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Identification of cattle carrying alleles associated with resistance and susceptibility to the Bovine Leukemia Virus progression by real-time PCR

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ABSTRACT

Previous studies have shown a significant association between polymorphisms of the BoLA DRB3 gene and Bovine Leukemia Virus (BLV) infection profile. The presence of allele ∗1501 has been associated with high proviral load in peripheral blood while allele ∗0902 has been associated with low proviral load. The purpose of this study was to develop allele-specific real-time PCRs to identify cattle carrying alleles associated with resistance (BoLA DRB3∗0902) or susceptibility (BoLA DRB3∗1501) to the BLV progression. Specific primers were designed and differential amplification was carried out by real-time PCR and monitored by SYBR® Green dye in DNA samples from peripheral blood. Conditions were also adjusted for traditional PCR amplification (end point amplification). These methods are rapid, simple and suitable for high throughput screening, and could aid in marker-assisted selection of BLV-resistant and susceptible cattle.

1. Introduction

The Major Histocompatibility Complex (MHC), only present in vertebrates, involves a cluster of genes related to antigen recognition and immune response. MHC class I and class II genes codify proteins involved in antigen processing and presentation to leucocytes. The DRB3 gene, which encodes the β chain of class II MHC molecule, is the only functional DRB gene in cattle. Exon 2 of the BoLA DRB3 gene is extremely polymorphic with over 100 different alleles described to date (Robinson et al., 2010).

Association between polymorphisms of the BoLA DRB3 gene with disease susceptibility and resistance, and productive traits has been extensively studied. Such associations include clinical and subclinical mastitis (Dietz et al., 1997a; Kelm et al., 1997; Sharif et al., 1998; Kulberg et al., 2007; Duangjinda et al., 2009; Zambrano et al., 2011), susceptibility to dermatophilosis (Maillard et al., 2002) and resistance to Bovine Leukemia Virus (BLV) induced persistent lymphocytosis (Xu et al., 1993; Zanotti et al., 1996) or a reduced number of infected circulating lymphocytes in BLV-infected cattle carrying resistance-associated alleles (Mirsky et al., 1998). An extensive study on 230 BLV-infected Holstein cattle showed a significant association between the presence of the allele DRB3∗16 (∗1501 according to the nomenclature adopted by the International Society of Animal Genetics) with high proviral load in peripheral blood, while BoLA DRB3∗0902, a subtype of the allele DRB3∗11 formerly associated with resistance to persistent lymphocytosis, was associated with low proviral load. Cattle with low proviral load seem to represent a state of resistance to BLV and it has been suggested that they would not transmit the infection under natural conditions. Therefore, the identification of these alleles could aid in the control of BLV infection by genetically-assisted selection (Juliarena et al., 2008).

Current methodology for typing BoLA DRB3 alleles include polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) (van Eijk et al., 1992), PCR sequence based typing (Takeshima et al., 2001), direct sequencing (Groenen et al., 1990) and multi-primer target PCR (MPT-PCR) (Ledwidge et al., 2001). Each of these methods has its own strengths and weaknesses but they are costly or time consuming when it is necessary to analyze a great number of samples. Calero et al. (2009) developed a fast, simple and reproducible method to detect mutated alleles responsible for genetic human prion disease, based on the use of allele-specific primers and real-time PCR monitored by SYBR® Green dye.

The aim of this study was to develop allele specific PCRs to identify cattle carrying alleles associated with resistance (BoLA

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DRB3∗0902) or susceptibility (BoLA DRB3∗1501) to the BLV progression.

2. Material and methods

2.1. Blood samples, DNA extraction and quantitation

Heparinized blood samples from 208 cattle belonging to the Argentinian Holstein dairy breed were obtained by jugular venipuncture. DNA was extracted from peripheral blood leucocytes, after lysis of erythrocytes with ammonium chloride solution (150 mM NH_4Cl, 8 mM Na_2CO_3 and 6 mM EDTA pH = 7) using the Illustra Blood Genomic Prep Mini Spin kit (GE Healthcare). Quantification of genomic DNA was carried out by measuring the absorbance at 260 nm. DNA samples were stored at −20 °C until used.

2.2. DNA amplification by PCR and real-time PCR

Specific primers able to amplify the alleles BoLA DRB3∗0902 and ∗1501 were designed based on sequences obtained from the European Molecular Biology Laboratory (EBI) database http://www.ebi.ac.uk/ipd/mhc/bola/index.html. Primer parameters as sequence specificity, Tm and secondary structures were considered to select the following primer pairs: 0902F: (5′-CCTGGAGTATTCTAAGAGCG-3′), 0902R: (5′-CGCCTCTCCTCCAGGATC-3′), 1501F: (5′-CGGGTCGCCGAGCAGTTGAACG-3′) and 1501R: (5′-CTCTCAACGACCCCGTAGTTGTG-3′). Primer location in the respective sequences is shown in Fig. 1.

PCR amplifications were carried out in a PT-100 thermal cycler (MJ-Research Inc.). Reaction mixture for the ∗1501-specific PCR contained 0.1 μg of total DNA, 4.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer and 0.5 U of Taq polymerase in Taq buffer. Amplification began with 4 min at 94 °C, followed by 35 cycles (40 s at 94 °C, 30 s at 63 °C, 30 s at 72 °C) and a final extension of 5 min at 72 °C. Reaction mixture for the ∗0902-specific PCR contained 0.1 μg of total DNA, 2.25 mM $MgSO_4$, 1 mM of each dNTP, 0.33 μM of each primer and 1.25 U of Taq polymerase in Taq buffer. Thermal cycling conditions were: 4 min at 94 °C, 35 cycles (40 s at 94 °C, 40 s at 63 °C, 40 s at 72 °C) and a final extension step of 5 min at 72 °C. The amplified products were observed and photographed under blue light transillumination after electrophoresis in 12% polyacrylamide gels stained with SYBR® Safe (Invitrogen) in TBE buffer.

Real-time amplification was carried out in duplicate for each sample in a 7500 Real Time PCR System (Applied Biosystems). Reaction mixture contained 50 ng of genomic DNA, 0.3 μM of each primer and Fast Start Universal Sybr Green Master Mix (Roche) in a final volume of 20 μl. The PCR cycling conditions were as follows: initial precycling stage at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were performed after each amplification.

2.3. Genotyping of BoLA DRB3 alleles

The genotyping was carried out by the PCR-RFLP method (van Eijk et al., 1992), PCR-sequence specific oligonucleotide polymorphism (PCR-SSOP) (Sala, 2009) or by amplification and sequencing from both ends of a 284 bp fragment (Baxter et al., 2008). In the latter case, allele assignment was done using the script Haplofinder (Miltiadou et al., 2003).

Performance of real-time PCRs in samples in which the BoLA-DRB3 genotype was determined by amplification and sequencing was analyzed by McNemar's test.

3. Results

DNA samples from Holstein cattle carrying a diversity of alleles were tested in both the ∗0902 and the ∗1501 PCRs. A total of 38 alleles different from ∗0902 were tested in the ∗0902-specific PCR and 16 alleles different from ∗1501 were tested in the ∗1501-specific reaction. As some of the BoLA DRB3 alleles only differ in a single nucleotide, an important issue to attend to was the specificity. To achieve this specificity an adjustment of parameters including primers and $MgCl_2$/$MgSO_4$ concentration and annealing temperature was carried out, to detect only carriers of the specific allele with enough sensibility to find heterozygous animals. In order to enhance specificity of the ∗1501-specific PCR reaction, adjuvants such as dimethyl sulfoxide, polyethylene glycol 8000, glycerol and formamide were tested, but raising annealing temperature in absence of adjuvants rendered the optimum condition. Samples from Holstein cattle carrying BoLA DRB3 most frequent alleles were tested in each reaction in order to test the specificity of both PCRs. As shown in Table 1 alleles with frequencies above 2% in Holstein cattle population from different countries (Dietz et al., 1997a,b; Sharif et al., 1998; Rupp et al., 2007; Juliarena et al., 2008) were tested in each reaction. The alleles presented on Table 1 also represent those alleles most prevalent (above 2%) in the Argentinian Holstein population (Juliarena et al., 2008). Samples from cattle carrying alleles found with lower frequencies in the Holstein population were also included: alleles ∗20011, ∗2002, ∗1601, ∗0801, ∗2901, ∗3601,∗4401, ∗0401, ∗2601, ∗2401, ∗2403, ∗4201, ∗4301, ∗0301, ∗4101, ∗2101, ∗2802, ∗ 2502, ∗25012, ∗3401 and ∗1902 were tested in the ∗0902-specific PCR and alleles ∗1601, ∗0301 and ∗2802 in the ∗1501-specific PCR.

Amplification products of 98 and 195 bp observed after electrophoresis are shown in Fig. 2. No amplification was observed in 66 from 69 samples from cattle without the allele ∗0902, two of them gave inconclusive result and the remainder resulted positive in the

>BoLA DRB3*1501
GGAGTATTCTACGAGCGAGTGTCATTTCTTCAACGGGACCGAGCGGGTGCGGTACCTGGACAGATACTT
CCATAATGGAGAAGAGTTCGTGCGCTTCGACAGCGACTGGGGCGAGTACCGGGCGGTGACCGAGCTAG
GGCGGCGGGTCGCCGAGCAGTTGAACGGCCAGAAGGACACCCTGGAGCGGGAGCGGGCCTATGTGGA
CACGTACTGCAGACACAACTACGGGGTCGTTGAGAGTTTCACTGTG

>BoLA DRB3*0902
CCTGGAGTATTCTAAGAGCGAGTGTCATTTCTTCAACGGGACCGAGCGGGTGCGGTTCCTGGAGAGATC
CTTCTATAATGGAGAAGAGAACGTGCGCTTCGACAGCGACTGGGGCGAGTACCGGGCGGTGACCGAGC
TAGGGCGGCCGGACGCCGAGTACTGGAACAGCCAGAAGGAGATCCTGGAGGAGAGGCGGGCCGAGG
TGGACAGGGTGTGCAGACACAACTACGGGGTCGGTGAGAGTTTCACTGTG

Fig. 1. Primer location in alleles BoLA DRB3∗1501 and BoLA DRB3∗0902. Sequences obtained from the IPD – MHC database were employed to manually design allele specific primers (underlined). Expected amplicon size for PCR∗1501 is 98 bp and for PCR∗0902 is 195 bp.

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Table 1
Diversity of BoLA DRB3 alleles tested on allele specific PCRs.

Allele designation		Frequency range[a]	Number of samples tested	
ISAG	PCR-RFLP		PCR∗0902	PCR∗1501
DRB3∗1201	8	6.74–21.3	9	15
DRB3∗0101	24	8.3–19.2	27	42
DRB3∗0901	11	7–18.2	4	2
DRB3∗0902			35	20
DRB3∗1101	22	7.9–15.5	7	7
DRB3∗1103			1	–
DRB3∗1501	16	6.7–14.35	20	40
DRB3∗2701	23	6.4–9.1	1	–
DRB3∗2703			2	11
DRB3∗1001	3	2.8–7	5	2
DRB3∗0201	7	2–5.32	5	8
DRB3∗1402	27	0.8–5.1	1	–
DRB3∗14011			1	2
DRB3∗1701	12	0.2–4.57	2	2
DRB3∗0601	26	1.4–3.26	6	1
DRB3∗0703	28	0.6–2.8	1	–
DRB3∗0701			1	–
DRB3∗1801	18	0–2.39	2	3
DRB3∗1802			1	–

BoLA DRB3 most frequent alleles (>2%) in Holstein population are shown. The number of samples tested in each PCR from cattle carrying each allele is shown.

[a] In Holstein cattle from Canadá, United States of America and Argentina. Frequency range is expressed in percentage (%).

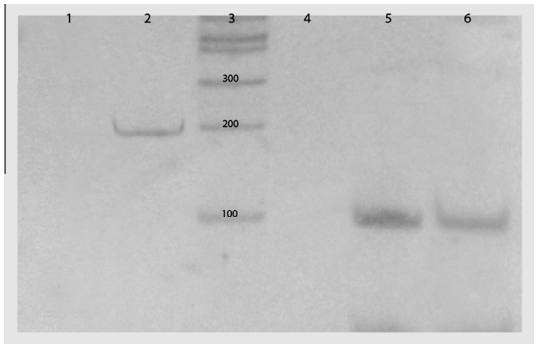

Fig. 2. BoLA DRB3 allele specific amplification. Gel electrophoresis (12% polyacrylamide gel) of ∗0902-specific (lanes 1 and 2) and ∗1501-specific (lanes 4–6) PCR products. Lanes 1: DNA from cattle not carrying the ∗0902 allele; 2: DNA from cattle carrying the BoLA DRB3∗0902 allele in heterozygosis; 3: MWM (sizes expressed on bp); 4: DNA from cattle not carrying the ∗1501 allele; 5: DNA from cattle carrying the BoLA DRB3∗1501 allele in homozygosis; 6: DNA from cattle carrying the BoLA DRB3∗1501 allele in heterozygosis.

∗0902-PCR. Absence of amplification was also verified in 4 samples from cattle carrying a subtype of the allele ∗11 (∗0901) different from ∗0902. The ∗0902-specific PCR detected 36 from 37 samples carrying the respective allele. Specificity of the ∗1501-specific reaction was tested in 55 samples representing the most prevalent alleles in the Holstein population as mentioned above. Fifty of them resulted negative in the ∗1501-specific PCR, 4 gave doubtful result and 1 sample was positive. Forty-two from 43 samples carrying the allele ∗1501 resulted positive in the ∗1501-specific PCR.

In order to adapt the PCRs to the real-time PCR system, a limited number of samples was tested by real-time PCR with the same pair of primers. These samples were representative of the BoLA DRB3 alleles found in the Holstein population with frequencies above 5% according to Table 1. The Cq value obtained in positive samples on real-time PCR∗0902 was between 21.42 and 23.57 (mean = 22.43, SD = 0.52), while samples without the allele were not amplified or resulted in *Cq* values between 32.77 and 38.18 (mean = 35.35, SD = 1.58). The difference in *Cq* values between positive and negative samples was always above 9.2 cycles (mean = 12.92, SD = 0.37). Therefore, the real-time PCR∗0902 was set in 30 cycles in order to obtain amplification exclusively in positive samples. Samples carrying the BoLA DRB3∗1501 allele were detected in the real-time PCR∗1501 with *Cq* values between 25 and 29, while negative samples were not amplified or resulted in *Cq* values above 36.52 (mean = 38.51, SD = 1.00). Consequently, the real-time PCR∗1501 was set in 35 cycles to avoid signal from negative samples, while amplification of positive samples was guaranteed. A slight difference in *Cq* values was observed between samples carrying the allele ∗1501 in homozygosis compared to those in heterozygosis (*Cq* mean = 26.81, SD = 0.67 vs *Cq* mean = 27.90, SD = 0.73, respectively). However, this difference did not allow us to differentiate between homozygous and heterozygous cattle. The difference in *Cq* values between positive and negative samples in the real-time PCR∗1501 was in average 10.77 (SD = 0.27) and always above 6.68 cycles. A representative experiment, showing the differential amplification of samples with or without the allele of interest in both the ∗0902 and the ∗1501 specific real-time PCR reaction is shown in Fig. 3. In order to explore the robustness of these methods, we carried out the real-time PCRs with variable amounts of input DNA. Negative samples did not show specific amplification in any of the PCRs when input DNA was between 5 and 100 ng, while in samples with the alleles of interest *Cq* varied according to the quantity of input DNA as expected, but was always under 25.40 for real-time PCR∗0902 and 31.43 for real-time PCR∗1501.

Real-time PCR performance is shown in Table 2. All 29 samples tested in real-time PCR∗0902 were correctly identified, except for 1 sample that was negative in the PCR and had the allele ∗0902 in heterozygosis. This sample was also negative in the traditional PCR∗0902. The observed difference was not statistically significant ($p = 0.3173$). All 24 samples carrying the allele ∗1501 were positive by real-time PCR∗1501, while 2 from 30 samples without the allele were amplified by the procedure. This difference was not statistically significant ($p = 0.1573$). The analysis of the melting curves for these 2 discrepant samples showed a peak undistinguishable from that of positive control. One of the samples that gave a false positive result in the real-time PCR∗1501 carried the allele BoLA DRB3∗1101 in combination with ∗0301, while the other carried the allele ∗0601 in combination with ∗1201. From the 28 samples that tested negative in real-time PCR∗1501, 8 carried the allele ∗1101 and 5 carried the allele ∗1201, while the allele ∗0301 was present in only 1 sample. Due to the fact that specificity of PCR∗1501 is based on the sequence of the forward primer, we analyzed the match between sequences of alleles present in false positive reactions on real-time PCR∗1501, with forward primer sequence. Alleles ∗1101, ∗1201 and ∗0301 showed 6 or more mismatches with this primer, but the allele ∗0601 only showed 2 mismatches, raising the possibility of a false positive reaction.

4. Discussion

Due to the wide dissemination of BLV among dairy cattle in Argentina and other countries, control and eradication programs based on the serological detection of BLV-infected cattle and the subsequent culling of infected animals are impracticable (Esteban et al., 2009). As vaccines to prevent BLV infection are not available, marker assisted selection of resistant cattle appears to be the only possible approach.

To implement such a program, methods for easily detect the presence of BoLA DRB3 alleles associated with resistance and susceptibility to BLV progression in large populations of cattle are desirable.

The allele specific real-time PCRs developed in the present study showed an adequate performance in samples representing

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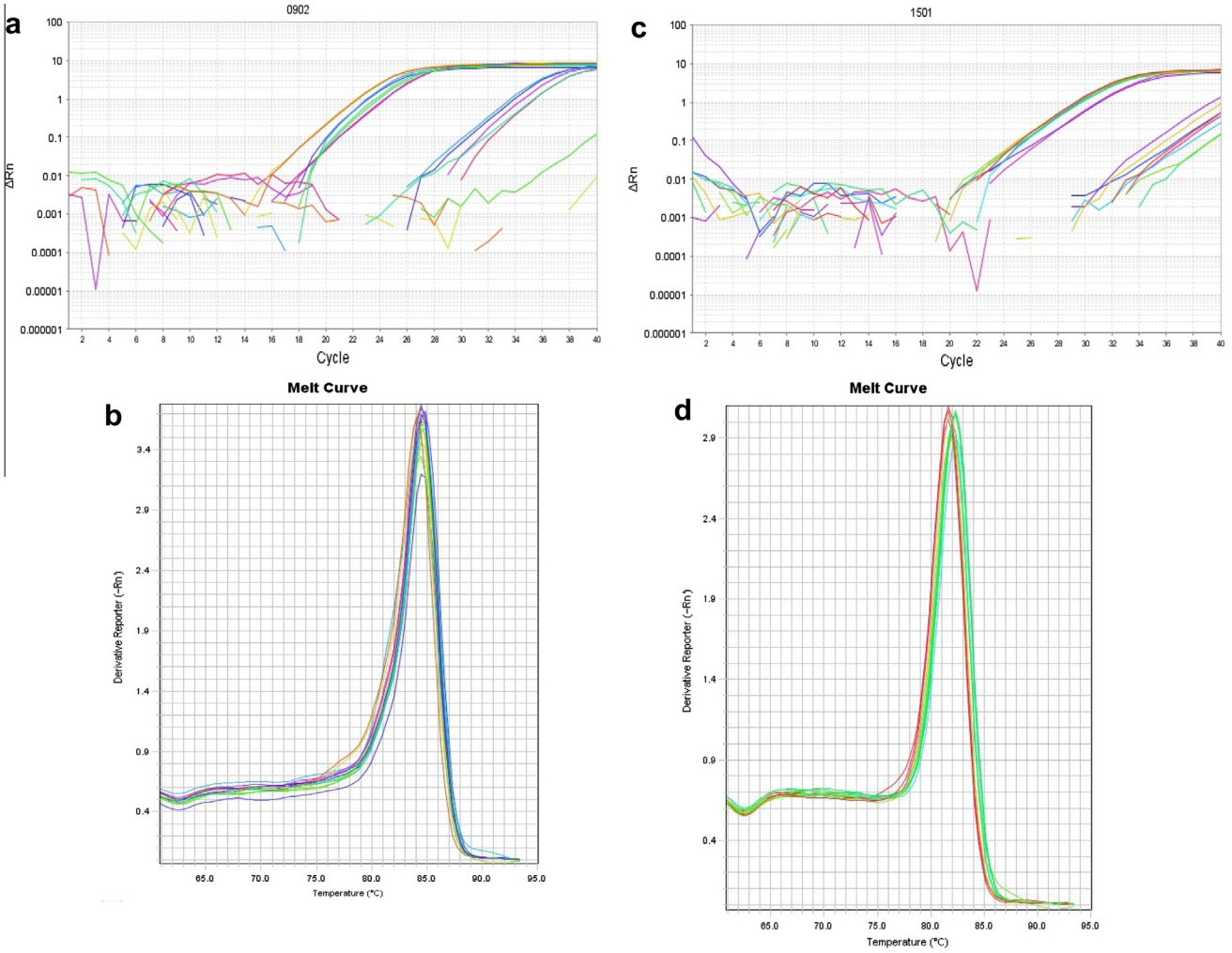

Fig. 3. Amplification of BoLA DRB3∗0902 and ∗1501 alleles by real-time PCR. Eight DNA samples (four with the allele of interest and four without it) and non-template control were tested in duplicate in each real-time PCR. *ΔRn* (an indicator of the magnitude of the reaction) is graphed against the number of cycles (Fig. 3a and c). The curves on the left correspond to the positive samples. The rate of change of the relative fluorescence units with time (derivative reporter) is plotted against the temperature in the melting curve analysis (Fig. 3b and d). (a) Real-time PCR amplification with ∗0902-specific primers. (b) Melting curve analysis of the ∗0902-specific reaction. (c) Real-time PCR amplification with ∗1501-specific primers. (d) Melting curve analysis of the ∗1501-specific reaction.

Table 2
Performance of real-time PCRs in samples presenting alleles with frequencies above 5% in Argentinian Holstein population. The BoLA DRB3 genotype was assigned after amplification and sequencing of a fragment of 284 bp of BoLA DRB3 gene and analysis by the script Haplofinder.

Samples	*n*	Positive	Negative
Real-time PCR∗0902			
With allele∗0902	15	14	1
Without allele ∗0902	14	0	14
Total	29		
Real-time PCR∗1501			
With allele∗1501	24	24	0
Without allele ∗1501	30	2	28
Total	54		

most frequent alleles found in the Holstein population worldwide. One of the two false-positive results obtained with the real-time PCR∗1501 was probably due to unspecific amplification of allele ∗0601, which appears with a frequency <5% in the Holstein population, and not to alleles ∗1101 and ∗1201, as they gave no specific amplification when present in other samples. We could not conclude about the origin of the other false positive reaction in the real-time PCR. In spite of these discrepancies, the performance of the real-time PCRs resulted acceptable for a screening method. Compared to the other methods available for genotyping BoLA DRB3 alleles, the real-time PCR is simple and thus adequate to be applied to large number of samples. Hence, the method fulfills the requirements to be used in a marker-assisted selection program of resistant cattle to control BLV infection. In the case of laboratories that lack the equipment necessary for real time amplification, the reaction can also be made in a conventional cycler, although more time consuming and laborious.

The control of BLV infection on the basis of genetic resistance is being carried out at an experimental farm in Argentina. Preliminary results show that low proviral load BLV-infected cattle carrying the allele ∗0902 do not transmit the virus to BLV free cattle under natural conditions in a commercial dairy farm (Juliarena et al., article in preparation). The availability of methods for the quick and easy screening of large numbers of cattle for the presence of certain BoLA DRB3 alleles is beneficial to such program aimed at controlling BLV infection.

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