

## ORIGINAL ARTICLE

# A pooled testing system to rapidly identify cattle carrying the elite controller *BoLA-DRB3\*009:02* haplotype against bovine leukemia virus infection

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As genetically resistant individuals, the “elite controllers” (ECs) of human immunodeficiency virus infection have been focused on as the keys to developing further functional treatments in medicine. In the livestock production field, identifying the ECs of bovine leukemia virus (BLV) infection in cattle is desired to stop BLV transmission chains on farms. Cattle carrying the bovine leukocyte antigen (*BoLA*)-*DRB3\*009:02* allele (*DRB3\*009:02*) have a strong possibility of being BLV ECs. Most of cattle carrying this allele maintain undetectable BLV proviral loads and do not shed virus even when infected. BLV ECs can act as transmission barriers when placed between uninfected and infected cattle in a barn. To identify cattle carrying *DRB3\*009:02* in large populations more easily, we developed a pooled testing system. It employs a highly sensitive, specific real-time PCR assay and TaqMan MGB probes (*DRB3\*009:02*-TaqMan assay). Using this system, we determined the percentage of *DRB3\*009:02*-carrying cattle on Kyushu Island, Japan. Our pooled testing system detected cattle carrying the *DRB3\*009:02* allele from a DNA pool containing one *DRB3\*009:02*-positive animal and 29 cattle with other alleles. Its capacity is sufficient for herd-level screening for *DRB3\*009:02*-carrying cattle. The *DRB3\*009:02*-TaqMan assay showed high-discriminative sensitivity and specificity toward *DRB3\*009:02*, making it suitable for identifying *DRB3\*009:02*-carrying cattle in post-screening tests on individuals. We determined that the percentage of *DRB3\*009:02*-carrying cattle in Kyushu Island was 10.56%. With its ease of use and reliable detection, this new method strengthens the laboratory typing for *DRB3\*009:02*-carrying cattle. Thus, our findings support the use of BLV ECs in the field.

## KEYWORDS

allele-specific PCR, *BoLA-DRB3\*009:02*, bovine leukemia virus, elite controllers, pooled samples, TaqMan assay

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## 1 | INTRODUCTION

Retroviruses infect a wide range of mammals and cause various disease processes in them including immunodeficiencies, neurological disorders, and tumor development.<sup>1</sup> The pathogenicity is heterogenous and depends on the virus–host interaction. Interestingly, there are rare patients with the in-built ability to keep human immunodeficiency virus (HIV) proviral loads below the detection limits of viral load assays without receiving antiviral therapy, the so-called “elite controllers” (ECs).<sup>2</sup> They are understood to have specific HLA haplotypes (*B\*27*, *B\*57*, and *B\*14* alleles).<sup>3–5</sup> Although attention has been directed at ECs as potential keys to opening further functional treatments in medicine, the percentage of ECs in the population is less than 1%.<sup>6</sup> The desirability of identifying the ECs of retroviruses also exists in the livestock production field. Therefore, identifying them would help to stop the spread of bovine leukemia virus (BLV), for which no vaccines or treatments are available. To identify these seldomly observed ECs in animals requires the development of a high-throughput genotyping identification strategy.

BLV, a *Deltaretrovirus* genus member within the *Retroviridae* family, causes the malignant B-cell lymphoma known as enzootic bovine leukosis. The similar structure and properties of BLV make it closely related to human T-lymphotropic virus type 1. BLV transmits via infected lymphocytes from infected cattle to uninfected ones. Infected cattle remain infected over their lifetimes. Upon infection with BLV, 30% of cattle develop persistent B-cell lymphocytosis, and fewer than 5% develop lymphosarcoma after a long latent period.<sup>7–9</sup> BLV infection, a chronic wasting disease, is responsible for reduced milk productivity and fertility rate decline, and is a lifelong illness even when the disease caused by it (enzootic bovine leukosis) becomes subclinical.<sup>10–13</sup>

The high global prevalence of BLV makes its eradication in the field by culling all the cattle infected with it economically impractical.<sup>14</sup> Therefore, keeping BLV-infected cattle requires the establishment of a BLV control strategy. The current BLV control strategies used for within-farm transmission are based on isolating BLV-infected cattle from the herd or barn.<sup>15</sup> They also involve avoiding other iatrogenic factors such as the repeated use of contaminated needles, dehorning, and rectal palpation via the use of a common sleeve.<sup>16–18</sup> Implementing these control strategies, however, requires enough space to separate the animals and this can be costly. Thus, effective BLV control is limited by a farm's size capacity.

Another control option for BLV involves the use of BLV ECs, which focuses on halting the within-herd BLV transmission chain.<sup>12</sup> Haplotypes of the bovine leukocyte

antigen (*BoLA*) class II-*DRB3* exon 2 region are associated with disease susceptibility in individuals.<sup>19</sup> Cattle carrying the *BoLA* class II-*DRB3\*009:02* allele (*DRB3\*009:02*) are known to be strongly associated with BLV dissemination resistance. Most of cattle carrying this allele results in an undetectable provirus status and arrested disease progression, even when infected.<sup>20–24</sup> A previous field study reported that cattle carrying *DRB3\*009:02* with absence of provirus were not the transmission sources of BLV.<sup>23</sup> Based on these knowledge, property of carrying *DRB3\*009:02* is useful marker for the screening of BLV ECs. On the other hands, minor population of cattle carrying *DRB3\*009:02* have been reported to have detectable BLV provirus and progress lymphoma<sup>25–28</sup> because BLV resistance seems to be determined by the combination of *DRB3* heterozygous alleles.<sup>29,30</sup> It is important to confirm not only *DRB3\*009:02* but also undetectable provirus level in BLV infected cattle. BLV ECs could be used for BLV transmission control by using them as barriers between BLV-infected and uninfected cattle, even when the farmer lacks enough space to separate them. However, at less than 10% of a population, cattle carrying this allele are rare.<sup>24</sup> Hence, genotyping all cattle to identify the relevant allele in a large population has huge costs and is laborious. To tackle this problem, pooled testing is a potentially useful screening method for identifying the rare target animals in a large population because of its time and cost advantages. Indeed, it has been used for surveying, monitoring, and epidemiological disease investigations.<sup>31–34</sup> Therefore, the objective of this study was to develop a pooled testing system with which to identify *DRB3\*009:02*-carrying cattle based on highly sensitive, specific real-time PCR with TaqMan minor groove binder (MGB) probe design (*DRB3\*009:02*-TaqMan assay).

## 2 | MATERIALS AND METHODS

### 2.1 | Samples used for evaluating the diagnostic sensitivity and specificity of the *DRB3\*009:02*-TaqMan assay

Genomic DNA samples, which we extracted from the whole blood samples of 150 cattle in Japan, comprised 25 samples from a farm in Hokkaido prefecture, 116 from five farms in Miyazaki prefecture, and nine from a farm in which the presence of cattle carrying *DRB3\*009:02* had already been confirmed in Oita prefecture.<sup>24</sup> Genomic DNA was extracted from each whole peripheral blood sample using the Wizard<sup>®</sup> Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA) or the magLEAD Consumable Kit with an automated nucleic

acid extraction system (magLEAD 12gC; Precision System Science Co., Ltd., Chiba, Japan) in accordance with the manufacturer's instructions.

To determine the *DRB3\*009:02*-TaqMan assay's performance for alleles that were not present in the current field samples, we used an artificially synthesized plasmid DNA containing the sequences of these alleles. For *DRB3\*009:01*, the pcDNA3.1D/V5-His-TOPO<sup>®</sup> vector (Invitrogen, Thermo Fisher Scientific Inc., MA, USA), which contains the cattle *DRB3\*009:01* (ACNO: MT890683) cDNA that was constructed previously, was used. This plasmid DNA contained the sequence of each allele including the primer and probe sites designed in this study.

## 2.2 | Primer-probe design

We designed primers and probe-based selective assays for *DRB3\*009:02* (ACNO: LR797970). For the primer-based selection, HiDi Taq DNA polymerase (myPOLs Biotec Konstanz, Germany), which has a discrimination ability at the 3' terminal nucleotide of the primer, was used. For the probe-based selection, the assay was designed to obtain a high-melting temperature ( $T_m$ ) value to enhance the MGB probe's specificity. Allele-specific primers and probe were designed based on the 357 *DRB3* sequences obtained from the IPD-MHC (major histocompatibility) database.<sup>35</sup> These FASTA format sequences were first imported into MEGA X software,<sup>36</sup> and sequence alignments were performed using ClustalW.<sup>37</sup> On the basis of such alignments, minor nucleotides in *DRB3\*009:02* were chosen as candidates for the 3' termini of the forward and reverse primer sites. We checked the internal sequences of the primer sets to identify the most suitable sequence with which to obtain a high-specificity probe. Finally, the primer sequence was obtained by keeping the 3' terminal nucleotide and adjusting the  $T_m$ . The primers and probe were custom-made by Eurofins Genomics (Eurofins Genomics K.K., Tokyo, Japan).

## 2.3 | Using conventional PCR to check the specificity of the primers

To check the specificity of the designed primer set, conventional PCR (cPCR) was performed with HiDi 2x PCR Master Mix (myPOLs Biotec). This reagent uses HiDi DNA polymerase, which has the same activity as HiDi Taq DNA polymerase. Genomic DNA from cattle heterozygous for *DRB3\*009:02* and 015:01, *DRB3\*034:01* and 005:03, or *DRB3\*001:01* and 014:01:01, and *DRB3\*009:01*-encoding plasmid DNA, were tested. We

tested *DRB3\*009:02* and 009:01 because the complete sequences of the forward and reverse primers are identical and both will therefore be amplified. *DRB3\*034:01* was tested to check the discriminative ability of the primers because the forward primer's sequence is identical to *DRB3\*009:02*, and only the 3' terminal of the reverse primer's nucleotide differs from *DRB3\*009:02*. DNA from cattle carrying *DRB3\*001:01* and 014:01:01 was tested to check for nonspecific reactions because these alleles do not have primers targeting their 3' terminal nucleotides. The components of the reaction mix were set in accordance with the manufacturer's instructions from the HiDi 2x PCR Master Mix as well as the amplification profile.

## 2.4 | *DRB3\*009:02*-TaqMan assay

PCRs were optimized in reaction mixes containing 0.3  $\mu$ l of HiDi Taq DNA polymerase, 2.5  $\mu$ l of 10  $\times$  HiDi reaction buffer, 200  $\mu$ M of dNTPs (TOYOBO Co., Ltd., Osaka, Japan), 0.6  $\mu$ M of each primer, 0.3  $\mu$ M of probe, 0.1  $\mu$ l of 50x Rox reference (Invitrogen, Thermo Fisher Scientific Inc.), 50 ng of template DNA, and PCR-grade water up to 25  $\mu$ l. The detailed quantity of each reagent used in the reaction mix is shown in Table S1. The conditions used for genome amplification involved an initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 10 s, and annealing and elongation at 65°C for 1 min. Real-time PCRs were conducted using the QuantStudio 3 system (Applied Biosystems, Thermo Fisher Scientific Inc.). Samples were considered to be positive when  $C_t$  values of 0.15  $\Delta R_n$  (an indicator of fluorescence) were obtained with fewer than 40 cycles and a positive signal.

To check the sensitivity and specificity of the *DRB3\*009:02*-TaqMan assay, genomic DNA from *DRB3\*009:02* and 015:01 heterozygous cattle and the plasmid DNA encoding the *DRB3\*009:01* sequence were tested. *DRB3\*009:01* was tested to check the probe's specificity because three nucleotides in the probe differ from *DRB3\*009:02* and both primer sequences were identical.

## 2.5 | Identification of *DRB3* exon 2 alleles in field samples using PCR-RFLP and sequencing-based genotyping

We used PCR-RFLP and DNA sequencing to identify *DRB3* exon 2 (*DRB3.2*) alleles in 150 field samples. The PCR-RFLP method is frequently used for *DRB3.2* because it provides information on candidate alleles in samples; it can also distinguish heterozygous and homozygous alleles.<sup>24,29,38</sup> The resultant PCR products were digested

with *RsaI*, *HaeIII*, and *BstYI* (New England Biolabs, MA, USA). First-round *DRB3.2* PCRs were performed in 20  $\mu$ l volumes each containing 0.2  $\mu$ l of TaKaRa Ex Taq HS (TaKaRa Bio Inc., Shiga, Japan), 2  $\mu$ l of 10x Ex Taq buffer (TaKaRa Bio Inc.), 1.6  $\mu$ l of dNTP Mix (TaKaRa Bio Inc.), 0.2  $\mu$ l of HL030 (5'-ATCCTCTCTCTGCAGCACATTTTC-3') and HL031 (5'-TTTAAATTCGCGCTCACCTCGCCGCT-3') primers (10  $\mu$ M each), 14.8  $\mu$ l of PCR-grade water, and 1  $\mu$ l of template DNA. Second-round PCRs were performed to increase the PCR product yield and obtain higher specificity. The 40  $\mu$ l volumes each contained 0.2  $\mu$ l of TaKaRa Ex Taq, 4  $\mu$ l of 10x Ex Taq buffer, 3.2  $\mu$ l of dNTP Mix, 0.2  $\mu$ l of HL030 and HL032 primers (5'-TCGCCGCTGCACAGTGAAGTCTC-3') (10  $\mu$ M each), 30.2  $\mu$ l of PCR-grade water and 1  $\mu$ l of the amplicon from the first-round PCR. PCRs were performed using an initial denaturation of 98°C for 30 s, followed by 10 cycles (first-round) or 35 cycles (second-round) of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, elongation at 72°C for 30 s, and a final extension at 72°C for 7 min. Second-round amplicons (10  $\mu$ l) were incubated with *RsaI* and *HaeIII* at 37°C for 6 h in 15  $\mu$ l volumes containing 0.5  $\mu$ l of enzyme (equal to 5 U), 1.5  $\mu$ l of CutSmart Buffer (New England Biolabs), and 3  $\mu$ l of PCR-grade water. For the *BstYI* reaction, each second-round PCR amplicon was incubated with *BstYI* at 60°C for 5 h in 15  $\mu$ l volumes containing 0.5  $\mu$ l of *BstYI* (equal to 5 U), 1.5  $\mu$ l of NEBuffer 2.1 (New England Biolabs), 10  $\mu$ l of the second-round PCR amplicon, and 3  $\mu$ l of PCR-grade water. The digested second-round PCR amplicons were electrophoresed on 6% polyacrylamide gels to obtain their restriction patterns. Using this method, the restriction pattern numbers which represents *DRB3* alleles in same classification according to the combination of restriction pattern of these enzymes (defined in Reference 38), we identified were 1, 2, 3, 6, 7, 8, 10, 11, 15, 16, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 32, 41, and 45. This indicates that the diversity of *DRB3.2* alleles in the samples used in this study were not biased toward specific alleles. Of note, samples that produced an E pattern with *BstYI* indicate that the *DRB3.2* allele 11 is represented by either *DRB3\*009:01*, *009:02*, or *009:03*. We identified 17 samples with this pattern.

Sanger sequencing was used for *DRB3.2* allele genotyping. The second-round amplicons obtained from the PCR-RFLP were extracted from 2% agarose gels using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). HL030 and HL032 primers were used for sequencing with the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Thermo Fisher Scientific Inc.) and the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific Inc.) in accordance with the manufacturer's

TABLE 1 Allele combinations for PCR-RFLP heterozygous allele 11

ID	Allele 1	Allele 2
#1	009:02	016:01
#2	009:02	015:01
#3	009:02	015:01
#4	009:02	002:01
#5	009:02	015:01
#6	009:02	007:01
#7	009:02	015:01
#8	009:02	015:01
#9	009:02	007:01
#10	009:02	007:01
#11	009:02	016:01
#12	009:02	016:01
#13	009:02	016:01
#14	009:02	016:01
#15	009:02	016:01
#16	009:02	010:01
#17	009:02	010:01

instructions. The resulting data were analyzed using GENETYX Ver. 15 software (GENETYX Corp., Tokyo, Japan). Heterozygous base-calling was used for the samples with heterozygous alleles. In such cases, *DRB3.2* alleles were determined by merging the sequence data and PCR-RFLP patterns. For the 17 *DRB3.2* allele 11-positive samples, we found that they were all were heterozygotes, including *DRB3\*009:02* (Table 1).

## 2.6 | Evaluating the diagnostic sensitivity and specificity of the *DRB3\*009:02*-TaqMan assay

Altogether, 150 field samples were used to evaluate the diagnostic sensitivity and specificity of the *DRB3\*009:02*-TaqMan assay compared with using combined PCR-RFLP and sequencing analysis. The *DRB3\*009:02*-TaqMan assay was performed twice in independent reactions for all samples. Ct values at 0.15  $\Delta$ Rn were obtained.

To determine the diagnostic sensitivity and specificity of the *DRB3\*009:02*-TaqMan assay with combined PCR-RFLP-Sanger sequencing, the kappa value was calculated to measure the agreement between the two different methods; that is, whether the samples were positive or negative in the *DRB3\*009:02*-TaqMan assay versus

*DRB3\*009:02* or not in the combined PCR-RFLP–Sanger sequencing.

## 2.7 | SYBR Green assay for *DRB3\*009:02*

To compare the *DRB3\*009:02*-TaqMan assay's performance with the previously described SYBR Green assay,<sup>39</sup> 150 field samples were tested using both methods. SYBR Green detection of *DRB3\*009:02* was performed in 20 µl reactions containing 10 µl of FastStart Universal SYBR Green Master (Rox) (Hoffmann-La Roche Ltd., Basel, Switzerland), 0.6 µl of forward (5'-CCTGGAGTATTCTAAGAGCG-3') and reverse (5'-CGCTCTCCTCCAGGATC-3') primers (10 µM each, equal to 0.3 µM), 6.8 µl of PCR-grade water, and 2 µl of 25 ng/µl DNA (equal to 50 ng/reaction mixture). PCRs were performed using initial pre-cycling (50°C for 2 min), initial denaturation (95°C for 10 min; 40 cycles of denaturation, 95°C for 10 s), and annealing and extension (60°C for 1 min). Post-amplification melting curve analysis was performed. The real-time PCRs were conducted using the QuantStudio 3 system. Ct values at 0.2 ΔRn were obtained. All samples were tested twice independently.

## 2.8 | Application of *DRB3\*009:02*-TaqMan assay to the pooled testing system

The *DRB3\*009:02*-TaqMan assay's performance on pooled DNA samples was determined to further identify *DRB3\*009:02*-carrying cattle in a population with a low percentage of this allele. One *DRB3\*009:02*-positive sample was pooled with 29 *DRB3\*009:02*-negative samples (1:29 pooling ratio) (*DRB3\*009:02*-containing DNA pool). This sample was used as a template for the *DRB3\*009:02*-TaqMan assay in the PCRs at 500, 250, 100, 50, 10, and 1 ng. Of note, 500, 250, 100, 50, 10, and 1 ng of the *DRB3\*009:02*-containing DNA pool contained 16.7, 8.3, 3.3, 1.67, 333, and 33.3 pg of DNA with *DRB3\*009:02*, respectively. A DNA pool of 29 samples without *DRB3\*009:02* (*DRB3\*009:02*-NOT-containing DNA pool) was also tested concurrently as the negative control. The PCR-RFLP patterns of the 30 samples used in this test are shown in Table S2.

## 2.9 | Regional survey of the percentage of *DRB3\*009:02*-carrying cattle in Kyushu Island, Japan

To determine the percentage of *DRB3\*009:02*-carrying cattle on Kyushu island, we employed the pooled testing

system and individual testing based on the *DRB3\*009:02*-TaqMan assay. Altogether, 180 cattle blood samples from 115 farms in Kyushu Island were collected in ethylenediaminetetraacetic acid tubes from July to August 2021. The age of 173 of these cattle was 6–10 months. The others were more than 15 months old. All samples were stored at 4°C in the laboratory of the University of Miyazaki until testing.

Next, batches containing 30 individual blood samples were mixed together in Eppendorf tubes. Genomic DNAs from these pooled bloods were extracted using the Wizard<sup>®</sup> Genomic DNA Purification Kit or the magLEAD Consumable Kit with an automated nucleic acid extraction system in accordance with the manufacturer's instructions. The *DRB3\*009:02*-TaqMan assay identified the presence of the *DRB3\*009:02* allele in each pool. Finally, genomic DNA from all 180 blood samples was extracted using the methods described above and the *DRB3\*009:02*-TaqMan assay was performed individually on the samples. We calculated the percentage of cattle carrying *DRB3\*009:02* alleles by dividing the number of PCR-positive individuals by the total number of samples × 100.

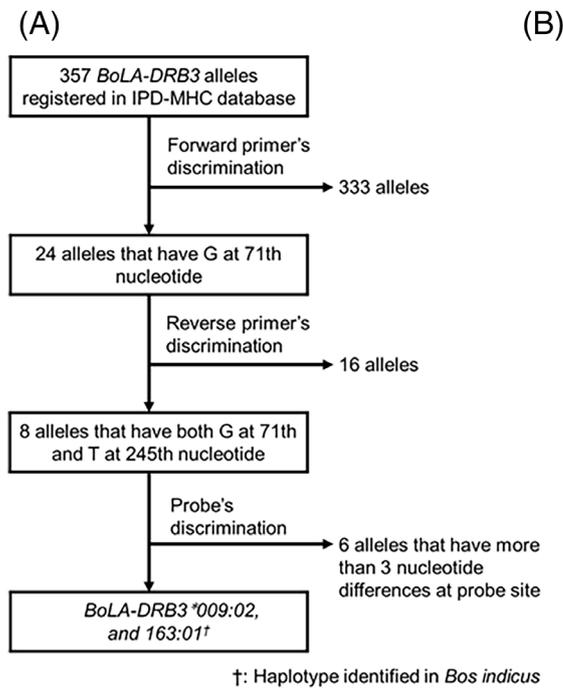
## 3 | RESULTS

### 3.1 | Primer/probe sequences and the performance of the *DRB3\*009:02*-TaqMan assay

On the basis of the discrimination ability at the 3' terminal nucleotide in the primer site by HiDi Taq DNA polymerase, the 71st G and the 245th T in the 270-bp *DRB3\*009:02* nucleotide sequence were selected for the 3' terminal nucleotides of the forward and reverse primers, respectively (Figure 1A). The forward primer (5'-GGGTGCGGTTCTGGAG-3'), reverse primer (5'-CGCTGCACAGTGAAACTCTCA-3') and probe (5'-FAM-AAGGAGATCCTGGAGGAGAGGC-MGB-Eclipse-3') were designed (Figure 1B). By aligning eight alleles that reacted to restriction digests with the forward and reverse primers, we confirmed that the probe's specificity sufficiently discriminated *DRB3\*009:02* from other alleles, except *DRB3\*163:01* (Figure 1C).

As shown in Figure 2, the designed primer set only amplified genomic DNA from cattle that were heterozygous for *DRB3\*009:02* and 015:01 or the plasmid DNA encoding *DRB3\*009:01* in the cPCRs. As expected, genomic DNA from cattle that were heterozygous for *DRB3\*034:01* and 005:03 and heterozygous for *DRB3\*001:01* and 014:01:01 failed to amplify. Thus, we used the designed primer set for the *DRB3\*009:02*-TaqMan assay.

**FIGURE 1** Primer and probe design for discriminating *DRB3\*009:02* from other alleles. (A) Schematic diagram showing the discrimination strategy used for *DRB3\*009:02*. The efficiency of HiDi Taq DNA polymerase decreased dramatically when the 3' terminal nucleotides in the primers and sample DNA differed. We therefore selected the 71st G for the target 3' terminal nucleotide in the forward primer and 245th T for the target 3' terminal nucleotide in the reverse primer. From 357 alleles, eight alleles including *DRB3\*009:02* were amplifiable by this primer. By merging the probe's discrimination ability, only *DRB3\*009:02* and *DRB3\*163:01* generated positive signals. (B) The primer and probe positions in the *DRB3\*009:02* sequence are shown. Gray highlight indicates the primer sites and underlining indicates the probe sites. The 3' terminal nucleotides in the primers are indicated in red. (C) Sequences in the probe's site for eight alleles that were amplified by the primers are shown. Gray highlight indicates the different nucleotides in *DRB3\*009:02*



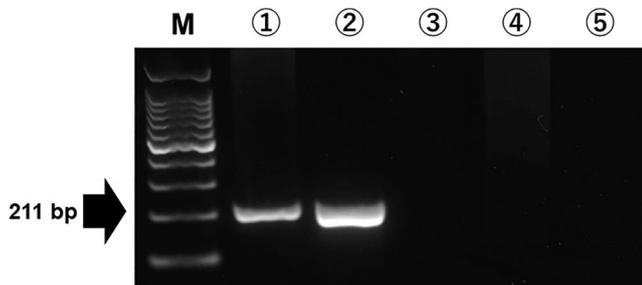
(B)

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    5'-
    CACATTTCTGGAGTATTCTAAGA
    GCGAGTGTCATTTCTTCAACGGG
    ACCGAGCGGGTGCGGTTCTCTGG
    AGAGATCCTTCTATAATGGAGAA
    GAGAACGTGCGCTTCGACAGCG
    ACTGGGGCGAGTACCGGGCGGT
    GACCGAGCTAGGGCGGCCGGA
    CGCCGAGTACTGGAACAGCCAG
    AAGGAGATCCTGGAGGAGAGGC
    GGGCCGAGGTGGACAGGGTGT
    GCAGACACAACACTACGGGGTTCGG
    TGAGAGTTTCACTGTGCAGCGG
    CGAG-3'
  
```

(C)

Allele	Sequence at probe site
009:02	A A G G A G A T C C T G G A G G A G A G G C
163:01	A A G G A G A T C C T G G A G G A G A G G C
009:01	A A G G A G A T C C T G G A G C G G A A G C
027:15	A A G G A C T T C C T G G A G C A G A G G C
003:01	A A G G A C T T G C T G G A G C A G A A G C
080:01	A A G G A C T T C C T G G A G C G G A A G C
034:03	A A G G A C A T C C T G G A G C G G A A G C
073:01	A A G G A C A C C C T G G A G C G G G C G C

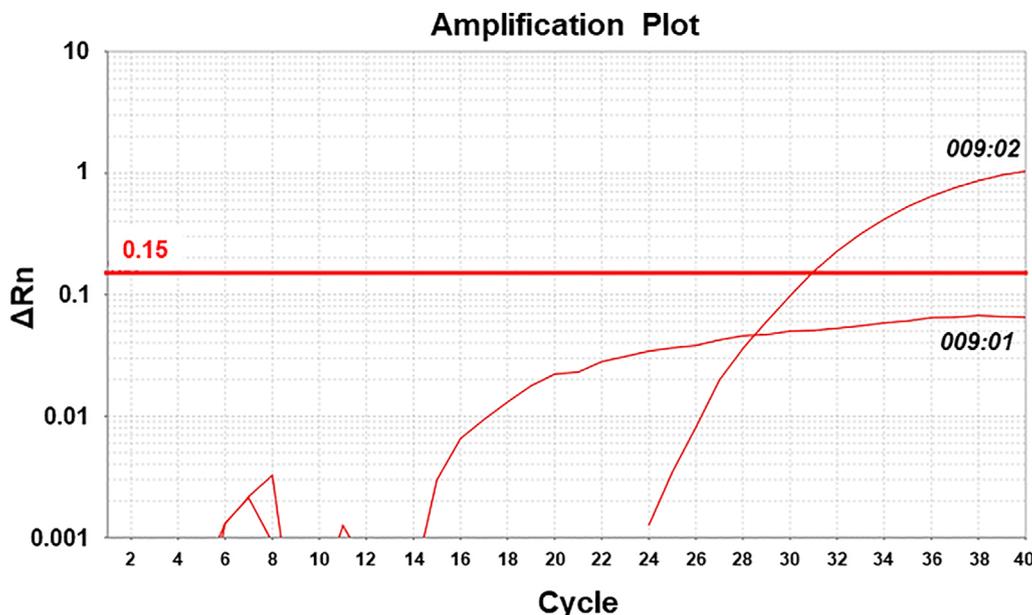


**FIGURE 2** Performance of the designed primer set in cPCR. Electrophoresis results from cPCR using the designed primer set. 1: Genomic DNA from cattle heterozygous for *DRB3\*009:02* and *015:01*. 2: Plasmid DNA encoding *DRB3\*009:01*. 3: Genomic DNA from cattle heterozygous for *DRB3\*034:01* and *005:03*. 4: Genomic DNA from cattle heterozygous for *DRB3\*001:01* and *14:01:01*. 5: Negative control (PCR-grade water)

With the *DRB3\*009:01*-encoding plasmid DNA, the *DRB3\*009:02*-TaqMan assay produced negative signals in quantities varying from 1 fg to 100 ng, indicating a limited hybridization of the *DRB3\*009:02*-specific probe to *DRB3\*009:01*. The amplification plot based on 50 ng genomic DNA samples from cattle that were heterozygous for *DRB3\*009:02* and *015:01* and that from 100 ng of plasmid DNA encoding the *DRB3\*009:01* sequence is shown in Figure 3.

### 3.2 | Diagnostic sensitivity and specificity of the *DRB3\*009:02*-TaqMan assay

As shown in Table 2 the results from the *DRB3\*009:02*-TaqMan assay versus PCR-RFLP-Sanger



**FIGURE 3** Discriminating *DRB3\*009:02* from 009:01 using the *DRB3\*009:02*-TaqMan assay. Amplification plot for the 50 ng samples of genomic DNA from cattle heterozygous for *DRB3\*009:02* and 015:01 and for 100 ng of plasmid DNA encoding the *DRB3\*009:01* sequence. Only genomic DNA from the cattle heterozygous for *DRB3\*009:02* and 015:01 produced positive signals

		PCR-RFLP and sequencing	
		<i>DRB3*009:02</i>	Others
<i>DRB3*009:02</i> -TaqMan assay	Positive	17	0
	Negative	0	133

**TABLE 2** Comparison between *DRB3\*009:02*-TaqMan assay versus combined PCR-RFLP–Sanger sequencing

sequencing completely matched; hence, the kappa value was 1. From these 150 field samples, 17 showed *DRB3\*009:02* positivity by producing positive signals (mean Ct value: 31.34; standard error, SE: ±0.20) in the *DRB3\*009:02*-TaqMan assay, whereas the others did not. Figure 4A shows the amplification plot from 13 of the *DRB3\*009:02*-positive samples and 51 other samples.

### 3.3 | Comparing the *DRB3\*009:02*-TaqMan assay with the SYBR Green assay

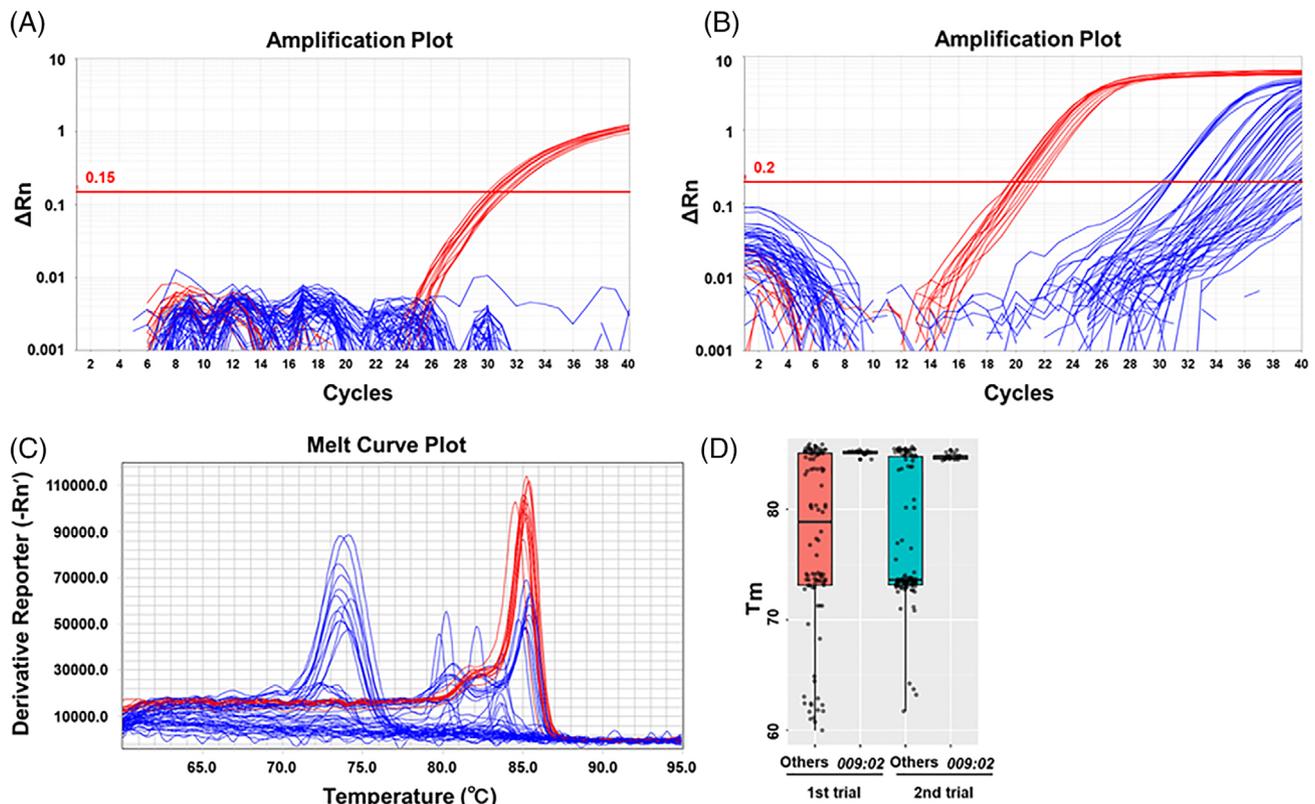
As shown in Figure 4B, the fluorescence signals from *DRB3\*009:02* in samples occurred early on in the SYBR Green assay. The mean Ct value in the 17 samples containing *DRB3\*009:02* was 21.12 (SE: ±0.32), whereas when *DRB3\*009:02* was absent in the DNA samples the mean Ct value was 35.06 (SE: ±0.18). All the *DRB3\*009:02*-lacking DNA samples generated fluorescence signals exceeding Ct values of 29.50, or no signals at all.

A comparison of the Ct values from the 17 *DRB3\*009:02*-positive samples in the two trials

between the *DRB3\*009:02*-TaqMan assay and the SYBR Green assay is shown in Table 3. One sample (sample #10) had a Ct value of 30.58 in the second test in the SYBR Green assay. When checking the Tm values for the melting peaks, we noticed that some DNA samples that did not contain *DRB3\*009:02* had similar Tm values as those from the *DRB3\*009:02*-positive DNA samples (Figure 4C,D). Thus, we were unable to differentiate DNA samples containing *DRB3\*009:02* from those with false negative signals and others using the Tm values of the melting peaks. However, the *DRB3\*009:02*-TaqMan assay showed superior Ct reproducibility in both trials (Table 3).

### 3.4 | Performance of the pooled testing system based on the *DRB3\*009:02*-TaqMan assay

The performance of the *DRB3\*009:02*-TaqMan assay on pooled samples was determined using pooled DNAs. One DNA pool contained DNAs from one animal carrying *DRB3\*009:02* and 29 other cattle without *DRB3\*009:02*



**FIGURE 4** Performance of the *DRB3\*009:02*-TaqMan assay versus the SYBR Green assay. Amplification plots for 13 *DRB3\*009:02*-positive samples and 51 other samples. (A) *DRB3\*009:02*-TaqMan assay. (B) SYBR Green assay. (C) Post-PCR melting curve for the samples obtained after PCR of (B). Red and blue lines in (A)–(C) indicate samples with *DRB3\*009:02* and other alleles, respectively. (D) Box and scatter plot of the  $T_m$  values from the melting peaks from the SYBR Green assay for 17 *DRB3\*009:02*-positive samples versus 133 others. The Y-axis indicates the  $T_m$  and the gray points indicate each sample. Red and blue bars indicate the first and second trials, respectively. The black bars in the boxes indicate the median  $T_m$  values

(the *DRB3\*009:02*-containing DNA pool). Another DNA pool contained DNAs from cattle without *DRB3\*009:02* (the *DRB3\*009:02*-NOT-containing DNA pool). As shown in Figure 5, the *DRB3\*009:02*-TaqMan assay produced positive signals in the 500, 250, 100, 50, and 10 ng *DRB3\*009:02*-containing DNA pool, but failed to detect *DRB3\*009:02* with 1 ng of DNA in this pool. This indicates that the *DRB3\*009:02*-TaqMan assay can detect *DRB3\*009:02* in DNA in a pooled sample when at least 333 pg of it is contained in the reaction mixture. Furthermore, none of the DNA quantities (1 ng–500 ng) in the *DRB3\*009:02*-NOT-containing DNA pool produced positive signals, indicating that no nonspecific amplification occurred in the tested pooled samples.

### 3.5 | Percentage of cattle carrying *DRB3\*009:02* in Kyushu Island, Japan

Six pools from 180 samples were tested. As shown in Table 4, all the pools produced positive signals.

Individual testing revealed that 19 samples were *DRB3\*009:02* allele-positive and that all the pools contained blood from cattle carrying *DRB3\*009:02* (Table 4). The *DRB3\*009:02* status of each sample is shown in Table S3. The percentage of cattle carrying *DRB3\*009:02* in the Kyushu Island was 10.56% (19/180 cattle, 95% confidence interval: 6.64%–16.22%).

## 4 | DISCUSSION

Cattle carrying the *DRB3\*009:02* allele are strongly associated with BLV dissemination resistance, as shown by having undetectable provirus levels and a lack of disease progression when infected with BLV, compared with other cattle.<sup>20–24</sup> By considering the presence of minor population of cattle carrying *DRB3\*009:02*, which have detectable BLV provirus,<sup>25–28</sup> property of this allele should be used for the screening of BLV ECs. BLV ECs can be characterized by satisfaction of both this allele and undetectable provirus. Using BLV ECs to control

**TABLE 3** Ct values for genomic DNA samples from *BoLA-DRB3\*009:02*-carrying cattle from *DRB3\*009:02*-TaqMan and SYBR Green assays

ID	<i>DRB3*009:02</i> -TaqMan assay		SYBR Green assay			
	Ct in 1st trial	Ct in 2nd trial	Ct in 1st trial	Tm in 1st trial	Ct in 2nd trial	Tm in 2nd trial
#1	35.09	34.97	22.00	85.16	22.06	84.97
#2	32.76	32.03	19.78	85.27	19.66	85.17
#3	31.57	31.82	20.73	85.16	20.70	85.17
#4	31.97	31.71	21.10	85.14	21.14	85.36
#5	31.26	31.71	21.39	84.52	21.34	84.43
#6	30.25	30.54	20.13	85.02	20.72	84.68
#7	30.50	30.36	20.51	85.24	20.88	84.74
#8	31.09	31.65	20.43	85.31	22.48	84.76
#9	31.54	31.59	20.33	85.41	22.47	84.71
#10	31.26	30.73	19.61	85.26	30.58 <sup>a</sup>	84.86 <sup>a</sup>
#11	30.60	31.05	19.73	85.26	20.35	84.86
#12	30.20	30.72	20.17	85.21	20.81	84.76
#13	30.76	31.23	21.70	85.04	21.89	84.50
#14	29.95	30.31	19.94	85.20	21.64	84.55
#15	30.32	30.45	21.15	85.04	20.21	84.55
#16	30.56	30.55	20.00	84.99	20.07	84.55
#17	32.37	32.21	20.80	84.99	21.56	84.50

<sup>a</sup>Indicates a sample that contains *DRB3\*009:02* but had a false negative signal.

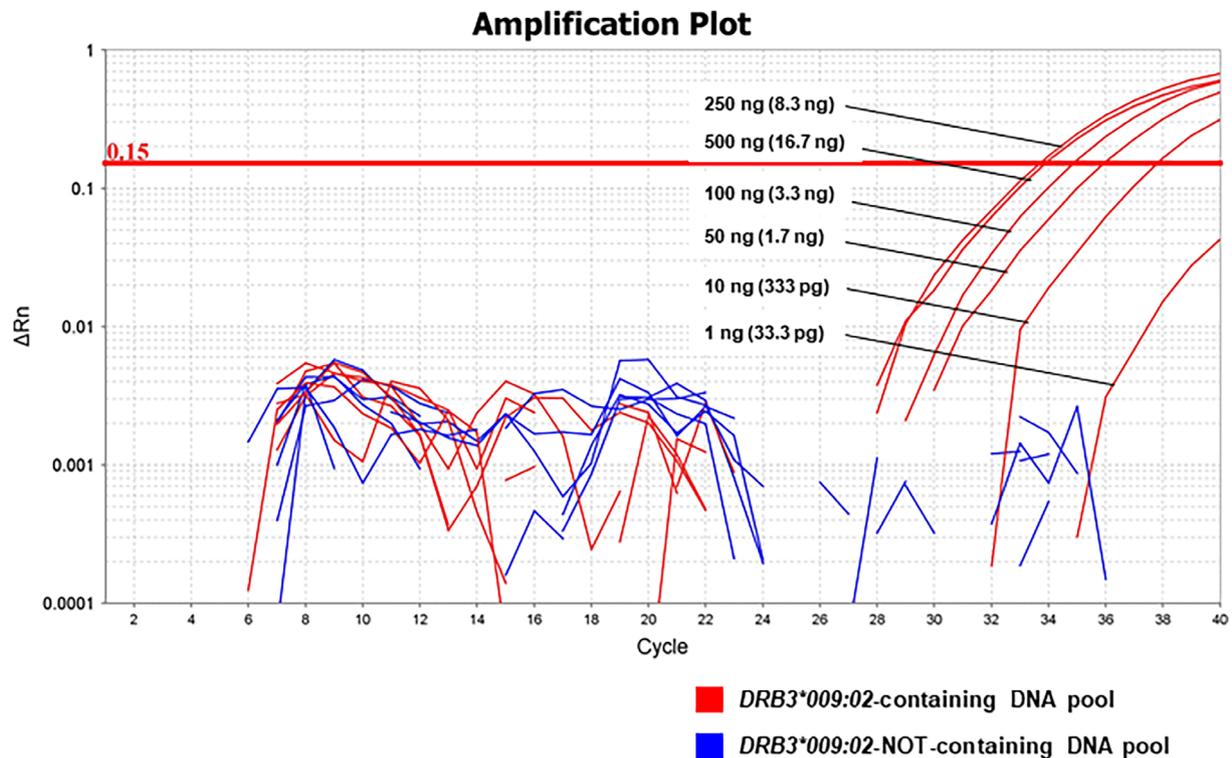
BLV infection will necessitate genotyping cattle for the presence of this allele. However, the percentage of cattle in the population carrying the *DRB3\*009:02* allele is low.<sup>24</sup> To identify cattle with *DRB3\*009:02* alleles in a large population rapidly, we found that employing a pooled testing system based on the *DRB3\*009:02*-TaqMan assay was useful for screening in terms of time and cost.

Currently, 357 *DRB3* alleles are registered in the IPD-MHC database. *DRB3* is one of the most polymorphic regions in the bovine MHC and many similar sequences exist. Our *DRB3\*009:02*-TaqMan assay detected *DRB3\*009:02* in field samples with high accuracy and reproducibility, as compared with the previously described SYBR Green assay.<sup>39</sup> The specificity of the TaqMan MGB probe combined with primer discrimination based on HiDi Taq polymerase was sufficient for *DRB3* genotyping because of its high specificity. This method is applicable in medicine to identify individuals with genetic resistance against other pathogens, such as the ECs of HIV, with more specificity.

Two other methods have been used to identify cattle carrying *DRB3\*009:02*: sequence-based typing (SBT)<sup>40–42</sup> and PCR-RFLP.<sup>24,29,38</sup> SBT combines PCR with allele specific-primers and uses a computer-controlled algorithm to interpret the Sanger sequencing results. Although this method enables high-throughput *DRB3* genotyping, the

problems of uncorrected genotyping results from heterozygous base-calling remain. This method also depends on the quality of the Sanger sequencing base-calling. In contrast, the PCR-RFLP method is a platform for *DRB3* genotyping in that it uses the restriction fragment patterns from the PCR amplicons. However, this method is unable to distinguish alleles with the same restriction pattern. Our team previously observed three cattle with *DRB3\*009:01* and 57 with *DRB3\*009:02* alleles among 60 cattle with the E pattern of *Bst*YI restriction.<sup>24</sup> Therefore, SBT and PCR-RFLP are hampered by specificity. Unlike them, by offering specificity benefit, the *DRB3\*009:02*-TaqMan assay is a practical diagnostic genotyping tool satisfying its requirement.

BLV infections have reportedly occurred in more than 56 countries.<sup>14</sup> In the current epidemic situation, completely eradicating BLV from the field by culling infected cattle is not economically feasible. Appropriate BLV control that involves keeping BLV-infected cattle is needed. One solution is to isolate BLV-infected cattle from the main herd or barn.<sup>15</sup> This control strategy, however, requires enough space and is therefore limited by the capacity of the farm. To heighten BLV control under this situation, we recommended that BLV ECs are used as a barrier against BLV transmission by positioning them between infected cattle herds and uninfected ones.



**FIGURE 5** Performance of the *DRB3\*009:02*-TaqMan assay at detecting *DRB3\*009:02*-carrying cattle DNA from pooled DNA. Amplification plots for the 500, 250, 100, 50, 10, and 1 ng of *DRB3\*009:02*-containing DNA pool and *DRB3\*009:02*-NOT-containing DNA pool are shown. Red and blue lines indicate the *DRB3\*009:02*-containing DNA pool and *DRB3\*009:02*-NOT-containing DNA pool, respectively. The blank area underneath the DNA amounts in the reaction mixtures indicates the amount of DNA used for detecting *DRB3\*009:02*. Ct values above 0.15  $\Delta Rn$  in the *DRB3\*009:02*-containing DNA pool were 33.85 (500 ng), 33.66 (250 ng), 34.82 (100 ng), 35.91 (50 ng), and 37.74 (10 ng); 1 ng = not detected

**TABLE 4** Detection of cattle carrying *DRB3\*009:02* using the pooled testing system and individual testing

Pool No.	No. of pooled samples	Pooled testing results	No. of positive animals in pooled samples
1	30	Positive	2
2	30	Positive	3
3	30	Positive	3
4	30	Positive	5
5	30	Positive	1
6	30	Positive	5

Thus, sufficient numbers of BLV ECs should be secured. We determined that the percentage of *DRB3\*009:02*-carrying cattle in Kyushu Island was 10.56% (19/180 cattle, 95% confidence interval: 6.64%–16.22%) (Table 4). This result is consistent with our previous study indicating that cattle carrying *DRB3\*009:02* existed in 6.8% of the cattle population in the same region.<sup>24</sup> Cattle carrying *DRB3\*009:02* have been seen in Japan and across the world.<sup>43–50</sup> On the other hands, not all cattle carrying *DRB3\*009:02* can be BLV ECs. Previous reports showed that minor population of cattle carrying this allele had detectable BLV provirus and progressed lymphoma.<sup>25–28</sup>

This is most likely because BLV resistance is determined by not only a single *DRB3* allele but also combinations of *DRB3* heterozygous allele and/or other factors, even when *DRB3\*009:02* seems to strongly affect BLV dissemination resistance.<sup>29,30</sup> Furthermore, cattle carrying *DRB3\*009:02* have detectable provirus in the early stage of BLV infection.<sup>51</sup> To avoid using cattle carrying *DRB3\*009:02* with detectable BLV provirus, we recommend characterizing BLV ECs by *DRB3\*009:02* and absence of BLV provirus. BLV ECs maintain status of undetectable BLV provirus in a long span.<sup>52</sup> We expect that identifying BLV ECs and the challenge of global

BLV control should be made easier by using a pooled testing system based on the *DRB3\*009:02*-TaqMan assay.

The breeding strategy employed for cattle is also a key aspect of disease control. After identifying *DRB3\*009:02*-carrying cattle, maintaining this allele in the cattle continuously will be needed. Thus, selective breeding based on the genetic information of the parents will be required. The appropriate use of genetic screening or engineering in food animals would help with the food safety of cattle products, one example of which is the production of prion gene-knockout cattle.<sup>53</sup> However, maintaining genetic species diversity is also important for retaining the various characteristics associated with disease resistance. Hence, producing cattle carrying *DRB3\*009:02* alleles by selective breeding while keeping cattle with other *DRB3* alleles is a good option.

This study has one limitation. The *DRB3\*009:02*-TaqMan assay cannot discriminate *DRB3\*163:01* among the alleles registered in the IPD-MHC database. *DRB3\*163:01* was identified in *Bos indicus*. Therefore, the *DRB3\*009:02*-TaqMan assay is recommended as a screening test for *DRB3\*009:02* when testing *B. indicus*. Of note, the SYBR Green assay also has difficulty differentiating *DRB3\*163:01* from *DRB3\*009:02*, but PCR-RFLP can discriminate these alleles. The sequence homology between *DRB3\*163:01* and *DRB3\*009:02* is 98.1%, and the loci of the different nucleotides within *DRB3\*009:02* are sporadic. We are interested in determining the susceptibility to BLV of cattle carrying the former allele.

## 5 | CONCLUSIONS

A pooled testing system is available for easy screening of cattle carrying *DRB3\*009:02* at herd-level with low cost and without excessive labor. The *DRB3\*009:02*-TaqMan assay shows high-discriminative sensitivity and specificity toward *DRB3\*009:02*, making it suitable for identifying cattle carrying *DRB3\*009:02* in individual post-screening tests. This reliable diagnostic laboratory tool is applicable in selective breeding. This strategy should contribute to BLV control through the use of BLV ECs.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## AUTHOR CONTRIBUTIONS

Kosuke Notsu contributed to funding acquisition, data collection, and analysis, manuscript writing. Hala El Daous contributed to data collection, analysis, and manuscript review. Shuya Mitoma contributed to analysis and manuscript review. Junzo Norimine reviewed the manuscript. Satoshi Sekiguchi contributed to conceptualization, funding acquisition, and supervision. All authors have read and approved the final manuscript.

## DATA AVAILABILITY STATEMENT

All the data generated or analyzed in this study are included in the published article and its additional files.

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