D-Immunologic Detection of Microorganisms

Immunologic methods take advantage of the specificity of antigen-antibody binding. For example, known antigens and antibodies are used as diagnostic tools in identifying microorganisms. In addition, serologic detection of a patient's immune response to infection, or antigenic or nucleic acid evidence of a pathogen in a patient's body fluids, is frequently useful. Immunologic methods are useful when the microorganism is difficult or impossible to isolate, or when a previous infection needs to be documented.

SPECIMEN COLLECTION & PREPARATION

1. Collect 5ml whole blood samples aseptically from the patient.
2. Allow blood to clot(leave it at room temperature or in incubater or in waterbath).
3. Remove serum ,by centrifugation for 5 min., as soon as possible to prevent haemolysis.
4. Repeat centrifugation to make sure that the serum clear without fibrin(give fales result)
5. Store serum at 2-8°C for 48 hours before performing the test. For longer periods of time the serum must be frozen.

A. Detection of microbial antigen with known antiserum:
- **Capsular swelling reaction:** e.g. serotypes of *S. pneumoniae*, *H. influenzae* type b, and *Neisseria meningitidis* groups A and C.
- **Slide agglutination test:** e.g. *Salmonella* species, can be identified by agglutination (clumping) of a suspension of bacterial cells on a microscopic slide. Agglutination occurs when a specific antibody directed against the microbial antigen is added to the suspension, causing cross-linking of the bacteria.

B. Identification of serum antibodies:
Detection in a patient's serum of antibodies that are directed against microbial antigens provides evidence for a current or past infection with a specific pathogen.
• **Direct agglutination**: to evaluate patients with fever of unknown origin, or when a suspected organism is difficult or dangerous to culture in the laboratory. e.g. *Brucella*.

• **Direct hemagglutination**: Antibodies directed against red blood cells can arise during the course of various infections. e.g. Epstein-Barr virus, *Treponema pallidum* and *Mycoplasma pneumoniae*.

C. **Other tests used to identify serum antigens or antibodies**

• **Latex agglutination test**: Latex and other particles can be readily coated with either antibody (for antigen detection) or antigen (for antibody detection). Addition of antigen to antibody-coated latex beads causes agglutination that can be visually observed (Figure 4.13). e.g. see the tests below.

• **Enzyme-linked immunosorbent assay (ELISA)**:

• **Fluorescent-antibody tests**:

![Figure 4.13 A. Schematic representation of antigens agglutinating latex beads with bound antibody. B. Photograph of agglutination reaction.](image-url)
ASO-Titer

PRINCIPLE
Anti-Streptolysin O antibodies are produced during *Streptococci pyogenes* infections, due to the presence of the Streptolysin O (SLO) liberated from the bacteria. Information on the extent and degree of the infection can be obtained from the latex agglutination of the antibodies serum level.

PROCEDURE
a) Qualitative method
1. Bring reagents and serum samples to room temperature.
2. Place one drop of undiluted serum onto a slide black area.
3. Add one drop of positive control and one drop of negative control in separate circles.
4. Swirl the ASO-latex reagent gently before using and add one drop next to the sample to be tested, one drop next to the negative and one drop next to the positive.
5. Mix the drops with a stirrer, spreading them over the entire surface of the circle. Use different stirrers for each sample.
6. Observe the presence or absence of agglutination within a period not longer than 3 minutes.

b) Semi-quantitative method
Prepare serial two-fold dilutions in physiological saline and proceed for each dilution as in the qualitative method. Then, observe the presence or absence of agglutination. The approximate ASO concentration in serum sample can be calculated by the following formula:

\[ [\text{ASO}] (\text{IU/ml}) = \text{highest positive dilution} \times 200 \] (as the reagent sensitivity is 200 IU/ml)

READING TEST RESULTS
1. Positive: The agglutination appears within 3 minutes.
2. Negative: No agglutination appears within 3 minutes.

Normal range: up to 200 I.U.
RA LATEX Test

Is a rapid agglutination procedure for semi-quantitative determination of rheumatoid factors (rheumatoid arthritis) in human serum samples.

**PRINCIPLE**

Detecting rheumatoid factor using a suspension of fine plastic granules coated with human gamma globulins which were agglutinated in the presence of RA. The presence or absence of a visible agglutination indicates the presence or absence of RF in the samples tested.

**PROCEDURE**

**Qualitative method**

1. Allow each component to reach room temperature,
2. Gently shake the lalex reagent to disperse the particles.
3. Add one drop of the latex reagent using the dropper provided (40µl) to each of the required circles of the agglutination slide.
4. Using the pipette stirrer provided, place a drop of undiluted serum onto a circle of a test slide.
5. Spread the reagent and serum sample over the entire area of the test circle using a separate stirrer for each sample.
6. Gently tilt the test slide backwards and forwards approximately once every two seconds for two minutes. Interpret results immediately after 2 minutes. Extended incubation may lead to false results. Positive and negative controls should be included.
7. At the end of the test rinse the test slide with distilled water, dry and store in a sealed bag.
Semi-quantitative determination
This can be performed in the same way as the qualitative test using serial dilutions of the serum in saline as follows:

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>1/2</th>
<th>1/4</th>
<th>1/8</th>
<th>1/16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample serum</td>
<td>100μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saline</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
</tr>
<tr>
<td>Volume of sample</td>
<td>50μl</td>
<td>50μl</td>
<td>50μl</td>
<td>50μl</td>
</tr>
<tr>
<td>8 x Titre</td>
<td>8x2</td>
<td>8x4</td>
<td>8x8</td>
<td>8x16</td>
</tr>
<tr>
<td>I.U./ml</td>
<td>16</td>
<td>32</td>
<td>64</td>
<td>128</td>
</tr>
</tbody>
</table>

Normal Levels: Adults < 8 I.U./ml

**CALCULATION OF RESULTS**
The titre is expressed as the reciprocal of the highest dilution showing microscopic agglutination: e.g. if this occurs in dilution 3, the titre is 8 corresponding to a concentration 64 I.U./ml.

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**CRP-Test**

Is a protein, which serum levels increase an acute phase status and in most forms of different tissue injuries, like malignant tumor, rheumatic fever, myocardial infarct, inflammations.
**PRINCIPLE**

The CRP reagent is a suspension of polystyrene latex particles coated with the gamma globulin fraction of antihuman CRP specific serum. When CRP is present in the sample, presence of agglutination indicates a content of CRP equal or greater than 6 mg/l, without previous sample dilution.

**PROCEDURE**

**Qualitative method**

1. Bring reagents and specimens to room temperature before use.
2. Place one drop (50µl) of the positive control on field 1 of the reaction slide, Place one drop (50µl) of the negative control on field 2. Using a pipette, place one drop (50µl) of each undiluted test specimen on successive field.
3. Gently resuspend the Latex reagent and add one drop (50µl) to each test field. Use a stirrer to spread reaction mixture over entire test field. Use different stirrers for each sample.
4. Rotate the slide (80-100 r.p.m.) for 2 minutes and read immediately under direct light.

**Semi-quantitative method**

1. Bring reagents and specimens to room temperature before use.
2. Make serial two fold dilutions of the sample in saline.
3. Proceed for each dilution as in the qualitative method.

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>1/2</th>
<th>1/4</th>
<th>1/8</th>
<th>...</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample serum</td>
<td>100µl</td>
<td>100µl</td>
<td>100µl</td>
<td>...</td>
</tr>
<tr>
<td>Saline</td>
<td>100µl</td>
<td>50µl</td>
<td>50µl</td>
<td>...</td>
</tr>
<tr>
<td>Volume of the sample</td>
<td>50µl</td>
<td>50µl</td>
<td>50µl</td>
<td>...</td>
</tr>
</tbody>
</table>

**READING THE RESULT**

A negative reaction is indicated by a uniform milky suspension with no agglutination as observed with the negative control.

A positive reaction is indicated by any observable agglutination in the reaction mixture. The specimen reaction should be compared with the positive control.
The titer, in the semi-quantitative method, is defined as the highest dilution showing a positive result concentration will be reciprocal of positive reading dilution x 6:

Normal Levels = Adults up to 6 mg/1

Rose Bengal Test

Brucella antigens are bacterial suspensions for use in slide agglutination tests to detect the presence of bacterial antibody like agglutinins associated with bacterial infection or previous exposure to a related organism. This test is a screening procedure only to establish the presence or absence of homologous antibody.

PRINCIPLE

Agglutinins combined with *Brucella* antigen (agglutinogen) under controlled conditions is capable of causing agglutination. The Rose Bengal stained *Brucella* antigen is used for the early detection of *Brucella* agglutinins (*Brucella abortus*, *B. melitensis*, *B. suis*).

PROCEDURE

1. Allow reagents and serum samples to reach room temperature for testing.
2. Shake the antigen bottle gently to insure a uniform suspension.
3. Place 50µl sample serum onto the selected ring of the slide.
4. Place one drop of the Rose Bengal antigen onto serum sample.
5. Mix serum sample with Rose Bengal antigen using stirring stick.
6. Repeat these steps using the positive and negative controls instead of serum sample.
7. Gently rock the slide for 2 minutes (automatic rotator can also be used).
8. Observe for agglutination after 4 minutes from beginning of shaking.

False positive results could appear if the test is read later than 2 minutes.

RESULTS

Negative: No agglutination
Positive: Agglutination and the titer is 1/80.
If positive, repeat the centrifugation of the sample serum and repeat the procedure, if the result also positive we should know if the infection active or inactive? by 2MET (2 merkapto ethanol broken IgM not IgG):
In tube, place 50µl sample serum + 50µl 2ME reagent, incubate for 1h..repeat procedure if positive=active infection(IgG) if negative=inactive infection(IgM)

Typhoid Fever Test (Widal Test)

The detection of specific antibodies of Salmonella, somatic (O) and flagellar (H) forms the basis for Widal test. This test dedicates that a serum with high levels of agglutinating antibodies to O and H >1/160 is indicative of the infection with this microorganism.

PRINCIPLE
The Bacterial Antigens is a slide agglutination test for the qualitative and semi-quantitative detection of antibodies anti-Salmonella in human serum. The reagents are standardized suspensions of killed and stained bacteria. When the antibodies are present in serum, a clear agglutination becomes evident.

PROCEDURE
Slide agglutination (qualitative)
1. Bring reagents and specimens to room temperature before use.
2. Gently shake the reagent to disperse the particles until you get a homogeneous mixture.
3. Place 50 µl of undiluted serum and 1 drop of Positive and Negative controls onto separate circles of the slide.
4. Add a drop (50 µsl) of the Reagent next to the drop of serum.
5. Mix both drops spreading them over the full surface of the circle
6. Rotate the slide manually or with mechanical shaker (80 - 100 rpm) during 1 minute.

RESULTS

*Slide agglutination method*

Examine microscopically the presence or absence of clumps within 1 minute, after removing the slide from the rotator comparing the test results with the control sera. If a reaction is found, the titre is 1/160 and establish the titer by a tube test.

**Pregnancy Test (PT)**

*Human chorionic gonadotropin(hCG)* is a glycoprotein hormone secreted by the developing placenta shortly after fertilization. The appearance of hCG in the urine and serum soon after conception and its subsequent rise in concentration during early gestational growth, make it an excellent marker for the early detection of pregnancy. The Pregnancy Test is a rapid, high sensitive, specific, qualitative test used to detect the presence of hCG in urine or serum.

**PRINCIPLE**

The sample is applied to the card and reacts initially with the specific, anti-hCG conjugate on the test membrane. This mixture moves along the membrane, by capillary action, and reacts with a specific anti-hCG in the test region. If hCG is present in the sample, the result is the formation of a colored band in the test region. If there is no hCG in the sample, the area will remain white. The sample continues to flow to the control region and forms a pink to purple color, indicating the test is working and the result is valid.

**SAMPLE COLLECTION**

URINE: Collect specimen in a clean, dry glass or plastic container. It is not necessary to obtain a first morning specimen, except in watery urine, however concentrations of hCG may be higher in this specimen. In cause of
turbidity or bloody urine, it should be centrifuged then procedure done. The sample can be refrigerated up to 72 hours prior to testing. A refrigerated sample must be allowed to warm to room temperature and mixed before testing.

**PROCEDURES**

**FOR STRIP TEST:**
1. Bring all materials and specimens to room temperature.
2. Remove test strip from the sealed foil pouch.
3. Dip the test strip into the sample with the arrows pointing toward the specimen.
4. The sample level should reach the maximum line marked on the strip, but must not exceed the maximum line.
5. Hold the strip in the sample until a reddish color appears at the lower edge of the test membrane (approximately 10 seconds).
6. Withdraw the strip and place it face up on a clean, dry surface.
7. Read the result between 3 and 10 minutes after adding the sample.

**FOR CARD TEST:**
1. Bring all materials and specimens to room temperature.
2. Remove test card from the sealed foil pouch.
3. Place the test card on a flat dry surface.
4. Using the provided plastic dropper, dispense 100ml sample (3 drops) to the sample well of the test card. Start timing.
5. Read result between 3 to 10 minutes after adding the sample.
READING THE TEST RESULTS

Negative: One (1) pink/purple band forms in the control region. No band is found in the test region.

Positive: Two (2) pink/purple bands form. In addition to the control band, a pink/purple band also appears in the test region.

Invalid: If there are no pink/purple bands in the control or test region, the test result is invalid. Retest the sample using a new device.

VDRL Test

Venereal Disease Research Laboratory (VDRL) test is a slide flocculation test employed in the diagnosis of syphilis, a sexually transmitted infection caused by Treponema pallidum, that is also used to assess response to therapy. Since the antigen used in this test is cardiolipin, which is a lipoidal extracted from beef heart, This test is classified as non-specific or non-treponemal or standard test. The antibodies reacting with cardiolipin antibodies have been traditionally termed “regain”.

**Principle:**
Patients suffering from syphilis produce antibodies that react with cardiolipin antigen in a slide flocculation test, which are read using a microscope. It is not known if the antibodies that react with cardiolipin are produced against some lipid component of Treponema pallidum or as a result of tissue injury following infection.

**Procedure:**
Patients’ serum is inactivated by heating at 56 °C for 30 minutes in a water bath to remove non-specific inhibitors (such as complement). The test can be performed both qualitatively and quantitatively. Those tests that are reactive by qualitative test are subjected to quantitative test to determine the antibody titres.

**Qualitative test:**
0.05 ml of inactivated serum is taken into one well. 1/60 th ml (or 1 drop from 18 gauge needle) of the cardiolipin antigen is then added with the help of a syringe (unbeveled) to the well and rotated at 180 rpm for 4 minutes. Every test must be accompanied with known reactive and non-reactive controls. The slide is then viewed under low power objective of a microscope for flocculation. The reactive and non-reactive controls are looked first to verify the quality of the antigen. Depending on the size the results are graded as weakly reactive (W) or reactive (R). Reactive samples are then subjected to quantitative test.

**Qualitative test:**
this is performed to determine the antibody titres. The serum is doubly diluted in saline from 1in 2 to 1:256 or more. 0.05 ml of each dilution is taken in the well and 1/60 ml of antigen is added to each dilution and rotated in a rotator. The results are then checked under the microscope. The highest dilution showing flocculation is considered as reactive titre.
CSF VDRL:

VDRL test may also be performed on CSF samples in the diagnosis of neurosyphilis. Quantitative VDRL is the test of choice on CSF specimens. However, there are some variations in this test. The antigen is diluted in equal volumes with 10% saline, CSF must not be heated (or inactivated), the volume of antigen solution taken is 0.01 ml (or 1 drop from 21 gauge needle) and rotation time is 8 minutes. Rest of the procedure remains same.

**Significance of VDRL test:**
VDRL test becomes positive 1-2 weeks after appearance of (primary lesion) chancre.

The test becomes reactive (50-75%) in the late phase of primary syphilis, becomes highly reactive (100%) in the secondary syphilis and reactivity decreases (75%) thereafter. Treatment in the early stages of infection may completely suppress production of antibodies and result in non-reactive tests. Effective treatment in the primary or secondary stages results in rapid fall in titre and the test may turn non-reactive in few months. Treatment in latent or late syphilis has very little effect on the titre and the titres may persist at low levels for long periods. Since the titre falls with effective treatment, it can be used for assessment of prognosis. VDRL test is more suitable as a screening agent than a diagnostic tool.

VDRL test is also helpful in the diagnosis of congenital syphilis. Since passively transferred antibodies through placenta may give false reactive test in serum of the infant, a repeat test after a month showing no increase in titre may help rule out congenital syphilis.

**Result and Interpretation of VDRL test**
VDRL test is positive in most cases of Primary Syphilis and are almost
always positive in Secondary Syphilis. It has a very good sensitivity for syphilis, except in late Tertiary Syphilis form.

The titer of reagin antibodies decreases with effective treatment, so VDRL test can be used to determine the treatment response of Syphilis.

A positive test result may mean have syphilis. If the test is positive, the next step is to confirm the results with fluorescent treponemal antibody-absorption (FTA-ABS) test, *T. pallidum* hemagglutination assays (TPHA), and the microhemagglutination assay (MHA-TP) which is a more specific syphilis test.

**False positive VDRL test result**

Reagin antibodies may be produced in response to nontreponemal diseases of an acute and chronic nature in which tissue damage occurs such as:

1. Leprosy
2. Hepatitis B
3. Infectious Mononucleosis
4. HIV
5. Certain types of pneumonia
6. Malaria
7. Systemic lupus erythematosus
8. Rheumatic fever
9. Rheumatoid arthritis
10. Tuberculosis