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REVIEW ARTICLE

A Comprehensive Review on HLA and its Detections by Polymerase Chain Reaction Technique

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ABSTRACT

The HLA or histocompatibility antigens play predominant role in acceptance or rejection of transplanted organs and in the regulation of immune response as well as in susceptibility or resistance to a large number of diseases. This system is highly polymorphic. Perfect HLA testing is also essential for anthropological studies having different racial groups, for blood component therapy as well as for research application in the development of MHC based vaccines. Now-a-days molecular (PCR) based HLA technique is recommended for HLA typing. Sequence-Specific Primer PCR (PCR-SSP) technique is based on the specificity of the primers in which a 3' single-base mismatch inhibits the priming of a non-specific reaction. Among the different molecular method usually PCR-SSP is done as it is comparatively easier and cost effective. Sequence Specific Oligonucleotide Probe Hybridization (PCR-SSOP) is more amenable to high-throughput HLA typing than PCR-SSP. In Reverse Line Strip (RLS) or PCR-SSO reverse assay multiple oligonucleotide probes specific for alleles of interest are immobilized on a single membrane which is hybridized with amplified DNA and the detection of HLA alleles is possible by colour development using a specific substrate. Luminex technology is another variation of PCR-SSO reverse assay where membrane is replaced with 100 different colours of polystyrene microbead. Reference strand–mediated conformational analysis (RSCA) is a conformational method for high-resolution typing class I and II genes using native polyacrylamide gels. Genomic DNA sequencing–based typing (SBT) methodology utilizes locus- or group-specific amplification followed by cycle-sequencing the desired number of exons on both strands.

KEYWORDS

PCR-SSP, PCR-SSOP, RLS, RSCA, Luminex Technology

INTRODUCTION

The term *HLA* stands for human leucocyte antigens as these antigens were initially detected on the leucocytes[1].

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These antigens play predominant role in acceptance or rejection of transplanted organs - so they are called as *histocompatibility antigens*[2]. They also play an important role in the regulation of immune response as well as in susceptibility or resistance to a large number of diseases. The main function of the HLA molecules is to present the antigen to the T cells and initiate the specific immune response[3]. The

genes responsible for these antigens are complex in structure and are clustered on a small segment of about 2 CM (centimorgan) in length on the short arm of chromosome 6 (p.21.3)[4] which spans about 4 megabases or 4 x 10^6 nucleotides, only a few of them are concerned with histocompatibility (called as *MHC* – a major histocompatibility complex). It is equivalent to *murine H-2 complex*. The HLA class I and class II genes are known as the most polymorphic genes in the human genome. The remarkable extent of the allelic diversity at these loci has been revealed over the past three decades by molecular genetic analyses, made possible by the development of recombinant DNA technology, sequencing, and, more recently, by PCR amplification[5].

HLA Molecules

This system is highly polymorphic *i.e.* there are several alternative forms (*alleles*) at each locus. These major histocompatibility complex (MHC) gene products are classified into three types according to their structure, distribution and function[6].

Class I Antigens

The class I antigens comprises the polymorphic class Ia having HLA-A, B and C antigens and the relatively non-polymorphic class Ib, HLA-E, F, G and Hfe (HLA-H) antigens. These are coded by three loci (closely linked) called as *HLA- A, B* and *C*. These are heterodimer having polymorphic glycoprotein linked non-covalently to a non-polymorphic peptide, called β2-microglobulin, which is encoded by gene on chromosome 15[7,8]. These class 1 antigens are distributed virtually on all nucleated cells as well as on platelets.

Class II Antigens

These are coded by a region called as *HLA-D*. Recent studies have identified further three sub-regions called as *DP, DQ* and *DR* in the originally defined HLA-D region. Chemically, class II antigens exist as bimolecular structure - each constituent is polymorphic polypeptide chains. They are called as α and β. Class II antigens are distributed mainly on antigen presenting cells such as macrophages, monocytes, dendritic cells etc., B cells and some activated T cells. Some cells may be induced (vascular endothelium, fibroblast, renal tubular epithelial cells etc.) to express class II antigens by *gamma interferon*, a cytokine produced by activated T-cells.

Class III Antigens

These are mainly the components of *complement system* like *C4, C2*, etc. Recent studies have shown that some cytokine like TNF-α, TNF-β are also encoded within the MHC. These are not related to histocompatibility, though these are genetically linked to class I and II antigens.

Class I and II genes are highly polymorphic. Each of the several alleles at different loci is expressed by a number like HLA-A1, HLA-B5 etc. Some of the alleles which have not yet been fully characterized are identified by '*w*', such as HLA-BW5. All class I antigens and most of the class II antigens can induce the formation of humoral antibodies in genetically non-identical individuals, which is the basis to type these antigens by conventional serological techniques. HLA system contains about 240 potential genes, where about 40% of the total HLA genes have immune related functions and there are 6 major HLA loci encoded within the human MHC. Class I region span about 2 megabases, class II & III region span 1 megabase, respectively.

Methods of Laboratory Testing

This testing is usually carried out for cases requiring organ (like kidney) and bone marrow or stem cells transplantation in order to find a suitable matched donor from the family members or the unrelated donor pool to avoid the Graft-versus-host disease (GVHD) and graft rejection. This HLA testing is also done to determine the disease susceptibility so for diagnostic or prognostic purpose[9,10,11]. Perfect HLA testing is also essential for anthropological studies having different racial groups, for blood component therapy as well as for research application in the development of MHC based vaccines.

This testing can be done by –

i. Peripheral blood - viable lymphocytes for serology testing or DNA extractions for PCR based testing.
ii. Plasma or serum - for detection of anti HLA antibodies.
iii. Tissue - for PCR based tests after DNA extraction.
iv. Lymph node or spleen - extracted DNA for PCR based tests and extracted lymphocytes (for mainly cadaver donor typing) for serology testing.

HLA testing are of several types, *viz.*-

i. Serological - microlymphocytotoxicity testing.
ii. Biochemical - by gel electrophosis.
iii. Molecular methods - several techniques are available like PCR-SSP, PCR-SSOP, PCR-SSCP, ARMSPCR, RFLP, RBH/RLS.
iv. Sequence based typing.
v. Microarry or DNA chip technology.

Among the different molecular method usually PCR-SSP is done as it is comparatively easier and cost effective. PCR based tests depend on the quality of DNA extracted and rely on the identification of nucleotide differences among the HLA alleles at various levels of resolution *viz.* low, intermediate or high. Molecular (PCR based) technique is more reliable and accurate; it is also fast and reliable than serological testing. PCR based technique has also some limitation like: (a) it is comparatively costly, (b) sequences available are mostly form the studies carried out in Caucasian and oriental populations, so there is chance of missing of some novel or rare alleles found in other ethnic population, which can be solved by following more than one procedure, (c) there is also chance of false positive and negative reactions due to failure of amplification. Usually the following molecular techniques (PCR based) are recommended for HLA class I & II genes:

Class I Genes: PCR-SSP, RBH/RLS, PCR-SSCP.

Class II Genes: RFLP, PCR-SSOP, PCR-SSP.

So PCR-SSP can be employed for both types and it is commonly used excepting in some rare cases as diseased earlier.

PCR Based Molecular Techniques of HLA Typing

Now-a-days molecular (PCR) based HLA technique is recommended for HLA typing[12]. These are of several types.

Sequence-Specific Primer PCR (PCR-SSP)

PCR primers are designed so that their 3'-most 1-2 nucleotides are complementary to base positions within Class I and II genes that differ for different alleles. The technique is based on the specificity of the primers in which a 3' single-base mismatch inhibits the priming of a non-specific reaction. Because *Taq* polymerase lacks 3' to 5' exonuclease activity, even if the primer pair does anneal non-specifically, they do not amplify efficiently. This was called amplification refractory mutation system (ARMS)[13]. HLA alleles within a group have a characteristic 'patchwork of single-nucleotide polymorphisms (SNPs)'. This technique was applied to HLA where the 3' base specificity in forward and reverse primers was ideally suited to define antigens or groups of alleles. For a particular SSP-PCR, productive DNA amplification occurs if an allele perfectly complementary to the two primers chosen is present in genomic DNA. By choosing a series of primers assessing polymorphism at the relevant regions within exons 2-3 and exon 2 of the Class I and II genes respectively, the HLA typing can be accomplished. SSP-PCR requires about 100 simultaneously performed PCR assays per patient to identify HLA-A, -B, -C, -DRB and –DQB1 allele groups at serological equivalent resolution from around 10 µg of DNA using 144 primer mixes and an agarose gel documentation system to record the tissue type. More reactions are required if higher resolution typing is desired. In each reaction a second set of primers directed at a non-HLA

locus serves as a positive control. The presence or absence of PCR products of the correct size is then assessed by gel electrophoresis. Because of the large number of reactions and possible alleles, most laboratories use local or commercial software for analysis and calling alleles.

There are many commercial kits available today with low-resolution and high-resolution capabilities, but this method has become laborious and consuming of both time and DNA. A commercial SSP tray of HLA Class I and II contains multiple pairs of PCR primers that are designed to anneal within DNA regions present in certain alleles or groups of alleles. Innovative versions of the PCR-SSP technology are coming into use. For example, 192 split well complete HLA type in one thermal cycler block (Texas Biogene, Richardson, TX, USA), gel-less analytic systems (Innotrain, Frankfurt, Germany) and quicker, more convenient methods of aliquoting PCR mixes, enzyme and DNA (Biofortuna, Bromborough, UK).

Sequence Specific Oligonucleotide Probe Hybridization (PCR-SSOP)

The PCR-SSOP technique is more amenable to high-throughput HLA typing than PCR-SSP[14, 15]. Polymorphic regions of the Class I and Class II genes are amplified from genomic DNA with the PCR, using primers that anneal to 5' and 3' flanking regions that are conserved among individuals. Care is taken in choosing primers that will result in roughly equal amplification of the two alleles in a heterozygous individual. Following PCR, the amplified DNA is used in standard dot-blot hybridization assays with sequence-specific oligonucleotide probes chosen to bind with polymorphic regions. High stringency conditions allow detection of single nucleotide differences between alleles. Alleles are assigned on the basis of patterns of positive and negative hybridization reactions with oligonucleotides specific for particular allele or sequence. SSOP typing requires a substantial number of oligonucleotide probes to detect and distinguish among the large number of known Class I and II alleles.

Reverse Line Strip (RLS) or PCR-SSO Reverse Assay

This technique is reverse of the SSOP protocol. Multiple oligonucleotide probes specific for alleles of interest are immobilized on a single membrane which is hybridized with amplified DNA and the detection of HLA alleles is possible by colour development using a specific substrate[16]. There have been several variations of SSO-rev: the first method a poly-T tail to attach the oligonucleotide to the membrane.

Subsequently the specific probe hybridises to the PCR product which has incorporated biotinylated primers during the amplification process. Streptavidin horseradish peroxidase conjugate is added and positive reactions are detected using a colored soluble substance. Another method following the same principle is performed in a 96-well tray. The DNA to be investigated is amplified as in PCR with the 3' primer biotinylated in order for one to be able to attach the PCR product directly to the avidin-coated tray. In this case the oligonucleotides are produced with an amino group at the 5'-end and conjugated with alkaline phosphatase. The binding between alkaline phosphatase probes is detected by an amplified colorimetric assay.

Various formats have been seen over the years including ELISA in plates (*e.g.* Biotest 'Elpha' system) and line probe assay (LIPA) (Innogenetics) with probes immobilized on nitrocellulose. Commercial kits included machinery or robots which enabled temperature-controlled washing of plates or membranes and delivery of enzymes and coloured substrates. As more and more HLA alleles have been discovered[17], the PCR-SSOP technique has proved to be more adaptable than PCR-SSP.

Luminex Technology

This is another variation of PCR-SSO reverse assay where membrane is replaced with polystyrene microbead or sphere of 100 different colours. The basic principle of the Luminex technology is its ability to measure multiple analytes simultaneously in a single reaction well[18]. Oligonucleotide probes,

covalently linked to a different set of polystyrene carboxylated microbeads, were designed to specifically detect the nucleotide sequences at the polymorphic sites of the HLA specificities[19]. The microbeads are discriminated on the basis of gradations in the incorporated fluorescent dye, enabling more than 100 distinct beads to be linked to the same number of probes. Hybridized amplicons are labelled with fluorescent Streptavidine R-Phycoerytrin molecules and the reactions are then acquired on flow cytometer LABScan™ 100 (Luminex) to identify the fluorescent intensity of phycoerythrin on each bead. One laser excites the colors inside the microbeads to identify whichever microbead is being read. The second laser excites the color on the microbead surface, *i.e.* the labelled reporter tag. Finally, the color signals are detected by an advanced optical system, and the signals are processed as data for each reaction. Luminex is the predominant technology – as the number of HLA alleles increases, Luminex kit vendors add more probes and, presumably, more beads are provided by Luminex when necessary. Luminex is developing a new platform, FLEXMAP 3D, which will enable testing on 500 beads (Luminex Corporation).

Reference Strand–Mediated Conformational Analysis (RSCA)

Reference strand–mediated conformational analysis is a conformational method for high-resolution typing class I and II genes using native polyacrylamide gels[20,21]. In this method, amplified unknown sample is mixed with dye-labelled amplified reference allele (same locus), the mixture is heated to 95°C, and the strands are allowed to reanneal in a thermal cycler. The resultant mixture of homo- and heteroduplexes is separated by electrophoresis through a native polyacrylamidegel system with a laser to detect fluorescence. Homoduplexes migrate faster through the gel than heteroduplexes, and only fluorescent duplexes are observed. With fluorescently labelled DNA standards run in the gel, it is possible to accurately type the samples at the allele level.

Genomic DNA Sequencing–Based Typing (SBT)

DNA sequencing, using the Sanger method of dideoxy chain termination, for HLA typing became the preferred method for allelic HLA typing through the discovery of locus- and antigen-specific polymorphisms in the noncoding introns flanking the polymorphic exons[22,23]. The methodology utilizes locus- or group-specific amplification followed by cycle-sequencing the desired number of exons (on both strands).

PCR SSP is commonly followed molecular techniques. Usually PCR-SSP as discussed is commonly done for molecular based HLA-typing (both for type I & II). Various commercial test kits are available. Equipments required are usually thermocycler, gel-doc system and sometimes some commercial kits also provide the software for interpretation of the result including the chart for manual interpretation. A flow chart of the method is as follows: extraction of DNA and its optimum quantification and then preparation of master mix as per requirement, then measured amount of the master mix is taken in the small tube and sealed, then run in thermocycler (which is to be performed as per the kit manual) for specified period and then the product is run in the prepared agarose gel (2%) for specified time and finally the result is obtained after visualization of the band appeared under transilluminator. Finally the interpretation of the test result is done by software or by the supplied interpretation chart manually. At each case corresponding controls to be used.

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