ORIGINAL ARTICLE

The detection of HLA-B27 allele types by quantitative real-time polymerase chain reaction with melting curve analysis

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Abstract

Human leucocyte antigen B27 (HLA-B27) is a major histocompatibility complex class 1 molecule that is strongly associated with ankylosing spondylitis. The polymerase chain reaction with sequence specific primers (PCR-SSP) is currently used for the discrimination of HLA-B27 allele types resulting from single base substitutions. However, this technique is a multi-step, time-consuming task and increases the chance of post-PCR contamination. The main objective of this study is to determine the HLA-B27 genotypes using quantitative real-time polymerase chain reaction (Q-PCR) followed by melting curve analysis. Genomic DNA (gDNA) was isolated from 50 outdated peripheral blood samples stored at -20 °C in the Molecular Genetics Laboratory, Department of Pathology, Phramongkutklao College of Medicine, Bangkok, Thailand. These blood specimens were previously collected from 25 patients each who were HLA-B27 negative and positive in PCR-SSP. The genotyping of HLA-B27 was detected in the isolated gDNA by a Q-PCR assay with melting curve analysis. All HLA-B27-positive blood samples yielded either a single peak or double peaks at 87.28 ± 1.36 °C. Only one peak for an endogenous reference gene (human *beta-globin* gene) was detectable at 84.90 ± 0.09 °C in all HLA-B27-negative blood samples. The Q-PCR followed by melting curve analysis is reliable for the DNA typing of the HLA-B27 alleles and could be used to an alternative to a conventional PCR-SSP method.

Keywords: human leucocyte antigen B27; melting curve analysis; quantitative real- time polymerase chain reaction

Introduction

Ankylosing spondylitis (AS) is a form of arthritis that effects the spine[1]. It causes inflammation of the spinal joints that can lead to severe and chronic pain. Human leukocyte antigen B27 (HLA-B27) is associated with AS, including ocular diseases, rheumatic diseases and inflammatory bowel disease. Approximately 95% of patients with AS have HLA-B27 compared with only 8% of healthy individuals. Several methods have been developed for the identification of the HLA-B27 allele[1], i.e. serology, polymerase chain reaction (PCR), including the standard PCR with sequence-specific primers (SSP). However, PCR is a time-consuming process and also requires post-PCR manual procedures[2]. Currently, the application of real-time PCR or quantitative PCR (qPCR) with a double-stranded DNA binding SYBR Green dye has facilitated the rapid detection and amplification of PCR products[3,4]. The aim of this study was to develop of the qPCR analysis for HLA-B27 allele test in routine work.

Materials and Methods

Samples:

Fifty outdated peripheral blood samples were enrolled in this study. They are comprised of 25 HLA-B27-negative and 25 HLA-B27-positive blood samples. All blood samples were kept at -20 ℃ until DNA isolation and analysis[5]. Genomic DNA was isolated from 500 µL of whole blood using the AxyPrep™ Blood Genomic DNA Miniprep Kit (CA, USA) according to the manufacturer's instructions.

Real-time PCR analysis:

The real-time PCR was performed on qTower^3-Real-Time PCR thermocycler (Analytik Jena AG, Germany). A total volume of 12 µL of each mixture consisted of 5 µl of SYBR Green I (EXPRESS SYBR™ GreenER™ qPCR Supermix, universal; Invitrogen, USA); 4 µL of detection mix containing the primer E91S and 136AS specific for HLA-B27, primer BG1 and BG2 specific for ß-globin[3]; 1 µL of distilled water; and 2 µl of DNA template. The real-time PCR amplification protocol for this reaction consisted of an initial denaturation step at 95 ℃ for 5 sec, and 45 cycles of 95 ℃ for 4 sec, 68 ℃ for 1 min, 95 ℃ for 45 sec, 58 ℃ for 1 sec and final extension at 72 ℃ for 33 sec. After amplification was complete, a final melting curve analysis (T_m) was followed by the generation of a thermal gradient from 60 ℃ to 95 ℃ with a ramp rate of 5 ℃/s. HLA-B27 positive samples give a unique melting peak at 87.28 ± 1.36 ℃. Therefore, HLA-B27 negative samples showed a single temperature curve at 84.90 ± 0.09 ℃ representing the ß-globin[5,6].

The results were confirmed on the agarose gel. The melting peak for ß-globin showed 268 bp PCR product and the melting peaks for HLA-B27 alleles showed 136 bp PCR product representative of HLA-B27-positive samples[5].

Results

The results of real-time PCR showed that among 50 samples, 25 samples were HLA-B27-positive and 25 samples were HLA-B27-negative. All negative samples had only one peak at 84.90 ± 0.09 ℃ (*Figure 1A*). All positive samples showed single or double peaks at 87.28 ± 1.36 ℃ (*Figure 1B*). This signal was absent for the HLA-B27 negative samples. The melting peaks for ß-globin (T_m = 84.90 ± 0.09 ℃) and the 268 bp PCR product were visible on the agarose gel correlated with HLA-B27-negative samples (*Figure 2A*). The melting peaks for HLA-B27 (T_m = 87.28 ± 1.36 ℃) and the 136 bp PCR product are visible on the agarose gel correlated with HLA-B27-positive samples (*Figure 2B*). Despite the lower fragment length, the HLA-B27 specific PCR product (136 bp) yielded a higher T_m value compared with the ß-globin 268 bp product because of a higher GC content[5].

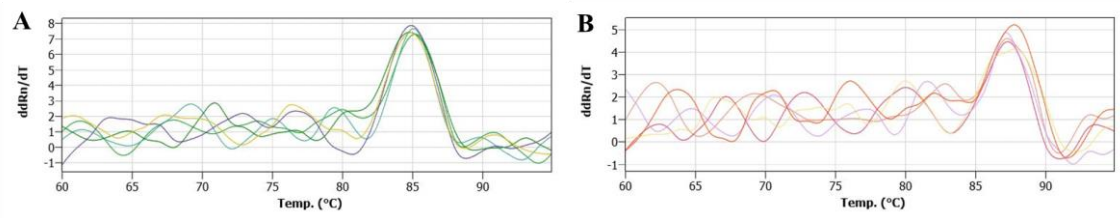

Figure 1 Melting curve analysis (T_m) from HLA-B27 negative samples (A) and HLA-B27 positive samples (B). The T_m of negative samples at 84.90 ± 0.09 ℃ results from the ß-globin. The T_m of positive samples at 87.28 ± 1.36 ℃ results from the HLA-B27.

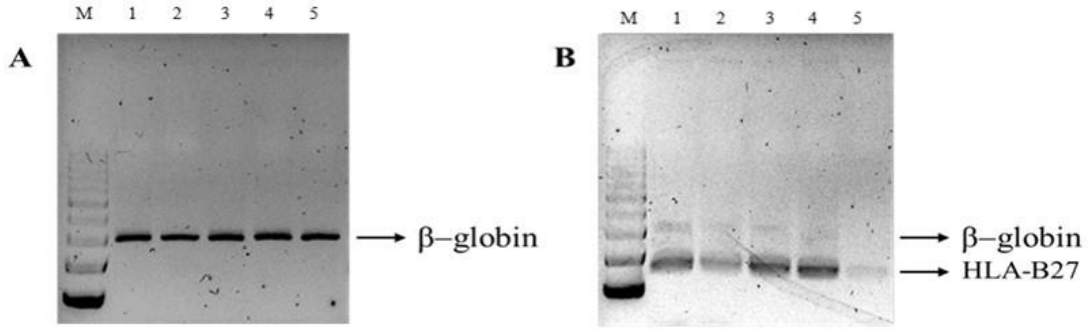

Figure 2 Gel electrophoresis on 2% agarose of PCR products from after real-time PCR. PCR products of HLA-B27 using E91S, 136AS primers and β-globin. The 268 bp PCR product specific for HLA-B27 negative samples **(A)**. The 136 bp PCR product specific for HLA-B27 positive samples **(B)**. Lane M, DNA ladder ranges from 100 to 1000 bp.

Discussions

We compared the results of 50 genotyped individual with our previous original PCR method. All genotypes were completely concordant. However, conventional PCR requires post-PCR manipulations that increase the risk of cross-contamination between samples. These post-PCR steps are laborious, especially when large numbers of samples and cost-effective[6,7]. Thus, we are starting point for development of a real-time PCR. Real-time PCR is one of the methods used to detect for HLA-B27 allele in patients suspected to have AS and related diseases. Real-time PCR is much more accurate than PCR. Real-time PCR substantially reduced the labor-intensive steps and the total processing time when compared to the usual time required for PCR. It is therefore a suitable in routine laboratory practice[8].

Conclusion

The real-time PCR assay is possibly reliable for the detection of HLA-B27.

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