대한진단검사의학회지 제29권 제6호 2009
Korean J Lab Med 2009;29:589-93
DOI 10.3343/kjlm.2009.29.6.589

실시간 PCR을 이용한 두 가지 상품화된 HLA-B27 검사의 평가

조은해 · 이상곤 · 석정호 · 박보야나 · 이은희

녹십자의료재단

Evaluation of Two Commercial HLA-B27 Real-Time PCR Kits

Eun Hae Cho, M.D., Sang Gon Lee, M.D., Jeong Ho Seok, M.D., Bo Ya Na Park, M.S., and Eun Hee Lee, M.D.

Greencross Reference Laboratory, Yongin, Korea

Background : The standard PCR with sequence-specific primers (SSP) is a widely used method of HLA-B27 typing in clinical practice. The aim of our study was to evaluate 2 Korean HLA-B27 kits with different real-time PCR chemistries.

Methods : To validate the accuracy of real-time PCR kits, we selected 28 HLA-B27-positive samples and 33 HLA-B27-negative samples with a wide range of different HLA-B specificities typed by standard PCR-SSP. The 2 real-time PCR kits used were the AccuPower® HLA-B27 real-time PCR kit (Bioneer, Korea) with TaqMan probes and the Real-Q™ HLA-B*27 detection kit (BioSewoom, Korea) with SYBR Green I dye for melting curve analysis.

Results : All 61 samples typed by PCR-SSP demonstrated a perfect concordance with the 2 real-time PCR assays. It was possible to clearly discriminate between HLA-B27-positive and -negative samples in both real-time assays.

Conclusions : In summary, both real-time PCR assays for HLA-B27 were fast, reliable, well-adapted for routine laboratory testing, and attractive alternatives to the conventional PCR-SSP method. *(Korean J Lab Med 2009;29:589-93)*

Key Words : *Real Time PCR, HLA-B27, PCR-SSP*

INTRODUCTION

Ankylosing spondylitis (AS) is associated with B27 with a relative risk of 95%, which is the highest value of relative risk among all HLA-disease associations [1]. The HLA-B27 allele is present in about 5% to 6% of Korean populations [2, 3]. According to the data published in the international ImMunoGeneTics database (IMGT, Release 2.24.0), 45 subtypes of HLA-B27 have been detected. A strong association has been found between the subtypes B*2705, B*2704, B*2702, and B*2707 and AS, whereas other subtypes are not associated with AS or may provide protection against the disease [4]. In Koreans, alleles of B*2705 and B*2704 are the most prominent subtypes [5, 6].

Various methods have been developed for the identification of the HLA-B27 allele. The PCR-based HLA-typing methods, including the standard PCR with sequence-specific primers (SSP), have become widely used alternatives to

Received : March 13, 2009 Manuscript No : KJLM09-042
Revision received : July 23, 2009
Accepted : September 8, 2009
Corresponding author : Eun Hae Cho, M.D.
Greencross Reference Laboratory, 314 Bojeong-dong, Giheung-gu, Yongin 449-913, Korea
Tel : +82-31-260-9216, Fax : +82-31-260-9638
E-mail : ssea74@yahoo.co.kr

serologic methods in clinical practice; however, these techniques are time-consuming and laborious. The drawbacks of the current methods, such as long processing time and the requirement for post-PCR manual procedures, have been overcome by the introduction of real-time PCR. Recently, 2 real-time PCR-based Korean HLA-B27 typing kits have been developed, of which one kit uses a TaqMan probe specific for HLA-B27 and the other uses SYBR Green I dye and melting curve analysis. The aim of our study was to evaluate the 2 Korean HLA-B27 kits with real-time analysis.

MATERIALS AND METHODS

1. Materials

In this study, we analyzed 61 samples that had been previously typed with the conventional PCR-SSP method; of these, 41 blood samples had been obtained from Koreans and 20 from HLA proficiency surveys of the College of American Pathologists (HLA-B27-A, 2008; HLA-B27-B, 2008; ML-C, 2007; and ML-B, 2008). Genomic DNA was extracted using the QIAamp blood kit (Qiagen, Hilden, Germany) from 200 μL of peripheral blood, which had been anticoagulated with ethylenediamine tetraacetic acid (EDTA), according to the manufacturer's instructions. Among the HLA-B27-negative samples, a wide range of different HLA-B specificities were investigated. HLA-B specificities of the 33 HLA-B27-negative samples that were tested in this study were as follows: HLA-B7, -8, -13, -18, -35, -37, -38, -39, -41, -44, -46, -48, -51, -52, -54, -55, -56, -57, -58, -59, -60, -61, -62, -64, -65, -67, -70, -71, and -75. The 28 HLA-B27-positive samples tested in this study were heterozygous with the following HLA-B specificities: HLA-B35, -44, -46, -49, -51, -53, -54, -55, -58, -61, -62, -65, -67, and -71.

2. Methods

1) Real-time PCR using the TaqMan probe

Real-time PCR reactions were performed using an Exicycler™ 96 Real-time Quantitative Thermal Block (Bioneer, Daejeon, Korea) and the AccuPower® HLA-B27 real-time PCR kit (Bioneer, Daejeon, Korea). The following 2 detection probes were used: an HLA-B27-specific probe and a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific probe, which were labeled with a fluorescent reporter dye FAM (6-carboxyfluorescein) and TAMRA (6-carboxytetramethyl-rhodamine) at the 5′ end, respectively and a fluorescent quencher dye BHQ (black hole quencher) at the 3′ end, for both. The real-time PCR reaction was performed in a total volume of 50 μL with the HLA-B27 Pre-Mix (Bioneer, Daejeon, Korea), which contains HLA-B27-specific primers targeting exon 2 of HLA-B gene, *GAPDH*-specific primers, dual-labeled fluorogenic probes, DNA polymerase, deoxynucleotide triphosphates (dNTPs), and stabilizer, with 5 μL of template DNA and 45 μL of 0.1% diethyl pyrocarbonate-treated distilled water. The amplification protocol for this reaction consisted of an initial denaturation step at 95℃ for 5 min, followed by 35 amplification cycles of denaturation for 5 sec at 95℃, annealing and extension for 15 sec at 65℃. During the PCR, the HLA-B27 target region and an internal control region for *GAPDH* were amplified simultaneously. Fluorescence signals were monitored in real-time to determine the threshold cycle number (C_t). A cut-off value was assigned on the basis of C_t values for FAM and TAMRA reporter dyes to interpret the results. Specimens yielding C_t FAM value of <30 and a C_t TAMRA value of <27 were interpreted as being HLA-B27-positive. Samples yielding a C_t FAM value of >30 were considered HLA-B27-negative if the internal control signal was above the assigned cut-off (C_t TAMRA, <27). A sample was considered to contain inhibitory substances or degraded DNA, if the C_t TAMRA value was >27. HLA-B27 positive and negative controls were coamplified.

2) Real-time PCR with melting curve analysis

Real-time PCR reactions were performed on a Rotor-Gene™ 6500 system (Corbett Research, Sydney, Australia) by using the Real-Q™ HLA-B*27 detection kit (BioSewoom, Seoul, Korea). For melting curve analysis, SYBR Green I dye was used after performing combined amplification with specific primers for HLA-B27 and β-globin. HLA-B27 pri-

mers targeting exon 2 of HLA-B27 were designed to amplify a 150-base pair PCR product. The PCR reaction was performed in a total volume of 25 μL (12.5 μL of the PCR reaction mixture, 4 μL of primer mixture, 3.5 μL of distilled water, and 5 μL of template DNA). The amplification protocol for this reaction consisted of an initial denaturation at 95℃ for 10 min, followed by 35 amplification cycles (30 sec of denaturation at 95℃, 30 sec of annealing at 63℃, and 30 sec of extension at 72℃. Melting curve analysis was followed by the generation of a thermal gradient from 50℃ to 99℃, with 5-sec intervals at 1℃ after 30 sec of the first step. HLA-B27 alleles were assigned to the characteristic peaks formed at 86.5±1℃. Hence, HLA-B27-negative samples showed a single temperature curve, corresponding to β-globin (peak at 83.8±1℃), whereas B27-positive samples showed 2 peaks at 86.5±1℃ and 83.8±1℃ or one peak around 86.5±1℃. For quality control, HLA-B27-positive and -negative controls were co-amplified.

3) Conventional PCR-SSP

The determination of HLA-ABC antigens by the conventional PCR-SSP was performed using the HLA-A, -B, and -C SSP tray kit (Biotest AG, Dreieich, Germany) according to the manufacturer's instructions.

4) Sub-typing of HLA-B27-positive samples

To validate the capability of detection of HLA-B27 subtypes, 9 HLA-B27-positive samples were subtyped by PCR with sequence-based typing. Of 9 samples, 4 samples showed the subtype HLA B*2705, 3 samples showed the HLA B*2706 subtype, and 2 samples showed HLA*2704 and HLA *2707 subtypes, respectively.

RESULTS

1. Real-time PCR with specific probes

All positive samples had C_t FAM values <28. All samples had C_t TAMRA values <26 (Fig. 1). HLA-B27-positive and -negative samples were discriminated with a clear cut-off value (C_t FAM value) (Fig. 2).

2. Real-time PCR with melting curve analysis

All positive samples showed double or single peaks at 86.0-87.0℃. All negative samples had only one peak at 83.5-84.3℃ (Fig. 3). A graphical representation of our results revealed 2 clearly distinct populations corresponding to HLA-B27-positive and -negative samples.

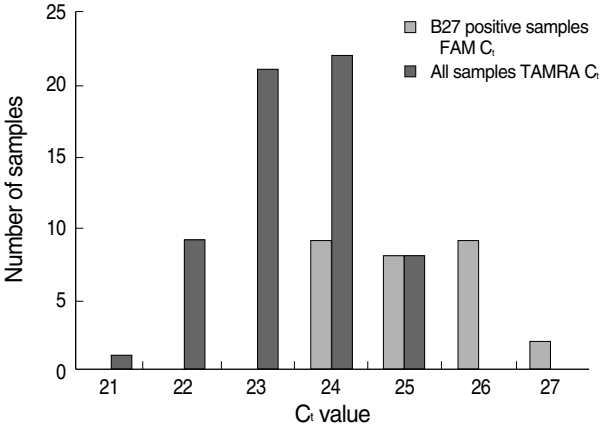

Fig. 1. Histogram representation showing the distribution of C_t FAM (HLA B-27) and C_t TAMRA (*GAPDH*) from 61 samples on genotyping by Taqman allele-specific amplification. All samples had C_t TAMRA values <26, and 28 positive samples had C_t FAM values <28.
Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

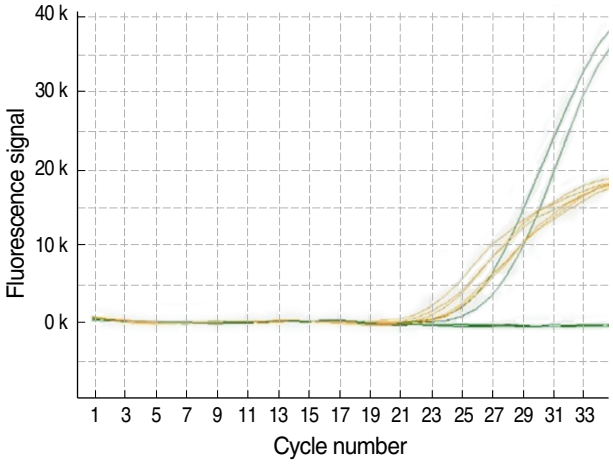

Fig. 2. Fluorescence signal versus cycle number plot during the amplification of 2 HLA-B27 positive cases out of six different human DNA samples with primers specific for HLA-B27 (green signal) and *GAPDH* (yellow signal).
Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

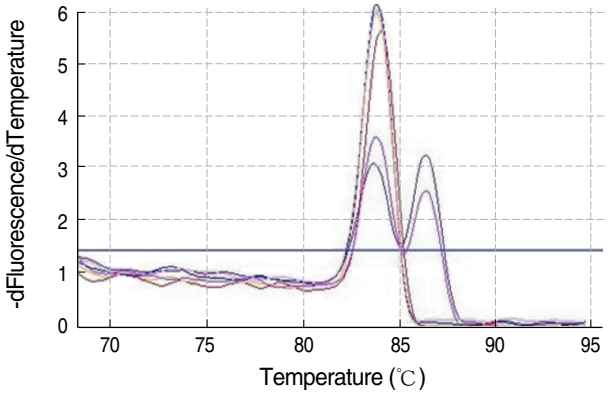

Fig. 3. Melting peaks specific for HLA-B27 around 86.5℃ and β-globin around 83.8℃ during the melting curve analysis of 2 HLA-B27 positive cases out of 6 different human DNA samples with SYBR Green I dye.

3. Comparison of three methods

Twenty-eight HLA-B27-positive samples analyzed by PCR-SSP were HLA-B27-positive by both real-time PCR methods, and the other 33 HLA-B27-negative samples were found to be HLA-B27-negative by both real-time methods. Thus, the results of the 3 assays were identical. A successful amplification was achieved in 100% of all the 61 samples tested. The conventional PCR-SSP method using gel electrophoresis takes approximately 5 hr. In comparison, the 2 real-time PCR systems can analyze 96 samples in <2 hr.

DISCUSSION

Serological methods, such as the microlymphocytotoxicity test, flow cytometry, and enzyme immunoassays used for typing of HLA-B27, lack specificity for the antigens that cross-react with HLA-B27, such as HLA-B7 [7, 8]. Therefore, several molecular methods have been developed for HLA-B27 genotyping. An approach widely used to detect HLA-B27 involves the use of SSP for PCR. However, conventional PCR-based genotyping requires post-PCR manipulations that increase the risk of cross-contamination between samples; further, these post-PCR steps are laborious, especially when genotyping large numbers of samples. Therefore, real-time PCR, which allows the simultaneous amplification and detection of a specific DNA target, is an attractive alternative to conventional PCR-SSP. Two real-time PCR formats for the genotyping of HLA-B27 were recently reported [9-12]. These methods used a real-time PCR with a TaqMan probe [11] and a LightCycler PCR machine (Roche Diagnostics, Indianapolis, IN, USA) with SYBR Green I dye or a fluorescence resonance energy transfer probe [9, 10, 12]. One major problem with SYBR Green-based detection is that non-specific amplification products and primer-dimers couldnot be distinguished from specific amplification products. However, a certain combination of specific primers and melting curve analysis of the PCR product results in an accurate determination of the HLA-B27 genotypes. Two Korean real-time PCR typing methods compare favorably with other real-time PCR-based methods previously described, because both methods accurately determined HLA-B27 genotypes and substantially reduced the labor-intensive steps and the total processing time (2 hr, including interpretation) when compared to the usual time required for PCR-SSP (5 hr). The additional cost incurred for obtaining the fluorescence probes that were necessary for real time PCR is compensated by the reduction in manual labor in the post-PCR steps.

A recent study of HLA-B27 subtypes in a Korean population revealed that HLA-B*2705 and B*2704 were the predominant subtypes; these subtypes were found in 244 (91.7%) and 22 (8.3%) of 266 AS patients [6]. Although the HLA-B*2704, B*2705, B*2706, and B*2707 subtypes were successfully identified, false-negative PCR results with rare HLA-B27 subtypes containing mismatches with the primers cannot be excluded. The HLA-B*2712,*2716,*2718, and *2723 subtypes, which had no known association with AS, had sequence variations at the primer binding site of the AccuPower® HLA-B27 real-time PCR kit. Nonetheless, these 2 real-time PCR approaches have the potential of becoming standard methods for diagnostic typing of HLA-B27, especially when HLA-B27 subtype frequencies in Korean populations and rare alleles of HLA-B27 that are not associated with AS are taken into account.

In conclusion, these real-time PCR methods appear to be reliable and very fast in the detection of HLA-B27 and

could be potential alternatives to conventional PCR methods.

REFERENCES

1. Gonzalez S, Martinez-Borra J, Lopez-Larrea C. Immunogenetics, HLA-B27 and spondyloarthropathies. Curr Opin Rheumatol 1999; 11:257-64.
2. Whang DH, Yang YS, Hong HK. Allele and haplotype frequencies of human leukocytes antigen-A, -B, and -DR loci in Koreans: DNA typing of 1,500 cord blood units. Korean J Lab Med 2008;28:465-74. (황동희, 양윤선, 홍혜경. 한국인에서 Human Leukocyte Antigen-A, -B, -DR 대립유전자와 일배체형 빈도: 제대혈 1,500단위의 DNA 형별검사. 대한진단검사의학회지 2008;28:465-74.)
3. Roh EY, Kim HS, Kim SM, Lim YM, Han BY, Park MH. HLA-A, -B, -DR allele frequencies and haplotypic associations in Koreans defined by generic-level DNA typing. Korean J Lab Med 2003;23:420-30. (노은연, 김현수, 김선미, 인영미, 한복연, 박명희. DNA 형별검사에 의한 한국인의 HLA-A, -B, -DR 형별 및 일배체형의 분포. 대한진단검사의학회지 2003;23:420-30.)
4. Khan MA, Mathieu A, Sorrentino R, Akkoc N. The pathogenetic role of HLA-B27 and its subtypes. Autoimmun Rev 2007;6:183-9.
5. Park KS, Kang SY, Lee WI. HLA-B27 subtypes in Korean patients with ankylosing spondylitis. Korean J Lab Med 2008;28:46-52. (박경선, 강소영, 이우인. 한국인 강직성 척추염 환자의 HLA-B27 아형 연구. 대한진단검사의학회지 2008;28:46-52.)
6. Lee SH, Choi IA, Lee YA, Park EK, Kim YH, Kim KS, et al. Human leukocyte antigen-B*2705 is the predominant subtype in the Korean population with ankylosing spondylitis, unlike in other Asians. Rheumatol Int 2008;29:43-6.
7. Dunky A, Neumuller J, Hubner C, Fischer GF, Bayer PM, Wagner E, et al. HLA-B27 determination using serological methods. A comparison of enzyme immunoassay and a microlymphocytotoxic test with flow cytometry and a molecular biological assay. Rheumatol Int 1996;16:95-100.
8. Levering WH, Wind H, Sintnicolaas K, Hooijkaas H, Gratama JW. Flow cytometric HLA-B27 screening: cross-reactivity patterns of commercially available anti-HLA-B27 monoclonal antibodies with other HLA-B antigens. Cytometry B Clin Cytom 2003;54:28-38.
9. Bon MA, van Oeveren-Dybicz A, van den Bergh FA. Genotyping of HLA-B27 by real-time PCR without hybridization probes. Clin Chem 2000;46:1000-2.
10. Faner R, Casamitjana N, Colobran R, Ribera A, Pujol-Borrell R, Palou E, et al. HLA-B27 genotyping by fluorescent resonance emission transfer (FRET) probes in real-time PCR. Hum Immunol 2004;65: 826-38.
11. Sylvain K, Aurelie H, Marc M, Christophe R. Rapid screening for HLA-B27 by a TaqMan-PCR assay using sequence-specific primers and a minor groove binder probe, a novel type of TaqMan trade mark probe. J Immunol Methods 2004;287:179-86.
12. Tiemann C, Vogel A, Dufaux B, Zimmer M, Krone JR, Hagedorn HJ. Rapid DNA typing of HLA-B27 allele by real-time PCR using lightcycler technology. Clin Lab 2001;47:131-4.