Methods of identifying unknown microbes fall into three categories:

1. Phenotypic – observable microscopic and macroscopic characteristics
2. Genotypic – genetic make up
3. Immunological – serology; antibody-antigen reactions
Phenotypic Methods

- Microscopic morphology – fresh or stained microorganisms from specimen; shape, size, stain reaction, cell structures
- Macroscopic morphology – colony appearance; texture, size, shape, pigment, growth requirements
- Physiological/biochemical characteristics – detection of presence or absence of particular enzymes or metabolic pathways
- Chemical analysis – analyze specific chemical composition; cell wall peptides, cell membrane lipids
Genotypic Methods

- Assess genetic make-up
- Culture is not necessary
- Precise, automated methods, quick results
Immunological Methods

• Specific antibodies are used to detect antigens
  – Easier than testing for the microbe itself
17.2 Specimen Collection and Laboratory Methods

- Sampling body sites or fluids for suspected infectious agent
- Results depend on specimen collection, handling, transport, and storage
- Aseptic procedures should be used
Figure 17.1 Sampling sites
TABLE 17.1 General Guidelines for Specimen Collection*

<table>
<thead>
<tr>
<th>Type of Specimen</th>
<th>Collection Method</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcess of skin or membrane or decubitus ulcer</td>
<td>Debride surface with sterile wipe; suction fluid with sterile needle and syringe.</td>
<td>Aspiration of tissue samples preferred to swab; transfer to anaerobic transport system.</td>
</tr>
<tr>
<td>Anaerobic cultures</td>
<td>Collect from deeper tissue below the surface, using sterile syringe.</td>
<td>Sample must not be exposed to air; transported immediately in anaerobic system to lab</td>
</tr>
<tr>
<td>Blood</td>
<td>Prep area of skin with iodine; use vacutainer blood collection tubes.</td>
<td>Inoculate blood culture bottle immediately.</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Prepare site for surgical incision; extract sample with special needle.</td>
<td>Most samples taken from sternum or ilium; placed in blood bottle for culture</td>
</tr>
<tr>
<td>Cerebrospinal fluid (CSF)</td>
<td>Remove CSF aseptically by lumbar puncture.</td>
<td>Place CSF into sterile tubes; do not refrigerate; transport immediately to lab for processing.</td>
</tr>
<tr>
<td>Feces</td>
<td>Take a small specimen into sterile container, cover, and transport.</td>
<td>Refrigerate samples held over one hour; culture is mainly to rule out enteric pathogens; special kits available to detect cysts and trophozoites of protozoans and ova and larvae of intestinal worms</td>
</tr>
<tr>
<td>Genital/urinary tract</td>
<td>Sterile swab of cervical mucus; swab urethral membrane or insert into lumen and twist.</td>
<td>Plate directly onto selective culture media with high CO₂ atmosphere; or collect with anaerobic transport swab system; process immediately.</td>
</tr>
<tr>
<td>Respiratory tract, lower</td>
<td>Have patient cough to loosen phlegm and expectorate sputum into sterile cup for transport.</td>
<td>Patient should brush teeth and rinse mouth with water prior to any sampling. If coughing does not work, induce sputum by having patient inhale sterile saline from a nebulizer.</td>
</tr>
<tr>
<td>Respiratory tract, upper</td>
<td>Cleanse and debride oral cavity membranes; vigorously swab area of lesion; throat cultures are taken by swabbing the posterior pharynx, tonsils, and inflamed sites.</td>
<td>Tissue aspirates of infected tissue preferred; throat samples are processed for strep throat; culturing for other throat pathogens (except viruses) is done when specifically requested; care must be taken to avoid normal tissue and saliva.</td>
</tr>
<tr>
<td>Urine</td>
<td>Sampling is usually done with sterile catheter or by clean midstream catch into a sterile container.</td>
<td>Patient must wash hands and clean external genitalia, separate labia (female) or pull back foreskin (male) and urinate for a short time, followed by collection of 100–200 cc sample (midstream); samples not immediately processed must be refrigerated.</td>
</tr>
</tbody>
</table>

*C for a detailed account of collecting and managing specimens, log on to http://www.medicine.uiowa.edu/path_handbook/Appendix/Micro/mi, and http://www.dhs.state.tx.us/LAB/bac_guidelines.shtml#guidelines
Overview of Laboratory Techniques

• Routes taken in specimen analysis
  – Direct tests (microscopic, immunologic, or genetic)
  – Cultivation, isolation, and identification (general and specific tests)

• Two categories of results
  – Presumptive
  – Confirmatory
Figure 17.2  Scheme of specimen isolation and identification

- **Specimen**
  - **Direct Testing**
    - *Microscopic*
      - Gram stain
      - Acid-fast stain
      - Fluorescent Ab stain
      - Gene probes
    - *Macroscopic*
      - Direct antigen
      - Gene probes
  - **Culture/Isolation**
    - *Tests on isolates*
      - Biochemical
      - Serotyping (slide)
      - Antimicrobial sensitivity
      - Gene probes
      - Phage typing
      - Animal inoculation

- **Patient**
  - Immunologic and serological tests (antibody titer) are performed on blood and other fluids
  - Clinical signs and symptoms
  - *In vivo* tests for reaction to microbe
17.3 Phenotypic Methods

- Immediate direct examination
  - Microscopic – differential and special stains – Gram, DFA, direct antigen testing
Phenotypic Methods

• Cultivation of Specimen
  – Colony appearance, growth requirements, appropriate media

• Biochemical testing
  – Physiological reactions to nutrients as evidence of the absence or presence of enzymes
Figure 17.4 Rapid tests
Figure 17.5

Cocci

Gram (+)

Catalase (+), irregular clusters, tetrads

- Strictly aerobic: Micrococcus
- Facultative anaerobic: Staphylococcus, Planococcus

Catalase (-), pairs, chain arrangement

Streptococcus

Gram (-)

Aerobic, oxidase (+), catalase (+)

Neisseria, Branhamella, Moraxella

Anaerobic, oxidase (-), catalase (-)

Veillonella

Rods

Gram (+)

Sporeformer

- Acid-fast: Bacillus, Clostridium
- Not acid-fast: Mycobacterium, Nocardia, Lactobacillus, Listeria

Non-sporeformer

Gram (-)

Aerobic oxidase (+)

Motile: Escherichia, Enterobacter, Citrobacter, Proteus, Salmonella
Nonmotile: Shigella, Klebsiella

Facultative anaerobic, oxidase (-) (ferment glucose)
Phenotypic Methods

• Miscellaneous tests
  – Phage typing
  – Animal inoculation
  – Antimicrobial sensitivity
• Important to consider whether microbe recovered and identified is actually causing the disease or simply normal flora
17.4 Genotypic Methods

• DNA analysis
  – Hybridization
    • Probes complementary to the specific sequences of a particular microbe
  – PCR
    • DNA and RNA analysis
    • Ribosomal RNA
      – Comparison of the sequence of nitrogen bases in ribosomal RNA
17.5 Immunological Methods

- **Serology** – *in vitro* diagnostic testing of serum
  - Antibodies have extreme specificity for antigens
- Visible reactions include precipitates, color changes, or the release of radioactivity
- Tests can be used to identify and to determine the amount of antibody in serum – *titer*
Figure 17.7 Basic principles of serological testing using antibodies and antigens

(a) In serological diagnosis of disease, a blood sample is scanned for the presence of antibody using an antigen of known specificity. A positive reaction is usually evident as some visible sign, such as color change or clumping, that indicates a specific interaction between antibody and antigen. (The reaction at the molecular level is rarely observed.)

(b) An unknown microbe is mixed with serum containing antibodies of known specificity, a procedure known as serotyping. Microscopically or macroscopically observable reactions indicate a correct match between antibody and antigen and permit identification of the microbe.
Figure 17.8 Specificity and sensitivity in immune testing
Agglutination and Precipitation Reactions

- **Agglutination testing** – antibody crosslinks whole-cell antigens, forming complexes that settle out and form visible clumps
  - Blood typing, some bacterial and viral diseases

- **Precipitation tests** – soluble antigen is made insoluble by an antibody
  - VDRL
  - Most precipitation reactions are carried out in agar gels
Figure 17.9 Agglutination and precipitation reactions

*Although IgG is shown as the Ab, IgM is also involved in these reactions.
In one method of setting up a double-diffusion test, wells are punctured in soft agar, and antibodies (Ab) and antigens (Ag) are added in a pattern. As the contents of the wells diffuse toward each other, a number of reactions can result, depending on whether antibodies meet and precipitate antigens.

Example of test pattern and results. Antigen (Ag) is placed in the center well and antibody (Ab) samples are placed in outer wells. The control contains known Abs to the test Ag. Note bands that form where Ab/Ag meet. The other wells (1, 2) contain unknown test sera. One is positive and the other is negative. Double bands indicate more than one antigen and antibody that can react.

Actual test results for detecting infection with the fungal pathogen Histoplasma. Numbers 1 and 4 are controls and 2, 3, 5, and 6 are patient test sera. Can you determine which patients have the infection and which do not?
Figure 17.11 Immunoelectrophoresis of normal human serum

Step 1  Serum sample separated next to trough

(+)

Direction of electrophoresis

(−)

Serum sample

Step 2  Antiserum added to trough

Albumin  Alpha globulin (α globulin)  IgM  Gamma globulin (γ globulin) (IgG)
The Western Blot for Detecting Proteins

- Electrophoretic separation of proteins, followed by an immunoassay to detect these proteins
- Highly specific and sensitive
- Sites of specific antibody binding will appear as a pattern of bands
- Second test used to verify HIV status
Figure 17.12 The Western blot procedure

Successive tests on an HIV+ patient over 30 days reveals an increase in band intensity over time. This is due to continued formation of anti-HIV antibodies.
Complement Fixation

- Lysin mediated hemolysis
- Test uses four components
  - Antigen, antibody, complement, and sensitized sheep RBCs
- Steps of test
  1. Test antigen reacts with test antibody
  2. Contents of tube from (1.) are mixed with sheep RBCs
     - Complement used up in first stage, no hemolysis
     - Unfixed complement, hemolysis
Figure 17.13 Complement fixation test

<table>
<thead>
<tr>
<th>Stage 1</th>
<th>Reaction System</th>
<th>Stage 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive patient’s serum</td>
<td>Sheep red blood cells with lysins on surface</td>
<td>Complement fixes antibodies; RBCs do not lyse</td>
</tr>
<tr>
<td>Ab + Ag</td>
<td>Complement</td>
<td>Lysins (unrelated to Ab in stage 1)</td>
</tr>
<tr>
<td>Ab/Ag complex</td>
<td>Complement fixed to Ab</td>
<td></td>
</tr>
</tbody>
</table>

| Negative patient’s serum | | |
| No Ab + Ag | Complement | |
| No Ab/Ag complex | | |
| No fixation | | |

Complement fixes RBCs; hemolysis occurs

(+) Antibody

Hemolysis

(−) No antibody present
Fluorescent Antibody and Immunofluorescent Testing

• Fluorescent antibody
  – Monoclonal antibody labeled by a fluorescent dye

• Two ways FABs are used
  – Direct testing
  – Indirect testing
Figure 17.14 (a, b)

(a) Direct Testing

- Unknown antigen (usually cell or tissue)
- Antibody labeled with fluorescent dye

(b) Indirect Testing

- Known Ag
- No Ab in serum

Visible fluorescence

Fluorescent microscopy

Ab2 fluorescently labeled; specific for Ab1

Ab2 attaches to Ab1—visible fluorescence

Positive

Ab2 attaches to Ab1—visible fluorescence

Negative

Ab2 cannot attach—no fluorescence

(c) Indirect Testing
Figure 17.14 (c)

(c) Indirect Immunofluorescence of specimen

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Immunoassays

- Extremely sensitive to detect trace antigens and antibodies
- **Radioimmunoassay** (RIA) – antigens or antibodies labeled with radioactive isotopes
- **Enzyme-linked immunosorbent assay** (ELISA) – enzyme-antibody complex produces a colored product when an enzyme-substrate reaction occurs
  - Indirect
  - Capture
Figure 17.15 Methods of ELISA testing

(a) Indirect ELISA, comparing a positive vs. negative reaction. This is the basis for HIV screening tests.

(b) Microtiter ELISA Plate with 96 Tests for HIV Antibodies. Colored wells indicate a positive reaction.

(c) Capture or Antibody Sandwich ELISA Method.
Note that an antigen is trapped between two antibodies. This test is used to detect the measles virus.

- Known antigen is adsorbed to well.
- Serum samples with unknown antibodies.
- Well is rinsed to remove unbound (nonreactive) antibodies.
- Indicator antibody linked to enzyme attaches to any bound antibody.
- Wells are rinsed to remove unbound indicator antibody. A colorless substrate for enzyme is added.
- Enzymes linked to indicator Ab hydrolyze the substrate, which releases a dye. Wells that develop color are positive for the antibody; colorless wells are negative.
- Antibody specific to test antigen is adsorbed to well.
- Test antigen is added; if complementary, antigen binds to antibody.
- Enzyme
- Enzyme-linked antibody specific for test antigen then binds to different antigenic site forming a sandwich.
- Enzyme's substrate (D) is added, and reaction produces a visible color change (•).
Tests that Differentiate T Cells and B Cells

• Rosette formation
  – Mix T cells with sheep red blood cells

• Fluorescent techniques
  – Differentiates T and B cells and subgroups
In vivo Testing

- Antigens are introduced directly into the body to determine the presence or absence of antibodies
  - Tuberculin skin test, allergy testing
A Viral Example

- Viruses present special difficulties because they are not cells
- Viruses are labor intensive to culture in the laboratory
Figure 17.17 Diagnosing viral infections

1. Cells infected with herpes simplex virus
2. Cells infected with influenza virus
3. Cells taken from patient are examined for evidence of viral infection, such as cytopathic effects (c) or virus antigen detected by fluorescent staining (d).
4. Culture techniques: Viruses require a living host to multiply.
5. Electron microscope is used to view virus directly. Viruses are sufficiently unique in structure that they can be differentiated to family or genus.
7. Western blot for HIV