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Intitulé

Limitations of Microlymphocytotoxicity technique for HLA class I typing

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Complement dependent lymphocytotoxicity (CDC);DNA based techniques; HLA class I; HLA typing;

ABSTRACT

HLA typing is a technique used to match recipients and donors for both bone marrow and organ transplants. Two HLA typing methods are performed in various laboratories including serological methods and DNA-based techniques. Our study was taken to identify the limits and strengths of serological typing by CDC technique particularly in class I typing. HLA class I were typed by standard microlymphocytotoxicity test using the commercially prepared Terasaki trays One Lambda Inc.b.USA. The results have shown that misreads and dropouts are highly common as B15 was the most ambiguous antigen misreads and dropouts 17 times as well as B27 was misread/dropout 15 times on serology. The false serological test can be a powerful tool to decrease the sensitivity of microlymphocytotoxicity tests. By DNA-based technique, however, we were able to identify almost all the ambiguous antigens obtains with the CDC technique.

MOTS CLÉS

complément de lymphocytotoxicité dépendante (CDC); techniques basées sur l'ADN; HLA classe I; typage HLA;

RÉSUMÉ

Le typage HLA est une technique utilisée pour apparier les receveurs et les donneurs de moelle osseuse et de greffe d'organe. Deux méthodes de typage HLA sont effectuées dans divers laboratoires, y compris la méthode sérologique et les techniques basées sur l'ADN. Notre étude a été prise pour identifier les limites du typage sérologique par technique de CDC en particulier dans le typage de classe I. HLA classe I ont été identifier par test de microlymphocytotoxicité standard en utilisant les plateaux de Terasaki préparés commerciaux One Lambda Inc.b.USA. Les résultats ont montré que les erreurs de lecture et les abandons sont très fréquents car B15 était l'antigène le plus ambigu et les abandons 17 fois ainsi que B27 a été mal lu / abandons 15 fois sur la sérologie. Test sérologique faux peut être un outil puissant pour décéase la sensibilité des tests

de microlymphocytotoxicité. Par la technique basée sur l'ADN, cependant nous avons pu identifier presque tous les antigènes ambigus obtenus avec la technique de CDC.

الكلمات الرئيسية

, تحليل الجسم المضاد 1تقنية اللمفاوية المتعمدة, التقنية المعتمدة على الحمض النووي, الجسم المضاد البشري اللمفاوي نوع
البشري اللمفاوي

ملخص

طباعة المضاد البشري اللمفاوي هي تقنية تستخدم لمطابقة المتلقين والمتبرعين لكل من النخاع العظمي وزرع
الأعضاء ,ويوجد طريقتان لطباعة لمضاد اللمفاوي بما في ذلك الطرق المصلية والتقنيات القائمة على الحمض النووي. وقد
وخاصة في الفئة الأولى من الطباع .microlymphocytotoxicity أخذت دراستنا لتحديد حدود استعمال الطباعة المصلية
بواسطة تقنية
طبع المضاد اللمفوي بالتقنية microlymphocytotoxicity باستخدام الصحن المختص المعد تجاريا Terazaki من طرف
.One Lambda Inc.b.USA الاساسية المصلية
اخطأ في 15وأظهرت النتائج أن خطأ قراءة و تخطي طباعة المضاد البشري اللمفاوي هو أمر شائع الى حد كبير حيث أن ب
مرة في التقنية المصلية15 اخطأ في القراءة/ و تخطى في الطباعة 27 مرة كما ان ب17القراءة/ و تخطى في الطباعة
يمكن أن يكون الاختبار المصلي الكاذب أداة قوية لتقليل حساسية اختبارات المصلية الدقيقة حيث ان نسبة الاختبارات الكاذبة
لها تأثير على مصداقية النتائج المحصلة. ومع ذلك ، فبالتقنية المستندة إلى الحمض النووي ، تمكنا من التعرف على كل
المضادات الغامضة تقريبا مع تقنية

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INTRODUCTION

The human leucocyte antigen (HLA), also known as the major histocompatibility complex was found on chromosome 6 in humans. it is considered as the most polymorphic gene with major functional and medical implications and has been one of the most regarding parameter of investigation in transplantation medicine and auto-immunity. Primary, genetic diversity at these loci were analyzed by HLA serologic method. This technique was developed in the 1964 by Terasaki and McClelland to significantly reduce the volume of typing reagents required to define an individual phenotype. This technique was the most commonly used method in routine clinical testing as a gold standard method.[1]

Several studies has shown that typing by serology may not be sufficiently reliable due to the increase of errors and ambiguities in results. In recent years, DNA-based typing has surpassed serology in many aspects. It uses synthetic products Therefore, there is no need to obtain live serum/cells from people who have undergone multiple blood transfusions as in CDC. At present, the serological method and reagents can not detect all currently known HLA alleles, for this reason this method is often followed by the molecular methods.

In our laboratory, we routinely type bone marrow transplant and organ transplant pairs for HLA class I initially by Microlymphocytotoxicity. HLA class II is performed using SSO or SSP typing techniques. nevertheless, errors may occur during the first serological workup, requiring the use of the DNA based techniques.

In this study, we need to identify the CDC technique limitations in HLA class I typing. in addition, we have to be able to detect troublesome antigens that are commonly misinterpreted in serology. moreover, we compared our serological results with DNA techniques results in order to identify few strengths of CDC technique.

CHAPTER 1

Bibliographical Study, Objects and Thesis in General

Human cells are known for the multiple receptors presented on their surface for many reasons. The main function of these receptors is to present antigens to other cells which is an important process in the immune system. The antigen presentation function is provided by the Major Histocompatibility Complex (MHC) molecules. The extreme diversity of the MHC also makes it the determining factor acceptance or rejection of transplants between donors and which was at the origin of his discovery by Jean Dausset. The human MHC is called HLA (Human Leukocyte Antigen) because the first histocompatibility molecule identified had been identified as an antigen leukocyte.[1]

1. HLA Immunology And Transplantation

Variety of transplantation antigens have been discovered including the MHC, minor histocompatibility antigens, ABO blood antigens. The sensitization to MHC antigens can caused by transfusion, pregnancy, or previous failed grafts which lead to the development of anti-human leukocyte antigen antibodies that are the main factor responsible for graft rejection in organ transplantation and play an important role in post-transfusion. [1]

1.1 CMH antigen and immunology of Transplantation

Transplantation is the operation of transferring cells, organ, or tissues from one person to another. The failure of an organ system can be repaired with transplantation of the organ as kidney, liver, heart, lung, or pancreas from a donor. Moreover, the immune system remains the most alarming barrier to transplantation as a routine medical treatment. our immune system has developed successful tools to fight foreign bodies. These tools are involved in the transplantation rejection that are recognized as a foreign agent by the patient's immune system.

A recognition of the non self agents will activate the immune response and will restorative the production of antigen specific antibodies that mark cells to be destructed by the immune system and help increase the immune response.

The HLA antigens presented on the cell surface are two types, HLA class I is a molecule presented on all the nucleated cells and it is composed of three alpha subunits and beta macroglobulin. On another hand, HLA class II is found only on the antigen-presenting cells and it is made up of two alpha units and two beta units.

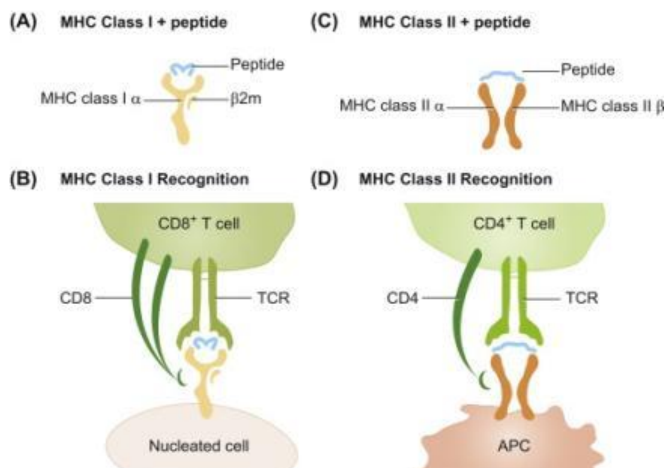


Figure 1: Recognition of MHC Class I and II Molecules by T Cells

a) The transplantation

The power of the immune reaction against the organ or tissue, additionally usually alluded to as graft, will depend on the type of graft being transplanted and the genetic difference between the donor and recipient. To minimize the risk of rejection, the donor and recipient are gently matched for genetic compatibility before transplantation. However, the small pool of qualified donors can make it hard to find a donor recipient match and there will always be a level of rejection against the graft. [2]

There are various types of transplantation including organs and tissues:

Auto-graft: Transplantation of tissues, cells or organs between sites of the same individual for example skin graft

Allograft: Transplantation of organs or tissues from a donor to a non genetically identical individual of the same species. Allograft is the most common type of transplant.

Xenograft**:** Transplantation of organs or tissues between two different species. Pig valves are commonly used to repair or replace a malfunction heart valve in humans. Xeno-transplantation of a whole organ is not currently available, although it is a field of huge scientific research as a possible solution for the existing critical under supply of enough organs.

Stem cell transplant: Stem cells are cells that have the capacity to develop into different types of the body cells Blood stem cells called haematopoietic stem cells can develop into all the cells found in the blood and are donated to restore destroyed or damaged blood cells. Haematopoietic

stem cell transplants are used to treat certain types of cancer for example some types of leukaemia, and blood diseases where the bone marrow has been damaged preventing the production of normal blood cells. These stem cells can be gathered either directly from the bone marrow or from the cord blood which is a type of blood from the placenta and umbilical cord from consenting mothers following childbirth.

b) Immunobiology of Rejection

The immune reaction to a new transplanted organ include both cellular (lymphocyte mediated) and humoral (antibody mediated) mechanisms. Although other cell types are also involved, the T cells have the major role in the rejection of grafts. The rejection reaction consists of the sensitization stage and the effector stage.

Sensitization stage

In this stage, the CD4 and CD8 T cells, via their T cell receptors, recognize the allo-antigens expressed on the foreign graft cells. Two signals are required for this recognition of an antigen, the primary is provided by the interaction of the T cell receptor with the antigen presented by MHC molecules, the following by a co-stimulatory receptor/ligand interaction on the T cell/APC surface. Of the multiple co-stimulatory pathways, the interaction of CD28 on the T cell surface with its APC surface ligands, B7-1 or B7-2 -commonly known as CD80 or CD86- has been analysed the most. additionnally, cytotoxic T lymphocyte–associated antigen-4 also attachs to these ligands and gives an inhibitory signal. Other co-stimulatory molecules include the CD40 and its ligand CD40L (CD154).

Ordinarily, helices of the MHC molecules form the peptide binding furrow and are occupied by peptides got from typical cell proteins. central tolerance mechanisms and peripheral tolerance mechanisms confirm that these self peptide MHC complexes are not recognized by the T cells, as a result preventing auto immune responses.

Allo-recognition consist of two pathways: the direct and indirect pathways. Each lead to a generation of different position of allo-specific T cell clones.

Direct pathway

Recipient's T cells recognize allo MHC molecules on the surface of cells in the donor's organ. systematically, host T cells notice the allo MHC molecule/allo-peptide complex as being similar in shape to self-MHC/foreign peptide complex consequently recognize the donor organ as foreign. This pathway is mostly the dominant pathway involved in the early allo immune

response. The transplanted organ hold a variable number of traveller APCs in the form of interstitial dendritic cells. APCs have a high density of allo MHC molecules and they are capable of directly stimulating the recipient's T cells. The comparative quantity of T cells that multiply in contact with allo genetic or donor cells is remarkably high compared with the quantity of clones that target antigen presented by self APCs. therefor, this pathway is important in acute allo rejection.[2]

Indirect pathway

T cells recognize refined allo antigen presented as peptides by self APC. The following responses similarly to responses that appear in late or chronic acute rejection are associated with T cell proliferative responses a more factor collection, including peptides that were already immunologically quiet. Such an adjustment of the example of T cell reactions has been named epitope exchanging or spreading.[3]

A connector between self MHC/allopeptide-primed T cells and the development of acute vascular type rejection has been proved to be mediated in part by accelerated allo-antibody production. futhermore, chronic allo-graft vasculopathy can be mediated by T cells primed by the indirect pathway.[4]

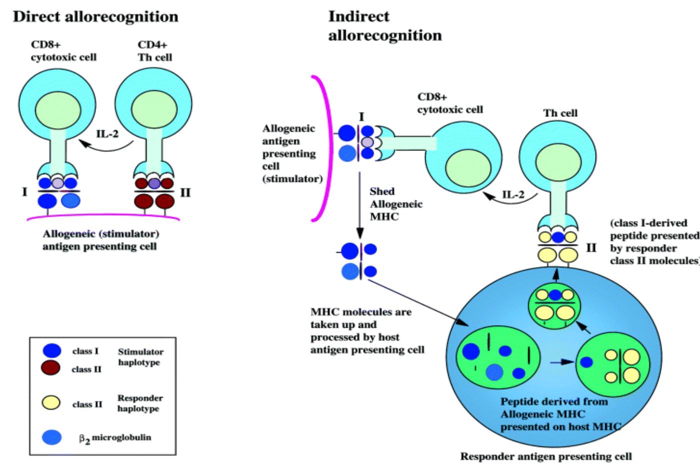


Figure 2: Diagrammatic representation of the direct and indirect pathways of allorecognition

1.2 HLA genetics and inheritance

The human MHC guides to the short arm of chromosome 6 (6p21) and ranges around 3,600 kilobases of DNA.[4] The human MHC is separated into three regions.

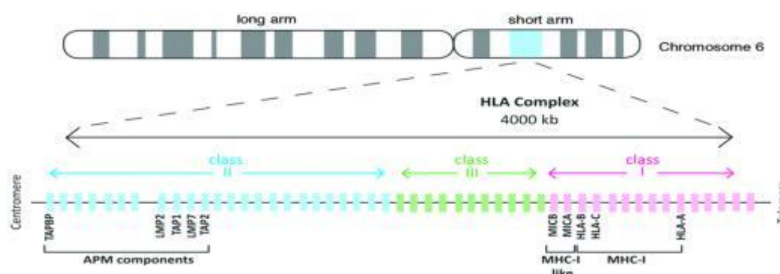


Figure 3: human MHC on the short arm of chromosome 6

The class I region carries the traditional HLA-A, HLA-B, and HLA-C genes that encode the heavy chains of class I molecules. The class II region comprises of a progression of subregions, each containing A and B genes encoding α and β chains, respectively.4 The DR quality family comprises of a single DRA gene and up to nine DRB genes (DRB1 to DRB9). The DRA gene encodes a constant α chain and it ties different β chains encoded by the DRB genes.

HLA-DR antigen specificities (DR1 to DR18) are directed by the polymorphic DRβ1 chains encoded by DRB1 alleles. HLA haplotypes of certain DRB1 alleles carry unequivocally connected DRB3, DRB4, or DRB5 locus. The DP and DQ groups have one expressed gene for α and β chains and an additional unexpressed pseudo-gene. The DQA1 and DQB1 gene products accomplice to shape DQ molecules, and the DPA1 and DPB1 product structure DP molecules.[5]

a) HLA haplotypes

HLA genes are exactly linked and the entire MHC is inherited as an HLA haplotype in a Mendelian law from each parent. The isolation of HLA haplotypes inside a family can be appointed by HLA family studies (Figure). Two siblings have a 25% chance of being genotypically HLA identical, a 50% chance of being HLA haplo-identical (sharing one haplotype), and a 25% chance sharing no HLA haplotypes.[5]

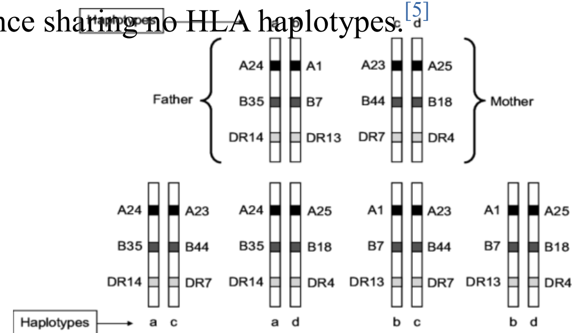


Figure 4: Mendelian inheritance of a family.

Possible irregular blends of antigens from various HLA loci on an HLA haplotype are huge, yet certain HLA haplotypes are discovered more much of the time in certain populations than expected by some coincidence. This event is called the linkage disequilibrium.[5]

b) Expression of HLA

HLA class I molecules are expressed on the surface of the nucleated cells. Class II molecules are expressed on the antigen-presenting cells (monocytes, macrophages, and dendritic cells), activated T lymphocytes, and B lymphocytes.[5]

1.3 HLA And Diseases association

Certain diseases, particularly the autoimmune nature, are related with specific HLA types.[6] The perception that few diseases are unmistakably more common in individuals with a specific HLA allele or haplotype permitted studies on HLA and disease associations. There are various examples of these associations found in table 1 below. The strongest association is shown by ankylosing spondylitis: B27 antigens are present in approximately 90% of patients. Those types of diseases have ambiguous etiology, are generally have familiar recurrence, and are supported by polygenic and environmental factors, their relation with HLA alleles identifies only one of the predisposing genetic factors.[7]

Disease	HLA antigens	Patients %
Ankylosing spondylitis	B27	89
IDDM	DR3	52
	DR4	74
Rheumatoid arthritis	DR4	68
Celiac disease	DR3	79
	DR7	60
Multiple sclerosis	DR2	51
Narcolepsy	DQ6	> 95

Table 1: HLA and disease associations

HLA associated diseases are divided by their pathogenetic mechanisms into three groups (Table 2).

Group	Disease
No autoimmune etiology	21-Hydroxylase deficiency Hemochromatosis
Autoimmune etiology	Ankylosing spondylitis Rheumatoid arthritis Celiac disease Insulin-dependent diabetes mellitus Multiple sclerosis
Unknown etiology	Narcolepsy

Table 2: pathogenetic mechanism of HLA associated diseases en haut

1.4 Clinical Uses of HLA Typing

a) Organ Transplant

The main application of HLA typing is in organ transplantation. Concerning the liver and heart transplants, very often the vital emergency concerning the recipient does not allow the typing of the donor before the transplant.

For the kidney transplant, the donor and the recipient are typed for HLA-A, HLA-B, and HLA-DR at the generic level. It is rare to find perfectly identical donors, also mismatches or mismatches (difference at the level of an allele) are tolerated.[8] But a pairing on the HLA-DR, then the HLA-B, then the HLA-A will be preferred.[9,10] A prerequisite for transplantation is research previous HLA allo-immunization, following an immunizing event such as pregnancy, trans fusion or a first transplant. The recipient of an anti-HLA antibody of directed specificity against a graft antigen must have the graft refused. An ultimate test called cross-match taking place before the transplant allows contact the donor's lymphocytes and the recipient serums, and detect immunization in based on the LCT reaction.[11,12]

b) Bone marrow allografts

Specific HLA typing is of great interest when the realization of bone marrow allografts, in order to determine the compatibility between the donor and its recipient. Generally, the search for an HLA donor compatible is done in 2 steps: a donor is all first sought among the siblings of the patient. If related individuals are not compatible, the clinician can then decide to search for a voluntary, anonymous bone marrow donor on one of the global files. In 2005, there were more than 9.5 million bone marrow donors on these files.[13] If possible, find a compatible donor in allelic typing for HLA-A, B, C, DR, DQ and possibly DP loci.

c) Hla and diseases

The HLA system has an interest as a marker of susceptibility to a given disease in population data. The risk is quantified in terms of relative risk, which is the ratio of the proportion of patients in individuals at risk and in patients in the unexposed. The relative risk is used to express how often the risk of developing a given disease is increased in subjects with the risk allele compared to those without it. When significantly different from 1, the risk factor is associated with the disease.[14] The association between HLA-B27 antigen and spondylo-arthropathies (ankylosing spondylitis and reactive arthritis) has been known for more than 20 years.[15]

2. HLA Class I And Class II Typing

Clinical HLA labs perform different tests to help the transfer programs; including HLA typing of the recipient and the donor, screening and, identification of HLA antibodies in the recipient, and

detection of antibodies in the recipient that are reactive with lymphocytes of a prospective donor (crossmatching).

2.1 HLA Typing By Serological Methods

The LCT technique has been utilized as a norm for serological typing of HLA class I and class II antigens.[16] HLA composing sera are principally gotten from multiparous alloimmunized and their HLA specificities are compared with a different panel of lymphocytes with previously known HLA types. ocassionally, the use of monoclonal antibody reagents derived from immunized mice is needed.

Peripheral blood lymphocytes express HLA class I antigens and are used for serological typing HLA-A, HLA-B and HLA-C.HLA class II typing is performed with B lymphocytes isolated from PBLs because these cells express class II molecules. HLA typing is performed in multi-well trays, each well containing one serum of HLA specificity. Lymphocytes are spread in the well and incubated, complement is added to mediate the lysis of bound antibodies.

Routine screening of serum from prospective transplant recipients to determine the presence of HLA antibodies to determine the degree of HLA alloimmunization. The Screening is performed on a panel of 30 to 60 cells, which represent most of the antigens encountered in the general population, using complement-dependent cytotoxicity (CDC) assay.

Lymphocytes from a group of donors are mixed with the recipient's serum, and complement is added to determine whether the recipient has antibodies that bind to the donor cells and activate the complement and membrane attack complex to cause cell death. The result is reported as the percentage of panel cells killed by reacting with HLA antibodies in the patient's serum, hence the name PRA (Panel Reactive Antibody). The CDC method is a non-specific test. A positive result indicates the presence of anti-donor antibodies in the recipient's serum. However, depending on the nature of the cells used in the panel, the specificity of anti-HLA antibodies may be determined.[17]

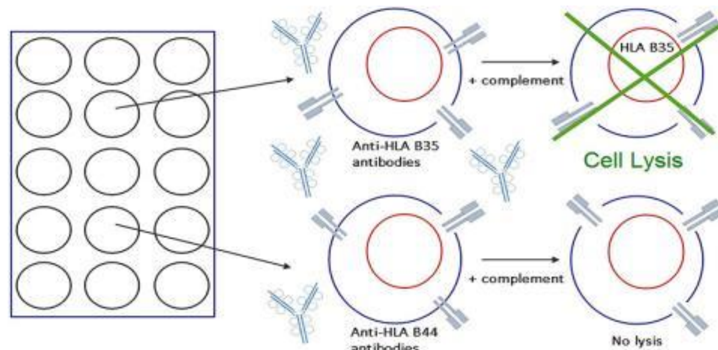


Figure 5: example of a serological reactiong using CDC technique. The lysis of the lymphocytes by serum containing anti-HLA B35 positivity of the cells.

2.2 HLA Typing By Molecular Methods

Currently, DNA-based assays are the method of choice due to their greater precision, sensitivity, specificity. These assays based on the chain reaction use oligonucleotide primers which define a single locus, one or a group of HLA alleles.

a) DNA isolation

Numerous DNA purification methods justify isolation of crude leukocytes to remove heme proteins associated with blood cells which are known to inhibit the PCR reaction. classic purification methods have generally been categorized as one of four classifications: (1) using enzymes (including proteinase K and RNAse A); (2) using enzymes and organic solvents (phenol and chloroform); (3) using solvents only; and (4) using resins or affinity gels. Most of these methods include the following steps: leukocytes or lysis of red blood cells, nuclear lysis, treatment with RNAse A and DNA precipitation. Many commercial products are available for high quality genomic purification from whole blood stains and other sources. Genomic DNA can be stored at 4 ° C up to 6 or stored between -20 ° C and -80 ° C for long-term storage.

b) PCR amplification: Sequence-Specific Primer

The SSP strategy is a simple PCR-based method that utilizes sequence-specific primers for DNA-based HLA typing.[18] SSP includes DNA extraction followed by the amplification of the particular HLA locus such as HLA-A, HLA-B. this technique relies on DNA amplification which uses allele specific primers and detects the correct size of the amplification product by gel electrophoresis. PCR- SSP used for the detection of polymorphisms using the lack of 5' to 3'exonuclease activity of Taq DNA polymerase used in the PCR amplification.

In the presence of the target allele and the primer carries the identical sequence, the amplification starts by the base at 3' of the primers in order to the synthesis of the new DNA strand. As a final

step, the visualization takes place using agarose gel electrophoresis with ethidium bromide under ultraviolet light. The SSP typing performed in less than 3 hours makes it the quickest method of obtaining an HLA typing compared to other molecular methods. 20 In the other hand, PCR-SSP is a comparatively expensive technique by using massive amounts of DNA and consuming large quantities of DNA polymerase.

Figure 6: Sequence-Specific Primer (SSP) Typing. The left panel is a picture of an agarose gel electrophoresis showing the pattern of allele/group-specific PCR products corresponding to a low-resolution HLA class I and class II genotyping. The right panel shows the interpretation scheme

SSO method was initially for HLA class II but then it was also developed for HLA class I. this technique is based on DNA extraction followed by the amplification of a particular locus using group-specific prime xons 2 and 3 are co-amplified as PCR d containing the genes regions of the ved to a nylon membrane which is bind. after the hybridization and wa lementary DNA sequence in the amplified DNA immobilized on the membrane. The probes is labeled with streptavidin conjugated to an enzyme and hybridization is detected with chromogenic or chemiluminescent substrates. Alternatively, probes can be labeled with digoxigenein and detected with antidigoxigenein antibodies. For the determination of the HLA type of an individual, a panel of probes specific for particular sequences to distinct HLA alleles would be hybridized to the locus specific PCR amplified sample. [19]

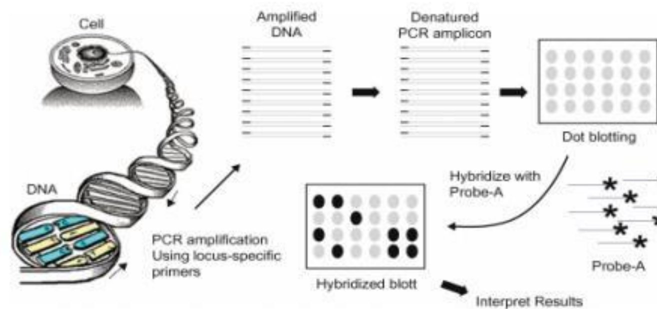


Figure 7: DNA from a panel of samples (96- or 384-well format) are PCR amplified using locus-specific primers. The PCR products are denatured and blotted on a series of nylon membranes and probed with a panel of allele/group-specific probes. Positive hybridization of probes labeled with digoxigenin are detected by chemiluminescence and exposed to x-ray film.

c) The Luminex technology

The Luminex innovation technology for HLA typing method is a fluid bead based reverse SSO technique. A biotinylated locus-specific amplicon is created from the sample gDNA by PCR and later denatured. This product is mixed with a cocktail of color coded polystyrene beads. Each bead is covered with an exceptional allele or group specific oligonucleotide probes. The beads introducing the match primers to the amplicon will hybridize. Amplicons tempered to the conjugated primers are recognized by streptavidin–phycoerythrin (SAPE) chemistry. This chemical mark, bound to the biotinylated amplicon, is excited by one of the two lasers on the Luminex instrument. The second laser identifies the associated bead color. The combined data is deciphered by a computer software, identifying positive reaction beads and their respective color for allele group assignment[19]

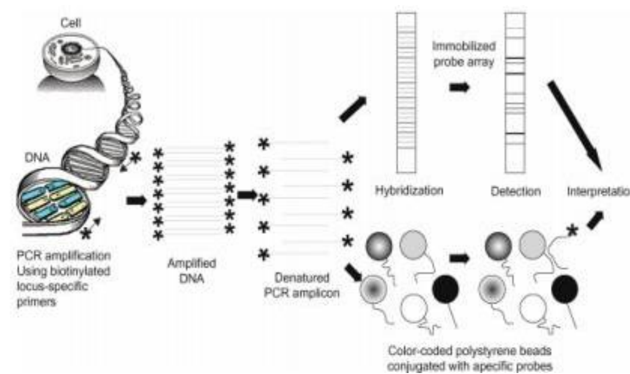


Figure 8: Reverse SSOP Hybridization Methods .DNA samples are PCR amplified using biotin-conjugated locus-specific primers and the amplicons are enzymatically labeled with a biotin molecule during the PCR reaction.

d) Sequence Based Typing

The SBT method has the highest resolution possible, which is important for identifying genetically compatible hematopoietic stem cell donor and recipient pairs and also for discovering new alleles. SBT uses locus- or group-specific amplifications of the polymorphic exons, followed by direct sequencing of the PCR products. The PCR amplification products are run on a 2% agarose electrophoresis to verify the presence of amplified products of the perfect size.

Sequencing is perfomed using DYEnamic ET terminator chemistry or BigDye chemistry on an automated sequencer. The sequences of both strands are imported into sequence alignment and analysis software for edition or quality control when needed.The sequence generated from the forward and reverse sequences is compared with a library of existed sequences, and alleles are assigned. PCR-SBT is the only technique that directly detects the nucleotide sequence of an allele, there for it can be accurately assigned. It requires very expensive equipment and complex laboratory methods to provide clear, high quality gDNA.[19]

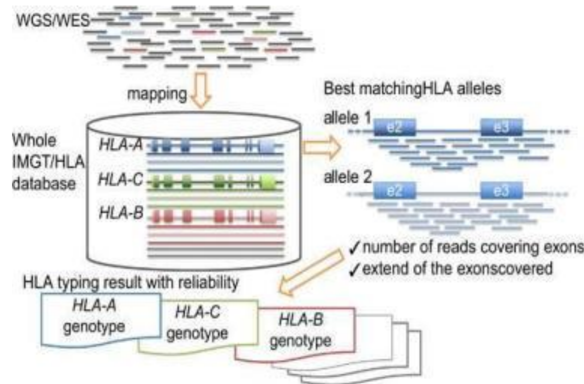


Figure 9: Sequence based technique's major steps. WGS: Whole Genomic Sequence. WES: Whole Exon Sequence

e) PCR/RFLP (restriction fragment length polymorphism)

RFLP analysis includes digestion of Genomic DNA with endonuclease enzymes, these enzymes cleaved a specific nucleotide sequence motif to generate different fragments of diverse lengths. This variation is detected using Southern blot analysis of the digestions fragments. RFLP probes for detection are cloned cDNA or genomic DNA sequences complementary to mainly HLA class II regions. DNA amplified and then incubated with a restriction enzyme which has the ability to recognize the specific nucleotide region. the digestion is performed whether a polymorphism is existed or not producing amplicon fragments of different lengths. The final product is analyzed using gel electrophoresis.

This technique may lead to inconsistencies during HLA typing due to the complexity of manipulation steps and incomplete enzyme digestion, moreover, it is incapable to detect multiple recognition sites concurrently.

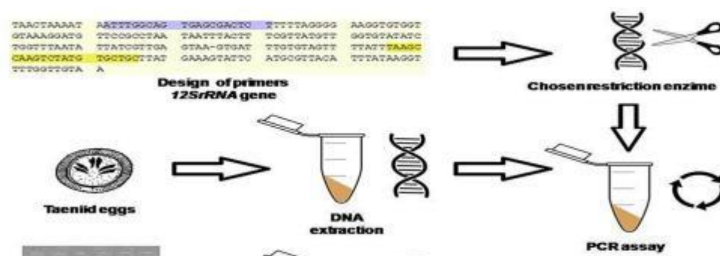


Figure 10: PCR-RFLP assay schema for a microbiological study

f) PCR/RSCA (reference strand-mediated conformational analysis)

RSCA technique achieves high-resolution results with no ambiguities. It is based on the principles that DNA fragments that differ in nucleotide composition exhibit various motilities after separation by PAGE (polyacrylamide gel electrophoresis). The amplified alleles are hybridized with fluorescent-labeled strands, forming dsDNA with special conformation. PCR/RSCA is able to resolve even single base alterations and identification of new mutations because of the different spatial structures of the formed double-stranded DNA[20]

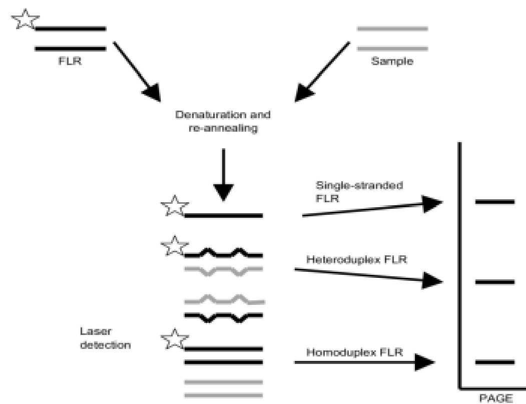


Figure 11: representation of the RSCA method.(FLR = fluorescence labeled reference)

3. Thesis Objectives and Aims

the micro-lympho cytotoxicity have been developed by the scientist terazaki and his colleague McClelland in 1964. the technique was a method of choice for many years in various laboratories for HLA typing, thus HLA testing has an important role in organ and bone marrow transplantation. numerous studies manifest that assigning HLA-class I by techniques involving DNA analysis indicates that serological typing is not sufficiently reliable.

Our laboratory is primarily typed the patients using serology for HLA class I, if serology was insufficient, the usage of DNA technology is then required. After deep observations, discrepant

results occurs with the serological method, and multiple samples with incomplete or ambiguous were found.

In order to identify these inadequate results, we collected patient data for 7 years which were performed by the serology and DNA techniques. Lymphocyte separation was done by density gradient centrifugation of heparinized sample and DNA extraction was done manually.

After analyzing all the data, the serological discrepancies were three types: false negative, false-positive reactions, and cross-reactivity group therefore serology has doubtful results which can affect the recipient's transplantation. This analysis confirms the low ability of serology to detect all currently known alleles for this cause, serology must be followed by molecular methods.

These ambiguous results can be associated with a wide range of errors, first, the purity of the T lymphocyte suspension as any contamination with other cells influences the viability of the results. On another hand, different antigen frequencies in various populations and different antisera used for serological identification can be associated with these serological discrepancies. A small particle of debris due to inadequate centrifugation may confuse the readers which increases the reading errors.

The serological limitations for class I typing include, at first all the commercial plates for HLA typing are designed for specificities frequently found in Caucasians while the plate for typing different other populations are expensive and not always available. Secondly, the quality and availability of antisera reactive are often inadequate to carry out reliable HLA typing.

Materials And Methods

HLA polymorphism can be studied through a serological approach based on complement dependent lymphocytotoxicity (CDC) technique (study of antigens on the surface of cells), or a molecular biology approach the DNA-based typing relies on sequence specific primers (SSP), sequence specific oligonucleotide probes (SSOP), and sequence-based typing (SBT). (definition alleles at the DNA level by the general study of exons two and three for class I and exon two for Class II). In our routine at the laboratory, we serologically type bone marrow transplant and organ transplant pairs for HLA class I initially by Microlymphocytotoxicity. HLA class II is performed using SSO or SSP typing techniques. nevertheless, errors may occur during the first serological workup, requiring the use of DNA based techniques.

1.Complement Dependent Cytototoxicity (CDC) technique

HLA class I were typed by standard microlymphocytotoxicity test using the commercially prepared terasaki trays One Lambda Inc.b.USA. The T lymphocytes separation was done by FluoroBeads®-T isolation method also called Immunomagnetic bead separation from the heparinized blood, these beads are superparamagnetic particles that have the possibility to attached to monoclonal antibodies. The Viable lymphocytes are incubated for a further 60 min with HLA specific antibodies found in Terasaki trays, this leads to an immunology reaction, if the antibodies recognize the antigens carried by the T lymphocytes the antibody will bind to the cell and this leads to cell lysis. for the stain, we used the FluoroQuench Acridine Orange/Ethidium Bromide to fluorescently stain the lymphocytes and to block cytotoxicity reaction. The trays are visualized using the Inverted microscope.

a) Serological typing Materials

- whole blood (from heparin tube)
- Magnetic FluoroBeads-T
- T Developer 1X diluted with PBS
- FluoroQuench stain
- Terasaki trays One Lambda
- RPMI 1640 medium
- Phosphate buffered saline (PBS)
- inverted fluorescence microscope

figure 12: CDC reagents. a) Phosphate buffered saline: is used for dilution, washing cell suspensions as well as additive to cell culture media. b) RPMI medium is used for growth of human lymphocytes. c) Fluorobeads T lymphocytes are superparamagnetic particles that have the possibility to attached to monoclonal antibodies, Fluorobeads T developer

b) CDC Method: lymphocytes T isolation

- Transfer 2 ml of whole blood in a 5 ml capacity tube
- Before use, agitate the Fluorobeads-T for 10 seconds on vortex machine
- Add 100 µl of FluoroBeads-T to the blood simple. close the tube immediately and shake with turnaround movement during 3 minutes at 20-25ºC. Do not exceed 4 minutes.
- Add 2 ml of T Developer 1X (dilution with PBS < 9 ml of PBS + 1 ml of T Developer >)
- close the tube again and return 2 to 3 times to mix perfectly
- Open the tube and place it into a strong magnet for 3 minutes
- Throw out the supernatant with a pasteur pipette. Pull the tube out of the magnet.
- Resuspend the cells in 1 to 2 ml of PBS. Pat gently to disperse the beads and place the tube into the magnet for 1 minute
- throw out the supernatant again . Repeat two times
- put the suspended cells in 500 µl of RPMI medium
- divide 1 µl of suspended cells inside the terazaki tray wellsand add 5 µl of FluoroQuench
- verify the cell concentration using an inverted Fluorescence Microscope
- adjust the concentration at 2×10^6 cells/ml

figure 13: a). inverted fluorescence microscope used to adjust the concentration and read the results. b) magnet with a strong magnetic fields for T lymphocyte-beads isolation. c) tissue typing syringes

2. DNA Typing Methods

2.1 DNA extraction

The Buffy Coats is used which were already prepared in the Eppendorf tubes and the manipulation was carried out using the Qiagen Germany commercial kit.

- In an Eppendorf tube, put 200µl of prepared buffy coat
- Add 20 µl QIAGEN protease and 200µl of AL buffer
- Mix gently for 15 seconds using vortex machine. incubate 10 minutes at +56°C
- Add 200 µl of Ethanol (96-100%) and Mix the tube again for 15 seconds on vortex machine then pour the mix in a QIAamp colon
- centrifuge for a minute at 6000g next Transfer the colon into a new collector tube
- Add 500 µl of tampon AW1. centrifuge for 1 minute at 20000g
- Transfer the QIAamp colon again into a new collector tube. Add 500 µl of tampon AW2
- Centrifuge 3 minutes at 20000g and transfer the QIAamp colon into a Eppendorf tube
- Add 50 µl extra pure water to the QIAamp colon
- incubate for 5 minutes at Room temperature then centrifuge 1 minute at 6000g
- trow the QIAamp colon, store the extracted DNA at -20°C

The purity of the DNA was done by measuring the absorbance at 260/280 using spectrometer.

2.2 PCR-Sequence specific primer

For SSP method, the amplification was done by the Micro SSP kit One Lambda Inc.USA using the PCR technique, which was taking place in Thermocycler T100 for 1H and 16 minutes..the PCR product was analyzed on 2.5% agarose gel containing ethidium bromide and visualized under ultraviolet (UV) light.

a) Sample preparation

- defrost the SSP kit as well as the number of DNA samples at room temperature (20-25ºC)
- agitate the DNA samples using vortex machine and place the PCR plate on an adapted rack
- take the Taq polymerase out of the freezer and keep it in ice until use
- add 1µl of extra pure water in the negative control tube of the primer plate
- add the Taq polymerase (5 U/µl) in the D-mix tube: SSP1L= 5.6 µl / SSP2L = 2µl
- agitate on vortex machine during 5 seconds and centrifuge during a short moment
- add 9 µl of reaction mixture (D-mix/Taq) to the negatif control tube
- add the DNA sample in the reaction mixture (D-mix/Taq): SSP1L=111µL / SSP2L= 39µL
- agitate the reaction mixture (D-mix/Taq/DNA)on vortex during 5 seconds
- centrifuge several times during a short moment
- distribute 10 µl of the reaction mixture (D-mix/Taq/DNA) In every reaction tube, except for the negative control one
- cover the reaction tubes with a provided adhesive sheet
- verify that the tubes are perfectly covered to avoid the evaporation during PCR reactionwith
- place the plate into the thermocycler

b) PCR Reaction

- turn on the thermocycler T100 and write your program number; file; HLA, protocol: PCR-SSP
- verify the temperature and the reaction volume 10 µl
- click on run to start the program. The PCR-SSP duration is 1h and 16 minutes
- when the PCR is done: move out the plate from the thermocycler

- withdraw delicately the adhesive film with maintaining the plate on a horizontal plan
- transfer the PCR product on an arose gel at 2.5%

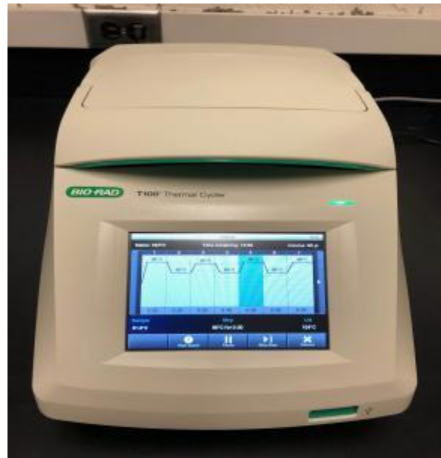


figure 14: thermocycler T100 used for PCR reaaction for both SSO and SSP

2.3 PCR-Sequence Specific Oligonucleotide Primer

We applied reverse SSO using Luminex technology with IMMUNOCOR SSO HLA typing kits. initially, the DNA is amplified with PCR method using a group specific primer, later the PCR product is biotinylated facilitated the detection using SAPE. the biotinylated PCR product is denatured and allowed to rehybridize to the DNA probe coupled to fluorescence microsphere. The IMMUNOCOR LUMINEX 200 is used to identify the fluorescent intensity of PE by the flow analyzer.

a) DNA amplification

- take the master Mix kit out of the refrigerator and leave it at room temperature (18-30)
- prepare the number of tubes needed for PCR reaction (TUBE CORNING IMMUCOR)
- DNA preparation: **a)** vortex the DNA 10 to 20s and put between 40-100ng in every well
- a mix for locus preparation: **b)** reaction mixture

  locus master mix: 6µl

  water nucleas free: depends on DNA volume

  c) take the Taq out of the freezer. Vortex for 10s and put in the Mix(0.2µl/1U for well)

  d) vortex the compete Mix

  e) pour the mix in the tubes contain DNA
- close the tubes perfectly to avoid the evaporation: place it immediately in thermocycler and assure that the PCR tubes are touching the thermocycler's bottom, if not add 1 to 2 pressure pad

- place the samples in the thermocycler then start the amplification program: assure that Ramp speed is at 3,9 ºC/S in all steps and that the LID is at 98 ºC.

b) Hybridization

- activate dilution buffers before use:5 minutes at 45ºC in Marie bath
- turn on the Luminex, warm up the beads in marie bath between 55 and 60 ºC for 5 to 10 minutes
- vortex the beads for 30s then distribute 5µl of PCR product for each well in a costar plate
- add 5µl of beads in each well, cover the plate with a thermoresistant film, place it in the thermocycler (add two or three pressure PAD)
- start the hybridization program: assure that the Ramp speed is at 3,9 ºC/S in all steps and that the LID is at 98 ºC.

During the hybridization: prepare the SA-PE dilution / for each well: 170µl dilution buffer + 0.85µl of SA-PE

after the hybridization: leave the plate in the thermocycler at 56ºC and distribute the diluted SA-PE in the 5 minutes after the final cycle.

c) Results and interpretation

- take the plate out of the thermocycler and place it in the Luminex
- press on start reading
- results interpretation is done using the Quick-Type or Match it DNA programs.

Figure 15: Luminex technology from IMMUCOR GAMMA®

CHAPTER 3

Results And Interpretation

The serological trays are visualized using the Inverted microscope. The cells with the positive reaction took up the red stain and appeared red under the microscope. The negative reaction appeared green under the microscope. the results scores depend on the number of lysed cells present in a single well; the scores are from negative to eight (all cells are lysed).

We were able to identify the CDC technique limitations in HLA class I typing. in addition, we were able to detect troublesome antigens that are commonly misinterpreted in serology based on the cross-reactivity group and the tray analysis summary of the LAMBDA Monoclonal typing tray set for class. moreover, we compared our serological results with DNA techniques results in order to identify the misreads antigens. More than 200 patients were analyzed for both organ and bone marrow transplants and they were class I typed using standard microlymphocytotoxicity test. we included in our study the patients whose serological results needed DNA-based typing. the serological and DNA results were analyzed in the EXCEL file caring the technique results, the date, reference, and the type of transplant needed.

1. Misreads and dropouts antigens detected on serology for HLA class I

The data was collected from 2014 to 2021 for both serological and DNA typing. All the HLA typing patients required either bone morrow or organ transplant in order to find a pair who is serologically matched at HLA class I locus. Figure below shows the number of misreads or dropouts on serology for both HLA-A and HLA-B loci and they were identified using DNA techn

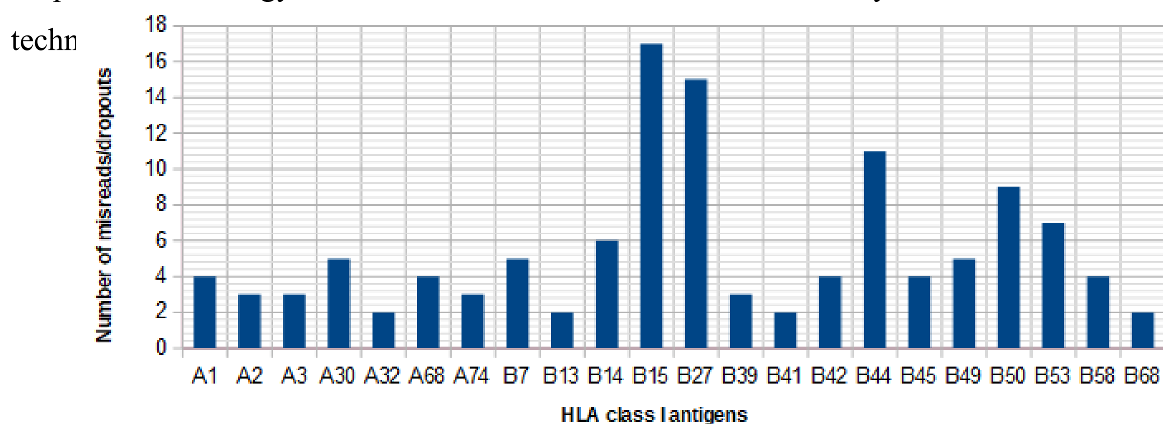


Figure 16: number of misreads/dropouts for HLA class I on serology

At first, HLA-A carries the lowest number of misreads/dropouts antigens, in the other hand HLA-B has the most ambiguous antigens. B15 was misreads and dropout 17 times on serology which make it the highest number among the others. B27 and B44 were misreads/dropouts 15 and 11 respectively, thus CDC was more sensitive for HLA-A detection and that was predected because HLA-A has a small number of variants compared to HLA-B. B41,B39 and B68 were misreads and dropouts between 2-3 times, this may due to different antigen frequencies in various population.

Both SSO and SSP techniques were able to detect these antigens. Figure below shows the SSP results for a particular patient containing the possible allele code as well as the serological worksheet carries the reaction scores and the specificity for each tray.

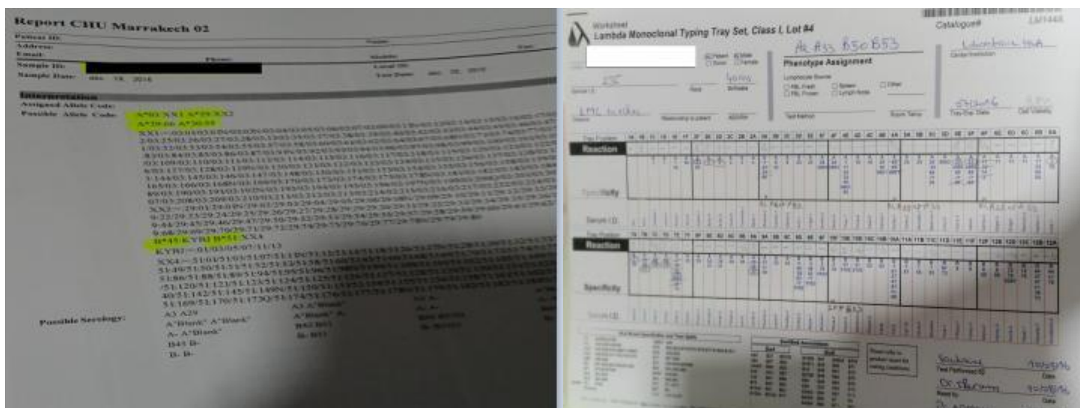


Figure 17:Left. DNA worksheet for SSP method and HLA genotype. Right. LCT worksheet for result registration quality

we calculated the number of misreads and dropouts separately for HLA-A and HLA-B (graph below):

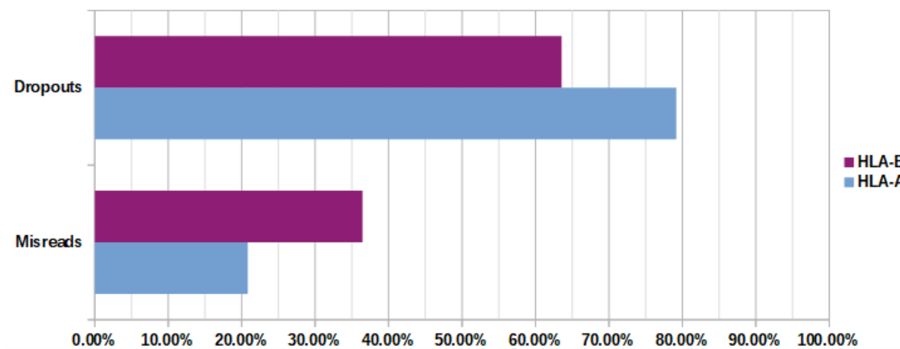


figure 18: total percentage of dropouts and misreads for HLA A and HLA B

HLA-A dropped out on serology nearby 80% which is lower than 63.5% in HLA-B. contrary for misreads, HLA-B showed more misreads than HLA-A therefore dropouts were more frequent than misreads may due to the low cellular expression on cells surface.

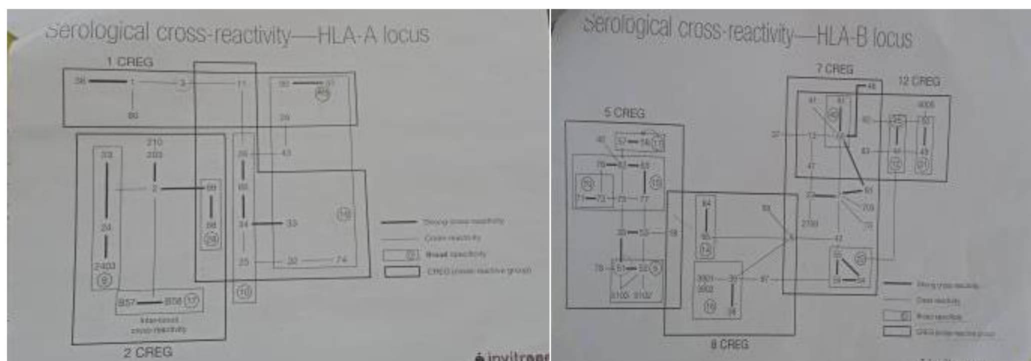
2. Cross Reactivity Group and its influence on serological results

Although CDC technique had considered as a gold standard, small parameters may modify the viability of the results including the Cross-Reactivity Group. Table 3 shows the influence of CREG on our serology results for both HLA-A and HLA-B loci.

HLA antigen	Cross reactive group	CDC technique results	Terazaki plate tray	catalog type
A1	cross reactivity with A30,	~90% cells are lysed	8B	LM144A
A2	cross reactivity with A68,A24	~65% cells are lysed	3F,7E	LM144A
A3	cross reactivity with A11,	~85% cells are lysed	6F	LM144A
A11	cross reactivity with A3	~55% cells are lysed	6F	LM144A
A29	cross reactivity with A30,	~81% cells are lysed	5C;8D	LM144A
B7	cross reactivity with B27,B60	~65% cells are lysed	9E;10B	LM144B
B8	cross reactivity with B18,B27,B54,B42,B14	~64% cells are lysed	5B;1F;11B;7F;6A	LM144B
B14	Cross reactivity with B8,B38	~75% cells are lysed	7C;1F;2F;5B;3E	LM144B
B17	cross reactivity with B51,B52,B35,B72,B78	~72% cells are lysed	4E;5D;8B	LM144B
B18	cross reactivity with B39,B8,B35,B51	~50% cells are lysed	4F;11B;5B;12F;8B	LM144B
B27	Cross reactivity with B81,B7,B8,B42	~80% cells are lysed	10B;12B;7F	LM144B
B35	Cross reactivity with B53,B63,B51,B17,B18	~82% cells are lysed	12F;9A;9C;5D;5B	LM144B
B41	Cross reactivity with B50,B42,B,B17,B18	~52% cells are lysed	11C;9F	LM144B
B44	cross reactivity with B49	~60% cells are lysed	6E	LM144B
B49	cross reactivity with B44,B41,B40	~66% cells are lysed	6E;11C;11A	LM144B
B50	cross reactivity with B41,B60,B49	~75% cells are lysed	11C;9A;6E;9D	LM144B
B51	cross reactivity with B17,B35,B53,B46,B78	~80% cells are lysed	4E;9D;8A;11F,9F	LM144B
B53	cross reactivity with B51,B17,B35	~91% cells are lysed	8A;4E;9A	LM144B
B57	cross reactivity with B35,	~60% cells are lysed	12F;	LM144B
B63	cross reactivity with B70,B77,B35	~59% cells are lysed	12F;8B;9A	LM144B
B72	cross reactivity with B15,B17	~57% cells are lysed	3B;5D	LM144B

Table 3: cross reactivity group of certain antigens and its influence on serology results (cell lysis errors)

Terazaki plate tray can be specific for one antigen as well as one terazaki tray can detect multiple antigens at the same time. Considering both HLA-A and HLA-B, CREG has a huge impact on the cell lysis in a specific tray. Five antigens integrated in cross reactivity for HLA-A, meanwhile more than 16 antigens were responsible for the cross reactivity in HLA-B. Furthermore HLA-B has the highest number of cross reactivity antigens compared to HLA-A which had a low number of cross reactivity antigens. in addition, ~90% of the cells for A30 and B53 were lysed which make CREG an importance parameter to take into consideration on serological typing. B72, A11 and B41 considered as the weakest antigens belong to Cross reactive group due to the low number of cell lysis (<=57%). The antigens belong to the same CREG present a high percentage of cell lysis including A3, B35, B51 and A29 in a field superior than 80%. Meanwhile, B8, B17,B35 and B51 has the biggest number of the antigens belonged to the same cross-reactive group more than 4 antigens per CREG. Images below present an example of cross reactivity



catalog used to help interpret the serological results.

Figure 19: Left: Serological cross reactivity catalog_ HLA-A. Right: Serological cross reactivity catalog_ HLA-B

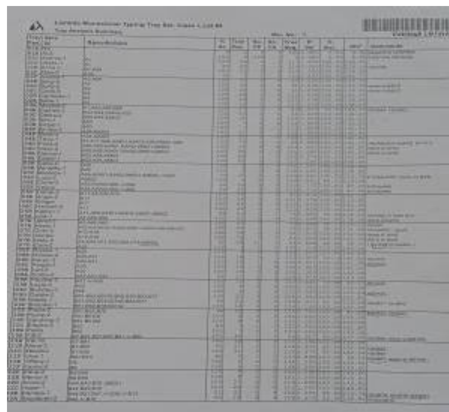
3. Observation of false positive and false negative reactions in Terazaki trays

Including few patients, negative results can turned out to be positive and the positive results may turned out to be negative on serology test. These interpretation are based on tray analysis summary from Lambda monoclonal tray set used in HLA typing by serology. Table 4 shows the false-positive and the false-negative serological HLA detected in a particular antigen.

HLA antigens	False-negative	False-positive	Repeated times
A2	....	11 fp A2; 1 fp A2	18
A23	1 fn A23 ; 2 fn A23	....	9
A29	....	1 fp A29;	6
A30	1 fn A30 ; 5 fn A30	....	29
A68	6/20 fn A68	....	4
B7	....	8/18 fp B7;	5
B27	1 fn B27	2 fp B27	8
B35	....	8 fp B35 ; 3 fp B35; 4/8 fp b35	13
B39	2 fn B39	4/9 fp B39 ; 4 fp b39	9
B44	2 fn B44 ; 1 fn B44	3 fp B44 ; 8 fp B44	27
B49	2/4 fn B49 ; 1 fn B49	1 fp B49 ;	7
B51	1 fn B51	....	2

Table 4: repeated times of false negative and false positive serological HLA typing reaction

Several patients including children were originally typed by serology gave a false positive or negative reactions in different trays. Furthermore, a tray can carry 9 false positive reactions for example 7 of them are due to B35 and 2 others are due to B44 as well as in false negative reactions. most of the false reactions were either containing respectively A30, B44 or A2. For example we can find 8/18 fp means that 8/18 reactions where positive and 10/18 of the reactions where negative. various antigens can be false-positive and false-negative such as B39, B44 and B49. A30 was false negative 29 times in numerous patients (tray number 09A in HLA-A catalog have a 5 fn A30 reactions, meanwhile tray number 08E have 1 fn A30 reactions. Figure below). B44 was 27 times false negative and false positive 7 of them belong to fp reactions as well as 20 of them where fn reactions. HLA-B has the highest number of the false tests compared to HLA-A which confirm that the serology is more sensitive for HLA-B typing. Figure below shows the tray analysis summary catalog HLA-A and HLA-B



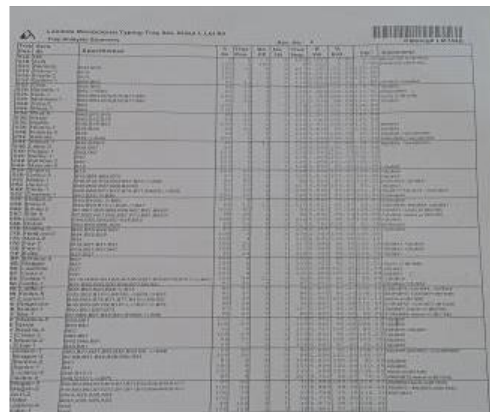


figure 20: tray analysis summary OneLamda serology tray set

CHAPTER 4

Discussion and perspectives

1. Thesis Discussion

Thanks to DNA-based techniques, however, we were able to identify almost all the ambiguous antigens obtains with the CDC technique. Our study has shown that very often some antigens were not determined by the serological method Microlymphocytotoxicity. this method was considered as a standard gold technique for HLA typing, hence there are various ambiguous antigens that need to be identified using other techniques including SSO, SSP, and SBT. Most of the antigens that had been misread/dropout in figure 16 were then detected using either SSO or SSP technique which confirms that serology has a high percentage of failure in detecting HLA antigens and this can be explained by either the serological typing shows only the molecules that are actually expressed on the cell and the molecular polymorphisms, which code for the different allelic variants or because of the problems of poor cell viability or poor surface HLA expression.

we obtained 120 misreads or unidentified antigens by serology most of them were HLA-B that was predicted because this locus is the most polymorphic locus among all HLA loci as Mishra el al also found that HLA-B was identified in 55 % of the cases. [21,22]A30 was the most frequent misread and missed antigen on HLA-A, while B27 and B15 were misread several times in serology for HLA-B and were detected in molecular methods as well as I S Gourley et al found [23] and we also found the B44 and B45 were not detectable on serology. Riba Silva et al have also found B45 antigen to be problematic.[24]misreads were less common than dropouts, A32 was misread as A74, and B15 was misread as B72 six times. False serological tests may be due to the combination of low expression of HLA antigens which E Kulcsarova and al also found. 26 nearly all the antigens were misreads on serology were split of the same broad specificities, belong to the same cross-reactive group (CREG), or considered as a false serological test.

The serological method may include other problems including cells concentration, with less than required concentration, incorrect or uncompleted results occur during the process, on another hand, we observe that manipulated cells must be read on the same day as CDC scores may change if you leave it overnight.

The diversity of the ambiguous antigens may be due to different antigen frequencies in a particular population and different commercial plates used in serology tests. the individuals with a blank antigen should be retyped using the molecular method. the usage of DNA-based typing is

recommended in solving the Ambiguities on serology method. these Ambiguities are very common in HLA typing either on serology or DNA-based typing. all individuals must be typed by serology first, if the ambiguous antigens are present, DNA-based typing is needed. Our study indicates that serological typing is inadequate for daily HLA typing.

Numerous studies have shown that both SSO and SSP methods can fail to give a valid result. In this case, Using SBT is a more accurate methodology as it has the highest resolution and is the only way to directly sequence and identify new alleles.[26]

2. HLA typing perspectives

HLA typing future is now focusing on the second and third-generation sequencing which moved forward into personalized medicine, genetic diseases, and clinical diagnostics whichever reduce time, provide higher sensitivity and high-quality results. Second-generation sequencing relies on library preparation using PCR technique, sequencing steps, and data software assay.

2.1 Clonal amplification and cyclic array sequencing

ROCHE/454 sequencing by synthesis (single nucleotide addition)

Library preparation: the usage of the spray method to break DNA strands into small fragments and add 2 adapters at both ends. Differently, after denaturation use primers for amplification, clone in a specific vector, and construct a single-stranded DNA library.

Emulsion PCR: before DNA is amplified, an aqueous solution with all components of PCR will be saturated into the surface of mineral oil with high-speed rotation, and it formed a small bubble of water wrapped by oil. One bubble forms an independent PCR reaction, and each bubble contains one DNA template and one small bead on the beads surface, we find complementary oligos to match the DNA adapters thus the ssDNA can specifically bind to the bead.

Occasionally, the incubation system contains PCR reagents to make sure that each DNA fragment is bound to the bead can be a specific template for amplification. After the PCR reaction, the emulsion system can be destroyed and targeted DNAs could be collected. In the end, each fragment will be amplified more than 1 million times to achieve the amount level required for the sequencing procedure.

Pyrosequencing: during sequencing on a picotiter plate, only one nucleotide is added in each round then a series of enzymatic reactions lead to the release of inorganic pyrophosphate Ppi only when the correct dNTP is incorporated. PPI transforms ATP which changes luciferin into oxyluciferin that emits light. Data is stored in standard flow gram format files for data analysis.

The emitted light is detected by a charge-coupled diode camera which is then translated into a single peak per base using computer software.

This technology offers a long sequence of reads from 400 to 1000 amplicons as well as the speed needed for a complete run is performed (10 up to 24h) depending on the machine used. For example, 454 GS FLX Titanium can provide up to 1*106 reads per run. Although the advantages, disadvantages still exist including the high cost of pyrosequencing reagents, high error rate, however, insertions mutations are the most common error followed by deletions.

Illumina (MiSeq and MiniSeq) sequencing by synthesis; cycling reversible terminator CRT

Illumina uses a different type of sequencing named CRT. Instead of emulsion PCR, the system incorporates a glass slide with lanes called flow cells. Various primers are complementary to a sequence of adapters in DNA fragments that are already attached to the flow cell where the sequencing is performed. The amplification is done using a process called clustering, each fragment is amplified isothermally to generate a cluster of amplified fragments, this technique is called bridge amplification.

Sequencing begins with the binding of the first sequencing primer on every fragment of each cluster to produce the first read. All four nucleotides are fluorescently labeled, when a nucleotide is complementary to the original sequence is incorporated and a light signal has a unique wavelength and intensity is emitted. all unbound nucleotides are washed from the flow cell. The signal is captivated by a CCD camera and recorded by the computer. Due to the reverse terminator chemistry, the fluorescent molecule of each nucleotide incorporated needs to be cleaved before starting the second cycle. The length of the read depends on the number of cycles programmed by the user.

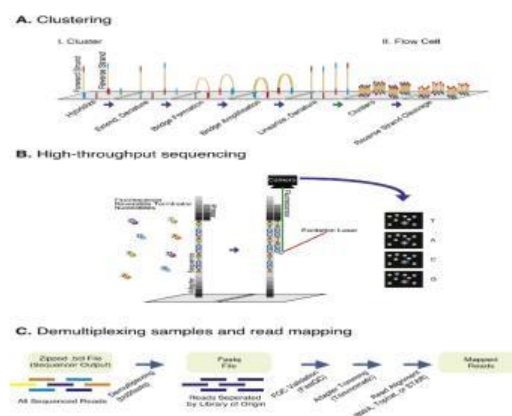


Figure 21: illumina sequencing and data processing workflow. A.clustering steps. B. fragment sequencing with reverse terminator nucleotides. C. read mapping and alignment

The MiSeq offers an option of 44-50M paired-end reads which is enough for HLA typing of many samples at the same time. CRT method overcomes the disadvantages of SNA, by incorporating only a single nucleotide at a time therefore the error rate of the machine increases. Sequencing errors accumulate against the read end, thus longer reads which can be trimmed are preferred compared to shorter ones.

Thermo-Fischer (Ion PGM)

The Thermo-Fischer machine relies on a ph-mediated sequencing detection technique. The addition of a new nucleotide on the extending DNA strand involves the formation of a covalent bond and the release of a positively charged hydrogen ion. The increase of PH level is detected by an ion-sensitive layer found on the bottom of the semiconductor chip, where sequencing happens. The read length is from 200 to 400 bp, depending on the chip for a minimum of 0.4 M and a maximum of 5.5 M reads can be exported from a run. This technique has no need for optical devices which increases the error calling, lower speed, higher cost. However, the run time range is from 2 to 7 hours considered as a positive characteristic against other competitors.

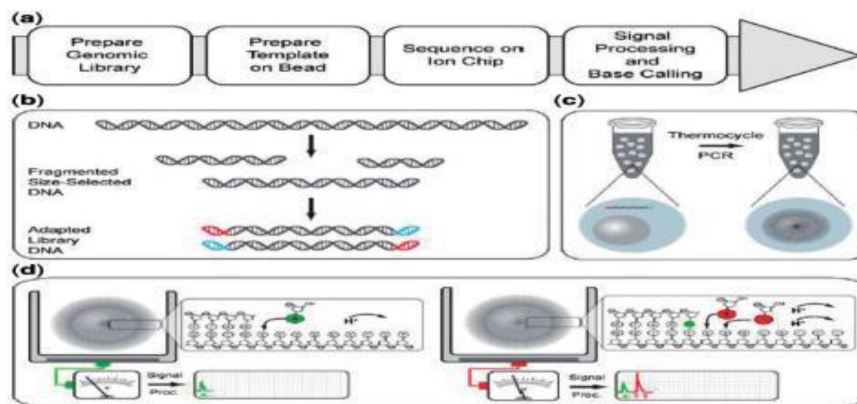


Figure 22: ion sequencing work flow. (a) semiconductor sequencing steps.(b) library preparation and adapter ligation.(c) emulsion PCR amplification.(d) PH detection and H proton deliverance

2.2 the third generation of sequencers (single molecule sequencing)

Since the development of second-generation sequencing is still going, the third generation is already on the field promising with a lower cost per base, faster sample preparation, simplified data analysis, and longer reads lengths thus longer reads simplify sequence assembly and facilitate SNP analysis, especially for HLA typing and ambiguities resolution. Furthermore, there is no need for PCR amplification which avoids errors and reduces preparation time.

Pacific biosciences – single molecule real-time sequencing (SMRT) move this

PacBio instruments use a flow cell with individual transparent bottom wells (zero-mode waveguide wells)

each one is caring 20 zeptoliters (1 zl = 10-21 l).

this technology utilizes short single-stranded hairpin adapters that bind to the ends of the DNA fragments. A unique DNA polymerase called phi29 anchored to the bottom of wells binds to a single DNA and starts extension. While nucleotide-binding the fluorophore emits light then visualized with a camera as well as the dye is cleaved and the polymerase starts incorporating a new nucleotide. Each type of color change at every well captured by the camera corresponds to a different nucleotide added to the sequence.

Every template is sequence multiple times in a circular way. These multiple times are used to generate a consensus read of insert called CCS circular consensus sequence.

The runtime and throughput of the instrument tuned by the user and longer templates require longer times. The pacbio template generation takes up to 6 hours a much less time compared to second-generation sequencing. Moreover, there is no need for PCR amplification which reduces errors and time. The major disadvantage of this method is the error rate due to the short interval between two nucleotide incorporation.

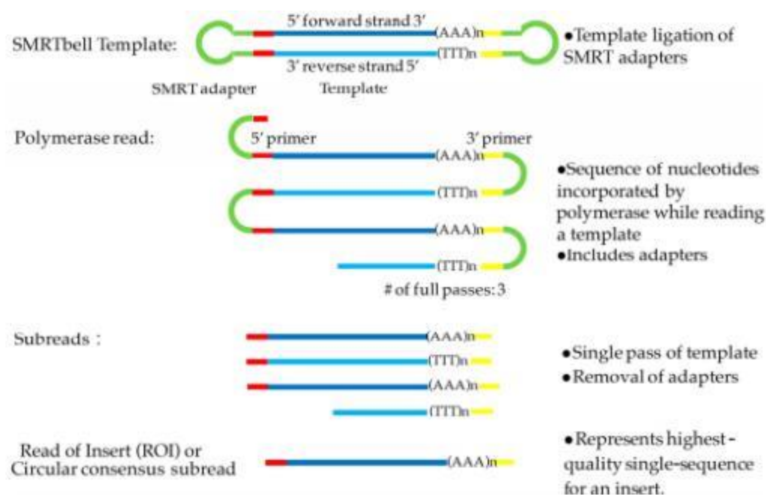


Figure 23: SMRT sequencing. DNA is labeled in blue and adapters in green.

Oxford Nanopore (MinION)

Oxford Nanopore uses an artificial bio-pore of nanoscale in diameter with an attached exonuclease. Each DNA end is bound to adapters, a leader, and a hairpin. The hairpin helps hold the ssDNA conformation, meanwhile, the leader adapter guides the DNA through the exonuclease.

The special character of each DNA base causes an observable disruption in electrical current, providing a readout of the underlying sequence. DNA can be sequenced by threading it through a nanopore in a membrane. Bases are identified by the way they change ions flowing through the pore from one side of the membrane to the other. The technique needs no polymerase, all it needs is ssDNA and two adapters making the cost of sequencing less than other techniques.

MinION is a Pocket-sized, portable device for biological analysis that contains up to 512 nanopore channels provides a short time of sample preparation within 20 min it is also adaptable to direct DNA or RNA sequencing. A disadvantage of the Nanopore is The large error rate of up to 30% due to the big amount of signals coming from the pore. [27]

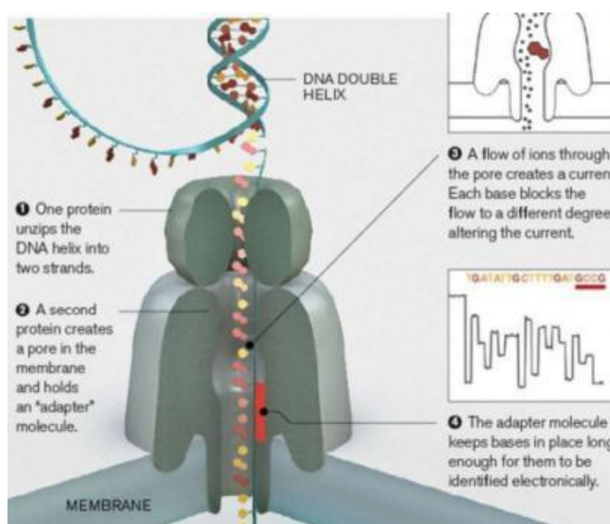


Figure 24: Oxford Nanopore technology. Illustration describe the steps and conformation of nanopore technique

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