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Validation of a Rapid, Robust, Inexpensive Screening Method for Detecting the HLA-B*58:01 Allele in the Prevention of Allopurinol-Induced Severe Cutaneous Adverse Reactions

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The HLA B*58:01 allele has been worldwide reported as a pharmacogenetic susceptibility to allopurinol-induced severe cutaneous adverse reactions (SCARs). To prevent these life-threatening conditions, the American College of Rheumatology hingly recommended that the HLA-B*58:01 be screened prior to the initiation of allopurinol therapy. Therefore, we developed a rapid, robust, inexpensive screening method using SYBR® Green real time PCR to detect the HLA-B*58:01 allele. A total of 119 samples were tested. The assay has a sensitivity of 100% (95% CI: 69.15%-100%), a specificity of 100% (95% CI: 96.67%-100%), a positive predictive value of 100% (95% CI: 69.15%-100%) and a negative predictive value of 100% (95% CI: 96.67%-100%). HLA-B*58:01 genotyping results showed 100% agreement with those obtained from Luminex SSO/SBT/SSP. The lowest limit of detection of this method is 0.8 ng/μL of DNA. The unit cost of the test is only $3.8 USD. This novel screening test using SYBR® real time PCR would be appropriate to identify individuals with the HLA-B*58:01 allele for the prevention of allopurinol-induced SCARs.

Key Words: Allopurinol hypersensitivity; HLA-B antigens; real time polymerase chain reaction; Stevens Johnson syndrome; toxic epidermal necrolysis; drug reactions with eosinophilia and systemic symptoms; severe cutaneous adverse reactions

INTRODUCTION

Allopurinol, a xanthine oxidase inhibitor, is more commonly used as a first-line therapy for the gout,[1] asymptomatic hyperuricemia-related cell-lysing therapy in malignancy diseases, and nephrolithiasis-associated hyperuricsuria.[2] However, allopurinol has been reported as a cause of cutaneous adverse reaction in 2%-3% of allopurinol users[3] and as the most common culprit medication of severe cutaneous adverse drug reactions (SCARs), including Steven-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug reactions with eosinophilia and systemic symptoms (DRESS) in Europe[4] and Asia.[5]

Since 2005, the HLA B*58:01 allele has been worldwide reported as a pharmacogenetic susceptibility to allopurinol-induced SJS/TEN/DRESS in diffirent populations.[6,7] The strongest association has been observed in a Taiwanese population (OR 580.3 [95% CI: 34.4-9,780.9]).[8] Although there are other risk factors for allopurinol hypersensitivity, such as higher dose, concurrent renal disease, and the use of diuretic medications[2] the HLA-B*58:01 allele contributes to the susceptibility and pathogenesis in a significant proportion of cases of allopurinol-induced SCARs. Subsequently, in 2012, the American College of Rheumatology recommended that HLA-B*58:01 be screened prior to the initiation of allopurinol treatment.[1]

To evaluate the benefit of screening tests, a number of studies were conducted and the results showed that screening for the HLA-B*5801 allele could potentially prevent SCARs, allopurinol - related fatalities, and be more cost-effective in comparison to

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the initiation of treatment without HLA screening.[9-11]

To apply HLA-B screening routinely in clinical practice, the key property of screening tests is simple and cost-effective,[12] while current various methodologies for HLA-B typing, such as polymerase chance reaction (PCR) using specific sequencing primers (SSP), sequencing specific oligonucleotide probe (SSO), and sequencing based-typing (SBT), are usually expensive and time-consuming[13] with the turnaround time up to 3 weeks.[14] In order to reduce time and cost of test, real time PCR might be a candidate for the screening method. Recently, we have just proposed a newly developed method for the detection of both HLA-B*58:01 and HLA-B*57:01 by using TaqMan® probe realtime PCR.[15] Herein, we present a rapid, robust, inexpensive approach to screen HLA-B*58:01 using SBYR® real time PCR.

MATERIALS AND METHODS

DNA samples

The samples for optimization and validation purposes (n=119) were randomly collected from those submitted for testing to ImmunoRheumatology Department, Pathology North (Australia) and from Vietnamese individuals in Sydney and Vietnam. The study protocol was approved by the the Northern Sydney Local Health District HREC, St Leonards, NSW, Australia (HREC/15/HAWKE/86).

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes collected into EDTA anti-coagulated tubes using 2 methodologies. All the blood samples were stored at -20°C until the day of extraction. The first methodology using the Wizard Genomic DNA extraction kit (Promega Corp, Madision, WI, USA) employs a modified version of the salting-out procedure as described by Miller *et al.*[16] The second methodology uses a silica-based method (AccPrep Genomic DNA Extraction kit, Bioneer Corp, Daejeon, Korea). DNA concentration was measured by using NanoPhotometer™. The average DNA concentration was approximately 46 ng/μL, and the A260/280 ratio was over 1.7 each in the 2 methodologies.

High resolution HLA-B typing using Luminex–SSO/SBT/SSP was performed in each sample at the New South Wales Transplantation and Immunogenetics Service (Australian Red Cross Blood Service).

Real time PCR using SBYR for the detection of HLA–B*58:01

A set of sequence specific oligonucleotides (Geneworks Pty Ltd, Thebarton, Australia) was modified from the set of primers previously published by Virakul *et al.*,[17] and SYBR® Green real time PCR was used to amplify the HLA-B*58:01 allele. This set of primers targets the region between exons 2 and 3 of HLA-B where HLA-B*58:01 polymorphisms are located and discriminated to the rest of HLA alleles, yielding an amplicon of 360 bps (B58:01–F: 5´-ACG-GAA-CAT-GAA-GGC-CTC-C-3´ and B58:01-R: 5´-GCC-ATA-CAT-CCT-CTG-GAT-3, respectively) (Fig. 1). The housekeeping gene actin (ACTB) was used as an internal control (ACTB-F 5´-CTG-TGC-TGT-GGA-AGC-TAA-GT-3´, ACTB-R 5´-GAT-GTC-CAC-GTC-ACA-CTT-CA-3´). A standard curve was prepared to calculate reaction efficiencies for the singleplex of B58:01 and ACTB by preparing a series of four 10-fold dilutions ranging from 80 to 0.08 ng/μL. A multiplex PCR was run to determine the lowest limit of detection by using dissociation curves. The annealing temperature of 65°C was identified as the optimal annealing temperature for both amplicons. The target amplicons that were amplified by the present method were confirmed by electrophoresis on 1.2% agarose gel (FlashGel® Cassette-Lonza, Allendale, NJ, USA) and by direct DNA sequencing using BigDye terminators v3.1 (Applied Biosystems, Waltham, WA, USA).

PCR reactions for both the target gene and internal control were initially prepared separately using the SensiMix™ SYBR No-ROX Kit (Bioline, Alexandria, Australia). PCRs were performed in a Rotor-Gene-Q cycler (Qiagen, Hilden, Germany). A multiplex PCR was performed using the same master mix, 400 nM of each B*58:01 specific primers and 200 nM of each ACTB primers. That was the best ratio determined for multiplexing reactions. The following PCR profile was used, initial hot-start 95°C for 3 minutes, followed by 40 cycles of 96°C for 15 seconds, 65°C for 15 seconds, and 72°C for 60 seconds.

Data analysis

Raw real time PCR data were analyzed using Rotor-Gene v6 software (Qiagen). Statistical analyses for the comparative and validation studies to calculate sensitivity, specificity; positive and negative predictive values were performed using MedCalc v12.7.0.0 (MedCalc Software, Ostend, Belgium). A Cohen's kappa for measuring agreement between the results obtained by using the screening method and the confirmatory test with Luminex SSO/SBT/SSB was analyzed using SPSS software version 22.0 (Chicago, IL, USA).

Raw data of direct sequencing obtained from Applied Biosystems 3130 Genetic Analyzer were analyzed using Chomas software 2.5.0 (Applied Biosystems).

RESULTS

Following the initial optimization of the reaction, testing on positive controls with the HLA-B*58:01 allele and negative control to identify an annealing temperature was performed for each set of primers for target and reference genes detection. Results demonstrated that 65°C was the ideal annealing temperature. To optimize the multiplex PCR, different ratios of the HLA-B*58:01 allele and ACTB primers concentrations were tested (data not shown). In comparing dissociation curves, the best ratio observed was 2:1 (400 nM of each B*58:01 specific primers

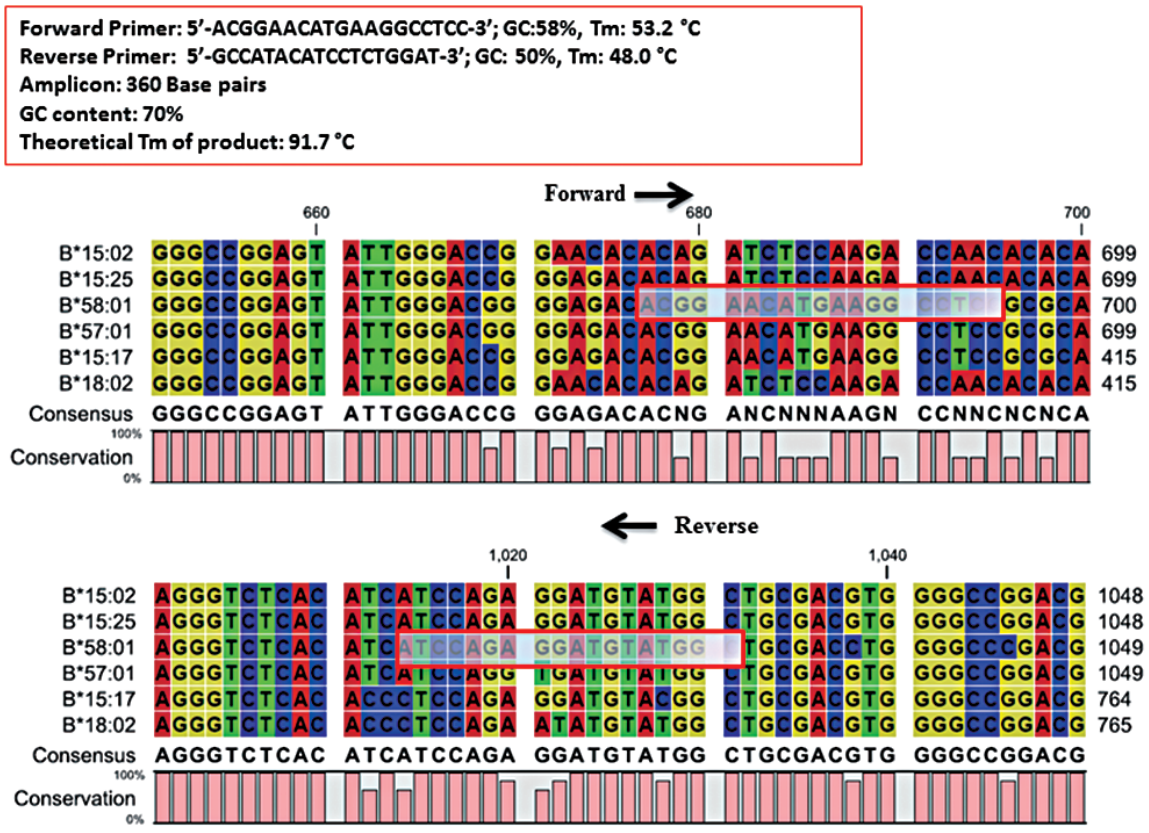

Fig. 1. Binding sites and properties of HLA-B*58:01 Primers.

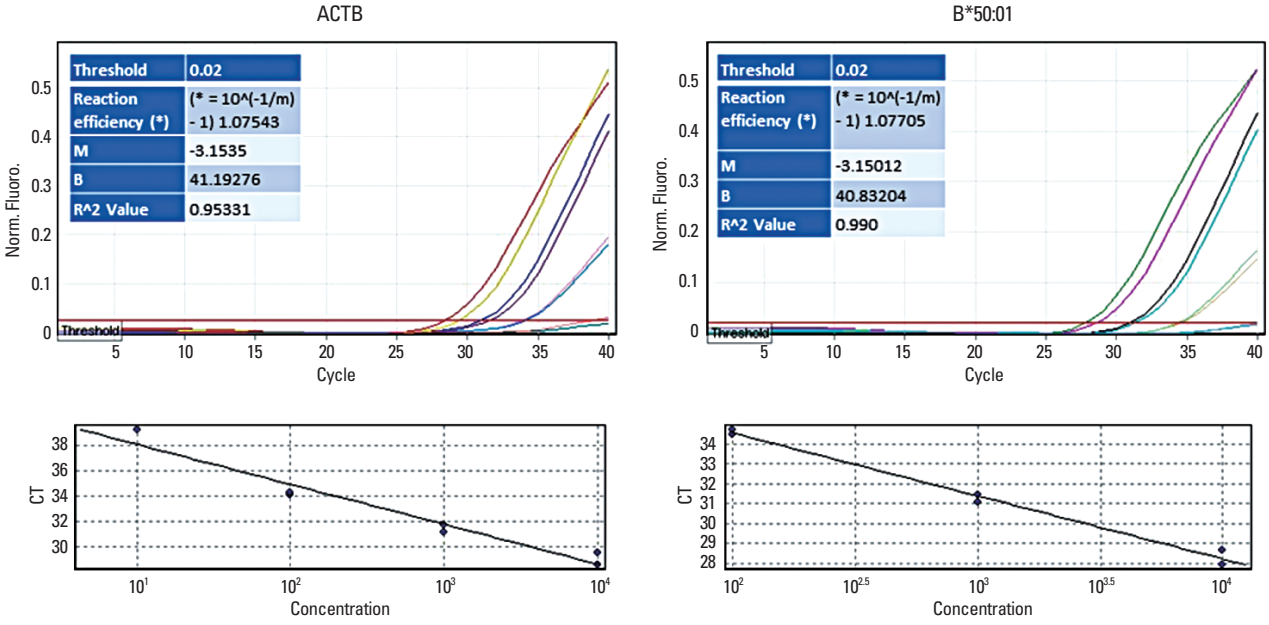

Fig. 2. PCR efficiencies of the HLA-B*58:01 allele and ACTB.

and 200 nM of each ACTB primers) and PCR efficiencies for HLA-B*58:01 was 107% (the lowest Ct: 28.53; highest Ct: 34.48; slope: -3.15; r^2=0.99), ACTB was 107% (the lowest Ct: 27.8; highest Ct: 39.2; slope: -3.15; r^2=0.95) (Fig. 2). The dissociation curve was used to determine the HLA-B*58:01 allele and ACTB with the theoretical TM of 91.7°C and 81.4°C, respectively. The

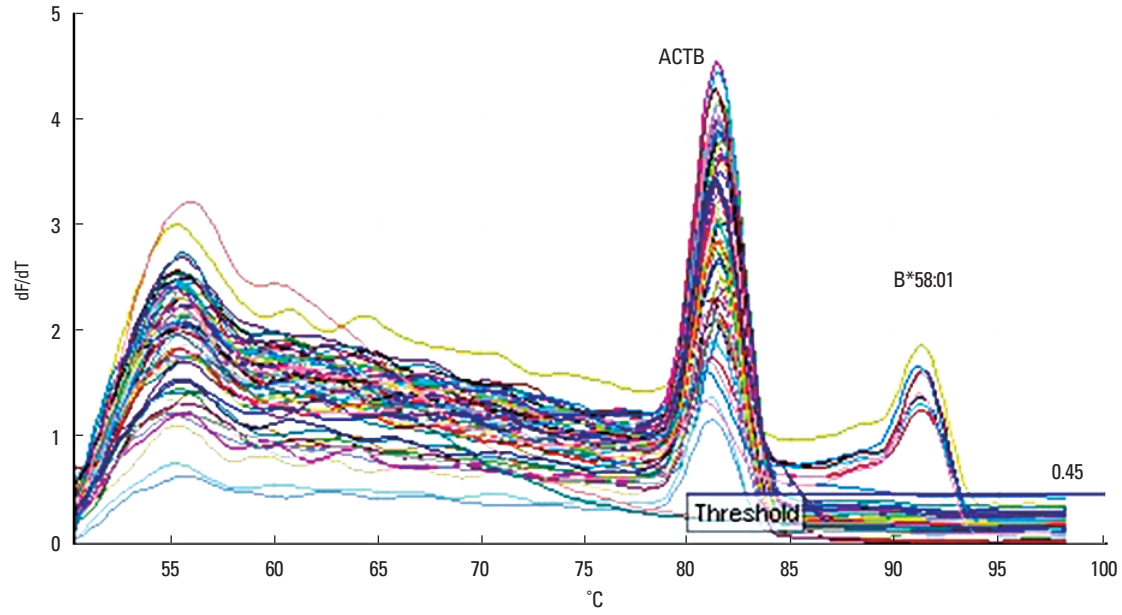

Fig. 3. Detection of the HLA-B*58:01 allele using dissociation curves of our screening method. The positive samples have 2 peaks of melting temperatures (ACTB and B*58:01); the negative samples have only 1 peak of ACTB.

Table. Comparison of the present method with Luminex SSO/SBT/SSP

SYBR real time PCR for HLA-B*58:01	Luminex SSO/SBT/SSP		
	HLA-B*58:01	Others	Total
Positive	10	0	10
Negative	0	109	109
Total	10	109	119

A sensitivity of 100% (95% CI: 69.15%-100%), a specificity of 100% (95% CI: 96.67%-100%), a positive predictive value of 100.0% (95% CI: 69.15%-100%), and a negative predictive value of 100.0% (95% CI: 96.67%-100%). One hundred percent agreement with Luminex SSO/SBT/SSP.

threshold for melting curves was set at 0.45 to avoid a false negative result. The assay was able to detect the target allele in a DNA sample with a concentration as low as 0.8 ng/μL, making it possible to use this assay for samples, such as buccal swabs.

The PCR product was confirmed by direct sequencing using BigDye terminators v3.1. The sequence of product had 100% matching with the sequence of the HLA-B*58:01 allele (HM 543722.1-EMBL-Bank Sequence Database).

From the total 119 samples tested, we detected 10 positive samples by using the present method. Two peaks were observed at 91.26°C±0.01°C (mean±standard error of mean; range 91.25°C-91.40°C) (HLA-B*58:01 allele) and 80.97±0.04 (ACTB). The 109 negative samples showed only 1 peak for ACTB (81.42°C±0.16°C) (Fig. 3). The comparative analysis between this assay using SYBR® Green real time PCR and Luminex SSO/SBT/SSP was performed by using MedCalc Software. Our screening assay had a sensitivity of 100% (95% CI: 69.15%–100%), a specificity of 100% (95% CI: 96.67%-100%), a positive predictive value of 100% (95% CI: 69.15%-100.0%), and a negative predictive value of 100% (95% CI: 96.67%-100%) (Table). HLA-B*58:01 genotyping results showed 100% agreement with those obtained from Luminex SSO/SBT/SSP.

DISCUSSION

In this study, we have reported a novel screening method for detecting the HLA-B*58:01 allele to prevent allopurinol-induced SCARs. Using SYBR® Green real time PCR, the target allele was amplified successfully and cost-effectively enabling it to be utilized in routine clinical practice.

In 2010, Virakul *et al.*[17] presented a method for detecting the HLA-B*58:01 allele using a touch down PCR-SSP. Although this method detected successfully the target allele from 200 samples, limitations of PCR-SSP are more time-consuming because post-PCR analysis is required. More importantly, we modified the reverse primer by removing 2 bases at the end of 3′ and 2 bases at the end of 5′ (Fig. 1). This advancement allowed our set of primers to amplify specifically the HLA-B*58:01 allele and minimized the risk of non-specific detection of the HLA-B*57:01 allele because a mismatch at the middle of the primer binding site was not robust enough for the latter one to eliminate the amplification of the HLA-B*57:01 allele (Fig. 1).

Recently, a similar approach was independently published for the detection of the HLA-B*58:01 allele using real time TaqMan® probe and specific primers.[3,13] Although real time PCR was also used, TaqMan® probe is less sensitive than SYBR® Green because of the probability of having false negative results due to mismatches at the probe binding site. To prevent SCARs,

the crucial properties of screening method are sensitivity and negative predictive values. A false negative result potentially could result in a life-threatening SCAR if a patient has been prescribed the medication. In our study, there were no false negative results. In addition, the studies using the TaqMan® probe were compared to the commercial SSP kit to determine validation of results. PCR–SSP is not a standard method of genotyping. In contrast, our study used high-resolution HLA-B typing using Luminex SSO/SBT/SSP and was performed independently and blindly at the New South Wales Transplantation and Immunogenetics Service (Australian Red Cross Blood Service). There was 100% agreement between our method and the Luminex SSO/SBT/SSP.

As compared to the commercially available testing kit (PG5801 Detection Kit; Pharmigene, Inc., Taipei, Taiwan), our method uses multiplexing reducing both time and costs. Multiplexing is also an optimal way to reduce the chances of obtaining a false negative result due to low quality of DNA or the presence of any potential PCR inhibitors. Further, it is more accurate and robust to detect the target PCR product using the melting curves rather than calculating delta Ct or loading PCR products on an agarose gel. Additionally, using our method, genomic DNA can be used to detect the target gene rather than to adjust concentration. Our assays are a robust and very sensitive technique that is able to detect the target allele at a very low amount of DNA (as low as 2 ng DNA/reaction), while the commercial kit requires 25-100 ng/reaction. Therefore, this method can be used for DNA that is extracted from specimens obtained by non-invasive methods, such as buccal swabs, which can have a lower yield of DNA. Finally, we calculated the unit price of our test, including DNA extraction kit, reagents for PCR that is only approximately 3.8 USD, being much cheaper than commercial kit (15 USD/kit), SBT (32 USD), SSO based typing (38 USD), and Loop-mediated isothermal amplification (6.4 USD) for the detection of the HLA-B*58:01 allele.[18]

A limitation of our assays is that since we tested and validated only 1 commercially available master mix (SensiMix™ SYBR No-ROX Kit), re-validation in an alternative mix will be required if this master mix is discontinued. Re-validation of the assay will also be required prior to implementation on other real time PCR cyclers although this could be of lesser concern because our method does not require the analysis of results using delta Ct and is only a qualitative one. Another limitation common to any other existing molecular test for HLA-alleles is related to the continuous need for sequence updates owing to the highly polymorphic nature of these genes. New polymorphisms that could be present in the primer-binding sites of the assay can always results in both false negative and false positive results.

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