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HLA class I A and B typing in the clinical laboratory using DNA-based techniques

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Abstract The potential for clinical HLA class I A and B typing utilizing the polymerase chain reaction combined with sequence-specific oligonucleotide probes (PCR-SSOP) was investigated. Two hundred and ten clinical samples for the HLA-B locus and 100 clinical samples for the HLA-A locus were typed by DNA-based methods and serology. For the HLA-B locus an improved SSOP typing system was developed which involved using HLA-B specific 5′ primers and two 3′ primers, in separate reactions. Using a panel of 30 digoxigenin-labelled SSOPs, HLA-B types were assigned for all 210 individuals with an improvement in resolution over previously described DNA-based systems and confirming serologically assigned types in all cases except one. In addition, using a single primer pair and a panel of 16 SSOPs, 100 samples were successfully HLA-A typed by PCR-SSOP resolving ambiguous serological types, including HLA-A19 subtypes and A2 homozygosity. In 25 samples, the assigned types were also confirmed by the amplification refractory mutation system (ARMS-PCR). These results indicate that non-urgent clinical HLA-A and -B typing may be performed by PCR-SSOP with a resolution at least equal to that of serology.

Key words Polymerase chain reaction · HLA-A · HLA-B · PCR-SSOP · Clinical transplantation

Introduction

The influence of patient-donor HLA matching on allograft survival in renal and bone marrow transplantation is well documented. This has been demonstrated by the highly successful survival rate of kidneys transplanted between either living related individuals or HLA identical siblings, 90 % of which have a graft survival in excess of 5 years. Martin and Dyer [12] and Opelz [18] have emphasized that a favourable HLA-DR and HLA-B match is the optimal requirement for a successful transplant. It is also known that the outcome of bone marrow transplantation critically depends on the degree of HLA matching, for example, one case has been reported in which T-cell mediated allograft rejection correlated with a single amino acid difference in an HLA-B allele (specifically HLA-B44) [5]. A further report by Steinle et al. [19] also suggested that single, naturally occurring, sequence variations in the HLA-B35 group of alleles influence allorecognition and could be of importance in clinical transplant matching. It is essential, therefore, that any HLA class I tissue typing method is ultimately capable of determining all allelic subtypes that may be of clinical importance and which may give rise to a rejection response.

Until recently, all routine HLA typing has been performed by serology, using the microlymphocytotoxicity test. However, the introduction of DNA-based testing for HLA class II typing has revealed that serological HLA-DR typing has error rates of up to 25 % [16]. The serological 'errors' incurred are mainly due to the limited availability of specific alloantisera and poor viability or low yield of lymphocytes isolated from the sample to be typed. These limitations do not apply to DNA-

based typing techniques. Thus, these methods can confirm homozygosity and identify additional genetic variation not detectable by serology. More recently, the availability of DNA sequence data for the HLA class I alleles has permitted the development of a number of PCR based typing methods for the HLA-A, -B and -C loci using the polymerase chain reaction [10, 17, 24], a technically more demanding task for class I than class II loci. Therefore, it is now possible to use DNA-based typing methods to investigate the accuracy of clinical HLA class I serological typing and from this, to develop more precise DNA-based methods for routine use.

The HLA class I A, B and C genes each consist of seven exons, of which exons II, III and IV code for the three external domains (α1, α2 and α3). The α1 and α2 domains are the most polymorphic, forming the peptide binding groove of the molecule which contains several hypervariable regions. These domains are encoded by exons II and III of the gene in question [11]. Identification of sequence polymorphism in these exons should form the basis of any DNA-based PCR typing system for the HLA class I loci.

The aim of this study, therefore, was to develop an appropriate system for clinical HLA-A and -B PCR typing of homozygous and heterozygous individuals, using panels of sequence-specific oligonucleotide probes (SSOPs) of a manageable size. For the HLA-B locus, the specific aim was to extend a recently developed DNA-based typing system [24], which was based only on exon II sequences. In order to further distinguish most clinically relevant specificities, this system was expanded into a more comprehensive form by extending the region amplified into exon III and by using a further 7 SSOPs specific for two hypervariable regions in this exon. In addition, typing of the HLA-A locus was performed using a protocol based on the method devised by Oh et al. [17] but with careful selection of the SSOPs used, reducing their number from 28 to 16. Further confirmation of certain assigned HLA-A types was achieved by supplementary use of a multiplex PCR amplification refractory mutation system (ARMS-PCR) [2, 21]. The development of these typing systems allowed a retrospective assessment of the accuracy of serological typing for the HLA-A and HLA-B loci and lead to the establishment of a manageable system for clinical HLA-A and -B PCR-SSOP typing.

Materials and methods

Cell lines

Initially, DNA from 36 B-lymphoblastoid cell lines [14, 22, 23], covering the majority of the WHO designated HLA-A and -B serological specificities, were used as control material. These cell lines were selected to give both positive and negative reactivities with all probes tested.

Patients and donors

One hundred and seventy renal donors and recipients (including 66 donor-recipient pairs transplanted between January 1992 and October 1993) were selected for inclusion in this study along with 40 bone marrow transplant-related samples (12 patients and 28 donors). All samples were HLA-B typed by a modified PCR-SSOP method. A further 100 samples (randomly selected bone marrow and renal, patients and donors) were HLA-A typed by PCR-SSOP, with 26 of these typed by both PCR-SSOP and ARMS-PCR. All 310 of these individuals were previously HLA-A and -B typed by serology using the standard microlymphocytotoxicity test [20].

DNA preparation

Genomic DNA was prepared from 1 ml of peripheral blood or 0.5 ml of frozen lymphocytes using a phenol/chloroform miniprep method [1] or by using a salting-out technique [15]. In both of the above methods the DNA was dissolved in 30 μl sterile double-distilled water.

HLA-B PCR primers and DNA amplification

Two HLA-B locus specific 5′-sided primers, CG4 (5′-GAC GAC ACC CAG TTC GTG A-3′) and CG5 (5′-GAC GAC ACG CTG TTC GTG A-3′), designed by Yoshida et al. [24], and derived from nucleotides 84–102 in exon II were employed, amplifying all HLA-B alleles except B54. The two 3′-sided primers, D1 (5′-GCC GCG GTC CAG GAG CT-3′) and D1X (5′-GCG GCG GTC CAG GAG CG-3′), were based on nucleotides 120–136 in exon III and amplify all alleles, regardless of a nucleotide mismatch at position 134 in exon III, generating a 560 bp PCR product. Each DNA sample was amplified using an equal mix of the 5′ primers and either the D1 or D1X 3′ primer in separate reactions to increase the resolution of HLA-B allele assignment over the original system described by Yoshida et al. [24].

Genomic DNA (1 μg) was amplified in a 50 μl reaction mix [67 mM TRIS-HCl pH 8.0, 16.6 mM ammonium sulphate, 6.7 μM EDTA, 0.017% BSA, 2 mM $MgCl_2$, 200 μM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 0.2 μM of each primer and 1 unit of Taq polymerase (Amplitaq, Perkin-Elmer Cetus, CA)]. The reaction mix was overlaid with mineral oil and amplified in a thermal cycler (Omnigene, Hybaid, Limited, Middlesex, UK) according to the following protocol: 5 cycles of 1 min denaturation at 95 °C, 1 min annealing at 65 °C and 2 min extension at 72 °C, followed by 25 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C and 2 min extension at 72 °C. Successful PCR amplifications were determined by agarose gel electrophoresis.

Table 1 Oligonucleotide probe sequences for exon II and III of the HLA-B gene

Hypervariable region/exon	Probe name	Sequence (5′–3′)	Corresponding nucleotide number
A/exon II	01	GAG GAA GGA GCC GCG GGC	128–145
	02	GAG GAC GGA GCC CCG GGC	128–145
	07	GAG GAT GGC GCC CCG GGC	128–145
	09	GAG TCC GAG AGA GGA GCC	122–139
B/exon II	03	ACA CGG AAC ATG AAG GCC	189–206
	04	ACA CAG ATC TCC AAG ACC	189–206
	24	GGG AGA CAC AGA TCT CCA	184–201
	25	GGA ACA CAC AGA TCT CCA	184–201
	05	ACA CAG ATC TTC AAG ACC	189–206
	08	ACA CAG ATC TGC AAG ACC	189–206
	10	GAT CTA CAA GGC CCA GGC	195–212
	11	GAA GTA CAA GCG CCA GGC	195–212
	12	ATC TGC AAG GCC AAG GCA	195–212
C/exon II	13	ACT GAC CGA GAG AGC CTG	216–233
	14	GAC TTA CCG AGA GAA CCT	215–232
	15	GAC TGA CCG AGA GAA CCT	215–232
	16	TTA CCG AGA GGA CCT GCG	218–235
	17	ACT TAC CGA GAG AGC CTG	216–233
	18	ACT GAC CGA GTG AGC CTG	216–233
D/exon II	20	AGC GGA GCG CGG TGC GCA	232–249
	21	CGG AAC CTG CGC GGC TAC	234–251
	22	CGG ACC CTG CTC CGC TAC	234–251
	23	CGG ATC GCG CTC CGC TAC	234–251
E/exon III	E1	CTC ACA CTT GGC AGA CGA	4–21
	E2	CAG AGC ATG TAC GGC TGC	15–32
	E3	CTG CGA CCT GGG GCC CGA	29–46
	E4	CTC ACA CTT GGC AGA GGA	4–21
	E5	CCA GTG GAT GTA TGG CTG	14–31
F/exon III	F1	CTC CGC GGG CAT GAC CAG	57–74
	F2	GGC ATA ACC AGT TAG CCT	64–81

HLA-A PCR primers and DNA amplification

PCR primers (5′-GAC GCC GCG AGC CAG AGG AT-3′ and 5′-TGC AGC GTC TCC TTC CCG TT-3′) and amplification protocols based on those of Oh et al. [17], amplified a 671 bp PCR product, spanning exons II and III.

Genomic DNA (0.5 μg) was amplified in a 50 μl reaction mix [10 mM TRIS-HCl pH 8.3, 50 mM potassium chloride, 0.01 % gelatin, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 0.2 μM of each primer and 1 unit of Taq polymerase]. The reaction mix was overlaid with mineral oil and amplified in a thermal cycler according to the following protocol: 5 min denaturation at 95 °C followed by 30 cycles of 1 min denaturation at 96 °C, 50 s annealing at 60 °C and 1.5 min extension at 72 °C. Successful amplifications were determined by agarose gel electrophoresis.

Sequence-specific oligonucleotide probes (SSOPs)

Initially, 23 SSOPs described by Yoshida et al. [24], designed to detect variations in sequences in four hypervariable regions in exon II of the HLA-B gene, were utilized. To expand the system into a more comprehensive form a further 7 probes (Table 1) were designed and used to detect sequence variations in two hypervariable regions of exon III of the HLA-B gene.

Originally, Oh et al. [17] described 28 SSOPs which were designed to detect sequences in the hypervariable regions of exons II and III of the HLA-A gene. However, in this study a simplified panel of 16 SSOPs (Table 2) was selected to provide a PCR-SSOP typing system with a resolution equivalent to that of serology.

The HLA-A and HLA-B oligoprobes were 15 and 18 bases in length, respectively. This allowed the use of tetramethylammonium chloride (TEMACl)-based buffers in single temperature hybridization and wash steps. All of the probes were also 3′-end labelled with digoxigenin-11-2′,3′-dideoxyuridine 5′-triphosphate (DIG-11-ddUTP; Boehringer Mannheim, Mannheim, Germany) using the enzyme terminal deoxynucleotidyl transferase (TdT; Boehringer Mannheim, Germany) according to the manufacturers instructions.

Dot-blot hybridization and signal detection

Successfully amplified DNA was spotted onto a series of replicate, positively charged, nylon-based membranes (Boehringer Mannheim, Germany) and then soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 5 min followed by a neutralizing solution (0.5 M TRIS-HCl pH 7.2, 1.5 M NaCl, 1 mM EDTA) for 1 min. The DNA was then immobilised by UV irradiation on a transilluminator for 1.5 min.

The membranes were prehybridized for 50 min, at either 52 °C for HLA-B or 46 °C for HLA-A, in 5 ml of hybridization buffer (3 M TEMACl, 50 mM TRIS-HCl pH 8.0, 2 mM EDTA and 0.1 % SDS). Subsequently, 10 pmol of the labelled SSOPs were hybridized with the membranes for 75 min at either 52 °C for HLA-B

Table 2 Oligonucleotide probe sequences for exon II and III of the HLA-A gene

Probe name	Exon II/III	Sequence (5′–3′)	Corresponding amino acid position
56R	II	GAG AGG CCT GAG TAT	55–59
62LQ	II	TGG GAC CTG CAG ACA	60–64
62G	II	GAC GGG GAG ACA CGG	61–65
62RN	II	GAC CGG AAC ACA CGG	61–65
62EG	II	GAG GAG ACA GGG AAA	62–66
73I	II	TCA CAG ATT GAC CGA	71–75
77S	II	GAG AGC CTG CGG ATC	76–80
114EH	III	TAT GAA CAG CAC GCC	113–117
131R	III	CGC TCT TGG ACC GCG	131–135
142TK	III	ACC ACC AAG CAC AAG	142–146
149T	III	TGG GAG ACG GCC CAT	147–151
150V	III	GAG GCG GTC CAT GCG	148–152
151R	III	GCG GCC CGT GTG GCG	149–153
156Q	III	GAG CAG CAG AGA GCC	154–158
161D	III	CTG GAT GGC ACG TGC	160–164
163R	III	GAG GGC CGG TGC GTG	161–165

or 46 °C for HLA-A. The prehybridization and hybridization stages were performed in a hybridization incubator (Hybaid Maxi Hybridization Oven, Hybaid Limited, Middlesex, UK). The membranes were then subjected to the following stringency washes: two 5 min washes in 2 × SSPE pH 7.4 (0.3 M NaCl, 0.02 M $NaH_2PO_4 \cdot H_2O$, 2 mM EDTA) and 0.1 % SDS at room temperature with constant agitation; and two 10 min washes in TEMACl wash (3 M TEMACl, 50 mM TRIS-HCl pH 8.0, 2 mM EDTA, 0.1 % SDS) at either 58 °C (exon II probes) or 62 °C (exon III probes) for HLA-B and at 50 °C for HLA-A.

The hybridized SSOPs were subjected to immunodetection of digoxigenin label using anti-DIG-AP fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim, Germany) and Lumigen-PPD (Boehringer Mannheim, Germany). The resulting chemiluminescent reaction was visualized by autoradiography and the allelic type assigned by probe reactivity (HLA-B Table 3; HLA-A Table 4).

HLA-A ARMS-PCR typing

The ARMS-PCR system (Cellmark Diagnostics, Cheshire, UK) used in this study for confirmation of certain assigned HLA-A types, utilized five separate multiplex PCR reactions. In brief, DNA samples were amplified by ARMS-PCR and the products detected by gel electrophoresis. The HLA-A type was then assigned after interpreting the resulting band patterns. Each reaction detects between 2 and 6 HLA-A locus specificities, with 20 different specificities being able to be detected in total [2, 21].

Results

HLA-B typing

All samples (36 control cell lines and 210 clinical samples) were successfully amplified by one or both of the two PCR reactions employed. All alleles with sequences complementary to the primers amplified. In addition, the 5′-sided primers amplified 15 alleles (0801, 1401/2, 3801, 3901/13/2/3, 5401, 5501/2, 5601/2, 1503, 7901) regardless of either a G/G primer/template mismatch at position 92 or an A/A mismatch at position 94 of exon II. A further allele (1801) also amplified successfully despite a C/A mismatch at position 88 of exon II. Similarly, the 3′-sided primers amplified all alleles including 8 alleles (2702/3/5/5w, 4101, 4701, 0801, 4201) with either a C/C or a G/G mismatch at position 134 of exon III.

The SSOP dot-blot hybridization patterns obtained usually had low backgrounds and a strong signal intensity. Twenty-three of the thirty SSOPs were highly specific and showed a strong hybridization signal intensity. Probe 13, although showing variable hybridization reactions with respect to the amount of non-specific hybridization, was still useful in assigning HLA-B alleles. Six probes (25, 8, 15, 16, 18 and F1) were of little use in assigning alleles because of the high degree of non-specific hybridization that occurred under the conditions used.

The types obtained for all 36 B-lymphoblastoid control cell lines agreed with published serological assignments except for the cell line FLE. This cell line is reported as a B63(15) in the literature [8], however, by PCR-SSOP typing it was found to be B*5701/2.

Of the 210 individuals typed, 17 were confirmed to be homozygotes and the remaining 193 were confirmed to be heterozygotes. Initially, the use of the two 3′ primers in separate reactions partially resolved some of the four ambiguous groups of alleles described by Yoshida et al. [24]. Subsequently, the use of seven extra oligoprobes improved the resolution further, as summarised in Table 5. Two ambiguous heterozygote combinations were also found to be resolved by using the probes based on sequences in exon III of the HLA-B locus (B44, B15 or B45/B50, B13 resolved as B44, B15 and B44, B1503/B4802/B7901 resolved as B44, B1503). Finally, one discrepancy between the molecular and serology types was found. This sample was typed by serology as B35, B70 and by PCR-SSOP as B35, B45/B50.

Table 3 HLA-B oligoprobe reactivities

Allele	0000 1279	0022001111 3445580123	11111 45678	2222 0123	EEEEE 12345	FF 12
D1 amplifications						
4701	+---	-++-------	--+--	--+-	-----	--
4401	+---	-++-------	+----	----	-----	--
4402/3	+---	-++-------	+----	+---	-----	--
4501	+---	-++-------	---+-	-+--	--++-	--
4901	+---	-++-------	+----	---+	--++-	--
5001	+---	-++-------	---+-	-+--	--++-	--
1801	-+--	-+-+------	---+-	-+--	-----	+-
3701	-+--	-++-------	--+--	--+-	-----	--
3501/3	-+--	----+-----	---+-	-+--	--+--	+-
3502/4/6	-+--	----+-----	---+-	-+--	--+--	--
3505	-+--	----+-----	---+-	-+--	-++--	+-
5301	-+--	----+-----	+----	---+	--+--	+-
5101/2/3	-+--	----+-----	+----	---+	+----	--
5104	-+--	----+-----	+----	---+	-----	--
5201/12	-+--	-++-------	+----	---+	+----	--
7801	-+--	----+----+	-----	-+--	+----	--
5801	-+--	+---------	+----	---+	--+--	+-
1301	--+-	-++-------	+----	+---	-----	-+
1302	--+-	-++-------	+----	+---	+----	-+
1501	--+-	-++-------	---+-	-+--	-----	+-
1502	--+-	-+-+------	---+-	-+--	-----	--
1504	--+-	-++-------	---+-	-+--	+----	+-
4601	--+-	-------+--	----+	-+--	-----	+-
5701	--+-	+---------	+----	---+	-----	+-
5702	--+-	+---------	+----	---+	-----	--
2702	---+	-----+--+-	-+---	---+	-----	--
2703/5/54	---+	-----+--+-	-----	--+-	-----	--
1401/2	---+	-----+---+	-----	-+--	----+	--
3801	---+	-----+----	+----	---+	-----	--
3901/13	---+	-----+---+	-----	-+--	-----	--
3902	---+	-++------+	-----	-+--	-----	--
3903	---+	-----+---+	-----	-+--	-+---	--
4802	---+	-++-------	---+-	-+--	--+--	+-
5501/2	---+	------+--+	-----	-+--	+----	-+
5601	---+	------+--+	-----	-+--	+----	-+
5602	---+	------+--+	-----	-+--	-----	-+
1503	---+	-++-------	---+-	-+--	-----	+-
7901	---+	-----+----	---+-	-+--	-----	+-
D1X amplifications						
4001/4	+---	-++-------	---+-	-+--	-----	--
4002/5	+---	-++-------	---+-	-+--	-+---	--
4003	+---	-++-------	---+-	-+--	-+---	+-
4101	+---	-++-------	---+-	-+--	---+-	--
2707	---+	-----+--+-	-----	--+-	-+---	--
0701	---+	------+--+	-----	-+--	-----	+-
0702/3	---+	------+--+	-----	-+--	-+---	+-
0801	---+	----+----+	-----	-+--	-+---	--
4201	---+	------+--+	-----	-+--	-+---	--
4801	---+	-++-------	---+-	-+--	-+---	--

HLA-A typing

All samples (36 control cell lines and 100 clinical samples) were successfully amplified despite an A/C mismatch in exon II at position 127 (0202/5) and 130 (0101, 3601) in the 5′ primer. The PCR products were dotted onto nylon membranes and hybridized with 16 SSOPs derived from hypervariable regions of exons II and III of the HLA-A locus. The hybridization patterns with all 16 SSOPs showed low backgrounds and a strong signal intensity.

All 36 B-lymphoblastoid cell lines were successfully typed and results agreed with those obtained by serology. All 100 clinical samples were also successfully

Table 4 HLA-A oligoprobe reactivities

Allele	56 R	62 LQ	62 G	62 RN	62 EG	73 I	77 S	114 EH	131 R	142 TK	149 T	150 V	151 R	156 Q	161 D	163 R
31011/2	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–
3001/2	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
4301	–	+	–	–	–	–	–	–	+	–	+	–	–	–	–	+
2901/2	–	+	–	–	–	–	–	–	+	–	–	–	+	–	–	–
0201–10	–	–	+	–	–	–	–	–	+	+	–	–	–	–	–	–
0211	–	–	+	–	–	+	–	–	+	+	–	–	–	–	–	–
0212	–	–	+	–	–	–	–	–	+	+	–	–	–	+	–	–
6801/2	–	–	–	+	–	–	–	–	+	+	–	–	–	–	–	–
6901	–	–	–	+	–	–	–	–	+	+	–	–	–	–	–	–
2501	–	–	–	+	–	–	+	–	+	–	+	–	–	–	–	+
2601	–	–	–	+	–	–	–	–	+	–	+	–	–	–	–	+
3401/2	–	–	–	+	–	–	–	–	+	–	+	–	–	–	–	–
6601	–	–	–	+	–	–	–	–	+	–	+	–	–	–	–	+
6602	–	–	–	+	–	–	–	–	+	–	+	–	–	–	–	–
3301	–	–	–	+	–	+	–	–	+	–	–	–	+	–	–	–
2301	–	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–
2401–3	–	–	–	–	+	–	–	–	+	–	–	–	–	+	–	–
3201	–	–	–	–	–	–	+	–	+	–	–	–	+	–	–	–
7401	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–
0101	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	+
0301	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–
0302	–	–	–	–	–	–	–	–	+	–	–	–	–	+	+	–
1101/2	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–	+
3601	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–

Table 5 Summary of resolved ambiguous allele groups

Allele group	Initial resolution using separate 3′ primers		Final resolution using seven extra oligoprobes	Number of cases
	Primer	Resolution		
'B7'	D1	B55,B56	No resolution possible	7
(B7,42,55,56)	D1X	B7,B42	B*07	69
			B*42	0
'B51'	D1	B51,B53	B*51	12
(B51,53)			B*53	0
'B14'	D1	B14,B39	B*14	9
(B14,39)			B*39	4
'B40'	D1	B45,B50	No resolution possible	2
(B40,41,45,50)	D1X	B40,B41	B*4001/4	11
			B*4002/3/5	8
			B*41	0

typed by PCR-SSOP, including 15 samples that could not be unambiguously typed by serology. Of these 15 samples, 5 were subtyped for the A*19 allele (1 × A*30, 3 × A*32, 1 × A*33), 1 was A*10 subtyped (A*26) and 3 had their A*03 status confirmed. In addition, 5 samples were also confirmed to be A*02 homozygotes and 1 was confirmed to be an A*02, A*28 heterozygote.

Further typing was performed on 26 of the 100 samples using ARMS-PCR. There was shown to be complete agreement between serology, PCR-SSOP and ARMS-PCR in 23 of these samples. However, 3 samples were found to be incorrectly typed by serology when comparing the results with PCR-SSOP and ARMS-PCR. These 3 samples were serologically typed as either A2, A2 or A2, A28 (2 cases) or A2, A29 (1 case), but were all confirmed to be A*02 homozygotes by PCR-SSOP and ARMS-PCR.

Discussion

Molecular typing of the HLA system has, until recently, been restricted to the class II region (HLA-DR, -DQ, -DP) because of the complexity and dispersed nature of the polymorphism of the class I loci [7]. In addition, ho-

mologous sequences shared between different class I loci, including pseudogenes, can lead to coamplification of these sequences, making locus-specific PCR or probing difficult. However, it has been previously reported that the HLA-B 5′ primers used in this study are unlikely to coamplify any pseudogenes [24]. The HLA-A primers, though, are known to coamplify three alleles of the HLA-H pseudogene [17], although the products obtained do not interfere with the typing results.

This study has improved upon the resolution reported in an earlier study by Yoshida et al. [24] for HLA-B typing, by using different 3′ primers (D1 and D1X) and extra oligoprobes derived from exon III of the HLA-B locus. In this original study, 22 unambiguous specificities could be identified, along with four unresolvable 'allele groups' (see Table 5). Initially, using the two 3′ primers in separate reaction mixes enabled certain of these 'allele groups' to be split up (Table 5); for example, the 'B7' group was subdivided into two groups, B55, B56 and B7, B42. The resolution was further improved by using seven extra oligoprobes; for example, the subgroup B7, B42 could be resolved into the alleles B*0701, B*0702/3 and B*4201. A parallel study [13] has also reported some improvements upon Yoshida et al.'s original HLA-B PCR-SSOP system. However, this parallel study used various amounts of probe and a range of stringency wash temperatures, resulting in a less manageable system than described in the present study which uses 10 pmol of probe throughout and only two stringency wash temperatures (58 °C and 62 °C). In addition, this latter study also reported a problem in differentiating between the alleles B*4201 and B*6701, and similarly B*1503 and B*4801. However, in the system described here we can distinguish between these alleles by using the 3′ primers in separate PCR reactions, thus improving the resolution of typing.

The number of HLA-B oligoprobes used in this study could also be slimmed down to 24 probes as 6 were found to be of little use in assigning alleles. The resolution of typing achieved by this system is comparable to serology utilizing 24 of the 30 oligoprobes tested, but can also be expanded into a higher resolution typing system. Such a high resolution typing system has been reported by Fernandez-Vina and colleagues [4] using different primers (for group-specific amplification) and many more probes. However, the number of group-specific PCR reactions (nine in total) in association with the number of probes required for typing (in excess of 40) gives rise to a system that is unwieldy for use in routine laboratory work. The system described here is more amenable for use in a clinical tissue typing laboratory and achieves a resolution at least equivalent to that of good serology.

However, there are a number of shortcomings. For example, it has been shown that the 5′ primers used in this study do not amplify HLA-B54 because of a nucleotide mismatch at the 3′-end of the primers. This could be overcome by using primers described by Cereb et al. [3] which are derived from locus-specific sequences in the non-coding introns flanking exons II and III. However, initial data published under the auspices of the 12th International Histocompatibility Workshop indicate that the 3′ HLA-B primers used in two separate PCR reactions in this study achieve a higher level of resolution compared with the intron primers used in a single reaction [9]. A possible solution (although not investigated here) may be achieved by combining the 5′ primer described by Cereb et al. [3] with the 3′ primers (D1 and D1X) in separate reactions, as used in this study.

Finally, in considering the application of the improved HLA-B PCR-SSOP typing system, developed in this study, the B-lymphoblastoid cell line FLE was found to type as a B*5701/2 with the oligoprobes used in this study, but had been previously reported as B63 in the literature [8]. The sequence data published by Hildebrand and colleagues [6] has designated B63 as B*1517 and, on comparison with previously published HLA class I nucleotide sequences, shows that this allele shares sequence motifs with B*5701/2 accounting for the identical oligoprobe reaction pattern and ambiguous typing using the current panel of probes. In addition, the discrepancy found between serology and PCR-SSOP in one sample remains to be resolved, but it is unlikely to be due to a failure of the PCR-SSOP typing system.

Generally, the HLA-A PCR-SSOP typing system described in this study is a simplified version of one described by Oh et al. [17]. Originally 28 SSOPs were used to type the HLA-A locus but this was reduced to 16 SSOPs for the purpose of this study. The results show that this modified version of the typing system can achieve a comparable level of resolution to that achievable by good serology. The use of the ARMS-PCR system on a small series of samples verified the PCR-SSOP results and also showed that the molecular methods could discriminate between alleles that can be difficult to assign by serology.

The application of improved PCR-SSOP methods for HLA-A and HLA-B as applied in this study shows it is possible to routinely type these loci in the laboratory with a resolution at least equal to that of serology. In addition, the PCR-SSOP typing methods can be used to supplement serological typing of 'problem' HLA-A and -B specificities, e.g. subtypes of A19. Both methods are an improvement over previously published systems offering a higher level of resolution without using a high number of probes for the B locus and a streamlined typing system, without significant loss of resolution, for the HLA-A locus.

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