Research in Veterinary Science 95 (2013) 991–995

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

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

A. Forletti [a,1], M.A. Juliarena [a,c,1], C. Ceriani [a,c], A.F. Amadio [b,c], E. Esteban [a], S.E. Gutiérrez [a,c,*]

[a] Laboratorio de Virología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Departamento SAMP, FCV – UNCPBA, Campus Universitario, 7000 Tandil, Buenos Aires, Argentina
[b] Instituto Nacional de Tecnología Agropecuaria, EEA Rafaela, Ruta 34 Km 227, 2300 Rafaela, Santa Fe, Argentina
[c] Researchers of CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina

ARTICLE INFO

Article history:
Received 8 May 2012
Accepted 16 July 2013

Keywords:
BoLA DRB3
Bovine Leukemia Virus
Resistance
Susceptibility
PCR
Real-time PCR

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

∗ Corresponding author at: Laboratorio de Virología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Departamento SAMP, FCV – UNCPBA, Campus Universitario, 7000 Tandil, Buenos Aires, Argentina. Fax: +54 249 4439850.
E-mail address: segutier@vet.unicen.edu.ar (S.E. Gutiérrez).
[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.rvsc.2013.07.018

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.