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ORIGINAL ARTICLE

A novel nested allele-specific PCR protocol for the detection of the *HLA-A*33:03*, a SCAR-associated allele, in Vietnamese people

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Abstract

Background: Severe cutaneous adverse drug reactions (SCARs) are rare but deadly drug reactions with severe damages to patients. One of the most well-known SCARs risk factors is the *human leukocyte antigen* (*HLA*) genes polymorphism. Among the *HLA* polymorphic alleles, the *HLA-A*33:03* allele has been found in association with SCARs induced by various drugs, especially in Asian people. There has not been any report on the specific detection protocol of the *HLA-A*33:03* allele.

Objective: This study aimed to design a nested AS-PCR protocol for detecting and distinguishing diplotype genotype of the *HLA-A*33:03* allele.

Methods: A nested allele-specific (AS)-PCR protocol with four primer sets was designed. The method was compared with the Sanger sequencing method on 100 samples of unknown genotypes of unrelated Vietnamese people.

Results: The nested AS-PCR method could identify the *HLA-A*33:03* allele and the *HLA-A*33:03* diplotype genotypes. Comparison with the Sanger sequencing method showed an absolute agreement ($\kappa = 1.00$, $p < 0.001$). The nested AS-PCR protocol had a sensitivity of 100% (95%CI: 92.13-100%) and a specificity of 100% (95%CI: 93.51-100%). The protocol was used for the determination of *HLA-A*33:03* allele distribution in 810 unrelated Vietnamese Kinh people, showing a frequency of *HLA-A*33:03* carriers of 19.6% and an allele frequency of 10.55%.

Conclusions: A novel nested AS-PCR method with a hundred-percent sensitivity and a specificity for the *HLA-A*33:03* allele detection was reported. The protocol can be applied for the stratification of patients at SCAR risks with various drugs.

Key words: SCAR, *HLA-A*33:03*, nested AS-PCR, protocol, Vietnam

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Abbreviations:

ADR	adverse drug reaction
AF	allele frequency
AS	allele specific
DRESS	drug reactions with eosinophilia and systemic symptoms
HLA	human leukocyte antigen
SCAR	severe cutaneous adverse drug reactions
SJS	Steven-Johnson syndrome
SNP	Single nucleotide polymorphism
SSOP	sequence specific oligonucleotide probes
TEN	toxic epidermal necrolysis

Introduction

Severe cutaneous adverse drug reactions (SCARs) are rare but deadly drug reactions including Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN) or drug reactions with eosinophilia and systemic symptoms (DRESS) with mortality rate of 10–40% and severe damages to patients.[1,2] One of the most well-known SCARs risk factors is the *human leukocyte antigen* (*HLA*) genes polymorphism. A number of *HLA* alleles have been used as pharmacogenetic markers in order to minimize the risk of SCARs. The *HLA-A*33:03* allele has recently been found to be associated with SCARs induced by various drugs in Asian populations, for instance, by allopurinol in Korean people (OR = 20.5, $p = 3.31\times10^{-6}$),[3] in Taiwanese people (OR = 7.3, $p = 0.047$)[4] and in Han Chinese people (OR = 12.0, $p < 0.001$)[5] or by strontium ranelate in Han Chinese people (OR = 19.4, $p = 0.006$).[6] A meta-analysis showed an association of the *HLA-A*33:03* allele with SJS/TEN induced by common cold medicines (OR = 2.28; 95%CI = 1.31–3.97).[7] Moreover, the *HLA-A*33:03* allele was reported as a risk factor for ticlopidine-induced cholestatic hepatotoxicity (OR = 36.5, $p = 7.32\times10^{-7}$).[8] Therefore, the *HLA-A*33:03* allele might be used as a molecular marker for personalized medicine in Asian people. Notably, the frequency of the *HLA-A*33:03* allele is relatively high in several Asian populations: 16.07% in Korean population,[9] 11.17% in Thai people.[10]

There has not been any report on a specific detection protocol of the *HLA-A*33:03* allele in clinical settings. Methods used for genotyping of the *HLA-A*33:03* allele such as sequencing[11] or sequence specific oligonucleotide probes (SSOP)[10,12] or multiplex real-time PCR,[13] scanning series of *HLA* genes, are very expensive and would be more suitable for research purposes.

In this study, we designed a novel nested AS-PCR protocol for detecting and distinguishing homozygous/ heterozygous genotype of the *HLA-A*33:03* allele. The protocol was used for the determination of *HLA-A*33:03* allele distribution in 810 unrelated Vietnamese Kinh people.

Methods

Human genomic DNA samples

Seven DNA samples of known *HLA-A* genotype supplied by the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand were used for the optimization of the nested AS-PCR protocol. The *HLA-A* genotypes of those samples were identified by SSOP method.[10]

For validation purpose, 100 DNA samples were collected from 100 unrelated Vietnamese Kinh people including 52 healthy volunteers and 48 allopurinol-induced SCAR patients admitted to Bach Mai Hospital, Hanoi, Vietnam.

For the determination of the *HLA-A*33:03* allele distribution, 810 DNA samples were collected from 810 unrelated Vietnamese Kinh people in the three main regions of Vietnam.

This study complied with the Declaration of Helsinki and was approved by the Ethics Committee of the Vietnam National Institute of Hygiene and Epidemiology (Approval Decision: IRB-VN01057-6/2018). All of the participants provided their written informed consents.

DNA isolation

The E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek, Atlanta, USA) was used for whole blood DNA isolation. The Nanodrop 2000 (Thermo Fisher, Waltham, USA) was used for DNA quantification and qualification. The samples at the concentration of 35–250 ng/µl and the A260/280 of 1.65–1.95 were chosen for the PCR reactions.

The nested AS-PCR method

There were two steps in the nested AS-PCR protocol. For the first PCR, each 20 µL of reaction mixture contained 40 ng of genomic DNA, 0.5 pM of each primer and 10 µL of GoTaq® Green Master Mix 2x (Promega Corporation, Madison, USA). The PCR conditions were 95°C for 3 minutes, followed by 26 cycles of 95°C for 30 seconds, and 63°C for 30 seconds, 72°C for 60 seconds; and finally 72°C for 7 minutes. 1 µL of the 100 fold-diluted PCR product was used as a template for the second step.

In the second step, three PCR reactions were performed in parallel in three separate tubes. Each 20 µL of the reaction mixture contained 1 µL of the diluted product of the first step, 0.5 pM of each primer and 10 µl of GoTaq® Green Master Mix 2x (Promega Corporation, Madison, USA).

For specificity enhancement, touchdown PCR cycles were used as follows: 95°C for 3 minutes; 5 cycles of 95°C for 30 seconds, 67°C for 30 seconds, 72°C for 30 seconds; 5 cycles of 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds; 10 cycles of 95°C for 30 seconds, 63°C for 30 seconds, 72°C for 30 seconds; 20 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds; and finally 72°C for 7 minutes.

Sequencing

The BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, USA) and an ABI™ 3500 analyzer (Applied Biosystems, Massachusetts, USA) were used for the Sanger sequencing. The primer sequences and sequencing procedures have been previously described.[14]

Data analysis

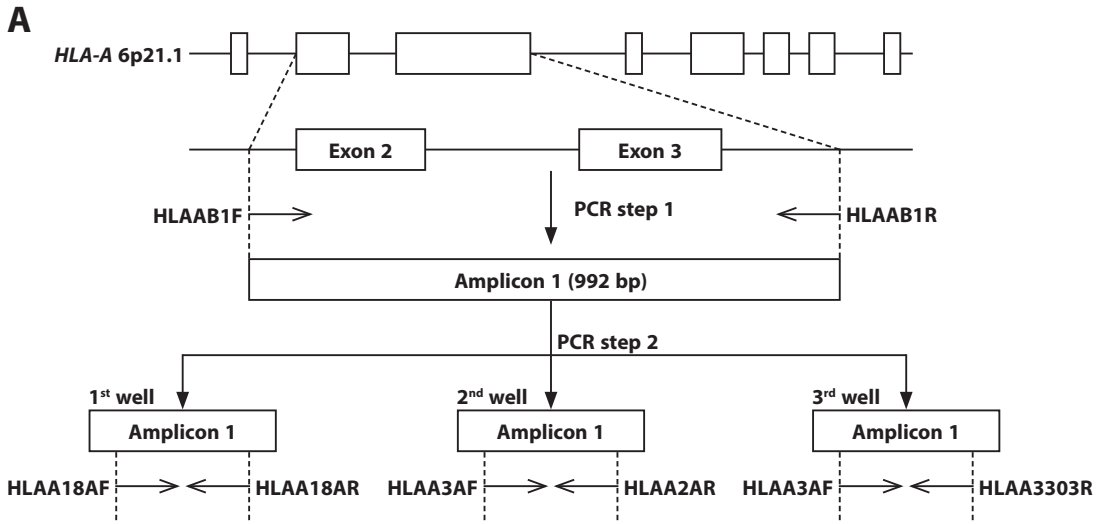
The sensitivity and specificity of the *HLA-A*33:03* allele detection were evaluated with the MedCalc v19.2.3 (MedCalc Software, Ostend, Belgium). Raw sequencing data were analyzed with the Bioedit 7.0.5.3 (Informer Technologies, Inc). Statistical comparisons between the nested AS-PCR method and the Sanger sequencing method were made with the SPSS 20 (Chicago, IL, USA).

Results

Figure 1 shows the principle of the detection of the *HLA-A*33:03* allele.

In the first step, the exon 2–3 region, which contains most of the SNPs of the *HLA-A* gene was amplified with the primer set HLAAB1F/ HLAAB1R. The second PCR step using three primer sets (**Table 1**) aimed to distinguish the *HLA-A*33:03* allele from the other 20 *HLA-A* alleles found in the Vietnamese population,[12] of which, there are two analogous alleles (*HLA-A*29:01* and *HLA-A*31:01*). **Figure 2** and **Figure 3** show the binding sites of these primer sets.

A



B

Result of electrophoresis after PCR step 2			Conclusion
HLAA18AF and HLAA18AR	HLAA3AF and HLAA2AR	HLAA3AF and HLAA3303R	
+	+	-	*HLA-A*33:03* negative
-	+	-	*HLA-A*33:03* negative
+	-	-	*HLA-A*33:03* negative
+	-	+	*HLA-A*33:03* heterozygote
-	+	+	*HLA-A*33:03* heterozygote
-	-	+	*HLA-A*33:03* homozygote

Figure 1. Diagram for detecting and distinguishing homozygous/heterozygous genotype of the *HLA-A*33:03* allele.
A. PCR procedures: Step 1. The primer set HLAAB1F/HLAAB1R specifically amplified the exon 2-3 segment of the *HLA-A* gene; Step 2. The 992 bp PCR product in step 1 was then used as a template for the PCR reactions in step 2 which used 3 primer sets. B. The different patterns can be obtained with the 3 primer sets in the second PCR and the according results of *HLA-A*33:03* diplotype genotypes.

Table 1. Primer sequences for detecting and distinguishing between the homozygous and heterozygous *HLA-A*33:03* genotypes.

Primer	Sequence (5′ – 3′)	Amplicon	Tm (°C)
PCR 1			
HLAAB1F	CGGCCTCTGTGGGGAGAAGCA	992 bp	70
HLAAB1R	GATATTCTAGTGTTGGTCCCAATTGT		72
PCR 2			
HLAA18AF	CCCACTCCATGAGGTATTACT	358 bp	62
HLAA18AR	CTCGGACCCGGAGACTGT		60
HLAA3AF	CCCACTCCATGAGGTATTACA	202 bp	62
HLAA2AR	GGCCTTCACATTCCGTATC		58
HLAA3AF	CCCACTCCATGAGGTATTACA	202 bp	62
HLAA3303R	GGCCTTCACATTCCGTATG		58

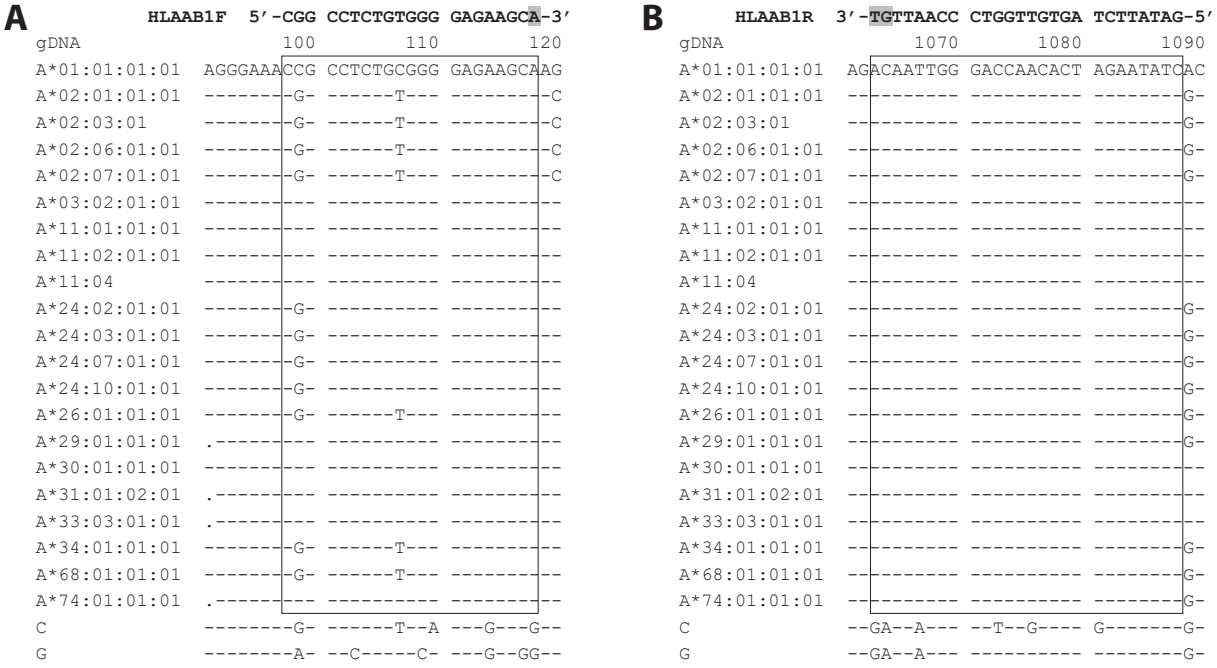


Figure 2. Binding sites of the primer set (HLAAB1F/ HLAAB1R) used in the first PCR.
A. The forward primer HLAAB1F of step 1 PCR. B. The reverse primer HLAAB1R of step 1 PCR. The nucleotides highlighted in grey of each primer would avoid nonspecific pairing with the exon 2-3 regions of the *HLA-C* and *HLA-G* genes.

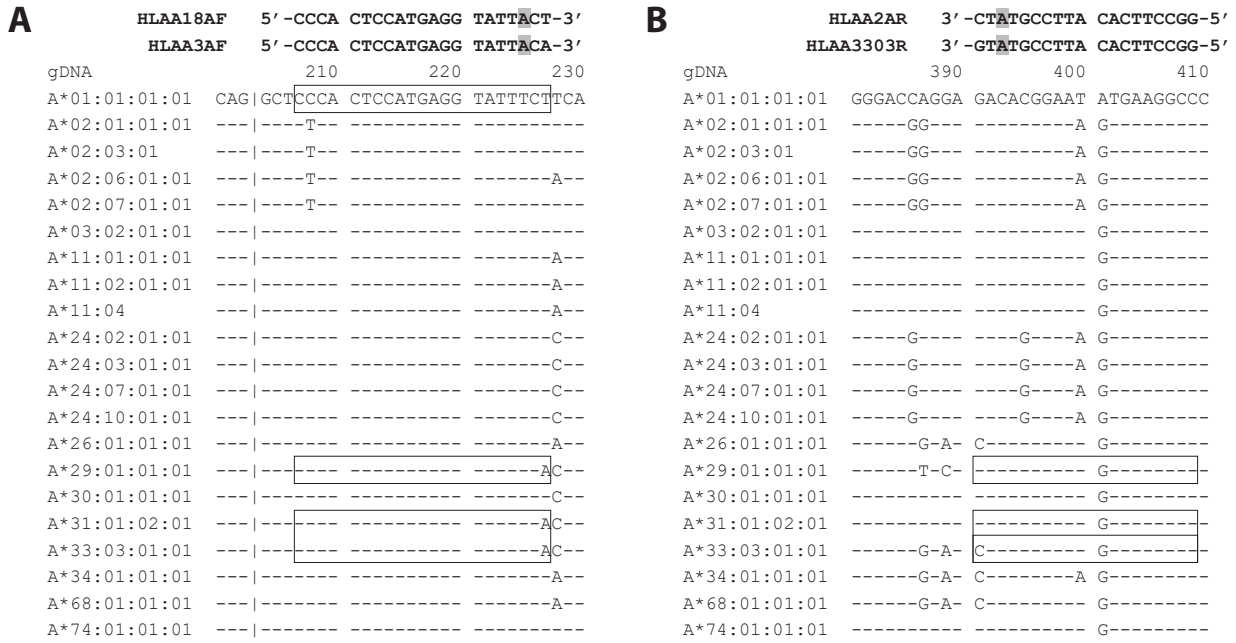


Figure 3. Binding sites of the primer sets used in the step 2 PCR.
A. HLAA18AF and HLAA3AF have one mismatch (replacement of T with A) at the third position from the 3′ terminus; B. HLAA2AR and HLAA3303R have one mismatch (replacement of G with A) at the third position from the 3′ terminus. The mismatches are highlighted in grey.

The protocol was tested on seven samples of known genotypes. The step 1 resulted in a single band of 992 bp with every single of the seven samples. The step 2 which aimed to amplify the 18 known *HLA-A* alleles (except for the three alleles *HLA-A*29:01*, *HLA-A*31:01* and *HLA-A*33:03*) in the Vietnamese population resulted in a single band of 358 bp with 6 samples. After the PCR reaction targeting the two homologous alleles (*HLA-A*29:01* and *HLA-A*31:01*), a single band of 202 bp was obtained with samples numbered 4, 5, 6, 7. After the PCR reaction targeting the *HLA-A*33:03* allele, a single band of 202 bp was obtained with samples numbered 1 and 2. The comparison between the electrophoresis results of the nested AS-PCR protocol and the known genotypes showed 100% accuracy.

After optimizing, this two-step nested AS-PCR protocol was validated with sequencing on 100 samples of unknown genotypes. We detected eight samples of homozygous *HLA-A*33:03* genotype, 38 heterozygous *HLA-A*33:03* carriers and 54 *HLA-A*33:03*-negative samples. The results of our protocol was absolutely consistent with that of the Sanger sequencing method ($\kappa = 1.00$, $p < 0.001$). The nested AS-PCR protocol had a sensitivity of 100% (95%CI: 92.13-100%) and a specificity of 100% (95%CI: 93.51-100%) in the *HLA-A*33:03* allele detection (**Table 2**).

The nested AS-PCR protocol was applied to evaluate the *HLA-A*33:03* allele distribution in 810 unrelated Vietnamese Kinh people, showing a frequency of *HLA-A*33:03* carriers of 19.6% and an allele frequency of 10.55% (**Table 3**).

Table 2. Comparison of the current method with sequencing.

The nested AS-PCR results for the *HLA-A*33:03* allele	Sequencing results for the *HLA-A*33:03* allele		
	Positive	Negative	Total
Positive	46	0	46
Negative	0	54	54
Total	46	54	100
Sensitivity	100.00% (95%CI: 92.13-100%)		
Specificity	100.00% (95%CI: 93.51-100%)		
Cohen's Kappa	$\kappa = 1.00$, $p < 0.001$		

Table 3. *HLA-A*33:03* allele distributions in 810 unrelated Vietnamese Kinh people.

Allele frequency (%)	10.55
*HLA-A*33:03* carriers (n, %)	159 (19.6)
Homozygous *HLA-A*33:03* genotype (n, %)	12 (1.5)
Heterozygous *HLA-A*33:03* genotypes (n, %)	147 (18.1)
Non-*HLA-A*33:03* carriers (n, %)	651 (80.4)

Discussion

The *HLA-A* gene is among the most polymorphic regions of the human genome with 5266 *HLA-A* alleles and 1716 distinct variant positions that had been reported.[15] Most of the SNPs of this locus are located in the exon 2–3 region.[16] For this reason, it is difficult to design PCR primers for the specific detection of each allele of this locus. This is the first report on a nested AS-PCR method for detecting and distinguishing homozygous/heterozygous genotype of the *HLA-A*33:03* allele.

One of the most significant challenges in the first PCR to amplify the exon 2–3 region of the *HLA-A* gene is the probability of nonspecific amplification of exon 2–3 regions of the *HLA-C* or *HLA-G* genes which are highly similar to that of the *HLA-A* locus. The first step primers (HLAAB1F/HLAAB1R) have 1-2 nucleotides at their 3′ terminus which are not complement to the equivalent nucleotides of the exon 2–3 regions of the *HLA-C* or *HLA-G* genes, preventing them from nonspecific pairing (**Figure 2 A and B**).

This nested AS-PCR protocol was customized for the Vietnamese population, with the 21 known *HLA-A* alleles.[12] In order to differentiate the *HLA-A*33:03* allele from the other 20 alleles, three primer sets were used in the second PCR. The exon 2–3 sequences of the *HLA-A*33:03*, **29:01*, and **31:01* alleles are very similar to each other. These three alleles differ from the other 18 *HLA-A* alleles in only one nucleotide (at position 227) (**Figure 3A**). Therefore, the two forward primers (HLAA3AF and HLAA18AF) were used to differentiate between the three homologous alleles and the rest 18 alleles. A mismatch (replacement of T with A) was placed at the third nucleotide from the 3′ terminus of the two forward primers (HLAA3AF and HLAA18AF) in order to increase the specificity.

The next issue we addressed was how to differentiate the *HLA-A*33:03* allele from the two homologous alleles (*HLA-A*29:01* and *HLA-A*31:01*). In the exon 2–3 sequence of these three alleles, there are three SNPs (at position 387, 389 and 391) that can be used for the differentiation of the *HLA-A*33:03* allele from the other two alleles (**Figure 3B**). The SNP at position 391 was used to design a reverse primer (HLAA2AR) which would specifically amplify the *HLA-A*29:01* and *HLA-A*31:01* alleles; and a reverse primer (HLAA3303R) which would specifically amplify the *HLA-A*33:03* allele. A mismatch (replacement of G with A) was placed at the third nucleotide from the 3′ terminus of the HLAA2AR and HLAA3303R primers in order to increase the specificity.

Comparative analysis of the newly developed PCR protocol and Sanger sequencing showed an absolute agreement. The nested AS-PCR protocol showed 100% sensitivity and specificity, ensuring the accuracy of the detection of patients at risk of SCARs due to the *HLA-A*33:03* presence. Moreover, this protocol can not only detect *HLA-A*33:03* carriers but also differentiate between homozygous and heterozygous genotypes. Most of the published PCR-based methods for the specific detection of *HLA* alleles cannot differentiate homozygous and heterozygous genotypes due to the fact that the *HLA* sequences are highly polymorphic.[17]

The protocol is quite complex with two PCR steps, thus, an important limitation of this method is the time required for the detection of PCR products on an agarose gel. The second PCR step requires three separate reactions. However, these reactions have the same touchdown PCR cycle, allowing them to be performed simultaneously on the same thermal cycler. Another typical limitation of nested PCR methods is the risk of cross-contamination which may happen in the preparation of template for the step 2 PCR. However, this risk can be minimized by using separate rooms for each PCR step.

The nested AS-PCR protocol was applied for determination of the *HLA-A*33:03* allele distribution in 810 Vietnamese Kinh people. The *HLA-A*33:03* allele frequency (AF) in this study was 10.55%, that was nearly as much as that reported by Hoa et al. in 170 Vietnamese Kinh people in Hanoi, the northern city of Vietnam (11.5%).[12] The slight difference may be explained by the notably bigger sample which distributed evenly in the three main regions of Vietnam of our study. The *HLA-A*33:03* AF of the Vietnamese Kinh population was lower than that of the Korean people (16.07%),[9] while as much as that of Han Chinese people in the Southern China (11.7%)[18] and Thai people (11.17%);[10] and much higher than that of Japanese people (6.7%),[19] European American people (0.36%)[20] or African American people (5.05%).[21] The *HLA-A*33:03* carrier frequency in the Vietnamese population was significantly high (19.6%), and similar to that in the Thai population (21.06%).[10] These comparisons show that the *HLA-A*33:03* allele variably distributes among various populations, even the Asian ones.

This nested AS-PCR protocol can be used for the *HLA-A*33:03* allele detection in Vietnamese people and with some necessary modifications, in some certain Asian populations with similar genetic characteristics such as the Thai or Chinese populations.

The use of pharmacogenetic markers, especially those involving adverse drug reactions (ADRs), for personalized therapy is an essential trend of modern medicine, in order to minimize ADRs and enhance therapeutic efficiency.[22] There have been multiple reports on the association of the *HLA-A*33:03* allele and severe ADRs induced by different drugs such as allopurinol,[3,4,5] strontium ranelate,[6] common cold medicines,[7] ticlopidine.[8] Therefore, our protocol can be used as a screening test for detection of patients with ADR risks, thereby, change the prescription toward a more suitable regimen. For this clinical purpose, we may not need to differentiate homozygous and heterozygous genotypes of patients. So that, only one PCR reaction is needed in step 2 for detection of the *HLA-A*33:03* allele, resulting in reduced cost. PCR-based genotyping methods are very popular in clinical laboratories, especially in pharmacogenomics. This nested AS-PCR protocol can be easily performed in small local hospitals with basic laboratory equipment and personnel. The cost for reagents in this protocol is around $2 per sample which is affordable for patients even in developing countries, enabling them to access a simple test which can help to prevent severe adverse drug reactions.

Conclusions

A novel nested AS-PCR method with a hundred-percent sensitivity and specificity for the *HLA-A*33:03* allele detection was reported. The protocol can be applied for the stratification of patients at SCAR risks with various drugs.

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Disclosure

The authors declare no conflict of interest.

Author Contributions

- P.T.T.H did the research
- T.Q.B co-supervised and edited the manuscript
- S.C., N.V.D, C.C.H, D.T.Q.N, T.N.P.M and N.H.H contributed to the research designed, collected the samples, and edited the manuscript
- P.T.H designed the research, co-supervised and drafted the manuscript.

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