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Reverse-SSO Hybridization Provides an Accurate and Simple HLA-DR Typing

—A Comparative Study with HLA-DR Serologic Typing—

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The HLA-DR molecule is a polymorphic membrane glycoprotein and one of the key molecules causing allograft rejection and graft-versus-host disease in organ transplantation. Serologic typing using DR specific alloantisera has long been used, but several problems have limited its application. The purpose of this study was to establish an efficient reverse-SSO typing system that detects DRB1 and DRB3/B4/B5 alleles on a single membrane. A DR typing membrane was prepared by immobilizing 21 dT-tailed sequence specific oligonucleotides (SSOs) on a nylon membrane and was used in a hybridization assay with digoxigenin-labeled PCR-amplified target DNA. The positive signals were detected on X-ray film using chemiluminescence. A comparison study with serology using DNAs from 105 unrelated individuals demonstrated that the reverse-SSO typing system was superior to serologic typing in terms of accuracy (100% vs 90.5%), simplicity, range of application, rapidity, and cost of the test. These data indicate that the reverse-SSO typing system can replace serology as a routine DR test, and will be useful in time-restricted solid organ transplantation and in selection of an unrelated marrow donor prior to bone marrow transplantation.

Key Words : *HLA-DR, Reverse-SSO typing system.*

INTRODUCTION

HLA-DR, one of the human leukocyte antigens (HLAs), has long been the focus of interest in the organ transplantation field. This is due to the fact that HLA-DR type matching between donor and recipient is the crucial factor affecting the clinical outcome of kidney (Ting, 1988) and bone marrow transplantation (Hansen et al., 1992). HLA-DR, the major HLA class II molecule expressed on antigen presenting cells such as B cell, is a heterodimeric membrane glycoprotein consisting of an α and a β chain encoded in DRA and DRB1 genes respectively on the short arm of human chromosome 6. Two distinct characteristics of DRB1 genes have been elucidated. One characteristic is its enormous polymorphism. More than 100 different DRB1 alleles (DRB1*0101-DRB1*1001) have been characterized by DNA sequence analysis and 18 different DR specificities (DR1-DR18) have been defined by serology (Bodmer et al., 1994 ; Marsh and Bodmer, 1992). A comparison of DRB1 sequences shows that the majority of polymorphism is confined to three variable regions (codons at 9-13, 26-33, and

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67-74) of the second exon of the DRB1 gene and that entire sequences in variable regions are frequently shared between alleles (Bell et al., 1989).

Another characteristic is its strong linkage to one of the second expressed DRB genes (DRB3, DRB4, and DRB5) resulting in coexpression of the second DR molecule (Bodmer et al., 1994). Alleles encoding DR2 (DR15 and DR16) serologic specificities are linked to a DRB5 gene encoding a DR51 molecule ; alleles encoding DR3 (DR17 and DR18), DR5 (DR11 and DR12), and DR6 (DR13 and DR14) serologic specificities are linked to a DRB3 gene encoding a DR52 molecule ; alleles encoding DR4, DR7, and DR9 serologic specificities are linked to a DRB4 gene encoding a DR53 molecule. However, alleles encoding DR1, DR8, and DR10 serologic specificities are not linked to a second DRB gene. This DRB1-DRB3/B4/B5 linkage has been used to assign accurate DR types in tissue typing laboratories.

Identification of DR types in individuals has been performed by complement mediated lympholysis using alloantisera specific for DR molecules. This serologic typing system is simple and rapid enough for clinical use. However, the requirement for purified B cells expressing a reasonable level of DR molecules on the cell surfaces and inaccuracy originating from the crossreactivity of the sera used, have limited its application (Lee et al., 1990 ; Zmijewski, 1994).

These disadvantages can be overcome by recently developed DNA typing methods : PCR-SSOP (Wordsworth et al., 1990), reverse-SSO (Eliaou et al., 1992 ; Buyse et al., 1993), PCR-RFLP (Uryu et al., 1990), PCR-SSP (Zetterquist and Olerup, 1992), and PCR-direct sequencing (Santamaria et al., 1992). Among these methods, the reverse-SSO typing system utilizing hybridization of PCR amplified and labeled target DNA to a set of membrane bound SSOs, is known to be a simple and time-saving system, and appropriate for the laboratories managing a moderate number of samples each day. There have been two reports on DRB reverse-SSO typing thus far. One report focused only on defining DRB1 types, thus, it carries some risk of mistyping without information on the linked second DRB genes (DRB3/B4/B5) (Eliaou et al., 1992). The second reverse-SSO typing system used 31 SSOs dotted on two separate membranes to be washed at two different temperatures (Buyse et al., 1993). This system is also not ideal in terms of simplicity and the cost of the test.

The purpose of this study was to establish an efficient reverse-SSO typing system that detects DRB1 and DRB3/B4/B5 alleles on a single membrane. A DR typing membrane was prepared by immobilizing 21 dT-tailed SSOs on a nylon membrane and was used in a hybridization assay with digoxigenin-labeled PCR-amplified target DNA. The established reverse-SSO typing system was evaluated by a comparison study with serology using DNAs from 105 unrelated individuals.

MATERIALS AND METHODS

Cells

Fifteen B-lymphoblastoid cell lines (Table 1) that have been characterized by many other reports (Bodmer et al., 1994 ; Kimura et al., 1992) were used as a source of reference DNA to confirm the specificity of the SSOs used in this study. As a further evaluation of our typing procedure, stored leukocytes from 105 unrelated Korean individuals that had been DR typed by serology using a commercial typing tray (Terasaki second DR (60) Tissue typing tray, One Lambda Inc. USA), were also included.

DNA extraction

Most of the genomic DNAs were obtained from B-lymphoblastoid cell lines and stored leukocytes using a salting-out method (Miller et al., 1989). Several stored leukocytes were subjected to a quick phenol/chloroform extraction method to isolate genomic DNAs (Grimberg et al., 1989).

DRB gene amplification

The generic DRB primers, DRampA (5′) and DRampB (3′), amplified the four expressed DRB genes (DRB1, DRB3, DRB4, DRB5) as described in the previous study (Kimura et al., 1992 ; Lee et al., 1993). DRB gene amplification and digoxigenin-labeling of target DNA were performed by a single standard PCR procedure using a DNA Thermal Cycler (Perkin-Elmer Cetus Corp., Norwalk, CT). Briefly, 100 ul of PCR mixture containing 0.5 ug genomic DNA, 50 pmol of each primer, 200 uM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 0.5 nmol of digoxigenin-11-dUTP, 2.5 units Taq DNA polymerase (Boehringer Mannheim, USA), and

Table 1. B Lymphoblastoid cell Lines Used in This Study[a]

Gell Line	HLA-DR Type[b]		HLA-DRB Allele[c]			
	DR	DR52/53	DRB1 *	DRB3 *	DRB4 *	DRB5 *
EHM	DR1	—	0101	—	—	—
PGF	DR15(2)	—	1501	—	—	0101
RML	DR16(2)	—	1602	—	—	0202
AVL	DR3	DR52	0301	0101	—	—
LKT3	DR4	DR53	0405	—	0101	—
SPOO10	DR11(5)	DR52	1101	0202	—	—
BM16	DR12(5)	DR52	1201	0202	—	—
HHKB	DR13(6)	DR52	1301	0101	—	—
WT47	DR13(6)	DR52	1302	0301	—	—
EK	DR14(6)	DR52	1401	0202	—	—
AMALA	DR14(6)	DR52	1402	0101	—	—
BURK	DR7	DR53	0701	—	0101	—
MADURA	DR8	DR52	0801	—	—	—
DKB	DR9	DR53	0901	—	0101	—
RAJI[d]	DR10/DR3	DR52	1001/0301	ND[e]	—	—

[a] : Bodmer et al., 1994 ; Kimura et al., 1992.
[b] : Types defined by serology.
[c] : Types defined by nucleotide sequence analysis.
[d] : Heterozygous B cell line.
[e] : Not defined.

PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin) were subjected to an initial denaturation step of 4 min at 94℃ followed by 30 cycles of incubation for 1 min at 94℃, 30 sec at 55℃, and 30 sec at 72℃. A final extension step of 4 min at 72℃ was added. Gene amplification was confirmed by agarose gel electrophoresis. Two additional 5′ primers were used along with primer DRampB to amplify specific DRB1 genes for clarification of DR assignment in several cases. Primer 3116GF (5′-GTTTCTTGGAGTACTCTACGTC-3′ ; 56℃ annealing temperature) was used for selective amplification of DRB1 alleles from DR3, DR11, and DR6 positive cells (Lee, 1993). Primer DRamp-2 (5′-TTCCTGTGGCAGCCTAAGAG-G-3′ ; 55℃ annealing temperature) was used for selective amplification of DR2 DRB1 alleles (Wade et al., 1993).

Sequence Specific Oligonucleotides (SSOs)

Based on the DRB allelic nucleotide sequences, 21 SSOs were designed to identify the generic DR type of the cell. Table 2 lists the sequences and specificities of SSOs used in this study. SSOs could be grouped into mono-specific and multi-specific based on their hybridization to a single or multiple DR types. The panel was designed so that each of the DR types except DR10, would hybridize to at least three SSOs in the panel to assist in accurate DR type assignments. A positive control (T-ALL) detecting all amplified DRB genes was used to determine the efficiency of the overall test procedure.

All SSOs were poly-dT tailed (approximately 400-mer) according to the method described by Saiki and colleagues with some modifications (Saiki et al., 1989). Briefly, a 100 ul mixture containing 200 pmol SSO, 80 nmol dTTP, 60 units terminal deoxynucleotidyl transferase (Boehringer Mannheim), 0.5 mM $CoCl_2$, 200 mM potassium cacodylate, 25 mM Tris-HCl, and 0.25 mg/ml bovine serum albumin (pH 6.6) was incubated for 1 hr at 37℃. The reaction was stopped by addition of 10 mM EDTA (100 ul).

Preparation of the typing membrane

The dT-tailed SSOs were diluted in 10X SSPE (1.5 M NaCl, 0.1 M Na_2HPO_4, 10 mM EDTA) to give a strong positive signal for each SSO and applied to a Zeta probe membrane (Bio-Rad, USA) using a dot blot apparatus (Bio-Rad). SSOs were covalently bound to the membrane by UV irradiation (302 nm, Spectroline, USA) for 5 min. Membranes were washed for 30 min at 55℃ in 5X SSPE/0.5% SDS to remove unbound SSOs, briefly rinsed in ddH_2O,

Table 2. SSOs Applied on the DRB Typing Membrane[a]

SSO Name	Sequence	Specificity	Hybridization Efficiency[d]
T-ALL	5'-TGGAACAGCCAGAAGGAC-3'	All DRB	+++
T-B3	5'-GAGCTGC(T,G)TAAGTCTGAG-3'	DRB3	++
T-B4	5'-GCGAGTGTGGAACCTGAT-3'	DRB4	+++
6-B5	5'-TCTTGCAGCAGGATAAGTAT-3'	DRB5	+
T-3116	5'-CTCTACGTCTGAGTGTCA-3'	DRB1*03, *11, *13, *14[b]	++
T-812	5'-CTCTACGGGTGAGTGTTA-3'	DRB1*08, *12, *1404	++
T-1414	5'-CTCCTGGAGCAGAGGCGG-3'	DRB1*01, *0405, *14[b]	+++
T-1479	5'-CCTCGGCCCGCCTCCGCT-3'	DRB1*04, *07, *09, *14[b]	+++
T-1	5'-TTCTTGTGGCAGCTTAAGTT-3'	DRB1*01	++
T-2	5'-CAGCAGGATAAGTATGAG-3'	DRB1*02	++
T-15	5'-GACATCCTGGAGCAGGCG-3'	DRB1*02(*15)[c]	++
T-3	5'-GGCCGGGTGGACAACTAC-3'	DRB1*03, DRB3*0101	+++
T-4	5'-GAGCAGGTTAAACATGAG-3'	DRB1*04, *1410	++
T-11	5'-GCCTGATGAGGAGTACTG-3'	DRB1*11	++
T-12	5'-CCAGGAGGAGCTCCTGCG-3'	DRB1*12	+
T-13	5'-GACATCCTGGAAGACGAG-3'	DRB1*13, *0402, *1102	++
T-14a	5'-GCCTGCTGCGGAGCACTG-3'	DRB1*14[b]	+++
T-7	5'-CCTGTGGCAGGGTAAGTATAAG-3'	DRB1*07	++
T-8	5'-GAAGACAGGCGGGCCCTG-3'	DRB1*08	++
T-9	5'-AAGCAGGATAAGTTTGAG-3'	DRB1*09	++
T-10	5'-GAGGAGGTTAAGTTTGAG-3'	DRB1*10	+

[a] : Each SSO was dT-tailed prior to application onto a membrane(see Materials and Methods).
[b] : See Table 3.
[c] : Hybridizes to cells carrying DR2 using a sample amplified with generic primers but exhibits hybridization to only DRB1*15(not DRB1*16) in the assay using samples amplified with DRB1*02 specific PCR primers.
[d] : Determined based on signal intensities on an autoradiograph obtained from a hybridization assay using digoxigenin-labeled PCR mixture containing all DRB generic types(DR1-DR14). Concentration of each SSO on the membrane was adjusted as follows ; +++ ; 3-15 pmol/dot, ++ ; 25-30 pmol/dot, + ; 50-90 pmol/dot.

and stored at room temperature in a sealed plastic bag until needed.

Hybridization and washing of the membrane

For hybridization, 20 ul PCR product was denatured by mixing with an equal volume of 400 mM NaOH/10 mM EDTA solution and applied to a typing membrane soaked in 5X SSPE/0.5% SDS solution. The typing membrane was agitated at 55℃ for 30 min. The membrane was then briefly rinsed twice at room temperature with 2X SSPE/0.1% SDS and washed once for 10 min at 55℃ in 2X SSPE/0.1% SDS. Finally, the membrane was rinsed in 2X PBS at room temperature.

Detection of positive signals

Visualization of positive signals was performed according to the method described previously (Shaffer et al., 1992). Nonspecific binding sites on the membranes were blocked by agitation for 30 min in TN buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 4% blocking reagent (Boehringer Mannheim). Agitation was continued for 30 min after addition of 1 unit anti-digoxigenin antibody-alkaline phosphatase conjugate (Boehringer Mannheim). The membrane was washed twice at room temperature with TN buffer for 15 min, and rinsed with TNM buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$). In a dark room, the membrane was soaked in Lumi-Phos 530™ (Boehringer Mannheim) for 1 min, sealed in a plastic bag, and warmed at 37℃ for 30 min before exposure to X-ray film (X-Omat K, Kodak, USA) for approximately 30 sec-1 min.

Stripping of the membranes

The typing membrane could be reused multiple times if stripped. The used membrane was washed

twice in 0.1X SSC/0.5% SDS at 95℃ for 20 min each. After rinsing briefly with ddH_2O, the membrane was stored in a sealed plastic bag at room temperature until needed.

Table 3. Expected Hybridization Patterns in Reverss-SSO DR typing[a]

DRB1 Type	dT-SSO																				
	ALL	B3	B4	B5	3116	812	1479	1414	1	2	15	3	4	11	12	13	14a	7	8	9	10
DRB1∗01[b]	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
DRB1∗0103[c]	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−
DRB1∗02	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
DRB1∗03	+	+	−	−	+	−	−	W	−	−	−	+	−	−	−	−	−	−	−	−	−
DRB1∗04[d]	+	−	+	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−
DRB1∗0401/09	+	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
DRB1∗0402[c]	+	−	+	−	−	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−	−
DRB1∗11[e]	+	+	−	−	+	−	−	W	−	−	−	±	−	+	−	−	−	−	−	−	−
DRB1∗1102[c]	+	+	−	−	+	−	−	W	−	−	−	±	−	+	−	+	−	−	−	−	−
DRB1∗12	+	+	−	−	−	+	−	W	−	−	−	±	−	−	+	−	−	−	−	−	−
DRB1∗1301/02/04	+	+	−	−	+	−	−	W	−	−	−	±	−	−	−	+	−	−	−	−	−
DRB1∗1303/05/06/07[c]	+	+	−	−	+	−	−	W	−	−	−	±	−	−	−	−	−	−	−	−	−
DRB1∗1401/07	+	+	−	−	+	−	+	W	−	−	−	±	−	−	−	−	+	−	−	−	−
DRB1∗1402/06/09	+	+	−	−	+	−	−	+	−	−	−	±	−	−	−	−	−	−	−	−	−
DRB1∗1403	+	+	−	−	+	−	−	W	−	−	−	±	−	−	−	−	−	−	+	−	−
DRB1∗1404[c]	+	+	−	−	−	+	+	W	−	−	−	±	−	−	−	−	+	−	−	−	−
DRB1∗1405/08	+	+	−	−	+	−	+	W	−	−	−	±	−	−	−	−	−	−	−	−	−
DRB1∗1410[c]	+	+	−	−	−	−	+	W	−	−	−	±	+	−	−	−	+	−	−	−	−
DRB1∗07	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−
DRB1∗08	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
DRB1∗09	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−
DRB1∗	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

[a] : Obtained based on DNA sequence information(Marsh and Bodmer, 1992).
[b] : All except DRB1∗0103.
[c] : Patterns not observed in this study.
[d] : All except DRB1∗0401, ∗0402, ∗0409.
[e] : All except DRB1∗1102.
W : Exhibits weak hybridization due to crossreactivity.
± : Cells carrying DRB3∗0101 are expected to be positive.

Table 4. Comparison of DR Assigned by Serology and Reverse-SSO Typing(N=105)

DR type[a]	DRB1 type[b]	Number[c]	False Positive		False Negative		Sensitivity		Specificity	
			Serology	R-SSO[d]	Serology	R-SSO[d]	Serology	R-SSO[d]	Serology	R-SSO[d]
1	∗01	9	0	0	0	0	1.00	1.00	1.00	1.00
2	∗02	21	0	0	0	0	1.00	1.00	1.00	1.00
3	∗03	4	1[e]	0	0	0	1.00	1.00	0.994	1.00
4	∗04	44	0	0	0	0	1.00	1.00	1.00	1.00
11	∗11	5	0	0	0	0	1.00	1.00	1.00	1.00
12	∗12	23	1[e]	0	1[e]	0	0.957	1.00	0.994	1.00
6	∗13 & ∗14	35	0	0	2[e]	0	0.935	1.00	1.00	1.00
7	∗07	11	0	0	0	0	1.00	1.00	1.00	1.00
8	∗08	21	1[e]	0	1[e]	0	0.952	1.00	0.994	1.00
9	∗09	13	0	0	0	0	1.00	1.00	1.00	1.00
10	∗10	6	0	0	3[e]	0	0.500	1.00	1.00	1.00

[a] : Types defined by serology.
[b] : Types defined by reverse-SSO typing
[c] : Number of occurences of each DR type among the study population by reverse-SSO typing.
[d] : Reverse-SSO typing.
[e] : See Table 5.

Assignment of DR type and data analysis

DRB1 type was assigned based on the expected pattern of hybridization to a panel of SSOs as shown in Table 3. The DRB3, DRB4 and/or DRB5 association was one of the major considerations in DRB1 assignment. HLA-DR types identified by serology and reverse-SSO typing are listed in Table 4. DR types of discordant samples identified by serology and reverse-SSO typing were confirmed by PCR-SSOP typing as previously described (Lee et al., 1993). Two concordant DR types among three obtained by the three different methods (serology, reverse-SSO typing, and PCR-SSOP) was counted as the correct DR type. The sensitivity and specificity of the tests were calculated based on the methods designed for determining the accuracy of a diagnostic test (Ingelfinger et al., 1983).

RESULTS

Adjustment of the SSO concentration on the typing membrane

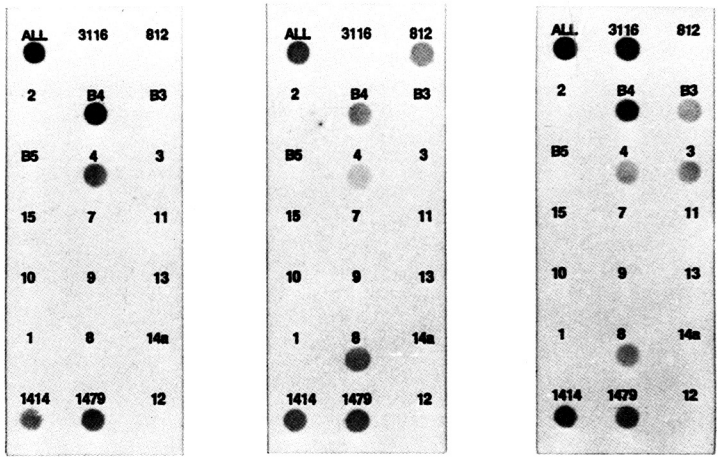
In order to maintain that positive signals on typing membrane revealed similar levels of intensities under the same experimental conditions, it was necessary to adjust the concentration of each SSO on the membrane. In the preliminary study, a constant concentration (15 pmol/dot) of each SSO was applied on a membrane and then was subjected to a hybridization assay with a digoxigenin-labeled standard PCR mixture containing all DRB generic types (DR1-DR14) in order to obtain positive signals for all SSOs on a membrane. SSOs were sorted as strong (+++), medium (++), and weak (+), based on signal intensities of each SSO. As seen in Table 2, six SSOs (T-ALL, T-B4, T-1414, T-1479, T-3, and T-14a) exhibited strong signals while three SSOs (T-B5, T-12, and T-10) exhibited weak signals. Concentration of SSOs on the membrane was adjusted to yield similar levels of signal intensities on an autoradiograph, and used in further studies.

Confirmation of specificities of SSO in DRB reverse-SSO typing

To confirm that SSOs retained their specificities under T tailing and UV fixation onto a nylon membrane, fifteen reference DNAs (Table 1) displaying different HLA-DR generic specificities were chosen and used in a hybridization assay after digoxigenin-labeled DRB gene amplification by PCR. As seen in Fig. 1~4, the positive and negative signals were clear, and all SSOs except T-1414 exhibited a sequence specific pattern. SSO T-1414 exhibited crosshybridization with DRB3 alleles that carry sequences with a one nucleotide mismatch at codon 71 (G/A). But it was still possible to discriminate whether the signal comes from perfect hybridization (Fig. 1) or crosshybridization (Fig. 2), based on their signal intensities. Results were repeatedly consistent.

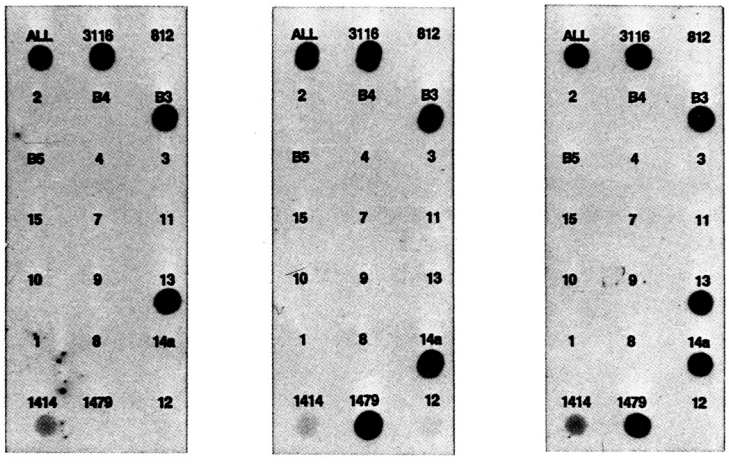
All the patterns obtained from reference DNAs were identical to the expected patterns as shown in Table 3. All of the types except DR10 were identified by their unique patterns by hybridization with multiple SSOs instead of a single SSO. Fig. 1 (left) shows an example of the unique hybridization pattern obtained from a DR4 reference DNA that hybridized to SSO on T-ALL, T-B4, T-4, T-1414 and T-1479. Although patterns became more complicated in heterozygous individuals, DR assignment was fairly easy in virtue of signals obtained from SSOs detecting DRB1-linked DRB genes (T-B3, T-B4, and T-B5) and sequences shared between members of a group (T-3116, T-812, T-1414, and T-1479) as well as information on Korean DRB1 allelic frequencies. For example, DRB1*1403 which carries a DR8 specific sequence at codons 70-74 could potentially be mistyped as DR8 without information on its DRB3 linkage. In this study, DRB1*1403 hybridized to SSOs, T-ALL, T-3116, T-B3, T-3 and T-8 (Fig. 1, right), and could easily be distinguished from DRB1*08 that hybridized to T-ALL, T-812 and T-8 only (Fig. 1, center). Another good example of accurate assignment in a heterozygous individual by reverse-SSO typing was observed from sample #9251. This sample was assigned as DR6 by serology using commercial typing tray but it was difficult to determine which of the DR6 splits, DR13 or DR14. Sample #9251 was revealed as a heterozygote carrying DRB1*13 and DRB1*14 alleles by the reverse-SSO typing system (Fig. 2).

Based on unique hybridization patterns (Table 3), the method could subgroup DR4 into three groups based on reactivity of SSOs T-1414 and T-13, and DR14 into six groups based on reactivity of SSOs T-3116, T-812, T-1479, T-1414, T-4, and T-14a. However, only two DR4 and four DR14 groups were observed in this study using Korean DNA samples. The pattern obtained from one of the samples (#



DRB1*	0405	04, 08	04, 1403
DR	4	4, 8	4, X
Cell	LKT3	#9432	#153

Fig. 1. Autoradiographs obtained from reference cell and samples (LKT3, #9432, and #153) by the reverse-SSO typing using DRB typing membranes prepared in this study. The name of the SSO is indicated above each signal. DRB1 types were obtained by reverse-SSO typing and DR types were obtained by serolgy. 'X' in Sample #153 indicates an undetectable type by serology. Information on the DR and DRB1 types of cell LKT3 was obtained from the reference(Kimura et al., 1992). DRB1*1403 was discriminated from BRB1*08 by hybridization with T-3116, T-B3, and T-3, and no hybridization with T-812.



DRB1*	1302	1402	13, 14
DR	13	14	6
Cell	WT47	EK	#9251

Fig. 2. Autoradiographs obtained from reference cells and sample(WT47, EK, and #9251) by the reverse-SSO typing using DRB typing membranes prepared in this study. The name of the SSO is indicated above each signal. DRB1 types were obtained by reverse-SSO typing and DR types obtained by serology. Information on the DR and DRB1 types of cells WT47 and EK comes from the reference(Kimura et al., 1992). SSO T-1414 exhibits weak hybridization due to the crossreactivity with DRB3*0101. Sample #9251, typed as DR6 by serology, exhibits a combined hybridization pattern identifying DRB1*13 and DRB1*14.

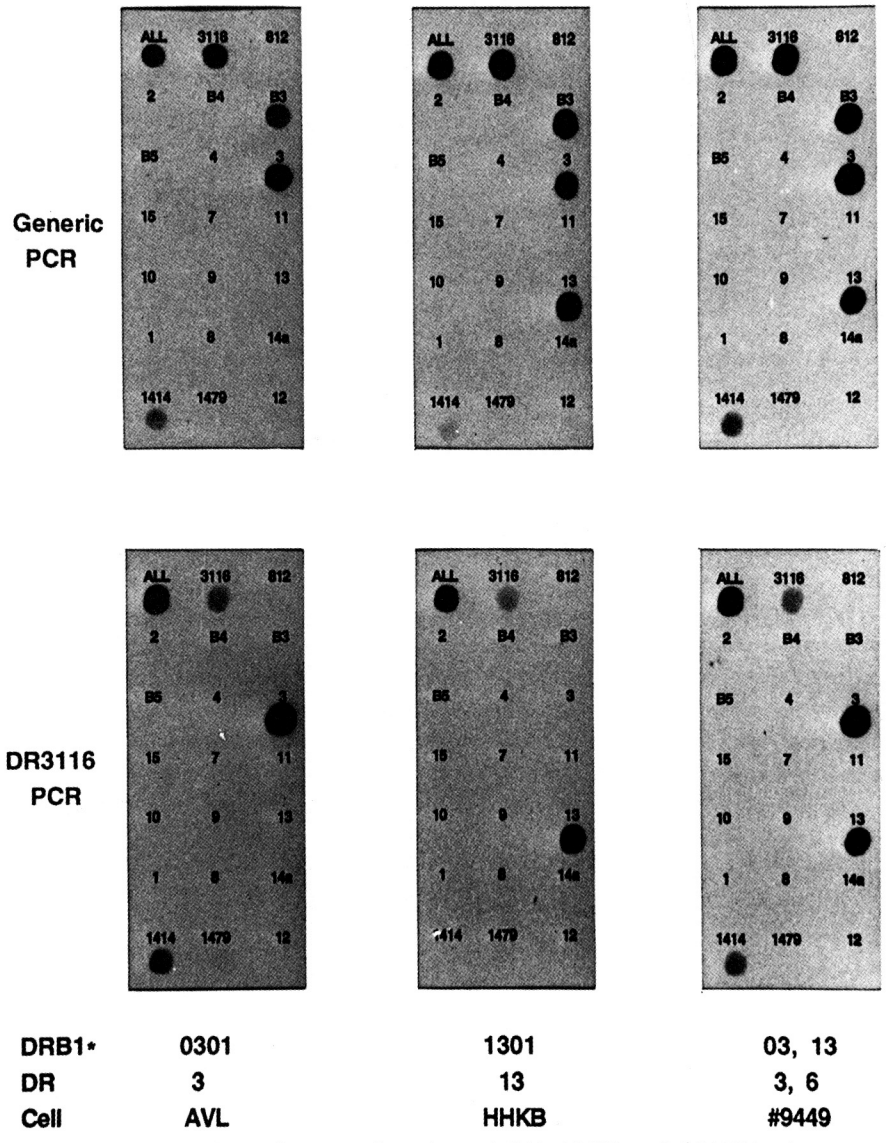


Fig. 3. Autoradiographs obtained from reference cells and sample(AVL, HHKB, and #9449) by the reverse-SSO typing using DRB typing membranes prepared in this study. The name of the SSO is indicated above each signal. DRB1 types were obtained by reverse-SSO typing and DR types obtained by serology. Informations on the DR and DRB1 types of cells AVL and HHKB come from the reference(Bodmer et al., 1994). Due to the identical patterns, DR types of sample # 9449 could not able be identified as DR13 homozygote or DRB1*03/DRB1*13 heterozygote using a generic PCR amplification (Top). Selective DRB1 gene amplification using 5′ PCR primer 3116GF clarified the types of sample #9449 as DRB1*03, DRB1*13 (bottom).

9449) was difficult to assign since DRB1*13 homozygote and DRB1*13/DRB1*03 heterozygote exhibited identical hybridization patterns (Fig. 3, top). This ambiguity arose from the shared sequence in DRB1*03 and DRB3*0101 alleles, at codons 71-77 probed with SSO T-3. Thus, DNAs from DR13 positive cells carrying DRB3*0101 hybridized to T-3. This ambiguity resolved by utiliz-

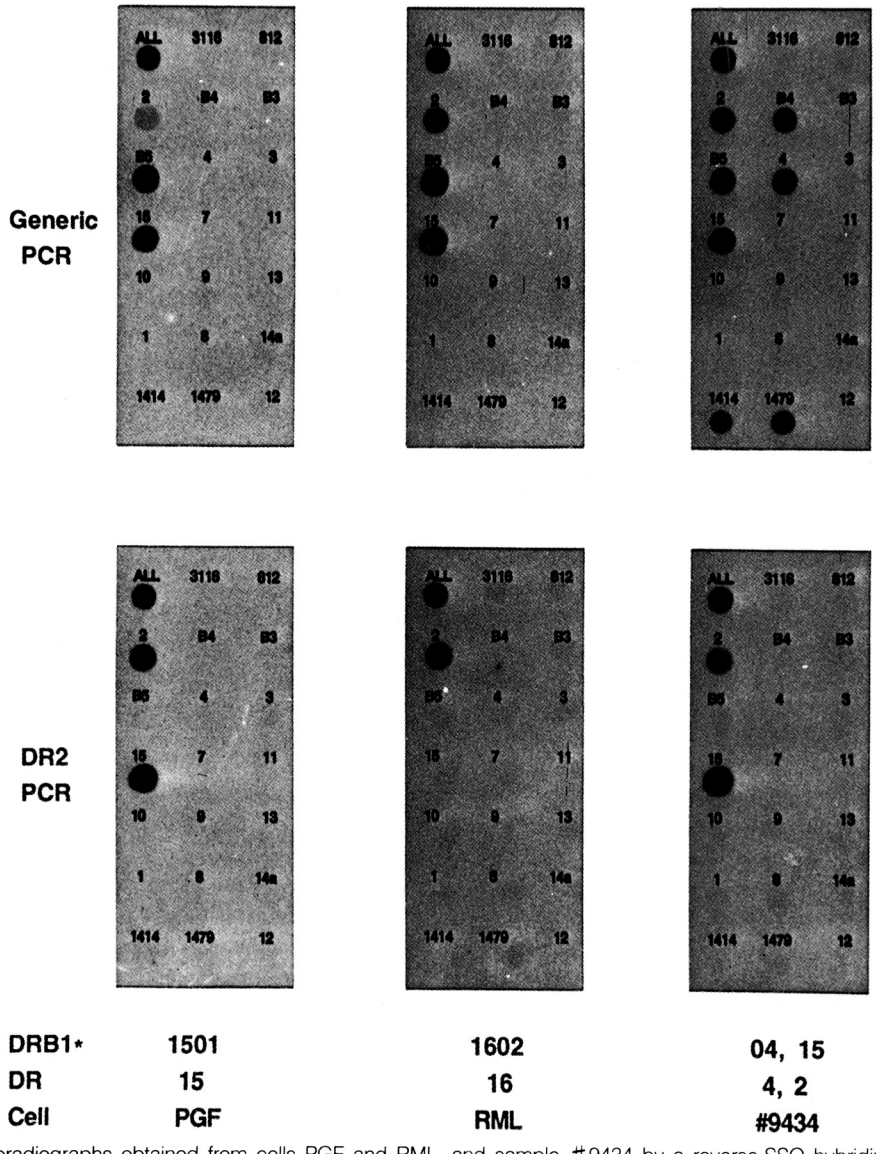


Fig. 4. Autoradiographs obtained from cells PGF and RML, and sample #9434 by a reverse-SSO hybridization assay using DRB typing membranes prepared in this study The name of the SSO is indicated above each signal. DRB1 types were obtained by reverse-SSO typing and DR types obtained by serology. Information on the DR and DRB1 types of cells PGF and RML comes from the reference(Bodmer et al., 1994). Discrimination of DRB1*15 and DRB1*16 was possible using DR2 DRB1 amplified DNA(Bottom), although it was not possible using a generic PCR amplification (Top). Sample #9434 typed as a heterozygous carrying DRB1*04 and DRB1*15 allele.

ing selectively amplified DRB1 PCR sample in the hybridization assay (Fig. 3, bottom). This selective amplification used the group specific PCR primer, 3116GF, which amplifies only DRB1*03, *11, *13 and *14 alleles. Likewise, DR2 splits, DRB1*15 and DRB1*16, could be discriminated by utilizing the DRB1*02 selectively amplified PCR sample in the hybridization assay (Fig. 4).

Table 5. DR Typing Results Obtained from Discordant Samples

Sample #	Reverse Typing (BRB1*)	PCR-SSOP[a] (DRB1*)	Serology (DR)
9242	12, 14	12, 14	12, 3
9255	12	12	12, 8
9368	08	08	8, 12
9223	04, 12	04, 12	4, X[b]
9360	02, 1403	02, 1403	2, X[b]
9432	04, 08	04, 08	4, X[c]
153	04, 1403	04, 1403	4, X[b]
9357	02, 10	02, 10	2
9379	1403, 10	1403, 10	6
9414	04, 10	04, 10	4

[a] : Lee et al., 1993.
[b] : Cells were DR52 positive but the DR types could not be assigned.
[c] : The DR type could not be assigned due to overall weak reactivity with multiple alloantisera.

Comparison with serology

DNAs from 105 unrelated individuals, whose HLA-DR generic types had been determined by serology, was characterized by DRB reverse-SSO typing. DRB genes from all DNA samples including those prepared by quick DNA preparation method were successfully amplified and typed. Table 4 shows a comparison of the serology and reverse-SSO typing results. The overall concordance rate was 90.5% (95/105) and complete agreement was observed for DR1, DR2, DR4, DR11, DR7, and DR9. Thirty five DR6-positive samples assigned by serology could be clearly subdivided into DR13 (20 subjects) and DR14 (15 subjects) in the reverse-SSO typing system. There were ten cases of discordance, involving DR3, DR12, DR6, DR8 and DR10. To confirm the DR types of discrepant samples, DNAs were subjected to generic PCR-SSOP typing. As shown in Table 5, the results obtained by PCR-SSOP typing were in complete concordance with those obtained by reverse-SSO typing. Of the 10 differences found, 7 corresponded to serologic misinterpretation or ambiguity in one allele of each heterozygote. Two of the serologically ambiguous samples were DRB1*1403. The remaining three were serologic blanks which typed as DR10 using DNA methods (sensitivity 50%), suggesting that the serologic typing reagents were not adequate to define DR10. In addition to the accuracy, specificity, and sensitivity of the test, reverse-SSO typing was superior to serology in many other aspects as discussed below.

DISCUSSION

Extensive polymorphism of DR alleles coupled with the sharing of modular sequence variations among alleles make it difficult to accurately define DR allelic types in a single step procedure. A two step strategy is, therefore, inevitable (Mach and Tiercy, 1991) : 'Low level typing' identifies DR generic types (DRB1*01-DRB1*14) and, if necessary, 'high level typing' characterizes DRB1 allelic types (DRB1*0101-DRB1*1001) based on the information obtained from 'low level typing'. Serologic typing, the most extensively utilized method, is the equivalent of 'low level typing'. While this typing system is simple and rapid, two main problems limit its application. It requires purified B cells which are difficult to obtain in certain cases (e.g., patients with hematologic disorders, pediatric patients, and cadaveric organ donors) and alloantisera used as reagents exhibit crossreactivity resulting in inaccuracy (Tiercy et al., 1991 ; Hopkins et al., 1992 ; Zmijewski, 1994). Recently developed DNA typing methods utilizing in vitro gene amplification by the PCR technique overcome these disadvantages. These DNA typing methods will soon replace serology in clinical laboratories (Ng et al., 1993 ; Verduyn et al., 1993), because these methods generally have improved DR typing in terms of accuracy, precision, and the range of application. The PCR-SSOP, reverse-SSO, PCR-RFLP and PCR-SSP are examples of 'low level typing', and high level PCR-SSOP and PCR-direct sequencing are examples of 'high level typing'.

In this study, we established an efficient reverse-

SSO typing system using a reasonable number of SSOs that detects DRB1 and DRB3/B4/B5 alleles on a single membrane. A DR typing membrane was prepared by immobilizing 21 dT-tailed SSOs on a nylon membrane. The membrane was used in a hybridization assay with digoxigenin-labeled PCR-amplified target DNA. The positive signals were detected on X-ray film using chemiluminescence. The efficiency of the reverse-SSO typing system was confirmed by a comparison study with serology using 105 Korean DNA samples.

The established reverse-SSO typing system exhibited several advantages. First, the most important advantage of this system was high accuracy (100%). This should be compared to the 90.5% accuracy for serologic typing. Included SSOs detecting the second expressed DRB genes (T-B3, T-B4, and T-B5) and sequence shared among groups of DRB1 alleles (T-3116, T-812, T-1414, and T-1479) on DR typing membrane were the crucial factor of the high accuracy of this typing system. One of the previous reports on DR reverse-SSO typing focused only on defining DRB1 types, thus, it carried some risk of mistyping without information on the linked second DRB genes (DRB3/B4/B5) (Eliaou et al., 1992). For example, the DR molecule specified by allele DRB1*1403 exists at a relatively high frequency in the Korean population (Lee, 1993), but is difficult to detect by serology (Obata et al., 1990). This allele is identical to DRB1*1402 except at codons 70-74. The sequence of codons 70-74 is shared with DR8 and could lead to the wrong assignment as DRB1*08 using certain systems of DNA typing. The same difficulties would apply to newly characterized DRB1 alleles, DRB1*1412 (Hashimoto et al., 1994), DRB1*0412 (Gao et al., 1992) and DRB1*1604 (Laforet et al., 1994) which also carry DR8 sequences. As shown in Figure 1, this problem was solved by including SSOs that detected the second expressed DRB genes and sequences shared among groups of DRB1 alleles on the DR typing membrane.

Second, utilization of the DNA allowed the test to be simple by omitting B cell purification procedure, the major labor-intensive step in HLA-DR serologic typing. Also, the clear positive and negative signals led to simple and clear DR assignment. Third, requirement of small volume of blood sample and utilization of the DNA instead of B cell could extend the range of application of the DR test. In contrast to the serologic typing requiring 20-30 ml blood sample, 0.5 ml blood would be more than enough for reverse-SSO typing. Also, the DNA can be extracted independent of cell viability, level of DR expression on the cell surface, and the cell type as long as cell carries nucleus. Therefore, reverse-SSO typing is useful for the HLA-DR typing of patients whose B cell purification is difficult (e.g., hematologic disorder patients) or large volume of blood sampling is not available (e.g., pediatric patients). Forth, using a pre-made typing membrane in a hybridization assay helped to obtain the results rapidly. The test time could be reduced to 4.5 hrs from DNA extraction to final result. This advantage would be specifically useful in time-restricted organ trasplantation. This also increases the number of the samples that each technician can handle in a day. Last, multiple utilization of the typing membranes reduced the testing costs. Instead of preparing new membrane, the most expensive step of reverse-SSO typing, simple stripping procedure provided typing membrane available each time. Stripping of the used membranes was performed by incubation of the membrane for 40 min in the stripping solution at high temperature. Each typing membrane could be reused about 50 times.

There are several factors to be considered in the establishment of an appropriate DNA typing system for the clinical typing laboratory : (1)the clinical requirements in terms of level of precision and time restriction,(2) the number of samples to be typed on a routine basis, and(3) the DR allelic frequency of the population to be typed. Considering these factors, the established reverse-SSO typing in this study is an appropriate low level typing for the laboratories serving time-restricted organ transplantation and managing a moderate number of samples each day.

In summary, the established reverse-SSO typing in this study is superior to serology in terms of accuracy, simplicity, range of application, rapidity and cost of the test. This system is an appropriate low level DR typing system and can replace serologic typing as a routine DR test. This reverse-SSO typing system will be useful in time-restricted organ transplantation and in identifying an appropriate marrow donor prior to unrelated bone marrow transplantation.

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REFERENCES

Bell JI, Todd JA, McDevitt HO . *Molecular structure of human class II antigens. In : Dupont B, ed. Immunobiology of HLA. Histocompatibility Testing 1987, volume II. New York : Springer-Verlag, 1989 ; 40-9.*

Bodmer JGE, Marsh SG, Albert ED, Bodmer WF, Dupont B, Erlich HA, Mach B, Mayr WR, Parham P, Sasazuki T, Schreuder GMT, Strominger JL, Svejgaard A, Terasaki PI . *Nomenclature for factors of the HLA system, 1994. Tissue Antigens 1994 ; 44 : 1-18.*

Buyse I, Decorte R, Baens M, Cuppens H, Semana G, Emonds MP, Marynen P, Cassiman JJ . *Rapid DNA typing of class II HLA antigens using the polymerase chain reaction and reverse DOT blot hybridization. Tissue Antigens 1993 ; 41 : 1-14.*

Eliaou J, Palmade F, Avinens O, Edouard E, Ballaguer P, Nicolas J, Clot J . *Generic HLA-DRB1 gene oligotyping by a nonradioactive reverse dot-blot methodology. Hum Immunol 1992 ; 35 : 215-22.*

Gao X, Veale A, Serjeantson SW . *HLA class II diversity in Australian aborigines ; unusual HLA-DRB1 alleles. Immunogenetics 1992 ; 36 : 333-7.*

Grimberg J, Nawoschik S, Belluscio L, McKee R, Turck A, Eisenberg A . *A simple and efficient non-organic procedure for the isolation of genomic DNA from blood. Nucleic Acids Res 1989 ; 17 : 8390.*

Hansen JA, Anasetti C, Petersdorf EW, Choo SY, Mickelson EM, Martin PJ . *The status of clinical marrow transplantation and current issues in donor matching. In : Tsuji K, Aizawa M, Sasazuki T, eds. HLA 1991 : Proceedings of the eleventh international histocompatibility workshop and conference, volume 2. Oxford University Press, Oxford. 1992 ; 13-9.*

Hashimoto M, Kaneshige T, Kinoshita T, Murayama A, Asai S, Yamasaki M, Nojima M, Ichikawa Y, Fukunish T . *A new DR14-related DRB1 allele, DRB1*1412, which differs from DRB1*1403 only at codon 86. Tissue Antigens 1994 ; 43 : 133-5.*

Hopkins KA, Maguire MG, Fink NE, Bias WB . *Reproducibility of HLA-A,B, and DR typing using peripheral blood samples : results of retyping in the collaborative corneal transplantation studies. Hum Immunol 1992 ; 33 : 122-8.*

Ingelfinger JA, Mosteller F, Thibodeau LA, Ware JH . *Biostatistics in Clinical Medicine. New York : Macmillan Publishing, 1983 ; 4-10.*

Kimura A, Dong RP, Harada H, Sasazuki T . *DNA typing of HLA class II genes in B-lymphoblastoid cell lines homozygous for HLA. Tissue Antignes 1992 ; 40 : 5-12.*

Laforet M, Urlacher A, Tongio MM . *A new HLA DR16 allele (DRB1*1604) with a short DR8 sequence. Tissue Antigens 1994 ; 43 : 257-60.*

Lee KW, Hurley CK, Hartzman R, Johnson AH . *The complexity of DRw6 and DR5 haplotypes in American blacks demonstrated by serology, cellular typing, and restriction fragment length polymorphism analysis. Hum Immunol 1990 ; 29 : 202-19.*

Lee KW . *DR6 in Koreans : DR11 frequently acts as a recipient gene to create DR13 alleles. Hum Immunol 1993 ; 37 : 229-36.*

Lee KW, Jung KS, Choi ES, Park CJ, Cho HC, Lee KM, Kim H, Lee MK, Hyun IG . *Pulmonary tuberculosis is not associated with HLA-DR phenotypes in the Korean population. Korean J Clin Pathol 1993 ; 13 : 643-51.*

Mach B, Tiercy JM . Genotypic typing of HLA class II . *from the bench to the beside. Hum Immunol 1991 ; 30 : 278-84.*

Marsh SGE, Bodmer JG . *HLA class II nucleotide sequences, 1992. In : Tsuji K, Aizawa M, Sasazuki T, eds. HLA 1991 : Proceedings of the eleventh international histocompatibility workshop and conference, volume 1. Oxford : Oxford University Press, 1992 ; 32-62.*

Miller SA, Dykes DD, Polesky HF . *A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988 ; 16 : 1215.*

Ng J, Hurley CK, Baxter-Lawe LA, Chopek M, Coppo PA, Hegland J, KuKuruga D, Monos D, Rosner G, Schmeckpeper B, Yang SY, Dupont B, Hartzman RJ . *Large-scale oligonucleotide typing for HLA-DRB1/3/4 and HLA-DQB1 is highly accurate, specific, and reliable. Tissue Antigens 1993 ; 42 : 473-9.*

Obata F, Abe A, Ohkubo M, Ito I, Kaneko T, Otani F, Watanabe K, Kashiwagi N . *Sequence analysis and oligonucleotide genotyping of HLA-DR "JX6", a DR "blank" haplotype found in the Japanese population. Hum Immunol 1990 ; 27 : 269-84.*

Saiki RK, Walsh PS, Levenson CH, Erlich HA . *Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc Natl Acad Sci USA 1989 ; 86 : 6230-4.*

Santamaria P, Boyce-Jacino MT, Lindstrom AL, Barbosa JJ, Faras AJ, Rich SS . *HLA class II "Typing" ; direct sequencing of DRB, DQB, and DQA genes. Hum Immunol 1992 ; 33 : 69-81.*

Shaffer AL, Falk-Wade JA, Tortorelli V, Cigan A, Carter C, Hassan K, Hurley CK . *HLA-DRw52-associated DRB1 alleles. identification using polymerase chain reaction-amplified DNA, sequence-specific oligonucleotide probes, and a chemiluminescent detection system. Tissue Antigens 1992 ; 39 : 84-90.*

Tiercy JM, Goumaz C, Mach B, Jeannet M . *Application of HLA-DR oligotyping to 110 kidney transplant patients with doubtful serological typing. Transplantation 1991 ; 51 : 1110-4.*

Ting A . *HLA matching and crossmatching in renal transplantation. In : Morris PJ, ed. Kidney transplantation : Principles and practice. Philadelphia : Principles and*

practice, 1988 ; 183-213.

Uryu N, Maeda M, Ota M, Tsuji K, Inoko H . *A simple rapid method for HLA-DRB and-DQB typing by digestion of PCR-amplified DNA with allele specific restriction endonucleases. Tissue Antigens 1990 ; 35 : 20-31.*

Verduyn W, Doxiadis II, Anholts J, Drabbels JJ, Naipal A, D'Amaro J, Persijn GG, Giphart MJ, Schreuder GMT . *Biotinylated DRB sequence-specific oligonucleotides- : comparison to serologic HLA-DR typing of organ donors in eurotransplant. Hum Immunol 1993 ; 37 : 59-67.*

Wade JA, Hurley CK, Hastings A, Ehrenberg P, Johnson AH, Martell RW, du Toit ED . *Combinatorial diversity in DR2 haplotypes. Tissue Antigens 1993 ; 41 : 113-8.*

Wordsworth BP, Allsopp CE, Young RP, Bell JI . *HLA-DR typing using DNA amplification by the polymerase chain reaction and sequential hybridization to sequence-specific oligonucleotide probes. Immunogenetics 1990 ; 32 : 413-8.*

Zetterquist H, Olerup O . *Identification of the HLA-DRB1*04,-DRB1*07, and-DRB1*09 alleles by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. Hum Immunol 1992 ; 34 : 64-74.*

Zmijewski CM . *HLA : The major histocompatibility complex-Methodologies. In : McClatchey KD, ed. Clinical laboratory medicine. Maryland ; Williams & Wilkins, 1994 ; 771-99.*