Overview on HLA and DNA typing methods

REVIEW

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ABSTRACT

The term HLA refers to the Human Leukocyte Antigen System, which is controlled by genes on the short arm of chromosome six. The HLA loci are part of the genetic region known as the major histocompatibility complex (MHC). The MHC has genes (including HLA) that form part of the normal function of the immune response. The essential role of the HLA antigens lies in the control of self-recognition and thus defense against microorganisms. The HLA loci, by virtue of their extreme polymorphism ensure that few individuals are identical and thus the population at large is well equipped to deal with attacks. Because some HLA antigens are recognized on all body tissues (rather than just blood cells), the identification of HLA antigens is described as "Tissue typing". In the last twenty years there has been an exponential growth in the application of DNA technology to the field of Histocompatibility and Immunogenetics. The development and application of several different DNA methods by many laboratories has resulted in that nearly every Histocompatibility and Immunogenetics laboratory performs some DNA typing to detect HLA alleles.

Key words: Human MHC, HLA antigens, genetic of HLA, HLA nomenclature, polymerasa chain reaction (PCR), DNA typing, sequence specific oligo nucleotide (SSO) probes, sequence specific primers (SSP), sequence based typing (SBT)

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RESUMEN

HLA y métodos de tipaje basados en ADN. El término HLA se refiere al Sistema de Antígenos Leucocitarios Humanos, el cual está controlado por genes que se encuentran localizados en el brazo corto del cromosoma 6. Los loci HLA forman parte de la región conocida como Complejo Mayor de Histocompatibilidad (MHC). Este complejo comprende genes (como los HLA) que integran la función normal del sistema inmune. La función esencial de los antígenos HLA comprende el reconocimiento y la defensa contra microorganismos. Los loci HLA debido a su polimorfismo extremo aseguran que muy pocos individuos sean idénticos y que las poblaciones sean por tanto lo suficientemente polimórficas para enfrentar ataques de microorganismos. Debido a que los antígenos HLA son reconocidos en todos los tejidos del organismo, la identificación de los antígenos HLA se ha descrito como "Tipaje de tejidos". En los últimos 20 años ha habido un crecimiento exponencial en la aplicación de la tecnología de ADN en el campo de la Histocompatibilidad e Inmunogenética. El desarrollo y aplicación por muchos laboratorios de diversos métodos de ADN han permitido que todos los laboratorios de Histocompatibilidad e Inmunogenética apliquen algún método de tipaje de ADN para la detección de los alelos HLA.

Palabras claves: MHC humano, antígenos HLA, genética del HLA, nomenclatura del HLA, reacción en cadena de la polimerasa (PCR), tipaje por métodos de ADN, bandas de oligo secuencia especifica (SSO), secuencia de primers específicos (SSP), tipaje basado en secuenciación (SBT)

*I*ntroduction

The term HLA refers to Human Leukocyte Antigens. These are proteins found in the membranes (outer coating) of nearly every cell in the body (all cells that have a nucleus). These antigens are in especially high concentrations on the surface of white blood cells (leukocytes) and are controlled by genes on the short arm of chromosome six. The HLA loci are part of the genetic region known as the major histocompatibility complex (MHC) [1]. The MHC has genes (including HLA) that form part of the normal function of the immune response.

The essential role of the HLA antigens lies in the control of self-recognition and thus defense against microorganisms. HLA antigens are the major determinants used by the body's immune system for recognition and differentiation of self from non-self (foreign substances). There are many different major histocompatibility (HLA) proteins, and individuals possess only a small, relatively unique set that is inherited from their parents. It is unlikely that 2 unrelated persons will have the same HLA make-up.

A person, on the average, will have one-half of the HLA antigens that match with one-half of their mother's antigens; the other half of the antigens will match with one-half of their father's antigens. This is particularly important in identifying good "matches" for tissue grafts and organ transplants, such as a kidney or bone marrow transplant.

Many HLA molecules exist, but some are of special interest because they are more common in certain autoimmune diseases. For example, the HLA-B27 antigen is found in 80-90% of the patients with ankylosing spondylitis and Reiter's syndrome and can aid in the diagnoses of these diseases. HLA-B27 is also present in 5-7% of the persons without autoimmune diseases. Thus, the mere presence of this HLA molecule is not an indication of disease.

HLA types can also be used to determine the parents of a child when in doubt. However, newer, more specific genetic testing is now available for this purpose.

1. Hugh H, Fudenberg JRL, An-Chuan Wang, Ferrara GB. Basic immunogenetics. 3rd ed., London: Oxford UniversityPress, 1984.

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History

The early development of HLA typing sprang from attempts by red cell serologists to define antigens on leucocytes using their established agglutination methods. These methods, however, had many technical problems and could not determine the extreme polymorphism of the system. Although Jean Dausset reported the first HLA antigen, MAC (HLA-A2, A28) in 1958, the poor reproducibility of leucoagglutination was hindering the progress. It was five years later that the polymorphic nature of the HLA system first appeared [2]. The definition of the 4a14b series by Jan van Rood in 1963 and the definition of LA1, LA2 and LA3 (HLA-Al, HLA-A2, HLA-A3) indicated a need for International Standardization and a series of International Workshops, were then started in 1964 [3].

HLA antigens

Based on the structure of the antigens produced and their function, there are two classes of HLA antigens, termed accordingly, HLA Class I and Class II. The overall size of the human MHC complex is of approximately 3.5 million base pairs (Figure 1). Within this the HLA Class I genes and the HLA Class II genes each spread over approximately one third of this length. The remaining section, sometimes known as Class III, contains loci responsible for the complement, hormones, intracellular peptide processing and other developmental characteristics [4]. Thus the Class III region is not actually a part of the HLA complex, but is located within the HLA region, because its components are either related to the functions of HLA antigens or are under similar control mechanisms to the HLA genes [5].

HLA Class I antigens

The cell surface glycopeptide antigens of the HLA-A,-B and -C series are called HLA Class I antigens [6]. A listing of the currently recognized HLA Class I antigens are expressed on the surface of most nucleated cells in the body. Additionally, they are found in a soluble form in plasma and are adsorbed onto the surface of platelets. Erythrocytes also adsorb HLA Class I antigens to varying degrees depending on the specificity (e.g. HLA-B7, A28 and B57 are recognizable on erythrocytes as the so called “Bg”. antigens). Immunological studies indicate that HLA-B (which is also the most polymorphic) is the most significant HLA Class I *locus*, followed by HLA-A and then HLA-C. There are other HLA Class I loci (e.g. HLA-E, F, G, H, J, K and L), but most of these may not be important as *loci* for “peptide presenters” [7].

The HLA Class I antigens comprise a 45 Kilodalton (Kd) glycopeptide heavy chain with three domains, which is non-covalently associated with the f3-2 microglobulin, which plays an important role in the structural support of the heavy chain. The HLA Class I molecule is assembled inside the cell and ultimately it is on the cell surface with a section inserted in the lipid bilayer of the cell membrane and having a short cytoplasmic tail.

2. Terasaki PI. History of HLA: Ten Recollections. ed., Los Angeles: UCLA Tissue Typing Laboratory Press, 1990.

3. Glenn E. HLA beyond tears. Introduction to Human Histocompatibility. Second edition. Atlanta: De Novo Inc, 1991.

4. Sanfilippo F, Amos DB. An interpretation of the major histocompatibility complex. In: Rose NR, Friedman H, Fahey JL, editors. Manual of clinical laboratory immunology, 3rd ed. Washington: ASM Press, 1986.

5. De Jong MM, Nolte IM, de Vries EG, Schaapveld M, Kleibeuker JH, Oosterom E, Oosterwijk JC, van der Hout AH, van der Steege G, Bruinenberg M, Boezen HM, Te Meerman GJ, van der Graaf WT. The HLA class III subregion is responsible for an increased breast cancer risk. Hum Mol Genet 2003;12(18):2311-9.

6. Roitt M, Brostoff J, Male DK. T-cell receptors and major histocompatibility complex molecules. In: Immunology. 5th ed. London: Churchill Livingston, 1998.

7. Lotteau V. Assembly and intracellular transport of HLA molecule antigen presenters. Ann Rech Vet 1992;23(3):268-74.

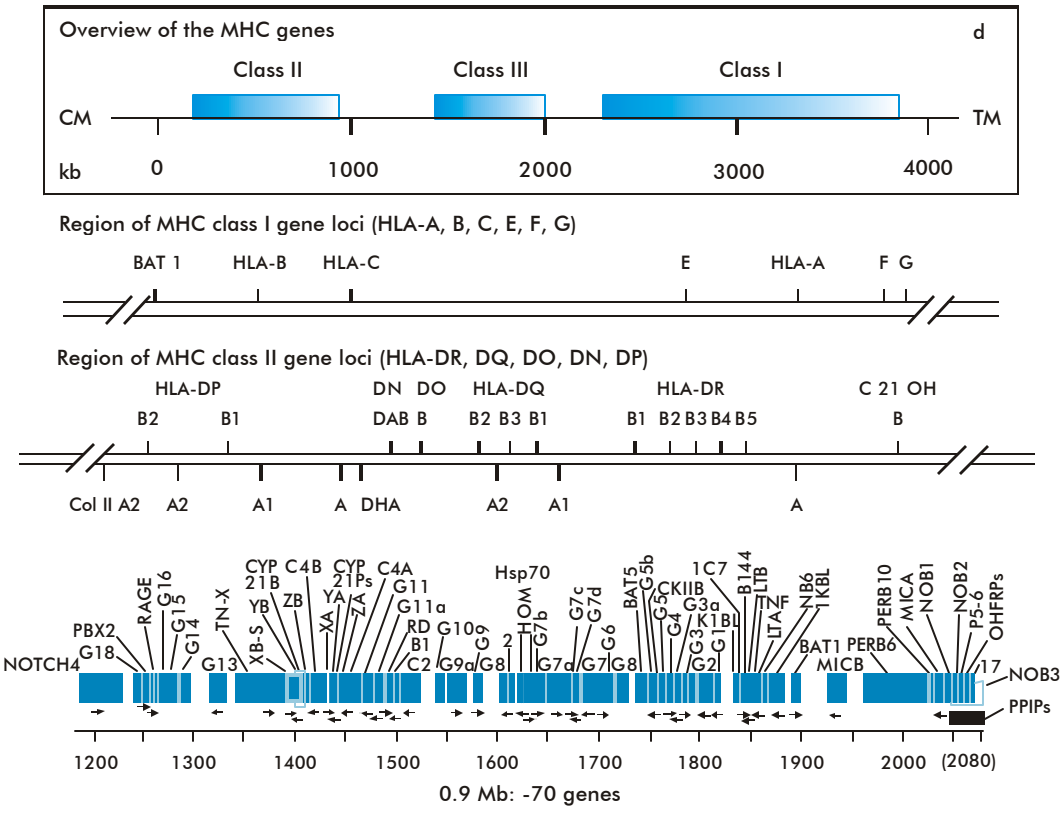

Figure 1. Genomic structure of the human MHC.

The general structure of HLA Class I, HLA Class II and IgM molecules show such a similarity in their subunits, that there is likely a common link between HLA and immunoglobulins, through some primordial cell surface receptor. The full 3-dimensional structure of HLA Class I molecules has been determined from X-ray crystallography [8]. This has demonstrated that the molecule has a cleft on its outermost surface, which holds a peptide. In fact, if a cell becomes infected with a virus, the virally induced proteins within the cell are broken down into small peptides and these are then inserted into this cleft during the synthesis of HLA Class I molecules. The role of HLA Class I molecules is to take these virally enhanced peptides to the surface of the cell and by linking them to the T-Cell receptor of a cytotoxic (CD8) T cell, demonstrate the presence of the virus. The CD8 T cell will now be "educated" and it will be able to initiate the process of killing cells which subsequently have that same viral protein/HLA Class I molecule on their surface. This role of HLA Class I, in identifying, changed cells (e.g. virally infected), is the reason why they must be present on all cells [6].

HLA Class II antigens

The cell surface glycopeptide antigens of the HLA-DP, -DQ and -DR loci are termed HLA Class II [3]. The tissue distribution of HLA Class II antigens is confined to the "immune competent" cells, including B-Lymphocytes, macrophages, endothelial cells and activated T-lymphocytes. The expression of HLA Class II on cells, which would not normally express them, is stimulated by cytokines such as interferon γ and in a transplant; this is associated with acute graft de-struction. HLA Class II molecules consist of two chains each encoded by genes in the "HLA Complex" on chromosome 6. The T cells, which link up to the HLA Class II molecules, are helper (CD4) T -cells. Thus the "education" process, which occurs from HLA Class II presentation, involves-the helper-function of setting up a general immune reaction that will involve cytokines, cellular and humoral defense against the bacterial (or other) invasion. This role of HLA Class II, in initiating a general immune response, is the reason why they need only to be present on "immunologically active" cells (B lymphocytes, macrophages, etc.) and not on all tissues [6].

HLA nomenclature

There are a number of ways of writing an HLA antigen. For example, it may be expressed as HLA-DR3, HLA-DRI7, HLA-DRB* 03 or HLA- DRBI*0301 [9]. These could all refer to the same antigen (Table 1). Firstly, "HLA" is the name for the gene cluster which tends to be inherited en-bloc. These HLA antigens are responsible for the presentation of "foreign" peptides (antigens) to the immune competent cells of the immune system [10].

The second part -e.g. DR- is the name of the specific *locus*. There are 6 loci (Figure 1) normally referred to. These are A, B, C, DR, DQ and DP. The HLA-A, B and C loci produce molecules (antigens) that normally present peptides of viral origin and are expressed on all nucleated cells. The HLA-A, B, C antigens are termed Class I. The HLA-DR, DQ and DP loci produce antigens that normally present peptides which have been broken down from bacterial or other proteins that have been engulfed by the cell in a process of immune surveillance. They are only expressed on cells actively involved' in the immune response, e.g. B lymphocytes monocytes and activated T lymphocytes.

Table 1. HLA Nomenclature.

Nomenclature	Indicates
HLA	The HLA region and prefix for an HLA gene
HLA-DRB1	A particular HLA loci i.e DRB1
HLA-DRB1*13	A group of alleles that encode the DR13 antigen
HLA-DRB1*1301	A specific HLA allele
HLA-DRB1*1301N	A null allele
HLA-DRB1* 130102	An allele that differs by synonymous mutation
HLA-DRB1* 13010102	An allele that contains a mutation outside the coding region
HLA-DRB1* 13010102N	A null allele that contains a mutation outside the coding region
HLA-DRB1*13X	High resolution not done
HLA-DRB1*13?	High resolution done but not discriminant

The HLA-DR, DQ and DP antigens are termed Class II. There are other Class I loci besides A, B and C and there are other Class II loci besides DR, DQ and DP. However, these loci are not normally tested for and their significance is not entirely clear. The third part, the number, e.g. 3, 17, 03, 0301, refers to the actual antigen at the locus. For example, the DNA in the gene region that we call the HLA-DR locus tends to be different from person to person. This difference will result in a different type of HLA-DR molecule. These different types of HLA-DR molecules are given names, such as DR17. Actually, HLA-DR 17 is the former way of writing this antigen-based on using antibodies that react to the antigens on the cells. Now we can look directly at the DNA and therefore the accuracy is much greate. The problem is that now we can see a lot more variation between the different antigens and so we need a different way of writing them. Hence, when we look at the antigens above: -HLA-DR3 is the broadest description of the antigen. It is the name for a specific group of antigens. The DR3 group can be divided into HLA-DR17 and HLA-DR18 by using antibodies (serology). When we look at this antigen at the DNA level we call the DR locus DRB 1 (because there are others termed A and B2, B3, etc.) and the antigen 03 (for the general antigen) and 01 for the specific variant of the 03. So, HLA-DR17 is now called HLA-DRB1*0301. This is similar for other antigens in the system, at either HLA Class II or Class I e.g. HLA-B60 (HLA-B*4001 molecularly).

How many HLA loci are there? The currently recognized loci are given in Table 2 [11]. Notice that HLA-DRB1 is the normal DR locus and the former DRw52 and DRw53 are DRB3 and DRB4 respectively.

The common HLA antigen and their molecular activity

The number of alleles that can now be recognized by molecular techniques is huge and is being increased

8. Browning M, Me Michael A. HLA and MHC: genes, molecules and function. London: Bios Scientific Publishers.1996.

9. Marsh SG, Albert ED, Bodmer WF, et al. HLA Nomenclature Report. Tissue Antigens 2002;60:407-64.

10. Tiercy JM, Marsh SG, Schreuder GM, Albert E, Fischer G, Wassmuth R. Guidelines for nomenclature usage in HLA reports: ambiguities and conversion to serotypes. Eur J Immunogenet 2002;29(3):273-4.

11. Tiercy JM, Marsh SG, Schreuder GM, Albert E, Fischer G, Wassmuth R. EFI Guidelines for Nomenclature usage in HLA Reports. European Journal of Immunogenetics 2002;29:273-4.

Table 2. Currently recognized HLA loci and those that are being routinely typed.

HLA Class	HLA-loci (genes)	Routinely typed?
Class I	HLA-A	Yes
Class I	HLA-B	Yes
Class I	HLA-C	Yes
Class I	HLA-E	
Class I	HLA-F	
Class I	HLA-G	
Class I	HLA-H	
Class I	HLA-J	
Class I	HLA-K	
Class I	HLA-L	
Class II	HLA-DRA	
Class II	HLA-DRBI	Yes
Class II	HLA-DRB2	
Class II	HLA-DRB3	Yes
Class II	HLA-DRB4	Yes
Class II	HLA-DRB5	Yes
Class II	HLA-DR86	
Class II	HLA-DRB7	
Class II	HLA-DRB8	
Class II	HLA-DRB9	
Class II	HLA-DQAI	
Class II	HLA-DQBI	Yes
Class II	HLA-DQA2	
Class II	HLA-DQB2	
Class II	HLA-DQB3	
Class II	HLA-OOB	
Class II	HLA-DMA	
Class II	HLA-DMB	
Class II	HLA-DNA	
Class II	HLA-DP AI	
Class II	HLA-DPBI	Not routine
Class II	HLA-DPA2	
Class II	HLA.DPB2	

rapidly. In table 2 we show the most frequent molecular variants and the alleles frequently typed.

Genetics of HLA

Routine tissue typing identifies the alleles at the three HLA Class I loci (HLA-A, -B, and -C) and the three Class II loci (HLA-DR, -DP and -DQ). Thus, as each chromosome is found twice (diploid) in each individual, a normal tissue type of an individual will involve 12 HLA antigens [12]. These 12 antigens are inherited co-dominantly that is to say, all 12 antigens are recognized by current typing methods and the presence of one does not affect our ability to type for the others. There are a number of genetic characteristics of HLA antigens, they are: Polymorphism, Inheri- tance, Linkage disequilibrium, Cross-reactivity [8].

Polymorphism

The polymorphism at the recognized HLA loci is extreme. Since the role of HLA molecules is to present peptides from invasive organisms, it is likely that this extreme polymorphism has evolved as a mechanism for coping with all of the different peptides that will be encountered. That is to say, each HLA molecule differs slightly from another in its amino acid sequence this is what we see as different HLA antigens. This difference causes a slightly different 3 dimensional structure in the peptide binding cleft, because different peptides have different shapes and charges. Human beings must have a large array of different HLA antigens, each with different shaped peptide binding area clefts to cope with all of these peptides. However, since the polymorphism is population specific, the frequent HLA antigens in different populations are clearly different [13]. For example, HLA-A34, which is present in 78% of Australian Aborigines, has a frequency of less than 1% in both Australian Caucasoid and Chinese. This has been reported in several HLA studies from various populations of the World [14, 15] and India [16-18]. Thus HLA antigens are of great significance in anthropological studies. Populations with very similar HLA antigen frequencies are clearly derived from a common stock [14, 15]. Conversely, from the point of view of grafts, which will be discussed later, it is very difficult to match HLA types between populations.

Inheritance of HLA

The normal way to present a tissue type is to list the HLA antigens as they are detected. There is no attempt to show which parent has passed on which antigen. This way of presenting the HLA type is referred to as a Phenotype [19]. An HLA phenotype is for example: HLA-A1, A3; B7, B8; Cw3, Cw4; DRI5, DR4. When family data is available, it is possible to assign each one of the antigens at each locus to a specific grouping known as a haplotype. A haplotype is the set of HLA antigens inherited from one parent. For example, the mother of the person whose HLA type is given above may be typed as HLA-A3, A69; B7, B45; Cw4; Cw9; DRI5, DRI7; Now it is evident that the A3, B7, Cw4 and DR15 were all passed on from the mother to the child above. This group of antigens is a haplotype.

In the absence of genetic crossing over, two siblings who inherit the same two HLA chromosomes (haplotypes) from their parents will be HLA identical. There is a *one* out of four chance that this will occur and therefore in any family with more than four children at least two of them will be HLA identical. This is because there are only two possible haplotypes in each parent.

Linkage disequilibrium

Basic Mendelian genetics states that the frequency of alleles at one locus does not influence the frequency of alleles at another locus (Law of independent segregation). However in HLA genetics this is not true. There are a number of examples from within the HLA system of alleles at different loci occurring together at very much higher frequencies than would be expected from their respective gene frequencies. This is termed linkage disequilibrium. The most extreme example is in Caucasians where the HLA-A1, B8, DR3 (DRBI*O301), DQ2 (DRB1*O201) haplotype is so conserved that even the alleles at the complement genes (Class III) can be predicted with great

12. Sullivan KA, Amos DB. The HLA system and its detection. In: Rose NR, FriedmilO H, Fahey JL, editors. Manual of clinical laboratory immunology, 3rd ed Washington: ASM *Press,* 1986.

13. Jin P, Wang E. Polymorphism in clinical immunology|From HLA typing to immunogenetic profling. J Transl Med 2003; 1:1-11.

14. Piancatelli D. Human leukocyte antigen-A, -B, and -Cw polymorphism in a Berber population from North Morocco using sequence-based typing. Tissue Antigens 2004. 26:78-87.

15. Clayton J, Lonjou C. Allele and haplotype frequencies for HLA loci in various ethnic groups. In: Charron D, editor. Genetic diversity of HLA, functional and medical implications. Paris: EDK Publishers, 1997;1:665-820.

16. Shankarkumar U, Pednekar SV, Gupte S, Ghosh K, Mohanty D. HLA antigen distribution in Marathi speaking hindu population from Mumbai, Maharashtra, India. J Hum1999;105:367-72.

17. Mehra NK, Taneja V, Kailash S, Raizada N, Vaidya MC. Distribution of HLA antigens in a sample of North Indian. Hindu population. Tissue Antigens1986; 27:64-74.

18. Pitchappan RM, Kakkanaiah VN, Rajasekar R, Arulraj N, Muthukkaruppan VR. HLA antigens in South India: I. Major groups of Tamil Nadu. Tissue Antigens 1984;24:190-6.

19. Thomas DG, Francis SC, David G, editors. Principles of medical genetics. Baltimore: Williams and Wilkins, 1998.

accuracy. Similar haplotypes are observed in selected caste groups and tribal groups of India [11]. Also, at HLA Class II, this phenomenon is so pronounced, that the presence of specific HLA-DR alleles can be used to predict the HLA-DQ allele with a high degree of accuracy before testing. Because of linkage disequilibrium, a certain combination of HLA Class I antigen, HLA Class II antigen and Class III products will be inherited together more frequently than would normally be expected. It is possible that these "sets" of alleles may be advantageous in an immunological sense, having a positive selective advantage.

Cross-reactivity

Cross-reactivity is the phenomenon whereby one antibody reacts with several different antigens, usually at one locus (as opposed to a mixture of antibodies in one serum) [20]. This is not surprising since it has been demonstrated that different HLA antigens share exactly the same amino acid sequence for most of their molecular structure. Antibodies bind to specific sites on these molecules and it would be expected that many different antigens would share a site (or epitope) which will bind to a specific antibody. Thus cross-reactivity is the sharing of epitopes between antigens.

The term CREG is often used to describe "Cross reacting groups" of antigens. It is useful to think in terms of CREG's when screening sera for antibodies, as most sera found are "multi-specific" and it is rare to find operationally monospecific sera. The rarity of monospecific sera means that most serological tissue typing uses sera that detects more than one specificity, and a typing is deduced by subtraction. For example, a cell may react with a serum containing antibodies to HLA-A25, A26, and A34 and be negative for pure A26 and pure A25 antisera. In this case, HLA-A34 can be assigned, even in the absence of pure HLA-A34 antisera.

HLA typing

Traditionally, HLA antigens have been defined using serological techniques. These techniques rely on obtaining viable lymphocyte preparations (for HLA Class II typing, B lymphocytes are needed) and the availability of suitable antisera to recognize the HLA antigens. During the last few years, DNA-based typing techniques have begun to replace the serological techniques in clinical applications. The DNA methods were initially applied to Class II typing, but more recently they have been used to determine Class I alleles. While serology performed adequately in typing family members, it proved unsatisfactory in typing unrelated donors for bone marrow transplantation, once the extent of polymorphism was known within "serologically identical" specificities. DNA typing also proved invaluable when serological typing was difficult (poor cell viability or expression) and in confirming or refuting phenotypic homozygosity.

Although DNA techniques were introduced to many Clinical Tissue Typing Laboratories with their involvement in the 10th International Histocompatibility Working Group (IHWG) in 1987 [21], studies in a limited number of research laboratories had preceded the Workshop by several years (Table 3).

Table 3. Milestones in the development of DNA-based typing for HLA technological events and major applications.

Year	Event
1980-82	cDNA sequencing
1987	10th International Histocompatibility Workshop
	New York _ RFLP
1987	advent of PCR
1987-89	class II SSOP
1988	RFLP class II typing with one enzyme
1988-91	DNA techniques for HLA typing in diseases
1990	heteroduplexes
1991	optimization of graft survival using RFLP
1991	11th International Histocompatibility Workshop
	Yokohama - class II SSOP evaluation
1992	HLA-DNA typing in Anthropology studies
1992	class II SSP
1992-onward	exponential increase in number of new HLA alleles identified
1992-93	class I SSOP
1993	class I SSP
1993-94	Sequencing Based Typing (SBT)
1995	HLA-DNA typing in bone marrow transplantation
1996	DNA typing for non-classical HLA loci
1996	12th International Histocompatibility Workshop
1996	Paris _ class I SSP and SSOP evaluation
1997	Reference-Strand Conformational Analysis (RSCA)

Restriction fragment length polymorphism (RFLP)

The 10th IHWG RFLP protocol included a total of 12 enzymes and 13 probes, defining a large number of restriction fragments [22]. RFLP bands identified with a DR probe correlated not only with serologically defined DR antigens and splits but also sometimes with Dw typing. Taq I was recommended as the enzyme of choice because of its ability to identify a large number of polymorphisms with DR, DQ and DQa hybridisation. The recognition site of Taq I includes the nucleotide dimer CpG and restriction sites containing this dimer show a higher frequency of polymorphism in human DNA than other restriction sites [23].

One of the novel ideas (and a sign of the future) was the use of 19 base pair oligonucleotides as probes based on sequence information [24-28]. As the probes also hybridized to other genomic sequences, restriction enzyme digestion and gel electrophoresis were required to separate the target sequence from the bulk of the DNA. In addition, due to the small number of available copies of the relevant DNA, this approach lacked sensitivity. A refinement of this method was to use the oligonucleotides to probe total RNA, as nonspecific bindings were not found on Northern blots [29, 30].

20. Shankarkumar U. Studies on the immunogenetic basis of selected diseases in South India. Ph. D. thesis. Madurai: Madurai Kamaraj University.1994.

21. Dupont B, Ed.: Immunobiology of HLA. New York: Springer-Verlag. 1989.

22. Bignon D, Semana G, Tiercy M, Simons M, Lalouellm, Cohen D. DNA-RFLP analysis: HLA-DR beta workshop report. In: Dupont B, ed. Immunobiology of HLA. New York: Springer-Verlag, 1989:851-60.

23. Barker D, Schafer M, White R. Restriction sites containing CpG show a higher frequency of polymorphism in human DNA. Cell 1984:36:131-8.

24. Angelini G, Prev Al Cd, Gorski J, Mach B. High-resolution analysis of the human HLA-DR polymorphism by hybridization with sequencespecific oligonucleotide probes. Proc Natl Acad Sci USA 1986;83: 4489-93.

25. Gorskij, *et al*. HLA-DRa, DXa and DR_III gene association studies in DR3 individuals. Hum Immunol 1987;20: 273-8.

26. Amar A, Holbeck Sl, Nepom G. Specific allelic variation among linked HLA class II genes. Transplantation 1987;44:831-5.

27. Le Gall I, *et al*. Two DR_ allelic series defined by exon II specific synthetic oligonucleotide genomic hybridization: a method of HLA typing Proc Natl Acad Sci (USA) 1986;83:7836-40.

The RFLP methods also had their disadvantages. They did not directly identify the polymorphic coding sequences within the second exon of DR, DQ and DQa, but relied on polymorphic restriction sites generally located outside these exons. In addition, they required the use of DR-DQ associations to discriminate between certain DR alleles that had identical DR_ RFLP patterns. Thus, care was needed to apply the system to non-Caucasian populations. The method was cumbersome and could take up to 16 days to produce results for only 24 samples. A non-radioactive RFLP method was described [31] using digoxigenin and chemiluminescence, but by this time fundamentally different techniques were being developed. Eventually, RFLPs were replaced, but not before the results from their use had stimulated the development of better methods for DNA typing.

Polymerase chain reaction (PCR)

Emergence of nucleotide sequence data for the alleles of HLA genes permitted the rapid development of many PCR-based techniques and reagents. Conversely, the PCR technique greatly reduced the effort required in subsequent sequencing of new alleles [32, 33]. PCR-based methods may be broadly classified into three categories: (i) those which generate a product containing internally located polymorphisms which can be identified by a second technique (e.g. PCR-sequence specific oligonucleotide (SSG) probing, PCR-RFLP, PCR followed by sequencing); (ii) those in which the polymorphism is identified directly as part of the PCR process, although there are post-amplification steps (e.g. PCR-sequence specific primer (SSP)); and (iii) conformational analysis in which different mutations generate specific conformational changes in PCR products. The latter are identified by electrophoretic analysis, e.g. heteroduplex analysis, single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE). The two methods most frequently adopted in clinical histocompatibility laboratories have been SSG and SSP, although at regular intervals a novel method or a novel variation of an existing method is reported [34].

Sequence specific oligonucleotides (SS0)

Hybridisation of PCR-amplified DNA with sequence specific oligonucleotide (SSO) probes was the first molecular typing method used to detect HLA Class II alleles. Specificity for a particular HLA locus was achieved by selecting PCR primers specific for a sequence in the conserved region of the second exon. The literature contains many alternative methods for SSO typing. The main differences between these were the length, the DNA sequence of oligonucleotide probes, the reporter molecule and its detection method. Initially, 32P-labelled allele-specific oligonucleotides were hybridized to an amplified conserved region of exon 2 of the HLA-DQa gene [35], but soon afterwards biotin was used as a label [36]. The PCR-SSO method was quickly applied to other loci, DP_[37], DQ_ [38, 39] and DR_[40, 41], with various procedures using [32]p, biotin or horseradish peroxidase-labelled probes. Methods in the clinical laboratory have tended to use either a substrate in a staining development system [42] or a substrate that generates a chemiluminescent signal [43].

The SSO method can be customized for each application. For example, the approximation of HLA-DR serological specificities requires the detection of shared polymorphic sequences which encode the epitopes detected by antibodies. These shared sequences identify families of alleles that belong to the same serologically-defined specificity groups. This level of typing is often referred to as "low resolution" or "generic" SSO. Alternatively, "high resolution" SSO typing can distinguish all known alleles. High resolution SSO usually requires selective amplification of a group of related alleles. For example, all HLA-DRBI *04 alleles are specifically amplified with selected PCR primers and then the DNA is hybridized with a panel of probes that distinguish each HLA-DRBI *04 allele [44]. The SSOP typing strategy for HLA-DP has been especially difficult because of the complex sharing of multiple sequence motifs between different DP alleles. The use of either a large number of probes, or the separation of the alleles by the digestion of the PCR-amplified DNA with a restriction enzyme, was suggested to overcome this [34]. Allele-specificity of certain heterozygotes has also been problematic (and as discussed later even greater for Class I typing) because of the ambiguous interpretations inferred from the hybridisation patterns of the SSO probes. One solution has been the use of primers differing from each other at the 3' end to prevent the amplification of DPwl alleles, but allowing the amplification of closely homologous alleles DPw3 and DPw6, to reduce the number of oligo-nucleotide probes required [45].

Today, most laboratories prepare one membrane for each probe in the assay. This procedure is facilitated using a 96-well manifold. The use of automation not only eliminates the reuse of membranes after hybridization, but it also minimizes sample to sample variation in loading and provides a relatively large surface area to aid in the evaluation of hybridisation dots of varying intensities. Other laboratories have used a robotic work station for both this and the amplification aspect of the technique [46].

Many laboratories reduce the number of different washing temperatures by including tetramethylammonium chloride (TMAC), which reduces the effect of the GC content on the stability of the hybrids and, providing the probes are of the same length, it enables the membranes to be washed at the same temperature. Some laboratories avoid the use of TMAC due to its toxic properties. Although this could lead to a requirement for many water-baths, the problem can be minimized by working simultaneously with those membranes which require the same washing temperature [47].

The SSO technique has proven to be very reliable, robust and accurate. Good amplification always gives a clean and clear-cut SSOP hybridisation, while almost all of the problematic typing results are due to poor amplification [48]. In a quality control exercise, the percentage of correct HLA typing was 99.8% for HLA-DR and 99.8% for HLA-DQB1 based on 9 244 HLA-DRBI and 7 244 HLA-DQB1 assignments [49]. A recent study using two different SSOP methods in

28. Tiercy JM, Gorski J, Betuel H, Freidel AC, Gebuhrer L, Jeannet M, Mach B. DNA typing of DRw6 subtypes: correlation with DRB1 and DRB3 alleleic sequences by hybridization with oligonucleotide probes. Hum Immunol1989;24:1-14.

29. Ucla C, Van Rood J, Gorski J, Mach B. Analysis of HLA-D micropolymorphism by a simple procedure: RNA oligonucleotide hybridization. J Clin Invest 1987;80: 1155-9.

30. Freeman SM, Noreen HJ, Bach FH. Oligonucleotide probing. Applications to HLA typing. Arch Pathol Lab Med 1988;112:22-7.

31. Bidwellll, *et al*. HLA-DR and -DQ ty-ping by DNA-RFLP analysis. In: Hill KM, Bidwell IL, eds. Handbook of HLA typing techniques. London: CRC Press 1993; 71-98.

32. Gyllensten VB, Erlich HA. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc Natl Acad Sci (USA) 1988;85:7652-6.

33. Ennis Pd, Zemmourj, Salter Rd, Parham P. Rapid cloning of HLA-A, B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. Proc Natl Acad Sci (USA) 1990;87:2833-7.

34. Entz P, Toliat MR, Hampe J, Valentonyte R, Jenisch S, Nurnberg P, Nagy M. New strategies for efficient typing of HLA class-II loci DQB1 and DRB1 by using Pyrose-quencing. Tissue Antigens 2005;65(1): 67-80.

35. Saiki RK, Buga Wan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified-globin and HLA- DQa DNA with allele-specific oligonucleotide probes. Nature 1986;324:163-6.

36. Saiki R, Chang CA, Levenson CH, Boehm CD, Kazazian RH, Erlich HA. Rapid genetic analysis of enzymatically-amplified DNA with non-radioactive allele-specific oligo-nucleotide probes. Am J Hum Genet 1987;41:A237.

37. Bugawan TL, Horn GT, Long CM, Mickelson E, Hansen JA, Ferrara GB, Angelini G, Erlich HA. Analysis of HLA-DP alleleic sequence polymorphism using the in vitro enzymatic DNA amplification of DPa and DP-loci. J Immunol1988;141:4024-30.

38. Horn GT, Buga Wan TL, Long CM, Erlich HA. HLA class II antigens and susceptibility to insulin-dependent diabetes mellitus. Proc Natl Acad Sci (USA) 1988;85: 6012-16.

39. Morel C, Arlen F, Jeannet M, Mach B, Tiercy JM. Complete analysis of HLA-DQB1 polymorphism and DR-DQ linkage disequilibrium by oligonucleotide typing. Hum Immunol 1990;29:64-77.

40. Erlich HA, Scharf SJ, Long C M, Horn GT. Analysis of isotypic and allotypic sequence variation in the HLA-DRb region using the *in vitro* enzymatic amplification of specific DNA segments. In: DUPONT B, ed. Immunobiology of HLA. New York: Springer-Verlag 1989;181-7.

41. Baxter-Lowe LA, Hunter B, Casper JT, Gorski J. HLA gene amplification and hybridization analysis of polymorphism. J Clin Invest 1989;84:613-8.

different laboratories found an error rate of 0.1 % in testing 2 604 samples for HLA-DP [50].

Reverse dot blot

Although the SSO technique is perfectly suited for analyzing large numbers of samples, it is not suitable for analyzing individuals or small numbers. The alternative technique of reverse dot blotting uses the same amplification procedure and SSO probes, but the panel of probes is bound to a solid support and the amplified sample is labeled and hybridized to that support. A single hybridisation and stringency wash allows the detection of sequence polymorphisms present in the chosen sample. There are several variations of the reverse dot blot. One method uses poly-T tails to attach the oligonucleotide to the membrane, thus increasing the binding efficiency of the oligonucleotide and sparing the specific binding sequence from forming the attachment bonds. The immobilized array of probes hybridizes to the PCR product which has incorporated biotinylated primers during the amplification process [51]. Streptavidin horseradish peroxidase conjugate is added and positive reactions are detected using a colored soluble substrate. Alternatively, positive signals are visualized by chemiluminescence [52]. In another method, oligonucleotides are coated to a microtitre plate by passive absorption and are used as capture probes [53]. Two invariant oligonucleotides covalently linked to horseradish peroxidase are used as detection probes. The hybridisation of the denatured amplified DNA sample with the bound capture probes and fixation of the detection probes to the DNA are carried out in a single step.

A third method is performed in a 96-well tray. The DNA to be investigated is amplified as in a normal PCR, with the 3' primer biotinylated in order to be able to attach the PCR product directly to the avidin-coated tray [54]. The synthetic oligonucleotides are produced with an amino group at the 5' end and conjugated with alkaline phosphatase (ALP). Binding of the ALP-labeled probes is detected by an amplified colorimetric assay, whereby the ALP generates a product that initiates a secondary cyclic enzyme reaction and results in a 30- to 50-fold greater sensitivity. A similar method uses digoxigenin labeling of the DNA during PCR and hybridisation to a panel of biotinylated SSO probes [55]. The capture of the target DNA by the SSO is revealed by an anti-digoxigenin-horseradish peroxidase conjugate. The hybridisation protection assay uses chemiluminescent acridinium-ester-Iabelled oligonucleotide probes [56]. Perfect matching with the amplified DNA stabilizes the acridinium-ester against hydrolysis. Thus, only fully matched hybrids emit chemiluminescence which is detected by automated luminometry.

Class I SSO

DNA typing techniques were initially applied to Class II rather than Class I genes for several reasons. The requirement for replacing Class II serology was thought to be more urgent as serological typing for Class II antigens was difficult (HLA-DR and -DQ) or impossible (HLA-DP). Class II was thought to be more important for transplantation and disease association. Furthermore, DNA typing for Class I was destined to be more complex because sequence polymorphisms in Class I genes are located in two exons [57, 58].

The greatest problem in Class I typing has been the large number of probes required to give unambiguous results for certain heterozygote individuals, i.e. instances where two or more pairs of alleles give the same probe reaction pattern. The choice in SSO for Class I has basically come down to whether a laboratory prefers performing one PCR amplification and using a very large number of probes, or using a two stage typing system (as in HLA-DR) with an initial medium resolution followed by a high resolution, depending on the results of the first typing. Regardless of which method is used, allele typing is much more difficult for HLA-B than HLA-A, due to the vast polymorphism at HLA-B. Some laboratories use a two-stage system. For example, 27 probes for HLA-A medium resolution. Using 27 probes are used results in eight theoretical ambiguous probe patterns which involve alleles from different serology groups. However, in a Caucasian population of 5 000 typed by PCR-SSO, only four of these patterns occurred on a total of 19 occasions [59]. Then use four high resolution systems composed of group-specific amplification at exons 2, 3 and 4 to define all HLA-A alleles. A similar scheme has been developed for HLA-B alleles. After the first stage medium resolution, the high resolution is composed of six separate systems using a total of 159 probes. This keeps the number of probes at a minimum and has the flexibility of typing to the level required, according to the nature of the sample. These high resolution systems also remove the previously mentioned hete-rozygote ambiguities [10].

Other laboratories use a battery of probes that enable the initial detection at the allele level. For example, one report used 91 HLA-A locus probes to identify all HLA-A alleles with the exception of A *0201 from A *0209, and A *0207 from A *0215N, which only differ in exon 4 [60]. A compromise between these two strategies has been devised by another group [61]. To obtain high resolution and yet limit the number of probes, they used variations at the HLA-B locus in codons 45 and 46 for amplification and initial screening with SSG probes spanning codons 40 to 48. Once the group of alleles was determined, the corresponding group-specific amplifications were performed. So far, the Class I systems developed have concentrated on identifying sequence differences within exons 2 and 3. In 1995, it was predicted that amplifying exons 2 and 3 would lead to the non-identification of only one of 1081 pairs of HLA-A alleles, three of 4851 pairs of HLA-B alleles and one of 528 for HLA-C [62] . This is because polymorphisms in exon 4 are linked with polymorphisms in exons 2 and 3. However, the mutations found in exon 4 which lead to a profound functional difference between null and expressed alleles will make typing, to include exon 4, desirable in the future [63, 64].

Now that some laboratories have SSOP technology available for Class I as well as Class II, the SSOP method has become popular in typing potential donors for marrow donor registries, especially in the USA.

42. Nevinny-Stickel C, Bettinotti MD, Andreas A, Hinzpeter M, Muhlegger K, Schmitz G, Albert ED. Non-radioactive HLA class II typing using polymerase chain reaction and digoxigenen-11-2'-3'dideoxyuridinetriphosphate-labelled oligonucleotide probes. Hum Immunol 1991;31:7-13.

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

44. Gao X, Fernandez-Vina M, Shumway W, Stastny P. DNA typing for class II HLA antigens with allele-specific or group -specific amplification. 1. Typing for subsets of HLA-DR4. Hum Immunol 1990;27:40-50.

45. Fugger L, Morling N, Ryder Lp, Odum N, SveGaard A. Technical aspects of typing for HLA-DP alleles using allele-specific DNA *in vitro* amplification and sequence-specific oligonucleotide probes. Detection of single base mismatches. J Immunol Methods 1990;129:175-85.

46. Cailla TS, Garchon HJ, Bach F. Automation of large-scale HLA Class II oligotyping using a robotic work station. In: Tsuki K, Alzawa M, Sasasuki T, eds. HLA 1991. Oxford: Oxford University Press, 1992:346-7.

47. Oh SH, Fleischhauer K, Yang SY. Isoelectric focusing subtypes of HLA-A can be defined by oligonucleotide typing. Tissue Antigens 1993;41(3):135-42.

48. Shankarkumar U, Ghosh K, Badakere H, Mohanty D. Novel HLA Class I Alleles Associated with Indian Leprosy Patients. Journal of Biomedicine and Biotechnology 2003;3:208-11.

49. Ng J, Hurley CK, Carter C, Baxter-Lowe LA, Bing D, Chopek M,, et al. Large-scale DRB and DQB1 oligonucleotide typing for the NMDP registry: progress report from year 2. Tissue Antigens 1996;47:21-6.

50. Noreen H, Steiner L, Davidson M, Johnson S, Segall M, Begovich Ab. Six new DPB1 alleles identified in a study of 1,302 unrelated bone marrow donor-recip-ient pairs. Tissue Antigens 1997;49:512-6.

51. Buga W An TL, Begovich AB, Erlich HA Rapid HLA-DPB typing using enzymatically amplified DNA and non-radioactive sequence-specific oligonucleotide probes. Immunogenetics 1990;32:231-41.

52. Buyse I, Decorte R, Baens M, Cuppens H, Semana G, Emonds MP, et al. Rapid DNA typing of class II HLA antigens using the polymerase chain reaction and reverse dot blot hybridization. Tissue Antigens 1993;41:114.

53. Cros P, Allibert P, Mandrand B, Tiercey LM, Mach B. Oligonucleotide genotyping of HLA polymorphism on microtitre plates. Lancet 1992;340:870-3.

54. Lazaro AM, Fernandezvina MA, Lru Z, Stastny P. Enzyme-linked DNA oligotyping. A practical method for clinical HLA-DNA typing. Hum Immunol 1993;36:243-8.

55. Nevinny-Stickel C, Albert Ed. HLA class II typing in a microtitre plate formate using digoxigenin-labelled amplified DNA and biotin-labelled oligonucleotide probes. Eur J Immunogenet 1993;20:419-27.

Complementary strand analysis

Any new allele can be accommodated by a single-step typing system, but the technique becomes unwieldy due to the large number of probes required. Madrigal and colleagues have attacked the problem at its roots with the "complementary strand analysis", i.e. they decided to separate the allelic products after a locus-specific PCR amplification. They developed a method that uses 40 oligonucleotide probes complementary to universal sequences of Class I alleles, termed the universal recombinant site targeting oligonucleotide (ORSTO) [65]. This method allowed the identification of 201 of the 221 known Class I alleles at that time. Six alleles could not be identified as their substitutions are outside exons 2 and 3 and the other 14 allele pairs required additional probes for identification.

Ligation-based typing

Another unique variation of PCR and oligonucleotide probes is Ligation-based typing. Initially applied to Class II alleles [66] but more recently to HLA-B27 testing [67], the method requires group-specific PCR amplification. Subsequently, an aliquot of this product is incubated with a heat stable ligase and with a pair of oligonucleotide probes designed to hybridize adjacently to the specific sequences of the amplified DNA. If the probes are perfectly complementary, they become ligated and are detected through their different labels by an enzyme-linked immunosorbent assay (ELISA). Otherwise, they remain separate and the ELISA is still negative.

PCR-RFLP

Initially, this method used the availability of sites in the nucleotide sequences to employ restriction endonucleases which recognized allelic variations, to digest PCR-amplified HLA genes (HLA-DR, -DQ, -DP) [68-70]. However, small bands located close to each other on the polyacrylamide gels may sometimes obscure the precise analysis and some heterozygotes cannot be discriminated [71]. These problems have been overcome for HLA-DR by a modified PCR-RFLP method using informative restriction enzymes, which have a single recognition site present in some alleles but not in others, and using group-specific primers to avoid cross-hybridisation with other genes [72, 73]. This method was also applied to HLA-DQB1, -DQA1 and -DPB1 ge-nes [74, 75] and, in some instances, the simultaneous digestion of amplified DNA with two or more enzymes has been applied [76]. One of the first indications that HLA-DP matching may be important in bone marrow transplantation was reported using the PCR-RFLP method [77]. A recent innovation was the use of consecutive rounds of PCR-RFLP. After the first digestion of the PCR product, the cleaved fragment was extracted from the gel and used as a template for a second PCR-RFLP [78].

Sequence based typing (SBT)

One of the drawbacks of SSO or RFLP is that, although they are capable of detecting a single base difference in DNA sequence between two alleles, they are not likely to detect a new undefined allele, unless the variation happens to be at the specific site detected by the probe or the enzyme used for restriction. Methods based on sequencing have come foreward. Initially, these were manual sequencing methods [79-81]. The initial and more comprehensive of these methods did not require any additional typing information, e.g. serology, and was later applied to Class I alleles [82]. After the isolation of RNA (in itself a limitation of the applicability of the technique) and reverse transcription to cDNA, six simultaneous PCR sequencing reactions were performed, four for DRB and one each for DQB1 and DQA1, before direct sequencing of the double stranded amplified DNA. The SBT technology advanced with the introduction of dye-labeled primers and fluorescent automated sequencing [83]. Group-specific amplification is performed to limit the number of allele sequences in any sequencing template; otherwise DNA from both haplotypes would be present. This, in turn, simplifies the software-based allele assignments-suitable computer software that was one of the greatest problems with this technique[84].

In a recent comparative study between two centers, it was concluded that neither the manufacturer of the DNA sequencer nor the sequencing method influenced the result. The most critical step was the amplification reaction [85, 86]. Sometimes, preferential amplification was observed, whereby one allele was more likely to be amplified than another when they were present in the heterozygote state.

There are several reports of the application of SBT in typing Class I alleles. In one study, it was shown that, despite the strong linkage disequi-librium between HLA-B and -C alleles, 12.5% of HLA-A, -B, -DR, -DQ identical bone marrow transplant pairs had an HLA-C disparity detected by the sequencing strategy [87]. In another study by the same group, the previous sequence of HLA-B*4402 was corrected and the cell which had originally been sequenced as B*4401 was found to express B*4402 [88] instead.

The great advantage of SBT is its accuracy. It is the only technique which directly detects the nucleotide sequences of an allele, thus allowing an exact assignment. It requires very expensive equipment and sophisticated laboratory approaches in order to give unambiguous high resolution typing results. Nevertheless, it should be only a matter of time until generally accepted, easy to perform protocols will be available, thus leading to a wider use of SBT.

Sequence specific primers (SSP)

In the early 1990s, publications appeared on a method called PCR-SSP [89, 90], the design of which was based on the amplification refractory mutation system (ARMS) [91]. The principle of this method is that a completely matched primer will be more efficiently used in the PCR reaction than a primer with one or several mis-matches. Specificity is determined by the use of sequence specific primers in which a 3' single-base mismatch inhibits the priming of non-specific reactions [79]. Because Taq polymerase lacks 3' to 5' exonuclease activity, even if primer pairs do anneal non-specifically, they will not amplify efficiently. Thus only the desired allele or alleles will be amplified and the amplified product can then be detected by agarose gel electrophoresis.

56. Matsubara K, Koide Y, Kobayashi A, Kaida R, Takeda S, Matsuda E, et al. A rapid and sensitive method for HLA-DRB1 typing by acridinium-ester-labelled DNA probes. Hum Immunol1992;35:132-9.

57. Etokebe GE, Opsahl M, Tveter AK, Lie BA, Thorsby E, Vartdal F, Spurkland A. Physical separation of HLA-A alleles by denaturing high-performance liquid chromatography. Tissue Antigens 2003; 61:443-50.

58. Cox ST, McWhinnie AJ, Robinson J, Marsh SG, Parham P, Madrigal JA, Little AM. Cloning and sequencing full-length HLA-B and C genes. Tissue Antigens 2003; 61(1):20-48.

59. Williams F, Mallon E, Middleton D. Development of PCR-SSOP for HLA-A typing of bone marrow registry donors. Tissue Antigens 1997;49:61-6.

60. Shankarkumar U, Prasanavar D, Ghosh K, Mohanty D, et al. HLA A*02 allele frequencies and B haplotype associations in Western Indians. Hum. Immunol. 2003;64:552-556.

61. Fernandez-Vina M, Lazaro AM, Sun Y, Miller S, Forero L, Stastny P. Population diversity of B-locus alleles observed by high-resolution DNA typing. Tissue Antigens 1995;45:153-68.

62. Parham P, Adams EJ, Arnett KL. The origins of HLA-A, B, C polymorphism. Immunol Rev 1995;143:141-80.

63. Ishikawa Y, Tokunaga K, Tanaka H, Nishimura M, Muraoka M, Fujii Y, et al. HLA-A null allele with a stop codon, HLA-A*0215N, identified in a homozygous state in a healthy adult. Immunogenetics 1995;43:1-5.

64. Magor KE, Taylor EJ, Shen SY, Martinez-Naves E, Valiante NM, Wells RS, et al. Natural inactivation of a common HLA allele (A *2402) has occurred on at least three separate occasions. J Immunol, 1997;158: 5242-50.

65. Arguello R, Avakian H, Goldman LM, Madrigal A. A novel method for simultaneous high resolution identification of HLA-A, HLA-B and HLA-Cw alleles. Proc Natl Acad Sci (USA) 1996;93:10961-5.

66. Fischer GF, F Ae I, Petrasek M, Moser S. A combination of two distinct *in vitro* amplification procedures for DNA typing of HLA-DRB and -DQB1 alleles. Vox Sang 1995;69:328-35.

67. Fischer GF, Fae I, Moser S, Petrasek M, Bassler HM, Menzel GR, Mayr WR, et al. Ligation based HLAB*27 typing. Tissue Antigens 1996;48:148-52.

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

69. Maeda M, Murayama N, Ishii H, Uryu N, Ota M, Tsuji K, Inoko H. A simple and rapid method for HLA-DQA1 genotyping by digestion of PCR-amplified DNA with allele specific restriction endonucleases. Tissue Antigens 1989;34:290-8.

70. Maeda M, Uryu N, Murayama N, Ishii H, Ota M, Tsuji K, Inoko H. A simple and rapid method for HLA-DP genotyping by digestion of peR-amplified DNA with allele specific restriction endonucleases. Hum Immunol, 1990;27:111-21.

Other researchers have used multiplex PCR, i.e. having several primer pairs in the same reaction [92]. Sizing of the PCR product is necessary for interpretation, requiring a longer run of the gel to separate the PCR fragments. Most researchers have found it unnecessary to perform nested PCR amplifications to obtain specific typing results, as suggested by others [93].

A multi-centre report analyzed PCR-SSP for HLA-DR and -DQ [94]. Whereas the agreement was 98% for HLA-DR typing, it was 91% for HLA-DQ typing. The authors concluded that a possible missed allele cannot be ruled out when only one allele is defined, a situation that has always been of concern. The nine laboratories all had pre-pipetted PCR amplification trays and one of the reasons given for the lower rate of agreement for DQ testing was the loss due to diffusion of pre-pipetted PCR reaction mixes. A similar reason may have been the cause of the problem with the SSP technique encountered by several laboratories during the 12th IHWC. Class I SSP techniques were being tested for the first time by a large number of laboratories and this may also have been a contributing factor. During a recent comparison of SSO and SSP typing on kidney graft pairs (zero mismatched by serology for HLA-A, -B), the primer pair used to amplify HLA-A*02 failed with several samples [95].

The SSP method is ideal for typing individual samples, but is costly and requires high capacity thermal cyclers for larger numbers of samples. One laboratory reduced this problem by instigating a two-stage technique-low resolution followed by high resolution according to the first result [96]. Since the method takes less than five hours, it can be applied to cadaveric grafting.

Sequence specific priming, exonuclease-released fluorescence (SSPERF)

The SSP method suffers from the disadvantage that the end-step of gel electrophoresis is not suitable for large numbers of samples or for automation. A novel method has been reported which removes the electrophoresis and combines high throughput with speed and high resolution. The method uses fluorogenic probes, each of which has a reporter and a quencher dye. When the probe is intact, the proximity of the two dyes results in the suppression of the reporter fluorescence. During PCR-SSP, if the target of interest is present, the probe specifically anneals between the forward and reverse primer site. The nucleolytic activity of the Taq polymerase cleaves the probe, resulting in an increase in fluorescence. Taq polymerase does not cleave the free probe, the enzyme requires sequence complementarity between the probe and template for cleavage to occur. After cleavage, the shortened probe dissociates from the target and polymerization of the strand continues. This process occurs in every cycle and allows a direct detection of the PCR product. Using the 14 sequence specific primer pairs selected originally by Olerup, *et al.* and three probes, Trucco and his colleagues applied the method to HLA-DQB1 allelic typing [97]. Other researchers have applied this method to Class I typing using two different fluorogenic probes and 24, 48 and 16 primer mixes for HLA-A, -B and -C, respectively [98].

Heteroduplexes

During the primer-annealing stage of each cycle of the PCR, a proportion of coding strands of each DRB locus allele may hybridize to the non-coding strands of a different DRB locus allele and vice versa. This double-stranded DNA will thus be mismatched in some regions (heteroduplexes), leading to alterations in the conformation of the DNA molecule. This conformation varies for each DR haplotype and can be detected by modified migration in non-denaturing polyacrylamide gels, as the heteroduplex will move more slowly than the homoduplexes (complementary strands). A single mismatch of the nucleotide can cause a marked electrophoretic retardation and thus even subtypes involving a single substitution can be detected, although irrelevant silent mutations can complicate the band patterns. PCR-heteroduplex analysis was first applied to HLA matching in 1990, in an analysis of HLA-DR allotypes encoded by the second exons of HLA-DRB genes [99].

Additional bands are formed in heterozygotes which are not present in either of the patterns of the individual alleles and are caused by heteroduplex formation in trans, i.e. between PCR products from two different haplotypes. This phenomenon was used in the DNA cross match test whereby DNA from two different individuals are co-amplified in the PCR. If the individuals are identical for HLA-DR, then the banding pattern of the mixture will be the same as the banding pattern of each one of the individuals; if the individuals are not identical, the mixture will contain extra bands. This method has proven to be very useful in analyzing HLA Class I identical individuals for HLA-DR disparity in the selection of unrelated donors to be used for bone marrow transplantation [100]. A modification of heteroduplex analysis is the inclusion of third party DNA to generate new heteroduplexes (spiking) which enhance the discrimination between otherwise similar patterns [101]. A further refinement was the use of temperature gradient gel electrophoresis which analyzes DNA not only on the basis of differential heteroduplex formation between different single strands, but also according to the thermal stability of the homo duplex, which is directly dependent on its nucleotide sequence [102].

Reference strand conformation analysis (RSCA)

Reference strand conformation analysis (RSCA), initially termed double-strand conformation analysis (DSCA), is a modification of complementary strand analysis [102]. DNA from a homozygous reference sample is amplified using primers, one of which is fluorescent-labeled at its 5' end. The sample under test is amplified and the PCR product is mixed with the reference PCR product to form heteroduplexes. These are resolved in an automated DNA sequencer with only the fluorescent-labeled duplexes being observed and identified according to the distance they have migrated.

Use of denaturing gradient gel electrophoresis (DGGE)

Another method involving allele separation has recently been applied to the HLA-B locus [104]. After

71. Alerup 0. HLA class II typing by digestion of PER-amplified DNA with allele-restriction endonucleases will fail to unequivocally identify the genotypes of many homozygous and heterozygous individuals. Tissue Antigens 1990;36:83-7.

72. Yunis I, Salazar M, Yunis EJ. HLA-DR generic typing by RFLP. Tissue Antigens 1991;38:78-88.

73. Ata M, Seki T, Fukushima H, Tsuji K, Inoko H. HLA-DRB1 genotyping by modified PCR-RFLP method combined with group-specific primers. Tissue Antigens 1992;39:187-202.

74. Nomura N, Ata M, Tsuji K, Lnoko H. HLA-DQBl genotyping by a modified RFLP method combined with group specific primers. Tissue Antigens 1991;38:53-9.

75. Ata M, et al. Modified PCR-RFLP method for HLA-DPB1 and -DQA1 genotyping. Tissue Antigens 1991;38:60-71.

76. Witzke SA, Witzke SA, Wardrh. HLA-DPB typing using co-digestion of amplified fragments allows efficient identification of heterozygous genotypes. Tissue Antigens 1992;40:175-81.

77. Nomura N, Ata M, Kato S, Inoko H, Tsuji K. Severe acute graft-versus-host disease by HLA-DPBl disparity in recombinant family of bone marrow transplantation between serologically HLA-identical siblings: an application of the polymerase chain reaction-restriction fragment length polymorphism method. Hum Immunol 1991;32:261-8.

78. Nieto A, Tobes R, Martin J, Pareja E. Allele walking: a new and highly accurate approach to HLA-DRB1 typing. Application to DRBl *04 alleles. Tissue Antigens 1997;49:141-51.

79. Santamaria P, Boyce-Acino MT, Lindstrom AL, Barbosa JJ, F Aras AJ, Rich SS. HLA class II "typing": direct sequencing of DRB, DQB and DQA genes. Hum Immunol 1992;33:69-81.

80. Spurkland A, Knutsen I, Markussen G, V Artdal F, Egeland T, Thorsby E. HLA matching of unrelated bone marrow transplant pairs: direct sequencing of *in vitro* amplified HLA-DRB1 and -DQB1 using magnetic beads as solid support. Tissue Antigens 1993;41:155-64.

81. Knipper AJ, Hinney A, Schuch B, Enczmann J, Uhrberg M, Wemet P. Selection of unrelated bone marrow donors by PCR-SSP typing and subsequent non-radioactive sequence-based typing for HLA DRBl/3/4/5, DQBl and DPBl alleles. Tissue Antigens 1994;44:275-84.

82. Santamaria P, Lindstrom AL, Boyce-Jacino MT, Myster SH, Barbosa JJ, Faras AJ, Rich SS. HLA class I sequence-based typing. Hum Immunol 1993;37:39-50.

83. Mcginnis MD, Conrad MP, Bouwens AG, Tilanus MG, Kronick MN. Automated, solid-phase sequencing of DRB region genes using T7 sequencing chemistry and dye-labeled primers. Tissue Antigens 1995;46:173-9.

84. Duquesnoy RJ. HLA Matchmaker: a molecularly based algorithm for histocompatibility determination. Description of the algorithm. Hum Immunol 2002;63:339-52.

85. Wang L, Marincola FM. Applaying pyrosequencing to HLA-typing. ASHI Quarterly 2003;27:16-8.

reverse transcription of RNA, most exon 2 and all exon 3 are amplified and the products separated using DGGE, which separates DNA fragments based on their sequence composition. Amplified products are excised from the gel and the eluted DNA is re-amplified and directly sequenced. Theoretically, 92 of the 118 HLA-B alleles known at the time of Eberle *et al.*'s study [92] could be typed by this method. If ambiguous pairs are still present, heterozygous sequencing is performed on a short segment at the beginning of exon 2. This increased the number of alleles which could be typed to 111. The method uses Taq FS dye primer chemistry which combines advantages of Taq and Sequenase into one enzyme, Taq FS, which has the thermo stability of Taq and the uniform nucleotide incorporation ability of sequenase. The same group recently applied this method to the HLA-DR locus [105].

Single strand conformation polymorphism (SSCP)

This technique is similar to that of heteroduplex analysis but uses single-stranded DNA [106, 107]. The technique is useful for the detection of HLA-DQA1, -DQB1, -DPA1 and DPB1 polymorphisms. Heteroduplex analysis can be used for these loci but requires spiking. Polymorphism at HLA-DRB loci can be determined by SSCP, but heteroduplex analysis is more informative. The SSCP analysis was originally introduced to detect point mutations in oncogenes and sequence polymorphisms in the human genome, based on the finding that the electrophoretic mobility of single stranded nucleic acid in a non-denaturing polyacrylamide gel depends not only on size, but also on sequence [108].

There is a limitation on the size of the DNA fragment to 200-400 base pairs, making SSCP unsuitable for Class I typing, and electrophoresis is a labor-intensive method which cannot be easily automated. During the 11th IHWC, improvements in this technique were reported that avoided the use of radioactive materials and specialized cooling equipment [109].

To improve the resolution of the system, some researchers have advocated the use of restriction enzyme cleavage of the amplified product to resolve some patterns which had been difficult to differentiate [110]. Others have used SSP amplification to divide a complex series of alleles into groups followed by SSCP to distinguish the alleles of HLA-DRB3 and -DQB1 [111].

Choice of method

Many molecular methods currently in use in the histocompatibility laboratory have been described. The use of a specific technique will depend on the laboratory's requirements. The choice will be influenced by clinical urgency and requirement, sample numbers, availability of equipment, staff skills and budget. Some laboratories, depending on their needs, may use a combination of methods. All techniques need to be continuously updated to allow for the detection of newly discovered alleles.

According to the clinical application, high or low resolution typing may be required. Kidney transplant candidates, for instance, do not necessarily have to be typed at a high resolution level, because only the "broad" serological specificities (e.g. DR1-DR10) are usually taken into consideration for organ allocation. For bone marrow transplantation purposes, high resolution typing is recommended. In July 1997, a survey of techni-ques used in 28 clinical histocompatibility laboratories in the United Kingdom showed the following: of 24 replies, only one laboratory did not use a DNA me-thod; of 23 laboratories using DNA methods, 13 used SSP, 9 used both SSP and SSO and one used SSO only; additionally, six laboratories used PCR-RFLP, mainly for testing family relationship for the UK. Human Organ Transplant Act, which by law then required an RFLP test, two performed SSCP, one performed heteroduplexing and one SBT. Interestingly, only two of the 24 laboratories never performed serology. Several of the laboratories which performed serology did so for cadaveric donors only [59].

An increasing number of methods are filtering into the clinical laboratory in the form of kit-based products. The popularity of these kits is increasing as Class I kits become available to complement the Class II kits. Nearly all of these kits are in a form suitable to *type* one individual at a time. Very few laboratories have the technology to prepare their own reverse dot blot methods. Several of those that have, have cooperated with commercial companies to take the product to the market.

86. Sharon D, Kathleen C, Barracchini, Chen D, Robbins F, Stroncek D, Francesco M. Marincola Ambiguous Allele Combinations in Sequence-based Typing. ASHI Quarterly Fourth Quarter 2003.

87. Petersdorf EW, Stanley F, Martin PJ, Hansen JA. Molecular diversity of the HLA-C locus in unrelated marrow trans-plantation. Tissue Antigens 1994;44:93-9.

88. Petersdorf EW, Setoda T, Smith AG, Hansen JA. Analysis of HLA-B*44 alleles encoded on extended HLA haplotypes by direct automated sequencing. Tissue Antigens 1994;44:211-6.

89. Alerup A, Zetterquist H. HLA-DR ty-ping by PCR amplification with sequence-specific primers (PCR SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 1992;39:225-35.

90. Alerup A, Zetterquist H. DR "low-resolution" PCR-SSP typing a correction and an update. Tissue Antigens 1993;41:55-6.

91. Newton CR, Graham A, Heptinstall LE. Analysis of any point mutation in DNA. The amplification refractory system (ARMS). Nucleic Acids Res 1989;17:2503-16.

92. Park MS, Tonal R. Phenotype frequencies of the class II (DR, DQ) DNA alleles by the patterns of sequence-specific primer mixtures (SSPM) in four different populations and the probable haplotypes between DRB1 allele and DQBl allele. In: Terasaki PI, Cecka M, eds. Clinical transplants 1992. Los Angeles: UCLA Tissue Typing Laboratory, 1992;475-500.

93. Bein G, Glaser R, Kirchner H. Rapid HLA-DRB1 genotyping by nested PCR amplification. Tissue Antigens 1992;39:68-73.

94. Zetterquist H, Bengtsson M, Backstrom G, Egle-Jansson I, Ekdahl AM, Grunnet N, et al. Report from the HLA class II typing by PCR-SSP multicentre study. Eur J Immunogenet 1997;24:191-9.

95. Mytilineos J, Lempert M, Middleton D, Williams F, Scherer S, Apelz G. HLA class I DNA typing of 215 "HLA-A, -B, -DR zero mismatched" kidney transplants. Tissue Antigens 1997;50:355-8.

96. Savelkoul PHM, De Bruyn-Geraets DP, Van Den Berg-Loonen EM. High resolution HLA-DRB1 SSP typing for cadaveric donor transplantation. Tissue Antigens 1995;45:41-8.

97. Fass SJ, Menon R, Braun ER, Rudert WA, Trucco M. Sequence-specific priming and exonuclease-released fluorescence detection of HLA-DQB1 alleles. Tissue Antigens 1996; 48:97-112.

98. Alms Camps M, Ludeck H, Sla Tev AK, Blascyk R. The taqman fluorotyping technique -a new way for differential automated detection of amplified products in the HLA-A, Band C PCR-SSP. Eur J Immunogenet 1997; 24:126.

99. Bidwell L, Hui KM. Human HLA-DR/Dw allotype matching by analysis of HLA-DRB gene PCR product polymorphism (PCR "fingerpints"). Technique 1990;2:93-9.

100. Clay TM, Bidwell L, Howard MR, Bradley BA. PCR-fringerprinting for selection of HLA matched unrelated marrow donors. Lancet 1991;337:1049-52.

101. Bidwell, JL, Clay, TM, Wood, NAP, Pursall, MC, Martin, AF, Bradley, BA and Hui, KM. Rapid HLA-DR-Dw and DP matching by PCR fingerprinting and related DNA heteroduplex technologies. In: HUI KM, BIDWELL]L, eds. Handbook of HLA typing techniques. London: CRC Press, 1993;99-115.

102. Uhrberg M, Hinney A, Enczmann J, Wernet P. Analysis of the HLA-DR gene locus by temperature gradient gel electrophoresis and its application for the rapid selection of unrelated bone manow donors. Electrophoresis 1994;15:1044-50.

103. Madrigal, J.A., Argüello, R., Scott, I. and Avakian, H. High resolution HLA Class I and II typing for unrelated bone marrow donors. Eur J Immunogenet 1997;24:70.

104. Eberle M, Knapp LA, Iw Anaga KK, Domanicom J, Aiyer K, Watkinsdl HLA-B ty-ping by allele separation followed by direct sequencing. Tissue Antigens 1997;49:365-75.

105. Knapp LA, Lehmann E, Hennes L, Eberle ME, Watkinsdl. High resolution HLA-DRB typing using denaturing gradient gel electrophoresis and direct sequencing. Tissue Antigens 1997;50:170-7.

106. Carrington M, Miller T, White M, Gerrard B, Stewart C, Dean M, Mann D. Typing of HLADQA1 and DQB1 using DNA singlestrand conformation polymorphism. Hum Immunol 1992;33:208-12.

107. Summers C, Fergusson W, Gokhale D, Taylorm. Donor-recipient bone marrow matching by single strand conformation polymorphism analysis. Lancet 1992; 339:621.

108. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sri USA 1989;86:2766-70.

109. Kimura A, Hoshino S, Yoshida M, Harada H, Sasazukl T. Polymerase chain reaction-single strand conformation polymorphism (pCR-SSCP) analysis of HLA genes: a practical HLA-matching system. In: Tsuji K, Aizawa M, Sasazuki T, eds. HLA 1991. Oxford: Oxford University Press 1992;511-4.

110. Hayashi T, Seyama T, Ito T, Kusunoki Y, Hirai Y, Nakamura N, Akiyama M.. A simple and rapid method for HLA-DQA1 genotyping by polymerase chain reaction-single strand conformation polymorphism and restriction enzyme cleavage analysis. Electrophoresis 1992;13:877-9.

111. Lo Y, Patel P, Mehal WZ, Fleming KA, Bell JI, W Ainscoa T Js. Analysis of complex genetic systems by ARMS-SSCP: application to HLA genotyping. Nucleic Acids Res 1992; 20:1005-9.

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