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The Crux

BIMONTHLY FORUM FOR THE LABORATORIANS

CONTENTS

Editorial

Diphtheria is an infection caused by the bacterium *Corynebacterium diphtheriae*. Signs and symptoms may vary from mild to severe. They usually start two to five days after exposure. Symptoms often come on fairly gradually, beginning with a sore throat and fever. In severe cases, a grey or white patch develops in the throat[1] This can block the airway and create a barking cough as in croup. The neck may swell in part due to large lymph nodes. A form of diphtheria that involves the skin, eyes, or genitals also exists. Complications may include myocarditis, inflammation of nerves, kidney problems, and bleeding problems due to low blood platelets. Myocarditis may result in an abnormal heart rate and inflammation of the nerves may result in paralysis.

Diphtheria is usually spread between people by direct contact or through the air. It may also be spread by contaminated objects. Some people carry the bacteria without having symptoms, but can still spread the disease to others. The three main types of *C. diphtheriae* cause different severities of disease.The symptoms are due to a toxin produced by the bacteria. Diagnosis can often be made based on the appearance of the throat with confirmation by microbiological culture. Previous infection may not prevent against future infection.

A diphtheria vaccine is effective for prevention and available in a number of formulations. Three or four doses, given along with tetanus vaccine and pertussis vaccine, are recommended during childhood. Further doses are recommended every ten years. Protection can be verified by measuring the antitoxin level in the blood. Treatment is with the antibiotics erythromycin or benzylpenicillin. These antibiotics may also be used for prevention in those who have been exposed to the infection. A surgical procedure known as a tracheotomy is sometimes needed to open the airway in severe cases.

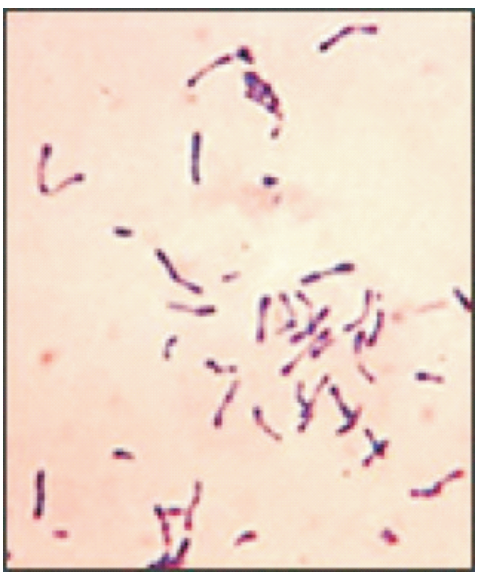
The **"DISEASE DIAGNOSIS"** segment diiscusses all clinoco-diagnostic aspects of Diphtheria. Rightly so, **"TROUBLESHOOTING"** delves deep into the right method of Gram's Staining and **"INTERPRETATION"** highlights Acid Fast Bacilli for you. **BOUQUET** is turning more and more pictorial!. Take a peep….

DISEASE DIAGNOSIS

DIPHTHERIA

Background

C diphtheria is responsible for both endemic and epidemic diseases, and it was first described in the 5th century BC by Hippocrates. Diphtheria manifests as either an upper respiratory tract or cutaneous infection and is caused by the aerobic gram-positive bacteria, *Corynebacterium diphtheria.* The infection usually occurs in the spring or winter months. It is communicable for 2-6 weeks without antibiotic treatment.People who are most susceptible to infection are those who are not completely immunized or have low antitoxin antibody levels and have been exposed to a carrier or diseased individual. A carrier is someone whose cultures are positive for the diphtheria species but does not exhibit signs and symptoms. Studies show that as the number of asymptomatic carriers decrease, the number of diphtheria cases consequently decline. *C diphtheria* is a nonencapsulated, nonmotile, gram-positive bacillus (see below). Pathogenic strains can result in severe localized upper respiratory infection, localized cutaneous infections, and rarely systemic infection.



Photomicrograph shows a number of gram-positive Corynebacterium diphtheriae bacteria,

Exotoxins are associated with both invasive localized and systemic forms of this disease; however, case reports of invasive disease in absence of the exotoxin release have been documented.Exotoxins are encoded in viral bacteriophages, which are transmitted from bacteria to bacteria. The 3 isolated strains of *C diphtheria* include gravis, intermedius, and mitis. Intermedius is thought to be responsible for systemic elaboration of the disease, as it is most often associated with the exotoxin. However, all 3 strains are capable of producing toxins. *Corynebacterium ulcerans* is a relatively rare species, which more frequently causes cutaneous diphtheria; however, this species may rarely cause respiratory symptoms. Severity of disease is dependent on exotoxin production. *C ulcerans* has also been linked to zoonotic transmission to humans and has been most frequently seen in agricultural communities associated with livestock.

Pathophysiology

Overcrowding, poor health, substandard living conditions, incomplete immunization, and immunocompromised states facilitate susceptibility to diphtheria and are risk factors associated with transmission of this disease.Human carriers are the main reservoir of infection; however, case reports have linked the disease to livestock.Infected patients and asymptomatic carriers can transmit *C diphtheria* via respiratory droplets, nasopharyngeal secretions, and rarely fomites.In the case of cutaneous disease, contact with wound exudates may result in the transmission of the disease to the skin as well the respiratory tract. Immunity from exposure or vaccination wanes over time. Inadequate boosting of previously vaccinated individuals may result in increased risk of acquiring the disease from a carrier, even if adequately immunized previously. Additionally, since the advent of widespread vaccination, cases of nontoxigenic strains causing invasive disease have increased. *C diphtheria* adheres to mucosal epithelial cells where the exotoxin, released by endosomes, causes a localized inflammatory reaction followed by tissue destruction and necrosis. The toxin is made of two joined proteins.he B fragment binds to a receptor on the surface of the susceptible host cell, which proteolytically cleaves the membrane lipid layer enabling segment A to enter.Molecularly, it is suggested that the cellular susceptibility is also due to diphthamide modification, dependent on human leukocyte antigen (HLA) types predisposing to more severe infection. The diphthamide molecule is present in all eukaryotic organisms and is located on a histidine residue of the translation elongation factor 2 (eEF2). eEF2 is responsible for the modification of this histidine residue and is the target for the diphtheria toxin (DT). Fragment A inhibits an amino acid transfer from RNA translocase to the ribosomal amino acid chain, thus inhibiting protein synthesis is required for normal host cell functioning.DT causes a catalytic transfer of NAD to diphthamide, which inactivates the elongation factor, resulting in the inactivation eEF2, which results in protein synthesis blockage and subsequent cell death. Local tissue destruction enables the toxin to be carried lymphatically and hematologically to other parts of the body. Elaboration of the diphtheria toxin may affect distant organs such as the myocardium, kidneys, and nervous system. Nontoxigenic strains tend to produce less severe infections; however, since widespread vaccination, case reports of nontoxigenic strains of *C diphtheria* causing invasive disease have been documented.

Epidemiology

Frequency

International

According to the World Health Organization (WHO), diphtheria epidemics remain a health threat in developing nations.The largest epidemic recorded since widespread implementation of vaccine programs was in 1990-1995, when a diphtheria epidemic emerged in the Russian Federation, rapidly spreading to involve all Newly Independent States (NIS) and Baltic States. This epidemic caused more than 157,000 cases and 5000 deaths according to WHO reports.Disproportionately high rates of death were observed in individuals older than 40 years, and 5,000 deaths were reported. This epidemic accounted for 80% of cases reported worldwide during this time period. From 1993-2003, a decade long epidemic in Latvia resulted in 1359 reported cases of diphtheria with 101 deaths. The incidence fell from 3.9 cases per 100,000 cases in 2001 to 1.12 cases per 100,000 population in 2003. Most cases were registered in unvaccinated adults. From 1995-2002, 17 cases of cutaneous diphtheria due to toxigenic strains were reported in the United Kingdom. Overall rates of infection have decreased in Europe from 2000

to 2009, according to the Diphtheria Surveillance Network. This has been attributed to improved vaccination rates creating herd immunity. However, issues with vaccinations still occur, especially in eastern European countries and Russia, and are thought to contribute to the ongoing outbreaks. Many case reports in the literature describe epidemics in sub-Saharan Africa, France, India, and the United States.

Mortality/Morbidity: Before the introduction of vaccine in the 1920s, the incidence of respiratory disease was 100-200 cases per 100,000 population in the United States and has decreased to approximately 0.001 cases per 100,000 population. The most widely quoted diphtheria mortality rate is 5-10%. It may reach higher than 20% in children younger than 5 years and adults older than 40 years. Immunization patterns have the most influence on mortality patterns. Mortality rates have not changed significantly over the past few decades. Most deaths occur on days 3-4 secondary to asphyxia with a pharyngeal membrane or due to myocarditis. Mortality rates of 30-40% have been reported for bacteremic disease.

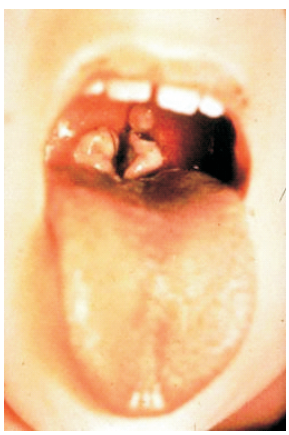
Race: No racial predilection for diphtheria has been reported.

Sex: No significant differences exist between the incidence of diphtheria in males and females. In certain regions of the world, however, women may have lower immunization rates than males. Female infants and young children account for the majority of deaths in endemic regions.

Age: Historically, diphtheria has been primarily a disease of childhood, affecting populations younger than 12 years. Infants become susceptible to the disease at age 6-12 months after their transplacentally derived immunity wanes.Since the advent of diphtheria vaccination, cases of pediatric disease have declined dramatically. Recently, however, diphtheria has shifted into the adolescent and adult population, most notably in ages 40 and older accounting for most new cases.This is primarily due to incomplete immunization status, including never being immunized, inefficient vaccine or response to vaccination, and not receiving a booster after previous vaccination. According to immunologic studies, one must have an antitoxin level of greater 0.1 IU/mL for adequate immunity.Additionally, adolescents and adults may exhibit an atypical presentation of the disease, thus potentially obscuring the diagnosis. Immunization schedules have recently changed requiring a toxoid booster at age 11-12 and every 10 years thereafter. The toxoid booster, without tetanus, is approved for pregnant women if their antitoxin titers are less than 0.1 IU/mL.

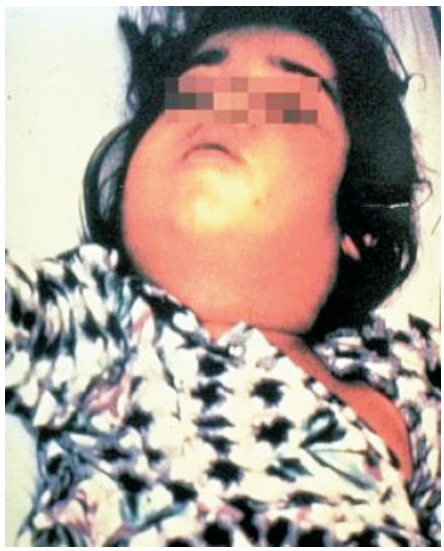
History

Onset of symptoms of respiratory diphtheria typically follows an incubation period of 2-5 days (range, 1-10 d).Symptoms initially are general and nonspecific, often resembling a typical viral upper respiratory infection (URI). Respiratory involvement typically begins with sore throat and mild pharyngeal inflammation. Development of a localized or coalescing pseudomembrane can occur in any portion of the respiratory tract. The pseudomembrane is characterized by the formation of a dense, gray debris layer composed of a mixture of dead cells, fibrin, RBCs, WBCs, and organisms; the pseudomembrane is shown in the image.



The characteristic thick membrane of diphtheria infection in the posterior pharynx.

Removal of the membrane reveals a bleeding, edematous mucosa. The distribution of the membrane varies from local (eg, tonsillar, pharyngeal) to widely covering the entire tracheobronchial tree. The membrane is intensely infectious, and droplet and contact precautions must be followed when examining or caring for infected patients. A combination of cervical adenopathy and swollen mucosa imparts a "bull's neck" appearance to many of the infected patients; this is shown in the image below. The most frequent cause of death is airway obstruction or suffocation following aspiration of the pseudomembrane.



Cervical edema and cervical lymphadenopathy from diphtheria infection produce a bull's neck appearance in this child.

Cutaneous diphtheria is a disease characterized by indolent, nonhealing ulcers covered with a gray membrane. The ulcers are often co-infected with *Staphylococcus aureus* and group A streptococci. This form of the disease is seen with increasing frequency in poor inner-city dwellers and alcoholics. The lesions of cutaneous diphtheria are infectious, and bacteria from cutaneous lesions have been found to cause pharyngeal infections and thus serve as a reservoir for infection.

Patients with diphtheria may present with the following complaints:

- Low-grade fever (rarely >103°F) (50-85%) and chills
- Malaise, weakness, prostration
- Sore throat (85-90%)
- Headache
- Cervical lymphadenopathy and respiratory tract pseudomembrane formation (about 50%)
- Serosanguineous or seropurulent nasal discharge, white nasal membrane
- Hoarseness, dysphagia (26-40%)
- Dyspnea, respiratory stridor, wheezing, cough

Respiratory diphtheria may quickly progress to respiratory failure due to airway obstruction or aspiration of pseudomembrane into the tracheobronchial tree.

Physical

General: Patient has a low-grade fever but is toxic in appearance, and also may have a swollen neck.

Pharyngeal diphtheria:

- Patients may present with general symptoms of fever, halitosis, tachycardia, and anxiety.

- Tonsils and pharynx: Pharyngeal erythema and edema, thick, gray, leathery membrane variably covers the tonsils, soft palate, oropharynx, nasopharynx, and uvula. Attempts at scraping the pseudomembrane causes bleeding of the underlying mucosa.
- Neck: Extensive anterior and submandibular cervical lymphadenopathy imparts a bull's neck appearance. The patient may hold his or her head in extension. It can occasionally also be associated with dysphonia.
- Respiratory distress manifesting as stridor, wheezing, cyanosis, accessory muscle use, and retractions.

Cardiac toxicity typically occurs after 1-2 weeks of illness following improvement in the pharyngeal phase of the disease. It may manifest as follows:

- Myocarditis is seen in as many as 60% of patients (especially if previously unimmunized) and can present acutely with congestive heart failure (CHF), circulatory collapse, or more subtly with progressive dyspnea, diminished heart sounds, cardiac chamber dilatation, and weakness.
- Atrioventricular blocks, ST-T wave changes, and various dysrhythmias may be evident.
- Endocarditis may be present, especially in the presence of an artificial valve.

Neurologic toxicity is proportional to the severity of the pharyngeal infection. Most patients with severe disease develop neuropathy. Deficits include the following:

- Cranial nerve deficits including oculomotor, ciliary paralysis, facial, and pharyngeal, or laryngeal nervous dysfunction.
- Occasionally, a stocking and glove peripheral sensory neuropathy pattern can be observed.
- Most *C diphtheriae* associated neurologic dysfunction eventually resolves.
- Peripheral neuritis develops anywhere from 10 days to 3 months after the onset of pharyngeal disease. It manifests initially as a motor defect of the proximal muscle groups in the extremities extending distally. Various degrees of dysfunction exist, ranging from diminished DTRs to paralysis.
- Other systems involvement: Diphtheria is occasionally seen in the female genital tract, conjunctivae, or ear.
- Invasive disease may manifest in multiple organ system disease, though this is rare.
- Cutaneous diphtheria begins as a painful lesion resembling an erythematous pustule, which breaks down to form an ulcer covered with a gray membrane.

Causes

The following factors may predispose to diphtheria infection:

- Incomplete or absent immunization, which is especially important in the adult population, and as well the pediatric population in underdeveloped countries, may predispose to infection. In some cases, immunity does not prevent infection but lessens the severity of the disease.
- Antitoxin titers decrease over time and immunity wanes, thus older people who have not received booster vaccination are more susceptible to contract the disease from carriers. Studies suggest if titer level is greater than 0.1 UI/mL, then an individual is characterized as immune from infection.
- Low herd immunity, possibly leading to increasing prevalence of diphtheria infections
- Travel to endemic areas or regions with current epidemics
- Immunocompromised states - Due to pharmacologic immune suppression, disease states including HIV, or relative compromise such as from diabetes or alcoholism
- Low socioeconomic status
- Large-scale population movements - Implicated in the spread of the epidemic in the Newly Independent States of the former Soviet Union
- Poor healthcare care system infrastructure
- Overcrowding - Military barracks, homeless shelters, jails
- Domestic animals such as cats may act as reservoir for human infection.

Differential Diagnoses

- Angioedema
- Emergent Management of Pediatric Epiglottitis
- Epiglottitis
- Infective Endocarditis
- Mononucleosis in Emergency Medicine
- Myocarditis
- Oropharyngeal/esophageal candidiasis
- Pediatric Pharyngitis
- Peritonsillar Abscess in Emergency Medicine
- Pharyngitis
- Retropharyngeal Abscess
- Rheumatic Fever in Emergency Medicine
- Septic Shock

Workup

Laboratory Studies

To establish the diagnosis of *C diphtheriae*, it is vital to both isolate *C diphtheriae* in culture media and to identify the presence of toxin production.

Bacteriologic testing

Gram stain shows club-shaped, nonencapsulated, nonmotile bacilli found in clusters. Immunofluorescent staining of 4-hour cultures or methylene blue–stained specimen may sometimes allow for a speedy identification.

Cultures

Inoculation of tellurite or Loeffler media with swabs taken from the nose, pseudomembrane, tonsillar crypts, any ulcerations, or discolorations. Identification is accomplished through observation of colony morphology, microscopic appearance, and fermentation reactions. Any diphtheria bacilli isolated must be tested for toxin production.

Obtain throat and pharyngeal swabs from all close contacts.

Toxigenicity

Toxigenicity testing is aimed to determine the presence of toxin production. Elek test detects the development of an immunoprecipitin band on a filter paper impregnated with antitoxin and then is laid over an agar culture of the organism being tested. Polymerase chain reaction (PCR) assays for detection of DNA sequence encoding the A subunit of tox+ strain are both rapid and sensitive. Once diphtheria infection has been established, the Centers for Disease Control and Prevention (CDC) should be contacted, and further testing may be requested.

Other laboratory studies

CBC may show moderate leukocytosis.

Urinalysis (UA) may demonstrate transient proteinuria.

Serum antibodies to diphtheria toxin prior to administration of antitoxin: Low levels cannot exclude the possibility of the disease; high levels may

protect against severe illness (concentrations of 0.1 to 0.01 IU are thought to confer protection).
Serum troponin I levels seem to correlate with the severity of myocarditis.

Imaging Studies

Chest radiograph and soft tissue neck radiography/CT or ultrasonography may show prevertebral soft tissue swelling, enlarged epiglottis, and narrowing of the subglottic region. Echocardiography may demonstrate valvular vegetations; however, this systemic manifestation of diphtheria is rare.
Other Tests: ECG may show ST-T wave changes, variable heart block, and dysrhythmia.
Procedures
The following procedures may be necessary:

- Endotracheal intubation
- Surgical airway - Cricothyroidotomy or tracheostomy
- Laryngoscopy, bronchoscopy as indicated in intubated patients
- Electrical pacing for high-grade conduction disturbances

Treatment

Prehospital Care

Careful assessment of airway patency and cardiovascular stability. Patients should be transported to the nearest hospital.

Emergency Department Care

Treatment of diphtheria should be initiated even before confirmatory tests are completed due to the high potential for mortality and morbidity. Isolate all cases promptly and use universal and droplet precautions to limit the number of possible contacts. Secure definite airway for patients with impending respiratory compromise or the presence of laryngeal membrane. Early airway management allows access for mechanical removal of tracheobronchial membranes and prevents the risk of sudden asphyxia through aspiration. Consider involving ENT or operating room personnel for intubation and securing of airway if there is suspicion for loss of the airway or respiratory failure. Maintain close monitoring of cardiac activity for early detection of rhythm abnormalities. Initiate electrical pacing for clinically significant conduction disturbance and provide pharmacologic intervention for arrhythmias or for heart failure. Provide 2 large-bore IVs for patients with a toxic appearance; provide invasive monitoring and aggressive resuscitation for patients with septicemia. Initiate prompt antibiotic coverage (erythromycin or penicillin) for eradication of organisms, thus limiting the amount of toxin production. Antibiotics hasten recovery and prevent the spread of the disease to other individuals. Neutralize the toxin as soon as diphtheria is suspected. Diphtheria antitoxin is a horse-derived hyperimmune antiserum that neutralizes circulating toxin prior to its entry into the cells. It prevents the progression of symptoms. The dose and route of administration (IV vs IM) are dependent on the severity of the disease. This antitoxin must be obtained directly from the Centers for Disease Control and Prevention (CDC) through an Investigational New Drug (IND) protocol. The patient must be tested for sensitivity to the antitoxin before it is given. Antitoxin is only available in the United States. Diphtheria disease does not confer immunity; thus, initiation or completion of immunization with diphtheria toxoid is necessary. Obtain throat and nasal swabs from persons in close contact with the suspected diphtheria victim; administer age-appropriate diphtheria booster. Initiate antibiotic therapy with erythromycin or penicillin for chemoprophylaxis in a patient with suspected exposure. Throat cultures should be repeated in 2 weeks after treatment.

Medication Summary

Patients with active disease as well as all close contacts should be treated with antibiotics. Treatment is most effective in the early stages of disease and decreases the transmissibility and improves the course of diphtheria. Additionally, close contacts, such as family members, household contacts, and potential carriers, must receive chemoprophylaxis regardless of immunization status or age. This entails treatment with erythromycin or penicillin for 14 days and post treatment cultures to confirm eradication. The CDC has approved macrolides such as erythromycin as first-line agents for patients older than 6 months of age. However, macrolide therapy has been associated with an increase in pyloric stenosis in children younger than 6 months, especially treatment with erythromycin. Intramuscular penicillin is recommended for patients who will be noncompliant or intolerant to an erythromycin course. The horse serum antitoxin is given to anyone suspected to have diphtheria and can be administered without confirmation from cultures, as it is most efficacious early during the course of the disease.

Transfer

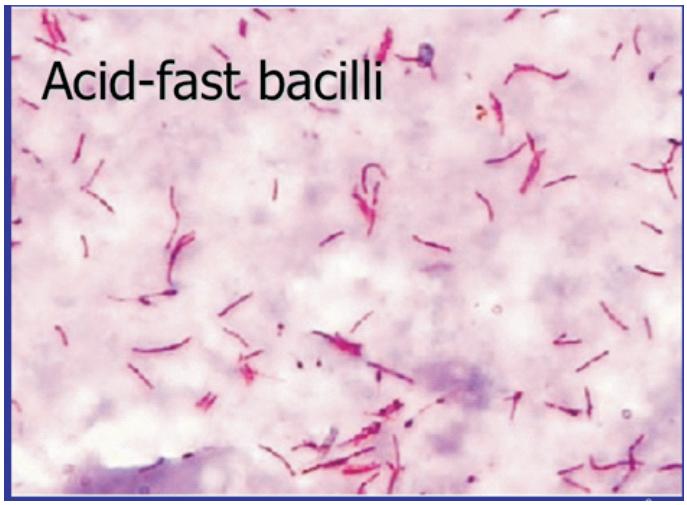
Intensive care unit admission is recommended for patients with impending respiratory compromise. Isolation may be indicated.

Deterrence/Prevention

The Global Pertussis Initiative formed in 2001 is the task force working towards global immunizations and disease prevention in infants, adolescents, and adults for diphtheria, pertussis, and tetanus. The 4 forms of the diphtheria toxoid are as follows: DTaP, Tdap, DT, and Td. The childhood vaccination is called DTaP. Adult vaccination form is Tdap. These toxoid vaccinations are combined with acellular pertussis and tetanus vaccine. DTap is given at 2 months, 4 months, 6 months, 15-18 months, and 4-6 years. The uppercase D denotes the full strength of tetanus toxoid (7-8 Lf units). DT does not contain pertussis and is given to children who have had previous adverse reactions to the acellular pertussis incorporated vaccine. Td is a vaccine for adolescents and adults given as a booster every 10 years or when an exposure has occurred. The lowercase d denotes reduced strength diphtheria toxoid (2.0-2.5 Lf units). It is given to those older than 7 years. Tdap is recommended for adolescents aged 11-12 years or in place of one Td booster in older adolescents and adults aged 19 years and older.In 2012, the CDC recommended patients 65 years and older receive Tdap if they have not received it previously. Boostrix is Tdap approved for adolescents aged 10 years and older, and Adacel is Tdap approved for those aged 11-64 years. For those 65 and older, the CDC recommends Boostrix. However, either Boostrix or Adacel may be used depending on availability. The CDC also recommends that pregnant patients greater than 20 weeks' gestation be given Tdap during pregnancy or shortly after delivery.CDC's Advisory Committee for Immunization Practices recently recommended that pregnant patients greater than 20 weeks' gestation receive Tdap regardless of previous Tdap history.This allows maternal antibodies to pass on to the fetus, giving protection for the few months of life. These immunization schedules have been modified due to trends of pertussis increasing in the adolescent and adult populations. Therefore, Tdap Boostrix, and Adacel are now recommended in the immunization schedule for prevention of endemics associated with pertussis and diphtheria.

INTERPRETATION

AFB



How is it used?

AFB testing may be used to detect several different types of acid-fast bacilli, but it is most commonly used to identify an active tuberculosis (TB) infection caused by the most medically important AFB, *Mycobacterium tuberculosis*. Mycobacteria are called acid-fast bacilli because they are rod-shaped bacteria (bacilli) that can be seen under the microscope following a staining procedure in which the bacteria retain the color of the stain after an acid wash (acid-fast).

A few different tests may be used to help identify AFB as the cause of an infection:

- An AFB smear is used as a rapid test to detect mycobacteria that may be causing an infection such as tuberculosis. The sample is spread thinly onto a glass slide, treated with a special stain, and examined under a microscope for "acid-fast" bacteria. This is a relatively quick way to determine if an infection may be due to one of the mycobacteria, such as *M. tuberculosis*. AFB smears can provide presumptive results within a few hours and are valuable in helping to make decisions about treatment while culture results are pending. However, this rapid test is less sensitive than culture to diagnosis a mycobacterial infection.
- A molecular test for TB called nucleic acid amplification test (NAAT) may be done in conjunction with an AFB smear. NAAT detects the genetic components of mycobacteria by amplifying/replicating pieces of the microorganisms' genetic material. These tests can help decrease the amount of time necessary for a presumptive diagnosis of tuberculosis to less than 24 hours. The testing can narrow the identification to a complex of mycobacteria (a combination, of which *M. tuberculosis* is the most common). They are fairly sensitive and specific when they are performed on specimens where acid-fast bacteria were seen on the smear. When they are done on samples that are AFB-negative by smear, they tend to be less sensitive. The test methods are approved for respiratory samples but must be confirmed with an AFB culture. They provide the health practitioner with a quick answer, allowing him or her to isolate potentially infectious people and minimize the spread of the disease. Guidelines from the Centers for Disease Control and Prevention recommend that people with signs and symptoms of TB have at least one sample tested using nucleic acid amplification in conjunction with AFB smear and culture.
- AFB cultures are used to diagnose active *M. tuberculosis* infections, infections due to nontuberculous mycobacteria, or to determine whether TB-like symptoms are due to another cause. They are used to help determine whether the TB is confined to the lungs (pulmonary disease) or has spread to organs outside the lungs (extrapulmonary disease). AFB cultures can also be used to monitor the effectiveness of treatment and can help determine when a person is no longer infectious. Though this test is more sensitive than an AFB smear, it takes longer for results to become available. Mycobacteria grow more slowly than other types of bacteria so positive identification may take days to several weeks, while negative results (no mycobacterial growth) can take up to 6 to 8 weeks to confirm.
- Susceptibility testing is usually ordered in conjunction with an AFB culture to determine the most effective antibiotic to treat the mycobacterial infection. *M. tuberculosis* may be resistant to one or more drugs commonly used to treat TB. In addition to routine susceptibility testing, there are now some molecular tests available that can identify the genes in bacteria that confer resistance to the most commonly prescribed drugs.

When is it ordered?

AFB testing is ordered when:

- Someone has symptoms that suggest pulmonary TB or other mycobacterial lung infection, such as:
  - Lingering, chronic cough that produces phlegm or sputum, sometimes with bloody streaks
  - Fever, chills
  - Night sweats
  - Loss of appetite
  - Unexplained weight loss
  - Weakness, fatigue
  - Chest pain
- A person has symptoms associated with a TB or other mycobacterial infection located outside of the lungs (extrapulmonary); the symptoms vary depending on the area of the body that is affected. Some examples include back pain and paralysis (spinal TB), weakness due to anemia (TB in the bone marrow), altered mental state, headache, and coma (TB meningitis), joint pain or abdominal pain.
- A TB screening test is positive and the person is at increased risk for active disease and/or characteristic signs are seen in an X-ray of the lung.
- Someone has been in close contact with a person who has been diagnosed with TB and the exposed person either has symptoms or has a condition or disease that puts him or her at a much higher risk of contracting the disease, such as HIV/AIDS. (Those with AIDS are more likely than other affected people to have extrapulmonary TB with a few, vague symptoms.)
- An individual is being treated for TB; AFB testing is usually ordered at intervals, both for evaluating the effectiveness of treatment and for determining whether or not a person is still infectious.

- An individual has a chronic skin infection that does not respond to the usual antibiotics given for a bacterial infection; NTM may be the cause of the infection since they do not respond to the same antibiotics used to treat a staphylococcal or streptococcal infection.

What does the test result mean?

AFB Smear and NAAT

A negative AFB smear may mean that no infection is present, that symptoms are caused by something other than mycobacteria, or that the mycobacteria were not present in sufficient numbers to be seen under the microscope. Usually three samples are collected to increase the probability that the organisms will be detected. Nevertheless, if AFB smears are negative and there is still a strong suspicion of a mycobacterial infection, then additional samples may be collected and tested on different days. A smear negative sample may still grow mycobacteria since the culture media allows low numbers of bacteria that cannot be seen in a microscopic examination to multiply and be detected. Positive AFB smears indicate a probable mycobacterial infection. However, a culture must be performed to confirm a diagnosis and identify the species of mycobacteria present. For people with signs and symptoms of an active TB infection, AFB smear results are considered together with results from NAAT for TB, as recommended by the Centers for Disease Control and Prevention. Though definitive diagnosis requires results from a culture, results from the smear and NAAT may be helpful in deciding what to do. For example, if there is a presumptive diagnosis of TB based on rapid test results, most health practitioners would treat. Interpretation of smear and NAAT results are summarized in the following table. Again, all results must be confirmed by results from culture.

AFB smear result	NAAT result for TB	Interpretation
Positive	Positive	Presumptive diagnosis for TB
Negative	Positive	NAAT is more sensitive than smear so this may occur in people with true disease; may test additional samples using NAAT. If more than one sample is positive by NAAT, this is a presumptive diagnosis for TB.
Positive	Negative	Questionable results for TB; an inhibitor may be present in the specimen or the AFB seen on the smear are not *M. tuberculosis*. A test for the inhibitor may be performed.
Negative	Negative	Symptoms probably not due to active mycobacterial infection.

AFB Culture

Positive AFB cultures identify the particular mycobacterium causing symptoms, and susceptibility testing on the identified organism gives the health practitioner information about how resistant it may be to treatment. A positive AFB smear or culture several weeks after drug treatment has started may mean that the treatment regimen is not effective and needs to be changed. It also means that the person is still likely to be infectious and can pass the mycobacteria to others through coughing or sneezing. A negative culture means that the person tested does not have an active AFB infection or that mycobacteria were not present in that particular sample (which is why multiple samples are often collected) or were present in numbers too low to be detected. Cultures are held for six to eight weeks before being reported as negative. The person tested may have a latent infection that caused a TB screening test to be positive but does not have active TB. If someone has a TB infection in another part of the body, a different type of sample may need to be collected and tested to identify the infection. A negative culture several weeks after treatment indicates that the TB infection is responding to drug treatment and that the person is no longer infectious.

Susceptibility Testing

Susceptibility testing results will list the antibiotics that will likely be most effective in treating the infection. Isoniazid and rifampin are two drugs commonly used to treat TB. If the bacteria are resistant to more than one or the primary drugs used for therapy, the organisms are called multidrug-resistant TB (MDR-TB), and if the organisms are resistant to multiple drugs approved for first and second lines of therapy, they are called extensively drug-resistant tuberculosis (XDR-TB).

Is there anything else I should know?

TB requires a lengthy course of multiple antibiotics to eradicate an active infection. People with inactive (latent) infections, although asymptomatic, may be treated with a single drug to reduce the risk of having an active infection in the future. A faster lab method to culture *Mycobacterium tuberculosis* has been developed. Culturing the sample in a liquid broth-based medium allows the organisms to be detected sooner. Some of the broth cultures require an automated instrument to detect the presence of the mycobacteria, while other methods can be read manually. A liquid culture method, called Microscopic-Observation Drug-Susceptibility (MODS) assay, takes only about 7 days to diagnose TB and detects bacterial resistance to antibiotics at the same time. Since this method can recognize the presence of multidrug-resistant TB (MDR-TB) much more quickly than conventional culture, it can help health practitioners diagnose and treat the disease at an earlier stage and has the potential to help control the spread of infectious TB. The benefits and limitations of this non-automated test are still being evaluated in resource-limited countries with high prevalence of TB. In December 2010, the World Health Organization recommended use of a fully automated, cartridge-based nucleic amplification assay that can simultaneously detect TB and rifampicin resistance directly from sputum in less than two hours. It was approved for marketing in the U.S. in July 2013 and this technology has been recently adopted by many laboratories. However, this NAAT test does not replace AFB cultures. All samples submitted for AFB testing should be cultured to ensure that any mycobacteria that are present are available for further testing, according to the Centers for Disease Control and Prevention.

Common Questions

1. Can I have a tuberculosis (TB) infection and not be sick?
2. What is the difference between multidrug-resistant TB (MDR-TB) and extensively-resistant TB (XDR-TB)?

3. Why is the doctor asking me to take my TB medication in the presence of a nurse?
4. Besides TB, what other types of mycobacteria can be identified with AFB testing?

1. Can I have a tuberculosis (TB) infection and not be sick?

Yes. There are many people in the United States and worldwide who have a latent form of TB infection. They have been exposed to the bacteria, but their body's immune system has confined it to a localized area in their lungs, in an inactive form. People with latent TB infections are not sick and they are not infectious, but the bacteria are still there and still alive. If those with latent infections are tested, most would have a positive TB skin test. The majority of people with latent TB infection, about 90%, will never progress to active tuberculosis disease. Those who do have active TB may not feel ill at first. Early symptoms may be subtle and, if the TB is extrapulmonary (outside of the lungs in organs such as the kidney and bone), the tuberculosis may be fairly advanced by the time it causes noticeable symptoms.

2. What is the difference between multidrug-resistant TB (MDR-TB) and extensively-resistant TB (XDR-TB)?

Both indicate strains of *Mycobacteria tuberculosis* that can be difficult to treat, but XDR-TB is resistant to more drug therapies. MDR-TB is resistant to the two most powerful drugs, isoniazid and rifampin. XDR-TB is currently defined by the Centers for Disease Control and Prevention and the World Health Organization as *M. tuberculosis* that is resistant to isoniazid and rifampin plus resistant to any fluoroquinolone and to at least one of three injectable "second-line" drugs (amikacin, kanamycin, or capreomycin). The emergence of XDR-TB is being closely watched by the world medical community and measures are being taken in hopes of limiting its spread.

3. Why is the doctor asking me to take my TB medication in the presence of a nurse?

The practice of taking TB medications in the presence of a health practitioner is known as direct observed therapy (DOT). DOT ensures that people are taking their medications and continuing their therapy for the required length of time. Unlike other bacterial infections that can be cured in 7-10 days, TB must be treated with two or more drugs for several months. People tend to forget to take their medication when they are feeling better. Since TB medications must be taken for many months, the risk of non-compliance is high. Having a health practitioner administer the medications weekly increases the likelihood that the entire regimen will be completed and decreases the likelihood that someone will relapse with a more resistant strain of TB.

4. Besides TB, what other types of mycobacteria can be identified with AFB testing?

Examples of other mycobacteria that can cause infections and are detected using AFB tests include:

- *Mycobacterium avium-intracellulare* complex (MAC)—can cause a lung infection in people with weakened immune systems, such as those with AIDS; this infection is not contagious but it can be difficult to treat as it tends to be highly resistant to antibiotics.
- Mycobacterial species, such as *Mycobacterium marinum*, grow in water, such as fish tanks, and can cause skin infections.
- *Mycobacterium fortuitum* and *Mycobacterium chelonae*, and other rapidly growing mycobacteria, cause skin and wound infections following cosmetic surgery, prosthetic device implantation, and visits to nail salons.
- A few mycobacteria, such as *Mycobacterium bovis*, can sometimes be transferred from animals to humans.

See the article on Nontuberculous Mycobacteria for more examples and details.

Nocardia species are not a type of mycobacteria but can be detected using some AFB laboratory tests. *Nocardia* can cause infections of the lungs, brain, or skin.

Brain Teasers

1. For clinical applications _____ is the preferred base matrix.

A. Human cerebrospinal fluid
B. Human serum
C. Human urine
D. Human plasma.

2. What are the commonly used stop solutions in ELISA-based systems?

A. 1N HCl
B. 4N H2SO4
C. NaOH
D. All of the above.

3. Which of the following can cause preanalytical errors in relation to ELISA-based systems?

A. Patient-based (smoking, etc.)
B. Specimen-based (time of collection)
C. Nature of the sample (matrix)
D. All of the above.

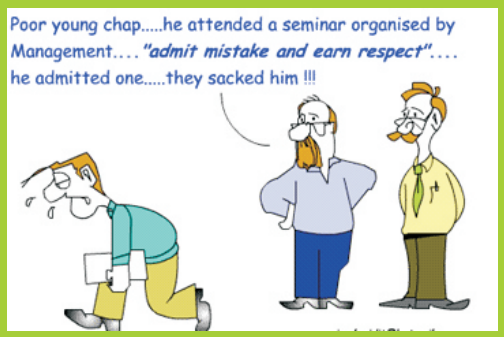
4. Which of the following would be included in analytical errors in relation to ELISA-based systems?

A. Washing error
B. Pipetting error
C. Equipment and procedural error
D. All of the above.

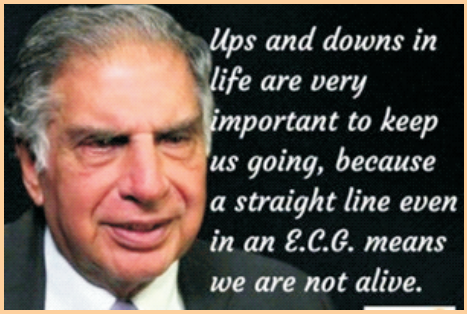
ANSWERS: 1. B, 2. D, 3. D, 4. D

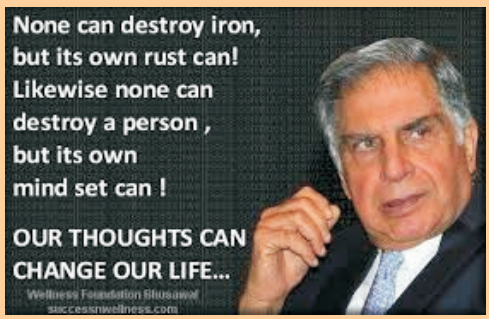
BOUQUET

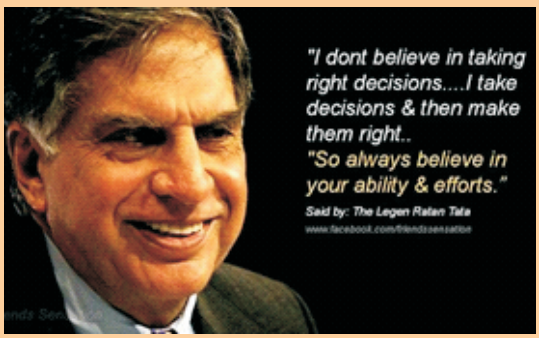
In Lighter Vein

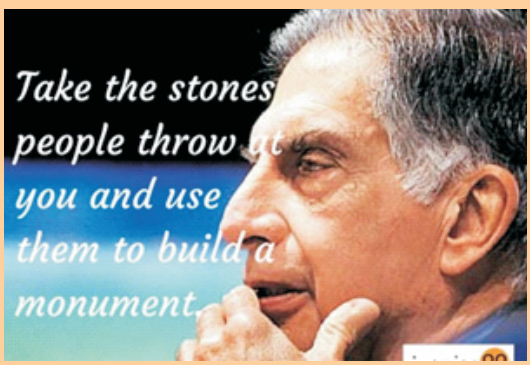


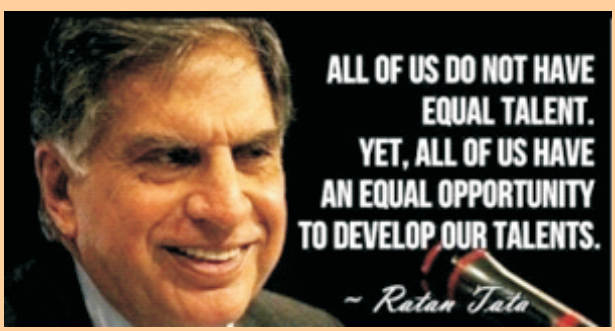
Wisdom Whispers











TROUBLESHOOTING

The Gram Stain

Gram staining is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. The method is named after its inventor, the Danish scientist Hans Christian Gram (1853-1938), who developed the technique in 1884 (Gram 1884). The importance of this determination to correct identification of bacteria cannot be overstated as all phenotypic methods begin with this assay.

The Basic Method

1. First, a loopful of a pure culture is smeared on a slide and allowed to air dry. The culture can come from a thick suspension of a liquid culture or a pure colony from a plate suspended in water on the microscope slide. Important considerations:
   - Take a small inoculum—don't make a thick smear that cannot be completely decolorized. This could make gram-negative organisms appear to be gram-positive or gram-variable.
   - Take a fresh culture—old cultures stain erratically.
2. Fix the cells to the slide by heat or by exposure to methanol. Heat fix the slide by passing it (cell side up) through a flame to warm the glass. Do not let the glass become hot to the touch.
3. Crystal violet (a basic dye) is then added by covering the heat-fixed cells with a prepared solution. Allow to stain for approximately 1 minute.
4. Briefly rinse the slide with water. The heat-fixed cells should look purple at this stage.
5. Add iodine (Gram's iodine) solution (1% iodine, 2% potassium iodide in water) for 1 minute. This acts as a mordant and fixes the dye, making it more difficult to decolorize and reducing some of the variability of the test.
6. Briefly rinse with water.
7. Decolorize the sample by applying 95% ethanol or a mixture of acetone and alcohol. This can be done in a steady stream, or a series of washes. The important aspect is to ensure that all the color has come out that will do so easily. This step washes away unbound crystal violet, leaving Gram-positive organisms stained purple with Gram-negative organisms colorless. The decolorization of the cells is the most "operator-dependent" step of the process and the one that is most likely to be performed incorrectly.
8. Rinse with water to stop decolorization.
9. Rinse the slide with a counterstain (safranin or carbol fuchsin) which stains all cells red. The counterstain stains both gram-negative and gram-positive cells. However, the purple gram-positive color is not altered by the presence of the counter-stain, it's effect is only seen in the previously colorless gram-negative cells which now appear pink/red.
10. Blot gently and allow the slide to dry. Do not smear.

What's Going On?

Bacteria have a cell wall made up of peptidoglycan. This cell wall provides rigidity to the cell, and protection from osmotic lysis in dilute solutions. Gram-positive bacteria have a thick mesh-like cell wall, gram-negative bacteria have a thin cell wall and an outer phospholipid bilayer membrane. The crystal violet stain is small enough to penetrate through the matrix of the cell wall of both types of cells, but the iodine-dye complex exits only with difficulty (Davies et al. 1983).

The decolorizing mixture dehydrates cell wall, and serves as a solvent to rinse out the dye-iodine complex. In Gram-negative bacteria it also dissolves the outer membrane of the gram-negative cell wall aiding in the release of the dye. It is the thickness of the cell wall that characterizes the response of the cells to the staining procedure. In addition to the clearly gram-positive and gram-negative, there are many species that are "gram-variable" with intermediate cell wall structure (Beveridge and Graham 1991). As noted above, the decolorization step is critical to the success of the procedure.

Gram's method involves staining the sample cells dark blue, decolorizing those cells with a thin cell wall by rinsing the sample, then counterstaining with a red dye. The cells with a thick cell wall appear blue (gram positive) as crystal violet is retained within the cells, and so the red dye cannot be seen. Those cells with a thin cell wall, and therefore decolorized, appear red (gram negative).

It is a prudent practice to always include a positive and negative control on the staining procedure to confirm the accuracy of the results (Murray et al 1994) and to perform proficiency testing on the ability of the technicians to correctly interpret the stains (Andserson, et al. 2005).

Excessive Decolorization

It is clear that the decolorization step is the one most likely to cause problems in the gram stain. The particular concerns in this step are listed below (reviewed in McClelland 2001)

1. Excessive heat during fixation: Heat fixing the cells, when done to excess, alters the cell morphology and makes the cells more easily decolorized.
2. Low concentration of crystal violet: Concentrations of crystal violet up to 2% can be used successfully, however low concentrations result in stained cells that are easily decolorized. The standard 0.3% solution is good, if decolorization does not generally exceed 10 seconds.
3. Excessive washing between steps: The crystal violet stain is susceptible to wash-out with water (but not the crystal violet-iodine complex). Do not use more than a 5 second water rinse at any stage of the procedure.
4. Insufficient iodine exposure: The amount of the mordant available is important to the formation of the crystal violet – iodine complex. The lower the concentration, the easier to decolorize (0.33% – 1% commonly used). Also, QC of the reagent is important as exposure to air and elevated temperatures hasten the loss of Gram's iodine from solution. A closed bottle (0.33% starting concentration) at room temperature will lose >50% of available iodine in 30 days, an open bottle >90%. Loss of 60% iodine results in erratic results.
5. Prolonged decolorization: 95% ethanol decolorizes more slowly, and may be recommended for inexperienced technicians while experienced workers can use the acetone-alcohol mix. Skill is needed to gauge when decolorization is complete.
6. Excessive counterstaining: As the counterstain is also a basic dye, it is possible to replace the crystal violet—iodine complex in gram-

positive cells with an over-exposure to the counterstain. The counterstain should not be left on the slide for more than 30 seconds.

Alternatives to the Gram Stain

Gram's staining method is plainly not without its problems. It is messy, complicated, and prone to operator error. The method also requires a large number of cells (although a membrane-filtration technique has been reported; Romero, et al 1988). However, it is also central to phenotypic microbial identification techniques.

This method, and it's liabilities, are of immediate interest to those involved in environmental monitoring programs as one of the most common isolates in an EM program, Bacillus spp., will frequently stain gram variable or gram negative despite being a gram-positive rod (this is especially true with older cultures). The problems with Gram's method have lead to a search for other tests that correlate with the cell wall structure of the gram-positive and the gram-negative cells. Several improvements/alternatives to the classical gram stain have appeared in the literature.

KOH String Test

The KOH String Test is done using a drop of 3% potassium hydroxide on a glass slide. A visible loopful of cells from a single, well-isolated colony is mixed into the drop. If the mixture becomes viscous within 60 seconds of mixing (KOH-positive) then the colony is considered gram-negative. The reaction depends on the lysis of the gram-negative cell in the dilute alkali solution releasing cellular DNA to turn the suspension viscous. This method has been shown effective for food microorganisms (Powers 1995), and for Bacillus spp (Carlone et al 1983, Gregersen 1978), although it may be problematic for some anaerobes (Carlone et al 1983, but also see Halebian et al 1981).

This test has the advantage of simplicity, and it can be performed on older cultures. False negative results can occur in the test by using too little inoculum or too much KOH (DNA-induced viscosity not noticeable). False positive results can occur from too heavy an inoculum (the solution will appear to gel, but not string), or inoculation with mucoid colonies. This can serve as a valuable adjunct to the tradition gram stain method (von Graevenitz and Bucher 1983).

Aminopeptidase Test

L-alanine aminopeptidase is an enzyme localized in the bacterial cell wall which cleaves the amino acid L-alanine from various peptides. Significant activity is found almost only in Gram-negative microorganisms, all Gram-positive or Gram-variable microorganisms so far studied display no or very weak activity (Cerny 1976, Carlone et al. 1983). To perform the test, the reagent is used to make a suspension (with the bacteria). Aminopeptidase activity of the bacteria causes the release of 4-nitroaniline from the reagent, turning the suspension yellow. The test is especially useful for non-fermenters and gram-variable organisms, and is a one step test with several suppliers of kits. Results of the test are available in 5 minutes.

Fluorescent Stains

A popular combination of fluorescent stains for use in gram staining (particularly for flow-cytometry) involves the use of the fluorescent nucleic acid binding dyes hexidium iodide (HI) and SYTO 13. HI penetrates gram-positive but not gram-negative organisms, but SYTO 13 penetrates both. When the dyes were used together in a single step, gram-negative organisms are green fluorescent by SYTO 13 while gram-positive organisms are red-orange fluorescent by HI which overpowers the green of SYTO 13 (Mason et al 1998). There are commercial kits available for this procedure, which requires a fluorescent microscope or a flow cytometer.

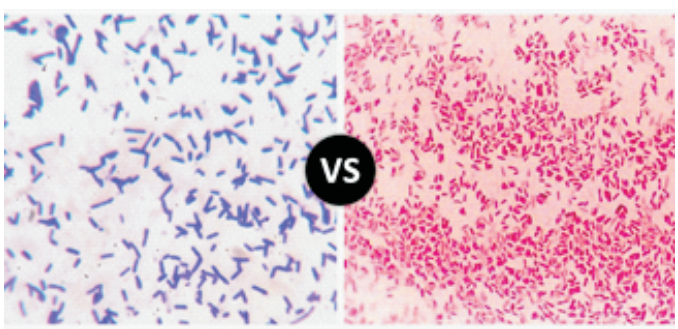
Sizemore et al (1990) developed a different approach to fluorescent labeling of cells. Fluorescence-labeled wheat germ agglutinin binds specifically to N-acetylglucosamine in the outer peptidoglycan layer of gram-positive bacteria. The peptidoglycan layer of gram-negative bacteria is covered by a membrane and is not labeled by the lectin. A variant of this method has also been used to “gram stain” microorganisms in milk for direct measurement by flow cytometry.

LAL-based Assay

Charles River Laboratories has just released a product to be used with their PTS instrument – the PTS Gram ID (Farmer 2005). This methodology makes use of the same reaction used for the chromogenic LAL test. Gram-negative organisms, with bacterial endotoxin, initiate the LAL coagulase cascade which results in activation of the proclotting enzyme, a protease. In the LAL test, this enzyme cleaves a peptide from the horseshoe crab coagulen, resulting in a clot. It can also cleave a peptide from a synthetic substrate, yielding a chromophore (p-nitroaniline) which is yellow and can be measured photometrically at 385 nm (Iwanaga 1987). Gram-positive organisms, lacking endotoxin, do not trigger the color change in this method, while gram-negative organisms do trigger it. Results are available within 10 minutes.

Summary

The differentiation of bacteria into either the gram-positive or the gram-negative group is fundamental to most bacterial identification systems. This task is usually accomplished through the use of Gram's Staining Method. Unfortunately, the gram stain methodology is complex and prone to error. This operator-dependence can be addressed by attention to detail, and by the use of controls on the test. Additional steps might include confirmatory tests, of which several examples were given. As with all microbiology assays, full technician training and competent review of the data are critical quality control steps for good laboratory results.



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