DEVELOPMENT OF PHOTOTYPING FOR HLA CLASS I (HLA-C)

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Molecular methods are now common practice for HLA typing and have replaced traditional serological methods in many histocompatibility laboratories around the world. HLA-Cw typing by standard serological techniques is associated with a high frequency of blanks and rarity of reliable typing reagents for several of the Cw specificities. The objective of this study is to develop a DNA based-typing method to detect HLA-C alleles by using sequence-specific primers (PCR-SSP) or "Phototyping" which is referred to by Bunce et al. (25-26). This thesis, 172 genomic DNA samples were typed and analyzed HLA-C alleles by using 43 SSP reaction mixtures for HLA-C alleles identification. The results of this study showed that the identification of HLA-C allele by this method could be more precise and accurate than those of the serologically defined HLA-C antigen (Cw1-Cw10). SSP was also possible to identify the so-called blank antigen by serology. These results were confirmed with HLA-C typing by PCR-SSOP method and compared with previously known HLA-Cw by serology and defining corresponding allele frequencies of these HLA-C alleles. The relative allele frequencies showed that Cw*0801 were the most frequently detected alleles, followed by Cw*0702.

However, those alleles known to show differences outside exon 2 and 3 need further development. Because HLA-C alleles that show difference outside exon 2 and 3 (that is exons 1, 4 and 5) could not be identified in this study. Phototyping of HLA-C that was developed and performed in this study has the advantages of a being simple and quick method that does not require any special equipment or reagents while producing well defined results and therefore suitable for routine DNA typing and for clinical transplantation. This method could be beneficial for investigation of HLA-Cw matching in allogenic solid organ and bone marrow transplantation.

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ปัจจุบันการตรวจ HLA มีวิธีการทางอณูชีววิทยาหลายวิธีที่สามารถนำมาใช้แทนที่การตรวจโดยวิธีทางซีโรโลยี(ซึ่งเป็นวิธีที่ยอมรับและนิยมกันมานาน) สำหรับการตรวจด้วยวิธีทางซีโรโลยีของ HLA-C พบว่า ผลการตรวจจำนวนมากไม่สามารถบอกชนิดอัลลีนของ HLA-C ที่จำเพาะและถูกต้องได้ ทั้งยังมีข้อจำกัดจากแอนตี้ซีร่าที่มีคุณภาพสำหรับใช้ในการตรวจสอบยังหายากอีกด้วย ดังนั้นวัตถุประสงค์ของการศึกษาครั้งนี้จึงได้ทำการพัฒนาวิธีการทางอณูชีววิทยาวิธีหนึ่งที่เรียกว่า Phototyping ซึ่งพัฒนามาจากวิธีของ Bunce et al. (25-26) โดยใช้ Sequence Specific Primer (SSP) อันประกอบด้วยชุดน้ำยาที่ใช้ในการเพิ่มขยายปริมาณยีนจำนวน 43 ชุด ทำการตรวจในกลุ่มตัวอย่างจำนวน 172 ราย โดยตรวจสอบความถูกต้องร่วมกับผลการตรวจด้วยวิธีการทางอณูชีววิทยาอื่นที่เรียกว่า Polymerase Chain Reaction - Sequence Specific Oligonucleotide Probe (PCR-SSOP) และเปรียบเทียบผลที่ได้กับผลการตรวจด้วยวิธีทางซีโรโลยี รวมทั้งทำการคำนวณหาค่าความถี่ของ HLA-C อัลลีน ในกลุ่มตัวอย่างที่ใช้ในการศึกษา ผลการพัฒนาวิธี Phototyping เพื่อใช้ในตรวจหาชนิดของ HLA-C อัลลีนในการศึกษาครั้งนี้ พบว่าผลการตรวจสามารถบอกชนิดของ HLA-C อัลลีนได้อย่างจำเพาะและถูกต้อง ผลการตรวจหาค่าความถี่ของอัลลีน พบว่า HLA-Cw*0801 มีค่าสูงสุดตามด้วย HLA-Cw*0702

จากการศึกษานี้แสดงให้เห็นว่าการตรวจหาชนิดของ HLA-C อัลลีนโดยวิธี Phototyping ที่พัฒนาขึ้นนี้เป็นวิธีที่มีขั้นตอนการทดสอบที่ง่าย สะดวก รวดเร็ว เครื่องมือและน้ำยาที่ใช้สามารถจัดหาได้ง่าย รวมทั้งให้ผลการตรวจที่จำเพาะและถูกต้อง ด้วยคุณสมบัติดังกล่าวจึงนับได้ว่าวิธีนี้เป็นวิธีที่ดีเหมาะสำหรับนำมาใช้เป็นวิธีตรวจหาชนิดของ HLA-C อัลลีนประจำห้องปฏิบัติการในงานเปลี่ยนอวัยวะ หากแต่ยังมีความจำเป็นต้องทำการพัฒนาต่อไป เพื่อให้สามารถตรวจหาชนิดของ HLA-C อัลลีนที่มีความแตกต่างที่ exon 1,4 และ 5 ทั้งนี้เพราะในการศึกษาครั้งนี้สามารถบอกชนิดของ HLA-C อัลลีนที่แตกต่างกันที่ exon 2 และ 3 เท่านั้น

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LIST OF ABBREVIATIONS

Abbreviation	Term
α	alpha
β2m	beta 2 microglobulin
μl	microlitre
AA	aplastic anemia
AIDS	Acquired Immunodeficiency Syndrome
AIH	autoimmune hepatitis
AlF	allele frequency
ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukemia
ARMS	amplification refractory mutation system
BD	behcet's disease
BMT	bone marrow transplant
bp	base pair
CMV	cytomegalovirus
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
Dig	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate

LIST OF ABBREVIATIONS (Continued)

Abbreviation	Term
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetraacetic acid
GVHD	graft versus host disease
HIV	human immunodeficiency virus-type I
HLA	human leukocyte antigen
IHWS	International Histocompatibility Workshop
Kb	kilobase
LTNPs	long term nonprogressors
M	molar
Mb	million bases
MHC	major histocompatibility complex
min	minute
ml	milliliter
mm	milimolar
NK	natural killer
nm	nanometre
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction restriction - fragment length polymorphism

LIST OF ABBREVIATIONS (Continued)

Abbreviation	Term
PCR-SSO	polymerase chain reaction - sequence specific oligonucleotide
PCR-SSP	polymerase chain reaction – sequence specific primer
Pmol	picomolar
Rpm	round per minute
RSCA	reference strand conformation analysis
RT	room temperature
SBT	sequence based typing
SDS	lauryl sulphate (sodium dodecyl sulfate) sodium salt
SSCP	single strand conformation polymorphism
SSO	sequence specific oligonucleotide
TE	Tris-EDTA
TMAC	tetramethylammonium chloride
UV	ultraviolet
WHO	World Health Organization

CHAPTER I

INTRODUCTION

The cell surface glycoproteins of the human leucocyte antigen (HLA) system are the products of a complex array of genes encoded with the human major histocompatibility complex (MHC). Analysis of the HLA complex have revealed the importance of typing and matching for allogenic solid organ and bone marrow transplantation (1-5) antigens, HLA-A, -B and –C which show a high level of polymorphism with frequent inter- and intralocus homology and only few or even nucleotide positions which determine their allele specificity (6).

HLA-C is the last well characterized of the HLA class I genes and the analysis of its polymorphism may provide importance clue on its biological, clinical and immunoregulatory role. However, putative roles of HLA-C in transplantation, immunobiology and disease association studies have perhaps been obscured by the difficulties in serological typing in comparison with the other classical human MHC class I loci HLA-A and –B. At present, ten HLA-C alleles are known to be identical in exon2 and 3 but show differences outside these exons. These alleles are Cw*0701/6, Cw*12021/2, Cw*15051/2, Cw*1701/02 and Cw*1801/02 cell surface expression of the Cw12-18 antigens which was found to be significantly reduced compared to HLA-A and B (7). HLA-C is known to be a highly polymorphic gene and more HLA-C alleles are still detected today.

Polymorphism of the HLA class I and class II loci have been traditionally detected by complement-dependent microcytotoxicity better known as serological method. Serology is a rapid method of tissue typing, suitable for phenotyping cadaver donors before organ transplantation but it is too often hindered by a lack of discrimination due to antibody cross-reactivity or a lack of reliable typing reagent.

DNA-based HLA class II typing methods such as restriction fragment length polymorphism (RFLP) (8, 9) polymerase chain reaction – sequence specific oligonucleotide (PCR-SSO (10), polymerase chain reaction – sequence specific primer (PCR-SSP) (11-12), nested SSP (13) and PCR-RFLP (14-15) have been used successfully to improve the quality of HLA typing. At present, DNA-based techniques are the methods of choice for HLA class II typing in many laboratories around the world including DNA-Laboratory at Siriraj Hospital.

Recently, DNA typing for HLA class I has advanced with PCR-SSP (16-21) and PCR-SSO methods (22-24). Bunce et al. (25-26) described complete DNA based typing method for HLA class I (A, B, C) by PCR-SSP. Therefore, complete DNA based typing for HLA class I can now be performed for HLA-A, B and C. The report of Bunce et al. marks a new era on which DNA-HLA typing will supercede the conventional HLA typing by serology.

The purpose of this study is to establish a Phototyping for HLA-C alleles, as described by Bunce (25-26), in our laboratory. It is often difficult to establish a new technique in the laboratory as there are many sources of variability between laboratories in establishing a method. We must be prepared to change certain parameters, and perform adequate controls to ensure correct operation as much as possible. At present, of the total 74 Cw alleles that have been identified and assigned, 25 alleles belong to the serologically undetectable Cw blank group (27). This method utilizes the same PCR protocols and parameters for all known alleles and the results of which can be visualized on a Polaroid photograph. The Phototyping for this study used the total of 43 SSP reaction mixtures to identified HLA-C alleles which included 24 SSP reaction mixtures for medium resolution typing and 2 SSP reaction mixtures for group specific of Cw*01, 03, and 07 while, 3 SSP reaction mixtures for Cw*04, *08, *16 and 4 reaction mixtures for Cw*15. By using these SSP reaction mixtures to perform typing on 172 genomic DNA samples to bring about an analysis of HLA-C alleles, the result of this method was then confirmed with HLA-C typing by PCR-SSOP method that were obtained from BSHI protocol in the 12th IHWS publications (28-29). In addition, corresponding allele frequencies of these HLA-C alleles were defined.

CHAPTER II

Literature Review

Human Major Histocompatibility Complex (MHC)

The MHC region composed of more than 200 genes located on the short arm of chromosome 6. Many of these genes have immunological functions in antigen processing and presentation. The MHC is divided into class I, class II and class III regions (Figure 1) (27,30-31).

Human Leukocyte Antigens (HLA)

The classical human MHC or HLA molecules are encoded by two highly polymorphic gene families (HLA class I and HLA class II) located in 3.5-3.8 centri-Morgans corresponding to 3,500 – 3,800 kb of the MHC region. The resulting HLA molecules are the most polymorphic found in human (32). The complete list of recognized serological and cellular HLA specificities is shown in Table 1 (27,30,33).

1. HLA Workshops and Nomenclature Reports

It is well known that the HLA system is one of the most extensive polymorphic gene systems known in mankind. For mutual understanding and good working environment in a highly complex phenomena, a common language is essential.

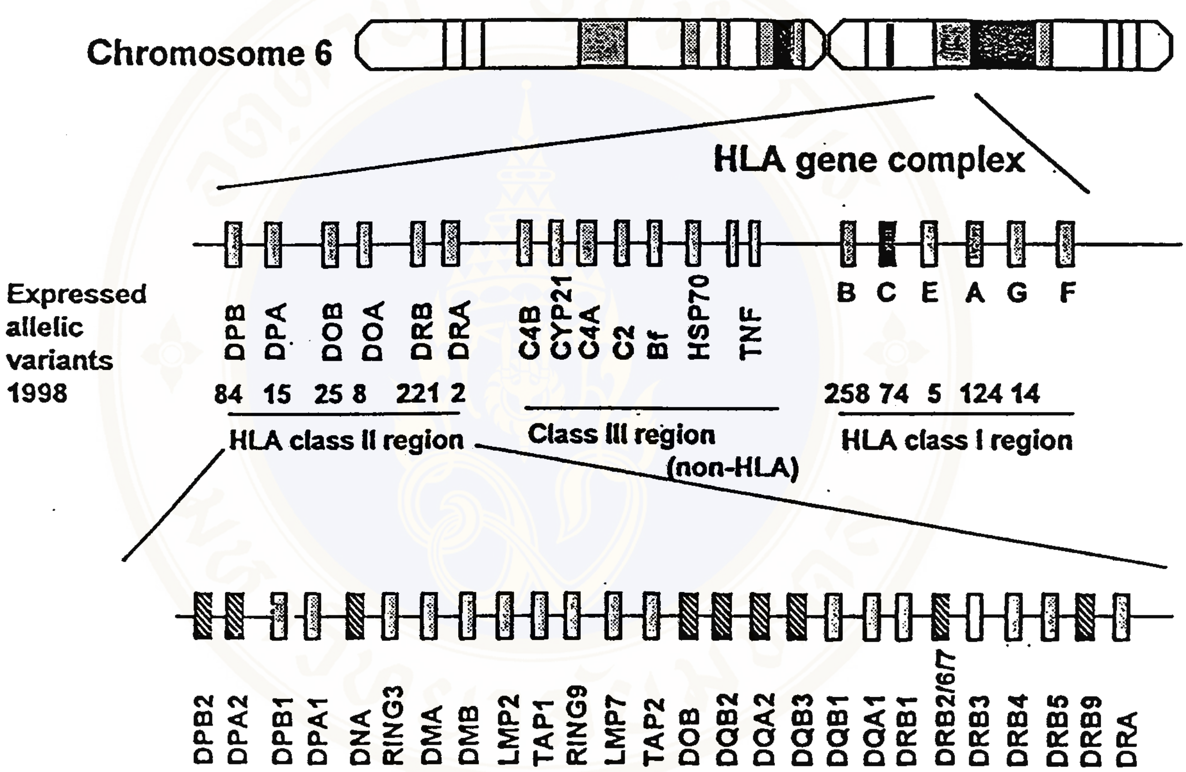


Figure 1. The HLA gene complex on the short arm of chromosome 6 (27,30)

Table 1. Complete listing of recognized serological and cellular HLA specificities from the report of Nomenclature for factor of the HLA System, 1996 and 1998 (27,33).

HLA-Class I				HLA-Class II			
A	**B**		**C**	**D**	**DR**	**DQ**	**DP**
A1	B5	B49(21)	Cw1	Dw1	DR1	DQ1	DPw1
A2	B7	B50(21)	Cw2	Dw2	DR103	DQ2	DPw2
A203	B703	B51(5)	Cw3	Dw3	DR2	DQ3	DPw3
A210	B8	B5102	Cw4	Dw4	DR3	DQ4	DPw4
A3	B12	B5103	Cw5	Dw5	DR4	DQ5(1)	DPw5
A9	B13	B52(5)	Cw6	Dw6	DR5	DQ6(1)	DPw6
A10	B14	B53	Cw7	Dw7	DR6	DQ7(3)	
A11	B15	B54(22)	Cw8	Dw8	DR7	DQ8(3)	
A19	B16	B55(22)	Cw9(w3)	Dw9	DR8	DQ9(3)	
A23(9)	B17	B56(22)	Cw10(w3)	Dw10	DR9		
A24(9)	B18	B57(17)		Dw11(w7)	DR10		
A2403	B21	B58(17)		Dw12	DR11(5)		
A25(10)	B22	B59		Dw13	DR12(5)		
A26(10)	B27	B60(40)		Dw14	DR13(6)		
A28	B2708	B61(40)		Dw15	DR14(6)		
A29(19)	B35	B62(15)		Dw16	DR1403		
A30(19)	B37	B63(15)		Dw17(w7)	DR1404		
A31(19)	B38(16)	B64(14)		Dw18(w6)	DR15(2)		
A34(10)	B39(16)	B65(14)		Dw19(w6)	DR16(2)		
A36	B3901	B67		Dw20	DR17(3)		
A43	B3902	B70		Dw21	DR18(3)		
A66(10)	B40	B71(70)		Dw22			
A68(28)	B4005	B72(70)		Dw23	DR51		
A74(19)	B41	B73					
A80	B42	B75(15)		Dw24	DR52		
	B44(12)	B76(15)		Dw25			
	B45(12)	B77(15)		Dw26	DR53		
	B46	B78					
	B47	B81					
	B48						
		Bw4					
		Bw6					

In this respect, the International Histocompatibility Workshop (IHW) is in its achievement, are absolute key to the orderly development of the HLA system and of its nomenclature.

MAC was one of the earliest antigens, defined by leukoaglutination in 1958 by Dausset (34). However, as a result of the unreliability of the leukoagglutination test, the existing of this specificity could not be clearly shown by the time of the 1st (IHW) in 1964. At the 2nd IHW, which was organized by van Rood in 1965, it was possible for the first time to see the association between specificities described independently by pioneer laboratories. Also at this workshop for the first time, it was note that the MAC antigen originally described by Dausset was also defined independently by five other laboratories. The description of LA-1, -2 and –3 by Payne et al. which was confirmed by the workshop is one of the classic descriptions of the allelism in the HLA region (L stand for leukocyte and A for first locus anticipating the possibility that there would be LB, LC, etc).

Dausset and his colleagues assigned the name HU-1, meaning human system 1. The compromise was reached at a meeting in America in 1968 followed by a report by Amos in a letter to Science (35), that was HL-A was a sort of double recombinant between HU-1 and LA. It took seven years to recognize the naming of the A, B, C and D loci before it could be agreed that the hyphen should be dropped to simplify the nomenclature.

Since they were the basis on which, by common agreement, new specificities and genes could be defined and the genetics of the system clarified, Nomenclature Committee's meetings and reports would follow each of the international workshops. Thus, the second nomenclature report in 1970 defined only four specificities. The third report, in 1972, introduced the concept of provisional definitions the letter "w" before the number – and recognized the problem of broad and narrow specificities reflected in the split of A9 into A23 and A24. The fourth report in 1975 clearly introduced on a well-defined genetic basis system. The HLA region have just been assigned to chromosome 6, HLA was the name of the region or system and follow a hyphen. The letters A, B, C, D etc designated the genes within the system whose alleles were A1, A2, etc. The 7th IHW in 1977, and its associated 5th nomenclature report, had as the major advance the naming of DR for D related and a 4a and 4b become Bw4 and Bw6 respectively (36).

The next major report in 1984 heralded the first major contribution of the molecular biology. The D region genes had been cloned, their two-chain structure identified and a map of the region had been established. The nomenclature DR, DP and DQ were introduced and the suggestion was made that the genes for the separate chains should be called DRA, DRB etc. (37). Thus, in addition to the definition of several new genes, nomenclature to relate the DNA sequence to the predominant serological specificities was introduced, for example; subtypes of B27 become B*2701 and B*2702. The emphasis was on the products that were expressed, but it was also recognized that there could be silent changes whose designation could be B*27011 (38).

At this stage, the asterisk between the gene name and the allele was introduced, since by that time many gene names ended with a number and the asterisk provided an appropriate spacer between the number defining a gene and that defining an allele.

All serological specificities will be named on the basis of correlation with an identified sequence, so there will be no need for a provisional "w" designation. The three exceptions are:

1) Bw4 and Bw6, to distinguish them as epitopes from other B-locus alleles;
2) The C-locus specificities where the "w" is retained to maintain the distinction between the HLA-C locus and the complement components;
3) The Dw and DP specificities, which were defined by mixed lymphocyte reaction (MLR) and primed lymphocyte typing (PLT).

2. Naming of new alleles

There are several required conditions for acceptance of new sequence for official names.

2.1 Where a sequence is obtained from cDNA or where PCR products are subcloned prior to sequencing, several cloned should have been sequenced.

2.2 If direct sequencing of PCR amplified material is performed, products from at least two separated PCR reactions should have sequenced.

2.3 Sequencing should be performed in both directions.

2.4 Where possible a novel sequence should be confirmed by a DNA typing method such as PCR-SSOP or PCR-SSP.

2.5 An accession number in a databank should have been obtained.

2.6 Full-length sequences are preferable though not essential; the minimum requirements are exon2 and 3 for an HLA Class I sequence and exon2 for an HLA Class II sequences.

2.7 Where possible, a paper should have been submitted for publication.

2.8 DNA or other material, in particular cell lines, should be made at least in the originating laboratory. The Nomenclature Committee will maintain documentation on this.

2.9 Submission of a sequence to the Nomenclature Committee should include a computer readable copy of the sequence. Researchers will be expected to complete a questionnaire relating to the sequence and provide a comparison of their new sequence with known related alleles.

Official designations are currently being assigned to new alleles in the period between Nomenclature Committee meetings provided they meet the criteria outlined above (39-41).

HLA-Class I

The class I region (2 million bases (Mb)) and contains a multigene family encoding human leukocyte antigen (HLA) class I molecules and many other non-HLA genes. HLA molecules play an important role in immune response by presenting peptide antigens to T lymphocytes. The genes encoding HLA-class I molecules are known to show an extensive degree of genetic polymorphism, which allows the HLA class I molecules to recognize diverse peptides derived from foreign antigens. At least 124 HLA-A, 258 HLA-B, 74 HLA-C, 5 HLA-E and 14 HLA-G alleles have been officially identified in the Nomenclature for factors of the HLA system, 1998.The full list of HLA Class I alleles as of August 1999 were shown in Table 2 (27, 33,42-46).

HLA-C Locus

HLA-C belongs to the HLA class I genes that are expressed together with β2 microglobulin on the cell surface of nucleated cells. HLA-C locus antigens have been detected after than HLA-A and -B determinants, probably because HLA-C alloantisera are only rarely found. This may be explained by the fact that both the density of these antigens on the cell surface and their immunogenicity are lower than those of other class I antigens. The analysis of their polymorphism may provide important clues on their biological role. There is some evidence of HLA-C antigens involvement in transplantation, NK-cell interactions, binding and present of peptides and disease associations (25, 47).

1. Location

HLA-C was located in the HLA class I region which has been mapped centromeric to HLA-A and telomeric to HLA-B, DR, DQ and DP. Among HLA genes, the HLA-C locus was the closest to HLA-B; therefore, the alleles of these two loci appear to be tightly associated (27-30).

Table 2. Full List of HLA Class I alleles assigned as of August 1999 (27,33)

HLA-A	HLA-A	HLA-B	HLA-B	HLA-B	HLA-C	HLA-E	HLA-F	HLA-G
A*0101	A*3007	B*07021	B*2709	B*4413	Cw*0102	E*0101	F*0101	G*01011
A*0102	A*31012	B*07022	B*2710	B*4414	Cw*0103	E*0102		G*01012
A*0103	A*3102	B*07023	B*2711	B*4415	Cw*0104	E*01031		G*01013
A*0104N	A*3103	B*0703	B*2712	B*4501	Cw*02021	E*01032		G*01014
A*0105N	A*3104	B*0704	B*2713	B*4502	Cw*02022	E*0104		G*01015
A*0106	A*3201	B*0705	B*2714	B*4601	Cw*02023			G*01016
A*02011	A*3202	B*0706	B*2715	B*4701	Cw*02024			G*01017
A*02012	A*3203	B*0707	B*2716	B*4702	Cw*0203			G*01018
A*02013	A*3204	B*0708	B*3501	B*4703	Cw*0302			G*0102
A*02014	A*3301	B*0709	B*3502	B*4801	Cw*003031			G*0103
A*0202	A*3303	B*0710	B*3503	B*4802	Cw*003032			G*01041
A*0203	A*3304	B*0711	B*3504	B*4803	Cw*03041			G*01042
A*0204	A*3305	B*0712	B*3505	B*4804	Cw*03042			G*01043
A*0205	A*3401	B*0713	B*3506	B*4805	Cw*0305			G*0105N
A*0206	A*3402	B*0714	B*3507	B*4806	Cw*0306			
A*0207	A*3601	B*0715	B*3508	B*4807	Cw*0307			
A*0208	A*4301	B*0716	B*35091	B*4901	Cw*0308			
A*0209	A*6601	B*0801	B*35092	B*5001	Cw*0309			
A*0210	A*6602	B*0802	B*3510	B*5002	Cw*0310			
A*0211	A*6603	B*0803	B*3511	B*5004	Cw*0311			
A*0212	A*68011	B*0804	B*3512	B*51011	Cw*04011			
A*0213	A*68012	B*0805	B*3513	B*51012	Cw*04012			
A*0214	A*6802	B*0806	B*3514	B*51021	Cw*0403			
A*0215N	A*68031	B*0807	B*3515	B*51022	Cw*0404			
A*0216	A*68032	B*0808N	B*3516	B*5103	Cw*0405			
A*02171	A*6804	B*0809	B*3517	B*5104	Cw*0406			
A*02172	A*6805	B*0810	B*3518	B*5105	Cw*0407			
A*0218	A*6806	B*1301	B*3519	B*5106	Cw*0501			
A*0219	A*6807	B*1302	B*3520	B*5107	Cw*0502			
A*0220	A*6808	B*1303	B*3521	B*5108	Cw*0602			
A*0221	A*6809	B*1304	B*3522	B*5109	Cw*0603			
A*0222	A*6810	B*1401	B*3523	B*5110	Cw*0604			
A*0224	A*6811N	B*1402	B*3524	B*5111N	Cw*0605			
A*0225	A*6812	B*1403	B*3525	B*5112	Cw*07011			
A*0226	A*6813	B*1404	B*3526	B*5113	Cw*07012			
A*0227	A*6814	B*1405	B*3527	B*5114	Cw*0702			
A*0228	A*6901	B*14061	B*3528	B*5115	Cw*0703			
A*0229	A*7401	B*14062	B*3529	B*5116	Cw*0704			
A*0230	A*7402	B*150110	B*3530	B*5117	Cw*0705			
A*0231	A*7403	B*150110	B*3531	B*5118	Cw*0706			
A*0232N	A*8001	B*15012	B*3532	B*5119	Cw*0707			
A*0233		B*15013	B*3533	B*52011	Cw*0708			
A*0234		B*1502	B*3701	B*52012	Cw*0709			
A*0235		B*1503	B*3702	B*5301	Cw*0710			
A*0236		B*1504	B*3801	B*5302	Cw*0711			
A*03011		B*1505	B*38021	B*5303	Cw*0712			
A*03012		B*1506	B*38022	B*5304	Cw*0713			
A*03013		B*1507	B*3803	B*5401	Cw*0714			
A*0302		B*1508	B*39011	B*5501	Cw*0801			
A*0303N		B*1509	B*39013	B*5502	Cw*0802			
A*0304		B*1510	B*39021	B*5503	Cw*0803			
A*11011		B*1511	B*39022	B*5504	Cw*0804			
A*11012		B*1512	B*3903	B*5505	Cw*0805			
A*1102		B*1513	B*3904	B*5507	Cw*0806			
A*1103		B*1514	B*3905	B*5508	Cw*12021			

Table 2. Full List of HLA Class I alleles assigned as of August 1999 (27,33) (continued)

HLA-A	HLA-A	HLA-B	HLA-B	HLA-B	HLA-C	HLA-E	HLA-F	HLA-G
A*1104		B*1515	B*39061	B*5601	Cw*12022			
A*1105		B*1516	B*39062	B*5602	Cw*1203			
A*2301		B*1517	B*3907	B*5603	Cw*12041			
A*2302		B*1518	B*3908	B*5604	Cw*12042			
A*2303		B*1519	B*3909	B*5605	Cw*1205			
A*2402101		B*1520	B*3910	B*5606	Cw*1206			
A*2402102L		B*1521	B*3911	B*5607	Cw*1301			
A*24022		B*1522	B*3912	B*5701	Cw*14021			
A*24031		B*1523	B*3913	B*5702	Cw*14022			
A*24032		B*1524	B*3914	B*5703	Cw*1403			
A*2404		B*1525	B*3915	B*5704	Cw*1404			
A*2405		B*1526N	B*3916	B*5705	Cw*15021			
A*2406		B*1527	B*3917	B*5706	Cw*15022			
A*2407		B*1528	B*40011	B*5801	Cw*1503			
A*2408		B*1529	B*40012	B*5802	Cw*1504			
A*2409N		B*1530	B*4002	B*5901	Cw*15051			
A*2410		B*1531	B*4003	B*67011	Cw*15052			
A*2411N		B*1532	B*4004	B*67012	Cw*1506			
A*2413		B*1533	B*4005	B*7301	Cw*1507			
A*2414		B*1534	B*4006	B*7801	Cw*1508			
A*2415		B*1535	B*4007	B*78021	Cw*1601			
A*2416		B*1536	B*4008	B*78022	Cw*1602			
A*2417		B*1537	B*4009	B*7803	Cw*16041			
A*2418		B*1538	B*4010	B*7804	Cw*1701			
A*2419		B*1539	B*4011	B*8101	Cw*1702			
A*2420		B*1540	B*4012	B*8201	Cw*1703			
A*2421		B*1542	B*4013		Cw*1801			
A*2422		B*1543	B*4014		Cw*1802			
A*2423		B*1544	B*4015					
A*2424		B*1545	B*4016					
A*2501		B*1546	B*4018					
A*2502		B*1547	B*4019					
A*2503		B*1548	B*4020					
A*2601		B*1549	B*4021					
A*2602		B*1550	B*4022N					
A*2603		B*1551	B*4023					
A*2604		B*1552	B*4024					
A*2605		B*1553	B*4025					
A*2606		B*1801	B*4101					
A*2607		B*1802	B*4102					
A*2608		B*1803	B*4103					
A*2609		B*1804	B*4201					
A*2610		B*1805	B*4202					
A*2611N		B*1806	B*4402					
A*2612		B*1807	B*44031					
A*2613		B*1808	B*44032					
A*2901		B*2701	B*4404					
A*2902		B*2702	B*4405					
A*2903		B*2703	B*4406					
A*2904		B*2704	B*4407					
A*3001		B*27052	B*4408					
A*3002		B*27053	B*4409					
A*3003		B*2706	B*4410					
A*3004		B*2707	B*4411					
A*3006		B*2708	B*4412					

2. Polymorphism

At the present time, 74 HLA-C alleles have obtained an official designation by Bodmer et al: Nomenclature for factors of the HLA system, 1998 (27, 33) (Table.2 and 3).

3. History

The existence of a third HLA class I series, subsequently named HLA-C, was first described in 1970 by Sandberg et al. (47) and has been confirmed by several groups of investigators (48). Whilst the detection of HLA-A and HLA-B antigens by serology is reasonably accurate and reliable, the serological detection of HLA-C has been poor with up to 50% of the population having only one detectable HLA-C antigen (49).

3.1 HLA-Cw1

HLA-Cw1 was first reported by Sandberg et al. (47) at the 4th IHW in 1970, and was formerly called AJ and T1. At the 8th IHW the gene frequency of Cw1 was determined for Caucasians to be 3.92%, for Orientals 15.74% and for Negroes 0.99%. Therefore this antigen was analyzed in detail in Orientals and Caucasians and was well defined by 9th IHW sera in Caucasians, Japanese and Chinese populations. There were strong associations of Cw1 with B27 in Caucasoid; with B54, B55 and B59 in Japanese and with B54, B55 and B40 in Chinese and Thais (50-56).

3.2 HLA-Cw2

HLA- Cw2 antigen, formerly 170, the molecular structure was studied by Sanry et al. (53) in 1977 and was suggested that the HLA-Cw2 antigen is expressed to a lower extent than the HLA-A or -B antigens and that essentially all of the beta2m of

Table 3. Designations of HLA-C Alleles (27)

HLA alleles	HLA specificity	Previous equivalents	Individual or cell line from which the sequence was derived	Accession number
Cw*0102	Cwl	Cwl.2, C1J1	T7527, AP, LCL721, KRC005, TTY, BRUG	M84171, Z46809, D50852, M16272
Cw*0103	Cwl	C1J2	ITOU	D64145
Cw*02021	Cw2	Cw2.2	MVL	M24030
Cw*02022	Cw2	Cw2.2	SWEIG, BDG, BRUG	M26712, D83029, M16273
Cw*02023	Cw2	-	KACD	Z72007
Cw*02024	Cw2	Cw2.4	HEL299, NM155, NM233, NM239, NM303, NM366, NM72, MAN527	U88838, U88839, U97346, U97347, Z96924
Cw*0203	-	-	NM3340	AF037449, AF037450
Cw*0302	Cw10(w3)	-	AP, JG	M84172
Cw*03031	Cw9(w3)	C3J1	GRC150, SJK	M99390, D50853
Cw*03032	Cw9(w3)	-	NM2688, NM3499	AF36554, AF036555
Cw*03041	Cw10(w3)	C3J2	KRC110, JD, SKA, JG	M99389, D64150, U44064, U31372, U31373
Cw*0342	Cw10(w3)	-	NM233, NM303, NM366	U97344, U97345
Cw*0305	-	MA083C, Cw*03MAC	MA083C, NM3214, NM3222, PAM	AF16303, AF009683, AJ005199
Cw*0306	-	-	NM133, NM627, NM2203, NM2415, NM2616	AF003283, AF003284
Cw*0307	Cw3	-	CTM-7980718	AF039198
Cw*0308	-	-	NM1931, TER0171	AF37074, AF037075, Y16411, Y16412
Cw*0310				
Cw*0311				
Cw*0309	-	-	NM4305	AF037076, AF037077
Cw*04011	Cw4	C4J1	C1R, KSE	M84386, X58536, D83030
Cw*04012	Cw4	Cw*04N	RN1238C	AF002271, AF017322
Cw*0402	Cw4	BeWo C.1	BeWo	M26432
Cw*0403 Cw*0404	- -	Cw4NM m126C, Cw*0401new	KW0010 m126C, NM157, NM187	L54059 U88251, AF017323, U96786, U96787
Cw*0405	-	Cw*0401New	NM2602	AF036556, AF036557
Cw*0406	-	TREC1	DM4, MP3	AF062587, AF062588, AF076476
Cw*0407				
Cw*0501	Cw5	Cw5N	OBL, RC, JME, LB129-SCLC	M58630, L24491, D64148, D83742, AJ010748
Cw*0502	Cw5	Cw5New	CTM-5957411	AF047366, AF047367

Table 3. Designations of HLA-C Alleles (27) (continued)

HLA alleles	HLA specificity	Previous equivalents	Individual or cell line from which the sequence was derived	Accession number
Cw*0602	Cw6	Cw6(W), C6J1	MS, G088, DJS, JOE, JD, TTU	M28206, X70857, Z22752-4, M28160, D64147
Cw*0603	-	-	NM779	AF019567, AF019568
Cw*0604	-	Cw6V	MA43, MA95	AB008136
Cw*0605				
Cw*07011	Cw7	-	MF, LCL721	M28207, Z46810, Y16418
Cw*07012				
Cw*0702	Cw7	JY328, Cw7J1, Cw7.5	JY, TID, KOK, WEHO	D38526, Z49112
Cw*0703	-	HLA-4	?	M11886
Cw*0704	Cw7	Cw7/8V	LB33-MEL, KR03/4, SSA, 40C	U09853, X83394, D49552, U38976
Cw*0705	-	39C	39C	U38975
Cw*0706	Cw7	Cw*07GB	GB92	X97321
Cw*0707	-	Cw7v	HAUP	Z79751
Cw*0708	-	RN2157C	RN2157C	AF017330, AF017331
Cw*0709	-	-	NM388	AF015556, AF015557
Cw*0710	-	-	NM1279	AF038573, AF038574
Cw*0711	-	Cw*0704x	LB129-SCLC	AJ010749
Cw*0712	-	Cw*0704N	TER#877, TER#878, TER#857	U60217, U60218
Cw*0713				
Cw*0714				
Cw*0801	Cw8	C8J1	02627, KNM, SFK, HTS	M84174, D64151
Cw*0802	Cw8	-	CGM1, LWAGS, WT51	M59865, M84173
Cw*0803	Cw8	C8J2	KRC103, SSK	Z15144, D50854
Cw*0804	-	-	NM313, NM914, C03	U96784, U96785, AF016304, AF009684, U60321, U60322
Cw*0805	-	Cw*08Var	NEQ2A10/97	Y15842
Cw*0806	-	-	EC22	AF082800, AF082801
Cw*12021	-	Cb-2	MT	M28172
Cw*12022	-	Cw*1202gyp, C12J1	G085, MSU, AKIBA	X70856,D64152, D83741
Cw*1203	-	Cw12New, C12J12	GB002, HNT	D64146
Cw*12041	-	Sy/9-2	M.H(9-2)	X99704
Cw*12042	-	Cw*12JD	NDS-JD, NM2018	Y11843, AF015558, AF015559
Cw*1205	-	Cw*12x16	ANDP	Z80228, Z83247
Cw*1206	-	-	NM1699	AF036552, AF036553
Cw*1301	-	CwBL18	TCC	M58631

Table 3. Designations of HLA-C Alleles (27) (continued)

HLA alleles	HLA specificity	Previous equivalents	Individual or cell line from which the sequence was derived	Accession number
Cw*14021	-	-	LUY, TC106, LKT2	U06487, Z47377, U41386, D49820, M28171
Cw*14022	-	-	NM1991	AF015554, AF015555
Cw*1403	-	Cx44	TID	D31817
Cw*1404	-	-	CTM-1986765	AF104218, AF104219
Cw*15021	-	C*X, Cw*6.2, CI.9, Cw15J1	AUCA#2, G085, G088, KUE, GM637	L20091, X67818, D83031, M24096
Cw*15022				
Cw*1503	-	-	GRC150	M99388
Cw*1504	-	Cw*15Sp	C047	X73518
Cw*15051	-	Cw*15v	LE023	X78343
Cw*15052	-	Cw*1505v	L7901	X87841
Cw*1506	-	Cw*15N	M001C, NM2732, JF	AF002270, AF017324, AF036550, AF036551, Y15745, Y15746
Cw*1507	-	-	PUSPAN	Y17064, Y17065
Cw*1508	-	Cw*15P	Peru-15	AJ010322, AJ010323
Cw*1601	-	CI.10	GM637, TC106	M24097, U41420, U56259, U56260
Cw*1602	-	Cw*16v	C073	X76189
Cw*16041	-	m183C, wt30L	BOJ, m183C, wt30C, NM290, NM633	Z75172, U88252, AF017326, U88253, AF017325, U96788, U96789
Cw*1701	-	Cw16New	RSH, GB86, BM21	U06835, X98742, Y10520
Cw*1702	-	Cw17N	KSU	D64149
Cw*1703				
Cw*1801	-	Cw*04GB, Cwx6	GB92, DUL	X96582, Z80227
Cw*1802	-	Cw*18GB	GB32	Y09156

the B lymphoblastoid cell line BR18 plasma membrane is associated with the HLA-A, B and C alloantigenic polypeptides.

At the 8th IHW, the population analysis gave frequencies of 9.8 for Caucasians, 20.3 for Negroes and a very low frequency (<1.5) for Oriental cells. Cw2 also showed expected linkage disequilibrium with HLA-B27 and to a lesser extent with B44 and B61. A frequent haplotype in Caucasians seems to be HLA-B61-Cw2-DR5. HLA-A2-B51-Cw2 also occurs in appreciable numbers in European Caucasians (54).

3.3 HLA-Cw3, Cw9, Cw10

HLA-Cw3 was first described by Svejgaard et al. (55) at the 5th IHW in 1972 that was initially defined by two sera, UPS from Copenhagen and Sa-50 from Gothenburg. This antigen originally called UPS and later was confirmed as belonging to the third HLA series at the 1975 IHW, where it was called Cw3. The expression of Cw1and Cw3 on the same haplotype in Orientals was observed by Payne et al. (48) and was later found to be strongly associated with B46.

As early as 1974, Mayr suggested that this antigen could be split into two parts, T3.1 and T3.2. This was confirmed at the Second Asia and Oceania Histocompatibility Workshop, where serum 537 from Aizawa showed that Cw3 could be split into Cw3.1 and Cw3.2 and latter was assigned as Cw9 and Cw10 respectively which was given by the WHO nomenclature committee at the 10th IHW.

The strong associations were observed for Cw9 with B55 and B62 and Cw10 with B62, B60 in Caucasians; Cw9 with B35, B55 and Cw10 with B40 in Japanese; and Cw9 with B15 and Cw10 with B58, B40 in Chinese and Thais (50,57).

3.4 HLA-Cw4

First evidence for the existence of this third series antigen was obtained in 1972 by Svejgaard et al. (55) by testing serum Rh315. The existence of Rh315 (T4) and its relationship to the third series could be demonstrated in the following years by serology. In the 1975 IHW T4-Rh315 could be determined by four strong and specific sera (169, 171, 172 and 174) and was designated as Cw4.

Cw4 was associated with B35 and was found in all populations, with the highest phenotype frequency in Negroes and the lowest frequency in Orientals. The strongest three-locus delta value was found for the A23-Cw4-B44 haplotype (58-60).

3.5 HLA-Cw5

Mayr et al. who described the antigen and its close linkage disequilibrium with the two B-locus antigens; B18 and B44 defined T5, subsequently named Cw5. The definition of Cw5 and Cw8 rather difficult because of the cross-reactivity between Cw5 and Cw8. Cw5 and Cw8 were nearly absent in non-Caucasian populations (61-63).

3.6 HLA-Cw6

The first illusion to the presence of a new third-locus antigen, T7, as the tail reactions of the Cw4 antiserum No.325 including B13 and B37 specificity was during the 6th IHW. In the 7th IHW, antisera 413 and 414 were used to assay this specificity and since the latter reacted infrequently with Cw4, the antigen was recognizable and was called Cw6 (60, 64-65). The association of psoriasis with Cw6 appears to be consistent in all populations, which may suggest that the primary association of the disease with DR7 is less likely (64).

3.7 HLA-Cw7

The antigen Cw7 was first defined by Dehay et al. (66) as CVE. They reported that CVE had a gene frequency of 0.163 in Caucasoid. It behaved as an allele of the HLA-C series. It capped independently from A and B antigens. The linkage disequilibrium was found with B7 and B8. Although, Tokunaga et al. reported a new antigen in C series (Cto-1) in the 27th Annual Meeting of Japanese Blood Transfusion Society in June 1979. At that time, the similarity of Cto-1 with CVE was not recognized (67-68). HLA-Cw7 is a common HLA-C antigen in many ethnic groups, with gene frequencies of 2%-39%.

3.8 HLA-Cw8

HLA-Cw8 was described by Mayr in 1978 which was called T8 and which was probably identical with T9 reported by Carvalho in 1979. Several groups during the 8th IHW confirmed the existence of T8. The definition of Cw8 was difficult, because of the cross-reactivity between Cw5 and Cw8 and the strong linkage disequlibrium with B14. The phenotype frequencies of Cw8 in Caucasians, Orientals and Negroes were 6%, 0% and 7% respectively (69-70).

3.9 HLA-Cw11 (the absent HLA-Cw antigen)

Cw11 was defined as a new Cw specificity during the 10th IHW. It was determined on haplotype reacting with both the Cw1 alloantisera and some of the Cw3 alloantisera. This specificity was in high linkage disequilibrium with B46. At the 11th IHW reported that the Cw11 allele does not exist. In fact, the HLA-C allele that is in high linkage disequilibrium with B46 is Cw1. The reactivity observed with some Cw3 alloantisera has to be attributed it a region of sequence identity between B46 and Cw3 in the α1 helix. Thus, sera previously called anti Cw9+Cw10+Cw11 should now be considered as anti-Cw9+Cw10+B46 (56, 71).

3.10 HLA-Cw*12 – Cw*18

Serological studies in the several IHW found that HLA-Cw1 to HLA-Cw10 were more or less adequately detected but the alleles Cw*12 to Cw*18 were largely undetected and showed as HLA-C blank, with the exception of Cw*15 which was subsequently serologically detected and described as "Cw6.2". Recently, molecular techniques have been applied to genotyping HLA-C leading to increase knowledge of HLA-C alleles and greater accuracy of HLA-C detection (49). A number of alleles encoding for these HLA-C "blanks" have recently been identified. HLA-Cw*12, was first identified in 1988 in a Japanese individual (72) and HLA-Cw*15 groups are so far the largest families which are composed of 7 and 8 different alleles respectively (73-74). The HLA-Cw*14 group has been found to posses four alleles (75-76). The following is Cw*16 group which composed three alleles (77) Cw*17 consists of two alleles, similar to the case of the Cw*18 group (78-80). The least group is Cw*13 which consist of a single allele, as determined to date.

4. Structure and Characteristic of HLA-Cw

In 1984, Sodoyer et al. (81) isolated genomic clones of HLA-C by screening a genomic library with an HLA class I gene probe. Mouse L cells transfected with one of the clones expressed a molecule that was serologically characterized as HLA-Cw3, which has been composed of at least 8 exons spanning approximately 3.5 Kb. The predicted 341-amino acid protein has a putative signal sequence and transmembrane domain. This provides, for the first time, data on the structure of HLA-C locus products and constitutes, together with that of the gene coding for HLA-A3, the first complete nucleotide sequences of genes coding for serologically defined class I HLA molecules.

The observation of locus-specific conservation of the class I promoter sequence is intriguing. Although few sequence variations were observed in the 450 bp upstream between alleles within the locus (1 to 4%), considerable interlocus variations were observed (9 to 20%). Sequence identity between HLA-A and -B, was far less than between HLA-B and -C, placing the HLA-B locus evolutionarily closer to HLA-C than to -A. The closer kinship between HLA-B and HLA-C was further supported by the observation that two loci share a large number of locus-specific nucleotides, suggesting that the diversification between HLA-B and -C may have been a relatively recent event (82).

Since the first description of HLA-C antigens in 1970 (47), the allelic polymorphism of the HLA-C locus and the biologic functions of its gene products have been characterized. Like HLA-A and -B the HLA-C locus contains 8 exons. /the polymorphic $\alpha1$, $\alpha2$ and $\alpha3$ domains of the heavy chain are encoded by exon 2, 3 and 4 are expressed together with $\beta2$ microglobulin on the cell surface. Although all three class I molecules have a similar structure, important difference distinguish HLA-C from HLA-A and -B alleles. HLA-C alleles have more similarity with each other than with HLA-A and -B. HLA-C variation is concentrated in positions away from the antigen recognition site, while epitopes forming the peptide-binding groove are conserved. Cell surface expression is lower for HLA-C antigens than for HLA-A and -B antigens. There are differences in efficiency of assembly and rate of exocytosis and stability of the C-locus. The α chain-β2 microglobulin peptide complex may account for the relatively low expression of HLA-C (51,83-85).

HLA-A, -B and -C antigens are polymorphic glycoproteins that function in presentation of foreign and self-antigens to cytotoxic T cells. In contrast to HLA-A and HLA-B, HLA-C antigens are cell surface expressed to a much lower extent (10%) that markedly impeded their serological and molecular characterization (86-87). Investigations of mechanisms that regulate surface expression of HLA-C antigens have suggested that the control of cell surface glycoprotein synthesis may occur at different levels in the cell. Transfer experiments of human class I genes into the class I mutant 721.221 have suggested that HLA-C expression be regulated at two levels; Transcription and post-transcription (88). First, the levels of HLA-C transcripts are lower than those found for HLA-A and HLA-B. Second, HLA-C heavy chains associate inefficiently with β2 microglobulin resulting in slower processing and consequently in lower expression HLA-C at the cell surfaces (89).

Although the cell surface expression of HLA-C locus molecules is lower than that of the HLA-A or HLA-B molecules, HLA-C appears able to elicit a cytotoxic response. It has also been reported that some natural killer (NK) cells can specifically recognize and lyse normal allogenic cells. The role of HLA-C in NK cell-mediated allorecognition was directly investigated by analyzing the effects produced by transfection of several HLA-C alleles on NK sensitivity of class I-deleted mutant cell lines. Transfection of cells with HLA-C alleles encoding Asn-77-lys-80 (including HLA-Cw2, Cw4, Cw5 and Cw6) inhibited the lysis of the targets by NK1-specific NK cells. Whereas HLA-C alleles encoding Ser-77-Asn-80 (including HLA-Cw3, Cw7, Cw8 and Cw13) protected the dominant inhibitory ligands that protect targets from lysis by these allospecific NK cells (90-95).

5. Function and Importance

HLA-C is well characterized of the HLA class I genes and the analysis of its polymorphism may provide important clues on its biological role. The main function of HLA-C that serves as HLA class I antigen is involved in the presentation of foreign antigens to the immune system (91). Consequently, the role of HLA-C in transplantation, immunoregulation and disease susceptibility is largely unknown. However, recently several functions have been elucidated which suggest that HLA-C does have a clinical and immunoregulatory role and has some evidence of HLA-C antigens involvement in transplantation, NK-cell interaction, binding and presentation of viral peptides and disease associations (96-97).

5.1 Transplantation

In transplantation, HLA-C can act as a target for hyperacute rejection. Cytotoxic HLA-C specific lymphocytes that are generated in vitro may be responsible in vivo for graft rejection (97). Grundschober C et al. (99) demonstrated that HLA-Cw disparity influences the incidence of severe graft versus host disease (GVHD) occurring after Bone Marrow Transplantation (BMT) with unrelated donors. It was generated from their experiment which showed that the relative risk (RR: 1.943) of severe GVHD (grad > II) for the patient who have been transplanted with HLA-Cw mismatched donor is twice greater than patients grafted with HLA-Cw identical donor.

5.2 Immunoregulation

For its immunoregulatory role, HLA-C acts as inhibitors for the lytic capacity of natural killer (NK) cells and non-MHC-restricted T-cells. It has been suggested by the finding that two natural killer (NK1 and NK2) cell specificities can be mapped to a diallelic sequence motif in the α1 domain of HLA-C (100-105). NK1 and NK2-specific natural killer cells are selectively inhibited by HLA-C alleles (Cw2, Cw4, Cw5, Cw6) with Asn 77- Lys 80 and by HLA-C alleles Cw1, Cw3, Cw7, Cw8, Cw13) with Ser 77-Asn 80 respectively (90, 106-111).

5.3 Disease susceptibility

Several researcher groups have reported HLA-C disease associations and were showed as following.

5.3.1 Psoriasis is a chronic hyperproliferative inflammatory disease. Several associations between psoriasis and specific human lymphocyte antigens (HLA) have been described. The strongest association is found between psoriasis and HLA-C antigens. The largest and most consistently reported relative risk has related to HLA-Cw6, which also shows a strong association with age of onset and family history (112-119). Especially, the association with alleles HLA-Cw*0602 was stronger in HIV-associated psoriasis (120).

5.3.2 Acquired Immunodeficiency Syndrome (AIDS), maximum HLA heterozygosity of class I loci (A, B and C) delayed AIDS onset among patients infected with human immunodeficiency virus-type 1 (HIV-1), whereas individuals who were homozygous for one or more loci progressed rapidly to AIDS and death. The HLA-class I alleles B35 and CW*04 were consistently associated with rapid development of AIDS-defining conditions (121). Nehete et al. (122) reported that their results support the role of the HLA-C locus in generation CTL responses and constitute the first report of an HLA-Cw7-restricted HIV-1 envelope-specific CTL response in HIV + long-term nonprogressors (LTNPs), which may be important in the control of HIV replication in vivo. Addition, Kuntz et al. (123) and Kaslow et al. (124) demonstrated that HLA-A1, Cw7, B8, DR3 has been postulated to be associated with rapid decline of T-helper cells in AIDS patients and with fast disease progression of AIDS.

5.3.3 Behcet's disease (BD) is a multisystem inflammatory disorder with recurrent manifestations including affecting eyes and mucocutaneous surfaces with occasional involvement of other organs including joints and the central nervous system. Sanz et al. (125) suggested that the Cw*1602-B51 haplotype could be the main HLA marker of this disease in the analyzed population.

5.3.4 Paranoid schizophrenia disease was found that HLA-Cw4 is increased in this disease (126-127).

5.3.5 Spondylarthritic disease, Kozin et al. (128) demonstrated that HLA-Cw1 and Cw2 are spondylitis-associated antigens.

5.3.6 Alcoholism and Alcoholic liver disease, HLA-Cw3 was served as immunogenetic markers of these diseases (129). On the other hand, HLA-Cw*0701 allele was associated and contribute to disease susceptibility with autoimmune hepatitis (AIH) (130).

5.3.7 Aplastic anemia (AA), Acute Myelogenous Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL), Sell et al. (131) had further observed that HLA-Cw1, HLA-DR52 and –DR53 antigens were shared protective markers against the development of these diseases.

5.3.8 Restenosis, the relationship between HLA-C locus and restenosis after coronary artery balloon angioplasty was showed that among HLA-C locus, Cw1 was negatively related to restenosis and may be a useful marker for the prediction of retenosis after percutaneous transluminal coronary angioplasty (PTCA) (132).

5.3.9 Bilateral Iridocyclitis with Retinal Capillaritis (BIRC) was reported by Matsuo et al. (133) and was showed that HLA-DR6 and HLA-Cw7 were associated significantly with the presence of this disease (chi square test, $p<0.0001$). In addition, Takakuwa et al. (134) found that these HLA antigens were also significantly greater with **severe preeclampsia** ($p<0.05$, chi square test)

5.3.10 Herpes Simplex Virus (HSV) infections, HLA-B27 and Cw2 was associated with symptomatic disease. Also HLA-Cw4 was significantly associated with HSV-2 infection. These associations indicate that immunologic factors linked to the MHC influence the risk of HSV-2 infection and disease expression (135).

5.3.11 C2 deficiency, was demonstrated by Clavijo et al.(136) that all patients with C2 deficiency carrying the haplotype (HLA-B18, SO42, DR2) were associated with HLA-Cw*1203. These conserved allelic combinations may become an important tool for the study of human evolution and may contribute to the expeditious selection of prospective donors in clinical transplantation.

5.3.12 Breast Cancer, the study of HLA in this was showed that the HLA-A11, A19 and B8 were found protective whereas, HLA-A2, B14 and Cw6 were a risk for breast cancer (137).

5.3.13 Cytomegalovirus (CMV) is ubiquitous in the world from the report of Machida et al. (138) was showed that incidence of HLA-A33 and HLA-Cw4 were significantly higher in seronegatives than control groups. This report suggested possible involvement of HLA type in CMV negativity.

6. Expression

HLA-C antigens are expressed on the surface of nucleated cells (139). Recent data on HLA-C expression was showed that in addition to HLA-G, the classical class I product HLA-C is also present in trophoblast (140-141). The expression of HLA- class I molecules in the trophoblast cells, forming the materno-fetal junctional zone is in homogeneous. It differs depending on the differentiation and location of trophoblast cells within the placenta and furthermore on the stage of gestation. On the transcriptional level HLA-A, -B, -C, -E and –G could be detected on individual trophoblast populations, whereas only HLA-C and HLA-G seem to be translated to protein. This give rise to the assumption that these two HLA class I molecules play an important role in the maintenance of pregnancy and could serve not only to block recognition by natural killer cells, but also to guide virus-specific HLA-C and possibly HLA-G restricted cytotoxic T-lymphocytes to their targets (142-144).

7. Detection

7.1 Serological Method

Complement-mediated microcytotoxicity (serology) using human alloantisera is the most widely used method of typing of the classical transplantation antigens including HLA-C. Serology for HLA-C is generally quick and reliable. However approximately 20 to 50% of HLA-C alleles are undetected and type as "blank" in most populations (96-97,145-146), due to their weak immunogenicity and the lack of specific reagents, serological typing of HLA-C determinants has never reached the level of sensitivity and specificity achieved with reagents for HLA-A and -B.

HLA-C gene products are the most poorly understood of the HLA class I molecules because they express at low level about 10% on the cell surface compared to HLA-A and –B (145-148). However, recent evidence shows that HLA-C molecules are functionally competent in eliciting T-cell responses and in controlling NK-cell recognition. At the present time, 74 HLA-C alleles have obtained an official designation (27). Among these, only 10 HLA-C antigen specificities can be detected by conventional serological methods.

7.2 Molecular Method

DNA-based typing procedures for HLA class I alleles have been developed and progressed rapidly since 1992 (149). More recently, several molecular techniques for HLA-C allele typing based on polymerase chain reaction (PCR) have proven to be accurate and efficient strategies for the analysis of HLA-C polymorphism. These include PCR-restriction fragment length polymorphism (RFLP) (150), PCR-sequence specific oligonucleotide probing (SSOP) (28-29), PCR-sequence specific primer (SSP) (96,151-156), amplification refractory mutation system (ARMS)-PCR (157-160) and single strand conformation polymorphism (SSCP) (66, 161). In addition, a novel fluorescence based PCR-SSP (162) and reference strand conformation analysis (RSCA) (159,163) molecular approach for HLA-C typing was recently described. Finally, sequencing based typing (SBT) technique was provided to increased resolution to the allelic level of HLA-C (164-170).

One of the interesting DNA-based typing for HLA-C was called "Phototyping" which was described by Bunce M et al. (25-26). They developed a multiple SSP typing system which utilize the same PCR protocols and parameters for class I and class II, the results of which can be visualized on a single Polaroid photograph in order to streamline DNA-based typing for class I and II. This method has an overall resolution equivalent to high-quality serological typing for HLA-C locus and can be completed with in 3 hours. In additional, the exciting news presented in their report concerned the design of an integrated DNA-Based typing system for HLA-C antigens that could readily improve and replace conventional HLA-typing by serology.

CHAPTER III

Materials and Methods

Samples

The samples of genomic DNA used for this study were obtained from 172 random donors at Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand.

DNA Isolation

For routine samples, genomic DNA was isolated from lymphocytes obtained from 5-10 ml of EDTA or ACD anti-coagulated blood. The DNA was prepared by an improved salting-out method (171) as in the following steps.

1. Centrifuge the tube(s) of whole blood at 1800 rpm for 10 minutes.
2. Transfer 250-500 ul of buffy coat or ficoll pellet to a 1.5ml microtube.
3. Add 1.0 ml of Solution A. Vortex and let stand for 2 minutes. Spin at 5000 rpm or 2000 g for 1 minutes. Discard the supernatant. (Repeat this step until all red blood cells (RBCs) are lysed however, do not repeat this step more than 3 times).
4. Vortex the pellet to prevent clumping. Approximate the pellet size and add the appropriate volumes of reagents as listed in the Table 4.

Table 4. List of the pellet size and the appropriate volumes of reagents (171)

Pellet Size	100-50 ul	50-25 ul	25-10 ul
Proteinase K	40 ul	20 ul	12 ul
ddH_2O	800 ul	400 ul	300 ul
10% SDS	300 ul	150 ul	105 ul
7.5M Guan.HCl	300 ul	150 ul	105 ul
Ethanol Precipitation			
Ethanol	4.0 ml	2.0 ml	1.0 ml

5. Add Proteinase K and vortex the sample.
6. Add ddH_2O and vortex the sample again.
7. Add 10% SDS and mix the sample gently by rocking the tube back-and-forth.
8. Add 7.5M Guanidine HCl and again mix the sample gently.
9. Incubate the sample at 68-70°C for 10 minutes
10. After the 10 minutes, mix the sample vigorously using plastic pasture pipettes until the mixture become homogeneous and try to avoid creating bubbles while mixing with pipettes.
11. Incubate the sample at 68-70°C for an addition 5 minutes.
12. After the 5 minutes, spin the sample at 10,000g for 4 minutes.
    12.1 If the pellet is compact and the supernatant is clear and free of debris, continue to the next step.

12.2 If the pellet is diffuse and the supernatant is cloudy, repeat steps 9-12.

13. Transfer the supernatant to the appropriate labeled tube by decanting or pipetting. Slowly add the appropriate volume of Ethanol to maintain the interface between the two phases. Gently rock the tube back-and-forth until cotton-like strands of DNA appear.

14. Vortex the sample to tighten the pellet. Transfer the DNA to another labeled 1.5ml microtube by drawing 800 ul of DNA-Ethanol using a blue pipette-tip.

15. Spin at 10,000g for 2 minutes. Discard the Alcohol supernatant.

16. Add 500 ul of 80% Ethanol to the sample, vortex to loosen the pellet and let the sample stand for 1 minute.

17. Spin the sample at 10,000g for 2 minutes. Discard as much of the supernatant as possible.

18. Add 200 ul of TE buffer or ddH_2O to the sample. Vortex and incubate at 68-70°C for 5 minutes with the cap open to evaporate the Ethanol.

19. Cap the tube and vortex the sample. If the sample is viscous, add 100 ul of ddH_2O and incubate for 2 minutes. Continue this procedure until a smooth, syrup-like consistency is achieved.

20. Adjust DNA concentration to 100 ug/ml. The concentration of DNA should be checked on agarose gel stained with Ethidiumbromine by comparison with known quantity of phage lambda DNA, because the estimation of DNA concentration by OD measurement may be inaccurate due to contamination of RNA during the preparation.

HLA-C Locus PCR-SSOP Typing

BSHI HLA Class I SSOP Kit, the 12th IHW Class I SSOP Typing (28-29).

1. HLA-C Amplification Condition

PCR was performed in 0.2ml sterile microtubes. The final volume of reaction was 100 ul and the final PCR reaction was as follows:

1x PCR Buffer for HLA-C locus, 200uM each of dNTPs, 1.0 mM $MgCl_2$, 0.2 uM 5'Primer (5CIn1-61), 0.2 uM 3'Primer (3BCIn3-12), 2 units Taq DNA Polymerase, 0.5-1.0 ug Genomic DNA Sample and Sterile ddH_2O up to 100 ul

and the sequence of primers was shown in Table 5.

Table 5. HLA-C locus PCR primer for SSOP typing (28-29)

Primer	Position	Sequence (5'-3')
5CIn1-61	Intron 1-22	AGC GAG G(GT)G CCC GCC CGG CGA
3BCIn3-12	Intron 3-12	GGA GAT GGG GAA GGC TCC CCA CT

The reaction tubes were briefly centrifuged to bring down all components to the bottom of the tube. The amplification was carried out in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus Corporation). The cycle parameter were as follows:

Two steps PCR program of	95°C	for 30 sec	30 Cycles
	65°C	for 1 min	
	72°C	for 30 sec	
Final Extension	72°C	for 5 min	

2. Detection of Amplified DNA

To determine if DNA was amplified by the PCR by visualization following gel electrophoresis. DNA is negatively charged due to its phosphate backbone. DNA migrates in an electric field toward the positive pole. The position of migration is determined by the size of the DNA fragment so that small fragments move faster than large fragments. The procedure were as follows:

2.1 Add 3 ul of gel loading buffer (GLB) to each well of an empty 96 well tray and 5 ul of amplified DNA, mix up and down three times and store remaining amplified DNA at 4°C.

2.2 Load the DNA sample to each well of the 1.5% agarose gel in 1xTBE buffer and run at 200 volts for 30 minutes.

2.3 Once electrophoresis is complete, remove the gel from the tank and photograph using UV light.

2.4. The size of amplified products was determined by comparison with a marker lane containing ΦX174 phage DNA digested by Hae III. The PCR product for HLA-C gene in this amplification was 909 bp.

3. Dot Blotting of Amplified DNA

To attach amplified DNA to a nylon membrane and to denature DNA for hybridization. Amplified DNA is treated with NaOH, which causes the double-stranded DNA to denature and become single stranded. The denatured DNA is then spotted on a nylon membrane. UV light is used to crosslink the DNA to the membrane so it dose not wash off during subsequent steps. DNA may be loaded onto the membrane manually using a pipette and a vacuum manifold. The process of dot blotting of amplified DNA were as follows:

3.1 Three ul of amplified DNA was diluted with 50 ul of 0.4 N NaOH, 25 mM EDTA and incubated for 10 minutes at room temperature, the chilled on ice.

3.2 Fifty ul of the diluted sample was load into the wells of the "Dot-Blotter" manifold containing a nylon membrane. The nylon membrane was cut to a desirable size (8 cm x 11 cm for 96 samples), and soaked in sterile distilled water and the in 10x SSPE for 15 minutes each before placing in the manifold.

3.3 Each DNA sample was applied by vacuum pressure onto nylon membranes.

3.4 TE buffer 100 ul was applied to each well to wash the applied DNA samples before removal of membranes.

3.5 Spot a few extra blots for repeats and place membrane on Whatman 3MM paper.

3.6 Place the membrane with the side containing the DNA facing down on the UV transluminator (Foto dyne, 305 nm) and crosslink the DNA to the membrane with approximately 2 minutes. The membrane was kept in sealed plastic bag at –20°C, awaiting hybridization.

4. Labeling of SSOP with Digoxigenin-ddUTP

4.1 The labeling was performed in 1.5ml sterile microtubes. The final volume of reaction was 25 ul and the reaction mix was prepared as follows:

SSO probe (10uM)	2	ul
10x Tailing buffer	2.5	ul
1 mM DIG-11-ddUTP	2.5	ul
1 mM DTT	1	ul
TdT (50 units/ul)	4	ul
ddH_2O upto	25	ul

The reaction mixture was incubated in water bath at 37°C for 2 hours.

4.2 The labeled oligonucleotide probe was precipitated with 2.5 ul of 4 LiCl and 75 ul of pre-chilled Absolute Ethanol mixed and incubated at –70°C for 30 minutes.

4.3 The reaction mixture was removed from –70°C incubation and centrifuged at 13,000g for 15 minutes in the refrigerated microcentrifuge at +4°C

4.4 The Ethanol was removed and the pellet was washed with 50 ul of 70% cold Ethanol, centrifuge at 13,000g for 5 minutes in the refrigerated microcentrifuge at +4°C, then the 70% Ethanol was removed.

4.5 The pellet was dried with a speedy vac and resuspend in 20 ul of sterile distilled water (probe concentration 1 pmole/ul).

4.6 The labeled probe was used immediately for hybridization or stored at

–20°C for long term storage.

The sequences and their specificity of SSO probes were shown in Table 6 and Table 7.

Table 6. HLA-C locus SSO probes (28-29)

Probe		Exon	Nucleotices	Sequence 5' - 3' specific changes underlined	Length
No.	Name				
1	C2EALL	2	151 - 166	GGG TGG AGC AGG AGG G	16
2	C2G1	2	226 - 242	TGA GCC TGC GGA ACC TG	17
3	C2G2	2	223 - 241	AGT GAA CCT GCG GAA ACT	18
4	C3A1	3	13 - 29	TCC AGT GGA TGT GTG GC	17
5	C3E12	3	127 - 142	GGA CCG CTG CGG ACA C	16
6	C3E1203	3	123 - 138	TCC TGG ACT GCC GCG G	16
7	C3A212	3	22 - 38	TGT ACG GCT GCG ACC TG	17
8	C3G2612	3	182 - 198	TGA GGC GGA GCA GTG GA	17.
9	C3H2	3	209 - 224	GGA GGG CGA GTG CGT G	16
10	C3J17	3	230 - 245	GCT CCG CGG ATA CCT G	16
11	C3G17712	3	162 - 177	CGC AAG TTG GAG GCG G	16
12	C3A7023	3	18 - 34	AGG ATG TCT TGG CTG CGA	17
13	C3G716	3	176 - 190	GGC CCG TGC GGC GGA	15
14	C3G16	3	183 - 198	GCG GCG GAG CAG CAG A	16
15	C3G16A	3	188 - 203	GGA GCA GCA GAG AGC C	16
16	C2D6	2	136 - 150	AGC CCC GGG CGC CGT	15
17	C3H3	3	209 - 224	GGA GGG CCT GTG CGT G	16
18	C3K3	3	242 - 259	CCT GAA GAA TGG GAA GGG	18
19	C2H303	2+ Intron2	257 - 270 2	CCA GAG CGA GGC CAG T	16
20	C3CA	3	65 - 1	GTA TGA CCA GTC CGC CT	17
21	C3A4	3	15 - 32	CAG AGG ATG TTT GGC TGC	18
22	C3B4	3	35 - 49	CCT GGG GCC GGA CGG	15
23	C3D58	3	102 - 118	GCC CTG AAT GAG GAC CT	17
24	C3A14	3	12 - 29	CTC CAG TGG ATG TTT GGC	18
25	C3C15	3	64 - 80	GGC ATG ACC AGT TAG CC	17
26	C3G8013	3	177 - 191	GCC CGT ACG GCG GAG	15

Table7. SSOP probe reaction patterns (28-29)

Probe	C2EALL	C2G1	C2G2	C3A1	C3E12	C3E1203	C3A212	C3G2612	C3H2	C3J17	C3G17712	C3A7023	C3G716	C3G16	C3G16A	C2D6	C3H3	C3K3	C2H303	C3CA	C3A4	C3B4	C3D58	C3A14	C3C15	C3G8013
HLA-Cw*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
0102	+	+		+																						
0103	+	+		+																						
1402	+	+																		+				+		
1403	+	+																		+				+		
0302	+	+															+	+		+						
0303	+	+															+	+	+							
0304	+	+															+	+								
0401	+		+																		+	+				
0402	+		+																		+	+				
0403	+		+																		+	+				
02021	+		+				+	+	+	-	-									+						
02022	+		+				+	+	+	-	-									+						
1701	+		+				-	-	+	+	+								+	-						
1702	+		+				-	-	+	+	+								+	-						
0501	+		+																				+			
0801	+	+																					+			+
0802	+	+																					+			
0803	+	+																					+			+
0602	+		+					+								+				+						
1602	+		+																	+						
1601	+	+																		+						
12021	+	+			+	-	+	+												+						
12022	+	+			+	-	+	+												+						
1203	+	+			-	+	-	+												+						
1301	+	+			+	-	+	+								+				+						
1502	+		+																						+	
1503	+		+																						+	
1504	+		+																	+						
15051	+		+																							
15052	+		+																							
0701	+	+									+	-	+	+						+						
0702	+	+									+	+	+	+						+						
0703	+	+									-	+	+	+						+						
0704	+	+									+	-	+	-		+				-						
0705	+	+									+	?+	+							+						
B22																						+				
B16							+																			
B14																						+				

5. **Hybridization**

5.1 The nylon membrane was prehybridized in plastic box, which contained pre-warm hybridization buffer at 51° C (10 ml per 100cm2 of membrane), for 20 minutes at 51°C in shaking water bath.

5.2 The 10 ul (10 pmoles) of DIG-11-ddUTP labeled probe was dissolved in 10-ml hybridization buffer.

5.3 Prehybridization solution was removed, and then the nylon membrane was hybridized in a plastic bag (plastic page protectors) with DIG-11-ddUTP labeled probe solution (10 ml per $100cm^2$ of membrane), and incubated with gentle agitation for 2 hours at 51°C.

6. **Stringency Washing**

Steps 6.1-6.4 were performed in plastic box with constant gentle agitation.

6.1. Discard the hybridization buffer and take out the membranes from the plastic bag and place it in the washing solution (100 ml of 2xSSPE, 0.1%SDS) and washed twice at room temperature for 5 minutes. (Several membranes can be washed together. For 10 membranes, about 500 ml of washing solution is required.)

6.2 The membrane was washed in 100 ml of TMAC solution for 10 minutes at room temperature.

6.3 The membrane was washed in 100 ml of TMAC solution for 30 minutes at 55°C.

6.4 The membrane was washed in 100 ml of 2xSSPE at room temperature for 10 minutes, twice, to remove the TMAC solution.

7. **Chemiluminescent Detection**

All steps were performed at room temperature with shaking using a platform shaker. The volumes of the solution were calculated for a membrane size of $100cm^2$ and adjusted for other membrane sizes.

7.1 The membrane was washed briefly with 50 ml of buffer 1 for 1-5 minutes, the non-specific protein binding sites were blocked by incubating the membrane for 30 minutes in 50 ml of Buffer 2.

7.2 During the blocking period, anti-DIG-alkaline phosphatase was prepared by dilution to 75 mU/ml (1:10,000) in 20 ml of buffer 2.

7.3 After the blocking solution was discarded, the membrane was incubated for 30 minutes in 20 ml of the Antibody solution prepared in step 2.

7.4 Nonspecific bound conjugate was rinsed off by washing twice with 100 ml of washing buffer 1 for 15 minutes per wash, and equilibrated for 5 minutes in buffer 3.

7.5 CSPD (10 mg/ml) was diluted to 0.1 mg/ml (1:100) in 10 ml of buffer 3.

7.6 The membrane was incubated with 10 ml of diluted CSPD solution for 5 minutes. (Diluted CSPD was stored at 4°C in the dark and reused at least 5 times, after use, the solution was filtered with 0.2 um filter.)

7.7 The membrane was drained of solution and blotted for a few seconds on a sheet of dry Whatman 3MM paper but not completely dried.

7.8 The damp membrane was sealed in a plastic bag and kept in the dark at room temperature for overnight in order to reach steady state for the alkaline phosphatase chemiluminescent reaction.

7.9 For detection of the chemiluminescent signal, the membrane was exposed to X-ray film (Kodak XAR) in a film cassette for 2-5 minutes at room temperature.

7.10 The exposed X-ray film was developed with an automatic film-processor and the hybridization signal were observed expose the top film for 2 minutes and check the intensity of the dots. Depending on these results process the second film accordingly.

Note: It may be necessary to re-expose the membrane to a third or fourth film for a future period of time, again depending on dot intensity.

8. Dehybridization of Probe

Removal of labeled probes from a membrane for the purpose of reprobing works for the another hybridization procedure and reasonably well for those membranes with negative results in the first hybridization.

All steps were performed at with shaking.

8.1 The membrane was incubated in plastic box with 100 ml (10 ml per 100 cm^2 of membrane) of 0.4N NaOH for 1 hour at 42°C

8.2 Then transfer to the 2xSSPE for 10 minutes.

8.3 Remove excess fluid on a filter paper

8.4 Subject it to autoradiography to confirm that the probe is removed sufficiently for rehybridization.

8.9 Put in a sealed plastic bag and store at 4°C until use.

9. Interpretation

For every SSOP, reference DNA should be included to serve as positive and negative controls. In general, for each SSOP, the exposure time is adjusted so that the negative controls show no spot or a very weak spot. The intensity of the spots should be represented by grades as follows:

0	=	not tested
1	=	negative (definite)
2	=	negative (probably)
4	=	indefinite
6	=	positive (probably)
8	=	positive (definite)

HLA-C Locus Phototyping (25-26)

The HLA-C typing component of Phototyping has also been tested on 172 DNA samples that was previously typed for HLA-C alleles by PCR-SSOP method (28-29).

1. Primers and Primer Mix (PM) Preparation

The amplification primer were purchased as crude oligonucleotide, the sequences are given in Table 8 and Table 9.

Table 8. Phototyping sense primer (25-26)

No.	Sense Primer	Locus	Exon	Position	Sequence 5' - 3'
1	130	HLA-B&C	3	59 - 76	CCG CGG GTA TGA CCA GTC
2	144	HLA-C	2	199 - 216	ACA AGC GCC AGG CAC AGG
3	159	HLA-C	2	252 - 270	TAC AAC CAG AGC GAG GCC A
4	160	HLA-C	2	253 - 270	ACA ACC AGA GCG AGG CCG
5	165	HLA-C	2	85 - 103	ACG ACA CGC AGT TCG TGC A
6	313	HLA-C	2	179 - 197	GGA CCG GGA GAC ACA GAA C
7	362	HLA-C	2	29 - 45	CGC TGT GTC CCG GCC CA
8	366	HLA-C	2	221 - 239	CCG AGT GAA CCT GCG GAA A
9	367	HLA-C	2	249 - 268	TAC TAC AAC CAG AGC GAG GA
10	368	HLA-B&C	2	210 - 228	CAC AGA CTG ACC GAG TGA G
11	369	HLA-C	2	123 - 140	AGT CCA AGA GGG GAG CCG
12	371	HLA-A&C	2	5 - 25	CCA CTC CAT GAG GTA TTT CTC
13	507	HLA-C	2	198 - 216	TAC AAG CGC CAG GCA CAG A
14	555(MM)	HLA-C	2	128 - 145	AAG AGT GGA GCC GCG GGA
15	556	HLA-C	2	166 - 183	AGT GGG AGG CGG CCC GTA
16	564	HLA-C	2	112 - 128	GCG ACG CCG CGA GTC CA
17	565	HLA-ABC	2	112 - 128	GCG ACG CCG CGA GTC CG
18	PN(0802)	HLA-C	3	123 - 142	TCC TGG ACC GCC GCG GAC AA
19	63	DRB	3	519 - 537	TGC CAA GTG GAG CAC CCA A

Table 9. Phototyping anti-sense primer (25-26)

No.	Anti-Sense Primer	Locus	Exon	Position	Sequence 5' - 3'
1	126	HLA-C	3	134 - 151	TGA GCC GCC GTG TCC GCA
2	127	HLA-B&C	3	18 - 36	GGT CGC AGC CAT ACA TCC A
3	143	HLA-C	3	25 - 42	GCC CCA GGT CGC AGC CAA
4	145	HLA-ABC	3	216 - 233	GAG CCA CTC CAC GCA CTC
5	146	HLA-A&C	3	196 - 214	CCC TCC AGG TAG GCT CTC T
6	147	HLA-B&C	3	65 - 84	TCG TAG GCT AAC TGG TCA TG
7	157	HLA-C	3	131 - 147	CCG CCG TGT CCG CGG CA
8	166	HLA-C	2	229 - 246	GCG CAG GTT CCG CAG GC
9	183	HLA-C	3	25 - 41	CCC CAG GTC GCA GCC AG
10	184	HLA-B&C	3	169 - 185	CGC ACG GGC CGC CTC CA
11	214	HLA-ABC	3	76 - 92	CTT GCC GTC GTA GGC GG
12	215	HLA-B&C	3	77 - 95	ATC CTT GCC GTC GTA GGC T
13	223	HLA-B&C	3	10 - 29	GCC ATA CAT CCT CTG GAT GA
14	238	HLA-ABC	3	216 - 233	GAG CCA CTC CAC GCA CAG
15	315	HLA-C	3	25 - 41	CCC CAG GTC GCA GCC AC
16	317	HLA-C	3	183 - 200	TCT CAG CTG CTC CGC CGT
17	377	HLA-B&C	3	195 - 213	CCT CCA GGT AGG CTC TCC A
18	378	HLA-C	4	234 - 251	CAG CCC CTC GTG CTG CAT
19	379	HLA-C	3	258 - 275	CGC GCG CTG CAG CGT CTT
20	382	HLA-C	3	195 - 213	CCT CCA GGT AGG CTC TCA G
21	388	HLA-C	3	18 - 36	GGT CGC AGC CAA ACA TCC A
22	389	HLA-C	3	246 - 265	AGC GTC TCC TTC CCA TTC TT
23	392	HLA-B&C	3	76 - 93	CCT TGC CGT CGT AGG CGA
24	456	HLA-ABC	3	252 - 270	GCT GCA GCG TCT TCT TCC T
25	566	HLA-ABC	3	252 - 269	CTG CAG CGT CTT CTT CCC
26	PN0402	HLA-C	3	192 - 208	AGG TAG GCT CTC CGC TC
27	64	DRB	4	579 - 598	GCA TCT TGC TCT GTG CAG AT

1.1 Testing Control primers and allele-specific primers

All primer combinations were initially tested at final concentration that were recommended by Bunce et al. (25-26); if the primer mix was weak or gave false positive amplifications the concentration of one or both primers was tested at different titration with positive and negative samples to ensure maximum efficiency and arrive at the optimal working concentrations of primer for each individual primer.

1.1.1 In this study, the concentration of control primers were titrated at titration: 0.2, 0.15, 0.1, and 0.05 uM.

1.1.2 All allele-specific primer combinations I each mixture was diluted from the original concentration at dilution: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256.

1.2 Preparation of primer mixes solution

In 5 ul primer mixes containing allele and control primers in distilled water were store at –70°C under 10 ul of mineral oil either in 200ul microtubes or 200ul strip-tubes.

2. Amplification Conditions

2.1 The final volume of all PCR reaction is 13 ul not including a 10 ul mineral oil overlay. PCR reaction mixtures consisted of as follows;

8 ul of 1xPCR buffer for Phototyping

200 uM of each dNTP

0.1 uM of DRB1 control primers (primer 63 & 64)

0.01-0.1 ug DNA sample

0.2 unit Taq DNA Polymerase

5 ul of Primer mix (already prepared)

2.2 PCR amplifications were carried out in a GeneAmp PCR system 9600 (Perkin-Elmer Corporation). The cycling parameter for 13 ul reaction in rapid-cycling PCR machine were as follows:

1 minute at 96°C followed by

5 cycles of 25 seconds at 96°C, 45 seconds at 70°C, 45 seconds at 72°C

followed by 21 cycles of 25 seconds at 96°C, 50 seconds at 65°C, 45 seconds at 72°C

followed by 4 cycles of 25 seconds at 96°C, 60 seconds at 55°C, 120 seconds at 72°C.

3. Gel Electrophoresis

3.1 PCR products were electrophoresis in 2% agarose gels containing 0.5 ug/ml ethidiumbromide after the addition of 5 ul of gel loading buffer.

3.2 The gels were run for 25 minutes (or until the dye had traveled 3 cm) at 15 v/cm in 1xTBE buffer and visualized using UV illumination and compared with the size marker ΦX174 phage DNA digested by Hae III.

3.3 Photographs of the gels were recorded.

4. Interpretation

The allele assignment for this typing was depended on the positive PCR products that was specific presented in each primer mix amplification. In this study, used a 24 primer mixes set for the medium-resolution HLA-C typing. Whereas, the subtyping for group specific of HLA-Cw*01,*03 and *07 was used 2 primer mixes set; 3 primer mixes set for HLA-Cw*04, *08, *16; and 4 primer mixes set for HLA-Cw*15.

Table 10. The working concentration of primer mix (PM) (25-26)

PM	Prev.PM in Ref.	Sense Primer		Anti-sense Primer	
			Conc. (uM)		Conc. (uM)
C1	86	368	1.7	315	0.7
C2	87	366	1.7	145	1.7
C3	88	368	3.4	389	3.4
C4	161	130	3.4	389	3.4
C5	201	160	3.4	389	3.4
C6	89	366	1.7	143	1.7
C7	242	564	1.0	143	1.0
C8	90	366	3.4	379	3.4
C9	91	367	3.4	127	3.4
C10	92	130	3.4	378	3.4
C11	93	313	1.7	184	1.7
C12	55N	565	0.7	183	1.7
C13	96	367	1.7	379	3.4
C14	182	165	1.7	166	1.7
C15	98	165	1.7	317	1.7
C16	276N	144	3.0	126	3.4
C17	102	369	3.4	126	3.4
C18	277N	144	3.0	157	3.4
C19	104	371	3.4	388	3.4
C20	243	565	0.7	143	1.0
C21	105	366	3.4	147	3.4
C22	107	366	3.4	382	3.4
C23	241	564	1.7	214	1.7
C24	111	366	1.7	377	1.7
C25		160	3.4	315	0.7
C26		368	1.7	392	1.0
C27	99	159	3.4	389	3.4
C28		368	3.4	223	3.4
C29	236	555MM	1.0	143	1.7
C30		367	1.7	PN0402	1.7
C31	251	362	1.7	143	1.7
C32	95	367	1.7	238	3.4
C33		144	3.0	184	1.7
C34	237	556	1.7	566	1.7
C35		PN0802	3.4	379	3.4
C36	238	556	1.7	456	1.7
C37	245	507	2.0	215	2.0
C38	246	144	3.0	215	3.0
C39	247	313	1.0	214	1.0
C40	248	313	1.0	392	1.0
C41	109	368	3.4	146	3.4
C42	110	366	3.4	146	3.4
C43	249	564	1.7	377	1.7

The sets of primer mix and their specificity for HLA-Cw* typing were shown in Table 11 and Table 12.

5. Statistical Analysis for Allele Frequency (Alf)

HLA-C allele frequency is calculated by dividing the number of individual (N) having the allele with twice the total number of individual (2N). Since each individual has two alleles inherited from the parent at a given locus, the some of all allele frequencies should be 1.0 or 100%.

Table 11. A set of primer mix of medium-resolution HLA-C typing (17,29)

PM	HLA-C alleles	Approx. Size (bp)
C1	Cw* 0102, 0103	340
C2	Cw* 0202, 1701, 1702	522
C3	Cw* 0302, 0303, 03041	564
C4	Cw* 0302	206
C5	Cw* 0302, 03041	522
C6	Cw* 0401, 0402, 0403, 1801, 1802	331
C7	Cw* 0401, 0402, 0403, 1403	440
C8	Cw* 0501	564
C9	Cw* 0602	297
C10	Cw* 0701 - 07 (Except *0704)	1062
C11	Cw* 0701, 0706, 0707	516
C12	Cw* 0702, 0703	442
C13	Cw* 0704	536
C14	Cw* 0801, 0802, 0803	160
C15	Cw* 0801, 0803	625
C16	Cw* 1202, 1301	462
C17	Cw* 1202	538
C18	Cw* 12042	458
C19	Cw* 1402, 1403,	551
C20	Cw* 1402, 1801, 1802	440
C21	Cw* 1502, 1503, (1505 weak)	374
C22	Cw* 0707, 1502 - 05, 1701, 1702	502
C23	Cw* 0202, 1202 - 03, 12042, 1205, 1301 1403, 1504, 1601 - 02, 1604	490
C24	Cw* 0202, 0602, 12041, 12042, 1205	502

Table 12. The sets of primer mix for HLA-Cw*01, *03, *04, *07, *08, *15, and *16 group specific subtyping (17,29)

HLA-Cw* Gruop Specific	PM	HLA-C alleles	Approx. Size (bp)
Cw*01	C25	Cw* 0102, 0103	300
	C26	Cw* 0103, 0801, 0802, 0803	394
Cw*03	C27	Cw* 0303	523
	C28	Cw* 0303, 03041	330
Cw*04	C29	Cw* 0401	424
	C30	Cw* 0402	471
	C31	Cw* 0403	543
Cw*07	C32	Cw* 0703	494
	C33	All Cw*07 (Except Cw*0703)	498
Cw*08	C34	Cw* 0801	103
	C35	Cw* 0802	153
	C36	Cw* 0803	104
Cw*15	C37	Cw* 1502	407
	C38	Cw* 1503	406
	C39	Cw* 1504, Cw*0701, 0706, 0707	423
	C40	Cw* 15051, 15052	424
Cw*16	C41	Cw* 1601	513
	C42	Cw* 1602	503
	C43	Cw* 16041, 0202, 1202, 1203, 12042, 1205, 1301	611

CHAPTER IV

RESULTS

The optimal working concentration of PCR-SSP reagents

It is often difficult to establish a new technique in the laboratory. There are many sources of variability between laboratories in establishing a method, we must be prepared to change certain parameters, and perform adequate controls to ensure the method operating correctly.

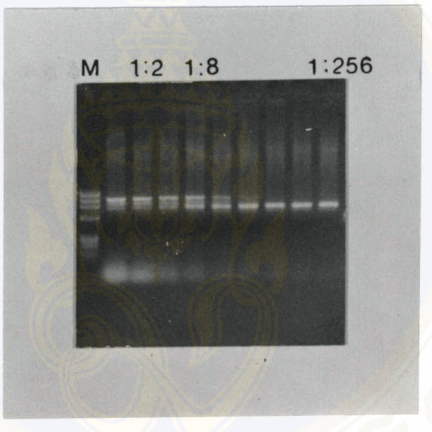
1. Control primers

Getting the right concentration of control primers (Sense primer 63 and Antisense primer 64 in the stock control solutions is of vital importance as these solutions are the standard for all the primer mixes. The concentration of primers must not be so high that the allele-specific amplicon is out-competed by the controls. On the other hand, if the concentration of control primers are too low then the control amplicon will be difficult or impossible to visualize and many primer mixes may appear to be not tested.

The good working concentration of the control primers in this study was found at 0.1 uM for each primer mix.

2. Allele-specific primers

The optimal dilution of concentration of the allele-specific primers were selected from the result of the best dilution (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256). The example of these dilution were shown in Figure 2 and the final dilution of all allele-specific primers in this study were summarized in Table 13.



The optimal dilution = 1:8

Figure 2. The selection of the optimal dilution for working concentration of primer mix

Table 13. The optimal working concentration of primer mix (PM)

PM	Prev.PM in Ref.	Sense Primer		Anti-sense Primer		Optimal Dilution
			Conc. (uM)		Conc. (uM)	
C1	86	368	1.7	315	0.7	1:2
C2	87	366	1.7	145	1.7	1:4
C3	88	368	3.4	389	3.4	1:4
C4	161	130	3.4	389	3.4	1:4
C5	201	160	3.4	389	3.4	1:4
C6	89	366	1.7	143	1.7	1:4
C7	242	564	1.0	143	1.0	1:4
C8	90	366	3.4	379	3.4	1:2
C9	91	367	3.4	127	3.4	1:4
C10	92	130	3.4	378	3.4	1:4
C11	93	313	1.7	184	1.7	1:4
C12	55N	565	0.7	183	1.7	undilute
C13	96	367	1.7	379	3.4	1:4
C14	182	165	1.7	166	1.7	1:4
C15	98	165	1.7	317	1.7	1:2
C16	276N	144	3.0	126	3.4	1:4
C17	102	369	3.4	126	3.4	1:4
C18	277N	144	3.0	157	3.4	1:4
C19	104	371	3.4	388	3.4	1:4
C20	243	565	0.7	143	1.0	1:4
C21	105	366	3.4	147	3.4	1:4
C22	107	366	3.4	382	3.4	undilute
C23	241	564	1.7	214	1.7	1:4
C24	111	366	1.7	377	1.7	1:2

Table 13. The optimal working concentration of primer mix (PM) (Continued)

PM	Prev.PM in Ref.	Sense Primer		Anti-sense Primer		Optimal Dilution
			Conc. (uM)		Conc. (uM)	
C25		160	3.4	315	0.7	1:2
C26		368	1.7	392	1.0	1:4
C27	99	159	3.4	389	3.4	1:4
C28		368	3.4	223	3.4	1:4
C29	236	555MM	1.0	143	1.7	1:4
C30		367	1.7	PN0402	1.7	1:4
C31	251	362	1.7	143	1.7	1:4
C32	95	367	1.7	238	3.4	1:4
C33		144	3.0	184	1.7	1:8
C34	237	556	1.7	566	1.7	undilute
C35		PN0802	3.4	379	3.4	1:32
C36	238	556	1.7	456	1.7	undilute
C37	245	507	2.0	215	2.0	undilute
C38	246	144	3.0	215	3.0	undilute
C39	247	313	1.0	214	1.0	1:4
C40	248	313	1.0	392	1.0	1:4
C41	109	368	3.4	146	3.4	undilute
C42	110	366	3.4	146	3.4	undilute
C43	249	564	1.7	377	1.7	1:2

HLA-C Phototyping

A phenotype is determined successfully when the control amplifications are positive and at least one allele is present. The most common form of individual PCR reaction failure is where random individual reactions fail to produce allele or control bands. Occasionally, samples have to be partially repeated because one or more of the reaction mixtures produce a very weak amplicon thus a confirmation is required.

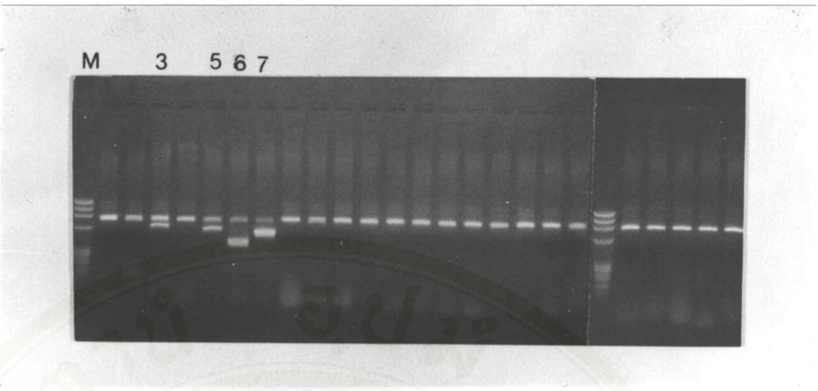
Amplification control primers, giving rise to a 796 bp fragment from the third intron of DRB1, are included in every PCR reaction. Some samples fail to produce a control band although they type satisfactorily. Control failure is not a problem if the genotype obtained is heterozygous for all alleles and the type is unequivocal. Homozygous samples in which the control has failed, normally require repeat typing with a new DNA sample.

In this study, 172 random donors were performed HLA-C typing and analyzed by the developed Phototyping. Figure 3 and 4 show the examples of medium resolution and group specific subtyping HLA-C Phototyping.

Electrophoresis migration is from top to bottom (- to +). Allele-specific amplificons are show as intense bands lower the 796 bp control e.g. :

Lane 3, 5, 6 and 7 of figure 3a show allelic amplicons corresponding to Cw*03041 (lanes 3, 5) and Cw*0401/02/03 (lanes 6,7).

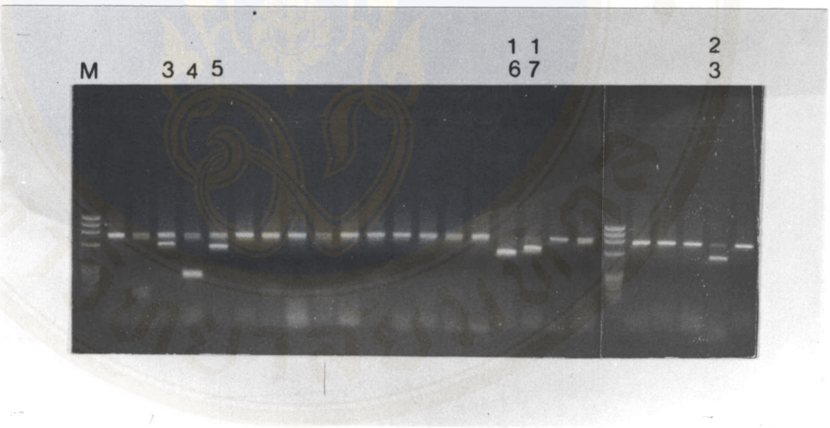
Lane 3, 4, 5, 16, 17 and 23 of figure 3b show allelic amplicons corresponding to Cw*0302 (lanes 3, 4, 5) and Cw*1202 (lanes 16,17, 23).



(3a)

Pos. reaction : 3, 5, 6, 7

SSP Type : Cw*0304, Cw*0401/2/3 SSOP Type : Cw*0304, Cw*0401/2



(3b)

Pos. reaction : 3, 4, 5, 16, 17, 23

SSP Type : Cw*0302, Cw*1202 SSOP Type : Cw*0302, Cw*1202/1301

Figure 3 : Medium resolution of HLA-C Phototyping

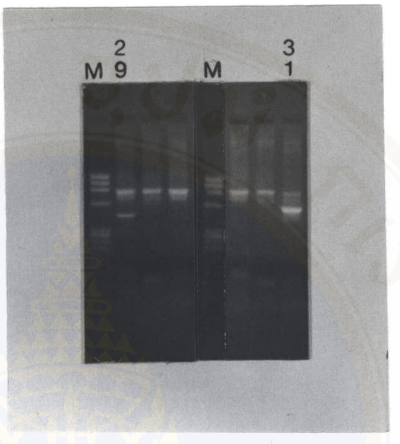


Figure 4 : Group specific subtyping of HLA-C Phototyping

Sample I : lane 29 show allelic amplicons corresponding to Cw*0401

Sample II : Lane 31 show allelic amplicons corresponding to Cw*0403

The HLA-C Phototyping results of 172 random donors are always compared with serological data, when available, and confirmed with SSOP typing results as shown in Table 14 and summary of the results can be seen in Figure 5. Eighty-one out of 145 serological assignments matched perfectly well with molecular typing. There were a total of 64/145 discrepancies between serology and Phototyping. Eleven differences involved underassigning the serologically problematic antigen Cw4, Cw6, Cw7 and Cw8, twenty four discordant results concerned serologically underdetectable alleles that were identified by molecular typing: alleles traditionally known as "Cw blank" (Cw*12, *14, *15, *16) and twenty-nine false serological assignments of HLA-Cw1, Cw2, Cw6, Cw7, Cw8, Cw10 and Cw14 antigens are shown in Table 15-17, respectively. Some Cw14 positive individuals showed cross-reactive with Cw1 antisera but never enough to lead to false assignment of Cw14 or positive proof that Cw14 can be detected serologically. Medium resolution typing performed by Phototyping agrees completely with medium resolution typing carried out by SSOP technique.

HLA-C allele frequencies

HLA-C allele frequencies were determined in 172 random donors. The phenotype frequencies are given in Table 18. Cw*0801 was found to be the most frequent allele, followed by Cw*0702. A number of alleles were absent in the random population sample: Cw*0103, *0402, *0501, *0703, *0705, *0707, *0802, *1204, *1205, *1301, *1403, *1503, *1504, *1601, *1604, *1701, *1702, *1801 and 1802.

Table 14. The results of serology typing, PCR-SSOP typing, and Phototyping ,for HLA-C

Sample No.	HLA No.	Serological						PCR-SSO Typing for HLA-C		Phototyping for HLA-C	
		HLA-A		HLA-B		HLA-C					
1	7644	3	33	55	58	1	9	0102	0302	**0102**	**0302**
2	6504	2	203	13	39	3	7	0302/04	0702	**03041**	**0702**
3	7181	2	24	7	58	3	7.2	0302/04	0702	**0302**	**0702**
4	5343	9.3	28	60	18	7	9	0304	0704	**03041**	**0704**
5	7650	24	26	18	27	7	9	0304	0704	**03041**	**0704**
6	367	33.2		44	58	10	7	0302/04	0701	**0302**	**0701/06**
7	884	2	2	76	77	3	8	0304	0801/03	**03041**	**0801**
8	3320	11.1	24	75	62	not tested		1203	0801/03	**0801**	**1203**
9	3321	207	31	39	48	7	8N	0702	0801/03	**0702**	**0801**
10	429	11.1	2	51	61	8N	14	0801/03	1402/03	**0801**	**1402**
11	444	2	11	27		7		0701	12021/2	**0701/06**	**1202**
12	4060	11.1	2	46	75	not tested		0102	0801/03	**0102**	**0801**
13	806	11.1	2	27	56	not tested		12021/2	1502/03	**1202**	**1502**
14	424	203	33	51	58	10	14	0302/04	1402/03	**0302**	**1402**
15	538	11.2	24	18	57	7	8	0701	12021/2	**0701/06**	**1202**
16	2264	2	33	58	46	not tested		0102	0302	**0102**	**0302**
17	591	2	33	44	60	7	9	0302/04	0701	**03041**	**0701/06**
18	535	11	33	13	27	3		0304	1502/03	**03041**	**1502**
19	909	203	33	55	75	1	8	0102	0801/03	**0102**	**0801**
20	749	2	2	60	46	1	7	0102	0304	**0102**	**03041**
21	3886	2	24	35	38	not tested		0303	0702	**0303**	**0702**
22	154	2	24	60	46	1	3	0102	0304	**0102**	**03041**
23	5342	33	24	35	62	4/6		0401/2/3	0401/2/3	**0401**	**0403**
24	1109	9.3	24	62	18	6	7	0401/2/3	0704	**0403**	**0704**
25	576	11.1		60	62	7	6	0401/2/3	0702	**0403**	**0702**
26	786	11	11	7	46	1	7	0702	1502/03	**0702**	**1502**
27	832	203	33	39	44	7	8	0701	0702	**0701/06**	**0702**
28	515	11.1	2sh	39	38	7		0702	0702	**0702**	**0702**
29	554	207	33	8	46	7	1	0102	0702	**0102**	**0702**
30	662	1	33	52	61	7		0702	1502/03	**0702**	**1502**
31	72	11.1	33.2	44	57	6	7	0602	0701	**0602**	**0701/06**
32	3264	11.2	26	40	51	7		0702	1601/2	**0702**	**1602**

Table 14. The results of serology typing, PCR-SSOP typing, and Phototyping ,for HLA-C (continued)

Sample No.	HLA No.	Serological						PCR-SSO Typing for HLA-C		Phototyping for HLA-C	
		HLA-A		HLA-B		HLA-C					
33	1110	11.1	66	60	75	4	8.2	0401/2/3	0401/2/3	**0401**	**0403**
34	3953	24	1	7	57	not tested		0602	0702	**0602**	**0702**
35	1140	11		7	61	7		0702	1502/03	**0702**	**1502**
36	804	11.1		18	27	9	7	0302/04	0701	**03041**	**0701/06**
37	983	2sh	1	39	37	7		0602	0702	**0602**	**0702**
38	639	11.2	9.3	7	18	7		0702	0704	**0702**	**0704**
39	577	11.1	207	35	44	4	7	0401/2/3	0701	**0401**	**0701/06**
40	4420	2	33	44	46	not tested		0102	0701	**0102**	**0701/06**
41	986	1	24	57	60	3	8	0302/04	0602	**03041**	**0602**
42	539	24	1	44	37	6	7	0602	0701	**0602**	**0701/06**
43	514	203	24	18	46	1	7	0102	0704	**0102**	**0704**
44	805	11.1	33	44	60	1	7	0102	0701	**0102**	**0701/06**
45	680	2		18	55	1		0102	0704	**0102**	**0704**
46	771	11.1	3	52	35	7		0401/2/3	0702	**0401**	**0702**
47	611	24	30	13	18	6	7.2	0602	0704	**0602**	**0704**
48	3963	24	33	7	44	not tested		0701	0702	**0701/06**	**0702**
49	1141	24	30	7	13	6	7.2	0602	0702	**0602**	**0702**
50	209	26	74	60	55	1	7.2	0102	0701	**0102**	**0701/06**
51	311	24	3	35	44	4	7.2	0401/2/3	0701	**0401**	**0701/06**
52	157	3	33	44	13	6		0602	0701	**0602**	**0701/06**
53	142	33		44		7		0701	0701	**0701/06**	**0701/06**
54	459	33.1	24	13	44	9	7	0302/04	0701	**03041**	**0701/06**
55	1886	24	33	76	44	7	9	0303	0701	**0303**	**0701/06**
56	3265	11.1		38	46	1	7	0102	0702	**0102**	**0702**
57	870	33	33	44	58	9	7	0302/04	0701	**0302**	**0701/06**
58	976	1	2	62	58	2		0602	1203	**0602**	**1203**
59	1138	11.2	33	60	58	9	7	0302/04	0702	**0302**	**0702**
60	1282	24		38	50	7	6	0602	0702	**0602**	**0702**
61	594	33		8	58	9	7	0302/04	0702	**0302**	**0702**
62	778	24	11	52	54	1	7	0102	0702	**0102**	**0702**
63	1747	207	24	35	46	not tested		0102	0401/2/3	**0102**	**0401**
64	233	33		5Y	44	7		0701	0702	**0701/06**	**0702**

Table 14. The results of serology typing, PCR-SSOP typing, and Phototyping ,for HLA-C (continued)

Sample No.	HLA No.	Serological HLA-A		HLA-B		HLA-C		PCR-SSO Typing for HLA-C		Phototyping for HLA-C	
65	1367	11.1		13	27	9		0304	1502/03	**03041**	**1502**
66	553	11.1		53	60	10		0304	1502/03	**03041**	**1502**
67	635	29	24	7	75	8		0801/03	15051/2	**0801**	**1505**
68	3952	11.1	74	39	60	not tested		0702	1502/03	**0702**	**1502**
69	19	207	207	46	27	1		0102	12021/2	**0102**	**1202**
70	18	24	11	75	60	8	4	0401/2/3	0801/03	**0401**	**0801**
71	7168	33	x	51	58	3	14	0302	1502/03	**0302**	**1502**
72	857	33.1	24	44	63	7		0701	0701	**0701/06**	**0701/06**
73	509	11.1	1	46	57	10	6	0602	0801/03	**0602**	**0803**
74	666	11	33	27	47	2	8	02021/2	0801/03	**0202**	**0801**
75	730	11.1	207	62	46	1		0102	0401/2/3	**0102**	**0403**
76	1533	24	2	27	46	9	1	0102	0304	**0102**	**03041**
77	1143	207	2	7	75	7	8	0702	0801/03	**0702**	**0801**
78	925	11.2	24	56	27	1	9	0102	12021/2	**0102**	**1202**
79	921	11.1	24	18	62	7	8	0704	1203	**0704**	**1203**
80	3546	11.1	24	27	45	not tested		0801/03	12021/2	**0801**	**1202**
81	615	11.1	33	58	46	10	8	0302/04	12021/2	**0302**	**1202**
82	3531	11.2	3	62	35	not tested		0401/2/3	1203	**0401**	**1203**
83	4155	2	2	13	36	not tested		0401/2/3	0701	**0403**	**0701/06**
84	1526	2sh	11	76	62	10		0302/04	1203	**03041**	**1203**
85	7124	10	33	52	61	7.2	X	0702	1502/03	**0702**	**1502**
86	7100	2	24V	35	62	1	7	0102	0704	**0102**	**0704**
87	867	33	31	13	58	not tested		0302/04	0602	**0302**	**0602**
88	4428	11.1		62	60	not tested		0401/2/3	1203	**0401**	**1203**
89	7131	2	X	46	60	1	3	0102	0304	**0102**	**03041**
90	7176	2	24	38	54	1	7.2	0102	0702	**0102**	**0702**
91	1149	2		13	46	1	6	0102	0401/2/3	**0102**	**0403**
92	4453	11.1		62	60	not tested		0401/2/3	0401/2/3	**0401**	**0401**
93	3263	11.1	1	40	46	1		0102	0102	**0102**	**0102**
94	1022	24	33	18	44	7		0701	0701	**0701/06**	**0701/06**
95	589	11.1	24	35	75	8		0801/03	1203	**0801**	**1203**
96	3258	11.1	33	58	62	6	9	0302	0401/2/3	**0302**	**0403**

Table 14. The results of serology typing, PCR-SSOP typing, and Phototyping ,for HLA-C (continued)

Sample No.	HLA No.	Serological						PCR-SSO Typing for HLA-C		Phototyping for HLA-C	
		HLA-A		HLA-B		HLA-C					
97	693	11	1	75	57	not tested		0602	0801/03	**0602**	**0801**
98	595	24	33	62	75	4	6	0401/2/3	0401/2/3	**0401**	**0403**
99	756	24	31	60	51	6	7	0702	1502/03	**0702**	**1502**
100	1148	11.1	2	58	75	10	8N	0302	0801/03	**0302**	**0801**
101	5385	11.1	24	52	56	BL	X	12021/2	12021/2	**1202**	**1202**
102	579	11.1	32	35		4	6	0401/2/3	0401/2/3	**0401**	**0401**
103	685	11.2	33	58	60	9		0302/04	0302/04	**0302**	**03041**
104	210	11	31	75	37	6		0602	0801/03	**0602**	**0801**
105	1722	31	24	38	75	not tested		0702	0801/03	**0702**	**0801**
106	741	2		77	7DT	8		0801/03	0801/03	**0801**	**0801**
107	975	11.1		18	75	7	8	0704	0801/03	**0704**	**0801**
108	521	11.1		75	46	1		0102	0801/03	**0102**	**0801**
109	694	11.1	9.3	76	75	9	8	0303	0801/03	**0303**	**0801**
110	512	11.1	24	77	51	8		0801/03	1402/03	**0801**	**1402**
111	1308	207	1	77	51	8		0801/03	1402/03	**0801**	**1402**
112	2717	11.1	2	46	46	8	1	0102	0102	**0102**	**0102**
113	1888	203	24	75	62	not tested		0401/2/3	0801/03	**0403**	**0801**
114	3943	2	24	46	62	1	8	0102	0401/2/3	**0102**	**0401**
115	1106	2	24	35	61	4/6		0401/2/3	1502/03	**0401**	**1502**
116	811	2		75	60	8	10	0304	0801/03	**03041**	**0801**
117	830	11.1	203	46	77	1	8	0102	0801/03	**0102**	**0801**
118	1525	11.1	11.1	75	60	9	8	0304	0801/03	**03041**	**0801**
119	3086	11	31	60	75	not tested		0304	0801/03	**03041**	**0801**
120	938	11.2	24	76	75	8	6	0401/2/3	0801/03	**0403**	**0801**
121	137	11.1	24	62		8.4	4	0401/2/3	0801/03	**0401**	**0801**
122	388	11.1	24	62	77	4	8	0401/2/3	0801/03	**0401**	**0801**
123	1020	11.1	24	75	77	6	8	0801/03	0801/03	**0801**	**0801**
124	4541	24	24	27	75	not tested		0702	0801/03	**0702**	**0801**
125	972	24		48	56	1	8N	0102	0801/03	**0102**	**0803**
126	324	24	33.2	58	75	10	8	0302	0801/03	**0302**	**0801**
127	119	3	24	13	77	6		0602	0801/03	**0602**	**0801**
128	469	3	33	75	77	not tested		0701	0801/03	**0701/06**	**0801**

Table 14. The results of serology typing, PCR-SSOP typing, and Phototyping ,for HLA-C (continued)

Sample No.	HLA No.	Serological HLA-A		Serological HLA-B		Serological HLA-C		PCR-SSO Typing for HLA-C		Phototyping for HLA-C	
129	4061	31	11.1	35	75	not tested		0401/2/3	0801/03	**0401**	**0801**
130	460	93	11	18	75	7	8	0704	0801/03	**0704**	**0801**
131	412	24	11.1	18	75	7.2	8N	0704	0801/03	**0704**	**0801**
132	545	1	74	39	57	6		0602	1502/03	**0602**	**1502**
133	5451	2	11.1	48	60	7	8N	0702	0801/03	**0702**	**0801**
134	7605	2	30	13	76	9		0303	0602	**0303**	**0602**
135	1968	33		58	48	9	8N	0302	0801/03	**0302**	**0801**
136	7637	26	11.1	51	62	14		0401/2/3	1402/03	**0403**	**1402**
137	8159	2		46		not tested		0102	0102	**0102**	**0102**
138	1715	1	31	37	38	6	8	0602	0702	**0602**	**0702**
139	8198	11.1	24	39	56	7		0702	1202	**0702**	**1202**
140	5725	11.1	X	54	55	1	X	0102	1203	**0102**	**1203**
141	7101	2	11.1	55	X	X	BL	1202	1203	**1202**	**1203**
142	7122	24	11.1	13	61	3	X	0304	0304	**03041**	**03041**
143	7133	2	33	58	46	1	3	0102	0302	**0102**	**0302**
144	7171	11.1	33	7	13	3	X	0302/04	0302/04	**0302**	**03041**
145	7187	11.2	33	58	60	3	X	0302/04	0302/04	**0302**	**03041**
146	5712	2	24	54	60	1	6	0102	0401/2/3	**0102**	**0403**
147	5390	11.1	33	13	58	3	X	0302/04	0302/04	**0302**	**03041**
148	5395	2	33	58	27	3	X	0302/04	12021/2	**0302**	**1202**
149	5397	11.2	24	52	39	7.2	X	0702	12021/2	**0702**	**1202**
150	7175	2	11.1	60	56	7.2	X	0702	0702	**0702**	**0702**
151	7185	2	24	7	61	7	8	0702	0801/03	**0702**	**0801**
152	7183	11.1	24	38	75	7.2	8	0702	0801/03	**0702**	**0801**
153	323	1	2	37	38	6	7	0602	0702	**0602**	**0702**
154	1289	203	11.1	13	54	1	6	0102	0401/2/3	**0102**	**0403**
155	1111	2		13	46	1	9	0102	0304	**0102**	**03041**
156	3667	24	2	13	62	not tested		0304	0401/2/3	**03041**	**0401**
157	1024	2	33.1	44	35	4	7	0401/2/3	0701	**0401**	**0701/06**
158	1093	203	11.1	46	62	1	6	0102	0401/2/3	**0102**	**0403**

Table 14. The results of serology typing, PCR-SSOP typing, and Phototyping ,for HLA-C (continued)

Sample No.	HLA No.	Serological						PCR-SSO Typing for HLA-C		Phototyping for HLA-C	
		HLA-A		HLA-B		HLA-C					
159	220	2SH	24	77	44	7	8	0701	0801/03	**0701/06**	**0801**
160	3275	11.1	11.1	13	39	4	9	0304	0401/2/3	**03041**	**0401**
161	926	11.1	34	62	56	4	9	0303	0401/2/3	**0303**	**0401**
162	919	11.1	68	55	60	1	7	0102	0702	**0102**	**0702**
163	610	24	11.1	62	60	7		0702	1203	**0702**	**1203**
164	723	24		76	51	9	14	0303	1402/03	**0303**	**1402**
165	1101	24	33	75	76	3		0303	0401/2/3	**0303**	**0403**
166	122	24		18	48	7	8N	0704	0801/03	**0704**	**0801**
167	506	29	33	7	75	not tested		0801/03	15051/2	**0801**	**1505**
168	734	3	11	62	35	not tested		1203	1203	**1203**	**1203**
169	561	68	30	13	62	6		0602	1203	**0602**	**1203**
170	252	68	332	8	46	1	7	0102	0702	**0102**	**0702**
171	507	24	11.2	75	46	10	8N	0801/03	12021/2	**0801**	**1202**
172	522	11.2	24	75	75	8N	8N	0801/03	12021/2	**0801**	**1202**

Figure 5. Summary of Phototyping results of 172 random samples

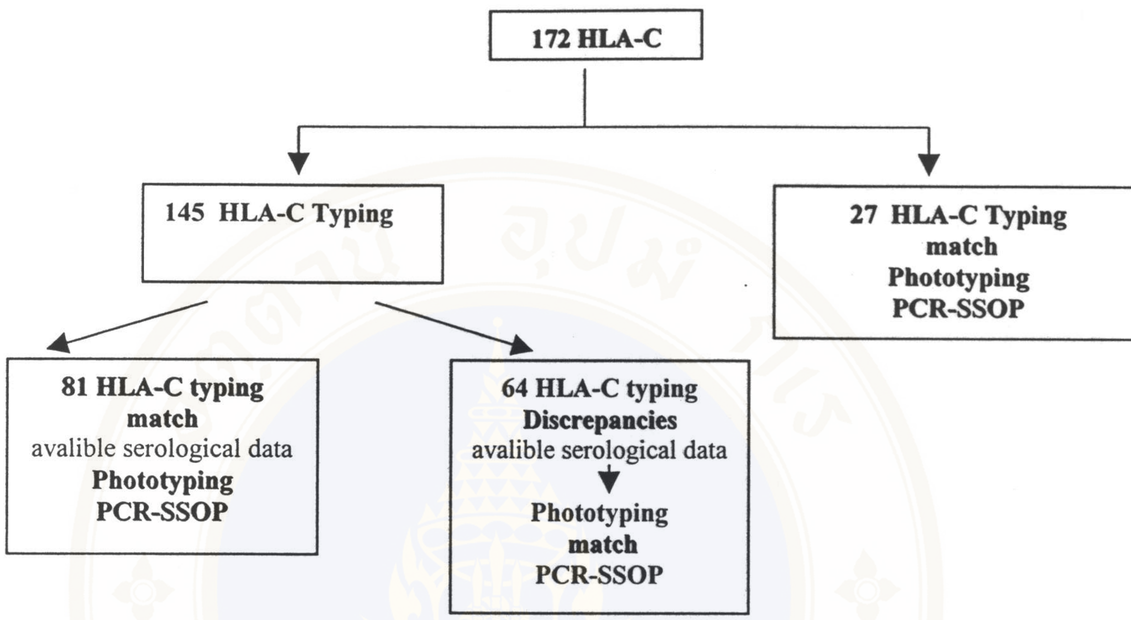


Table 15. The serological problematic antigens

No.	Sample.No.	HLA NO.	Serological					PCR-SSO Typing for HLA-C		Phototyping Typing for HLA-C	
			HLA-A		HLA-B		HLA-C				
Cw4											
1	46	771	11.1	3	52	35	7	0401/2/3	0702	0401	0702
2	75	730	11.1	207	62	46	1	0102	0401/2/3	0102	0403
3	136	7637	26	11.1	51	62	14	0401/2/3	1402/3	0403	1402
4	165	1101	24	33	75	76	3	0303	0401/2/3	0303	0403
Cw6											
1	37	983	2sh	1	39	37	7	0602	0702	0602	0702
2	134	7605	2	30	13	76	9	0303	0602	0303	0602
Cw7											
1	45	680	2		18	55	1	0102	0704	0102	0704
2	52	157	3	33	44	13	6	0602	0701	0602	0701/0706
Cw8											
1	104	210	11	31	75	37	6	0602	0801/3	0602	0801
2	108	521	11.1		75	46	1	0102	0801/3	0102	0801
3	127	119	3	24	13	77	6	0602	0801/3	0602	0801

Table 16. HLA-Cw blank group by serology

No.	Sample.No.	HLA NO.	Serological HLA-A		HLA-B		HLA-C		PCR-SSO Typing for HLA-C		Phototyping Typing for HLA-C	
1	11	444	2	11	27		7		0701	12021/2	0701/0706	1202
2	18	535	11	33	13	27	3		0304	1502/3	03041	1502
3	30	662	1	33	52	61	7		0702	1502/3	0702	1502
4	32	3264	11.2	26	40	51	7		0702	1601/2	0702	1602
5	35	1140	11		7	61	7		0702	1502/3	0702	1502
6	65	1367	11.1		13	27	9		0304	1502/3	03041	1502
7	66	553	11.1		53	60	10		0304	1502/3	03041	1502
8	67	635	29	24	7	75	8		0801/3	15051/2	0801	1505
9	69	19	207	207	46	27	1		0102	12021/2	0102	1202
10	84	1526	2sh	11	76	62	10		0302/4	1203	03041	1203
11	85	7124	10	33	52	61	7.2	X	0702	1502/3	0702	1502
12	95	589	11.1	24	35	75	8		0801/3	1203	0801	1203
13	101	5385	11.1	24	52	56	BL	X	12021/2	12021/2	1202	1202
14	110	512	11.1	24	77	51	8		0801/3	1402/3	0801	1402
15	111	1308	207	1	77	51	8		0801/3	1402/3	0801	1402
16	115	1106	2	24	35	61	4/6		0401/2/3	1502/3	0401	1502
17	132	545	1	74	39	57	6		0602	1502/3	0602	1502
18	139	8198	11.1	24	39	56	7		0702	1202	0702	1202
19	140	5725	11.1	X	54	55	1	X	0102	1203	0102	1203
20	141	7101	2	11.1	55	X	X	BL	1202	1203	1202	1203
21	148	5395	2	33	58	27	3	X	0302/4	12021/2	0302	1202
22	149	5397	11.2	24	52	39	7.2	X	0702	12021/2	0702	1202
23	163	610	24	11.1	62	60	7		0702	1203	0702	1203
24	169	561	68	30	13	62	6		0602	1203	0602	1203

Table 17. False serological assignments

No.	Sample.No.	HLA NO.	Serological						PCR-SSO Typing for HLA-C		Phototyping Typing for HLA-C	
			HLA-A		HLA-B		HLA-C					
1	24	1109	9.3	24	62	18	6	7	0401/2/3	0704	0403	0704
2	25	576	11.1		60	62	7	6	0401/2/3	0702	0403	0702
3	91	1149	2		13	46	1	6	0102	0401/2/3	0102	0403
4	96	3258	11.1	33	58	62	6	9	0302	0401/2/3	0302	0403
5	98	595	24	33	62	75	4	6	0401/2/3	0401/2/3	0401	0403
6	102	579	11.1	32	35		4	6	0401/2/3	0401/2/3	0401	0401
7	120	938	11.2	24	76	75	8	6	0401/2/3	0801/3	0403	0801
8	146	5712	2	24	54	60	1	6	0102	0401/2/3	0102	0403
9	154	1289	203	11.1	13	54	1	6	0102	0401/2/3	0102	0403
10	158	1093	203	11.1	46	62	1	6	0102	0401/2/3	0102	0403
11	99	756	24	31	60	51	6	7	0702	1502/3	0702	1502
12	123	1020	11.1	24	75	77	6	8	0801/3	0801/3	0801	0801
13	171	507	24	11.2	75	46	10	8N	0801/3	12021/2	0801	1202
14	172	522	11.2	24	75	75	8N	8N	0801/3	12021/2	0801	1202
15	138	1715	1	31	37	38	6	8	0602	0702	0602	0702
16	15	538	11.2	24	18	57	7	8	0701	12021/2	0701/0706	1202
17	27	832	203	33	39	44	7	8	0701	0702	0701/0706	0702
18	79	921	11.1	24	18	62	7	8	0704	1203	0704	1203
19	33	1110	11.1	66	60	75	4	8.2	0401/2/3	0401/2/3	0401	0403
20	41	986	1	24	57	60	3	8	0302/4	0602	03041	0602
21	81	615	11.1	33	58	46	10	8	0302/4	12021/2	0302	1202
22	112	2717	11.1	2	46	46	8	1	0102	0102	0102	0102
23	114	3943	2	24	46	62	1	8	0102	0401/2/3	0102	0401
24	20	749	2	2	60	46	1	7	0102	0304	0102	03041
25	26	786	11	11	7	46	1	7	0702	1502/3	0702	1502
26	58	976	1	2	62	58	2		0602	1203	0602	1203
27	71	7168	33	x	51	58	3	14	0302	1502/3	0302	1502
28	73	509	11.1	1	46	57	10	6	0602	0801/3	0602	0803
29	78	925	11.2	24	56	27	1	9	0102	12021/2	0102	1202

Table 18. Genotype and Phenotype frequencies in 172 random donors in this study

HLA-C allele	n	% Genotype frequency	% Phenotype frequency
Cw*0102	41	11.92	23.84
Cw*0103	0	0.00	0.00
Cw*0202	1	0.29	0.58
Cw*0302	21	6.10	12.21
Cw*0303	7	2.03	4.07
Cw*0304	28	8.14	16.28
Cw*0401	23	6.69	13.37
Cw*0402	0	0.00	0.00
Cw*0403	16	4.65	9.30
Cw*0501	0	0.00	0.00
Cw*0602	20	5.81	11.63
Cw*0701/06	29	8.43	16.86
Cw*0702	**44**	**12.79**	**25.58**
Cw*0703	0	0.00	0.00
Cw*0704	13	3.78	7.56
Cw*0705	0	0.00	0.00
Cw*0707	0	0.00	0.00
Cw*0801	**49**	**14.24**	**28.49**
Cw*0802	0	0.00	0.00
Cw*0803	2	0.58	1.16
Cw*1202	15	4.36	8.72
Cw*1203	13	3.78	7.56
Cw*1204	0	0.00	0.00
Cw*1205	0	0.00	0.00
Cw*1301	0	0.00	0.00
Cw*1402	6	1.74	3.49
Cw*1403	0	0.00	0.00
Cw*1502	13	3.78	7.56
Cw*1503	0	0.00	0.00
Cw*1504	0	0.00	0.00
Cw*1505	2	0.58	1.16
Cw*1601	0	0.00	0.00
Cw*1602	1	0.29	0.58
Cw*1604	0	0.00	0.00
Cw*1701	0	0.00	0.00
Cw*1702	0	0.00	0.00
Cw*1801	0	0.00	0.00
Cw*1802	0	0.00	0.00

CHAPTER V

DISCUSSION

The HLA system discriminates self from non-self entities and plays an important role in the interactions and activation of immunocompetent cells. HLA genes reside on the short arm of chromosome 6 and show a high degree of genetic polymorphism. During the last four decades, HLA allospecificity is mainly identified by serological typing, however, there are technical difficulties with HLA serotyping. Because many types of antiserum have to be prepared by screening an enormous number of multiparous women and yet not all specificities can be identified. Good serology can identify many antigens monospecifically but in some cases serological cross-reactivity confounds the accurate identification of one or both antigens. On the other hand, monoclonal antibodies appear to be a promising source of sufficient HLA antibodies, however, not all specificities can be produced. Another approach uses recombinant gene technology and cellular engineering to produce and detect HLA antibodies efficiently. The PCR based technique-developed recently-and can provide an effective mean of HLA genotyping. Its also makes genotyping possible even when samples are preserved under unfavorable conditions for serotyping. In addition, the PCR method allows more detailed typing to be performed in comparison to serological method.

Since serological determination of HLA-C remain somewhat complicate, molecular characterization is a valuable tool for the identification of HLA-C alleles.

Serotyping of HLA-C antigens is more difficult than that of other HLA class I antigens such as HLA-A and -B, and so serologically untypable blank antigens are much common in HLA-C locus. This study attempted to perform the genotyping of HLA-C alleles by establishing of "Phototyping" or "PCR-SSP" technique using 43 sequence specific primer sets and found that the genotype of HLA-Cw* allele could be identified easily and rapidly by this method. In addition, PCR-SSP genotyping was also possible for other HLA-C alloantigens, so-called blank antigens, that cannot be typed serologically due to lack of the relevant antiserum or lack of knowledge of the haplotype.

The principle of "Phototyping" for HLA-C is that each group of alleles or individual allele making up a serological specificity is amplified by a primer pair exactly matched to that group. By keeping the PCR conditions stringent, primer pairs will not non-specifically amplify with other related alleles. The amplification primers are designed with the specificity dependent nucleotide(s) on the terminal 3-primer end. Because Taq polymerase lack 3' to 5' exonuclease activity even if primer pairs do anneal non-specifically, they will not amplify efficiently. Identification of the alleles is based on the presence or absence of amplified products observed after agarose gel electrophoresis. All primer combinations were initially tested at the same final concentration as reported by of Bunce et al. (25, 26). If the primer mix is weak or gives false positive amplifications, the concentration of one or both primers is tested at different concentration against known positives and negatives control to obtain optimum results and arrives at the optimal working concentration of primer for each individual primer mix. However, these concentrations should be used only as a guide as primers synthesized by different methods or used under different conditions may require slightly different concentrations.

"Phototyping" produces reliable and consistent results. This technique is rapid (not more than 2.30 hours after already have DNA); simple and ideally suited for individual typing but could be less convenient when larger numbers of samples are to be typed in parallel. The experience of this study proves that, it was necessary to accurately qualify and quantify the concentration of DNA in solution after extraction and before diluting prior to incorporating in the PCR amplification. Failure to do so results in nonspecific amplification. The major drawback of this method is the rapidly increasing number of HLA-C alleles identified and hence the increasing number of primers needed for Phototyping.

The result of this study shows an impressive quantitative advantage of molecular typing for the detection of HLA-C alleles. There can be no doubt that serological typing is no longer a suitable method for organ transplantation purpose due to inadequate determination of HLA-C antigens. Recent reports show the associations between graft-versus-host disease (GVHD). Serologically undetectable HLA-C mismatches in bone marrow transplantation (172-173) suggest a functional relevance of molecular HLA-C matching. Whether matching for HLA-C has any influence on kidney graft survival will have to be clarified in future studies using molecular techniques. In addition, Phototyping for HLA-C should also be useful for forensic purposes, since the distribution of alleles makes this suitable for forensic statistic.

The developed "Phototyping" technique in this study has proven to be accurate and efficient for the detection of HLA-C alleles including the serological blank. Although, the allelic diversity of the HLA-C gene is predominantly present in the $\alpha 1$ and $\alpha 2$ domains of the heavy chain, encoded by exon 2 and 3 but at present, it is known that some HLA-C alleles show difference outside these exons. These are Cw*0701/06, Cw*12021/2, Cw*15051/2, Cw*1701/02 and Cw*1801/02 (7,80,96). Because of those clinical significance of HLA-C mentioned above, the high resolution of HLA-C typing techniques is necessary in order to identify polymorphism display in exon 1, 4 and 5 that allow the differentiation of Cw*0701 from Cw*0706, Cw*12021 from Cw*12022, Cw*15051 from Cw*15052, Cw*1701 from Cw*1702 and Cw*1801 from Cw*1802 which need further development.

CHAPTER VI

CONCLUSION

The results of this study showed that the identification of HLA-C allele by this method could be more precise and accurate than those of the serologically defined HLA-C antigen (Cw1-Cw10). It was also possible to identify the so-called blank antigen by serology. These results were confirmed with HLA-C typing by PCR-SSOP method and compared with previously known HLA-Cw by serology and defining corresponding allele frequencies of these HLA-C alleles. The relative allele frequencies showed that Cw*0801 were the most frequently detected alleles, followed by Cw*0702

HLA-C "medium-resolution" typing by this developed method is ideally suited for analyzing small numbers of samples simultaneously and can replace serological methods for HLA-C typing in routine clinical practices including donor-recipient matching in allogeneic solid organ and bone marrow transplantation. It has proven to be accurate and efficient for the detection of HLA-C alleles which show polymorphism inside exon 2 and 3. But it still needs further development (high resolution) in order to identify HLA-C alleles that show polymorphism outside exon 2 and 3 that is exon1, 4 and 5. Due to an ever increasing of new sequences every now and then, it is recommended that the test primers must always be developed to be able to assign new alleles.

The established "Phototyping" technique in this study is the first DNA based typing method to detect HLA-C developed in our DNA laboratory at the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital.

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APPENDIX I

EQUIPMENTS

1.	Autoclave	Hirayama	Japan
2.	Cassette for X-ray film (8"x10")	Okamoto	Japan
3.	Centrifuge (Nanofuge Hoefer)	Hoefer	USA
4.	Centrifuge (Sorvall GLC-2)	Sorvall	Germany
5.	DC Power Supply (PS 500XT)	Hoefer	USA
6.	DNA Thermal Cycler (GeneAmp 9600)	Perkin Elmer	USA
7.	Dot Blotter (Milli Blot-D)	Millipore Corporation	France
8.	Dri-Bath (Thermolyne 16500)	Barnstead/Thermolyne	USA
9.	Freeze Dry Centrifuge (SC 110)	Savant Instruments	USA
10.	Gel Electrophoresis System (BRL 11.14)	BRL	USA
11.	Hot Air Oven (Memmert UM 400)	Memmert GmbH Co.W	Germany
12.	Magnetic Stirrer	Barnstead/Thermolyne	USA
13.	Micro Centrifuge (Tomy MRX-150)	Tomy Seiko Co.Ltd.	Japan
14.	Microwave	National	Japan
15.	Multichannel Finnpipette (Digital 50-300)	Labsystems	Finland
16.	NANOpure Analytical (Type D4700)	Branstead/Thermolyne	USA
17.	pH Meter (model 410A)	Orion	USA
18.	Photo documentation (Fotodyne MP-4)	Fotodyne	USA
19.	Pipetman (P10, 20, 200, 1000)	Gilson	France

20.	Plastic Sealer (Krups Vacupack 2)	Krups	Sweden.
21.	Plate Form Rotator (TPM-2)	Sarstedt	Germany
22.	Shaking Water Bath (Haake SWB 20)	Haake	Germany
23.	Spectrophotometer (UV 160A)	Shimadzu Corporation	Japan
24.	Sterile GARD Hood Class II Type A/B3	The Baker Company.inc	USA.
25.	Transluminator (FD-33002, UV300)	Fotodyne	USA
26.	Vortex Mixer (Genie 2)	Scientific Industries INC	USA

APPENDIX II

SUPPLY

1.	Falcon tube (6 ml, 15 ml, 50 ml)	Becton Dickinson	USA
2.	Micro tube (0.5 ml, 1.5 ml)	Treff AG.	Switzerland
3.	Nylon Membrane Filter	Biotrace, Gelman	USA
4.	Nylon Membrane, Positive Charge	Boehringer Mannheim	Germany
5.	Overhead Projector Film 0.1 mm. PVP		Thailand.
6.	Pipette tip (Blue and Yellow)	Scientific Plastics	USA
7.	Polaroid Film	Polaroid	UK
8.	Thin Wall tube (0.2 ml)	Scientific Plastics	USA
9.	Whatman 3 mm paper	Whatman	USA
10.	X-OMAT AR Film	Kodak Company	USA

APPENDIX III

CHEMICALS AND REAGENTS

1.	Absolute ethanol	Merck	Germany
2.	Agarose (ultra PURE)	Gibco BRL	USA
3.	Ammonium acetate	Sigma	USA
4.	Anti-Digoxigenin-AP (Fab Fragment)	Boehringer Mannheim	Germany
5.	Blocking reagent	Boehringer Mannheim	Germany
6.	Boric acid	Sigma	USA
7.	Bovine Serum Albumin (Fraction V)	Boehringer Mannheim	Germany
8.	Bromphenol Blue (sodium salt)	Sigma	USA
9.	Cacodylic acid Dimethylarsinic acid	Sigma	USA
10.	Cobalt chloride	Sigma	USA
11.	CSPD	Boehringer Mannheim	Germany
12.	d GTP, d TTP, dCTP, dATP	Boehringer Mannheim	Germany
13.	Digoxigenin-11-ddUTP	Boehringer Mannheim	Germany
14.	Dithiothreitol (DTT)	Boehringer Mannheim	Germany
15.	EDTA (Disodium Salt)	Sigma	USA
16.	Ethidium bromide	Sigma	USA
17.	Ficoll 400	Pharmacia	Sweden

18. Gelatin from Porcine Skin	Sigma	USA
19. Isoamylalcohol	Merck	Germany
20. Magnesium chloride	Sigma	USA
21. Maleic acid	Sigma	USA
22. Mineral oil	Sigma	USA
23. N-Lauroylsarcosine	Sigma	USA
24. Nonidet P-40 (NP-40)	Sigma	USA
25. Phi X 174 DNA RF Hae III Digest	Biolabs	USA
26. Polyvinyl pyrolidon (PVP-40)	Sigma	USA
27. Potassium Chloride	Merck	Germany
28. Sodium Acetate, anhydrous	Sigma	USA
29. Sodium Chloride	Merck	Germany
30. Sodium Dihydrogen Phosphate	Merck	Germany
31. Sodium Dodecyl Sulphate (SDS)	Sigma	USA
32. Sodium Hydroxide	Merck	Germany
33. Tetramethylammonium chloride (TMAC)	Aldrich Chemical Company	USA.
34. Tris (hydroxymethyl) aminomethane	Sigma	USA
35. TRIZMA base	Sigma	USA
36. Tween 20	Amresco	USA

APPENDIX IV

ENZYMES

1.	Proteinase K	Boehringer Mannheim	Germany
2.	TAQ DNA Polymerase	BRL	USA
3.	Terminal Transferase (TdT)	Boehringer Mannheim	Germany

APPENDIX V

BUFFER AND SOLUTIONS

1. Solution A

Ammoniumchloride	6.35	gm
EDTA	1.33	gm
Trizma base	0.92	gm
add dd $H_2 0$ up to	1000	ml

2. 10% SDS

SDS	100	gm
add dd $H_2 0$ up to	1000	ml

3. 7.5 M Guanidine HCl

Guanidine HCl	216	gm
1M Tris-HCl pH 7.6	30	ml
add dd $H_2 0$ up to	300	ml

Filtered through 0.2 um filter membrane

4. 1 M Tris-HCl (pH7.6)

Tris-HCl	121.1	gm
dd H_20	900	ml
adjust pH to 7.6 with conc. HCl		
add dd H_20 up to	1000	ml
Sterilize by autoclaving		

5. 80% Ethanol

Absolute Ethanol	80	ml
dd H_20 up to	100	ml

6. 0.5 M EDTA (pH8.0)

EDTA disodium salt	186.1	gm
dd H_20 to	900	ml

adjust pH to 8.0 with NaOH pellets

7. Proteinase K (10mg/ml)

add dd H_20 up	1000	ml
Sterilize by autoclaving		

8. TE BUFFER (pH 8.0)

1 M Tris- HCl pH 8.0	10	ml
0.5 M EDTA pH 8.0	2	ml
add dd H_20 up to	1000	ml

9. 5 M NaCl

NaCl	146.1	gm
dd H_20 up to	500	ml

Sterilize by autoclaving

10. 1 M $MgCl_2$

$MgCl_2$	203.3	gm
add H_20	1000	ml

Sterilize by autoclaving

11. 1 M Tris-HCl (pH 8.0)

Tris-HCl	121.1	ml
dd H_20	900	ml

adjust pH to 8.0 with Conc. HCl

add dd H_20 up to	1000	ml

Sterilize by autoclaving

12. 5% EDTA (pH 7.4)

EDTA (disodium salt)	50	gm
add dd H_20	900	ml

adjust pH to 7.4 with NaOH

add dd H_20 up to	1000	ml

Sterilize by autoclaving

13. 1 M Tris-HCl (pH 8.4)

Tris-HCl	121.1	gm
dd H_20	900	ml
adjust pH to 8.4 with Conc.HCl		
add dd H_20 up to	1000	ml
Sterilize by autoclaving		

14. 10X PCR Buffer A (for SSOP method)

750 mM	Tris-HCl (pH9.0)
200 mM	$(NH_4)_2SO_4$
0.1%	Tween

15. 10X PCR Buffer (for Phototyping)

670 mM	Tris base pH8.8
166 mM	Ammonium Sulphate
20 mM	Magnesium chloride
1%	Tween 20

16. 5 mg/ml Ethidium Bromide

Ethidium Bromide	50	mg
dd H_20	10	ml

17. 10X TBE Buffer

TRIZMA base	54.0	gm
Boric acid	27.5	gm
0.5 M EDTA pH 8.0	20.0	ml
add dd H_20 up to	500	ml

18. 2% Agarose Gel (For Horizon 11.14)

agarose	1.5	gm
1X TBE	75	ml
Boil		
add 5 mg/ml Ethidium bromide	7.5	µl

19. Gel Loading Buffer

Glycerol	30	ml
Bromphenol Blue	100	mg
1X TBE up to	100	ml

20. 10X Tailing Buffer

Na - Cacodilate	0.0224	gm
1 M Tris - HCl (pH 7.2)	30	ml
$CoCl_2$	7.137	gm
dd H_20 up to	100	ml

21. 1 M Tris-HCl (pH 7.2)

Tris-HCl	121.1	gm
add dd H_20	900	ml
adjust pH to 7.2 with Conc. HCl		
add dd H_20 up to	1000	ml

22. 2 M Ammonium Acetate

Ammonium acetate	154.16	gm
dd H_20 up to	1000	ml

23. 1 M Ammonium Acetate

Ammonium acetate	77.08	gm
dd H_20 up	1000	ml

24. 30X SSPE

NaCl	263.25	gm
NaH_2PO_4	41.397	gm
EDTA disodium Salt	11.166	gm
dd H_20	800	ml
adjust pH to 7.4 with NaOH		
add dd H_20 up to	1000	ml

25. 50X Denhardt's Solution

Solution I

PVP 40	2	gm
Ficoll 400	2	gm
dd H_20	100	ml

autoclave 10 min at 120°C

Solution II

BSA (Fraction V)	2	gm
dd H_20	80	ml

adjust to pH 3.0 with 2 N HCl

Boil gently for 15 min and cool down in ice at 4°C

add dd H_20 up to	100	ml

Mix well Solution I and Solution II , store at -20°C

26. 2 N HCl

dd H_20	50	ml
Conc. HCl	16.67	ml
add dd H_20 up to	100	ml

27. 5 M Tetramethyammonium Chloride (TMAC)

TMAC	548	gm
add dd H_20 up to	1000	ml

28. TMAC Solution

1 M Tris - HCl pH 8.0	25	ml
5 M TMAC	300	ml
0.5 M EDTA pH 8.0	2	ml
10% SDS	5	ml
add dd $H_2 0$ up to	500	ml

29. 10% Lauryl Sarcosine

lauryl sarcosine	10	gm
add dd $H_2 0$ up to	100	ml

30. Hybridization Buffer (Store at -20°C)

30X SSPE	100	ml
50X Denhardt's solution	50	ml
10% N-Lauryl sarcosine	5	ml
10% SDS	1	ml
add dd $H_2 0$ up to	500	ml

31. 2X SSPE, 0.1% SDS

30XSSPE	40	ml
10% SDS	6	ml
add dd $H_2 0$ up to	600	ml

32. **Blocking Solution**

Blocking Reagent	10	gm
Buffer O up to	100	ml

Shaking and heating either on a heating block or in a microwave oven and sterilize by autoclaving.

33. **Buffer 1**

Tween 20	3	ml
add Buffer O up to	1000	ml

34. **Buffer 2**

Blocking Solution	100	ml
Buffer O up to	1000	ml

35. **Buffer 3**

1 M Tris - HCl (pH 9.5)	100	ml
1 M NaCl	100	ml
1 M $MgCl_2$	50	ml
add dd H_20 up to	1000	ml

Sterilize by autoclaving

36. **2 mM d NTP Mix**

100 mM d ATP	20	ul
100 mM d CTP	20	ul

100 mM d GTP	20	ul
100 mM d TTP	20	ul
dd H_20 up to	1000	ul

37. 1 M TRIS-HCl pH 9.5

Tris-HCl	121.1	gm
ddH_2O	900	ml

adjust pH ti 9.5 with Conc. HCl

Sterilize by autoclaving

38. Washing Solution

0.3% of Tween 20 in Buffer I

39. Marker

Phi X 174 Hae III fragment	20	ul
dd H_2O	150	ul
Loading Buffer	30	ul

40. 4N NaOH

NaOH	16	gm
dH_2O up to	100	ml

41. 250 mM EDTA

0.5 M EDTA	50	ml
ddH_2O up to	100	ml

42. Buffer For Dot Spot

4 N NaOH	10	ml
250 mM EDTA	10	ml
ddH_2O up to	100	ml

43. TE Buffer For Dot Spot

1 M Tris-HCl pH 8.0	10	ml
0.5 M EDTA pH 8.0	2	ml
ddH_2O to	900	ml
adjust pH to 8.0		
add ddH_2O up to	1000	ml

BIOGRAPHY

NAME	Mr. Rungrot Thongpradit
DATE OF BIRTH	7 January 1966
PLACE OF BIRTH	Ratchaburi, Thailand
INSTITUTIONS ATTENDED	Dhonburi Teachers College, 1985 – 1987 Bachelor of Education (General Science) Mahidol University, 1996-2000: Master of Science (Transfusion Science)
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