reaction-Sequence-Based Typing. Journal of Dairy Science **85**(6): 1630-1632.

Takeshima, S., N. Saitou, M. Morita, H. Inoko and Y. Aida (2003). The diversity of bovine MHC class II DRB3 genes in Japanese Black, Japanese Shorthorn, Jersey and Holstein cattle in Japan. Gene **316**: 111-118.

Takeshima, S. N., Y. Kitamura-Muramatsu, Y. Yuan, M. Polat, S. Saito and Y. Aida (2015). BLV-CoCoMo-qPCR-2: improvements to the BLV-CoCoMo-qPCR assay for bovine leukemia virus by reducing primer degeneracy and constructing an optimal standard curve. Arch Virol **160**(5): 1325-1332.

Takeshima, S. N., Y. Matsumoto, T. Miyasaka, M. Arainga-Ramirez, H. Saito, M. Onuma and Y. Aida (2011). A new method for typing bovine major histocompatibility complex class II DRB3 alleles by combining two established PCR sequence-based techniques. Tissue Antigens **78**(3): 208-213.

Thibodeau, J., M.-C. Bourgeois-Daigneault and R. Lapointe (2012). Targeting the MHC Class II antigen presentation pathway in cancer immunotherapy. Oncoimmunology **1**(6): 908-916.

Trolle, T., C. P. McMurtrey, J. Sidney, W. Bardet, S. C. Osborn, T. Kaever, A. Sette, W. H. Hildebrand, M. Nielsen and B. Peters (2016). The Length Distribution of Class I-Restricted T Cell Epitopes Is Determined by Both Peptide Supply and MHC Allele-Specific Binding Preference. Journal of immunology (Baltimore, Md. : 1950) **196**(4): 1480-1487.

Tsutsui, T., S. Kobayashi, Y. Hayama and T. Yamamoto (2016). Fraction of bovine leukemia virus-infected dairy cattle developing enzootic bovine leukosis. Preventive Veterinary Medicine **124**: 96-101.

Udina, I. G., E. E. Karamysheva, S. O. Turkova, A. R. Orlova and G. E. Sulimova (2003). Genetic Mechanisms of Resistance and Susceptibility to Leukemia in Ayrshire and Black Pied Cattle Breeds Determined by Allelic Distribution of Gene BoLA-DRB3. Russian Journal of Genetics **39**(3): 306-317.

Underwood, W. J., R. Blauwiekel, M. L. Delano, R. Gillesby, S. A. Mischler and A. Schoell (2015). Chapter 15 - Biology and Diseases of Ruminants (Sheep, Goats, and Cattle). Laboratory Animal Medicine (Third Edition). J. G. Fox, L. C. Anderson, G. M. Otto, K. R. Pritchett-Corning and M. T. Whary. Boston, Academic Press**:** 623-694.

Usui, T., S. Konnai, S. Tajima, S. Watarai, Y. Aida, K. Ohashi and M. Onuma (2003). Protective Effects of Vaccination with Bovine Leukemia Virus (BLV) Tax DNA Against BLV Infection in Sheep. Journal of Veterinary Medical Science **65**(11): 1201-1205.

Van den Broeke, A., M. Oumouna, T. Beskorwayne, M. Szynal, Y. Cleuter, S. Babiuk, C. Bagnis, P. Martiat, A. Burny and P. Griebel (2010). Cytotoxic responses to BLV tax oncoprotein do not prevent leukemogenesis in sheep. (1873-5835 (Electronic)).

Velickovic, Z. M., B. Delahunt and J. M. Carter (2002). HLA-DRB1 and HLA-DQB1 polymorphisms in Pacific Islands populations. Tissue Antigens **59**(5): 397-406.

Wang, H., K. M. Norris and L. M. Mansky (2003). Involvement of the Matrix and Nucleocapsid Domains of the Bovine Leukemia Virus Gag Polyprotein Precursor in Viral RNA Packaging. Journal of Virology **77**(17): 9431.

Wang, S. S., A. M. Abdou, L. M. Morton, R. Thomas, J. R. Cerhan, X. Gao, W. Cozen, N. Rothman, S. Davis, R. K. Severson, L. Bernstein, P. Hartge and M. Carrington (2010). Human leukocyte antigen class I and II alleles in non-Hodgkin lymphoma etiology. Blood **115**(23): 4820-4823.

Watanuki, S., S.-n. Takeshima, L. Borjigin, H. Sato, L. Bai, H. Murakami, R. Sato, H. Ishizaki, Y. Matsumoto and Y. Aida (2019). Visualizing bovine leukemia virus (BLV)-infected cells and measuring BLV proviral loads in the milk of BLV seropositive dams. Veterinary Research **50**(1): 102.

Wieczorek, M., E. T. Abualrous, J. Sticht, M. Á lvaro-Benito, S. Stolzenberg, F. Noé and C. Freund (2017). Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. Frontiers in Immunology **8**: 292.

Willems, L., H. Heremans, G. Chen, D. Portetelle, A. Billiau, A. Burny and R. Kettmann (1990). Cooperation between bovine leukaemia virus transactivator protein and Ha-ras oncogene product in cellular transformation. The EMBO journal **9**(5): 1577-1581.

Willems, L., P. Kerkhofs, F. Dequiedt, D. Portetelle, M. Mammerickx, A. Burny and R. Kettmann (1994). Attenuation of bovine leukemia virus by deletion of R3 and G4 open reading frames. Proceedings of the National Academy of Sciences of the United States of America **91**(24): 11532-11536.

Xu, A., M. J. v. Eijk, C. Park and H. A. Lewin (1993). Polymorphism in BoLA-DRB3 exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. (0022-1767 (Print)).

Yoshida, T., H. Mukoyama, H. Furuta, Y. Kondo, S.-n. Takeshima, Y. Aida, M. Kosugiyama and H. Tomogane (2009). Association of the amino acid motifs of BoLA-DRB3 alleles with mastitis pathogens in Japanese Holstein cows. Animal Science Journal **80**(5): 510-519.

Yuan, Y., Y. Kitamura-Muramatsu, S. Saito, H. Ishizaki, M. Nakano, S. Haga, K. Matoba, A. Ohno, H. Murakami, S. N. Takeshima and Y. Aida (2015). Detection of the BLV provirus from nasal secretion and saliva samples using BLV-CoCoMo-qPCR-2: Comparison with blood samples from the same cattle. Virus Res **210**: 248-254.

Zhang, J., Y. Chen, J. Qi, F. Gao, Y. Liu, J. Liu, X. Zhou, J. Kaufman, C. Xia and G. F. Gao (2012). Narrow Groove and Restricted Anchors of MHC Class I Molecule BF2*0401 Plus Peptide Transporter Restriction Can Explain Disease Susceptibility of B4 Chickens. The Journal of Immunology **189**(9): 4478.

Zhao, X. and G. C. Buehring (2007). Natural genetic variations in bovine leukemia virus envelope gene: Possible effects of selection and escape. Virology **366**(1): 150-165.
Zhou, H.-X. and X. Pang (2018). Electrostatic Interactions in Protein Structure, Folding, Binding, and Condensation. Chemical reviews **118**(4): 1691-1741.
Zhuang, W., S. Tajima, K. Okada, Y. Ikawa and Y. Aida (1997). Point mutation of p53 tumor suppressor gene in bovine leukemia virus-induced lymphosarcoma. (0887-6924 (Print)).
Zyrianova, I. M. and S. N. Kovalchuk (2020). Bovine leukemia virus tax gene/Tax protein polymorphism and its relation to Enzootic Bovine Leukosis. Virulence **11**(1): 80-87.

Summary

Chapter 1: Introduction and research aim

Bovine leukemia virus (BLV) infects cattle worldwide and causes enzootic bovine leucosis, the B-cell lymphoma in cattle. During infection, the DNA copies of BLV genome integrates into host genome called as provirus leading to lifelong infection. The provirus load (PVL) is positively related with viral transmission as well as disease progression. So far, no therapy and prevention methods have yet been established. Therefore, cattle breeding selection based on disease resistance gene might be a promising strategy to reduce BLV-induced damages. *BoLA-DRB3* is a highly polymorphic gene which is responsible for antigen presentation and therefore associated with several cattle diseases. This research aimed to investigate the relationship between *BoLA-DRB3* polymorphisms with BLV infection outcome (PVL level and lymphoma development) in cattle.

Chapter 2: Differential association between BoLA-DRB3 Polymorphism with BLV-Induced Lymphoma and Proviral Load

Polymorphism of *BoLA-DRB3* is reported to associate with PVL; however, little is known about the relationship of *BoLA-DRB3* polymorphism with BLV-induced lymphoma. Furthermore, whether or not PVL-associated *BoLA-DRB3* allele is linked to lymphoma-associated *BoLA-DRB3* allele has not been clarified. In this chapter, using Holstein cows as a model, I compared the association between *BoLA-DRB3* polymorphism with BLV-induced lymphoma and with PVL. I found that two *BoLA-DRB3* alleles were specifically associated with lymphoma resistance (**010:01* and **011:01*); Two other alleles, **002:01* and **012:01*, were associated with PVL resistance and susceptibility, respectively. In contrast, lymphoma and PVL shared two resistance-associated (*DRB3*014:01:01* and **009:02*) *BoLA-DRB3* alleles. Interestingly, we found that PVL associated alleles, but not lymphoma associated alleles, are related with the anti-BLV gp51 antibody production level in cows. Overall, this study is the first to demonstrate that the *BoLA-DRB3* polymorphism confers differential susceptibility to BLV-induced lymphoma and PVL.

Chapter 3: Association of BLV -induced lymphoma with BoLA-DRB3 polymorphisms at DNA, amino acid, and binding pocket property levels

The study In Chapter 2 shows that the DNA sequence polymorphisms of *BoLA-DRB3* allele have exhibited a correlation with BLV-induced lymphoma in Holstein cows. However, the association may vary between different cattle breeds and the information in Japanese black cattle is not yet available. In this chapter, I comprehensively analyzed the correlation between BLV-induced lymphoma and DRB3 allele types at DNA, amino acid, and binding pocket property levels in Japanese black cattle. I found that *DRB3*011:01* was identified as a resistance allele, whereas *DRB3*005:02* and *DRB3*016:01* were susceptibility alleles. Amino acid association studies showed that positions 9, 11, 13, 26, 30, 47, 57, 70, 71, 74, 78, and 86 were associated with lymphoma susceptibility. Structure and electrostatic charge modeling further indicated that binding pocket 9 of resistance DRB3 was positively charged. In contrast, alleles susceptible to lymphoma were neutrally charged. Altogether, this is the first association study of BoLA-DRB3 polymorphisms with BLV-induced lymphoma in Japanese black cattle. In addition, these results further contribute to understanding the mechanisms regarding how *BoLA-DRB3* polymorphisms mediate susceptibility to BLV-induced lymphoma.

Chapter 4: BoLA-DRB3 heterozygote advantage against the outcome of BLV infection

Host genetic heterozygosity at major histocompatibility complex is thought to have enhanced ability against infectious diseases due to recognizing a more divert antigen pool. However, whether heterozygote of *BoLA-DRB3* has advantage against BLV induced-lymphoma and PVL still unclear. In this chapter, I found heterozygote at *BoLA-DRB3* has advantage against BLV PVL and BLV-induced lymphoma in both Holstein cows and Japanese black cattle.

Chapter 5: General conclusions and perspectives

First, I found that BLV-induced lymphoma compared with PVL is associated with differential *BoLA-DRB3* polymorphisms in cattle. This result could be an important reference for accurate cattle breeding selection to combat BLV-induced lymphoma. Second, I identified the electrostatic charge difference between lymphoma resistant and susceptible *BoLA-DRB3* at the encoding DRBβ binding pocket 9 (a structure for antigen binding). This finding potentially contributes in future vaccine development for BLV-induced lymphoma as the charge of binding pocket is a key factor for antigen interaction and triggering effective immune response and therefore, the charge of peptide vaccine should be taken in account to fit with the charge of BoLA-DRB3 binding pockets. Third, I found that *BoLA-*

DRB3 heterozygous status was significantly associated with both PVL and lymphoma resistance irrespective of cattle breeds. This result suggests that cattle breeding in BoLA-DRB3 heterozygous setting could potentially reduce the occurrence rate/ level of BLV-induced lymphoma and PVL.