Rapid HLA typing by multiplex amplification refractory mutation system

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Abstract

Aims—To detect HLA susceptibility and protective alleles associated with insulin dependent diabetes mellitus (IDDM) using a multiplex amplification refractory mutation system (ARMS). These include DR3 and DR4 alleles at the DRB1 locus, presence or absence of aspartic acid at position 57 (Asp-57) of the DQB1 locus, and presence or absence of arginine at position 52 (Arg-52) of the DQA1 locus.

Methods—The ARMS approach was used to design allele specific primers for the detection of the major susceptibility and protective alleles for IDDM. These include DR3 and DR4 alleles at the DRB1 locus, Asp-57 and non-Asp-57 at the DQB1 locus, and Arg-52 and non-Arg-52 alleles at the DQA1 locus. The allele specificity of each set of primers was first tested separately using DNA samples from 15 individuals previously typed for the DRB1, DQB1, and DQA1 loci using the sequence specific oligonucleotide (SSO) technique. The possibility of using multiplex ARMS for typing multiple susceptibility/protective alleles for IDDM was further investigated by testing various combinations of allele specific primers, thereby reducing the number of separate polymerase chain reactions required to type all these alleles.

Results—A “three-tube” system worked well and gave accurate results. Tube 1 contained ARMS primers for the detection of IDDM susceptibility alleles DR3 and DR4; tube 2 contained ARMS primers for the detection of susceptibility alleles non-Asp-57 and Arg-52; and tube 3 contained ARMS primers for the detection of the protective alleles Asp-57 and non-Arg-52. DNA samples typed with this ARMS method were in complete agreement with those obtained using the SSO technique.

Conclusion—This method is rapid and has no requirement for radioactivity. It is an efficient method for population screening.

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alleles. Population studies have shown that IDDM is associated with HLA-DR3 and HLA-DR4 alleles, with the risk of developing IDDM particularly increased in DR3 and DR4 heterozygotes.^{1,2} The HLA DQ genes, which are in linkage disequilibrium with HLA DR, are also strongly associated with IDDM. The presence of DNA sequences coding for an amino acid other than aspartic acid at position 57 (non-Asp-57) of the HLA DQ β chain is associated with susceptibility to IDDM, whereas aspartic acid in this position (Asp-57) seems to be related to resistance to the disease.^{3,4} The DQ α chain alleles bearing arginine in position 52 (Arg-52) have also been reported as conferring susceptibility to IDDM.⁵

The typing of HLA DR3 and DR4, the presence or absence of Asp-57 at the DQ β chain, and the presence or absence of Arg-52 at the DQ α chain is typically done by hybridising sequence specific oligonucleotides (SSO) to dot blots of relevant polymerase chain reaction (PCR) products.^{3,5} The complexity of the HLA system and the large number of HLA alleles associated with IDDM necessitate the use of numerous SSO probes. For example, dot blot analysis for the identification of specific Asp-57 and non-Asp-57 alleles at the DQB1 locus requires nine SSO probes.⁶

Wu *et al*⁷ and Newton *et al*⁸ have recently described an allele specific PCR (AS-PCR), or the amplification refractory mutation system (ARMS), which is capable of discriminating a single nucleotide change. The principle of ARMS relies on the fact that *Taq* polymerase, the DNA polymerase commonly used in PCR, lacks a 3' to 5' exonuclease activity. Thus a 3' terminal mismatch of a PCR primer will retard amplification to a significant extent. The extent of the reduction in efficiency of amplification, however, depends on the type of mismatch.^{7,8} In instances where the mismatch is not sufficiently refractory to extension, a further deliberate mismatch near the 3' end will often deliver the specificity required.⁸ We used this approach to design allele specific primers for the detection of major susceptibility and protective alleles—DR3, DR4 alleles at the DRB1 locus, Asp-57, non-Asp-57 alleles at the DQB1 locus, and Arg-52, non-Arg-52 alleles at the DQA1 locus in IDDM. We used a multiplex ARMS system⁹ for the detection of these alleles, thereby reducing the number of separate PCR reactions required to type all the alleles.

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It is well established that susceptibility to insulin dependent diabetes mellitus (IDDM) is associated with particular HLA class II

Methods

DNA samples used in this study were kindly provided by Dr F Cornelis and were previously DRB1, DQB1, and DQA1 typed using the SSO technique.^{10–12}

Oligonucleotide primers were synthesised using an Applied Biosystems 380A DNA synthesizer (Applied Biosystems, Foster City, California, USA). Oligonucleotide primers specific for DR3, DR4, DQ β chain Asp-57, non-Asp-57, and DQ α chain Arg-52, non-Arg-52 alleles were designed using published sequences.¹³ Primer sequences are shown in the table. Deliberate mismatches to increase primer specificity are underlined. The number in parentheses following each primer indicates the codon number of the last base of the primer. The orientation of the primers (sense or anti-sense) is indicated after each primer.

HLA DR3-specific amplification was directed using DR3 specific sense primer PD3 and anti-sense primer PDR3. The DR3 specific PCR product was 250 base pairs. The DR4 specific amplification was directed using DR4 specific sense primer PD4 and anti-sense primer PDR4. The DR4 specific PCR product was 99 base pairs.

The aspartic acid at position 57 of the DQB1 locus was detected by the use of an ARMS anti-sense primer PAsp which is specific for alleles containing aspartic acid codon GAC or GAT at position 57. PAsp was designed such that both Asp-57 alleles containing GAC and GAT at position 57 would be amplified. The sense primer DB130 was previously shown to be specific for the DQB1 and would not amplify the related DQB2 locus.¹⁰ The Asp-57 specific PCR product was 199 base pairs. The non-Asp-57 alleles—that is, alleles that code for amino acids other than aspartic acid at position 57—were detected by using the sense primer DB130¹⁰ and three ARMS anti-sense primers PNAasp1, PNAasp2, and PNAasp3 specific for alleles containing non-aspartic acid codons GCC, GTT, or AGC at position 57. The non-Asp-

57 specific PCR product was 199 base pairs.

The amino acid arginine at position 52 of the DQA1 locus was detected by ARMS sense primers PArg1 and PArg2 which are specific for alleles containing the arginine codons AGA and CGC at position 52 and two anti-sense primers PCArg3 and PCArg4. These primers encompass all sequence variations. The Arg-52 specific PCR product was 138 base pairs. The non-Arg-52 alleles—that is, alleles that code for amino acids other than arginine at position 52 of the DQA1 locus—were detected by using two ARMS sense primers PNAArg1 and PNAArg2, specific for alleles containing non-arginine codons AGC or CAC at position 52 and two anti-sense primers PCArg3 and PCArg4, which encompass all the sequence variations. The non-Arg-52 specific PCR product was 138 base pairs.

Primers AAT-1 and AAT-2,¹⁴ which amplify a 360 base pair region of exon III of the human α -1-antitrypsin gene, were co-amplified in all ARMS reactions as an internal positive control.

PCR was carried out as described before¹⁵ in 100 μ l reaction volumes with appropriate primers (100 pmol each except for multiplex ARMS where the concentrations of Arg-52 and non-Arg specific primers were 30 pmol each and the concentrations of the AAT internal control primers were 20 pmol each). Genomic DNA (1 μ g) and 2.5 units of Ampli-Taq DNA polymerase, 100 μ M each dNTP, 10 mM TRIS-HCl (pH 8.3), 50 mM KCl (Perkin-Elmer Cetus) were used. $MgCl_2$ was kept at 1.5 mM. Thermal cycling was performed using a thermal cycler (Perkin-Elmer Cetus). The cycling profile for all reactions was 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute for 30 cycles. The temperature profile above was optimised by testing each set of primers at annealing temperatures ranging from 55°C to 60°C. Seven to ten microlitres of the PCR products were run on 1.5 % agarose gels. Following electrophoresis, the gel was stained with ethidium bromide and photographed under ultraviolet transillumination.

Results

Allele specificity of PCR primers is conferred by the 3'-terminal nucleotide.^{7,8,16} In some instances additional deliberate mismatches near the 3' end of certain primers were required to provide the necessary specificity. To test the allele specificity of the primers constructed to detect DR3, DR4, Asp-57, non-Asp-57, Arg-52 and non-Arg-52 alleles, each set of primers was tested separately using DNA samples from 15 individuals previously typed for DRB1, DQB1, and DQA1 using the SSO technique.^{10–12} The results were in complete concordance with those obtained by SSO analysis. Figure 1 shows the representative data on four DNA samples illustrating the specificity of the ARMS primers.

Sequence of primers

DR3 specific primers:

PD3: 5' CACGTTTCTTGAGTAC 3'(10) (sense)
PDR3: 5' CGT AGT TGT GTC GCG AGT AGT TGT CCA ACC 3'(74) (anti-sense)

DR4 Specific primers:

PD4: 5' CAG GTT AAA CAT GAG TGT CAT TTC TTA AAC 3'(19) (sense)
PDR4: 5' GCT GTC GAA GCG CAC GTA CTC CTC TTG GTG 3'(33) (anti-sense)

Asp-57 specific primers:

DB130: 5' AGG GAT CCC CGC AGA GGA TTT CGT GTA CC 3'(10) (sense)
PAsp: 5' TTC CTT CTG GCT GTT CCA GTA CTC GGC CT 3'(57) (anti-sense)

non-Asp-57 specific primers:

DB130: as above
PNAasp1: 5' TCC TTC TGG CTG TTC CAG TAC TCG CCG G 3'(57) (anti-sense)
PNAasp2: 5' TCC TTC TGG CTG TTC CAG TAC TCG GAA A 3'(57) (anti-sense)
PNAasp3: 5' TCC TTC TGG CTG TTC CAG TAC TCG GAG C 3'(57) (anti-sense)

Arg-52 specific primers:

PArg1: 5' GAG ACT GTC TGG TGT TTG CCT GTT CTC AGA 3'(52) (sense)
PArg2: 5' GAG ACT GTC TGG CAG TTG CCT CTG TTA CGC 3'(52) (sense)
PCArg3: 5' CTC ATT GGT AGC AGC GGT AGA GTT GTA GCG 3'(79) (anti-sense)
PCArg4: 5' CTC ATT GGT AGC AGC GGT AGA GTT GGA GCG 3'(79) (anti-sense)

non-Arg-52 specific primers:

PNAArg1: 5' GAG ACT GCC TGG CGG TGG CCT GAG TTA AGC 3'(52) (sense)
PNAArg2: 5' GAG ACT GTC TGG AAG TTG CCT CTG TTA CAC 3'(52) (sense)
PCArg3: as above
PCArg4: as above

Internal control primers:

AAT-1: 5' CCC ACC TTC CCC TCT CTC CAG GCA AAT GGG 3'
AAT-2: 5' GGG CCT CAG TCC CAA CAT GGC TAA GAG GTG 3'

Deliberate mismatches to increase primer specificity are underlined.

Figure 1 ARMS analysis of IDDM susceptibility and protective alleles. M is a pBR322 DNA digested with *MspI* (marker). C denotes the position of the AAT (360 base pairs) internal positive control. 250, 99 indicate the sizes in base pairs of the DR3 and DR4 specific PCR products, respectively. 199 indicates the size in base pairs of the Asp-57 and non-Asp-57-specific products and 138 indicates the size in base pairs of the Arg-52 and non-Arg-52-specific products. Numbers 1–4 above each track represent DNA from individuals previously typed by SSO technique. Case 1: DRB1* 0301/0404 (DR3/DR4); DQB1*0201/0302 (non-Asp-57/non-Asp-57); DQA1* 0301/0501 (non-Arg-52/Arg-52). Case 2: DRB1* 1101/1101 (DRX/DRX); DQB1* 0301/0301 (Asp-57/Asp-57); DQA1* 0501/0501 (Arg-52/Arg-52). Case 3: DRB1* 0401/07 (DR4/DRX); DQB1* 0201/0302 (non-Asp-57/non-Asp-57); DQA1* 0201/0301 (non-Arg-52/non-Arg-52). Case 4: DRB1* 0301/1401 (DR3/DRX); DQB1* 0201/0503 (non-Asp-57/Asp-57); DQA1* 0101/0501 (non-Arg-52/Arg-52). DRX denotes any allele other than DR3 or DR4.

We further investigated the possibility of using multiplex ARMS for typing many susceptibility/protection alleles for IDDM in a single PCR tube. The feasibility of multiplex ARMS has recently been shown for the DRB3 system.⁹ By designing allele specific primers, such that different HLA alleles yield PCR products of different length, a PCR using a mixture of different primers to type several different alleles can be performed in a single tube. We tested various combinations of ARMS primers and found that the PCR reactions divided into three sets worked well and gave accurate results. Hence a “three-tube” test strategy was subsequently used. IDDM susceptibility alleles DR3 and DR4

were typed in tube 1 containing the primers PDR3, PD3, PDR4 and PD4.

Susceptibility alleles non-Asp-57 and Arg-52 were typed in tube 2 containing the primers DB130, PNAsp1, PNAsp2, PNAsp3, PArg1, PArg2, PCArg3 and PCArg4. The IDDM protective alleles Asp-57 and non-Arg-52 were typed in tube 3 containing the primers DB130, PSAsp, PNArg1, PNArg2, PCArg3 and PCArg4 (fig 2, table). The same 15 DNA samples as above were typed using this strategy and the results were in complete agreement with those obtained by typing the alleles separately and with those obtained by the SSO technique. Figure 2 shows the representative data on four DNA samples.

Discussion

There is increasing interest in typing individuals and population groups for HLA alleles which are related to susceptibility to IDDM. In this study we have shown that it is possible to type for several major susceptibility and protective HLA alleles for IDDM by ARMS. These include the DR3 and DR4 at the DRB1 locus, Asp-57 and non-Asp-57 at the DQB1 locus, and Arg-52 and non-Arg-52 at the DQA1 locus. To type individuals for all these alleles using the standard SSO technique would be time consuming because of the need for dot blots and the large number of SSO probes each with its own washing condition. In contrast, the ARMS method for the detection of these alleles has the advantage of being rapid and non-radioactive.

The efficiency of the ARMS system may be further enhanced in a multiplex format. Thus we are able to type all common IDDM related HLA alleles using a simple three-tube assay. Compared with conventional multiplex PCR,¹⁷ multiplex ARMS requires more careful primer design and more stringent experimental conditions. Similar to conventional multiplex PCR, the ARMS products should be distinguishable from each other by length and by the fact that the primers should work together at a certain annealing temperature. This latter requirement may need some experimentation to achieve, because allele specificity is also temperature dependent. The primers should not interact to form spurious products. The design of relatively long primers (28–30 bases) with about 50% GC content permits the use of higher annealing temperature, thus minimising spurious product formation. Furthermore, as the primers are designed to bind to adjacent polymorphic regions of an HLA gene, they may form unwanted, but nevertheless, legitimate PCR products in combination. To circumvent this, as in the DR3 and DR4 systems, we used a double ARMS format^{18,19} in which both the 5' and 3' primers are allele specific and product formation by the 5' primer to one allele with the 3' primer to another allele is not possible. Finally, different primers have different amplification efficiencies—for example, the ARMS primers for Arg-52 consistently produce more PCR product than the

Figure 2 Multiplex ARMS analysis of IDDM susceptibility and protective alleles. M is pBR322 DNA digested with *MspI* (marker). C denotes position of the AAT (360 base pairs) internal positive control. Numbers 1–4 above each track represent the same four cases as figure 1. 1–3 under the gels represent the three sets of multiplex PCR necessary for full typing. Tube 1 represents the typing of DR3 and DR4-specific alleles; tube 2 represents the typing of non-Asp-57 and Arg-52-specific alleles; tube 3 represents typing of Asp-57 and non-Arg-52-specific alleles. 250, 99, 199 and 138 denote the same ARMS products described in figure 1.

non-Asp-57 primers. Therefore, in order for these primers to work together in a single tube, the efficiencies of these reactions have to be balanced by adjusting the relative primer ratio. Other potential solutions to this problem include varying the type of extra mismatch or the length of primers so as to match their amplification efficiency. Nevertheless, a well designed multiplex ARMS is a rapid one-stage system.

This new approach should prove very efficient for population screening. The system can also easily be modified to include further susceptibility alleles which may be discovered in the future. Multiplex allele specific PCR should be of value in studying the HLA susceptibility to other disorders and indeed for the rapid genotyping of many other multi-allelic systems.

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