

Polymerase chain reaction (PCR) and sequence specific oligonucleotide probes (SSOP) genotyping assay for detection of genes associated with rheumatoid arthritis and multiple sclerosis

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Abstract— In this paper an assay for the detection of genes associated with rheumatoid arthritis (RA) and multiple sclerosis, using polymerase chain reaction (PCR) and sequence specific oligonucleotide probes (SSOP) is presented, in order to be further applied in a portable Lab-On-Chip (LOC) device. A substantial part of these reagents were based on the literature (11th International Histocompatibility Workshop, IHW), bearing the advantage of proven successful implementation in genotyping, while others were designed for this study. More precisely, our methodology discriminates HLA-DRB1 as DRB1*01, *04 and *10, which include shared epitope (SE) alleles associated with RA and additionally DRB1*15 allele, including DRB1*1501 associated with MS (broad genotyping method). To further present the basic elements of the assay for high resolution genotyping of SE DRB1 alleles, we provide as an example the case of HLA-DRB1*10 alleles (HLA-DRB1*100101, *100102, *100103, *1002 and *1003). Regarding the methodology for developing a detection assay, for SNPs associated with RA or MS the basic steps are presented. DNA sequence data are obtained from IMGT/HLA and SNP database. Online software tools are used to define hybridization specificity of primers and probes towards human DNA, leading to hybridization patterns that uniquely designate a target allele and evaluate parameters influencing PCR efficiency. Respecting current technological limitations of autonomous molecular-based LOC systems the approach of broad genotyping of HLA-DRB1*01/*04/*10/*15 genes, is intended to be initially used, leaving, high resolution genotyping of SE alleles for future implementations. This method is easy to be updated and extended to detect additional associated loci with RA or MS.

I. INTRODUCTION

New diagnostic procedures based on Lab-on-a-chip (LOC)

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technologies[1] are capable of performing a wide range of genomic tests by using a sample of blood or other body fluid such as saliva. Portable miniaturized LOC devices aim to provide susceptibility and diagnosis of common and rare diseases at the point-of-care and other preferred healthcare environments. Rheumatoid arthritis (RA) is a disease characterized by initial inflammation of the joints and other autoimmune features. So far a single mendelian mechanism has not been proven as a causative factor (MIM 180 300)[2]. A significant genetic association has been established since 1970s, located in the HLA region on chromosome 6[3].

In this region, alleles associated with RA share an amino acid sequence motif, at residues 67-74 in the third hypervariable region of the DRB1 chain. This motif is defined as the shared epitope (SE), and can be found in HLA-DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408 and *1001[4, 5, 6]. It is very interesting that this motif is not found in other closely related alleles that are not associated with RA, indicating the importance of this region of DR molecule in rheumatoid arthritis disease pathogenesis[7].

Complexity characterizes the genetic base of the disease and there are more loci, in populations with European origin that have been associated with RA. More recent studies have shown an association with PTPN22[8], OLIG3 and TNFAIP3[9], a locus in region 6q23[10], a locus mapped between TRAF1 and C5[11] and STAT4[12].

The myelin loss, axonal pathology and progressive neurological dysfunction are main characteristics of multiple sclerosis (MS) which is a common inflammatory disease of the central nervous system[13]. The association between MS and MHC (major histocompatibility complex) class II, has been mapped to the extended haplotype HLA-DQA1*0102-DQB1*0602-DRB1*1501-DRB5*0101. This is the principal MHC haplotype that increases MS risk in individuals of Northern European descent[14].

In the study of Chao and coworkers[15] interactions between HLA-DRB1, HLA-A and HLA-B haplotypes were evaluated. HLA-A and -B alleles on haplotypes not bearing HLA-DRB1*15 were not over-transmitted[15]. MS association with SNPs in the interleukin-7 receptor (IL7R) has also been confirmed[16].

In this paper the methodology presented discriminates DRB1 alleles as DRB1*01, DRB1*04 and DRB1*10, which include SE alleles associated with RA and regarding MS, as

DRB1*15 including DRB1*1501 associated allele (broad genotyping method), based on polymerase chain reaction and sequence specific oligonucleotide probes (PCR-SSOP). Furthermore, the methodology of high resolution genotyping of SE alleles using as an example the case of DRB1*10 alleles (DRB1*100101, *100102, *100103, *1002 and *1003) is also presented. In addition, we incorporate the basic steps of the methodology for detection of SNPs associated with RA or MS.

II. METHODOLOGY OF GENES' GENOTYPING

The sequences of alleles used in this study are derived from IMGT/HLA (database version 2.28) [17,18,19,20,21,22]. Static text version of the sequence alignments is available from ftp://ftp.ebi.ac.uk/pub/databases/imgt/mhc/hla/DRB_nuc.fasta, provided by the HLA Informatics group.

The primers' sequence, used for the amplification of the second exon of HLA-DRB gene, generic and group specific, were given by the 11th International Histocompatibility Workshop (11th IHW)[23] cited in [24,25].

Furthermore, we test the generic and group specific primers against the HLA-DRB sequences using probe and primer search tool, online software from IMGT/HLA database website [17, 18, 19, 20] in order to define their specificities against the current database collection of HLA DRB alleles (<http://www.ebi.ac.uk/imgt/hla/probe.html>). This was necessary due to new entries in the IMGT/HLA database that followed their publication. Parameters influencing PCR efficiency are evaluated using NetPrimer free online software (PREMIER Biosoft Int., Palo Alto, Calif.). Oligonucleotide primers are analyzed for the presence of possible hairpins, secondary structure, melting temperature etc.

We also used Blast software [26,27] for three different reasons: i) to identify putative hybridization sites on human genome by the selected primer pairs, that could incorrectly amplify other DNA sequences apart from the targets (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastDescAd#), ii) the exact region of the HLA-DRB gene that the primers or the probes are complementary to, was designated using the BLASTn software (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&SHOW_DEFAULTS=on&BLAST_SPEC=blast2seq&LINK_LOC=align2seq) against static text version of the sequence alignments of DRB1 exon 2, available from IMGT/HLA database (ftp://ftp.ebi.ac.uk/pub/databases/imgt/mhc/hla/DRB_nuc.fasta), and iii) primer pair design for selected SNP, which is performed with software Primer-blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The computation is performed at NCBI using the BLAST network service.

The key components of the methodology for PCR-SSOP gene detection method, which are presented in the

forthcoming sections, are: (a) assessment of primers and probes along with the hybridization patterns for the detection of DRB1 alleles as DRB1*01/*04/*10 and (b) DRB1*15 (c) designation of primers and probes and hybridization patterns that facilitate the PCR-SSOP high resolution subtyping of the HLA-DRB1*10 gene as alleles DRB1*100101, *100102, *100103, *1002 and *1003 and (d) basic steps of PCR SSOP assay for SNP detection.

A. PCR-SSOP assay for DRB1*01/*04/*10 broad genotyping

Generic primer pair DRB amp-A (5' primer) and DRB amp-B (3' primer) (11th IHW)[23], amplifies the majority of HLA-DRB genes, between them the targets DRB1*01, DRB1*04 and DRB1*10, with the exception of alleles DRB1*010105, *010202, *040503, *040504. Concerning the amplification of alleles DRB1*010105, 040503 and 040504, which are target loci, two additional 3' primers could be generated. For this purpose, the sequence of primer DRB amp-B should be modified, so we design two alternative versions in order to cover all the SE alleles in IMGT-HLA database. We designate them as DRB amp-Ba and DRB amp-Bb and each of them contains a single base pair substitution compared with the initial 3' primer (DRB amp-B). So, we need to use a 3' primer mix comprised by three different 3' primers (table I). Conditions recommended by the 11th International Histocompatibility Workshop for PCR assay are presented in table II.

The DRB amp-1 5' primer (from the DR1-group specific amplification primers pair, 11th IHW)[23] hybridizes to all HLA-DRB1*01 genes apart from DRB1*0107 and DRB1*0122 which do not target SE alleles.

TABLE I

THE SEQUENCES OF THE GENERIC AND GROUP SPECIFIC PRIMERS USED FOR THE DRB GENE AMPLIFICATION AND THE PROBES SUPPORTING THE ALLELES' DETECTION. THE NUMBERS REFER TO THE CORRESPONDING NUCLEOTIDES OF DRB EXON 2. THE INDICATION (-8) REFERS TO THE LAST 8 NUCLEOTIDES OF INTRON 1. AS NUMBER ONE (+1) THE FIRST NUCLEOTIDE OF EXON 2 WAS CONSIDERED. THE UNDERLINED NUCLEOTIDES REFER TO THE BASE SUBSTITUTIONS OF DRB AMP-B THAT LED TO DRB AMP-BA AND DRB AMP-BB DESIGNED FOR THIS PAPER. PRIMERS/PROBES INDICATED WITH ONE ASTERISK ARE BASED ON PUBLISHED DATA FROM 11TH IHW [23], WHILE TWO ASTERISKS REFERS TO NEWLY DESIGNED SEQUENCES

	Primer/Probe (5' - 3')
5' primer	*DRBamp-A, -8 CCCCACAGCACGTTTCTTG 11
5' primer	*DRB amp-2, 6TTCCTGTGGCAGCCTAAGAGG26
3' primer	*DRB amp-B, 247 CCGCTGCACTGTGAAGCTCT 266
3' primer	**DRB amp-Ba, 247 CCGCTGCACCGTGAAGCTCT 266
3' primer	**DRB amp-Bb, 247 TCGCTGCACTGTGAAGCTCT 266
Probe 1	*DRB amp-1, 6 TTCTTGTTGGCAGCTTAAGTT 25
Probe 2	*DRB amp-4, 4GTTTCTTGGAGCAGGTTAAAC24
Probe 3	*DRB5704, 152GCCTGTGCGGAGCACTG169
Probe 4	*DRB7007, 184ACATCCTGGAAGACGAGC201
Probe 5	*DRB1008, 12GAGGAGTTAAGTTTGTAG29
Probe 6	**pr10a, 195CGGAGGCGCGCCGCGGTG212
Probe 7	**pr10b, 195CGGAGGCGGGCCGCGGTG212
Probe 8	**pr10c, 234TACGGGGTGTGGAGAGC251
Probe 9	**pr10d, 90GAGGAGTACGCGCGCTTC107

The DRB amp-4 5' primer (from the DR4-group specific amplification primers pair, 11th IHW)[23] hybridizes to all HLA-DRB1*04 genes apart from a few HLA-DRB1 alleles

that do not belong to subtypes of HLA genes defined as SE alleles, but also to non-SE alleles DRB1*1410 and *1457. We use DRB amp-1 and DRB amp-4 to probe DRB1*01 and *04 genes, respectively. Probing of the entire group of DRB1*10 alleles is performed by probe DRB1008 (11th IHW)[23]. To avoid contamination of our results with DRB1*14 genes due to probe DRB amp-4, we use additional probes DRB5407 and DRB7007 (11th IHW)[23], that among other genes hybridize to DRB1*1410 and *1457, respectively and we exclude their co-hybridization cases with DRB amp-4. Probes DRB5407 and DRB7007 additionally hybridize to other non-target genes.

TABLE II

CONDITIONS RECOMMENDED BY THE 11TH INTERNATIONAL HISTOCOMPATIBILITY WORKSHOP [23] FOR PCR AMPLIFICATION OF THE MHC CLASS II GENE TARGETS FOR PRIMER PAIRS DRB AMP-A /DRB AMP-B AND DRB AMP-2/DRB AMP-B (PRIMERS BASED ON PUBLISHED DATA, SEE TEXT)

Primer pair	Denaturation	Annealing	Extension	MgCl ₂ concentr.
DRB amp-A	96 ^o C-30sec	55 ^o C-1min	72 ^o C-1min	2.0mM
DRB amp-B				
DRB amp-2	96 ^o C-30sec	60 ^o C-1min	72 ^o C-1min	1.5mM
DRB amp-B				

TABLE III

HYBRIDIZATION PATTERNS OF DRB PROBES (11TH IHW) THAT LEAD TO DRB1*01/*04/*10 BROAD GENOTYPING. DRB ALLELES FROM [21, 22]. THE SYMBOL (+) REFERS TO THE HYBRIDIZATION OF THE PROBE TO THE ALLELE. IMGT/HLA PROBE SEARCH RESULTS, DATABASE VERSION: 2.28

DRB allele	DRB amp-1	DRB amp-4	DRB 5704	DRB 7007	DRB 1008
DRB1*01 (group a ₁)	+				
DRB1*0114	+		+		
DRB1*0103	+			+	
DRB1*04 (group a ₂)		+			
DRB1*1410		+	+		
DRB1*0462, *0469		+	+		
Group b ₁			+		
DRB1*1457		+		+	
DRB1*0402, *0414		+		+	
Group b ₂				+	
DRB1*10					+

Group a₁: all DRB1*01 alleles apart from *010202, 0107, 0122.

Group a₂: all DRB1*04 alleles apart from *040303, 0419-0423, 0425, 0427, 0432, 0435, 0444, 0449, 0452, 0464, 0466, 0485.

Group b₁: DRB non-SE alleles that hybridize to DRB5704 apart from those presented in the table.

Group b₂: DRB non-SE alleles that hybridize to DRB7007 apart from those presented in the table.

B. PCR-SSOP assay for DRB1*15

Group specific primer pair DRB amp-2 (5' primer) and DRB amp-B (3' primer) (11th IHW), amplifies HLA-DRB1*15 and HLA-DRB1*16 genes, with the exception of alleles DRB1*150203, *150205, *1506, *1528, *160102, *160202, *1604, *160501, *1607 and *1612. The sequences of the group specific primer pair are presented in table I and conditions recommended by the 11th International

Histocompatibility Workshop for PCR assay are presented in table II.

To exclude the alleles HLA-DRB1*16 we design specific probes pr16a (5'TCCTGGAAGACAGG3') and pr16b (5'TCCTGGAGGACAGG3'). The cases that are initially amplified by primer pair DRB amp-2/DRB amp-B and do not hybridize with pr16a and pr16b correspond to HLA-DRB*15 gene alleles apart from DRB1*1517N, 1521.

C. PCR-SSOP for high resolution genotyping of HLA-DRB1*10

Considering the alleles that initially hybridize to DRB1008, we designed 4 probes (pr10a, pr10b, pr10c and pr10d) that allow high resolution genotyping hybridizing respectively with DRB1*100102, *100103, *1002 and *1003. Thus, DRB1*100101 hybridizes only with DRB1008 and each DRB1*10 allele co-hybridizes with DRB1008 and one more probe. Probes pr10b, pr10c and pr10d, also hybridize with non-DRB1*10 genes, but we exclude these cases (table IV). Appropriate probe design under the same basic principles, allows high resolution genotyping for almost all allele subtypes in each SE HLA-DRB1 gene (data not shown).

TABLE IV

HYBRIDIZATION PATTERNS OF DRB PROBES THAT LEAD TO HIGH RESOLUTION GENOTYPING OF DRB1*10 GENE. ALLELES FROM [21, 22]. PROBE DRB1008 FROM 11TH IHW. THE SYMBOL (+) REFERS TO THE HYBRIDIZATION OF THE PROBE TO THE ALLELE. IMGT/HLA PROBE SEARCH RESULTS, DATABASE VERSION: 2.28

DRB allele	DRB 1008	Pr10a	Pr10b	Pr10c	Pr10d
DRB1*100101	+				
DRB1*100102	+	+			
DRB1*100103	+		+		
DRB1*1002	+			+	
DRB1*1003	+				+
Group z ₁			+		
Group z ₂				+	
Group z ₃					+

Group z₁: DRB non-SE alleles that hybridize to pr10b.

Group z₂: DRB non-SE alleles that hybridize to pr10c.

Group z₃: DRB non-SE alleles that hybridize to pr10d.

D. PCR SSOP assay basic steps for detection of SNP (associated with RA or MS)

In order to design primers which amplify a SNP associated with RA or MS, we obtain from SNP database <http://www.ncbi.nlm.nih.gov/SNP/>[28] the most recent version of DNA sequence containing the associated SNP and afterwards we use the Primer-blast software[26] defining as a region of interest approximately 130bp DNA sequence flanking SNP. After several tests we obtain the primer pair. Finally, we design the probe defining approximately, a 21 bp DNA sequence including SNP in the middle of the sequence. Using the Blast software[26,27], we check the hybridization of the probe towards the delineated region of interest to ensure that there is a unique hybridization site. Respecting the requirement of the miniaturized dimensions of the LOC, multiplex PCR reactions will be designed in order to extend the miniaturized LOC capabilities.

III. CONCLUSIONS

It is expected that the methodology presented in this study allows broad genotyping of HLA-DRB1 genes that include SE alleles, and high resolution SE alleles' genotyping as well as genotyping of SNP associated with RA and MS complex disorders. Considering the limitations of autonomous molecular-based LOC diagnostic devices - due to the relative small number of PCR and probes that they can host, we intend to adopt the approach of broad genotyping of HLA-DRB1 genes using generic primers and probes instead of group specific primers, or high resolution genotyping. McClure and co-workers (2009) have shown that in the case of broad HLA genotyping instead of full sub-typing the analysis of carriage of any DR*01, DR*04 or DR*10 allele denoted an individual as SE positive. The results are very similar to the others derived from analysis with SE subtypes[6]. The use of sequences based on the literature (11th International Histocompatibility Workshop, IHW), has the advantage of proven successful implementation in genotyping, while others were designed for this study and have to be tested in the laboratory. The design of this work is flexible and can be updated and extended to include more loci associated with RA and MS.

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