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RESEARCH ARTICLE

Evaluating novel effective primers to amplify heterozygous alleles of second, third and fourth exons of HLA-A; -B; -C; -DRB1 and -DQB1 loci using sequencing-based typing†

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Abstract: Human leukocyte antigen (HLA) typing is one of the most crucial steps that determines the success of an organ transplant. However, HLA typing is a challenging task due to the diversity of HLA alleles, which is caused by high polymorphism of the region and high number of guanine and cytosine bases that limits the degree of amplification. Low resolution serology typing that is currently employed in Sri Lanka may fail to identify subtle differences in certain alleles, which may affect the long-term survival of the organ recipient. Therefore, a low cost, high-resolution DNA-based typing method for the HLA loci of Sri Lankans was developed based on polymerase chain reaction (PCR) amplification followed by Sanger sequencing, which is considered to be the gold standard for HLA typing. With minimised PCR bias and equal chances of amplifying all the alleles curated so far, a novel set of primers were designed to amplify the second and third exons of alleles in group specific PCR. To increase the resolution of alleles further, the fourth exon was also amplified using novel primers designed in this study and primers reported in the literature. Touchdown PCR and hot-start PCR were used to optimise PCR conditions so that non-specific amplifications are minimal. SBTengine® (version 3.12.0.2724) software was used in assigning the sequence chromatogram to the allele sequence. Seventeen new primers were designed in this study to ensure the amplification and identification of both alleles in heterozygous individuals that were previously unable to be identified using primers reported in the literature.

Keywords: Alleles, human leukocyte antigen (HLA) typing, PCR, polymorphism, Sanger sequencing.

INTRODUCTION

The major histocompatibility complex (MHC) is one of the most polymorphic regions in the human genome (Erlich *et al*., 2011), which is located on the short arm of the sixth chromosome. MHC region in humans is called the human leukocyte antigen (HLA). This complete region is about 4 Mb in size. HLA genes are organised into three classes of molecules denoted as class I (containing HLA-A, -B and -C genes), class II and class III. Each set of HLA alleles are inherited as a set of genes called a haplotype, which follows the Mendelian rules of inheritance (Choo, 2007). Every individual inherits two haplotypes; one from the mother and the other from the father.

HLA typing is a test process that is used to find an individual's genomic identity of HLA alleles. These alleles are responsible for the production of HLA molecules that are extremely important in self and non-self-discrimination. This region contains thousands of HLA genes, which code for surface proteins that are involved in antigen peptide presentation in a cell. Therefore, this region mediates immune responses and is important in organ matching prior to transplantations. It also has a strong association in the development of autoimmune diseases such as multiple sclerosis, narcolepsy, celiac disease, rheumatoid arthritis, and

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type I diabetes (Wang *et al*., 2012). These alleles also act as protective factors in infectious diseases such as human immunodeficiency virus, and animal studies have shown that HLA genes contribute to disease susceptibility or resistance (Wang *et al*., 2012) and adverse drug reactions (Erlich *et al*., 2011).

Sequence diversity of alleles is localised in the second exon for class II locus and, in the second and third exons for class I locus (Erlich *et al*., 2001). Therefore, amplification of the corresponding exons is more important in identifying alleles. Each HLA allele name has a unique number, which spreads up to four sets of digits called fields, separated by colons. The length of the allele designation is dependent on the sequence of the allele and that of its nearest relative, which in turn is determined by the resolution of typing method.

Immense allelic diversity of the HLA loci is mainly accomplished by recombination, which results in a patchwork pattern of sequence motifs and makes HLA typing a challenging task, even at low resolution. In this situation, the numerous technological advancements in the application of DNA-based methodologies are of particular importance (Danzer *et al*., 2013).

Although different DNA-based methods are available for HLA typing, sequence-based typing (SBT) is recognised as 'the gold standard', as the alleles are assigned based on the nucleotide sequence, by comparison of the obtained sequence with all the known allele sequences of the particular HLA locus in the database and their heterozygous combinations (Voorter *et al*., 2007). When probe reactivity patterns or Sanger sequencing chromatograms result in more than one genotype, these genotypes are called ambiguous data. There are two types of ambiguity: allele level ambiguity and genotype ambiguity. As a result of continuous increase in the discovery of new HLA alleles, length of the ambiguity string also continues to increase (Erlich, 2012). This emphasises the need for better and newer methods in HLA typing. The level of ambiguity can be reduced by increasing the number of exons being analysed in HLA typing (Mahdi, 2013). The nomenclature of a HLA allele has the HLA gene (A, B, C or D) followed by four fields representing the allele group, HLA protein, any synonymous DNA substitutions within the coding regions and differences in a non-coding region, respectively. As higher fields are identified, the polymorphism of the allele is better defined, thus enhancing allele resolution.

The ImMunoGeneTics project/ Human Leukocyte Antigen (IMGT/HLA) database is the primary database of immuno polymorphism. It provides a locus-specific database for the identification of genes in HLA system (Robinson *et al*., 2013). This database has successfully provided free public access for retrieving and analysis of data through the web link, *http://www.ebi.ac.uk/imgt/hla/* (Robinson *et al*., 2000). Requirements of HLA typing mainly depend on the type of transplantation. The current standard of HLA typing requires only the investigation of HLA-A, HLA-B, HLA-C, HLA-DQB1 and HLA-DRB1 for transplantations (Jung, 2011).

In transplantations, the major effect in graft rejection occurs due to the effects of HLA-DR (HLA-DRB1, HLA-DRB3, HLA-DRB4, etc.) and HLA-B antigens. The most important effect during the first six months after transplantation is by HLA-DR antigens. The effect of HLA-B comes in the first two years and the effect of HLA-A comes in long-term graft rejection (Mahdi, 2013). That is, the strongest impact is due to HLA-DR mismatching and then HLA-B and HLA-A, respectively. In transplantations of early days, mismatches in HLA-C had not been subjected to much concern, but later, research experiments have proven that there is a correlation between HLA-C mismatches and graft rejection as well (Frohn *et al*., 2001). Therefore, matching of HLA-C locus is also important in organ transplantations. From class II, HLA-DR and HLA-DQ matching is important for bone marrow transplantations, and HLA-DR is important in solid organ transplantations (Erlich *et al*., 2001). Mismatches in class I HLA alleles are associated with increased risk of mortality, while matching in class II HLA alleles are associated with enhanced survival.

Two population HLA typing studies have been conducted in Sri Lanka so far, identifying commonly present HLA alleles in Colombo, the economic capital of Sri Lanka. The most recent study, a DNA-based assay involving locus-specific PCR amplification followed by next generation illumina sequencing, identified A * 33:03-B * 44:03-C * 07:01-DPB1 * 02:01-DQA1 * 02:01-DQB1 * 02:02-DRB1 * 07:01 as the most common haplotype found in the Colombo population with a frequency of 1.4 % (Grifoni *et al*., 2018).

Currently, serology-based HLA typing, a low resolution method is performed in Sri Lanka mainly due to the high cost associated with high resolution methods. However, these low-resolution methods are associated with high chances of mismatches in the lower fields, which could affect organ transplants especially in the long term. The objective of this study was to develop a DNA-based typing method to identify HLA alleles with less ambiguity and high resolution, especially in heterozygous individuals. Novel primers were designed targeting all the alleles currently curated

in IMGT/HLA database. However, to ensure non-preferential amplification of all alleles in PCR, a group-specific allelic amplification method was followed using the designed primers. Amplification of the fourth exon is important to improve resolution by providing further sequence data. PCR optimisation was done to minimise nonspecific amplifications, which could cause high basal noise in sequence chromatograms leading to ambiguous typing results.

METHODOLOGY

Selection of samples

Samples used in this study were blindly coded surplus blood samples (n = 10) of individuals, representing age groups 20 to 45 years, both male (n = 5) and female (n = 5) attended to laboratory diagnosis at the Durdans Hospital, Sri Lanka.

Oligonucleotide primers

All the allelic sequences downloaded from IMGT/HLA database were aligned using BioEdit software (version 7.2.5) (Hall, 1999). Most conserved regions in all the alleles were selected to design primers manually, following the guidelines for primer designing (Dieffenbach *et al*., 1993). At positions where the designed primer sequence was incompatible with the sequence of a particular allele/group of alleles, degenerate primers at the position were designed to ensure high degree of primer specificity for all the alleles and minimum preferential amplification in PCR due to primer bias. The new primer sequences were again aligned with the allele sequences curated in IMGT/HLA database to ensure that all alleles were targeted by the designed primers. Primers were synthesised by Integrated DNA Technologies (USA). The sequences of the primers are shown in Table 1.

Table 1: List of primers used in the typing process

	Primer name	Primer sequence
01	HLA-A-2,3F*	5' AAA CSG CCT CTG YGG GGA GAA GCA A 3'
02	HLA-A-2,3R1*	5' GCC CCG TGG CCC CTG GTA 3'
03	HLA-A-2,3R2*	5'CCC CGT GGC CCC TGGTAC 3'
04	HLA-A-4F1**	5' GTT TAG GCC AAA AAT CCC CCC 3'
05	HLA-A-4F2**	5' GTT TAG GCC AAA AAT TCC CCC 3'
06	HLA-A-4R**	5' CAG CGA CCA CAG CTC CAG 3'
07	HLA-B-2,3F*	5'CTG CVG GGA GGA GMR AGG GGA CCG CAG 3'
08	HLA-B-2,3R*	5'AGG CCA TCC CCG GCG ACC TAT 3'
09	HLA-B-4F***	5' TAC CCG GTT TCA TTT TCA GTT G 3'
10	HLA-B-4R1***	5' CCA CGA TGG GGA AGG TGG A 3'
11	HLA-B-4R2***	5' CCA CGA TGG GGA GGG TGG A 3'
12	HLA-C-2,3F****	5' GAG GKG CCC KCC CGG CGA 3'
13	HLA-C-2,3Ra****	5' GCT GAT CCC ATT TTC CTC CCC TCC TC 3'
14	HLA-C-2,3Rb*	5' GCT GAT CCC ATT TTC CTC CCT TCC TC 3'
15	HLA-C-4F2*	5' GCT GTT CCT CCC TCA GAG AC 3'
16	HLA-C-4R*	5' GGA AAG GAG GGG AAG GTG AG 3'
17	HLA-C-4Rb*	5' GGA AAA GAG GAG AAG GTG AG 3'
18	HLA-DRB1-2F*	5' AGA ACA CAA GGA AGT ATT AAA TCA CTC 3'
19	HLA-DRB1-2R*	5' TCC CCT CCC ACA ACA G 3'
20	HLA-DQB1-2F1*	5' GCG GGC GGT TCC ACA G 3'
21	HLA-DQB1-2F1b*	5' GCG GGC TGT TCC ACA G 3'
22	HLA-DQB1-2R1*	5' AGA ATG TTT ATT CCT GAA GTG GAT AG 3'
23	HLA-DQB1-3F*	5' AGA GAA TAA AGG AAA TGCAAT AAA GTG 3'
24	HLA-DQB1-3Fb*	5' AGA GAA TAA AGG AAA TGTGAT AAA GTG 3'
25	HLA-DQB1-3R*	5' TTT CCC TAG CAT CTG GAA AGG TG 3'

* Novel primers designed in this study; ** primers adapted from Swelsen *et al*. (2005); *** primers adapted from Vooter *et al*. (2002); **** primers adapted from Itoh *et al*. (2005)

Two approaches were followed in primer designing. In the first approach, each PCR mixture contained only one pair of primers, targeting only a specific group of alleles. Thus, a number of PCR reactions were done for the same locus, each targeting a specific group of alleles (Table 2). In the other approach, multiplex PCR was employed where all the primers (each targeting a different group of alleles) amplifying a certain region of a single locus were added to the same PCR tube (Table 3), to check whether a single PCR reaction can amplify both the alleles.

Table 2: Combinations of primers to amplify target regions in allele-group specific PCR

PCR mixture number	Amplified region	Primer name		Product size (bp)
		Forward primer	Reverse primer	
I	HLA-A, Exon 2, 3	HLA-A-2,3F	HLA-A2,3R1	910
II	HLA-A, Exon 2, 3	HLA-A-2,3F	HLA-A2,3R2	910
III	HLA-A, Exon 4	HLA-A-4F1	HLA-A-4R	1445
IV	HLA-A, Exon 4	HLA-A-4F2	HLA-A-4R	1445
V	HLA-B, Exon 2, 3	HLA-B-2,3F	HLA-B-2,3R	945
VI	HLA-B, Exon 4	HLA-B-4F	HLA-B-4R1	1428
VII	HLA-B, Exon 4	HLA-B-4F	HLA-B-4R2	1428
VIII	HLA-C, Exon 2, 3	HLA-C-2,3F	HLA-C-2,3Ra	965
IX	HLA-C, Exon 2, 3	HLA-C-2,3F	HLA-C-2,3Rb	965
X	HLA-C, Exon 4	HLA-C-4F2	HLA-C-4Ra	593
XI	HLA-C, Exon 4	HLA-C-4F2	HLA-C-4Rb	593
XII	HLA-DRB1, Exon 2	HLA-DRB1-2F	HLA-DRB1-2R	1237
XIII	HLA-DQB1, Exon 2	HLA-DQB1-2F1a	HLA-DQB1-2R1	1429
XIV	HLA-DQB1, Exon 2	HLA-DQB1-2F1b	HLA-DQB1-2R1	1429
XV	HLA-DQB1, Exon 3	HLA-DQB1-3Fa	HLA-DQB1-3R	937
XVI	HLA-DQB1, Exon 3	HLA-DQB1-3Fb	HLA-DQB1-3R	937

Table 3: Combinations of primers in multiplex PCR reactions

PCR mixture number	Targeted region	Primer name				Product size (bp)
		Forward primers		Reverse primers		
XVII	HLA-A, Exon 2, 3	HLA-A-2,3F		HLA-A-2,3R1	HLA-A-2,3R2	910
XVIII	HLA-A, Exon 4	HLA-A-4F1	HLA-A-4F2	HLA-A-4R		1445
XIX	HLA-B, Exon 4	HLA-B-4F		HLA-B-4R1	HLA-B-4R2	1428
XX	HLA-C, Exon 2, 3	HLA-C-2,3F		HLA-C-2,3Ra	HLA-C-2,3Rb	965
XXI	HLA-C, Exon 4	HLA-C-4F2		HLA-C-4Ra	HLA-C-4Rb	593
XXII	HLA-DQB1, Exon 2	HLA-DQB1-2F1a	HLA-DQB1-2F1b	HLA-DQB1-2R1		1429
XXIII	HLA-DQB1, Exon 3	HLA-DQB1-3Fa	HLA-DQB1-3Fb	HLA-DQB1-3R		937

DNA extraction

DNA was extracted using GenoSpin G™ genomic DNA extraction kit (CeyGen Biotech, Sri Lanka). Briefly, the cell pellet resulting from 200 µL of blood was treated with 200 µL of lysis buffer and 40 µL of proteinase K (20 mg/mL) and incubated at 56 for 30 min, to which 400 µl of binding buffer and 300 µL of absolute ethanol were added. The resulting mixture was then placed on a GenoSpin R™ column and centrifuged at 10,000 g for 1 min. The column was washed with 500 µL each of wash buffer-1 and wash buffer-2 by centrifuging at 10,000 g for 1 min. Then it was eluted with 20 µL of elution buffer to recover DNA, which was stored at -20 ºC until used. DNA concentration and purity were determined by Qubit®3.0 Fluorometer (Thermo Fisher Scientific).

PCR optimisation and fragment amplification

As the PCR products should be of high purity for downstream sequence analysis, the PCR reactions were optimised using thermal gradient, Touch-Down PCR (TD-PCR) and hot-start PCR methods, to minimise the formation of primer dimers and nonspecific products.

DNA amplification was carried out using the Applied Biosystems Veriti™ Thermal Cycler and optimised PCR conditions. Each PCR amplification was performed as hot-start PCR and was visualised by ultraviolet transilluminator after running on an ethidium bromide stained agarose gel.

Fragment sequencing and allele assignment

A volume of 40 µL of PCR product was sent for Sanger sequencing to Macrogen Inc., Korea. Thereafter, the sequence chromatograms were analysed using the SBTengine® software developed by GenDx Pvt. Ltd. (Netherlands), where the sequences were matched with the allele sequences in the IMGT/HLA database to assign alleles to the samples.

Compliance with ethical standards

In this study no patient identifiable data; human participants or animals were directly used. Further, approval was obtained from the Durdans Hospital to use the surplus samples for the study.

RESULTS AND DISCUSSION

PCR amplification of the polymorphic exons of HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 loci using the primers designed in this study (Table 1) was

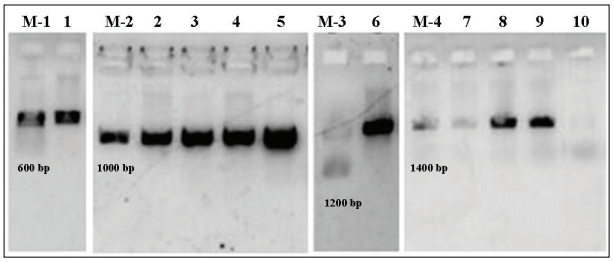

Figure 1: TD-PCR amplified fragments of HLA loci of the first subject (10 µL) after running on 0.8 % agarose gel at 80 V for 30 minutes, and staining with ethidium bromide. (Multiplex PCR for HLA-C, -DRB1 and -DQB1. Individual PCR for HLA-A). M-1, M-2, M-3 and M-4 are molecular weight markers of 600 bp, 1000 bp, 1200 bp and 1400 bp, respectively. Lane 1- 4th exon of HLA-C (593 bp); lane 2- 2nd and 3rd exons of HLA-A (928 bp); lane 3- 2nd and 3rd exons of HLA-B (945 bp); lane 4- 2nd and 3rd exons of HLA-C (965 bp); lane 5- 3rd exon of HLA-DQB1 (937 bp); lane 6- 2nd exon of HLA-DRB1 (1237 bp); lane 7- 4th exon of HLA-A (1445 bp); lane 8- 4th exon of HLA-B (1428 bp); lane 9- 2nd exon of HLA-DQB1 (1429 bp); lane 10- negative controller (migrated distance is not comparable among PCR products of different sizes).

Table 4: Alleles detected in two subjects by SBTengine® software, in each PCR mixture shown in Table 2

Analysis number	PCR mixture numbers	Detected alleles			
		Subject 01		Subject 02	
		Allele 1	Allele 2	Allele 1	Allele 2
01	I, III	HLA-A*33:03	-	-	HLA-A*33:01:05
02	I, IV	HLA-A*33:03	HLA-A*33:79	HLA-A*33:01:01	HLA-A*33:22
03	II, III	HLA-A*33:03	-	HLA-A*33:01:01	HLA-A*33:22
04	II, IV	HLA-A*33:03	-	HLA-A*33:01:01	HLA-A*33:01:05
05	V, VI	HLA-B*35:03:01	-	HLA-B*40:06	HLA-B*40:11:02
06	V, VII	HLA-B*35:03:01	-	HLA-B*40:06	HLA-B*40:11:02
07	VIII, X	HLA-C*04:102	HLA-C*04:102	HLA-C*08:02:07	HLA-C*12:127
08	VIII, XI	HLA-C*04:102	HLA-C*04:102	HLA-C*08:02:07	HLA-C*12:127
09	IX, X	HLA-C*04:102	HLA-C*04:102	-	-
10	IX, XI	HLA-C*04:102	HLA-C*04:102	-	-
11	XII	HLA-DRB1*15:01	HLA-DRBI*15:28	HLA-DRB1*15:01	HLA-DRB1*15:85
12	XIII, XV	HLA-DQB1*06:01	-	-	-
13	XIV, XV	HLA-DQB1*06:01	-	HLA-DQB1*06:209	-
14	XIII, XVI	-	HLA-DQB1*06:157	-	HLA-DQB1*06:01:07
15	XIV, XVI	HLA-DQB1*06:01:02	-	HLA-DQB1*06:209	-

Table 5: Alleles detected by the sequence chromatogram of each PCR mixture shown in Table 3

Analysis number	PCR reaction number	Detected alleles			
		Subject 01		Subject 02	
		Allele 1	Allele 2	Allele 1	Allele 2
16	XVII, XVIII	-	-	-	-
17	V, XIX	HLA-B*35:03:01	-	HLA-B*40:06	HLA-B*40:11:02
18	XX, XXI	HLA-C*04:102	HLA-C*04:102	Not examined	
19	XXII, XXIII	HLA-DQBI*06:01	HLA-DQBI*06:157		

successful. The gel images were used to ascertain that the PCR products were sufficiently devoid of non-specific amplifications such as primer dimers, prior to sending each product for Sanger sequencing (Figure 1).

The obtained sequence chromatograms were analysed. All the possible combinations of mixtures (where different exons of the same locus were amplified in different mixtures; Table 4) were then analysed separately in order to find out the best allele amplification method for each locus. For HLA-A and HLA-B loci, both alleles in heterozygous individuals could be detected by separate PCR reactions with primers designed in this study, targeting specific groups of alleles (described in the methodology as the first approach). For loci HLA-C, HLA-DRB1 and HLA-DQB1, the best amplification method was multiplex PCR (as both alleles could be successfully identified in heterozygous individuals in a single reaction mixture). The results obtained from both approaches are summarised separately in Tables 4 and 5.

The objective of this study was to develop a DNA-based typing method by designing sets of novel primers to identify HLA alleles especially in heterozygous Sri Lankan individuals. In achieving this objective, three main issues were addressed: (i) attempting to minimise detection of only a single allele in heterozygous individuals due to PCR bias and sequencing ambiguities, (ii) to resolve ambiguities associated with SBT typing of the second and third exons by further amplification of the fourth exon, (iii) to optimise PCR so that sequence quality will not pose a problem in allele identification. To ensure that both alleles are identified in heterozygous individuals, group specific amplification was carried out where different primers were designed to amplify the same target region with resolved degeneracy, targeting a group of alleles in a single PCR reaction. Thus, different PCR mixtures for each locus containing different primer combinations were used as shown in Table 2. This approach of targeting to amplify different alleles in different reaction mixtures is proven successful as both alleles could be detected for heterozygous loci of samples. The same alleles (detected using the DNA-based method) were identified (up to the second field) using serologic typing method, which was used to verify the accuracy of the developed assay.

The alleles identified in this study correspond to the commonly identified alleles in recent population study conducted in Colombo, Sri Lanka (Grifoni *et al*., 2018). Allele A*33:03, which had the highest allelic frequency (allelic frequency: 0.188, phenotype frequency: 33.9 %) in the population study was identified in Subject 1 of this study, while allele B*35:03 identified with an allelic frequency of 0.06 and phenotype frequency of 11.3 % in the Colombo population, was identified up to the third field resolution of B*35:03:01 in Subject 1 in this study. Similarly, allele B*40:06 present in an allelic frequency of 0.09 and phenotype frequency of 17.1 % in the population is identified in Subject 2 of this study. Alleles DRB1*15:01 and DQB1*06:01 present in allelic frequencies of 0.118 and 0.167 and phenotypic frequencies of 22.4 % and 30.3 %, respectively are identified in Subject 1. This shows that the individuals tested in this study have inherited commonly found HLA alleles in the Colombo population.

Heterozygosity was observed in HLA-A, HLA-DRB1 and HLA-DQB1 loci of Subject 1 and HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 loci of Subject 2. However, it was noted that both alleles were detected only in certain PCR reactions, indicating that it is necessary to carry out allele group-specific PCR reactions to ensure the detection of both alleles for certain loci (comparison of analysis number 2 of subject 1 with analysis number 1, 3 and 4, of the same subject in Table 4 where HLA-A was amplified). Multiplex PCR was done to ascertain whether such PCR bias actually exists or not (Table 3), and the results obtained vary with the loci (Table 5). For HLA-A locus, multiplex PCR seems to be unsuccessful as PCR mixture number XVII of Table 3, which was a multiplex PCR failed to yield any results. For HLA-C,

-DRB1 and –DQB1 loci, multiplex PCR has identified both alleles in a single reaction mixture (Table 5), which could be due to the comparatively low degree of polymorphism in these loci than in HLA-A and HLA-B loci. Therefore, it is more advantageous to use multiplex PCR to amplify all the alleles in a single reaction tube for those loci rather than using different reaction mixtures. It saves time, resources and is less cumbersome. The multiplex PCR reactions are tabulated in Table 3.

The degree of resolution of alleles could be improved by incorporating additional sequence information (fourth exon of HLA-A, HLA-B and HLA-C loci). All the alleles were identified up to the second field level. However in some alleles, the third field could also be identified, where applicable. Of the 18 alleles detected in the two subjects, eight were identified up to the third field level. The first field represents the serological antigen and the second field represents the subtypes based on the nucleotide substitutions that change the amino acid sequence of the encoded protein. Alleles whose numbers differ in the two sets of digits must differ in one or more nucleotide substitutions that change the amino acid sequence of the encoded protein. The third field level only appears if there are synonymous nucleotide substitutions (also called silent or non-coding substitutions) within the coding sequence. Alleles that only differ by sequence polymorphisms in the introns, or in the 5' or 3' untranslated regions that flank the exons and introns, are distinguished using the fourth set of digits (Marsh *et al*., 2010). Thus, alleles with synonymous nucleotide substitutions could be identified in eight alleles using additional sequence data, by identifying up to third field level.

It was also investigated whether the presence or absence of a group of alleles could be predicted prior to sequencing by the presence or absence of PCR product. However, from the sequencing results, it was clear that to ascertain the presence or absence of a particular allele, sequencing the PCR product is mandatory. The optimisation of PCR reactions with the utilisation of hot-start, TD-PCR and thermal gradient methods seems to be successful as most of the chromatograms obtained after Sanger sequencing are of high quality, with large contiguous reads. Also, the quality of the sequence traces remains a crucial factor in differentiating between two or more similar alleles. As only a minimum amount of primer dimers, which did not interfere in the process of sequencing were observed, additional purification of the PCR products prior to sequencing is unnecessary.

Expertise in analysing chromatograms is also required when using SBTengine® software, as base calling should be done manually at some crucial positions. Based on the results, it can be concluded that not all PCR reaction combinations or analysis combinations should be done in order to reveal HLA alleles. Either group specific or multiplex PCR could be employed depending on the locus.

CONCLUSION

Both alleles in heterozygous individuals could be identified to at least the second field level using the primers developed in this study by either group specific or multiplex PCR methods. This shows the employability of these primers to develop a low cost, high resolution HLA typing method using DNA sequencing.

Conflict of interest

All authors declare that they have no conflict of interests.

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REFERENCES

Choo S.Y. (2007). The HLA system: genetics, immunology, clinical testing, and clinical implications. *Yonsei Medical Journal* **48**: 11–23.
DOI: http://dx.doi.org/10.3349/ymj.2007.48.1.11

Danzer M., Niklas N., Stabentheiner S., Hofer K., Pröll J., Stückler C., Raml E., Polin H. & Gabriel C. (2013). Rapid, scalable and highly automated HLA genotyping using next-generation sequencing: a transition from research to diagnostics. *BMC Genomics* **14**: 221–231.
DOI: https://doi.org/10.1186/1471-2164-14-221

Dieffenbach C.W., Lowe T.M. & Dveksler G.S. (1993). General concepts for PCR primer design. *Genome Research* **3**: s50–s37.
DOI: https://doi.org/10.1101/gr.3.3.S30

Erlich H. (2012). HLA DNA typing: past, present, and future. *Tissue Antigens* **80**: 1–11.
DOI: https://doi.org/10.1111/j.1399-0039.2012.01881.x

Erlich H.A., Opelz G. & Hansen J. (2001). HLA DNA typing review and transplantation. *Immunity* **14**: 347–356.

Erlich R.L. *et al*. (11 authors) (2011). Next-generation sequencing for HLA typing of class I loci. *BMC genomics* **12**: 42–55.
DOI: https://doi.org/10.1186/1471-2164-12-42

Frohn C., Fricke L., Puchta J.C. & Kirchner H. (2001). The effect of HLA-C matching on acute renal transplant

rejection. *Nephrology, Dialysis, Transplantation* **16**(2): 355–360.
DOI: https://doi.org/10.1093/ndt/16.2.355

Grifoni A. *et al*. (17 authors) (2018). Sequence-based HLA-A, B, C, DP, DQ, and DR typing of 714 adults from Colombo, Sri Lanka. *Human Immunology* **79**(2): 87–88.
DOI: https://doi.org/10.1016/j.humimm.2017.12.007

Hall T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and anlysis program for Window 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Itoh Y., Mizuki N., Shimada T., Azuma F., Itakura M., Kashiwase K., Kikkawa E., Kulski J.K., Satake M. & Inoko H. (2005). High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-luminex method in the Japanese population. *Immunogenetics* **57**: 717–729.
DOI: https://doi.org/10.1007/s00251-005-0048-3

Jung H.L. (2011). Shedding a new light on the HLA matching. *The Korean Journal of Hematology* **46**: 1–2.
DOI: https://doi.org/10.5045/kjh.2011.46.1.1

Mahdi B.M. (2013). A glow of HLA typing in organ transplantation. *Clinical and Translational Medicine* **2**: 1–5.
DOI: https://doi.org/10.1186/2001-1326-2-6

Marsh S.G.E. *et al*. (24 authors) (2010). Nomenclature for factors of the HLA system. *Tissue Antigens* **75**: 291–455.
DOI: https://doi.org/10.1111/j.1399-0039.2010.01466.x

Robinson J., Halliwell J.A., Mcwilliam H., Lopez R., Parham P. & Marsh S.G.E. (2013). The IMGT/HLA database. *Nucleic Acids Research* **41**: 1222–1227.
DOI: https://doi.org/10.1093/nar/gks949

Robinson J., Malik A., Parham P., Bodmer J.G. & Marsh S.G.E. (2000). IMGT/HLA database-a sequence database for the human major histocompatibility complex. *Tissue* **55**: 280–287.
DOI: https://doi.org/10.1034/j.1399-0039.2000.550314.x

Swelsen W.T.N., Voorter C.E.M., Tilanus M.G.J., Kamps M.A.F. & van den Berg-loonen E.M. (2005). No HLA-A gene detectable on one of the haplotypes in a caucasian family. *Human Immunology* **66**: 155–163.
DOI: https://doi.org/10.1016/j.humimm.2004.10.009

Voorter C.E.M., Mulkers E., Liebelt P., Sleyster E. & van den Berg-loonen E.M. (2007). Reanalysis of sequence-based HLA-A, -B and -Cw typings: how ambiguous is today's SBT typing tomorrow. *Tissue Antigens* **70**: 383–389.
DOI: https://doi.org/10.1111/j.1399-0039.2007.00921.x

Vooter C.E.M., Swelsen W.T.N. & van den Berg-loonen E.M. (2002). B * 27 in molecular diagnostics : impact of new alleles and polymorphism outside exons 2 and 3. *Tissue Antigens* **60**: 25–35.
DOI: https://doi.org/10.1034/j.1399-0039.2002.600104.x

Wang C. *et al*. (11 authors) (2012). High-throughput, high-fidelity HLA genotyping with deep sequencing. *Proceedings of the National Academy of Sciences* **109**: 8676–8681.
DOI: https://doi.org/10.1073/pnas.1206614109