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
serologic methods in clinical practice; however, these tech-
niques 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. Rec-
ently, 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 ev-
aluatethe 2 Korean HLA-B27 kits with real-time analysis.

MATERIALS AND METHODS

1. Materials

In this study, we analyzed 61 samples that had been pr-
eviously 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 Am-
erican Pathologists (HLA-B27–A, 2008; HLA-B27–B, 2008;
ML–C, 2007; and ML–B, 2008). Genomic DNA was extract-
ed using the QIAamp blood kit (Qiagen, Hilden, Germany)
from 200 μL of peripheral blood, which had been anticoag-
ulated with ethylenediamine tetraacetic acid (EDTA), ac-
cording 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 he-
terzygous 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 Exi-
cycler™ 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 glyc-
eralddehyde–3–phosphate dehydrogenase (GAPDH)–spe-
cific probe, which were labeled with a fluorescent reporter
dye FAM (6–carboxyfluorescein) and TAMRA (6–carboxy-
tetramethyl–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 per-
formed 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 poly-
merase, deoxynucleotide triphosphates (dNTPs), and sta-
bilizer, 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 denatura-
tion step at 95°C for 5 min, followed by 35 amplification
cycles of denaturation for 5 sec at 95°C, annealing and ex-
tension for 15 sec at 65°C. 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 (Ct). A cut–off value was assigned on the basis of
Ct values for FAM and TAMRA reporter dyes to interpret
the results. Specimens yielding Ct FAM value of <30 and a
Ct TAMRA value of <27 were interpreted as being HLA–B27–positive. Samples yielding a Ct FAM value of >30 were
considered HLA–B27–negative if the internal control sig-
nal was above the assigned cut–off (Ct TAMRA, <27). A
sample was considered to contain inhibitory substances or
degraded DNA, if the Ct 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™ 6000 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°C for 10 min, followed by 35 amplification cycles (30 sec of denaturation at 95°C, 30 sec of annealing at 63°C, and 30 sec of extension at 72°C. Melting curve analysis was followed by the generation of a thermal gradient from 50°C to 90°C, with 5–sec intervals at 1°C after 30 sec of the first step. HLA-B27 alleles were assigned to the characteristic peaks formed at 86.5±1°C. Hence, HLA–B27–negative samples showed a single temperature curve, corresponding to β-globin (peak at 83.8±1°C), whereas B27–positive samples showed 2 peaks at 86.5±1°C and 83.8±1°C or one peak around 86.5±1°C. 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.

RESULTS

1. Real-time PCR with specific probes

All positive samples had C FAM values <28. All samples had C TAMRA values <26 (Fig. 1). HLA–B27–positive and –negative samples were discriminated with a clear cut-off value (C 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°C. All negative samples had only one peak at 83.5–84.3°C (Fig. 3). A graphical representation of our results revealed 2 clearly distinct populations corresponding to HLA–B27–positive and –negative samples.
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 could not 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


