University of Jordan
Faculty of Medicine

Medical Committee
The University of Jordan

Introduction to

Microbiology

Title :

Viral Test-2

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: 25

- ○ Slides
- ○ Handout
- ● Sheet

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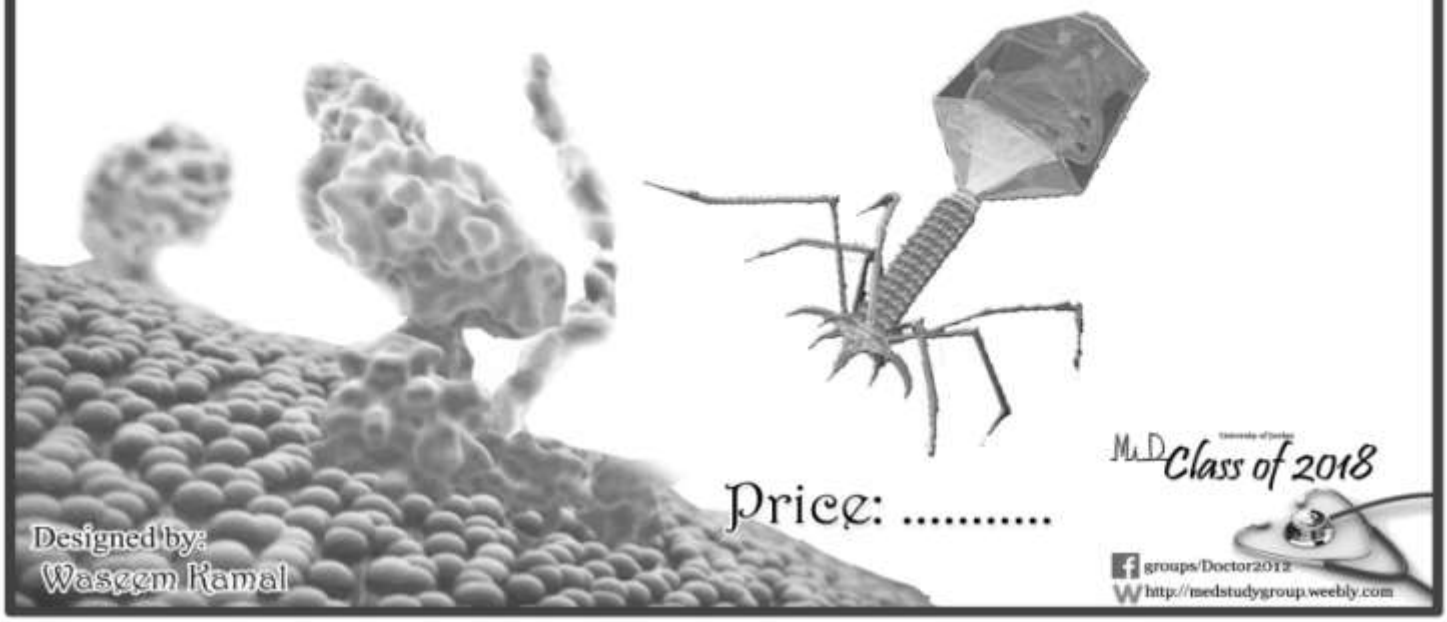

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We talked about nuclear methods and serological methods for viral diagnosis

Molecular methods based on the detection of viral genome .it is often said that molecular methods is the future direction of viral diagnosis ,nowadays it is the best choice for the viral detection because they are quick and its highly specific and has high sensitivity .
How ever in practice ,although the use of these methods is indeed increasing ,the role played by molecular methods in a routine diagnostic virus laboratory is still small compared to conventional methods. It need experts to perform this M. methods ,these methods are prone to contamination so they need specific location in the lab ,and specific equipment which only used in the M. methods

**we have too many classical molecular techniques but only the PCR (polymerase chain RxN) that we will talk about

Nucleic acid detection: short length of viral genome makes them ideal candidate for nucleic-acid based diagnosis ..so the template we will use is the genome whether RNA or DNA

PCR :
We have many types of PCR techniques
1-conventoinal PCR .. 2-real-time PCR

About the conventional PCR it's products run on agarose gel for detection of these products ..
agarose gel: a powder you put it in water in certain concentration ether 0.5% ,0.7% or 1% ,we mean by 1% that is 1g of powder per 100ml of water .most of the time these solution are put in the microwave ,it boils ,until it become combined after that you pure the gel into a tray and leave it to set with a comb ,after that when it set you remove the comb then you get a number of wells where you but the PCR samples with the dye in those wells and run the electrophoresis .

PCR allows the amplification of specific target DNA sequences by a factor 10^6 and Is those an extremely sensitive techniques
Why do we use the PCR?
For DNA amplification ,before putting the sample on the agarose gel we must amplify DNA ,because if we don't have enough DNA sample we will not see a good, strong band ,so to make high concentration of DNA\RNA to run in the agarose gel and can be seen .

**what are the *requirements in the PCR* tube to complete the reaction
1-nucleotides(A,T,C,G)
2-DNA polymerase
3-template (ether RNA or DNA)we said that PCR is used for amplification of the DNA ,but we can start with RNA using reverse transcription to DNA and then amplifying DNA . some times you but the materials in the PCR tubes but with RNA and the machine will reverse transcribe the RNA to DNA or you may firstly reverse transcribe the RNA to DNA and then you but the reverse transcribed RNA (DNA) to the reaction
4-nuclease free medium ,H2O :in order not to break the template that we started with
5-primer (the most important Part) it is a short sequence complementary to the start segment of the template we want to amplify ,the length vary from 15-50, but usually we use 20-25 bases in length .

”it is based on an enzymatic reaction involving the use of synthetic oligo-nucleotides flanking the target nucleic sequence of interest “ so these primers act as the oligo-nucleotides that bind to the beginning of the template ,including the taq polymerase (DNA polymerase)then we set the machine 30-40 cycles (30-40 PCR reactions) each cycle will double the # of templates ,we start with two strands then become (4,8,16,….)by the end of the 30 or 40 cycle we get from one million up to ten million copies of the template .

How do we set the machine of the PCR ??
1.***Dnaturation*** : (temp. 94,95,96)to un-wound or open the dsDNA (it takes 30 seconds)
2.***Annealing*** : binding of the primer to its complementary sequence at the beginning of the template ,temperature drops to from 95 to (65-55) depending on the primer and on the reaction ..it takes also 30 seconds .
3.***Extension (elongation***): when the DNA polymerase come and start making a complementary sequence starting from the primer (so the new strand composed of the primer + the sequence resulted from DNA polymerase) .so you set the machine on 95-94 C for 30 s ,followed by 60 C for 30or 15s then for 70or72,74 C also depending on the reaction and the primer .
**the length of the new strand depend on the length of the template ,(if its shorter than 1 kb-kilo base pairs- then it needs 30 s ,but longer it needs 1 minute)
Then the product of the PCR are put into the agarose gel.

“further sensitivity and specificity may be obtained by the nested PCR “ the denaturation and the annealing temp. may vary ,some primers may work at 60 , some at 72, some cannot at those two temp ..
NESTED PCR: when you set the same reaction in each tube ,but at different temp (e.g the first tube temp of the annealing 56,second 60,third 62 and so on) if the primer cannot bind at 56 ,others at 60 or 62.
”detection and identification of the PCR product is usually carried out by agarose gel electrophoresis” .

Advantages of the PCR:

- Extremely high sensitivity ,may detect down to one viral genome per sample volume
- Easy to set up .
- Fast turnaround time (most of the PCR techniques gives the product on 2.5-3 hours so it considered fast method for detection of the viral genome)

Disadvantages of the PCR :

- Extremely liable to contamination (the equipments of the rxn should ONLY be used for the PCR, and for the first trial ,so as not to contaminate with other templates from other trials)
- High degree operator skill required .(as we mentioned)
- Not easy to set up a quantitative assay .

*real –time PCR :in the same time you can do quantification of the amount of the template you are amplifying .
Referring to slide 33 :in conclusion ..1-target dsDNA .2-the denaturation step .3- the annealing 4-the extension step >>getting two new strands .

In the next cycle each strand will serve as template (in the first cycle after denaturation we have 2 template ,,the second cycle they become 4 templates and so on (doubling) .
In the PCR tubes the volume added 25 or 50 or 100 ml depending on the rxn and the amount of the product you need ,and what do you want it for .(if you want the product to run on the agarose gel then you put the lowest volume)

Serology:
In the last lecture we said that is the Detection of the rising titers (conc.)of the antibodies between the acute and the convalescence stage .
Referring to slide 36 :the first antibody to rise during the acute infection is the IgM .lets assume that it will rise in 5days ,get the peak on 7-8 days ,and then decrease in 2 weeks .around the peak of the IgM ,the IgG start to rise ,at this stage the symptoms might appear (symptoms of the acute infection)
**when the patient comes to your clinic complaining from the symptoms and in the lab they will look for the IgG titer (most of the time its not high , even if it was high its not diagnostic ,and does not mean anything AT THIS STAGE)this sample considered during the acute stage …after 7-10 days ,you take another sample to look also for the IgG (this sample considered during the convalescent stage) if there was 4 fold increase in the IgG titer between the acute and convalescent sample (first and second sample) ..so its now diagnostic and indicate the primary (acute) infection .
So, infection with specific virus rises the IgM ,followed by the IgG that peaks in 2-3 weeks ,after that it start to decline ..

**upon re-infection with the same virus .the response of the IgM and IgG is faster. because we have the memory B cells , which are sensitive to this virus ,and they produce the antibodies quicker ,and the IgG is going to rise ,and on the second or third exposure IgG will plateau at high reading ,and that what gives the body the protective immunity .Thus ,most of the time vaccines are given 2-3 doses in order to get the protective immunity (high titer of IgG).
***next year (enshallah)we will take the Hepatitis B vaccine by 3 doses (0-1-6)first dose –after a month-after 5 months ..respectively to get High IgG

So ..back to the criteria for diagnosing primary (acute) infection :

- 4 fold increase or more in the titer of IgG or total antibody between the acute and the convalescent stage (the convalescent stage means the recovery period ..here the symptoms are disappearing).
- The presence of IgM ,which s indicative for the acute infection

- Seroconversion :the presence of antibodies against certain antigen or virus (if we take a newborn,and he had not exposed to the influenza virus for example ..he will not has antibodies for this virus ..once he infected and produce the antibodies for this virus we say he has seroconversion .
- A single high titer of the IgG is very unreliable (as we said 2 readings with 4 fold increase NOT one reading).
-

Criteria for diagnosis of the re-infection :

- One or more increase in the titer of IgG or total antibodies between acute and the convalescent sera .(4 folds indicative for the acute ….1 or 2 fold is indicative for re-infection)
- Absence or slight increase in the IgM .

Serological methods :1-CFT. 2-western blot 3-ELISA. 4-the immunofluorescence assay.

1.complement fixation test (CFT):
The complement system is part of the immune system .the role of the complement proteins is to attach to the antibody-antigen complex and destroy them .-the Dr read slide #37 directly-.
How do we make the CFT ??-back to slide # 38- we take two serum samples ,the first one has antibodies against antigen of a specific virus ,but the second has not , then we add the antigen to both samples (antigen of the virus that we think it's the causative agent of infection).in the first sample the antigen will bind with the antibody ,but the second sample the antigen is free –since there are no antibodies to bind with-.then we add the sheep RBC plus the anti –sheep RBC to both samples .in the first sample the SRBC are left intact ,but in the second one they lysed (first rxn is REACTIVE ,the second one NOT reactive)
The explanation :in the first rxn we have antibody – antigen complex and we have the sheep RBC-anti- sheep RBC complex but the complement proteins will attach to the antigen –antibody complex and destroy it (they were deviated from the RBCs anti-RBCs complex) ,and the complement proteins will be used up >>>NO lysis of the RBC complex. In the second sample we don't have antigen-antibody complex ,so the complement proteins present in the serum will bind with the RBC complex(sRBCs-anti-sRBCs) and cause lysis their lysis {***we consider that binding of the RBC with their anti RBC as antigen -antibody complex that the complement system bind and destroy it ONLY in the absence of the normal antigen –antibody complex}

2-ELISA: enzyme linked immuno sorbent assay .
We have test plate with multiple wells (most of the time its 96 well-plate).Surface of the solid plate (microtiter plate) coated with antigen Or antibody depending on the ELISA assay either direct ,indirect ,sandwich or competitive – refer to slide #40.

- Direct assay: the bottom of the plate is coated with antigen ,then we add the primary antibody that bind to this antigen (mostly ,this assay is done in the labs).
- Indirect assay: this assay is used for testing the serum of the patient ,firstly we coat the plate with the antigen .then adding the serum sample ,if it contains the antibody for this antigen (we call it the Primary antibody)it will bind to it (e.g if the patient has antibodies against the HIV, and you add the antigen of the HIV to the plate ,then binding will occur).the exact steps are …adding the antigen >washing to remove the excess antigens>adding the serum of the patient >wait for two hours >also washing to remove the un-bound antibodies (primary antibodies which exist on the serum)>adding the secondary antibody(which is against the primary antibody NOT the antigen)this secondary antibody is labeled with enzyme{the antibodies often are labeled either with enzymes or fluorescent material}>washing to remove the excess secondary antibodies >add the substrate for the enzyme of the secondary antibodies >if ALL these things(antigen ,1ry antibody ,2ry antibody)are bound then the enzyme will transform the substrate changing its color >taking the samples to a certain machine at certain wave length >distribute them to the different wells ..RESULTS>> whether it's positive or negative

- Pic in slides >> the yellow color means + test (there is antibodies in the serum against the antigen we added)..colorless wells means that negative test ..and as the darkness of the yellowish color increased the positivity increase (more antibodies in the serum)
- Sandwich assay(capture): here we coat the plate with ANTIBODIES not like the direct and the indirect assay .>adding the antigen>adding the 1ry antibody> then the 2ry antibody which is also enzyme labeled antibody .

***note that in the case of the direct assay the enzyme-labeled antibody is the 1ry antibody ,,here we don't add a 2ry antibody ..

3-western blot :also called (SDA PAGE)-sodium dodecyl sulfate polyacrylamide gel electrophoresis -the idea here is to test for the presence of the viral proteins **,.SDS** are materials added to the gel in order to make it ,**polyacylamide** is one of the components of the gel ,**gel** is the result we get after pouring and leaving to set ..**electrophoresis**, to run a current .-then Dr read slide #42.
How the western blot works ??we start by making the gel >put it in the tubes of a tray >passing the current from the top of the tubes to the bottom .{the DNA gel we used before is thick ,but for the western blot its thin by putting it between two glass pieces }>>then you get wells in the gel where you can put the serum sample with dye >run the current for 2-3 hours >the result is separation of the proteins whither viral or cellular according to their M.W ,high M.W at the top ,low at the bottom .

**but how can you detect the presence of specific proteins e.g HIV??
Transfer the fragile gel that contain the proteins to a *nitrocellulose* membrane{white colored membrane that can tolerate the washing steps ,incubation with 1ry,2ry antibodies }>then run a current that is transferred from the gel to the membrane >adding antibodies against the protein you are looking for (HIV for example)>incubate for couple of hours or overnight at 4 degrees (temp.) with a rocker >washing >adding the 2ry antibodies {most of the time its immnuofluorescent labeled antibodies} >incubate>washing >move them to the scanner to detect the fluorescent antibodies >a band of the protein you need will appear .-then Dr read slides #43,44,45-.
The blot is incubated with ageneric protein (such as milk protein)before you add the 1ry or 2ry antibodies ,y have to add milk at a certain conc (2.5%) in order to block all the un-occupied spaces in the nitrocellulose membrane ,so when you scan the membrane you get a clear background ,,then after adding the milk put the antibodies ..
Refer back to slide #46:
in conclusion 1-separation of the proteins depending on the M.W ..
2-transferring to a nitrocellulose membrane ..
3-incubate with 1ry >then 2ry antibody which is immunofluorescent –labeled ..
4-once you put them in the machine ONLY the protein that is bound to the 1ry ,consequently to the 2ry antibody can be seen (the labeled ones) ,other proteins- which we are not looking for- will not appear after scanning .
In slide #47 ..
western blot for HIV ,different proteins in different samples ,you can see that C is positive for the HIV.

Samples that are used for detection of the virus :

- Blood>CMV
- Skin>HSV,VZV
- Feces>gastroenteritis viruses

- Nasopharyngeal aspirate>the upper respiratory tract viruses {“don’t memorize the viruses in the slides” Dr .said }

4.immunofluorescence :{they are very similar to those of the ELISA}

- Direct :we have antigen >binding to the primary antibody (the same sequence ..adding then washing ,,then adding and so on)
- Indirect :we put the cells >infect then with the virus >take the media >wash the cells >incubate with 1ry >then 2ry antibodies ,,to detect the antigen in the cells ..{if the cell is infected with the virus it will present the antigen of the virus on its surface that will bind to the 1ry >2ry antibodies }

***note that the direct immunofluorescence as the direct ELISA has only 1ry antibody which is labeled {direct elisa is labeled with enzyme but direct immunofluorescence is labeled with fluorochrome}, whereas the indirect elisa as the indirect immunfluorescence ,,the 2ry antibody is labeled with enzyme, fluorochrome respectively …

Advantages of the serological methods :
.results available quickly, but the Dr.said NEVER few hours ,*western blot* >2-3 days in order to finish all the incubation process .
*Immunofluorescence y*ou need to seat the cells ,infect them, do the labeling >so you need 3-4 days
ELISA: the quickest serological method but NOT few hours

***the potential problems in the slide 52 represent the disadvantages of the serological methods so please refer to them .

Usefulness of the serological methods :
How useful it depends on the individual viruses .(you have to know that the in most of the viral infections ,the development of the symptoms occur before the development of the antibodies {as we said before that when the patient complain from the symptoms and you test the sample for the IgG titer its usually low conc ,but the titer increase many folds after the symptoms start to disappear(during the convalescent stage)}
So once the titer of the antibodies rises ,its an advanced stage of the infection …BUT for certain viruses such as Rubella the development of the antibody and the symptom occur at the same time ,so better opportunity for the treatment earlier ..
There are also viruses that produce clinical disease months or years after seroconversion, e.g HIV and rabies ,,in these cases the serological methods are the most beneficial .
***Dr. did not read slide 54…he read slide 55(about the CSF antibodies ,he just read it)

Back to slides, not everything is included here