Bacterial Culturing

Culturing is routine for most bacterial and fungal infections but is rarely used to identify helminths or protozoa. Culturing of many pathogens is straight forward, for example, streaking a throat swab onto a blood agar plate in search of ***group A β-hemolytic Streptococcus***.

Certain pathogens are very slow growing (for example, ***M. tuberculosis***). Microorganisms isolated in culture are identified using such characteristics as ***colony size***, ***shape, color, and Gram stain***, ***hemolytic reactions*** on solid media, ***odor***, and ***metabolic properties***, also pure cultures provide samples for ***antimicrobial susceptibility testing***.

The success of culturing depends on ***appropriate collection*** and ***transport techniques*** and on ***selection of appropriate culture media***, because some organisms may require special nutrients. A. ***Specimen collection***

Many organisms are fragile and must be transported to the laboratory with minimal delay. For example, ***Gonococci*** and ***Pneumococci*** are very sensitive to heating and drying. Samples must be cultured promptly, or, if this is not possible, transport media must be used to extend the viability of the organism to be cultured. When anaerobic organisms are suspected, the patient's specimen must be protected from the toxic effect of oxygen.

All clinically important bacteria are ***heterotrophs*** (that is, they require organic carbon for growth). Heterotrophs may have complex or simple requirements for organic molecules ((Organisms that can reduce carbon dioxide and, therefore, do not require organic compounds for cell growth, are called ***autotrophs***)).

Most bacteria require varying numbers of growth factors, which are organic compounds required by the cell to grow, but which the organism cannot itself synthesize (for example, vitamins). Organisms that require either a large number of growth factors or must be supplied with very specific ones are referred to as ***fastidious***.

Bacteria can be categorized according to their growth responses in the presence and absence of oxygen. ***Strict aerobes*** cannot survive in the absence of oxygen and produce energy only by ***oxidative phosphorylation***.

Strict anaerobes generate energy by fermentation or by anaerobic respiration and are killed in the presence of oxygen.

Facultative anaerobes can grow in the absence of oxygen but grow better in its presence. ***Aerotolerant anaerobes*** have mechanisms to protect themselves from oxygen (therefore, being able to grow in its presence or absence) but do not use oxygen in their metabolism.

Microaerophiles require oxygen for their metabolism but cannot survive at atmospheric levels of oxygen, microaerophiles are found in lakes and wet soil where the oxygen concentration is within an acceptable range.

Two general strategies are used to isolate pathogenic bacteria, depending on the nature of the clinical sample:

1- First method uses ***enriched media*** to promote the nonselective growth of any bacteria that may be present

2-Second approach employs ***selective media*** that only allow growth of specific bacterial species from specimens that normally contain large numbers of bacteria (for example, stool, genital tract secretions, and

sputum). Isolation of a bacterium is usually performed on solid medium. ***Liquid medium is used to grow larger quantities of a culture of bacteria that have already been isolated as a pure culture.***

- ***Enriched media:*** Media fortified with blood, yeast extracts, or brain or heart infusions are useful in growing fastidious organisms. ***Blood agar*** contains protein sources, sodium chloride, and 5 % blood and supports the growth of most gram-positive and gram-negative bacteria isolated from human sources. ***Haemophilus influenzae*** and ***Neisseria gonorrhoeae*** are highly fastidious organisms. They require ***chocolate agar***, which contains red blood cells (RBCs) that have been lysed.

This releases intracellular nutrients, such as ***hemoglobin, hemin ("X" factor),*** and ***nicotinamide adenine dinucleotide ("V" factor)***, required by these organisms.

Enriched media are useful for culturing normally sterile body fluids, such as blood and CSF, in which the finding of any organisms provides reasonable evidence for infection by that organism. Failure to culture an organism may indicate that:

1- The culture medium is inadequate

2- The incubation conditions do not support bacterial growth

- ***Selective media:*** The most commonly used selective medium is ***MacConkey agar***, which supports the growth of most gram-negative rods, especially the Enterobacteriaceae, but inhibits growth of gram-positive organisms and some fastidious gram-negative bacteria, like *Haemophilus* and *Neisseria* species.

Growth on blood agar and chocolate agar but not MacConkey agar suggests a gram-positive isolate or a fastidious gram-negative species. On the other hand, most gram-negative rods often form distinctive colonies on MacConkey agar. This agar is also used to detect organisms able to metabolize lactose.

Clinical samples are routinely plated on blood agar, chocolate agar, and MacConkey agar. ***Hektoen enteric agar*** is also a selective medium that differentiates lactose / sucrose fermenters and non-fermenters as well as ***H2S producers*** and non-producers. It is often used to culture ***Salmonella*** and ***Shigella*** species.

Thayer-Martin agar is another selective medium composed of chocolate agar supplemented with several antibiotics that suppress the growth of nonpathogenic *Neisseria* and other normal and abnormal flora. This medium is normally used to isolate *Gonococci.*

When submitting samples for culture, the physician must alert the laboratory to likely pathogens whenever possible, especially when unusual organisms are suspected. This allows inclusion of selective media that might not be used routinely.

Bacterial Identification

The most widely used identification scheme involves determining the morphologic and metabolic properties of the unknown bacterium and comparing these with properties of known microorganisms. Alternate identification schemes using nucleic acid–based methods are used also. It is essential to start identification tests with pure bacterial isolates grown from a single colony.

Single enzyme test for bacterial identification

Different bacteria produce varying spectra of enzymes; e.g., some enzymes are necessary for the bacterium's individual metabolism, and some facilitate the bacterium's ability to compete with other bacteria or establish an infection.

Tests that measure single bacterial enzymes are simple, rapid, and generally easy to interpret. They can be performed on organisms already grown in culture and often provide presumptive identification.

1) ***Catalase test:*** The enzyme catalase catalyzes the degradation of hydrogen peroxide to water and molecular oxygen ($H_2O_2 \rightarrow H_2O + O_2$). Catalase positive organisms rapidly produce bubbles when exposed to a solution containing hydrogen peroxide. The catalase test is key in differentiating between many gram-positive organisms; e.g., ***Staphylococci*** are catalase positive, whereas ***Streptococci*** and ***Enterococci*** are catalase negative. The production of catalase is an important virulence factor because H_2O_2 is antimicrobial, and its degradation decreases the ability of neutrophils to kill invading bacteria.

2) ***Oxidase test:*** The enzyme ***cytochrome c oxidase*** is part of electron transport and nitrate metabolism in some bacteria. The enzyme can accept electrons from artificial substrates (such as a ***phenylenediamine derivative***), producing a dark, and oxidized product. This test assists in differentiating between groups of gram-negative bacteria. ***Pseudomonas aeruginosa***; e.g., is oxidase positive.

3) ***Urease:*** The enzyme urease hydrolyzes urea to ammonia and carbon dioxide ($NH_2CONH_2 + H_2O \rightarrow 2NH_3 + CO_2$). The ammonia produced can be detected with pH indicators that change color in response to the increased alkalinity. The test helps to identify certain species of ***Enterobacteriaceae***, ***Corynebacterium urealyticum***, and ***Helicobacter pylori***.

4) Coagulase test: Coagulase is an enzyme that causes a clot to form when bacteria are incubated with plasma. The test is used to differentiate ***Staphylococcus aureus*** (coagulase positive) ***from coagulase-negative Staphylococci.***

Automated systems for bacterial identification

Microbiology laboratories are increasingly using automated methods to identify bacterial pathogens; as in the ***Vitek System***, small plastic reagent cards containing micro liter quantities of various biochemical test media in 30 wells provide a biochemical profile that allows for organism identification. An inoculum derived from cultured samples is automatically transferred into the card, and a photometer intermittently measures color changes in the card that result from the metabolic activity of the organism.

The data are analyzed, stored, and printed in a computerized database. There are many commercial variants of these automated systems and several can be used for simultaneous identification and antimicrobial susceptibility determination.

Tests based on the presence of metabolic pathways (API; analytical Profile Index)

These tests measure the presence of metabolic pathways in a bacterial isolate, rather than a single enzyme. Commonly used assays include those for oxidation and fermentation of different

carbohydrates, the ability to degrade amino acids, and use of specific substrates. A widely used manual system for rapid identification of members of the family ***Enterobacteriaceae*** and other gram-negative bacteria makes use of twenty micro tubes containing substrates for various biochemical pathways. The test substrates in the micro tubes are inoculated with the bacterial isolate to be identified, and, after 5 hours incubation, the metabolic profile of the organism is constructed from color changes in the micro tubes. These color changes indicate the presence or absence of the bacteria's ability to metabolize a particular substrate. The results are compared with a data bank containing test results from known bacteria. The probability of a match between the test organism and known pathogens is then calculated.

- ***Immunological bacterial identification***

In the diagnosis of infectious diseases, immunologic methods take advantage of the specificity of ***antigen–antibody binding***, as known antigens and antibodies are used as diagnostic tools in identifying microorganisms.

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 infecting microorganism is difficult or impossible to isolate or when a previous infection needs to be documented***.

I- Detection of microbial antigen with known antiserum

These methods of identification are often rapid and show favorable sensitivity and specificity; unlike microbial culturing techniques, these immunologic methods do not permit further characterization of the microorganism, such as determining its antibiotic sensitivity or characteristic metabolic patterns:

1. Quellung reaction: Some bacteria having capsules can be identified directly in clinical specimens by a reaction that occurs when the organisms are treated with serum containing specific antibodies. The Quellung reaction ***makes the capsule more refractile and thus more visible, but the capsule does not actually swell***. This method can be used for all serotypes of ***S. pneumoniae***, ***H. influenzae type b***, and ***Neisseria meningitidis*** groups A and C.

2. Slide agglutination test: Some microorganisms, such as ***Salmonella*** and ***Shigella*** 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.

II-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; and it characterized by:

1) Antibody may not be detectable early in an infection

2) The presence of antibodies in a patient's serum cannot differentiate between a present and a prior infection

3) A significant rise in antibody titer over a 10 to14-day period does distinguish between a present or prior infection

Techniques such as ***complement fixation*** and ***agglutination*** can be used to quantitate antimicrobial antibodies.

1. ***Complement fixation:*** It is the older method but still useful method for detecting serum antibody directed against a specific pathogen employs the ability of antibody to bind complement. A patient's serum is first incubated with antigen specific for the suspected infectious agent, followed by the addition of complement. If the patient's serum does contain immunoglobulin (***IgG or IgM***) that target the specific antigen (***indicating past or current infection***), then the added complement will be sequestered in an ***antigen–antibody–complement complex ("complement fixation")***.

Then the sensitized (***antibody-coated) indicator sheep RBCs*** are added to the solution. If complement has been fixed (because the patient's serum contained antibodies against the added antigen), then little complement will be available to bind to the antibody–RBC complexes, and the cells will not lyse.

If complement has not been depleted by initial antigen–antibody complexes (because the patient's serum does not contain antibodies to the specific antigen), the complement will bind to the antibody–RBC complexes, causing the cells to lyse. ***As hemolyzed RBCs release hemoglobin, the reaction can be monitored with a spectrophotometer.***

2. Direct agglutination: Direct bacterial agglutination testing is sometimes ordered when a suspected pathogen is difficult or dangerous to culture in the laboratory. This test measures the ability of a patient's serum antibody to directly agglutinate specific killed (yet intact) microorganisms. This test is used to evaluate patients suspected of being infected by ***Brucella abortus*** or ***Francisella tularensis***.

3. Direct hemagglutination: Antibodies directed against RBCs can arise during the course of various infections; such antibodies are typically found during ***infectious mononucleosis caused by Epstein-Barr virus***. When uncoated (native) animal or human RBCs are used in agglutination reactions with serum from a patient infected with such an organism, antibodies to RBC antigens can be detected. The patient's antibodies cause the RBCs to clump. This test is, therefore, a direct hemagglutination reaction. In the case of some diseases, including pneumonia caused by ***Mycoplasma pneumoniae***, IgM auto antibodies may develop that agglutinate human RBCs at 4^{o} C but not at 37^{o} C ***((termed the "cold agglutinins" test))***

Other tests used to identify serum antigens or antibodies

1. 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; such methods are used to rapidly test CSF for antigens associated with common forms of ***bacterial or fungal meningitis***. When antigen is coated onto the latex bead, antibody from a patient's serum can be detected. Latex agglutination tests are widely used for the identification of ***β-hemolytic Streptococci group A***.

2. Enzyme-linked immunosorbent assay:

Elisa is a diagnostic technique in which antibody specific for an antigen of interest is bound to the walls of a plastic micro titer well. Patient serum is then incubated in the wells, and any antigen in the serum is bound by the antibody on the well walls. The wells are then washed, and a second antibody is added. This one is also specific for the antigen but recognizes epitopes different from those bound by the first antibody. After incubation, the wells are again washed, removing any unattached antibody.

Attached to the second antibody is an enzyme, which, when presented with its substrate, produces a colored product, the intensity of the color produced being proportional to the amount of bound antigen. Elisa can also be used to detect or quantitate antibody in a patient's serum; the wells are coated with antigen specific for the antibody in question. The patient's serum is allowed to react with the bound antigen, the wells are washed, and a secondary antibody (that recognizes the initial antibody) conjugated to a color product–producing enzyme is added to the well. After a final washing, substrate for the bound enzyme is added to the well, and the intensity of the colored product can be measured.

3. Fluorescent-antibody tests: Organisms in clinical samples can be detected directly by specific antibodies coupled to a fluorescent compound such as ***fluorescein***. In the direct Immunofluorescence antibody technique, a sample of concentrated body fluid (like CSF or serum), tissue scraping (like skin), or cells in tissue culture is incubated with a ***fluorescein-labeled antibody*** directed against a specific pathogen.

The labeled antibody bound to the microorganism ***absorbs ultraviolet light*** and emits visible fluorescence that can be detected using a ***fluorescence microscope***. A variation of the technique, the indirect Immunofluorescence antibody technique, involves the use of two antibodies. The first is unlabeled antibody (***the target antibody***), which binds a specific microbial antigen in a sample; and this clinical sample is subsequently stained with a fluorescent antibody that recognizes the target antibody. Because a number of labeled antibodies can bind to each target antibody, the fluorescence from the stained microorganism is intensified.

- ***Nucleic acid based tests***

The most widely used methods for detecting microbial DNA fall into three categories:

1) Direct hybridization (non-amplified assay)

2) Amplification methods using the polymerase chain reaction (PCR)

3) DNA microarrays

Although not likely to completely replace culture techniques in the near future, nucleic acid–based tests for the diagnosis of infectious diseases are gaining wider acceptance.

Direct detection of pathogens without target amplification

This highly specific method of pathogen detection involves identification of the DNA of the pathogen in a patient sample or, more commonly, organisms isolated in culture. The basic strategy is to detect a relatively short sequence of nucleotide bases of DNA sequence) that is unique to the pathogen. This is done by hybridization with a probe, a single-stranded piece of DNA (usually labeled with a fluorescent molecule) containing a complementary sequence of bases. In bacteria, DNA sequences coding for ***16S ribosomal RNA sequences (rRNA)*** are commonly used targets because each microorganism contains

multiple copies of its specific 16S rRNA gene, thereby increasing the sensitivity of the assay. Whenthe probe is bound to the target, the label will give off a signal after the free probe is washed away. A limitation of standard direct probe hybridization is the requirement for a 10^4 or greater number of copies of target nucleic acid for detection.