Serology Review
1393

By:

Ghasem Ghalamfarsa
PhD of Medical Immunology
Laboratory Quality Control

Discuss the quality control of serological assays
SEROLOGICAL ASSAYS

• Determination of immune status for epidemiological purposes

• Methods with highest possible sensitivity level should be employed

  – SENSITIVITY – Galen (1986) - “expressed as a percentage, indicates the frequency of positive test results in patients with a particular disease” – frequency of occurrence of positive tests
TEST QUALITY

• SENSITIVITY
  – defined as the proportion of subjects with the disease who have a positive test for the disease
  – describes ability of an immunologic reagent to detect small amounts of antigen

• SPECIFICITY
  – the proportion of subjects without the disease who have a negative test
  – describes the selective reaction between antigen and its corresponding antibody
SENSITIVITY, SPECIFICITY & SEROLOGICAL ASSAYS

- The sensitivity and specificity of the assays depend greatly on the antigen used.

- Assays that use recombinant protein or synthetic peptide antigens tend to be more specific than those using whole or disrupted virus particle.
PREDICTIVE VALUE

• Predictive value is determined by sensitivity & specificity of the test and the prevalence of disease in the population being studied.

• POSITIVE PREDICTIVE VALUE
  – the probability of disease in a patient with positive test result.

• NEGATIVE PREDICTIVE VALUE
  – the probability of not having the disease if the test result is negative or normal.
SPECIMENT HANDLING AND TRANSPORT

- Quality laboratory results begin with proper collection and handling of the specimen submitted for analysis.

- Correct patients preparation, specimen collection, specimen packaging, and transportation are of vital importance.
QUALITY SAMPLE

Hyperlipemic, hemolyzed, heat-inactivated samples as well as samples containing particulate matter or exhibiting obvious microbial contamination may cause erroneous results!
QUALITY CONTROL

“The aim of quality control is simply to ensure that the results generated by the test are correct. However, quality assurance is concerned with much more: that the right test is carried out on the right specimen, and that the right result and right interpretation is delivered to the right person at the right time”
# ACTIVITIES WITHIN EACH PHASE OF THE TOTAL TESTING PROCESS

*MMWR 2005, Vol.54/RR13*

<table>
<thead>
<tr>
<th>Before testing</th>
<th>During testing</th>
<th>After testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test ordering</td>
<td>Control testing / checks</td>
<td>Reporting results</td>
</tr>
<tr>
<td>Patient identification, preparation</td>
<td>Test performance</td>
<td>Documenting</td>
</tr>
<tr>
<td>Specimen collection, handling</td>
<td>Results interpretation</td>
<td>Confirmatory testing</td>
</tr>
<tr>
<td>Preparing materials, equipment, and testing area</td>
<td>Recording results</td>
<td>Patient follow-up</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Disease reporting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biohazard waste disposal</td>
</tr>
</tbody>
</table>
The Quality Assurance Cycle

Pre-Analytic
- Personnel Competency Test Evaluations
- Sample Receipt and Accessioning

Analytic
- Data and Lab Management
- Safety
- Customer Service
- Quality Control Testing
- Sample Transport
- Record Keeping
- Reporting

Post-Analytic
- Patient/Client Prep Sample Collection
PERFORMANCE OF TESTING
GOOD LABORATORY PRACTICE

- Precision
- Reliability
- Accuracy
LABORATORY QUALITY CONTROL

- **Quality Control** - QC refers to the measures that must be included during each assay run to verify that the test is working properly.

- **Quality Assurance** - QA is defined as the overall program that ensures that the final results reported by the laboratory are correct.
QUALITY ASSESSMENT

- Quality Assessment (also known as proficiency testing)
  - is a means to determine the quality of the results generated by the laboratory. Quality assessment is a challenge to the effectiveness of the QA and QC programs.

- Quality Assessment may be external or internal.
VARIABLES THAT AFFECT THE QUALITY OF RESULTS

- The educational background and training of the laboratory personnel
- The condition of the specimens
- The controls used in the test runs
- Reagents
- Equipment
- The interpretation of the results
- The transcription of results
- The reporting of results
The following items are essential elements of quality control that must be performed during every assay:

1. Each run must include one full set of controls
2. The controls for each test run must yield results within the limits of the manufacturer's criteria for acceptability and validity of the run.
3. All test kits must be used before the expiration date to ensure valid results
4. Physical parameters of the test such as incubation time and temperature must be followed to ensure proper performance.
SELECTING CONTROL MATERIALS CALIBRATORS

• Has a known concentration of the substance (analyte) being measured

• Used to adjust instrument, kit, test system in order to standardize the assay

• Sometimes called a standard, although usually not a true standard
SELECTING CONTROL MATERIALS

CONTROLS

• Known concentration of the analyte
  – Use 2 or three levels of controls
  – Include with patient samples when performing a test

• Used to validate reliability of the test system
TYPES OF CONTROLS

- INTERNAL CONTROLS
- EXTERNAL CONTROLS
INTERNAL CONTROLS

INTERNAL, PROCEDURAL, OR BUILT-IN CONTROLS

• Evaluate whether certain aspects of the test system are working properly.
• They are designed to verify that the test system is working as expected, that sufficient specimen was added and, for unitized test devices, whether is migrated through the test strip properly.
• Certain test systems might have electronic internal controls to monitor electronic functions.
INTERNAL CONTROL

Reference material:

– POSITIVE CONTROL
– NEGATIVE CONTROL
– CUT-OFF CONTROL
EXTERNAL CONTROLS

• Mimic patients specimens and monitor the testing process, from specimen application to result interpretation, to assure proper test performance.

• They might be provided as liquid or other materials similar to patient specimens and might be included with the test system or purchased separately.
FREQUENCY OF CONTROL TESTING

• In the test instructions you should specify **minimum frequency** for running controls, and include recommended levels of control materials that correspond to medical decision levels.

• The appropriate control testing frequency for each test system should not be less than specified in the product insert.

• Controls should be tested concurrent with patient specimens by the personnel who routinely perform patient testing.
CORRECTIVE ACTION WHEN CONTROL TESTING FAILS

• If controls were not performed as expected, the results should not be reported until the **problem is identified and corrected**. The product insert should provide information on procedures for handling unexpected control results, identifying source of error, and manufacturers contact address for technical assistance.

• **Documenting and monitoring** control testing results provides an indication that the test was properly performed.

• Record of control results should be **periodically** reviewed to detect shifts or changes in performance over time.
TEST RESULT INTERPRETATION

- When the test is complete, interpret the results according to instructions in the product insert, as

  - QUALITATIVE
    - determines whether the substance being tested for is present or absent

  - QUANTITATIVE
    - measures the amount of a substance present
ERRORS IN MEASUREMENT

- **TRUE VALUE** - this is an ideal concept which cannot be achieved.

- **ACCEPTED TRUE VALUE** - the value approximating the true value, the difference between the two values is negligible.

- **ERROR** - the discrepancy between the result of a measurement and the true or accepted true value.
**SOURCES OF ERROR**

- **Input data required** - such as standards used, calibration values, and values of physical constants.
- **Inherent characteristics of the quantity being measured** - e.g. CFT and HAI titre.
- **Instruments used** - accuracy, repeatability.
- **Observer fallibility** - reading errors, blunders, equipment selection, analysis and computation errors.
- **Environment** - any external influences affecting the measurement.
- **Theory assumed** - validity of mathematical methods and approximations.
EXAMPLES OF POTENTIAL SOURCES OF ERROR TO CONSIDER THE HAZARD ANALYSIS

• OPERATOR ERROR / HUMAN FACTORS

  • Use of incorrect specimen type
  • Incorrect application of the specimen on the device
  • Incorrect placement of device (e.g., non-level surface)
  • Incorrect placement of reagents including strips, or other components that contain reagents
  • Use of incorrect reagents, for example, reagents that are not specific for the particular device or lot, or generic reagents
  • Incorrect order of reagent application
  • Use of incorrect amount of reagent
  • Incorrect timing analysis (e.g., specimen application, running the test, or reading results)
  • Incorrect reading test results
• SPECIMEN INTEGRITY AND HANDLING

• Error in specimen collection
• Use of inappropriate anticoagulant
• Clotted specimen
• Error in specimen processing and handling
• Incorrect specimen transport and/or storage
• Presence of interfering substances
• Presence of bubbles in the specimen
• REAGENT INTEGRITY (REAGENT VIABILITY)

• Use of improperly stored reagents
• Use of outdated reagents
• Use of improperly mixed reagents
• Use of contaminated reagents
HARDWARE, SOFTWARE AND ELECTRONICS INTEGRITY

- Power failure
- Repeated plugging and unplugging of the device
- Hardware failure
- Software failure
- Electronic failure
- Physical trauma to unit
STABILITY OF CALIBRATION AND INTERNAL CONTROLS

- Factors that affect calibrator and calibration stability, including determination of calibration stability over time and after power failures
- Factors that may interfere with calibration
ENVIRONMENTAL FACTORS

- Impact of key environmental factors (heat, humidity, sunlight, surface angle, device movement, etc.) on reagents, specimens, and test results
- Impact of key environmental factors (including electrical or electromagnetic interference) on instruments, if appropriate.
RANDOM ERROR

- An error which varies in an unpredictable manner, in magnitude and sign, when a large number of measurements of the same quantity are made under effectively identical conditions.

- Random errors create a characteristic spread of results for any test method and cannot be accounted for by applying corrections.

- Random errors are difficult to eliminate but repetition reduces the influences of random errors.
  - Examples of random errors include errors in pipetting and changes in incubation period.

- Random errors can be minimized by training, supervision and adherence to standard operating procedures.
RANDOM ERRORS
SYSYSTEMATIC ERROR

• An error which, in the course of a number of measurements of the same value of a given quantity, remains constant when measurements are made under the same conditions, or varies according to a definite law when conditions change.

• Systematic errors create a characteristic bias in the test results and can be accounted for by applying a correction.

• Systematic errors may be induced by factors such as variations in
  – incubation temperature,
  – blockage of plate washer,
  – change in the reagent batch or
  – modifications in testing method.
SYSTEMATIC ERRORS
MONITORING QC DATA

- Use Shewart chart or Levey-Jennings chart

- Plot control values each run, make decision regarding acceptability of run

- Monitor over time to evaluate the precision and accuracy of repeated measurements

- Review charts at defined intervals, take necessary action, and document
SHEWHART CHART
or Levey-Jennings Chart

VZV IgG ELISA: Target Value = 49 U/ml
Traits of Immunogens (Antigens)

• Foreignness
  – Ex. Plant protein more immunogenic than animal protein

• Size
  – >10,000 daltons

• Complexity
  – Heterogeneity in building blocks that comprise the antigen
    – Proteins>polysaccharides>>lipids and nucleic acids
Haptens

- Small molecule that, by themselves, are NOT immunogenic.

- When coupled to a high molecular weight protein, the hapten becomes the antigenic determinant (epitope) for the antigen.
• Antibody
  – Please tell me you can define antibody by now.

• Heterophile antigen
  – Antigens that appear on the surface of tissues of several different species.

• Heterophile antibody
  – Antibodies that are generated in response to an antigen in one species, but are cross reactive with an antigen in another species.
    • Example: Epstein-Barr virus antibody detected in Monospot test.
IgG

IgG Structure

- Antigen-binding site
- Light chain
- Heavy chain
- Variable region
- Constant regions
Factors affecting Ag/Ab Reactivity

Effect of pH

\[ \text{pH 7.5} \quad \text{pH 7.0} \]

![Graph showing complex formation vs. antigen concentration with equivalence point, prozone area, antibody excess, and antigen excess regions.](image)
Factors affecting antigen antibody reaction

• Many factors affect the interaction between antigen and antibody; these include
  • Specificity
  • cross reactivity
  • Temperature
  • pH
  • ionic strength
  • Concentration
  • intermolecular specificity
**Specificity:**
The ability of a particular antibody to combine with one antigen instead of another is referred to as specificity.

This property depends on the antigen – binding fragment of an immunoglobulin molecule.

Antigen – antibody reactions can show a high level of specificity.
Cross reactivity:
Unrelated molecules can have antigens with similar antigenic determinants.

This means a proportion of the antibodies directed against one kind of antigen will also react with the other kind of antigen. This is called cross reactivity.

An example of cross reactivity is when; antibodies directed against a protein in one species may also react in a detectable manner with the homologues protein in another species.
**Temperature:**
The optimum temperature needed to reach equilibrium in an antibody – antigen reaction differs for different antibodies.

IgM antibodies are cold reacting, with a thermal range of 4-22°C, and IgG antibodies are warm reacting with an optimum temperature of reaction of 37°C.
pH:

- Although the optimum pH for all reactions has not been determined, a pH of 7.0 is used for routine laboratory testing.
**Ionic strength:**
The concentration of salt in the reaction medium has an effect on antibody uptake by the membrane bound erythrocyte antigens. Sodium and chloride ions in solution have an inhibitory effect. These ions cluster around the opposite charges on antigen and antibody molecules which partially neutralizes them.

This hinders the association of antibody with antigen.

Reducing or lowering the ionic strength of a reaction medium, such as low-ionic strength salt, can enhance antibody uptake.
Concentration:
Under normal conditions, the concentration of antigen and antibody should be optimal, but sometime this is not the case.

Excess antibody or antigen concentration will result in a false reaction, sometimes known as zonal reaction when the concentration of antigen is excess it is known as a Serology post zone reaction; excess antibody is referred to as a prozone reaction. This phenomenon can be overcome by serial dilutions until the optimum amount of antigen and antibody is present.
Prozone effects in antigen antibody reaction in agglutination reaction

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Agglutination Reaction</th>
<th>Antigen antibody ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>- Prozone</td>
<td></td>
</tr>
<tr>
<td>1:40</td>
<td>- Prozone</td>
<td></td>
</tr>
<tr>
<td>1:80</td>
<td>- Prozone</td>
<td></td>
</tr>
<tr>
<td>1:160</td>
<td>++ Equimolar</td>
<td></td>
</tr>
<tr>
<td>1:320</td>
<td>++++ Equimolar</td>
<td></td>
</tr>
<tr>
<td>1:640</td>
<td>++++ Equimolar</td>
<td></td>
</tr>
<tr>
<td>1:1280</td>
<td>++ Equimolar</td>
<td></td>
</tr>
<tr>
<td>1:2560</td>
<td>- postzone</td>
<td></td>
</tr>
</tbody>
</table>
Materials Necessary for Basic Serologic Tests

- Glassware
- Serological pipettes
- Test tubes
- Glass slide
- Water Bath
- Incubator
- Centrifuge
- Rotating machines
Quality control

- Positive and negative control should include in each test.
- Reaction time critical.
- (More than 3 min >>> drying of sample false positive result)
- Freezing the latex reagent lead to spontaneous agglutination ... so always bring reagent to room temp. before starting the test.
- Do not mix component form different kit or manufacturers.
- Always use new pipette when transferring sample.
- Do not use reagent after expiration date.
- Sample showing turbidity or hemolysis may yield incorrect result.
Classification of antigen-antibody interactions:

1. **Primary serological tests: (Marker techniques)** e.g.
   - Enzyme linked immuno sorben assay (ELISA)
   - Immuno fluorescent antibody technique (IFAT)
   - Radio immuno assay (RIA)

2. **Secondary serological tests: e.g.**
   - Agglutination tests
   - Complement fixation tests (CFT)
   - Precipitation tests
   - Serum neutralization tests (SNT)
   - Toxin-antitoxin test

3. **Tertiary serological test: e.g.**
   - Determination of the protective value of an anti serum in an animal.
A. Agglutination tests:

1. Agglutination/Hemagglutination
   When the antigen is particulate, the reaction of an antibody with the antigen can be detected by agglutination (clumping) of the antigen. The general term agglutinin is used to describe antibodies that agglutinate particulate antigens. When the antigen is an erythrocyte, the term hemagglutination is used. All antibodies can theoretically agglutinate particulate antigens, but IgM, due to its high valence, is particularly good agglutinin and one sometimes infers that an antibody may be of the IgM class if it is a good agglutinating antibody.

a. Qualitative agglutination test
   Agglutination tests can be used in a qualitative manner to assay for the presence of an antigen or an antibody. The antibody is mixed with the particulate antigen and a positive test is indicated by the agglutination of the particulate antigen.
For example, a patient's red blood cells can be mixed with antibody to a blood group antigen to determine a person's blood type. In a second example, a patient's serum is mixed with red blood cells of a known blood type to assay for the presence of antibodies to that blood type in the patient's serum.
b. Quantitative agglutination test

Agglutination tests can also be used to measure the level of antibodies to particulate antigens. In this test, serial dilutions are made of a sample to be tested for antibody and then a fixed number of red blood cells or bacteria or other such particulate antigen is added. Then the maximum dilution that gives agglutination is determined. The maximum dilution that gives visible agglutination is called the *titer*. The results are reported as the reciprocal of the maximal dilution that gives visible agglutination.
2-Passive hemagglutination:
The agglutination test only works with particulate antigens. However, it is possible to coat erythrocytes with a soluble antigen (e.g. viral antigen, a polysaccharide or a hapten) and use the coated red blood cells in an agglutination test for antibody to the soluble antigen. This is called passive hemagglutination.

The test is performed just like the agglutination test. Applications include detection of antibodies to soluble antigens and detection of antibodies to viral antigens.
Components:
1. Serum from patient (unknown Ab).
2. Specific diagnosticum – killed known pure culture of bacteria (known Ag).
3. NaCl solution.
Passive (indirect) agglutination

Principle

– precipitation reaction converted into agglutination
  - coating antigen onto the surface of carrier particles like red blood cells, latex, gelatin, bentonite
    • background clears

Examples of types

– latex agglutination
– co-agglutination
– passive hemagglutination (treated red blood cells made resistant)
Passive Hemagglutination Agglutination Test (PHAT)

Components:
1. Pair serum from patient (unknown Ab).
2. Specific erythrocyte diagnosticum – (known Ag on surface erythrocytes from sheep).
3. NaCl solution.

Dilution of serum from patient
1:20  1:40  1:80  1:160  1:320  Control

Agglutination  Non agglutination
Precipitation Tests

- One of the easiest of serological tests
- Relies on fact that antigens and antibody mixed in the proper proportion form large macromolecular complexes called **precipitates**
- Correct proportions are important to create precipitation
- Two techniques determine optimal antibody and antigen concentrations
  - Immunodiffusion
  - Immunelectrophoresis
Ring Precipitation Test

Components:

1. Material from patient (unknown Ag).
2. Specific precipitation serum – (known Ab).

Used:

For express-diagnosis of infection disease (ex. Anthrax)
Precipitation test in gel

Well containing antigen molecules

Line of precipitation

Well containing antibodies against the antigen

Agar

Zone of antigen excess

Zone of optimal precipitation

Zone of antibody excess

Well containing 4 different types of antigen

Lines of precipitation

Well containing different types of antibodies

Copyright © 2009 Pearson Education, Inc., publishing as Pearson Benjamin Cummings.
Liquid Precipitation

Precipitation in tubes
Gel Precipitation: Radial Immunodiffusion (RID)
Double Gel Diffusion: Ouchterlony
BRUCELLOSIS
Laboratory changes:

- C.B.C
- Bacteriologic evidence
- Radiographic changes
- Serologic tests
Laboratory changes

C.B.C

- Granulopenia in acute brucellosis
- Lymphocytosis in chronic brucellosis
- Atypical lymphocytosis
- Anemia due to hypersplenism
- Thrombocytopenia causing hemorrhage
- Moderate elevation of ESR
Bacteriological Diagnosis
Laboratory changes
Bacteriologic tests

- Efforts to grow Br. Abortus from blood, usually fail (30% pos.)
- When the illness is due to Br. Suis or Br. Melitensis blood culture usually succeeded (85% pos.)
Laboratory changes

Bacteriologic tests

- Taking **bone marrow** is more rewarding
- **Brucella** can be isolated from liver taken by biopsy
- **Human semen** may be positive
Laboratory changes

Bacteriologic tests

- Blood culture positivity decreases with increasing duration of illness
- Blood or bone marrow cultures should be incubated for at least 6 weeks
Laboratory changes

Bacteriologic tests

• Blood culture processed in radiometric detection systems may yield positive cultures in less than 10 days.

• The culture of brucella organisms is potentially hazardous to laboratory personal.
Serological Diagnosis
Laboratory changes

Serologic Tests

- Tube Agglutination
- 2ME Agglutination
- Coombs’ test
- Complement fixation
- Radioimmunoassay
- ELISA
- Rapid Agglutination
- Rose bengal test
Serologic Tests

IgM in acute brucellosis

- Rises first
- Is the only antibody for the first weeks
- Peaks at about 3 months
- Drop off after 3 months
DIAGNOSIS OF BRUCELLOSIS

SEROLOGIC TESTS

IgM in acute brucellosis:

- 1) Rises first
- 2) Is the only antibody for the first weeks\(^{(1)}\)
- 3) Peaks at about 3 months\(^{(4)}\)
- 4) Drop off after 3 months

DH-14

Acute
Up to 3 mo

Subacute
3 mo - 1 yr

Chronic
> 1 year

Exacerbation

IgM

IgG

IgA
Serologic Tests

IgG in brucellosis

- Begins to rise in the second week
- Remain elevated for > 1 year in treated patients falls by 6 months
- In unrecovered patients persists for a long time
DIAGNOSIS OF BRUCELLOSIS

SEROLOGIC TESTS

IgG in brucellosis:

1) Begins to rise in the second week
2) Remain elevated for > 1 year
   In treated patient falls by 6 months
3) In unrecovered patients persists for a long time

DH-15
Laboratory changes

Serologic Tests

- Serologic tests are based on rising and falling of IgM and IgG
- During re-infection or exacerbation, antibody titers become elevated
- In relapses, IgG may be the only antibody which rises
# Laboratory changes

## Serologic Tests

<table>
<thead>
<tr>
<th>Tests</th>
<th>Antibodies which can be detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>STA</td>
<td>IgM + IgG</td>
</tr>
<tr>
<td>2ME</td>
<td>IgG</td>
</tr>
<tr>
<td>COOMBS</td>
<td>1) If STA is negative and disease is chronic then only IgG</td>
</tr>
<tr>
<td></td>
<td>2) If STA is positive, IgM + IgG</td>
</tr>
<tr>
<td>C.F.</td>
<td>IgG</td>
</tr>
<tr>
<td>RIS</td>
<td>IgG &amp; IgM separately</td>
</tr>
<tr>
<td>ELISA</td>
<td>IgG &amp; IgM separately</td>
</tr>
</tbody>
</table>
Standard tube agglutination test

- Is the most frequently utilized test
- Measuring antibodies to brucella abortus antigen
- A fourfold or greater rise in titer to 1:160 or higher is significant
Standard tube agglutination test

- Titer of $=> 1:160$ is suggestive if there is symptoms consistent with brucellosis
- Acute brucellosis is most likely to be associated with a titer above 1:160
- A great majority of patients will have titers of 1:160 to 1:320
Standard tube agglutination test

- **By 3 weeks** of illness over 97% of patients demonstrate serologic evidence of infection
- This test detects antibody to Br. *Abortus*, Br. *Melitensis* and Br. *Suis* but not to Br.
- Significant STA titers can **persist** for up to 2 years in 5-7% of cases
Standard tube agglutination test

- Individuals with subclinical infection may demonstrate significant STA titers
- A diagnosis of brucellosis can not be established on the titers alone
- STA test can not differentiate persisting active infection from treated brucellosis
Standard tube agglutination test

- In chronic localized brucellosis STA titers may appear or
- It is not useful in differentiating relapsing infection from other febrile illnesses in patients with past brucella infections
Standard tube agglutination test

False positive results

- F. tularensis
- Y. enterocolitica
- V. cholera
- Salmonella
- Vaccine against F. Y. V & S.
- Brucella skin testing
- Stenotrophomonas maltophila
- E. coli O157
Standard tube agglutination test

False negative results

- Agammaglobulinemia
- First week of disease
- Disease due to Br. Canis
- Chronic brucellosis
- Prozone phenomenon
False-negative results in STA test
Brucella Canis infection

• The antigen used in the STA test does not react with brucella *Canis*
• If Br. Canis infection is suspected serologic tests specific for Br. Canis must be requested
False-negative results in STA test
Chronic brucellosis

• **STA test** may be positive in low titer or may be negative

• **Coombs** test and **C.F.** tests may be positive

• A positive coombs test and **CF** test at 1:16 in such cases is strung evidence of continuing infection
False-negative results in STA test
Prozone phenomenon

• The Prozone phenomenon appears to be related to the presence of IgG or IgA (Blocking antibodies)
• It can be eliminated if dilutions are carried out to at least 1:1280
Serologic Tests

2-Mercaptoethanol test (2ME)

- The STA test measures both IgM and IgG
- The addition of 2ME to the STA test results in the destruction of bonds of IgM
- 2ME test result is the detection of only IgG
Serologic Tests

2-Mercaptoethanol test (2ME)

- With prompt and adequate therapy IgG usually become undetectable within
- Those patients who develop persistent infection usually maintain elevated IgG agglutinins
Serologic Tests

2-Mercaptoethanol test (2ME)

- 2ME test will be negative if STA test is really negative
- 2ME test is less sensitive than STA test
- The prognosis of acute brucellosis may be predicted from the fall of 2ME
Serologic Tests  
A and M antigens

- Antigen A and M are common to the three main brucella species.
- In the Br. Abortus, there is more A antigen than M antigen.
- In the Br. Melitensis there is more M antigen than A antigen.
Serologic Tests
A and M antigens

• Specific antigen may show higher agglutinin titers in patients infected with brucella other than Br. Abortus

• Ideally in all countries prepared antigen for serological testing should consist of predominant species
Serologic Tests

A and M antigens

- In IRAN, we have human brucellosis nearly always due to Br. Melitensis but use Br. Abortus antigen.
- Br. Abortus antigen in our laboratories shows lower agglutinin titers.
Serologic Tests

A and M antigen

- In IRAN We must accept titers lower than 1:160 if there is signs and symptoms compatible with brucellosis
Serologic Tests

Coombs test

- Serum may contain brucella antibodies which do not produce agglutination
- Non-agglutinating antibodies are called incomplete antibodies
- Incomplete antibodies can be detected by addition of rabbit anti-human globulin
Serologic Tests

Coombs test

• If STA is negative and there are symptoms and signs compatible with chronic brucellosis, then combs titer of $=> 1:40$ should be considered positive

• Coombs test is not recommended when STA test is positive
Serologic Tests

Complement fixation test

- The CF test measures IgG antibodies
- Titers of => 1:16 should be considered positive
Serologic Tests

Radioimmunoassay

- RIA test determines anti-brucella IgM and IgG
- Avoids the difficulties with blocking or non-agglutinating antibodies
- Can differentiate between chronic and acute brucellosis
Serologic Tests

**ELISA test**

- ELISA test can distinguish acute cases from chronic cases
- In ELISA test cross reaction can occur with yersiniosis
Serologic Tests

Rose Bengal plate test

- Is an agglutination test in which the brucella cells are bound to a dye
- Is quick and easy to read
- It is a useful screening test
Brucella skin test (Brucellin)

**Demonstrate** delayed hypersensitivity

The antigen is a filtrate of a culture of brucella organisms or purified extract

**Injects** intradermally
Brucella skin test (Brucellin)

- The test is positive if local redness with induration is present after 24-48 hours
- Antigen can provoke an antibody response or a significant rise in a pre-existing response

*Brucellin test, should not be performed*
Interpretation of STA and 2ME tests

<table>
<thead>
<tr>
<th>STA =&gt; 1:160</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
</tr>
<tr>
<td>IgM + IgG</td>
</tr>
<tr>
<td>IgG</td>
</tr>
</tbody>
</table>

Addition of 2ME

<table>
<thead>
<tr>
<th>Negative</th>
<th>1:80</th>
<th>1:160</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Cured infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Contact with ag.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) First week of bru.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Acute</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Subacute</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) Exacerbation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late chronic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Serologic Testing for Syphilis
Treponema pallidum – The Agent of Syphilis

- Spirochete
- Obligate human parasite
- Transmission
  - Sexual
  - Trans-placental
  - Percutaneous following contact with infectious lesions
  - Blood Transfusion
    - No reported cases of transmission since 1964
Syphilis – The “Great Imitator”

- Infectious Dose: \(~57\) organisms\(^1\)
- Incubation Period – 21 days (median)
- 3 clinical stages of syphilis
  - **Primary:**
    - Painless sore (chancre) at inoculation site
  - **Secondary:**
    - Rash, Fever, Lymphadenopathy, Malaise
  - **Tertiary/Latent:**
    - CNS invasion, organ damage
- “The physician that knows syphilis knows medicine.”
  - Sir William Osler
Laboratory Diagnosis of Syphilis

The Uncommon Methods

- **Rabbit Infectivity Test (RIT)**
  - High Sensitivity and Specificity
  - Long turn-around-time
  - Limited to research settings
- **Dark Field Microscopy**
  - Useful only during primary infection
  - Technician expertise required
- **Immunostaining**
  - Direct fluorescent antibody or silver stain
- **Polymerase Chain Reaction (PCR)**
  - Not commercial available
Laboratory Diagnosis of Syphilis

The Common Methods

• Serology
  – Mainstay for syphilis testing
  – Two classes of serologic tests
    • Non-treponemal
    • Treponemal
Serologic Tests for Syphilis: Non-Treponemal Assays

- Principle:
  - *T. pallidum* infection leads to the production of reagin
    - **Reagin** – Antibodies to substances released from cells damaged by *T. pallidum*
  - Reagin reacts with cardiolipin
    - **Cardiolipin** – a phospholipid component of certain eukaryotic and prokaryotic membranes

- Examples of non-treponemal tests:
  - Rapid Plasma Reagin (RPR)
  - Venereal Disease Research Laboratory (VDRL)
Serologic Tests for Syphilis: Non-Treponemal Assays

- RPR and VDRL are agglutination assays
Serologic Tests for Syphilis: Non-Treponemal Assays

- RPR and VDRL are agglutination assays
Non-Treponemal Tests:

Advantages

• Rapid turnaround time – Minutes
• Inexpensive
• No specialized instrumentation required
• Usually revert to negative following therapy
  – Can be used to monitor response to therapy
Non-Treponemal Tests: Limitations

- Results are subjective
  - Intra- and Inter-laboratory variability
- Non-specific
  - False positive results can result from other infectious or non-infectious conditions
    - EBV, Lupus, etc.
- Limited sensitivity in early/primary syphilis and in late/latent syphilis
- Low throughput
  - Problematic for high volume laboratories
Non-Treponemal Tests: Limitations, continued

• Possibility for prozone effect
  – High levels of antibody may inhibit the agglutination reaction
  – To identify prozone, labs must serially dilute samples

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undilute</td>
<td><img src="image1" alt="Undilute" /></td>
</tr>
<tr>
<td>1:2</td>
<td><img src="image2" alt="1:2" /></td>
</tr>
<tr>
<td>1:4</td>
<td><img src="image3" alt="1:4" /></td>
</tr>
<tr>
<td>1:8</td>
<td><img src="image4" alt="1:8" /></td>
</tr>
<tr>
<td>1:16</td>
<td><img src="image5" alt="1:16" /></td>
</tr>
</tbody>
</table>
Serologic Tests for Syphilis: 
Treponemal Assays

• Principle:
  – Infection leads to production of specific antibodies directed against *T. pallidum*

• Treponemal tests detect IgG or total IgM/IgG antibodies directed against *T. pallidum*
Serologic Tests for Syphilis:
Treponemal Assays
- Microhemagglutination assay (MHA)
- Fluorescent treponemal antibody (FTA–ABS)
- *Treponema pallidum* particle agglutination (TP–PA)
- Enzyme Immunoassay (EIA)
- Multiplex Flow Immunoassay (MFI)
Treponemal Assays: Multiplex Flow Immunoassays

Patient Serum Added

Syphilis IgM
Syphilis IgG
Treponemal Assays:
Multiplex Flow Immunoassays

[Diagram showing the process of adding labeled anti-IgM and anti-IgG reporter antibodies, followed by patient serum addition, and finally reading the results from a plate.]
Treponemal Assays: Advantages

- High Specificity
- Possibly higher sensitivity during early and late syphilis stages compared to non-treponemal tests
- Newer Methods
  - Objective result interpretation
  - Automation option
  - High throughput
  - High reproducibility/precision
Treponemal Assays: Limitations

- Remain positive despite treatment
  - Cannot be used to monitor response to therapy
- Conventional Methods
  - Subjective interpretation requiring technician expertise to read
- Newer Methods
  - Expensive instrumentation
  - Higher cost/test
### Sensitivity & Specificity of Serologic Tests for Syphilis

<table>
<thead>
<tr>
<th>Test</th>
<th>Primary (%)</th>
<th>Secondary (%)</th>
<th>Latent (%)</th>
<th>Late (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nontreponemal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDRL</td>
<td>78 (74–87)</td>
<td>100</td>
<td>95 (88–100)</td>
<td>71 (37–94)</td>
<td>98 (96–99)</td>
</tr>
<tr>
<td>RPR</td>
<td>86 (77–100)</td>
<td>100</td>
<td>98 (95–100)</td>
<td>73</td>
<td>98 (93–99)</td>
</tr>
<tr>
<td><strong>Treponemal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTA-ABS</td>
<td>84 (70–100)</td>
<td>100</td>
<td>100</td>
<td>96</td>
<td>97 (94–100)</td>
</tr>
<tr>
<td>MHA-TP</td>
<td>76 (69–90)</td>
<td>100</td>
<td>97 (97–100)</td>
<td>94</td>
<td>99 (98–100)</td>
</tr>
</tbody>
</table>
### Conditions Associated with False Positive Serological Tests for Syphilis

<table>
<thead>
<tr>
<th>Nontreponemal Tests</th>
<th>Treponemal Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral infection</td>
<td>Pyoderma</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Skin neoplasm</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>Acne vulgaris</td>
</tr>
<tr>
<td>Acute or chronic illness</td>
<td>Mycoses</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>Crural ulceration</td>
</tr>
<tr>
<td>Recent immunization</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Drug addiction</td>
<td>Psoriasis</td>
</tr>
<tr>
<td>Leprosy</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Malaria</td>
<td>Pregnancy</td>
</tr>
<tr>
<td></td>
<td>Drug addiction</td>
</tr>
<tr>
<td></td>
<td>Herpes genitalis</td>
</tr>
</tbody>
</table>
Typhoid fever
Causes

1. Caused by the bacterium *Salmonella Typhi*.
2. Ingestion of contaminated food or water.
3. Contact with an acute case of typhoid fever.
4. Contact with a chronic asymptomatic carrier.
Salmonella typhi

- Rod shaped, **flagellated**, aerobic, Gram -ve bacilli.
- Refrigeration and freezing could slow their growth.
- **Pasteurizing and food irradiation** kill *Salmonella* for commercially-produced foodstuffs
- Foods prepared in the home from raw eggs can spread salmonella if not properly cooked before consumption.
How does the bacteria cause disease?

- Ingestion of contaminated food or water
- *Salmonella typhi*
- Carried by white blood cells into the liver, spleen, and bone marrow
- Multiply and reenter the bloodstream (Clinical illness)
- Bacteria invade the gallbladder, biliary system, and the lymphatic tissue of the bowel and multiply in high numbers
- Then pass into the intestinal tract (can be identified for diagnosis in cultures from the stool)
- Typhoid ulcers can cause perforation and hemorrhage
Symptoms

• **No symptoms** - if only a mild exposure; some people become "carriers" of typhoid.
• Poor appetite, Headaches and generalized pains,
• Fever, Lethargy
• Rose spots on chest wall
• Diarrhea / constipation and abdominal pain
• Chest congestion develops in many patients,
• slow heartbeat.
• Enlarged spleen and liver
Symptoms

- Aches and pains
- High fever
- Diarrhea
- Chest congestion
- Typhoid Meningitis
Diagnosis of typhoid fever is made by

- Clinical examination
- Blood, bone marrow, or stool cultures for *S. typhi*
- Serological Tests
Serodiagnosis of Typhoid:

1. Detection of Antibodies in serum:
   1. Widal test (Tube or Slide),
   2. Typhidot assay
   3. Tubex system,
   4. Dipstick assay.

2. Detection of Antigens in serum:
   1. Tubex system
   2. Countercurrent Immunoelectrophoresis (CIE).
   4. ELISA

3. Detection of Antigens in urine:
   1. Tubex system
   2. CIE,
   3. Latex agglutination
   4. Co-agglutination
Widal test
Antigenic structure of Salmonella

H (flagella) antigens
O (somatic) antigens
Vi (Virulence) capsular polysaccharide antigens
<table>
<thead>
<tr>
<th><strong>O (somatic) antigens</strong></th>
<th><strong>H (flagella) antigens</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• LPS in the cell wall;</td>
<td>▶ Present in flagella;</td>
</tr>
<tr>
<td>• Heat stable</td>
<td>▶ Heat labile;</td>
</tr>
<tr>
<td>• Less immunogenic</td>
<td>▶ Strongly immunogenic;</td>
</tr>
<tr>
<td></td>
<td>Induce rapid &amp; High Ab titres;</td>
</tr>
<tr>
<td></td>
<td>▶ Agglutination with antisera:</td>
</tr>
<tr>
<td></td>
<td>- Fine, compact, granular chalky clumps</td>
</tr>
<tr>
<td></td>
<td>▶ Agglutination with antisera:</td>
</tr>
<tr>
<td></td>
<td>- Large, loose, cotton wool clumps</td>
</tr>
</tbody>
</table>
Vi (virulence) antigen

- Capsular polysaccharide expressed on certain serotypes
- Heat labile;
- Poorly immunogenic, BUT antibodies are protective:
  1. Detection of Vi antibody not helpful in diagnosis
  2. Absence in a case of typhoid → poor prognosis;
  3. Persistence of Vi antibody: carrier state
WIDAL Test

- Tube agglutination test.
- Detects anti O and H antibodies in serum
- Diagnosis of Typhoid and Paratyphoid cases
- Carriers of typhoid bacilli possess antibody against the Vi antigen of S. typhi. (Vi tires seem to correlate better with the carrier state than do O or H titres).
- For this reason, the use of Vi agglutination for detection of carriers was suggested.
Widal test

• Significance
  ➢ 1st week negative.
  ➢ Titers raise in 2nd week
  ➢ Raise of titers is diagnostic
Materials

• **Antigens:**
  – Suspension of *S. typhi* "O" antigen, O
  – Suspension of *S. typhi* "H" antigen; H
  – Suspension of *S. paratyphi* A "H" antigen, PA
  – Suspension of *S. schottmuelleri* "H" antigen, PB

• **Antibody:** serum of suspected patient

• Normal saline

• Test tubes and pipettes
PROCEDURE
PROCEDURE

• Make the mark of tubes
• Dilute patient’s serum 1:10 (0.1 ml serum + 0.9 ml saline).
• Add reagents as the following:

<table>
<thead>
<tr>
<th>Reagent (ml)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>1:10 Patient serum</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>discard</td>
</tr>
<tr>
<td>Serum dilution</td>
<td>1:20</td>
<td>1:40</td>
<td>1:80</td>
<td>1:160</td>
<td>1:320</td>
<td>1:640</td>
<td>-</td>
</tr>
<tr>
<td>Bacteria suspension</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Final serum dil.</td>
<td>1:40</td>
<td>1:80</td>
<td>1:160</td>
<td>1:320</td>
<td>1:640</td>
<td>1:1280</td>
<td>-ve</td>
</tr>
</tbody>
</table>

Bacteria suspension: O H PA PB

Shake several times, put it in 37°C water bath for 16-18 hours. Then let it stand at room temperature over night.
Observation:

**Do not shake tubes before reading the results**

1. Control tube (Tube No. 7): no agglutination (-)
2. Lowest titer tube: absolutely agglutination (++++)
3. Other tubes:
   - $\frac{3}{4}$ agglutination +++
   - $\frac{1}{2}$ agglutination ++
   - $\frac{1}{4}$ agglutination (+)
   - no agglutination (-)

Interpretation:

**Agglutination titer:** the highest dilution of serum which appears (++) bacteria agglutination.
Agglutination how it appears after reactivity

- **Felix tube**
  - Round bottom
  - O agglutination

- **Dreyer’s tube**
  - Conical bottom
  - H agglutination

- **Compact granular agglutination**
- **Loose Cotton woolly clumps**

Observed for agglutination:

- H : Loose, cotton woolly clumps;
- O : Compact Fine granular agglutination;
- Supernatant should be clear;
How do you read Widal test results for typhoid fever?

• The highest dilution of the patients serum in which agglutinations occurs is noted, ex. if the dilution is 1 in 160 then the titer is 160.

• Agglutination in dilution up to <1:60 is seen in normal individuals.

• Agglutination in dilution 1:160 is suggestive of Salmonella infection.

• Agglutination in dilution of >1:320 is confirmatory of Enteric fever.
## Interpretation of results

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>$O &lt; 1:80$, $H &lt; 1:160$, $PH &lt; 1:80$</td>
<td>Normal value</td>
</tr>
<tr>
<td>$O \geq 1:80$ &amp; $H \geq 1:160$ or $O \geq 1:80$ &amp; $PH \geq 1:80$</td>
<td>Typhoid fever</td>
</tr>
<tr>
<td>$O \geq 1:80$ &amp; $PH \geq 1:80$</td>
<td>Paratyphoid fever</td>
</tr>
<tr>
<td>$O \geq 1:80$ &amp; $H &lt; 1:160$ or $O \geq 1:80$ &amp; $PH &lt; 1:80$</td>
<td>Early infection or other salmonella infections</td>
</tr>
<tr>
<td>$O &lt; 1:80$ &amp; $H \geq 1:160$ or $O &lt; 1:80$ &amp; $PH \geq 1:80$</td>
<td>Vaccination or nonspecific memory reaction</td>
</tr>
</tbody>
</table>
### Conclusion

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>O</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>PA</strong></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>PB</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1:40 1:80 1:160 1:320 1:640 1:1280

**N.B.**
- Single test not diagnostic (Except at high titers > 320).
- Paired samples tests with rising titer is diagnostic
- Diagnostic.
  - O > 1:80
  - H > 1:160
- H agglutinins appear first
Interpretation of Widal test

• Test results need to be interpreted carefully in the light of:
  1. Past history of enteric fever,
  2. Typhoid vaccination,
  3. General level of antibodies in the healthy populations in endemic areas of the world.
False Positive Reactions with WIDAL Test

1. patients who have had previous vaccination or infection with _S typhi_.
2. Cross-reaction with non–typhoidal _Salmonella_.
3. in association with some autoimmune diseases.
4. Infection with _malaria_
False Negative Reactions with WIDAL Test

1. Early treatment,
2. Relapses of typhoid fever.
3. Occasionally the infecting strains are poorly immunogenic.
C-Reactive Protein

- Non-specific protein that appears in serum as a response to inflammatory conditions
- Involved in opsonization and complement fixation
- Latex agglutination available
  - Coated with Anti-CRP
- Conditions elevated
  - Bacterial infection
  - Viral infection
  - Active rheumatic fever
  - Active rheumatoid arthritis
  - TB infections
  - Malignancies
  - Following surgeries
C-Reactive Protein

• ESR also used to gauge inflammation.
• C-RP has the following advantages over the ESR:
  – Rises quickly DURING inflammation.
  – Decreases quickly once inflammation resolved.
  – Not affected by anemia or abnormal serum proteins.
CRP-Latex

• **PRINCIPLE OF THE METHOD**
• The CRP-latex is a slide agglutination test for the qualitative and semiquantitative detection of C-Reactive Protein (CRP) in human serum.

• Latex particles coated with goat IgG anti-human CRP are agglutinated when mixed with samples containing CRP.
CLINICAL SIGNIFICANCE

• CRP is an acute-phase protein present in normal serum, which increases significantly after most forms of tissue injuries, bacterial and virus infections, inflammation and malignant neoplasia. During tissue necrosis and inflammation resulting from microbial infections, the CRP concentration can rise up to 300 mg/L in 12-24 hours.
• **Latex**
  Latex particles coated with goat IgG anti-human CRP, pH, 8.2. Sodium azide 0.95 g/L.

• **Control +**
  Human serum with a CRP concentration > 20 mg/L. Red cap Sodium azide 0.95 g/L.

• **Control -**
  Blue cap Animal serum. Sodium azide 0.95 g/L.
Reagents deterioration:

• Do not freeze: frozen reagents could change the functionality of the test.

• Presence of particles and turbidity.
SAMPLES

• Fresh serum. Stable 7 days at 2-8ºC or 3 months at –20ºC.

• Samples with presence of fibrin should be centrifuged before testing.

• Do not use highly hemolysed or lipemic samples.
1. High CRP concentration samples may give negative results (prozone effect). Re-test the sample again using a drop of 20 μL.

2. The strength of agglutination is not indicative of the CRP concentration in the samples tested.

3. Clinical diagnosis should not be made on findings of a single test result, but should integrate both clinical and laboratory data.
ADDITIONAL MATERIAL REQUIRED

• Stop-watch
• Isotonic saline
• Glass slide with clear/white background
  Only a clean and dry glass slides / tubes must be used.
• appropriate Pipettes/Micropipettes
• Mixing sticks
• a High intensity direct light source
Thanks for your attention