CHAPTER 2

RESERCH METHODOLOGY

MATERIAL

1. Chemicals

Chemicals	M.W.	Source
Ammonium Sulphate ((NH4)2SO4)	132.14	Merck
Boric acid		
Bromophenol Blue	-	Gibco BRL
2'-Deoxynucleoside 5'-triphosphate (dATP, dTTP, dGTP, dCTP)		Pharmacia
Ethidium Bromide	-	Sigma
Ethylenediaminetetraacetic acid, EDTA $(C_{10}H_{14}N_2O_8Na_2.2H_2O)$	372.2	Sigma
Glycerol $(C_3H_8O_5)$	92.09	Sigma
Magnesium chloride ; $MgCl_2$ $(Cl_2Mg.6H_2O)$	203.31	Fluka
Primer set :		
KKU HLA class II Typing Kit composed of primers		
HLA-DRB1	20 pairs	BSU
HLA-DQB1	17 pairs	BSU
Tris Hydroxymethylaminoethane; Trisamine $(NH_2C(CH_2OH)_3)$	121.14	USB
Tween 20 $(C_{58}H_{114}O_{26})$	1,227.54	Sigma

2. Enzymes

Enzymes	Sources
Taq DNA Polymerase	New England Biolabs, USA

3. Instruments and Equipment

Electronic analytical and precision balance, Sartorius, Germany

Gel Document 1000 or Polaroid camera, Fotodyne, USA

Horizontal gel electrophoresis set , Life technologies, USA

Hot air oven, MOV 212 F, Sunyo, Japan

Medical freezer (-30°C), MDF 0535, Sunyo, Japan

Microfuge E, Beckman, USA

PCR machine , Perkins Elmer 4800 and 9600

pH-meter, 520A, Orion, USA

Power supply, EC 135, E-C Apparatus, USA

Sterilizer autoclave, MLS-3000, Sunyo, JapanUV-transilluminator, TVC 312 A, Spectrolines, USA

UV Transiluminator, TVC 312A, Spectrolines, USA

Vortex-mixer, vortex genie 2, Scientific Industries, USA

4. Miscellaneous

Flat Top Microtubes , Treff , Switzerland

Pasteur pipettes

PCR tubes , Treff , Switzerland

Pipettes

Pipette tips

5. Sample subjects

All DNA samples used in this study were kindly provided by Dr. Suvina Ratanachaiyavong, Department of Biomedical Science, Faculty of Medicine, Songklanagarind University. Blood samples were collected from 124 patients with Graves' disease at Songklanagarind Hospital and then from 103 blood donors who served as a control group, who

were also interviewed for background information. DNA samples were extracted by a salting-out method modified from Miller et al., (1988) combined with phenol-chloroform extraction modified from Garcia-Glosas et al. (2001) the samples was precipitated with absolute ethanol, dissolved in TE, measured and stored at -70°C until use.

6. Reagents

6.1 Reagents for DNA extraction

(1)   White Cell Lysis Buffer: WCLB

10 mM Tris- HC1 (pH 8.0)

2 mM Na$_2$EDTA

0.4 M NaC1

(2)   Red Cell Lysis Buffer: Sucrose lysis buffer

0.32 M Sucrose

10 mM Tris-HC1 (pH 7.5)

5 mM MgCl$_2$

1% Triton X-100

autoclaved and stored at 4°C

(3)   Phenol-chloroform-isoamyl alcohol

a.   3 volume of melted phenol was mixed with 1 volume of chloroform-isoamyl alcohol (24:1).

b.   0.6 volume of 1 M Tris-HC1 (pH8.0) was added and mixed well.

c.   Mixture was spanned 10 min at 1500 rpm or let stand until the phenol phase becomes transparent.

d.   The upper aqueous phase was removed.

e.   0.6 volume of 0.1 M Tris-HC1 (pH 8.0) and 8-hydroxy-quinoline was added to a final concentration of 0.1 %.

f.   The mixture was mixed well and spanned 10 min at 1500 rpm or stand enough time to obtain transparency of the phenol phase

h.   The upper aqueous phase was remove.

(4)   10% (w/v) Sodium dodecyl sulfate (SDS)

(5)   10 mg/ml proteinase K dissolved in 1x TBE buffer

(6)   6M NaCl (saturated sodium chloride)

350.64g of sodium chloride was dissolved in 800mls of ddH_2O and then made up to one litre with ddH_2O.

(7)   Cold 100% and 75% ethanol

(6)   TE buffer: 10 mM Tris-HC1 mM EDTA (pH 8.0)

6.2 Reagents for Polymerase Chain Reaction

(1)   TQR buffer   950   μl

        10x PCR buffer (670 mM Tris, 166 mM Ammonium sulfate, 1%

        Tween)   180.0   μl

        dNTP (25mM)   12.8   μl

        25 mM MgCl2   109.2   μl

        Autoclaved distilled water 648.0   μl

(2)   Working DNA   160   μl

(3)   each primer   5   μl

Primers were reconstituted to 100 pmol/μl in ddH2O and store at -70 °C and diluted to 10 pmol/μl to made working primers and could be store at -20 °C.

(4)   *Taq* DNA Polymerase (5 units/μl)   12.7   μl

6.3 Reagents for gel electrophoresis

(1)   loading buffer (30% glycerol in TE , 0.05% bromophenol blue)

(2)   10xTBE

        Tris base   108   g

        Boric Acid   55   g

        0.5 M EDTA pH 8.0   40   ml

Stir and fill with DW to   1000   ml

950ml of distilled water was combined with 50ml of 10xTBE to make 0.5x TBE.

(3)   1% agarose (one litre).

Agarose gel   10   g

0.5x TBE   1   litre

10% ethidium bromide   12.5   μl

10g of agarose gel was dissolved by heating in a microwave in 400ml 0.5xTBE.

The solution was toped up to one litre with 0.5x TBE and 12.5ml of 10% ethidium bromide

solution was added.

METHODS

1. DNA sample preparation.

DNA was extracted according to Ratanachaiyavong S. et al. 1989, using a

method originally described by Kunkel et. al.1977 in combination with the salting out method

(Miller et al., 1988)   and the phenol-chloroform extraction. Detailed experimental procedures are

described.

(1) Cold sucrose lysis buffer was added to the samples in a ratio of approximately

4:1.

(2) The mixture was placed on ice for 15 min and spun at 12,000 rpm, 4°C for 15

min

(3) The pellet was collected and resuspended in 5ml nuclei lysis buffer. 300μl of

10% SDS and 100μl proteinase K were added and the solution was gently mixed.

(4) The homogenates were incubated at 37°C overnight or at 50°C for 2-3 hours.

(5) 2 ml of saturated NaCl were added into each sample. The mixture was shaken

vigorously for at least 30 sec before centrifugation at 2,500 rpm for 15 min.

(6) The supernatant was transferred to a new 20 ml tube. 4ml of phenol:

chloroform: isoamylalcohol (25:24:1) was added and the mixture shaken for 1 min before

centrifugation at 2,000 rpm for 15 min.

(7) The upper aqueous phase was transferred into a new tube. 2 volumes of cold absolute ethanol was added and the solution gently mixed by inversion to precipitate the DNA.

(8) The DNA strands were transferred to a new 1.5 ml Eppendorf tube , resuspended in 500 μl of 1xTE buffer and left to dissolve at $4^{\circ}C$ until completely dissolved.

(9) The tubes were then stored at $4^{\circ}C$ for short term use and $-21^{\circ}C$ for intermediate periods (1-6 mth) and $-70^{\circ}C$ for longer periods.

(10) The concentration and purity of DNA were determined by measuring the absorbance at wavelengths of 260 nm (OD260 for nucleic acids) and 280 nm (OD280 for protein concentrations). The ratio between the readings (OD260/OD280) provided an estimation of sample purity: pure preparations had values close to 1.8 and protein-contaminated samples were significantly lower. OD260 also allowed the calculation of nucleic acid concentrations of the samples. One OD is equivalent to 50μg /ml of double-stranded DNA.

2. **Typing of HLA - DRB1 and –DQB1 genotypes by Polymerase Chain Reaction –
    Sequence Specific Primers (PCR –SSP)**

Polymerase Chain Reaction – Sequence Specific Primers (PCR-SSP) was used on the DNA samples for the amplification based on the principle that a completely matched primer will be more efficiently used in the PCR reaction than one with one or several mismatches**.** An amplified PCR product was detected in ethidiumbromide-stained gel. The positive reaction is present in a correct length. DNA samples from individuals were added to all the different primer pairs, with the DNA further amplified only where the primer pairs were perfectly matched. The primer could be more specific by making them longer or by creating additional mismatches from the 3'-end. The HLA alleles were determined by reading the pattern of reactions. An internal positive control primer specific for human Growth hormone gene ensure that no specific HLA allele was missed from amplification failure. The primers and procedure used in this study were based on the KKU HLA class II Typing Kit, which includes 20 reactions for HLA-DRB1 typing and 17 reactions for HLA-DQB1 typing, as shown in Tables 3 and 4. A control primer specific to the growth hormone gene (439 base pairs) was added to each reaction.

The PCR parameters were adjusted to avoid non-specific amplifications so that all reactions could be run in the same cycler as in the cycling parameters.

(1) 2 min denaturation at 96°C

(2) 5 cycles of 96°C denaturation for 30 sec, 68°C annealing <u>at</u> 60 sec and 72°C extension at 40 sec

(3) 21 cycles of 96°C denaturation at 30 sec, 65°C annealing at 60 sec and 72°C extension at 40 sec

(4)   4 cycles of 96°C denaturation at 30 sec, 55°C annealing at 75 sec and 72°C extension <u>at</u> 120 sec

(5) 10 min of extension at 72°C

The PCR conditions :

Template	50	ng/ul
Control Primers (HGH)	0.1	uM
Specific Primers	2	uM
Taq DNA Polymerase	0.1	unit
dNTP	0.18	mM
pH	8.0	
Mg	1.5	mM
PCR cycle	32	cycles

Table 3 The HLA-DRB1 specific primers of the KKU HLA class II Typing Kit (Leelayuwat

C, et al, 2003)

		base	G+C	A+T	4(G+C)	2(A+T)	Tm
3A21	g C A C g T T T C T T g T g g C A g C T T A A g T T	26	12	14	48	28	76
3A20	C A C C g C g g C C C g C C T C T	17	14	3	56	6	62
3A22	g C C C g C T C g T C T T C C A g g A T	20	13	7	52	14	66
3B4	C A C g T T T C C T g T g g C A g C C T A A g A	24	13	11	52	22	74
3A28	C C g C g C C T g C T C C A g g A T	18	13	5	52	10	62
3A29	g C T C C g T C A C C g C C C g g T	18	14	4	56	8	64
3A25	g g A C g g A g C g g g T g C g g T A	19	14	5	56	10	66
3A24	C g C T g C A C T g T g A A g C T C T C C A	22	13	9	52	18	70
2J26	g g A C A g A T A C T T C C A T A A C C A g g A g g A g A	29	14	15	56	30	86
3A27	A g T T g T g T C T g C A g T A g T T g T C C A C C C	27	14	13	56	26	82
3A26	g C A C g T T T C T T g g A g C A g g T T A A A C	25	12	13	48	26	74
3B1	g C C g C T g C A C T g T g A A g C T C T C	22	14	8	56	16	72
3B30	g g T g C g g T T C C T g g A C A g A T A C T T C T	26	14	12	56	24	80
3B6	g T C C T T C T g g C T g T T C C A g T A C T C C T	26	14	12	56	24	80
1101/58.1	C T T C T g g C T g T T C C A g T A C T C C T	23	12	11	48	22	70
2J27	C C C A C A g C A C g T T T C T T g g A g T A C T C T A C	29	15	14	60	28	88
2J25	g g C T g T T C C A g g A C T C g g C g A	21	14	7	56	14	70
1300/67	g C C C g T C C g T C T T C C A g g A A	20	13	7	52	14	66
2J28	A C A g C A C g T T T C T T g g A g T A C T C T A C g T C	29	14	15	56	30	86
2J29	g g C T g T T C C A g T A C T C g g C g C T	22	14	8	56	16	72
3B2	g g C T g T T C C A g T g C T C C g C A g	21	14	7	56	14	70
5' 05-O	g T T T C T T g g A g T A C T C T A C g T C	22	10	12	40	24	64
5' 08-O	A g T A C T C T A C g g g T g A g T g T T	21	10	11	40	22	62
3' 11-O	T C T g C A A T A g g T g T C C A C C T	20	10	10	40	20	60
4J24	g C g g g T g C g g T T C C T g g A g	19	14	5	56	10	66
3B3	T g T g T C T g C A g T A g g T g T C C A C C g	24	14	10	56	20	76
3C7	C A C g T T T C C T g T g g C A g g g T A A g T A T A	27	13	14	52	28	80

		base	G+C	A+T	4(G+C)	2(A+T)	Tm
3C8	C A g C A C g T T T C T T g g A g T A C T C T A C g g	27	14	13	56	26	82
3C9	g T T g A g T C T g C A g T A g g T g T C C A C C A g	27	15	12	60	24	84
3C10	C A g C A C g T T T C T T g A A g C A g g A T A A g T T	28	12	16	48	32	80
3B29	C A A C C C C g T A g T T g T g T C T g C A C A C	25	14	11	56	22	78
3B5	g C g g T T g C T g g A A A g A C g C g	20	13	7	52	14	66
5A11	T T T C T T g g A g C T g C g T A A g T C	21	10	11	40	22	62
5A12	g T T T C T T g g A g C T g C T T A A g T C	22	10	12	40	24	64
5A13	C T g T T C C A g g A C T C g g C g A	19	12	7	48	14	62
5A14	g C T g T T C C A g T A C T C g g C A T	20	11	9	44	18	62
5' 53	g A g C g A g T g T g g A A C C T g A	19	11	8	44	16	60
3'048	C T g C A C T g T g A A g C T C T C C A	20	11	9	44	18	62

Table 4 The HLA-DQB1 specific primers of the KKU HLA class II Typing Kit(Leelayuwat
C, et al, 2003)

																		base	G+C	A+T	4(G+C)	2(A+T)	Tm
5' 01	C	g	g	A	g	C	g	C	g	T	g	C	g	g	g	g			16	14	2	56	4	60
3' 01	g	C	T	g	T	T	C	C	A	g	T	A	C	T	C	g	g	C	A	A	20	11	9	44	18	62
3' 02	T	g	T	T	C	C	A	g	T	A	C	T	C	g	g	C	g	C	T		19	11	8	44	16	60
5' 02	T	g	C	g	g	g	g	T	g	T	g	A	C	C	A	g	A	C			18	12	6	48	12	60
3' 03	g	C	g	g	C	g	T	C	A	C	C	g	C	C	C	g	A				17	14	3	56	6	62
5' 09	g	A	C	g	g	A	g	C	g	C	g	T	g	C	g	T	T	A			18	12	6	48	12	60
3' 04	C	A	C	C	g	T	g	T	C	C	A	A	C	T	C	C	g	C	T		19	12	7	48	14	62
5' 08	g	A	C	g	g	A	g	C	g	C	g	T	g	C	g	T	C	T			18	13	5	52	10	62
3' 13	A	A	C	T	C	g	C	C	C	g	g	g	T	C	C	C					16	12	4	48	8	56
5' 05	g	g	A	g	C	g	C	g	T	g	C	g	T	C	T	T	g	T	A		19	12	7	48	14	62
3' 05	g	C	T	g	T	T	C	C	A	g	T	A	C	T	C	g	g	C	A	T	20	11	9	44	18	62
5' 07	g	T	g	C	g	T	C	T	T	g	T	g	A	g	C	A	g	A	A	g	20	11	9	44	18	62
3' 07	g	C	A	A	g	g	T	C	g	T	g	C	g	g	A	g	C	T			18	12	6	48	12	60
3' 09	A	g	T	A	C	T	C	g	g	C	g	T	C	A	g	g	C	g			18	12	6	48	12	60
3' 08	C	T	g	T	T	C	C	A	g	T	A	C	T	C	g	g	C	g	g		19	12	7	48	14	62
3' 10	C	T	g	T	T	C	C	A	g	T	A	C	T	C	g	g	C	g	T		19	11	8	44	16	60
5' 11	C	A	C	C	A	A	C	g	g	g	A	C	C	g	A	g	C	g			18	13	5	52	10	62
5' 10	C	A	C	C	A	A	C	g	g	g	A	C	C	g	A	g	C	T			18	12	6	48	12	60
3' 11	g	g	T	A	g	T	T	g	T	g	T	C	T	g	C	A	T	A	C	g	20	10	10	40	20	60

Procedure

       (1) Primer was prepare to set for HLA-DRB1 and HLA-DQB1 typing.
Concentration of control primers and specific primers were 0.26 and 5.2 μM, respectively. 5μl of
each primer mix (37 mixes) were then transferred to a 0.2 ml PCR tube.

(2) Working DNA concentration was adjusted to approximately 50 ng/μl with sterile distilled water.

(3) Working PCR solution was prepared, using

TQR buffer	300	μl
Working DNA	50	μl
Taq Polymerase	5	μl (5 units /μl)

(4) 8 μl of the mixture were mixed and transferred to each tube of primer set, and then placed on ice. The final concentrations of control primers and specific primers were 0.1 and 2 μM, respectively.

(5) PCR test was performed using the cycling parameters as described above. The amplified product was kept at -20 $^{\circ}$C until use.

(Note: The advantage of using this PCR-SSP procedure is that alleles the other chromosome carries are uninfluenced the reactions. Also, the time used to perform the test is short, which makes this method suitable for HLA typing routine work.)

Detection of PCR products

(1) A typical 1.0% gel was made up as described above. The mixture was heated on a high microwave setting for 1-2 min until all solids or viscous agar had melted and the solution had started to boil.

(2) The mixture was left to cool to approximately 55°C.

(3) 12.5 μl of 10% Ethidium Bromide were added after the mixture had cooled, and gently mixed into the agar.

(4) Once the gel was assembled, the gel was poured into trough slowly and allowed to set. (30 to 40 min)

(5) The TBE running buffer was made using 5ml of 10x TBE + 45 ml ddH$_2$O The running buffer was added enough to submerge the gel.

(6) The buffer was poured over the gel. 5 μl of loading buffer was added to the PCR product and transferred to each well.

(7) After loading the samples, the gel was run at 150-200v 60mA (usually 15

min to 20 min) until bromophenol-blue loading buffer dye nearly reached the base of the gel or the second row of wells.

(8) The ethidium bromide stained gel was visualized under UV light and photographed.

Interpretation of the results

After the amplified PCR fragments were separated using gel-electrophoresis, the gel was photographed and the results interpreted. Positive allele-specific amplifications were identified by the presence of a correct-sized PCR allele-specific amplicon, whereas absence of an allele-specific amplicon indicated the absence of the desired alleles in a primer mix. The interpretations of the test were available in case of the presence of a control band (Human Growth Hormone gene) at 439 bps. If all of the reactions have failed then the whole result was repeated. Alleles were assigned by identifying the pattern of positive and negative reactions (fig.4, 5) and interpreting these with reference to the information given in Tables 5, 6, 7.
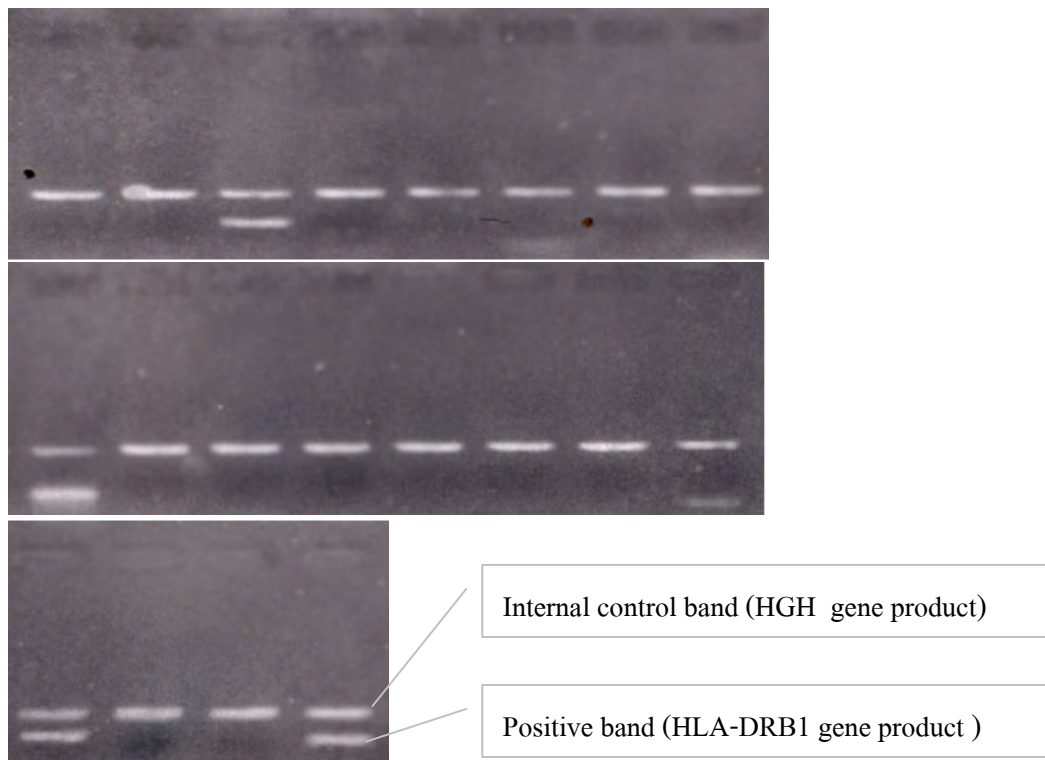
Table 5 Specificities of HLA-DRB1 primer mixes used in the KKU HLA Typing Kit

(Leelayuwat C, et al, 2003)

SPECIFICITY	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DRB1*01(01,02,04)	+																			
DRB1*0103		+																		
DRB1*15(01-03,06)			+																	
DRB1*16(01-08)				+																
DRB1*03(04,11)					+														+	
DRB1*03(01,06,08,10)					+	+													+	
DRB1*03(02,03,05,07,09)						+													+	
DRB1*04(01-22 not 0415)							+													+
DRB1*11(01-31 not								+											+	
DRB1*1122							+	+											+	
DRB1*0415							+	+												+
DRB1*12(01-03,05)									+					+					+	
DRB1*1302										+			+						+	
DRB1*13(01,05,06,09,10,16,18,20,27,28,31)										+									+	
DRB1*13(03,21,30)											+								+	
DRB1*1315										+				+					+	
DRB1*1319													+	+					+	
DRB1*1326										+			+	+					+	
DRB1*13(031,12,13,33)											+		+						+	
DRB1*1332										+	+		+						+	
DRB1*1307/14(03,09,12,16,22,25,29,31)													+						+	
DRB1*14(02,06,13,19,20)													+	+					+	
DRB1*14(01,04,05,07,08,11,14,18,23,26,28)												+	+						+	
DRB1*1410							+						+						+	
DRB1*1424										+			+	+					+	
DRB1*1427										+			+						+	
DRB1*1415													+			+			+	
DRB1*0701															+					+
DRB1*08(01-19, not 0805,0818)																+				
DRB1*0819									+							+				
DRB1*09012																	+			+
DRB1*1001																		+		
DRB3																			+	
DRB4																				+

Table 6 Specificities of HLA-DQB1 primer mixes used in the KKU HLA Typing Kit

(Leelayuwat C, et al, 2003)

SPECIFICITY	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
DQB1*0501	+																
DQB1*0502		+														+	
DQB1*0504		+															
DQB1*0503			+														
DQB1*0601				+													+?
DQB1*06(02,10,11,13)					+												
DQB1*06(04,05,06,09)						+											
DQB1*06(03,14)					+		+										
DQB1*0607							+										
DQB1*06(08,12)					+	+											
DQB1*02(01,02)								+		+							
DQB1*0203								+			+						
DQB1*0304									+								
DQB1*0301									+								+
DQB1*03(02,07)										+					+		
DQB1*0303											+				+		
DQB1*0306											+				+?		
DQB1*0305												+					
DQB1*0401													+				
DQB1*0402														+			

Figure 6 PCR-SSP analysis of HLA-DRB1 alleles. The DNA was amplified using 20 sequence-specific primers for DRB1 (lanes 1 - 18), DRB3 (lane 19) and DRB4 (lane 20)

The allele-specific products are visible as intensive bands below the internal positive control (PCR product of the human growth hormone gene) in lanes 3, 9, 17, 20 (DRB1*15(01-03,06), DRB1*09012, DRB4).

Internal control band (HGH gene product)

Positive band (HLA-DRB1 gene product)
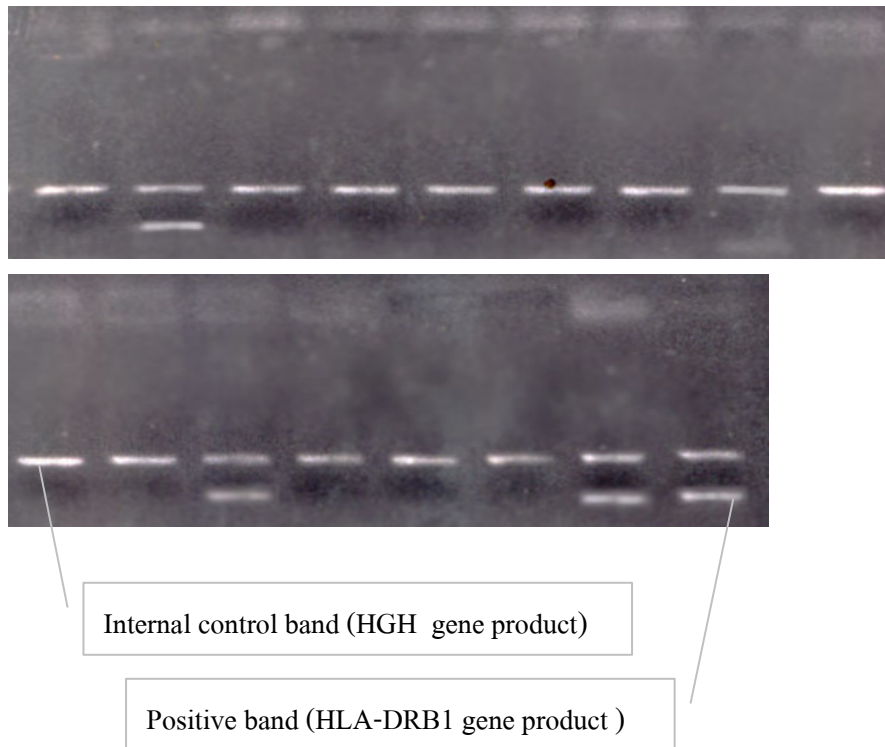
Figure 7 PCR-SSP analysis of HLA-DQB1 alleles. The DNA was amplified using 17 sequence-specific primers for DQB1 (lanes 1‑17). The allele-specific products are visible as intensive bands below the internal positive control (PCR product of the Human Growth Hormone gene in lanes 2, 11, 15, 16 (DQB1*0502, DQB1* 0303/0306).

scoring code:

8 = strong positive

6 = positive

4 = weak positive

2 = band apparent but very weak

1 = negative

0 = no positive band and control band failure

Table 7 HLA PCR-SSP typing worksheet; C= Internal control : 439 bp for Locus DR, DQ, T=

Test

SPECIFICITY DRB1*	bp	MIX	C	T
01(01,02,04)	212	1R		
0103	204	2R		
15(01-03,06)	202	3R		8
16(01-08)	143	4R		
03011,03012,03(04,06,08,10,11)	219	5R		
03(01-10) not 0304	166	6R		
04(01-22)/1410/1422	267	7R		
11(01-31 not 11(09,10,13,16,17,20)/0415	126	8R		
12(01-03,05)/0819	183	9R		8
13(01,02,05,06,09,10,15,16,18,20,26,27,28,31,32)/14(24,27)	136	10R		
13(03,12,13,21,30,32,33)	180	11R		
14(01,04,05,07,08,11,14,18,23,26,28)	224/215	12R		
14(01-31 not 14(17,21,30)/1302,13031,13(07,12,13,19,26,32,33)	147	13R		
12(01-05)?/13(15,19,26)/14(02,06,13,19,20,24)	178	14R		
0701	266	15R		
08(01-19 not 0805,0818)/1415	236	16R		
09012	245	17R		8
1001	208	18R		
DRB3	173	19R		
DRB4	213	20R		8
SPECIFICITY DQB1*	bp	MIX	C	T
0501	128	1Q		
0502/0504	156	2Q		8
0503	87	3Q		
0601	198	4Q		
06(02,03,08,10-14)	165	5Q		
06(04,05,06,08,09,12)	127	6Q		
06(03,07,14)	127	7Q		
02(01-03)	205	8Q		
03(01,04)	122	9Q		
02(01,02)/03(02,07)	129	10Q		
0203/03(03,06)	129	11Q		8
0305	139	12Q		
0401	200	13Q		
0402	200	14Q		
03(02,03,06?,07)	122	15Q		8
0502	117	16Q		
0301/0601?	130	17Q		8

Statistical analysis

The significance of an association between the HLA allele and Graves' disease was calculated using Fisher's exact test (two-tailed). The strength of the association is given as odds ratio, which is similar to relative risk, but more commonly used in a retrospective study such as this. An odds ratio was considered to be significant if the value of the lower 95% confidence interval did not fall below 1.00. An odds ratio was considered to be significant when the p-value <0.05.

1. Allele frequencies

$$AF = \underline{\text{sum of each individual allele}}$$
$$N$$

2. Significance testing

Chi-squared test

A 2x2 table was first constructed, actual numbers of occurrences placed, and the expected frequencies in each cell calculated. The expected frequency in a cell is the product of the relevant row and column totals divided by the sample size (grand total; N = a+b+c+d). For the cell with observed frequency 'a', for example, the expected value is (a+b)(a+c)/N. The difference between observed (O) and expected (E) values (residual) was calculated. The χ^2 was calculated as the sum of (O-E)2/E for all four cells: C2 = $[\sum$ (O-E) ^{2}/E] The general layout of a contingency table for a disease association study is as follows:

A 2x2 table		allele		Row totals
		Present	Absent	
outcome	Patients	a	b	a+b
	Controls	c	d	c+d
Column totals		a+c	b+d	N=a+b+c+d

3. Yates's correction for discontinuity

It may be necessary instructions to use the Yates's continuity correction for small samples. The Yates's correction helps make the discrete data generated by the test statistics $[\sum (O-E)^2/E]$ more closely approximate to the continuous Chi-squared distribution. This is achieved by changing the above formula to $[\sum (|O-E|-0.5)^2/E]$. By doing so, the discrete data distribution and continuous data distribution are approximated better. This will result in a smaller calculated value of χ^2 and reduce the risk of a type I error.

4. Relative risk / Odds ratio calculation

The calculation of RR conferred by an HLA antigen / haplotype / genotype is usually done by Woolf's method which was later modified by Haldane.

$$RR = a(b+d) / b(a+c)$$

Especially for small values of a and b compared to c and d, ad/bc (which is the OR) is a close approximation. If the probability of an event is p, then the odds of that event is p/(1-p).

5. Quality Control

The PCR reagents and conditions were tested for efficiency before routine typing is performed. For instance, the PCR buffer, dNTP's, magnesium chloride and Taq polymerase were tested for optimal concentration. The concentration of primers must be not so high that the allele-specific amplicon is out-competed by the controls and the concentration of control primers should be not too low since the control amplicon will be difficult or impossible to visualize. To establish a good working concentration, titration of the control and specific primers was recommended. For the reason that there is some variability in amplification efficiencies between batches of primer mixes so that every new synthesis of primer mixes must be optimized. In addition, several different DNA samples of different phenotypes should be trial to check that the PCR condition is efficient for most primer mixes. This is commonly caused by an imbalance in the

MgCl:dNTP ratio. The possible primer mixes should be tested with both positive and negative samples to assure for PCR contamination (fig.7), and all primer mixes should be batch tested, aliquots and stored frozen. Besides, all PCR reagents should be tested for contamination and the reagent must be discarded whenever PCR contamination is suspected. DNA preparation and pre-PCR steps should be performed in a different room than post-PCR handling to avoid PCR contamination and the laboratory equipment should be separated.
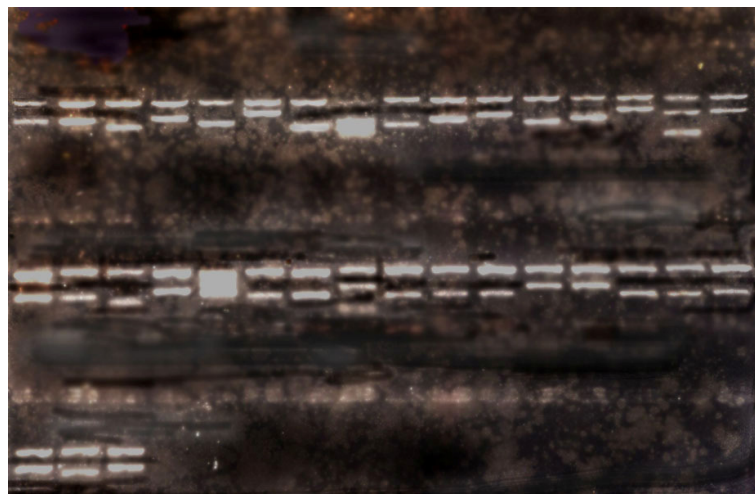

Figure 8 The positive allele DNA was tested with possible primer mixes for DRB1; 1R-20R mixes not 2R (lanes 1 - 19) and DQB1; 1Q-17Q mixes not 12Q (lane 20 - 35). The allele specific products are visible as intensive bands below the internal positive control (PCR product of the human growth hormone gene).