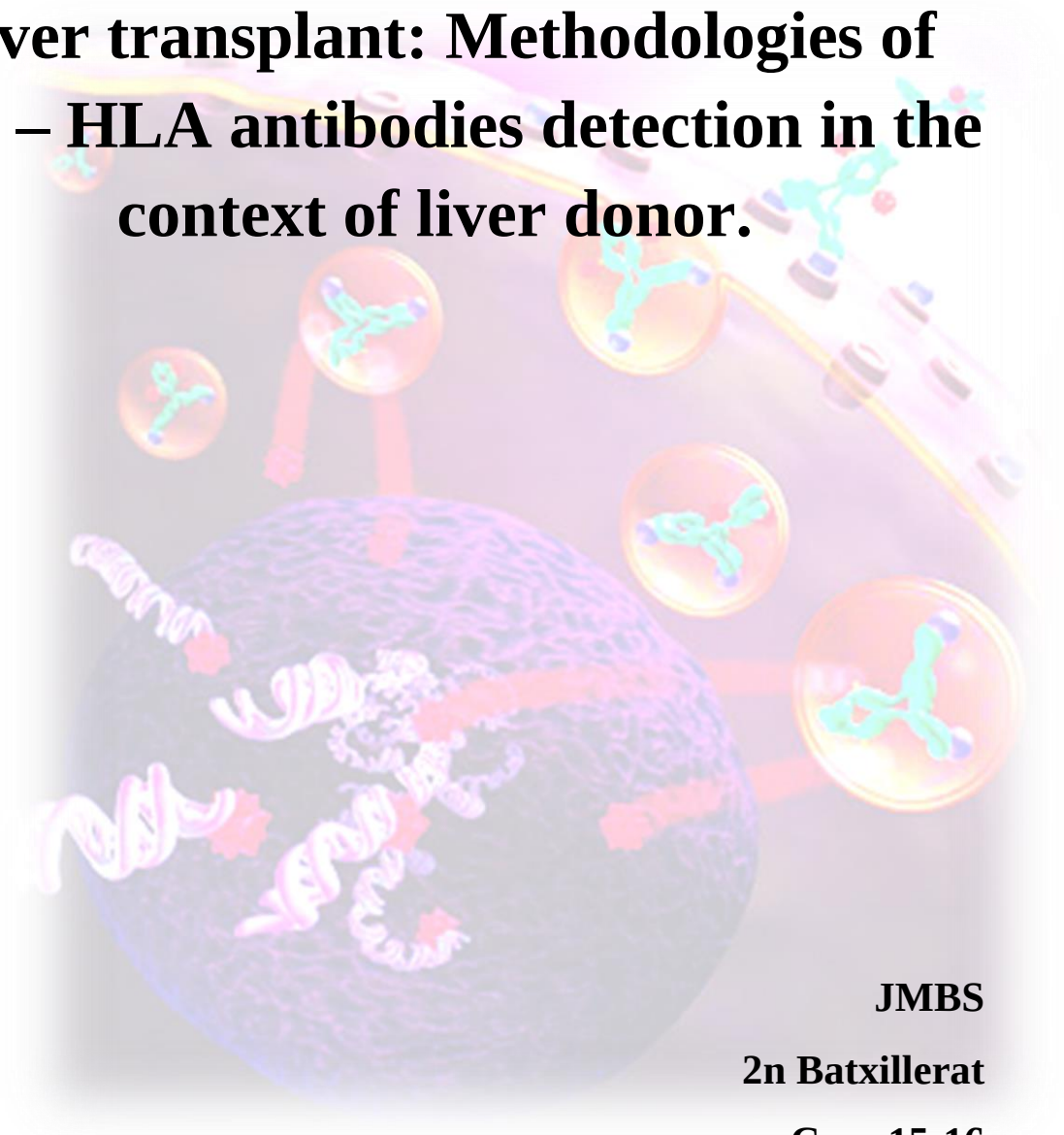
Liver transplant: Methodologies of anti – HLA antibodies detection in the context of liver donor.



JMBS

2n Batxillerat

Curs 15-16

{DEDICATED TO JOSEP MARIA BARRERA SALA}

Index:

1. INTRODUCTION

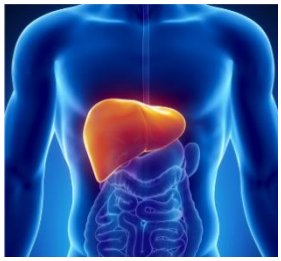
1.1. Motivation and expectations

Since I was 5 years old I have wanted to study medicine. My uncle was a doctor and worked at "Clínic" hospital, in Barcelona. He was hepatologist and he had done a lot of research projects all around the world. My vocation comes because I have always been interested in his job and all the works that I have seen him doing. I want to study it because I don't like to see people suffering and I feel so powerless when someone of my immediate surrounding or even someone I don't know has pain. I think that this project will help me to introduce myself into the wide human body world. I also will learn how to work in a real laboratory with human cells, not with chemical substances as before. I expect that I will reach experience of the "real life". I expect that because at the moment if I don't know how to solve a maths problem, the teacher explains me how to do it and it's all, but when I am working there I will have to try to solve my own problems like in the real jobs. Of course there will be people helping me, but it will be more realistic than just sitting at a table in classes. I decided to do this project in English because I thought that this language is being and it will be the language of the future. All the research nowadays is made in English so that everybody could understand it. I think that this may help me in a future if I work in a project similar to this, at least I will have a basis.

My research project will consist of 9 different parts. The first one will be the liver with its functions, the diseases that can suffer and the transplant. I'm going to start with this part to situate the reader in this certain organ although the HLA system is present in all our body. The second part will be focused in the HLA system and the different immunity responses against the graft. In the third part there is going to be explained the rejection of the organ. The rejection will be the topic of the third part. The next section will be about the different methodologies that could be used to detect anti-HLA antibodies. The fifth part will be focused in a real case of hepatic transplant, rejection and recovery: the interview to a transplanted person and how she lived the process, this part will be the most "human" part of the project. In the sixth part I will make a

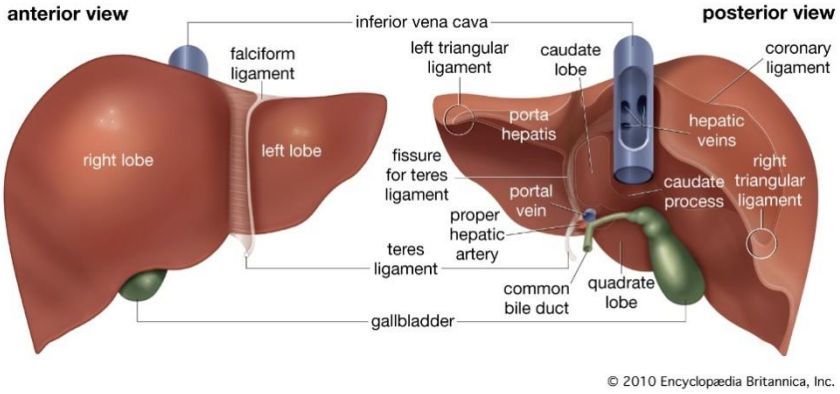
glossary with the explanations of some technical words, initials and some products, the seventh one consists in the acknowledgements. In the eight part there will appear the conclusion. The last part will be the webgraphy.

1.2 The liver



Anatomy:

- The liver is the largest organ in our body. It is located in the upper right side of our abdomen. This organ weights approximately 1’36 kg. It keeps about the 13% of body’s blood. It is made by a brown tissue and protected by the abdomen muscles.



- As it can be seen in the photo, the liver consists in two (the most important) different lobes, the biggest one is the right one, and it is six times bigger than the left lobe. The falciform ligament is the one that join together the two lobes. The porta hepatis is a fissure that divides the left lobe into 4 segments.
- The gallbladder helps with the digestion and keeps the bile produced in the liver. It is connected with the pancreas with the common bile duct. Near this duct there is the proper hepatic artery (originated in the common hepatic artery) which provides the liver and the gallbladder of oxygenated blood. The hepatic veins collect the deoxygenated blood.
- Teres ligament is the remaining portion of foetal umbilical vein. The coronary ligament connects the upper central part of the liver with the diaphragm. The left and the right triangular ligaments connect the upper ends of the organ with the diaphragm.

Basic functions:

- **Digestion**: It produces the bile, which helps in broking the fat into small pieces to be digested with more facility.
- **Metabolism**: The liver metabolizes lipids, carbohydrates and proteins into useful materials in the body.
- **Immunity**: This organ removes bacteria from our body producing immune factors.
- **Storage**: It is a reserve of glucose, iron, vitamins and minerals.
- **Detoxification**: It clears the body of harmful substances. It clearances bilirubin. Bilirubin is a substance caused by the deterioration of haemoglobin (red blood cells). It is used to detect some diseases such as hepatitis or cirrhosis. It is eliminated by bile and urine.
- **Production**: The liver produces proteins for the blood plasma, cholesterol and special proteins to help fat go around the body.

1.3 Most common diseases that can lead to a liver transplant:

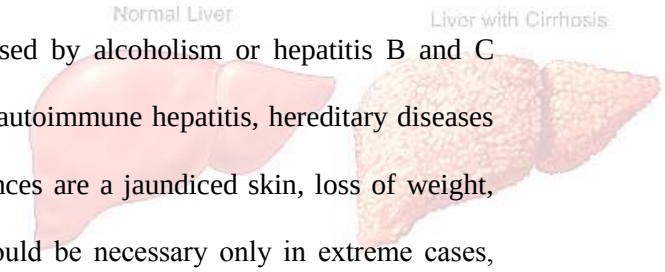
1.3.1 Hepatic insufficiency or liver failure: Is the state of the liver in which it can't work normally nor perform its normal functions. There are two types:

1.3.1.1. Acute: In acute liver failure, difficult complications appear such as hepatic encephalopathy or a decrease of proteins synthesis. These difficulties indicate that the liver has suffered enough and it has lost the function of 80-90% of its cells. It could be caused by drugs, as mushrooms, or by infections too.

1.3.1.2. Chronic: People with chronic liver failure have liver diseases at large stage. The following diseases are the ones that could appear.

I: Cancer: This is a disease that makes the cells divide without control. It is usually necessary to do a liver transplant if the cancer is spread in one to three tumours that cannot be entirely removed either because of the place they are located or because their liver function is poor. But the transplant is not always the solution, most of the times what they do is to remove part of the liver. A condition to make this type of surgery is that the person whose liver is going to be removed (part of it) has to be in good health conditions and their liver functions mustn't be poor.

II: Hepatic cirrhosis: The scar tissue makes the liver hard and unable to work correctly. It is usually caused by alcoholism or hepatitis B and C infection. Other causes might be autoimmune hepatitis, hereditary diseases or some medicines. Its consequences are a jaundiced skin, loss of weight, tiredness… A liver transplant would be necessary only in extreme cases, when cirrhosis leads to end-stage liver disease.



III: Hepatitis: Inflammation of the liver.

- **A:** It is caused by Hepatitis A virus. In general this type of hepatitis is self-limited and it doesn't cause chronic disease.
- **B:** It is caused by the HBV. In some cases it might be chronic and could lead to cirrhosis. Nowadays there are effective treatments with

antiviral that can control the viral replication. Only in exceptional cases of HBV infection, they could be object of transplants.

- There are vaccines against HAV[(1)] and HBV[(2)], the prevalence of the infection has decreased.

- **C:** This type of Hepatitis in most cases could lead to chronic disease that could evolved into HCC [(3)]. Although recently new antiviral drugs are effective in controlling the viral replication, there are some patients that didn't respond to the treatment and required a liver transplant.
- **D:** It's a type of hepatitis that only presents symptoms in case the patient has Hepatitis B too. The virus of Hepatitis D could deteriorate hepatitis B infection.

IV: Storage diseases: Some diseases like Wilson disease doesn't make some enzymes and the proteins storage in the liver disturb its function.

V: Sclerosing cholangitis: It's an inflammation, cicatrisation and destruction of the biliary conducts, inside and outside the liver. It may need a transplant but in early phases it could be treated by not surgical methods.

VI: Wilson disease: It is a genetic disease in which there is too much cooper in the tissues of the body. The liver usually filters extra cooper but in this disease liver doesn't filter cooper properly and it goes all around our body. High levels of cooper poison and can cause severe damage. This disease could be treated with medicines but if the liver damage is serious a transplant will be necessary.

VII: Metabolic disease: It affects the chemical activity in cells.

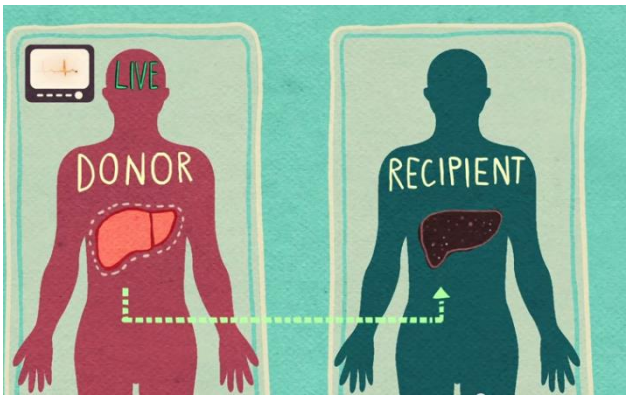
1.4. Transplantation

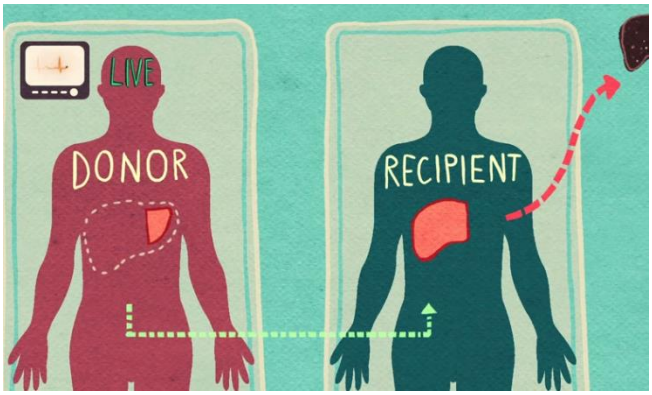
1.4.1. Definition:

- A transplant is an operation that consists in removing a damaged organ and replace it with a healthy organ. This kind of operations are very delicate because the doctors have to take into account a lot of things such as the compatibility of the organs, the level of blood in the body, if the healthy organ is healthy enough… They are complicated surgeries but most of the times they are successful.

1.4.2. Donors: Living donors or donors with encephalic death

- The organ can be from a person with encephalic death (the entire organ is removed) or a portion of the liver of an alive person. In the last case, the donor tends to be a family member but it can be an unrelated person too. The living transplant has three basic steps, the first one is remove part of the liver of a healthy donor, the second one is remove the entire liver of the donor and the last one is put the healthy liver part into the recipient's body. As all the organs in our body, the liver will regenerate and grow in both cases within a few weeks. There are some requirements to be a living donor.
    - The donors must be less than 55 years old because the liver doesn't grow back as well in older people.
    - Of course, they have to be compatible with the recipient.
    - They are asked to do some psychological tests and physical examination.



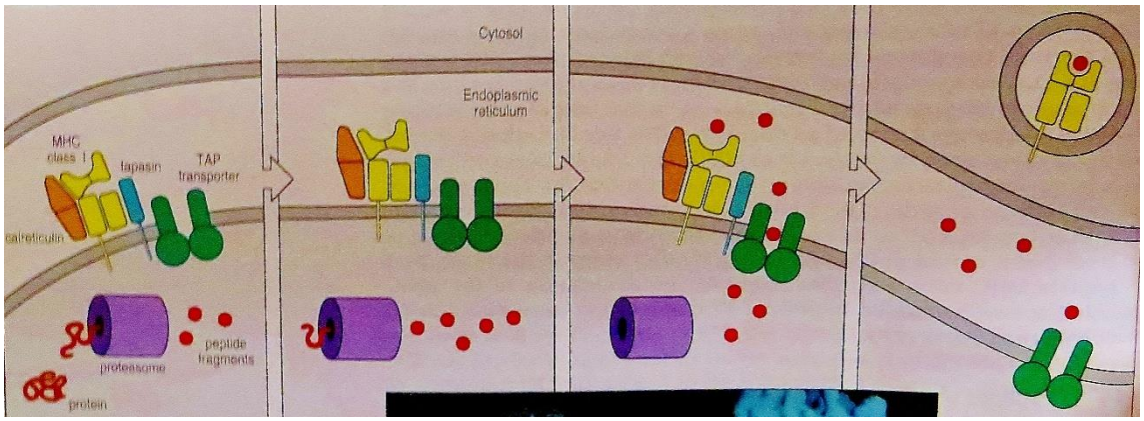


2. HLA [4] SYSTEM:

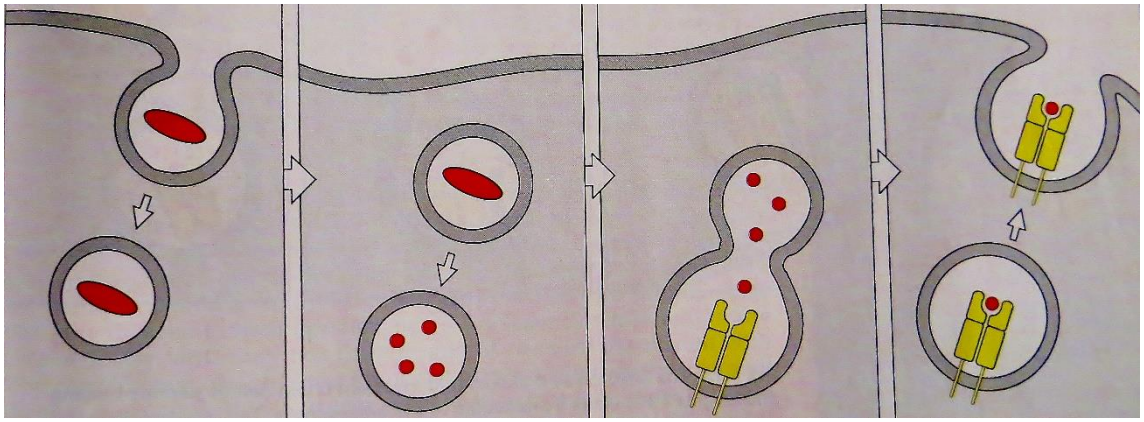
Major Histocompatibility Complex (MHC): It's the heterogeneous and polymorphic region of the short arm from the chromosome 6. It codes for the cell surface molecules (HLA). It is divided into three regions: the Class I, the Class II and the Class III. The first two classes coincide with the HLA classes but the third one doesn't contain any gene of the HLA, however it codes for important genes in the immune response.

HLA molecules: They are glycoproteins that have the function of presenting peptides to T-cells. The HLA genes code for a HLA molecule, and a group of these gens form the HLA system.

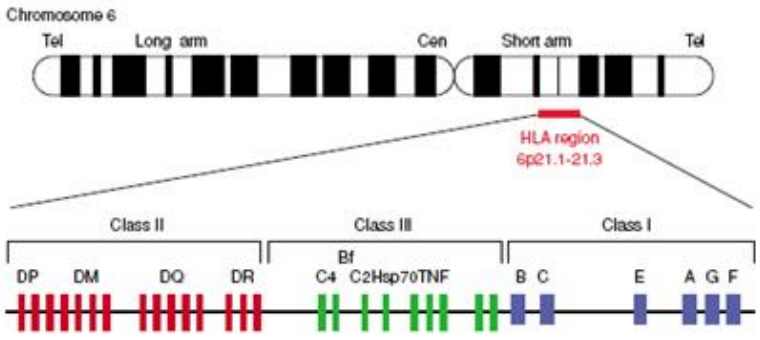
HLA system: It is a group of genes that are in the chromosome 6 and they are the responsible for the regulation of the immune system. This system has a lot of functions, including the rejection. What it does is to cause the rejection of the transplanted organ because its antigens detect anti-HLA antibodies coming from the donor and they detect them as foreigners. There are variables such as HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ and HLA-DP. These variables can be divided into different groups, the first one (Class I) includes HLA-A, HLA-B and HLA-C and the second one (Class II) includes HLA-DR, HLA-DQ and HLA-DP. Class I antigens are formed in the following way: A protein is degraded to peptide fragments by the proteasome [16], this process is done in the cytosol, then these fragments go inside the endoplasmic reticulum and join the MHC class I, after this the MHC class I with the peptide goes to the cytosol again.



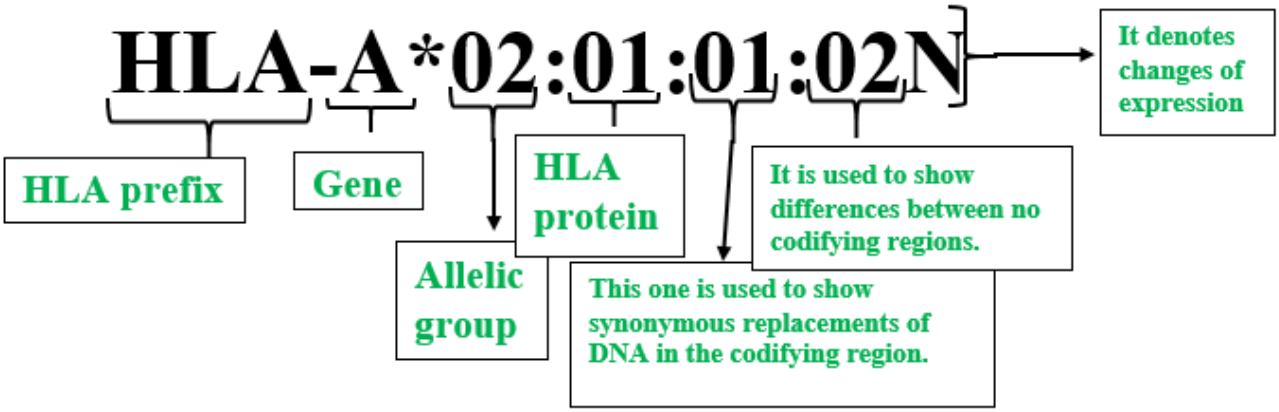
Class II antigens are formed in a different way: The proteins are taken from outside the cell to inside the vesicles in the cell, the acidification of these vesicles make the proteins break into peptide fragments, then a vesicle that contains MHC class II fuses with the vesicle containing the peptide fragments, the fourth step is when the peptide fragments join the MHC class II and it is transported to the surface.



The first class refers to the peptides from inside the cell whereas the second one is the ones that present antigens on the surface of the cell which come from the extracellular space. All the genes that code for the HLA molecules are identified as the MHC (Major Histocompatibility Complex). These molecules are really variable in every person and they make a wall for the transplants because these differences are recognized as aliens for the recipient. HLA system is inherited in blocks, each block is called haplotype [5].



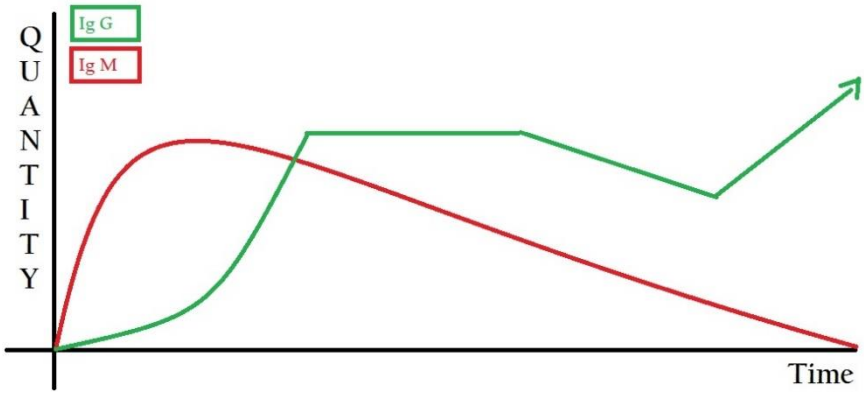
There is a specific nomenclature to call every antigen:



Immune response against the graft

- **Cellular immunity or cell-mediated response:** This type doesn't involve antibodies. It uses three mechanisms to protect the body. The first one consists in activate T cells [(6)] which can produce apoptosis [(7)] in the cells that present alien epitopes [(8)] on its surface. The second mechanism activates macrophages letting them destroy pathogens. The last one consists in stimulating some cells to make them produce cytokines which influence other cells activity that are involved in the immune answer of our body.
- **Humoral immunity or antibodies-mediated response:** It involves the participation of B cells [(9)]. In this sort of rejection what attacks antigens are macromolecules like antibodies or proteins of the complementary system.

There are different types of antibodies that can join the HLA system. IgM and IgG are two examples. IgM is the first antibody that is activated when there is an infection. This antibody will activate IgG that are the antibodies that will destroy the pathogen. There are different types of IgG; G1, G2, G3 and G4.



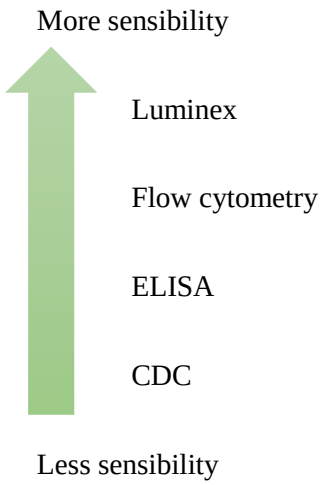
3. REJECTION

When a transplant is done, the cells from the recipient's body must accept the foreign liver but most of the times they eventually don't. This is because the immune system of the recipient realizes that the cells of the organ that has just been transplanted are different from its body's cells. This is called rejection. People who receive an organ have to take immunosuppressive [(10)] drugs to overcome the rejection. However, the immunosuppressant is not specific, the patient increases the risk of having infections. Liver transplant rejection is special because the risk of chronic rejection declines as time passes, so their life expectancy lengthen, however, the majority of the recipients have to take pills for the rest of their lifes.

- **Grades of rejection:** There are three different grades of rejection. Hyper acute rejection, acute rejection and chronic rejection.
    - **Hyperacute rejection:** It is mainly caused by donor's antibodies. In this type of rejection the cells that take part are the B cells. It happens after minutes or hours of the transplant. In the case of the liver, this type of rejection is very uncommon.
    - **Acute rejection:** This type of rejection could happen from the first week after the transplant to three months later. In all transplants there is an acute rejection in some degree. In liver transplant there is from 10 to 30% of probability of rejection. If this acute rejection is not detected on time, it could lead to chronic rejection. Most of the times, this type of rejection is mediated by cells, not antibodies.
    - **Chronic rejection:** It is an immunological injury of the organ that can occur after several episodes of rejection, clinically undetected. In comparison with other transplants, chronic liver rejection is uncommon but it still exists.

4. METHODOLOGIES OF DETECTION OF ANTI-HLA ANTIBODIES:

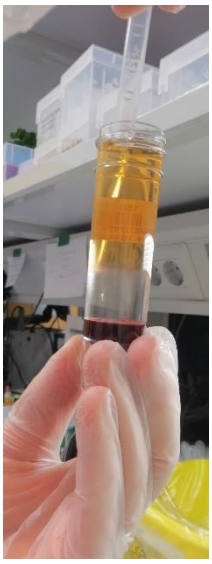
History: Some methods have been used all along the history. The investigation has made possible the discovery of more specific and sensible new methods.



4.1. ELISA: (Enzyme-linked immunosorbent assay). This method is used to find the presence or the absence of a certain protein with antibodies. Because of this, ELISA is called immunoassay. It consists in adding a substance in different samples with different proteins, this will lead to an enzymatic reaction causing the change of the sample colour. This change of colour will allow us to detect a certain protein and measure it. To perform this method we have two types of antibodies. The first one is the primary antibody which has more affinity with a specific epitope of the target protein. The second one is the secondary antibody whose function is to recognize the primary antibody. This secondary antibody has a specific pattern to be named. "Secondary antibody organism anti- primary antibody organism". The second antibody is marked with an enzyme that has to be incubated with a substance that changes its colour if the secondary antibody gets attached to the complex.

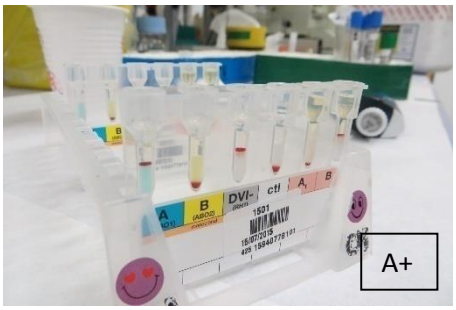
4.2. Cross-match: It is a method that could be made with two different tests. Cytotoxicity and Flow Cytometry. The first part in both cases is the same, so doing it only once is enough. This first part consists in obtaining the donor cells. The donor's blood can come from other hospitals or from inside the hospital. If it comes from other

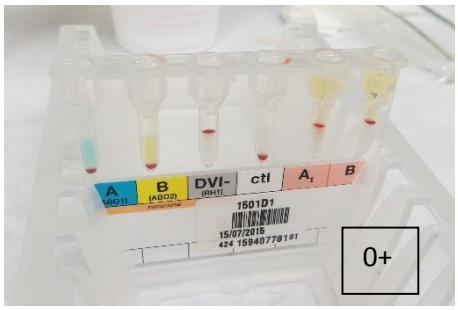
hospitals the blood comes inside EDTA [11] (see ANNEX 2.1) tubes whereas if it comes from inside the hospital, it comes in containers of 15-20 with defibrinated blood. First of all this blood is diluted at 1/2 with PBS (like physiologic serum). Then a liquid called ficole-Hypaque is added to separate the different phases of blood in centrifugation, this separation allows us to take the lymphocytes out of the blood. After that the tube is put into the centrifuge (see ANNEX 1.2 and 1.2.2) for 25 minutes at 1800 revolutions. When the tube is taken out from the centrifuge, the lymphocytes are taken out from the tube and put into another clean tube. We know where the lymphocytes are because they form a misted zone around the tube. Then PBS is added to the tube to make 3 washings. They are made by putting PBS inside the tube with the cells and centrifuging for 5 minutes at 1800 revolutions, when the tube is taken out from the machine, PBS is thrown away and this process is made 3 times. After that, the cells are counted by a machine (see ANNEX 1.3) and diluted to the decided concentration. Before starting the tests, a verification of the blood groups must be done. It's an easy test made with two blisters, one for the blood group of the recipient and the other one for the donor. The blister has 6 small tubes.



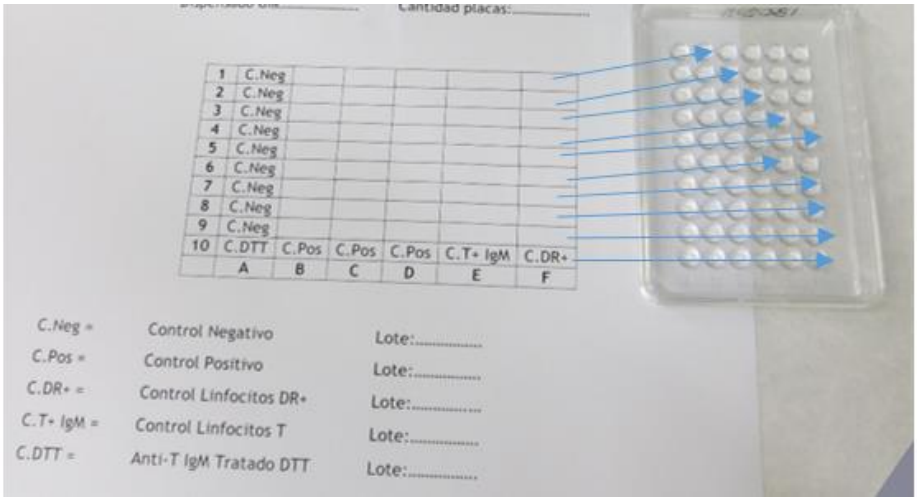
This tubes are the A and B direct, RH, a control and A and B indirect. Inside the A, B (direct) and the RH there are antibodies against each group. (Inside tube A there are antibodies against group A…). First of all 1 drop of a dilution of blood is added in the first three tubes (A, B direct and RH). Then 2 drops of serum are added in each remaining tube (A and B indirect). Then a red corpuscle solution is added. When all the products are inside the corresponding tube, the blister is put into the centrifuge. When the centrifuge finishes, some lines appear. Depending on where these lines are, it will be positive or negative and A, B, AB or 0. The line of the control has to be at its bottom to verify that the method had work correctly.

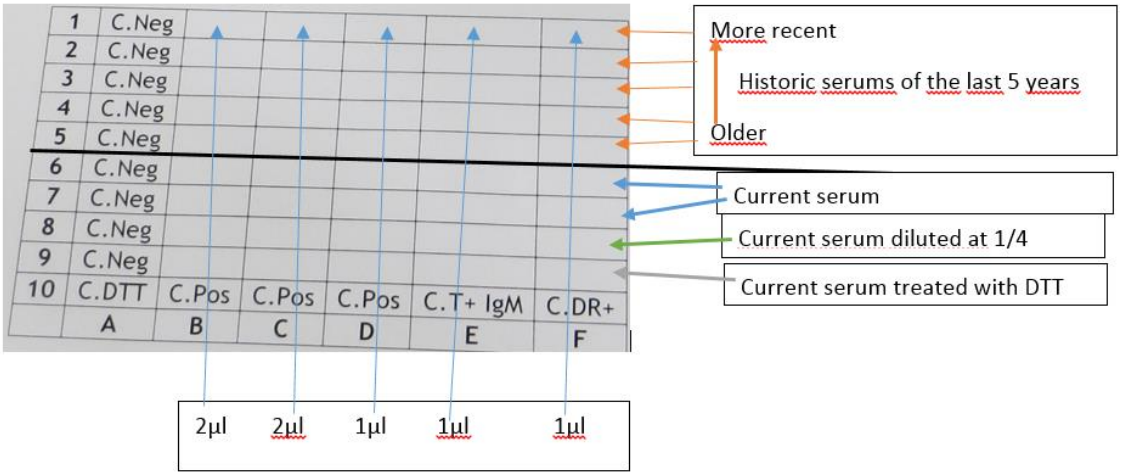
Results:





4.3. Cytotoxicity: With this method we could discover the antigen-antibody specificity of an only type of receptor. At the end the cells that are still alive are the ones with the suitable antigens, and this type of antigens will be right antigens to do the transplant and avoid the rejection. This method is made with 2 plates. The distribution of the plates is the following one:



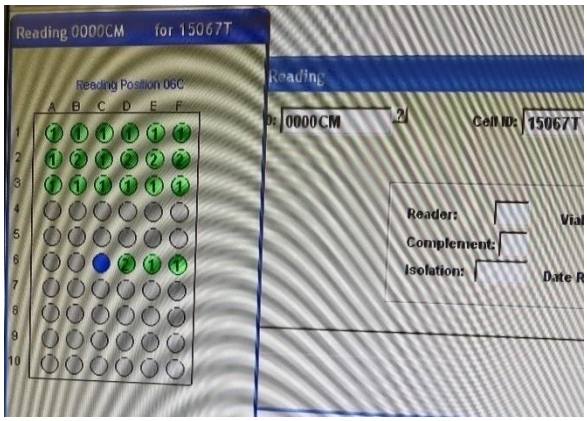


- **1-** While the blood is being centrifuged, 20 µL of DTT [12] are mixed with 180µL of recipient's serum. Then it is let to incubate for 20 minutes at 37ºC.
- **2:** When the cells have been counted by the machine we make a calculation to get the wanted concentration, we want 8 million cells/ml. To get that concentration we will make that calculation:

Initial volume (of cells) X final concentration = Final volume X initial concentration

- The final volume will be the volume in which there will be the decided concentration of cells. This will be the amount of PBS that we'll have to put. This quantity will be mixed with the cells and the mixture inside the tube will be ready to be put in the wells of the plate.
- **3:** 1 µL of the mixture of cells and PBS is put into every well from the plate. One plate is put under incubation at 4ºC and the other one at room temperature for 30 minutes. IgM is more reactive at 4ºC and IgG is more reactive at room temperature, that's why one plate incubates at one temperature and the other at the other temperature.
- **4:** After incubation, rabbit serum as complement (see ANNEX 2.4) source is added.
- **5:** Then the plate is incubated for 1 hour at room temperature.
- **6:** After this time, 3µL of eosin (colorant) (see ANNEX 2.3) and 6µL of formaldehyde [13] are added in every well.
- **7:** The plate is covered with the lid to avoid the evaporation of the samples.
- **8:** 10 minutes after, the plate could be read by the microscope (see ANNEX 1.5).

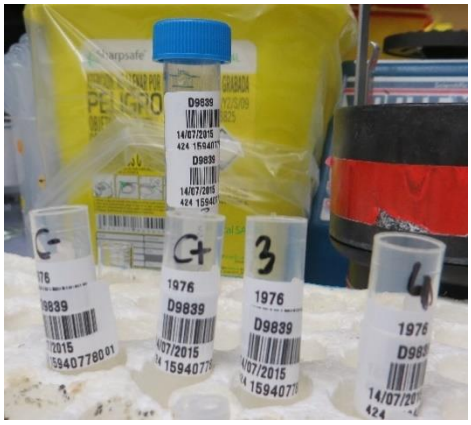
- **Reading the results** (This method has a big semblance with CDC, the difference is that in CDC lots of serums are mixed with lots of cells, I cytotoxicity with Xmatch only 1 serum is mixed with 1 donor cells. Due to that, the two methods are read by the microscope equally).
    - The plate is put in the microscope and the results are transferred to the computer manually. The computer is connected to a keyboard that has exclusively numbers, not letters. The person who is reading the results is who presses the numbers on the keyboard depending on the colour of the cells (this determines if they are alive or not).



If between a 0 and a 10% of the cells are dead, number 1 is pressed, if between a 10 and a 20% of the cells are dead, number 2 is pressed, if between 20 and 50% of the cells are dead, number 4 is pressed. When a number between 50 and 80% of cells are dead, it means that a lot of cells are dead and number 6 is pressed. If the 80-100% of the cells are dead, number 8 is pressed. The more dead cells there are, the more positive the result will be. If the results are really positive, it means that the antibodies have joined the antigens and the colorant has gone inside the cell, producing apoptosis. If the results are positive, the patient and the donor aren't compatible.

4.4. Flow cytometry: The basis of this method is to detect IgG joined to B or T receptor cells. T cells are identified because of the marking of a monoclonal antibody marked with Cy7 and B cells are marked with a specific anti CD19.

There are 4 tubes if the patient has historic serum (Positive control, negative control, historic serum, current serum). In case the patient has more than one historic serum, the one that would be used would be the most recent. In case the patient hasn't got any historic serum, only 3 tubes will be used. (Positive control, negative control and the actual serum).



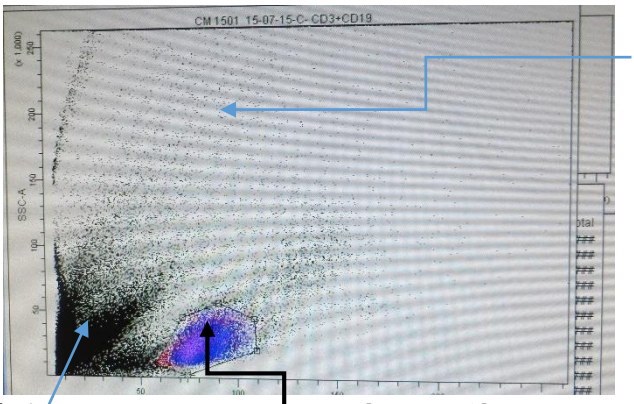
- **1:** 25µL of controls are put into each tube and 25 µL of serum is put into the corresponding tube.
- **2:** When the cells have already been separated from the blood, 50 µL of cells are put into the tubes.
- **3:** Then the tubes have to be under incubation at room temperature during 30 minutes.
- **4:** 2 washings are made. These washings are made with:

  1L of PBS + 20mL of foetal bovine serum + NaN_3

  3ml of this solution are put inside every tube. The tubes are put into the centrifuge for 5 minutes at 1800 revolutions. This process is repeated twice.
- **5**: The monoclonal is prepared. The cytometer (see ANNEX 1.4) could detect the colours thanks to this solution.
  - 100 µL of incubation buffer [14] +
  - 1 µL of FITC +

- 10 µL of CD19 (it is the colour used to detect B cells) +
- 10 µL of Cy7 (it is the colour used to detect T cells)

- **6:** When the washings have finished, 100 µL of monoclonal are added in each tube.
- **7:** The plate has to be under incubation during 30 minutes at 4ºC and in the dark because any light could made the fluorochrome deteriorate.
- **8:** 2 washings like the first ones are made.
- **9:** 100 µL of fixation tampon are added because in case that the tubes couldn't be read on the same day, the fluorochrome doesn't lose its intensity.
- **10:** The cytometer reads the samples and the results are transferred to the computer automatically.
- **Reading the results:** The results are read by the cytometer where the tubes are put. A needle sucks the cells and two lasers impact on them. One laser determines the size of the cells with the joined antibodies and the other determines the texture of the cells. While they are being read the image on screen is this.



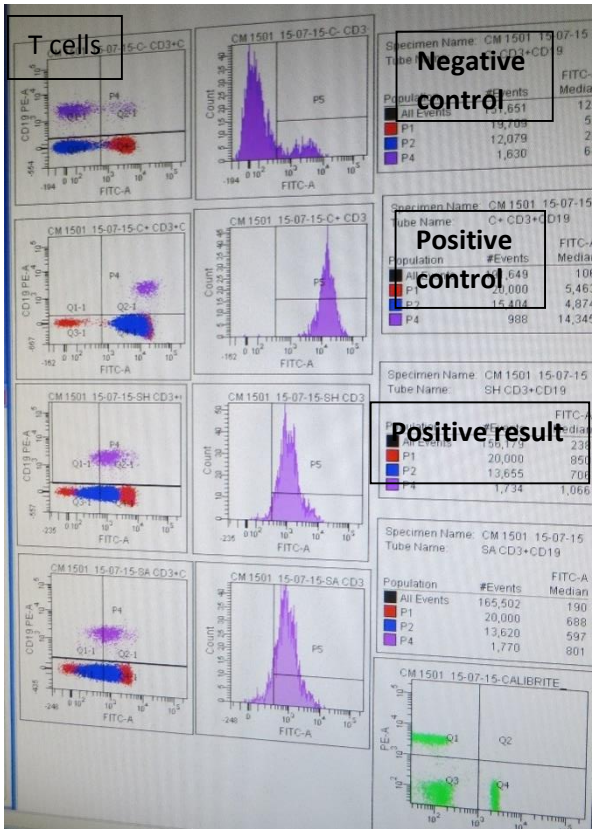
Granulocytes

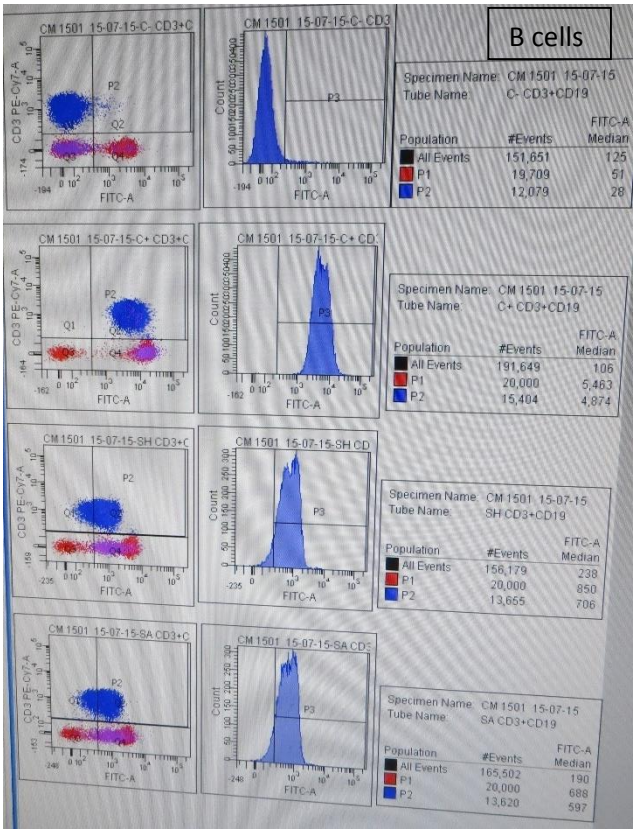
Hematite's

Lymphocytes (the ones we will study)

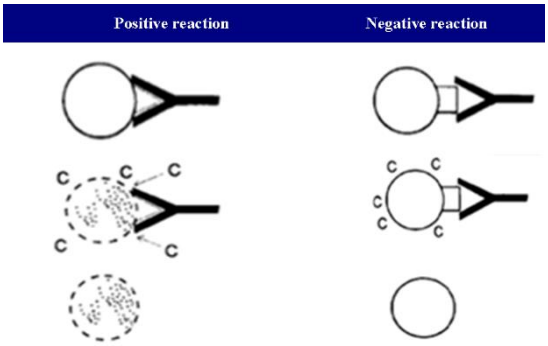
When all the samples (C+, C-, historic and current serum) have been read, the images on screen are these ones. One for T-cells and the other one for B-cells. Positivity in both T and B cells indicate the presence of anti-HLA antibodies of Class I while if the postivity is only for B cells the results

indicate the presence of anti-HLA antibodies of Class II. In the case of the hepatic receptors, a positive cytometry inidcates a bigger possibility of atresia biliar but it doesn't contraindicate the transplant.

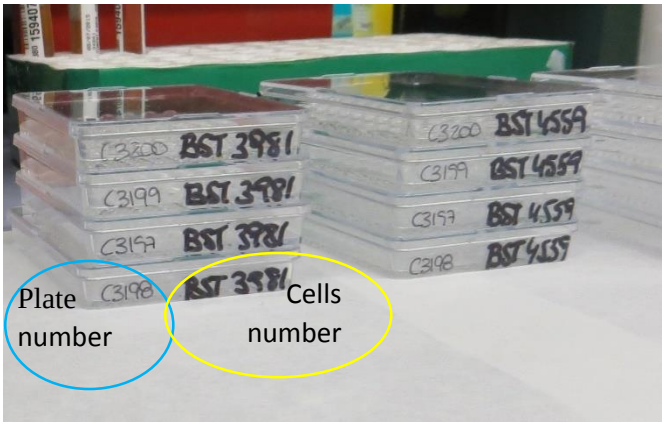




4.5. CDC (Complement Dependent Cytotoxicity): The key of this method is the coloration of the cells of different receptors that gives a positive result. It consists in putting cells in a plate with antiserums. If the antibody reacts with the cells the complement is added letting the colorant go inside the cell and leading it to apoptosis. If this result is positive and the cells die the transplant can't be done. If the antibodies and the surface HLA antigens don't react, the complement isn't fixed there so the cell doesn't get dyed. Although this method has some disadvantages too. We can't distinguish the important antibodies (anti-HLA) and the less important antibodies such as IgM, an autoantibody. IgM and IgG are complement fixers.



- Procedure:
  - The recipients' serum is mixed with known cells to see if they present antibodies against the antigens of the cells.
  - 1- First of all the serum samples are put into a panel, each sample in one well. There are 32 numbers that correspond to a plate which contain the serums inside. The panels are exposed to different cells every day.
    - Example: The C3200 plates have serums of 60 people. There are 32 C3200 panels and each one is exposed to different cells.
      - Day 1: 8 C3200 panels are exposed to 8 cells, each plate to each cell.
      - Day 2: 8 other C3200 panels are exposed to 8 different cells from the day before.
      - On day 4 all serums will have been exposed to all cells and we will be able to see their sensibility against the antigens of the different cells. The panels are marked on the left with the number of the serums. The 32 panels which have this equal number contain the same serums. On the right they have the number of the cells that the serums are going to be exposed to.

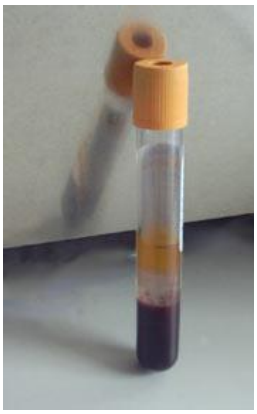


- 2-The cells are defrosted into a container with PBS with foetal and put into other tubes smaller than the first. The process has to be fast because the product used to freeze the cells is toxic.
- 3- The tubes are washed and centrifuged for 10 minutes at 1200 revolutions, this process is repeated 3 times. At the end the cells will rest at the bottom of the tube. (See ANNEX 3.1)
- 4- A machine counts the cells in every tube and sees how many types of antigens there are. In the end this machine does some statistics relating every parameter like lymphocytes.
- 5- The cells are added inside the serum and it has to rest for 30 minutes. If the antibodies of the serum react with the antigens of the cells they join.
- 6- After this time a machine adds the complement and it has to rest during an hour. This complement is made in the laboratory. Actually, its name is "Rabbit Complement" because it is made with the serum of rabbits' blood. If the antibodies had reacted and had joined the antigens the complement will break the cell's membrane with little wells. The complement will interact only with the cells that have the antibodies attached to their surface.
- 7- The next step is to add eosin and formaldehyde. They are colorants. When they have been added to all the wells, the cells which the complement has joined the antibody are dyed and then apoptosis is induced. This can be seen

with the microscope, the death cells will have a black colour whereas the normal cells will be brilliant and red.

4.6. LUMINEX: The fundament of the method is based on the ELISA method but with polystyrene beads (see ANNEX 2.2) instead of plates. This beads are read with the Luminex machine (see ANNEX 1.1) that has lasers that read the fluorescence in the red wave length and the green one, plotting the results on the computer.

There are two methods made with Luminex. Both methods are similar but not exactly the same. The first we have to do in both cases is to prepare the serum. Serum is plasma minus the coagulant factor. To do it we have to put blood in tubes that have a special gel inside. Then centrifuge it at 2500 rpm. When the serum is already separated from the rest of the blood, it is distributed into 4 capillaries (see ANNEX 3.4) being careful because it's not recommendable to fill the entire capillary because when it is put into the freezer the serum grows and it could open the cover of the capillary. One of the capillaries is used for Luminex method, another one is used in CDC and the two others are frozen and saved because sometimes the tests have to be repeated or made new tests. Frozen serum could be kept at -20ºC or at -80ºC if it is expected to be used in a longer period of time. All the samples from the same patient are identified by the same number using the bar code system.



Two more aliquots are put into the panels, the controls. They are used to check if the method works correctly. There is 1 positive and 1 negative control. If the results match the controls (the positive control has a positive result and the negative one has a negative result) means that the method is working correctly. They are treated as one sample more and all the products added to the aliquots of the samples are also added to the controls.

The second approximate to antibody detection is only made if the results of the first one are positive.

1st: Screening: Detects if the serum contains antibodies or not.

2nd: Single Antigen: It specifies which type of antibodies they are and what antigens are reactive.

- **Screening:** It is a method that detects if there are antibodies in the recipient serum.
    - **1:** The serum is homogenized in the Vortex (see ANNEX 1.6).
    - **2:** Place the capillaries inside the centrifuge (see ANNEX 1.2.1) for 90 seconds.
    - **3:** Fill with distilled water the Terasaki plate (that is made with a permeable membrane that allows the washings after reactions) to clean it and permeate the membrane. Then put the plate on a vacuum machine to extract the water.
    - **4:** Put 12µL of serum and 12µL of EDTA in every well of the Terasaki plate with EDTA pipette (see ANNEX 1.7).

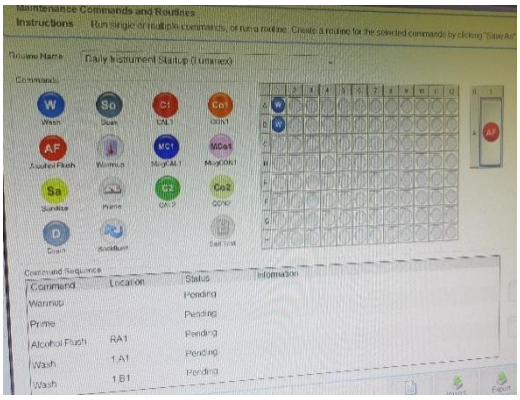
***** In Screening every well of the Terasaki plate that is filled with the serum is used to detect antibodies from both the Class I and the Class II whereas in Single Antigen we have to fill two wells with every serum to detect Class I and Class II separately. (see ANNEXOS 3.2 and 3.3)

- **5:** Incubate the Terasaki plate in a machine called agitator which shakes it for 15 minutes.
- **6:** After that, 3µL of polystyrene beads are added. Before adding them, they are put on vortex to avoid being attached ones to the others.

- **7:** When the beads have been added the Terasaki plate is put again in the agitator for 30 minutes to improve the interaction of the antibodies and the beads.
- **8:** Make a preparation with some products included in the kit.

22µL of buffer X number of samples (+2 or 3) + 3µL of conjugated X number of samples (+2 or 3)

- **9**: 100µL of buffer are added in each well with a multipipette (see ANNEX 1.8), then the whole plate is put into the vacuum machine. This step is called the washing. The polystyrene beads rest attached at the bottom of the plate and the buffer washes the rests of other products. The antibodies have already joined the antigens in the surface of the beads.
- **10:** The second step of the washing is to add 200µL of buffer to clean the plate, put into the vacuum machine to take out the buffer and rests of products. This step is repeated 3 times.

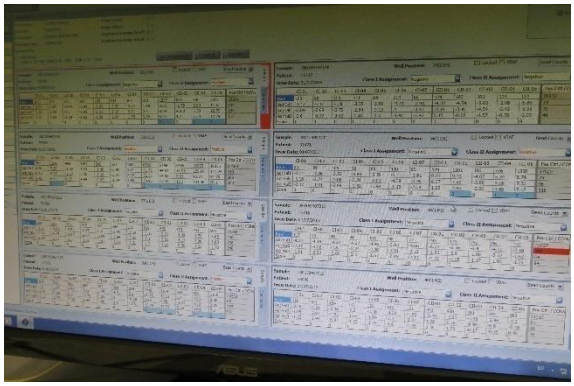


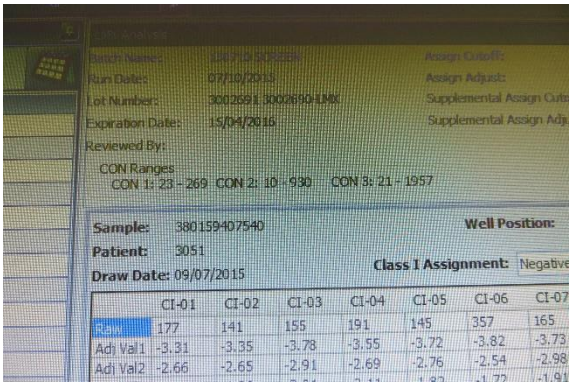
- **11:** Add 25µL of preparation in every sample of the Terasaki plate.
- **10:** The plate is put again 30 minutes in the agitator.
- **13:** 130µL of buffer are added into every well.
- **14:** The Luminex machine has to be cleaned every time before being used. It is made with a special programme of the computer. System liquid is put into two wells of the machine panel. Ethylic alcohol 70% is put in a little box. Then the needle of the machine takes the liquid and the alcohol and they run all around the machine cleaning it of the possible contaminating products.

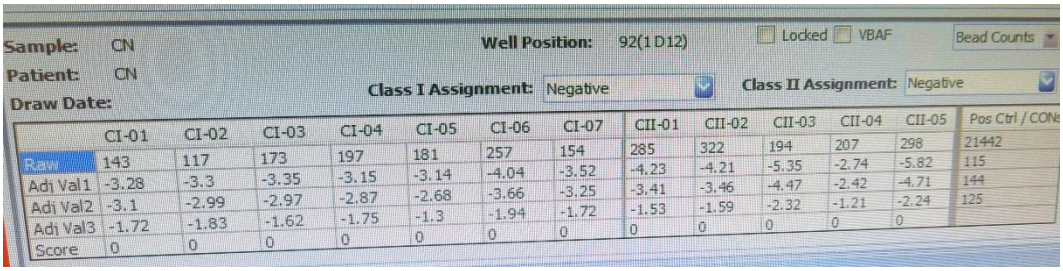
- **15:** The lists of the patients are transferred to the computer and the machine is configured with the PC.
- **16:** The plate is put into the Luminex machine. The needle takes the beads of each well and puts them in the middle of the lasers. This lasers determine the fluorescence of every bead, the more fluorescent they are the more positive the

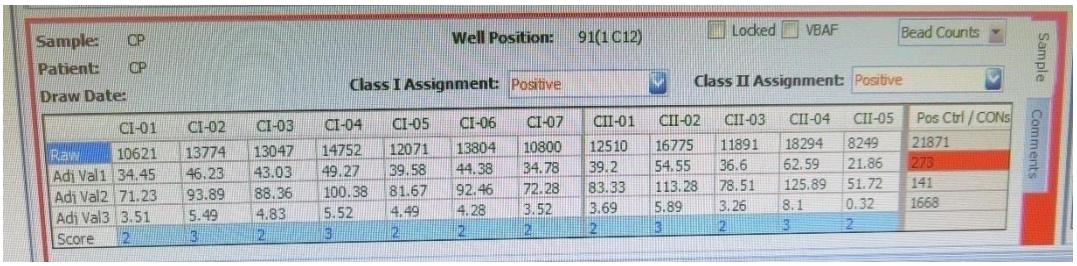
result is. We have to remember that in Screening the machine separates Class I and Class II because they are in the same well, in Single Antigen Class I and Class II are already separated.

- **17:** The results are transferred to the computer and the computer organizes the information and interprets it.









Results with the computer:

The positive control has to be a number bigger than 10.000 in the Raw. If the positive control is under 10.000 all the samples in the same plate where the control is have to be tested again because this means that the method hasn't run correctly. To be positive the parameters (Adj Val) have to be bigger than 5 and to determinate if the entire sample is positive, the 3 values have to be over 5. Then it is when SA has to be done to check if these detected antibodies are specific against the donor.

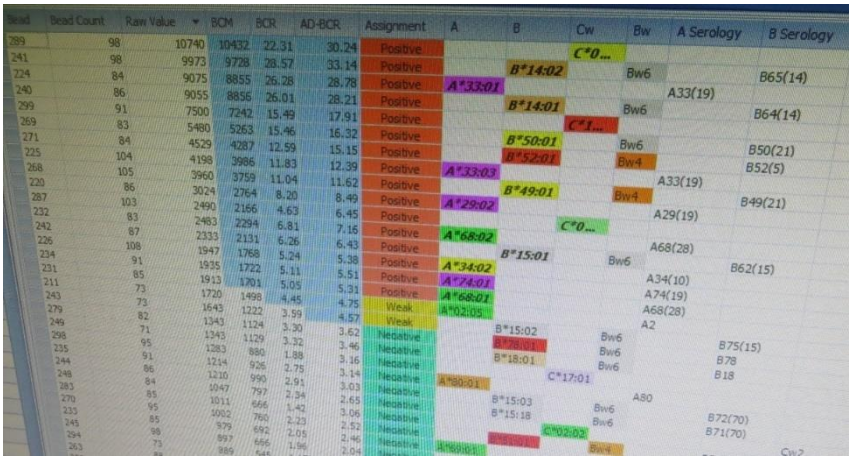
Sometimes the Pos Ctrls/CONs turns into red colour. This means that it is not between the values that it is supposed to be. (CON1: 23-269, CON2: 10-930, CON3: 21-1957). If only one of them is out from the range it hasn't importance but when the three are in red, the test has to be repeated but with one more step. Serum cleaner is added and the serum is diluted.

- **Single Antigen:** This method is used to discover the specificity of antibodies. It is only done when the results of Screening have been positive. In the plate we have to use 2 wells for each sample to difference Class I and Class II.
    - **1:** The serum is put into the aliquots after separating it from the rest of the blood.
    - **2:** This serum is centrifuged.
    - **3:** While it is being centrifuged the plate is cleaned with water, and with the vacuum machine this water is pulled out.
    - **4:** 5µL of EDTA are added in every well of the panel.
    - **5:** 5µL of serum are added in every well of the panel.
    - **6:** The plate is put into the agitator for 15 minutes.
    - **7:** A liquid made with buffer and conjugate (see ANNEX 2.2) is made and it is protected from the light with aluminium paper.

22,5 µL of buffer X number of samples (+2 or 3) + 2,5 µL of conjugate X number of samples (+2 or 3)

    - **8:** When the agitator finishes, 20µL of beads (polystyrene beads) are added in each well.
    - **9:** Then it is put into the agitator again for 30 minutes.
    - Then, 100 µL of buffer (there is a different buffer for Class I and Class II) are added in every well and then they are pulled out with the vacuum machine.
    - **10:** 200 µL of buffer are added and pulled out 3 times to wash the panel.
    - **11:** The diluted conjugate is added into the panel, 25 µL in each well.
    - **12:** The plate is put into the agitator again for 30 minutes.

- **13:** After this, 130µL of buffer are added into each well.
- **14:** The Luminex machine is washed with system cleaner and ethylic alcohol 70%.
- **15:** The list of patients is transferred to the computer and the Luminex machine is configured to do Single Antigen.
- **16:** The plate is put into the machine and the needle takes the beads of every well.
- **Reading the results:** The computer shows the sensibility of the antibodies against the antigens. The specificity with each antigen, if it's positive or negative.



The BCM parameter determines the fluorescence, when this result is over 1500 is positive. The BCR and AD-BCR parameters that are over 4 are positive. If the three values are positive the assignment (final result) is positive. If two or one of the values is positive but not the three, the final result is weak. (This final result has to be interpreted by the person doing the test, for example, if a lot of results are positive, this weak results are interpreted as positive results but if all the results or most of it are negative, they are interpreted as negative.)

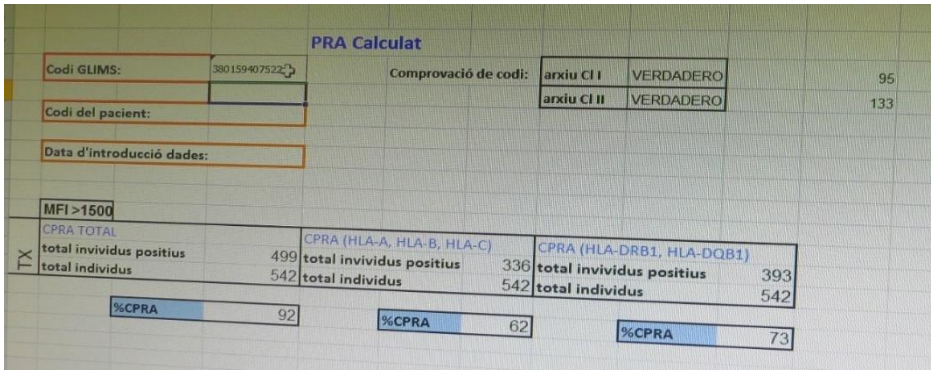
* The workday conjugate is prepared diluting the conjugate with the buffer for the number of samples we have, plus an extra to have enough amount.

*The Luminex machine has to be switched on 30 minutes before using it to let the lasers warm up.

*DILUTION AND SERACLEAN: When the results from the controls of Class II are really high, the samples have to be tested again but applying a treatment. This treatment consists in making a dilution with the serum with 25µL of BSA +PBS AND 25µL of serum. Sometimes it might be necessary to clean the serum with seraclean, then the treatment to follow is put 20 µL of serum (diluted or not) and 4µL of seraclean beads. The aliquots with the mixture are put in the Vortex machine, then they are protected from the light and at the end they are put into the agitator for 30 minutes. Then they are ready to be treated equal as other samples with the normal procedure.

4.7. C-PRA: There is another program that complements the results of the compatibility tests. This program makes statistics with donors and recipients. In C-PRA is seen the probability between 100 that a recipient has to find a compatible donor. The recipient is specific but the donors are a sample of the most frequent antigens of the population.

For example, if the result of the C-PRA is 47, that means that the receiver has antibodies against 47 people so there would be 53 donors of the 100 that could be compatible.



There are two matches of the recipient and the donors, one for class I and the other for class II. Then a third one is done and the donors that have positive antigens from class I and class II at the same time. On the photo above it is seen that the recipient has antibodies against 62 donors that have antigens of class I and against 73 donors that have antigens of class II. In the total result we could see that there are 92 donors that have antigens of class I and class II at the same time, so there are 92 people incompatible with this patient.

5. INTERVIEW WITH A PATIENT:

I've made an interview to Maria, a patient that received a transplant, she had rejection but thanks to some immunosuppressor drugs now she is stabilized.

Maria explained me her story, his husband was behind the camera helping her because she was really nervous to remember how horrible the days of her disease were. Now she is happy, healthy and doing a normal life. She has a son and a daughter and they have helped her in all the process. She caught HVC in Cardona when someone used a needle to treat her after using it in an infected person. In Manresa the life length that the doctors gave her was 3 months. Then, her daughter called us and we (my family) talked to our uncle (Dr. Josep Maria Barrera). He put her name in the waiting list because he thought that maybe in the future she would need a transplant and when the patients have more than a certain age they can't get in the list. After a time, she needed a transplant and a liver for her arrived, she went to the hospital but in the end, the liver wasn't good enough. Then, her name was pulled out from the list, however her daughter didn't stop fighting for her mother, she talked to the doctors and they told her that if a liver arrived, they would transplant it to her, because her life length was shorter and shorter. When they finally found a liver for her it was from an alive person. Before the transplant she lost her mind and she even didn't recognize the doctors. This transplant was an exceptional case, the man whose liver was transplanted to Maria, received a liver too. His liver was too small for him, he needed a bigger liver and when a liver from a dead person arrived for him, the doctors made a double transplant. They put the bigger liver in the man's body and the man's liver (smaller) into Maria's body.

Then a virus infected her liver but finally a treatment from the USA arrived in the CLINIC, this treatment was only for 4 people and one of them was Maria. She explained me how she felt in that moment and especially when the doctors told her that she was totally cured. In this interview we can see a person that has suffered a lot and has won the battle against the pain. She told me that she has had a lot of pain but she knew that all the suffering was to cure her. (See ANNEX 4)

6. ACKNOWLEDGEMENTS TO:

Josep Maria Barrera

Dra. Guadalupe Ercilla

Dr. Carles Serra

Montse Digón

Maria Muñoz

Mercè Baiget

Cristina Ferran

Carmen Domènech

Núria Palau

Rosa Tristany

My parents and my brother

Montse González and Xavier Barrera

Ester Casado and Antonio Casado

LINK TO THE ORAL PRESENTATION:

https://www.youtube.com/watch?v=YwGqiFy6oKs

7. CONCLUSIONS: When I started this project I didn't expect it would be so different of what I initially had thought. First of all, my idea was searching lots of graphics from Barcelona, Catalonia and Spain's hospitals and then compare them with each other. What I did not know when I had that idea is the quantity of work it will suppose to me to explain all the methods perfectly with all the details. So then I focused my project exclusively on the methods. But I wasn't satisfied, I needed something more, applications, at least one, of these methods. Then I saw clearly what I would do. I linked the methods with their direct application, the transplantation, and I explained the liver transplant, to be more specific. But I couldn't explain the liver transplant if I hadn't explained about the liver. So my final project is completely different from my first idea. If I had to do it again, I wouldn't change anything because it has been incredible for me the opportunity that this project has brought me.

My project hasn't got an experimental part or a material product result but I don't think it is necessary to do a good project. Although I haven't created anything new, I've learned a lot during my stay in "Hospital Clínic". About a 95% of the project is made with information learned in the lab while the other 5% is Internet information. These 15 days were fantastic and really productive. And thanks to this project I could speak to Maria, a survival and a fighter, and her story should be an example for all of us.

Every method has its own function, some methods are more sensible while the others are more specific. A lot of tests are done to verify the compatibility. For example, there are old methods that are still done because they are more specific than some newer and more sensible tests.

I think that the perfect test would be a machine with high sensibility and specificity. A machine that could detect if the antibody had joined the antigen and the complement had joined the antibody and then read the cells that were still alive with lasers after adding a fluorescent colorant. The lasers could define the type of antigens that are the ones that no antibody had joined them. This machine doesn't exist yet but in my opinion it will exist in a future because it would be very practical. Today, all the tests almost say the same, the methodology and the basis is different but the result, at the end, inform us of the compatibility of a certain antigen with a certain antibody.

With this new machine, we wouldn't need the other methods anymore. First of all we would get more precision because of the combination of the other methods' best parts. This new and incredible machine would be a union of: Cytotoxicity; with the normal method the cells are read manually by a person and it is definitely not as accurate as the new machine would be. The new engine would have detectors and the death cells wouldn't pass to the next step which would be the reading of them. Luminex fluorescence because the colorant wouldn't be eosin, it would be a fluorescent colorant. The exact methodology wouldn't be neither of Single Antigen nor Screening, in this case, the lasers would detect the type of surface antigens that any antibody have joined them, so, the ones with transplant possibilities. At the end, this new machine would transfer the results to C-PRA so that it could make statics with the possible donors and recipients according to the results of all the test process.

8. GLOSSARY:

1: HAV: Hepatitis A Virus

2: HBV: Hepatitis B Virus

3: HCC: Hepatocarcinoma

4: HLA: Human Leukocyte Antigen system.

5: Haplotype: It's the combination of an allele from a locus [(15)] with an allele from a different locus.

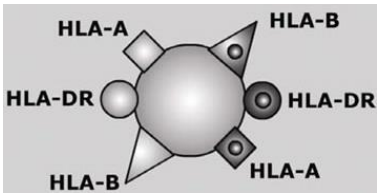
6: T-cells: This type of cells has an important role in the cellular immunity response. They can be distinguished from other lymphocytes because they have T-cell receptors

7: Apoptosis: It is the succession of some biochemical events that lead the cells' death. Unlike the necrosis, apoptosis has advantages during the vital cycle of an organism.

8: Epitopes: It's the part (specific region) of a macromolecule that the immune system recognizes, particularly T cells, B cells and antibodies.

9: B cells are a type of cells that have an important role in the humoral immunity response. They make antibodies against antigens.

10: Immunosuppressive: Type of pills that wither the immune system to avoid rejection.



11: EDTA: It is a liquid that avoids the complement activation. The mother solution comes into a reactive and it is prepared in the laboratory. It's a solution at the 6%. For every 6 g of EDTA there are 100ml of physiologic serum. To make the solution that we are going to work with, we have to mix 30µL of mother solution and 270µL of PBS+ BSA at the 5%. The final solution will be at 1:10.

12: DTT: It's a liquid that inhibits the effect of the IgM, it is used to detect IgG and not the IgM. IgM doesn't have specific HLA, it is a self-antibody.

13: Formaldehyde: It is a liquid that stabilizes the cells so that they could be read later with the same results.

14: Buffer: It's a liquid that dilutes the conjugate because it's too concentrated, it is also used to clean the plate with the beads inside.

15: Locus: It's the fixed position of a gen in a chromosome.

16: Proteasome: Enzymatic complex from the cytosol that breaks some proteins into peptides.

9.WEBGRAPHY:

- **Wikipedia**
  - http://en.wikipedia.org/wiki/Major_histocompatibility_complex
    - Last Update: 12 October 2015, at 23:01.
  - http://en.wikipedia.org/wiki/Liver_failure
    - Last Update: 5 August 2015, at 18:12
  - http://en.wikipedia.org/wiki/ELISA
    - Last Update: 7 October 2015, at 13:59
  - http://en.wikipedia.org/wiki/Porta_hepatis
    - Last Update: 9 June 2015, at 01:00
  - http://en.wikipedia.org/wiki/Liver#Gross_anatomy
    - Last Update: 18 October 2015, at 15:31
  - http://en.wikipedia.org/wiki/Round_ligament_of_liver
    - Last Update: 20 November 2014, at 12:51
  - http://en.wikipedia.org/wiki/Hepatitis_D
    - Last Update: 14 October 2015, at 09:59
  - http://en.wikipedia.org/wiki/Acute_liver_failure
    - Last Update: 25 July 2015, at 19:22
  - http://en.wikipedia.org/wiki/Transplant_rejection
    - Last Update: 2 October 2015, at 21:15
  - http://en.wikipedia.org/wiki/Liver_transplantation
    - Last Update: 1 September 2015, at 16:32
  - http://en.wikipedia.org/wiki/Bilirubin
    - Last Update: 13 October 2015, at 16:24
  - http://en.wikipedia.org/wiki/Galactose-alpha-1,3-galactose
    - Last Update: 5 September 2015, at 15:44
  - http://en.wikipedia.org/wiki/B_cell
    - Last Update: 24 September 2015, at 13:17
  - https://en.wikipedia.org/wiki/Cell-mediated_immunity
    - Last Update: 30 September 2015, at 15:39
  - https://en.wikipedia.org/wiki/Experiments_in_immunology
    - Last Update: 7 January 2015, at 11:32
  - https://en.wikipedia.org/wiki/Affinity_chromatography
    - Last Update: 1 April 2015, at 20:34
  - https://en.wikipedia.org/wiki/Haplotype
    - Last Update: 16 June 2015, at 13:06
  - https://en.wikipedia.org/wiki/Experiments_in_immunology
  - Last Update: 7 January 2015, at 11:32

Medline Plus:

- http://www.nlm.nih.gov/medlineplus/livertransplantation.html
  - Last Update: 30 September 2015
- http://www.nlm.nih.gov/medlineplus/ency/article/000255.htm
  - Last Update: 09 September 2015
- http://www.nlm.nih.gov/medlineplus/ency/article/000285.htm
  - Last Update: 09 September 2015
- http://www.nlm.nih.gov/medlineplus/ency/article/003006.htm
  - Last Update: 09 September 2015
- http://www.nlm.nih.gov/medlineplus/ency/article/000815.htm
  - Last Update: 09 September 2015

National Institute of Diabetes and Digestive and Kidney Diseases:

http://www.niddk.nih.gov/health-information/health-topics/liver-disease/liver-transplant/Pages/facts.aspx

Last Update: April 23, 2012

Wikiversity:

- https://en.wikiversity.org/wiki/Transplantation/The_HLA_System_and_HLA_Typing
  - Last Update: 19 June 2015, at 01:38
- https://en.wikiversity.org/wiki/HLA
  - Last Update: 30 September 2014, at 15:01

Essay made by the the medical centre and a private hospital from Córdoba on 12th May 2011.

ANNEXOS

Liver transplant: Methodologies of anti – HLA antibodies detection in the context of liver donor.

Annex 1: Machines

1.1: Luminex

1.2: Centrifuge

1.2.1: For little aliquots

1.2.2: For bigger tubes

1.3: Counting-cells machine

1.4: Cytometer

1.5: Microscope (used to read cells in CDC)

1.6: Vortex

1.7: EDTA pipette

1.8: Multipipette

Annex 2: Products

2.1: EDTA

2.2: Beads

2.3: Eosin

2.4: Complement.

Annex 3: Material of the methods

3.1: Aliquots with cells (CDC)

3.2: Single Antigen plate (Luminex)

3.3: Screening plate (Luminex)

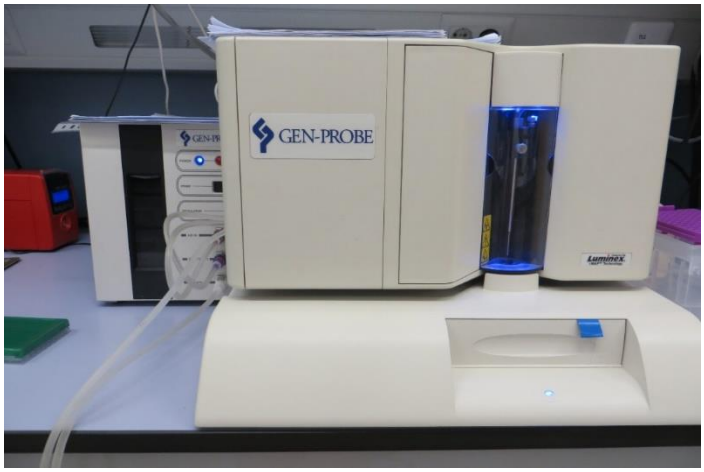
3.4: Different aliquots with the same serum

Annex 4: Link to the interview

Annex 5: Me at the hospital

Annex 1: Machines

1.1: Luminex needle and Luminex machine

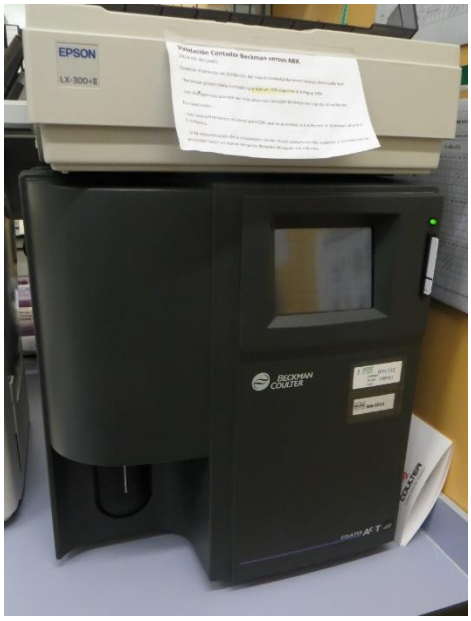


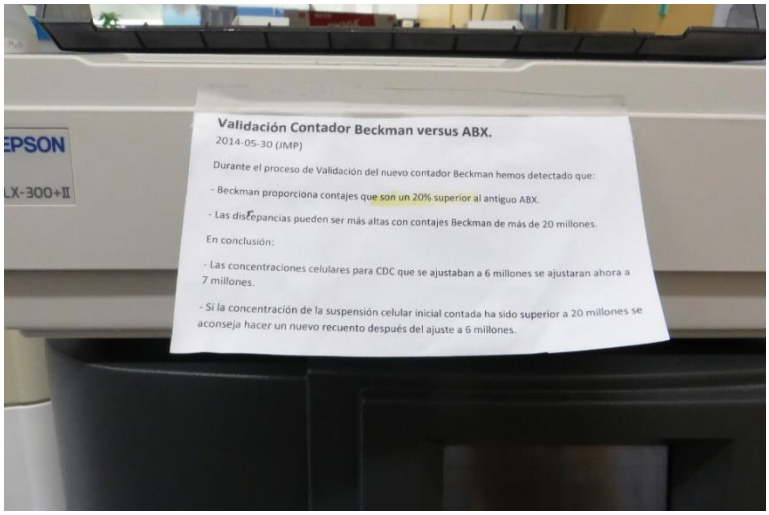
1.2: Centrifugue

1.2.1: For little aliquots

1.2.2: For bigger tubes

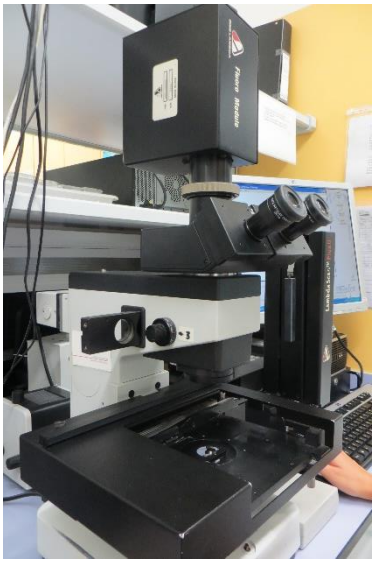
1.3: Counting cells machine





1.4: Cytometer

1.5: Microscope (used to read cells in CDC).



1.6: Vortex: This machine vibrates to detach the products inside the tubes of their bottom and to agitate them too.

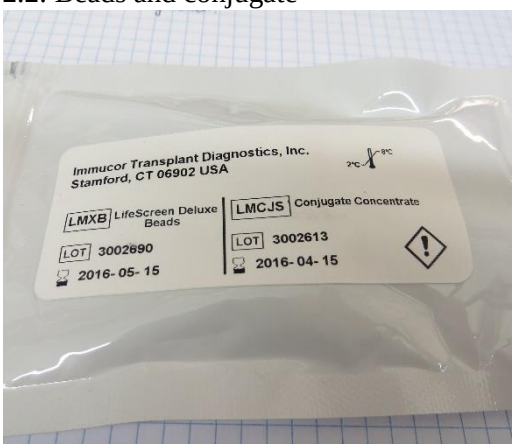
1.7: EDTA pipette: It is used to put the EDTA into the wells, it's automatic. The µl of EDTA wanted are written in the machine and then, manually, the button is pressed and the wanted µl of EDTA goes into each well.

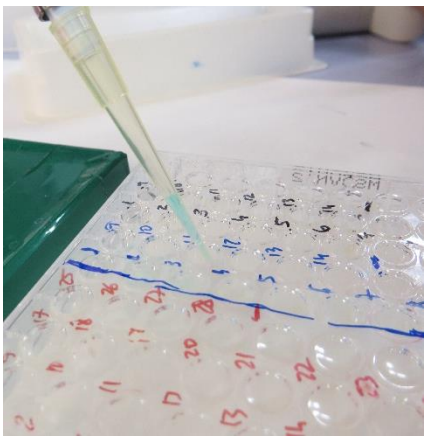
1.8: Multipipette: It is used to put different products into the wells, it's more useful than a normal pipette because with this one, a whole row of wells could be filled at the same time.

Annex 2. Products

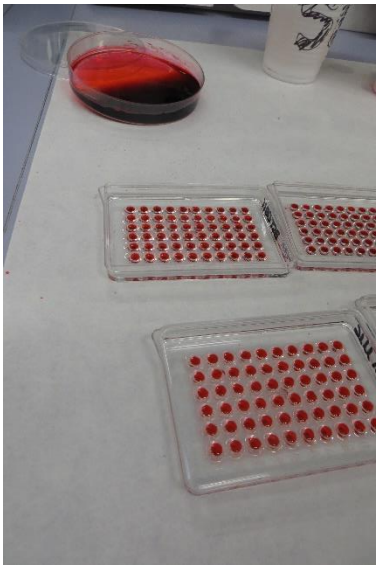
2.1: EDTA

2.2: Beads and conjugate





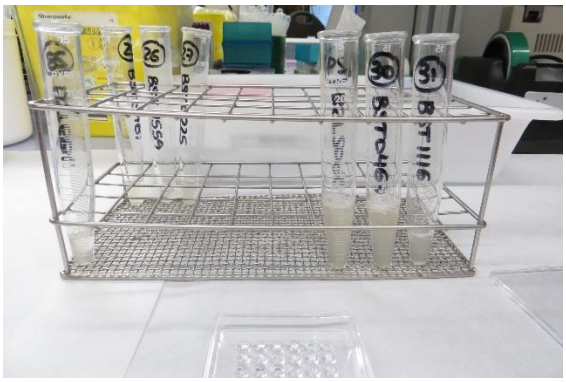
2.3: Eosin



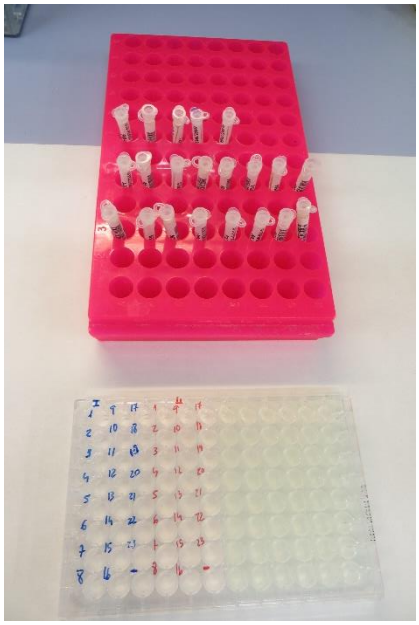
2.4: Complement

Annex 3: Material of the methods

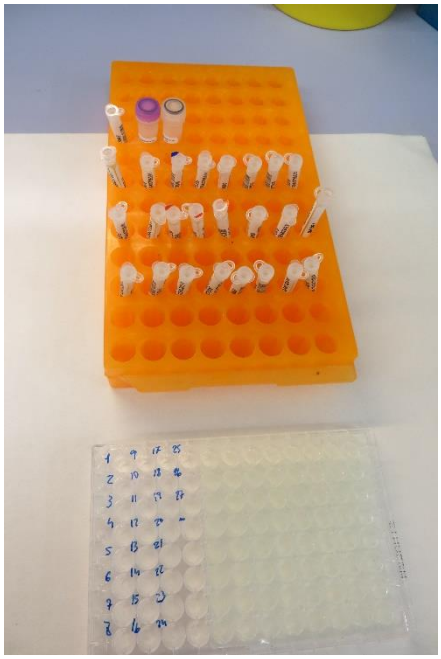
3.1: Aliquots with cells (CDC)



3.2: Single Antigen plate. Class I and Class II are read separately (in different wells). (Luminex)



3.3: Screening plate. Class I and Class II are read at the same time in the same well. (Luminex)



3.4: Different aliquots with the same serum (in each hole).

Annex 4: https://www.youtube.com/watch?v=Iw53nZCrMp4

Annex 5: Me at the hospital