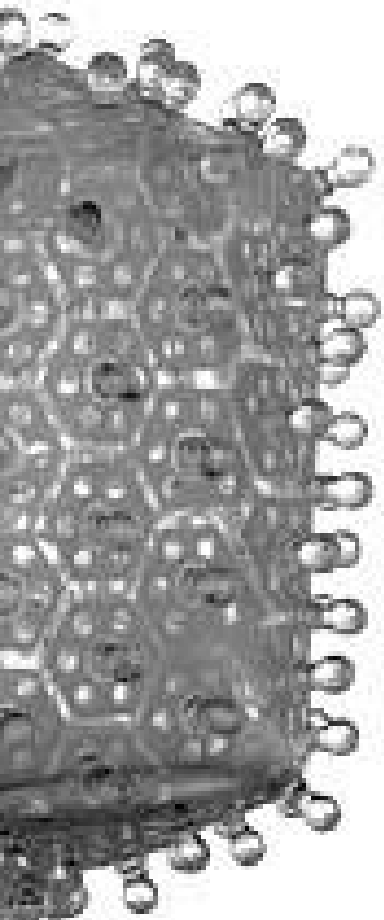

Microbiology

Lecture No:.....27.....

Dr Name:..Hamed Zoubi

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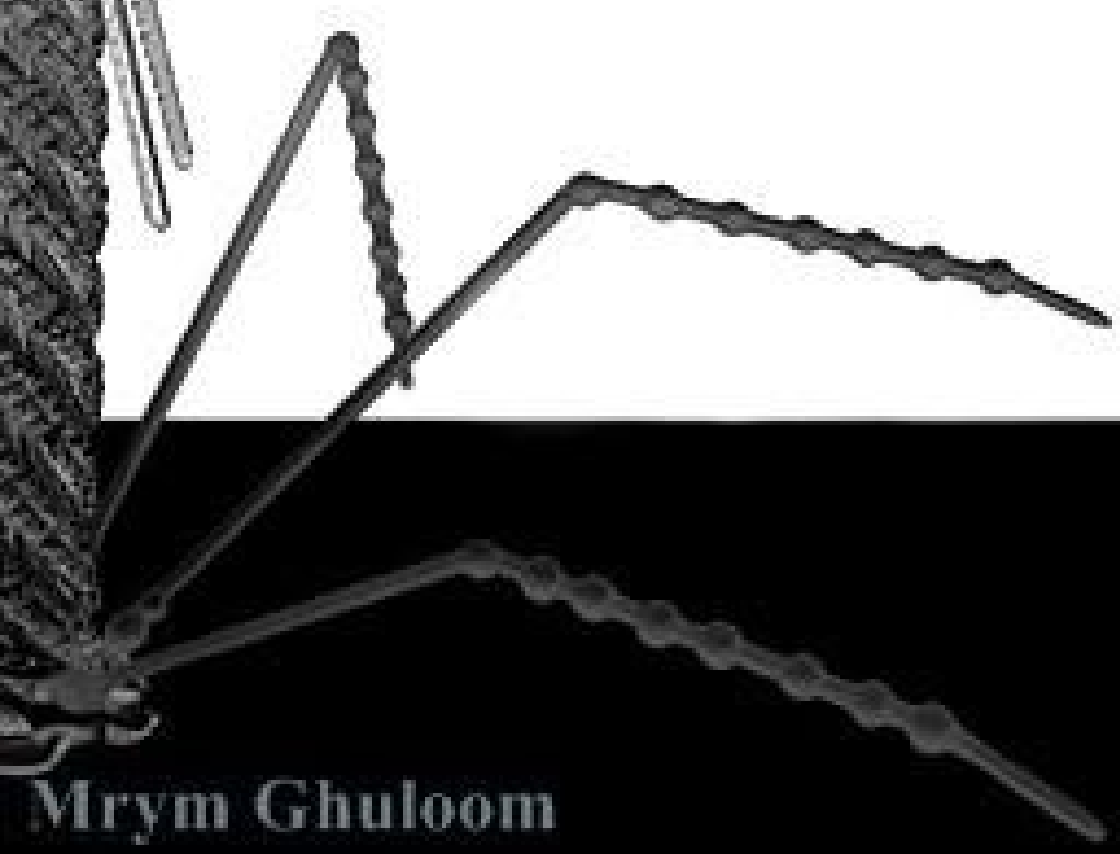
Sheet ■ Slide □



Mrym Ghuloom

Diagnosis (3)

بسم الله الرحمن الرحيم

Today's lecture is the last one in diagnosis.

In the previous 2 lectures we talked about many rapid diagnostic tests that are used to get rapid results .

Today we will talk about other methods that take more time than the rapid ones, so we do not call it rapid. Therefore, diagnosis methods are divided into rapid diagnostic tests and other tests that are not rapid.

Other diagnostic tests:

1-Virus isolation in cell culture. (for sure , cell culture needs at least two days, you cannot get the results immediately)

2-Detection of antiviral antibodies.*(needs days to weeks)*

Of course it is not a rapid method because antibodies will take weeks to months until they appear _they do not immediately appear_(The virus inter the body, then the antibodies will appear in few days or weeks later).

1-Virus isolation in cell culture

-It is not very common because it is expensive and need a well settings in the lab.(so not commonly used but it is *the gold standard* f or many infections; for example new emerging viral infections: SARS (sever acute respiratory syndrome and MERS corona virus (middle east respiratory syndrome corona virus)

For new emerging infections there is no pre-set test to detect the cause of the infection, so you need to go back to the classical way ,that is the cell culture.

-We have three cell types; that we use for cell culture: semi-continuous, continuous and lymphocyte culture.

1) Semi-continuous

Why it is called semi? because it is not continuous and you can only use it to a certain limit.

- The cells will divide , but to a certain limitation and after a certain number of divisions they will stop dividing , so this culture has a certain life span .

- they can be subcultured for 50 generations only , so it has a limited life span.

- Source of cells :Usually we take it from human or animal foetal tissues.

- **certain criteria**: The cells must be of the normal karyotype, they must have the correct number of chromosomes.

-**Seeds lot**:(a term used in the book):

the idea is that you store a lot of these cells after the 2nd or 3rd generation (law passage level) in liquid nitrogen (thousands of vials(قوارير)containing suspensions of these cells),then you can use it for many applications, you can take it out of the liquid nitrogen and use it.

- **Usages :** it is used mainly in making vaccines , especially for MMR vaccines (Mumps, Measles and rubella) .Classic cells are called WI -38 (from the Wistar Institute in Philadelphia) and MRC-9 (from the Medical Research Council laboratories) are used in this type of cultures .

  - These cultures are used to make vaccines *only* under two conditions *1- fresh low passage level* (i.e. you don't use cells from generation 40 or 50, it should be fresh , from the 1st five generations)

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2- *the karyotype must be normal* (you have to check the karyotype, the number of chromosomes must be normal), then you can use it in MMR vaccine production.

2) Continuous:

-From its name, cells divide indefinitely. (So if you think of tumor cells, one of their properties is that they divide indefinitely, there is no growth control on them).

Usage : These cells are mainly used for diagnostic purposes, and the karyotype in most of the cases is not normal (the cells have abnormal number of chromosomes)

Cell type (source) :

It could be **tumor cells** that divide indefinitely(without a control).Or you might subculture many **normal cells**, subculture the cells from the 1st generation, 2nd generation … until you reach an advanced generation, the probability of abnormal karyotype is higher ,so now **these cells will be transformed and will act as tumor derived cells .**

Some lines (cultures) from the continuous type, which we take from the kidneys of monkeys and dogs can be used for vaccine production for polio and influenza viruses , respectively .

Monkey kidney culture → polio vaccine
Dog kidney culture → influenza vaccine

3) Lymphocyte culture:

In general ,cells need special circumstances to divide.

1- One of the methods that is used in the lab is to *immortalize these cells* , using a virus called Epstein-Barr virus(EBV), infection of lymphocytes with EBV will make them divide as if they are tumor cells, Indefinitely .

So this is an example on immortalization of cells by a virus infection ,that is the Epstein-Barr virus (EBV).

2- Another way to stimulate the division of cells is to add certain chemicals to the medium .for example : Interleukin 2 (IL-2: it is a cytokine) stimulates the division of T lymphocytes (sometimes it is called T lymphocyte growth factor) .

-why do we have a lymphocyte line?

-We use it specifically for HIV (Human Immunodeficiency Virus) and HTLV (Human T-Lymphocyte Virus); to observe the cytopathogenic effect (CPE) of these viruses, so we can culture the HIV in lymphocyte line and see (CPE) which will be in the form of *syncytium formation* (which is the fusion of cells to form multinucleated giant cells).

- Test Procedure :

Usually to grow cells in a simple way ,you have to grow a **monolayer** of cells , just a mono-cellular layer (cell beside a cell, you do not want them to accumulate on each other), then you add the sample (whether it is from the CSF, the stool or from the respiratory tract) on this monolayer. If the virus is in the sample, then it may cause what we call cytopathogenic effect that can be observed , or it may not be visible or it may not cause CPE, so you can detect the presence of the virus in the sample using many techniques according to the characteristics of that virus as discussed later . (We will know what CPE means later).

- ✓ Viruses that cause CPE :

For this CPE to appear , it might take:

- 2 days (48 hours) in case of **herpes and enteroviruses.**

-from 10 to 14 days in case of **CMV (cytomegalovirus).**

This Cytopathogenic effect ,if you remember from lecuter # 5 could be:

1-Burster or lytic viruses: they well damage and kill the cells, this appear in the form of rounding up and cell lysis. example : **enterovirus**

2-Creepers (ex: *herpes and paramyxovirus*) : these viruses spread from cell to cell without bursting the cell (so we call them creepers –الزواحف-), you will see a **multinucleated giant cell** in couple of days (or syncytium) , and this is the CPE.

- ✓ Some viruses will cause no visible cytopathogenic effect , but they may inhibit the superinfection of these cells by another virus and that can be detected .
- ✓ Immunofluorescence is another method, if you cannot see the CPE then you can use immunofluorescence against the antigen of the virus.
- ✓ some viruses can be detected by their capacity to bind red blood cells (haemadsorption) , and this is another cytopathogenic effect.

For HIV (as we said lymphocyte culture needs a special technique , and multinucleated giant cell will be the effect.) In order to grow lymphocytes in the lab, we mention *immortalization using EBV* ,and also we can stimulate the cell to divide using *a plant lectin and interleukin 2.*

Procedure of cell culture:

A-In a simple way, you grow the cells in ***a growth medium*** (cells grow and divide on it so it is called growth medium) it contains salts at physiological

concentrations , glucose, amino acid, vitamins, antibiotics to inhibit fungal-bacterial contamination, it is buffered at pH 7.2-7.4 and fetal calf العجلserum is added *at concentration of 10-20%.*)

B-Once you get a monolayer of the cells, (you do not want them to divide further, in order *not* to have an overgrowth) , you discard the *growth medium* and replace it with *maintenance medium.*

- ✓ **<u>maintenance medium</u>**: is different from the growth medium in the fetal calf serum concentration (in maintenance it is 2-5%); in order to permit little or no further division of cells.

C-The monolayer that is formed will be attached to the plastic container or flask as we call it ,once you get the monolayer, you can detach it using *trypsin* .

D-Store these cells in the liquid nitrogen as we said, and then you can use it from time to time at a concentration of 10^6 (million cell)/ml in each diagnostic step.

****

The other part of our lecture is:

2- The detection of antiviral antibodies

It is not always true that if the virus is being there then it is the causative agent of the disease .Also , the patient is not always being symptomatic , he may carry the virus and infect others without having any symptoms.

These serological tests are another diagnostic confirmatory or helpful methods that are based on the detection of the antibodies made against the virus, and these antibodies usually appear later after the infection (after 2 weeks up to 9 months).

So you can detect these antibodies, (if you remember the antibodies start in the form of IgM and then IgG) by two main methods:

1- *Rising titer of the antibody* :

This method depends on taking a pair of samples .After the onset of the infection, take the first sample from the patient (a serum sample) and store it. 10 to 14 days later, you take another sample. And if there is a four -folds more increase in the titer of the antibodies >> it is an acute infection caused by that specific virus.

2- *Detect the IgM antibodies* , it is rapid and specific , and you can still detect it from the onset of infection up to 9 months, and if the antibodies are there , then there is a current or an acute infection .

If you remember we talked about the ELISA capture method in the previous lectures(actually it is in sheet 7, page 14) so we can use it to detect the IgM antibodies.

It is very sensitive if you follow each step with a washing step _to remove unbound and nonspecific agents_ and use the proper control (+ and -)

Back to the slide and read in brief the application of ELISA test to detect the IgM for rubella virus

-IgM antibody rises in the following cases: (both scenarios might give you false positive results)

1- Secondary infection (ex: reactivation of herpes)
2- In booster dose of vaccine الجرعة المدعمة:
antibodies produced due to vaccination decrease with time , so they give booster doses to stimulate the memory cells to produce more antibodies. Booster dose will start in the form of IgM , increasing it and giving wrong test result.

You are looking for infection not for a person who has a IgM due to a vaccine or due to reactivation).

3- Immunoblotting methods

▼ Southern blot (not related to the south, it is the name of discoverer): for DNA hybridization.

▲ Northern blot (as it is discovered after southern blot, just the opposite): for RNA hybridization.

◀◀ Western blot : for protein detection, we use it frequently for the diagnosis of HIV .

Western blot is one the doctor cares about.

We use if to further confirm diagnosis of HIV. It is not easily socially, personally and in all aspects to tell a patient that you are HIV positive. Because it might represents the end of the life for this patient so you have to be 100% sure that it is an HIV, so you must use more than one test: serological tests and immunoblotting(western blot).

What is western blot? (please read it from the slide)

1- Virus proteins are separated as bands according to their molecular weights by electrophoresis through a polyacrylamide gel . These proteins will be subjected to an electrical current from – to + , the sample contains the proteins of the virus (HIV Virus)and these proteins will move from the – to the + side because they are negatively charged, so they will move away from the cathode .

2- When you finish the electrophoresis, move these proteins (till now you do not see the proteins) to a nitrocellulose paper(ورق سكري) to anchor it more, press this paper to the gel with a specific buffer, so

the proteins will move from the gel to the nitrocellulose paper.(also till now you do not see the proteins)

3- Add to it the patient's serum, if the proteins of HIV are found(which is the antigen) and you have patient's serum(which has the antibodies for the proteins) they will react.

🔁Of course there is always a wash step after each step.

4-Add an antihuman antibody (anti-IgM) (recap the picture :we have the proteins of the HIV and the IgM from the patient's serum bound to it), you add the anti-IgM antibody that is labeled by an enzyme , then you add the substrate of the enzyme, so the color will change (for example to orange or blue).

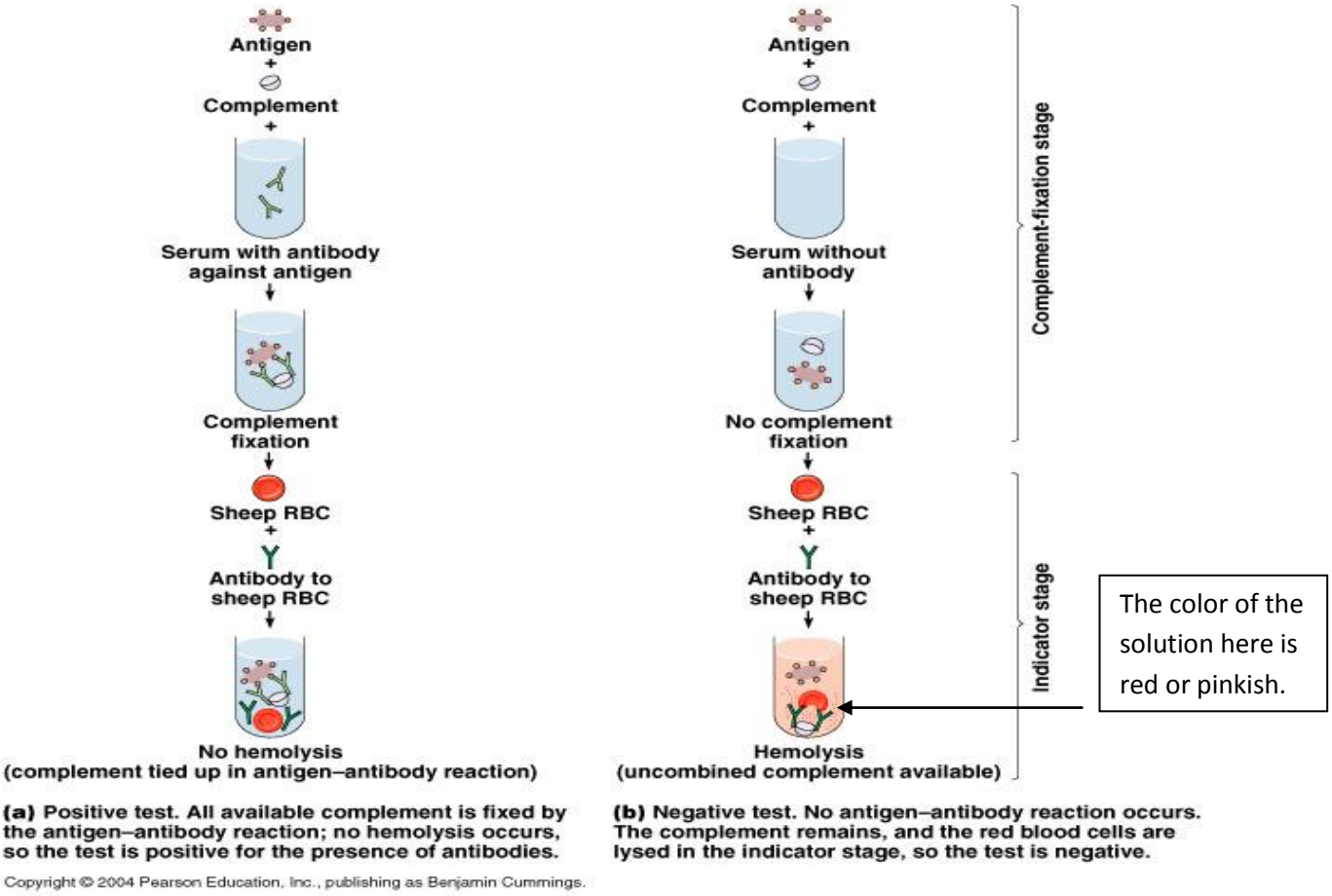
So ,We add the secondary antibodies and they will bind to the primary antibodies (human serum IgM), add the substrate, and the color is changed, that means the secondary antibody is there and the primary antibody is also there, so the patient is positive for HIV antibodies, so you confirm the diagnosis of HIV and you can release the result to the physician and to the patient through the physician .

4-Some traditional serological tests

These comprise complement fixation , radial haemolysis (screening test for rubella antibody), and haemagglutination inhibition to detect post-immunization and post-infection antibodies to influenza.

You have to know about them as they are mentioned in the book .

✨complement fixation



It is rarely used.(the doctor showed as a figure not from our book ,it is only for understanding , please go back to slides ,but I think the figure above is amazing *_*).

*What are complements ?

complements are proteins found in the blood or serum , their function is to bind to antigen-antibody complex, and if the antibody is found on the surface of RBCs forming another type of complex , binding of the complement to that complex will cause hemolysis).

Test procedure :

- If there are Antibodies in the serum , they will bind to the antigens(a reaction will occur between the antigens and the antibodies forming a complex).
- You add a complement , and the complement will bind to the antigen_ antibody complex .
- So the complement is now fixed (it is not free); because there are antibodies in the patient's serum that could form complexes with antigen.

*How to make sure that the complements are fixed and there is no free ones?

Add RBCs with antibodies. Again , they will form a complex that the complement will bind to if it was free ., but You will not find any free complement , why?
Because all of them are bound to the 1st complex (antigen-antibody). So the RBCs will precipitate in the bottom of the well , and they will not burst.

That means all the complements are fixed to antigen-antibody complexes, and the patient *is positive for the antibodies*, i.e. he has the antibodies .

-On the other hand , If the patient doesn't have the antibodies, the antigens added will not bind to antibodies as they are not there , and there will be no complex . Then we add the complement , it will not bind to the antigen alone, as it only binds to a complex, so the complement will be *free* .when you add RBCs - antibodies complex, the complement will bind to it immediately, *busting the RBCs*, which results in *pinkish or reddish color solution* . Which means *negative* result , i.e. the patient doesn't have the antibodies.

-The last test

✨**Radial hemolysis test** (it is a variant of complement fixation)

A) You link the *virus to the RBCs* (instead of linking the antibody to the RBCs as in the complement fixation)then mix it with the molten(ذائب) agarose and leave it to cool.

B) You pinch small wells within the agarose, and each one is filled with serum sample . so if the serum contains the antibody , the antibody will bind to the antigen found on the surface of RBCs to make a complex .

C) Incubate it overnight to allow the antibodies to diffuse and bind the antigen .

D) Add the complement : if there are antibodies, then there is a complex and the complement will be fixed, you will see a **zone of lysis** or clear zone of lysis surrounding the wells, and **the diameter of the zone is related to the amount of antibodies** (the more the diameter of the zone of inhibition or lysis means the more amount of the antibodies(direct proportionality علاقة طردية) .

So you can use this technique to quantify the antibodies.

- ✓ It is commonly used as **screening test for Rubella antibodies in females** and less for influenza virus
- ✓ it is not very accurate in its results ,But it can be used to screen lot of serum samples from pregnant women in antenatal clinics and ***if you find a positive sample*** then you have to send it to the lab to do the ELISA test or other more sensitive tests to have a more accurate result.

good luck all :D