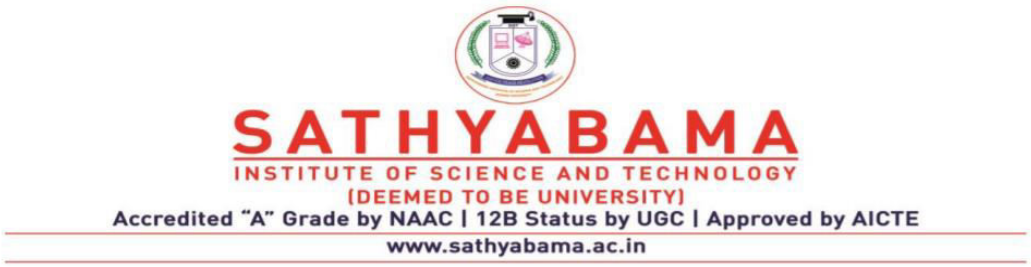


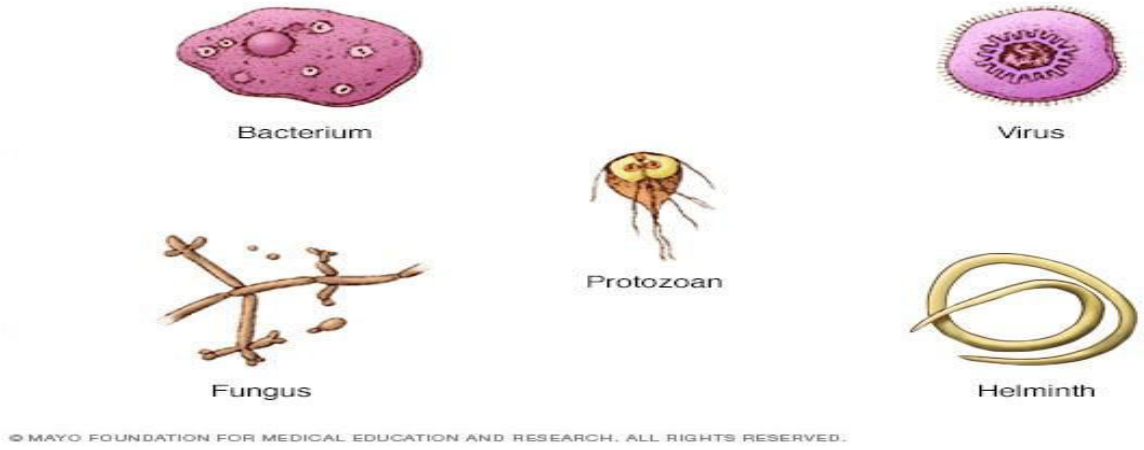
SCHOOL OF BIO AND CHEMICAL ENGINEEING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – MEDICAL LABORATORY TECHNOLOGY – SMB1607

1.Bacterial,Fungal,Viruses and Protozoan diseases of the body system

Infectious agents: From bacteria to worms



Types of infectious agents

Infectious agents come in many shapes and sizes. Categories include:

- Bacteria
- Viruses
- Fungi
- Protozoans
- Helminths

Bacteria

Bacteria are one-celled organisms that can be seen only with a microscope. They're so small that if you lined up a thousand of them end to end, they could fit across the end of a pencil eraser.

Not all bacteria are harmful, and some bacteria that live in your body are helpful. For instance, Lactobacillus acidophilus — a harmless bacterium that resides in your intestines — helps you digest food, destroys some disease-causing organisms and provides nutrients.

Many disease-causing bacteria produce toxins — powerful chemicals that damage cells and make you ill. Other bacteria can directly invade and damage tissues. Some infections caused by bacteria include:

- Strep throat

- Tuberculosis
- Urinary tract infections

Viruses

Viruses are much smaller than cells. In fact, viruses are basically just capsules that contain genetic material. To reproduce, viruses invade cells in your body, hijacking the machinery that makes cells work. Host cells are often eventually destroyed during this process.

Viruses are responsible for causing many diseases, including:

- AIDS
- Common cold
- Ebola virus
- Genital herpes
- Influenza
- Measles
- Chickenpox and shingles

Antibiotics designed for bacteria have no effect on viruses.

Fungi

There are many varieties of fungi, and we eat several of them. Mushrooms are fungi, as are the molds that form the blue or green veins in some types of cheese. And yeast, another type of fungus, is a necessary ingredient in most types of bread.

Other fungi can cause illness. One example is candida — a yeast that can cause infection. Candida can cause thrush — an infection of the mouth and throat — in infants and in people taking antibiotics or who have an impaired immune system. Fungi are also responsible for skin conditions such as athlete's foot and ringworm.

Protozoans

Protozoans are single-celled organisms that behave like tiny animals — hunting and gathering other microbes for food. Many protozoans call your intestinal tract home and are harmless. Others cause diseases, such as:

- Giardia
- Malaria
- Toxoplasmosis

Protozoans often spend part of their life cycles outside of humans or other hosts, living in food, soil, water or insects. Some protozoans invade your body through the food you eat or the water you drink. Others, such as malaria, are spread by mosquitoes.

Helminths

Helminths are among the larger parasites. The word "helminth" comes from the Greek word for worm. If these parasites — or their eggs — enter your body, they take up residence in your intestinal tract, lungs, liver, skin or brain, where they live off your body's nutrients. Helminths include tapeworms and roundworms.

Understanding infection vs. disease

There's a difference between infection and disease. Infection, often the first step, occurs when bacteria, viruses or other microbes that cause disease enter your body and begin to multiply. Disease occurs when the cells in your body are damaged — as a result of the infection — and signs and symptoms of an illness appear.

In response to infection, your immune system springs into action. An army of white blood cells, antibodies and other mechanisms goes to work to rid your body of whatever is causing the infection. For instance, in fighting off the common cold, your body might react with fever, coughing and sneezing.

Warding off germs and infection

What's the best way to stay disease-free? Prevent infections. You can prevent infections through simple tactics, such as washing your hands regularly, avoiding close contact with people who are

sick, cleaning surfaces that are touched often, avoiding contaminated food and water, getting vaccinations, and taking appropriate medications.

Hand-washing. Often overlooked, hand-washing is one of the easiest and most effective ways to protect yourself from germs and most infections. Wash your hands thoroughly before preparing or eating food, after coughing or sneezing, after changing a diaper, and after using the toilet. When soap and water aren't available, alcohol-based hand-sanitizing gels can offer protection.

Vaccines. Vaccination is your best line of defense for certain diseases. As researchers understand more about what causes disease, the number of diseases that can be prevented by vaccines continues to grow. Many vaccines are given in childhood. But adults still need routine vaccinations to prevent some illnesses, such as tetanus and influenza.

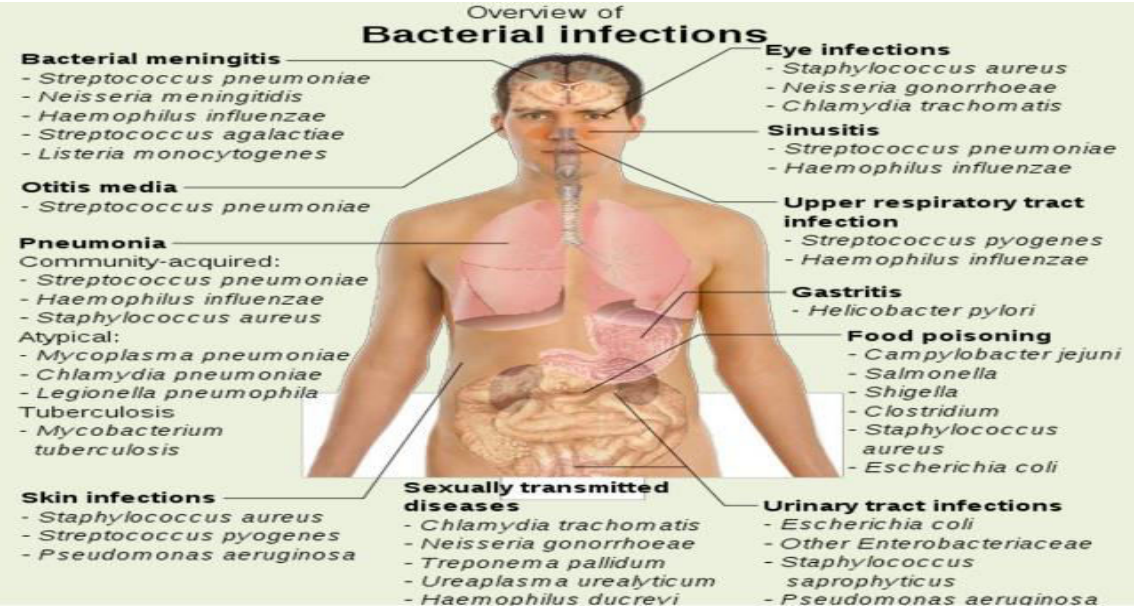
Medicines. Some medicines offer short-term protection from certain germs. For example, taking an anti-parasitic medication might keep you from becoming infected with malaria if you travel to or live in a high-risk area.

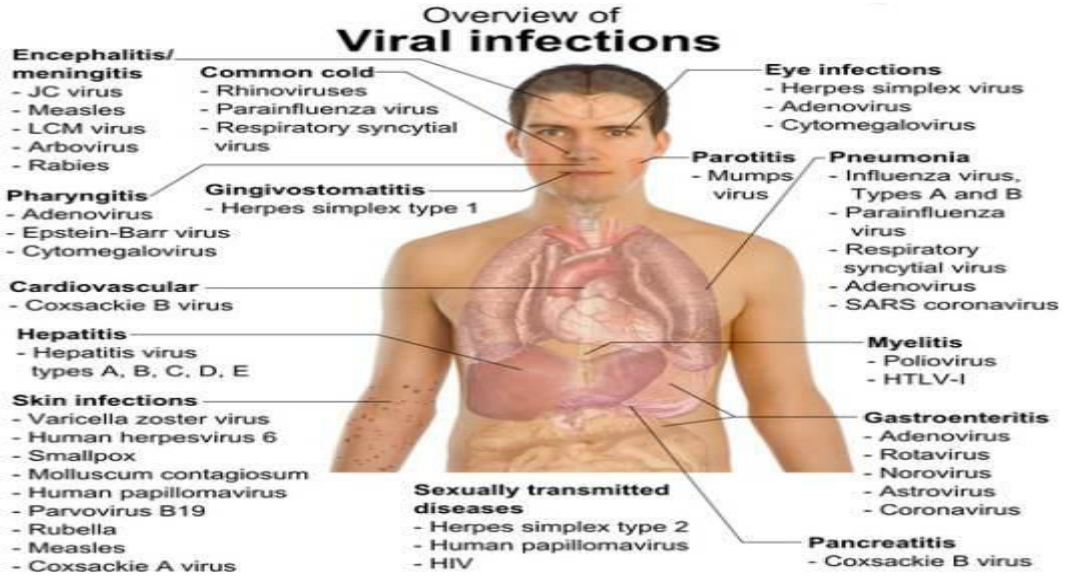
When to seek medical care

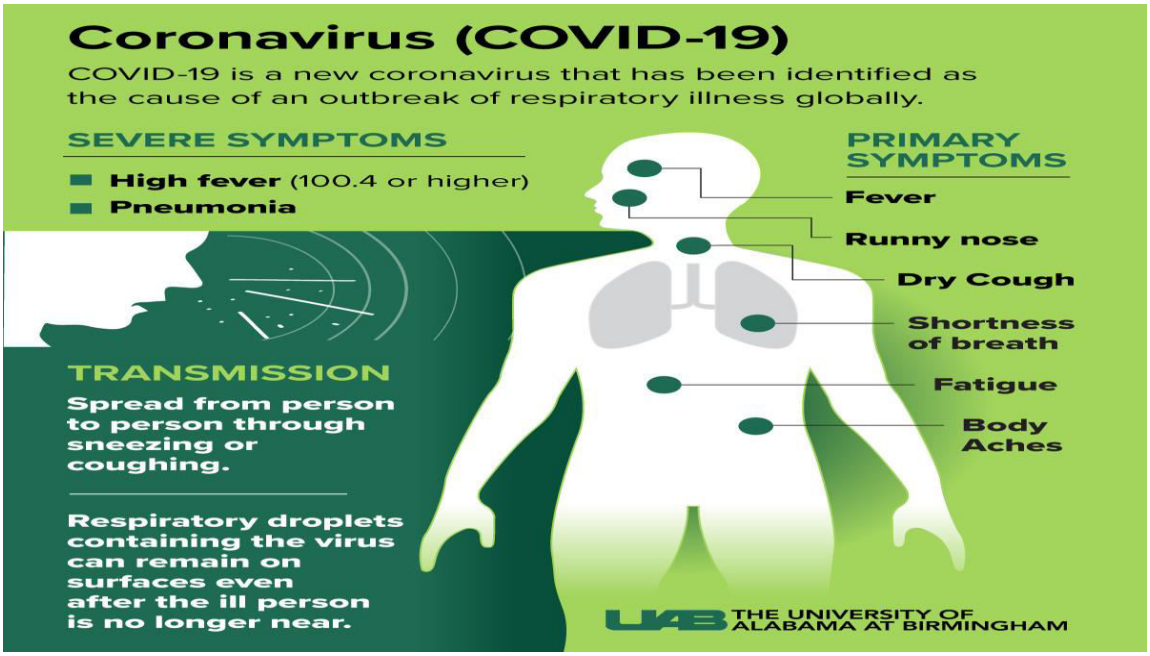
Seek medical care if you suspect that you have an infection and you have experienced:

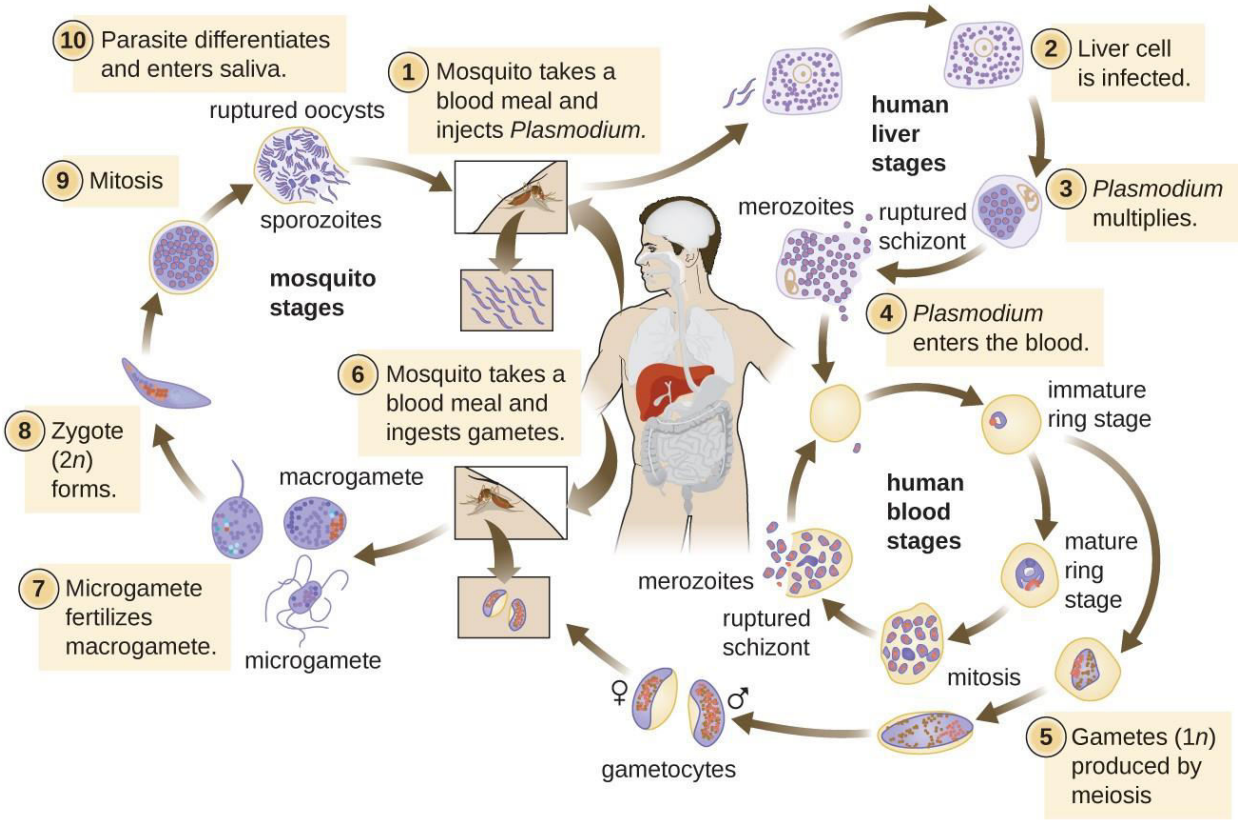
- An animal or a human bite
- Difficulty breathing
- A cough lasting longer than a week
- Periods of rapid heartbeat
- A rash, especially if it's accompanied by a fever
- Swelling
- Blurred vision or other difficulty seeing
- Persistent vomiting
- An unusual or severe headache

Your doctor can perform diagnostic tests to find out if you're infected, the seriousness of the infection and how best to treat that infection.


Overview of
Bacterial infections
Bacterial meningitis
- Streptococcus pneumoniae
- Neisseria meningitidis
- Haemophilus influenzae
- Streptococcus agalactiae
- Listeria monocytogenes
Otitis media
- Streptococcus pneumoniae
Pneumonia
Community-acquired:
- Streptococcus pneumoniae
- Haemophilus influenzae
- Staphylococcus aureus
Atypical:
- Mycoplasma pneumoniae
- Chlamydia pneumoniae
- Legionella pneumophila
Tuberculosis
- Mycobacterium tuberculosis
Skin infections
- Staphylococcus aureus
- Streptococcus pyogenes
- Pseudomonas aeruginosa
Sexually transmitted diseases
- Chlamydia trachomatis
- Neisseria gonorrhoeae
- Treponema pallidum
- Ureaplasma urealyticum
- Haemophilus ducrevi
Eye infections
- Staphylococcus aureus
- Neisseria gonorrhoeae
- Chlamydia trachomatis
Sinusitis
- Streptococcus pneumoniae
- Haemophilus influenzae
Upper respiratory tract infection
- Streptococcus pyogenes
- Haemophilus influenzae
Gastritis
- Helicobacter pylori
Food poisoning
- Campylobacter jejuni
- Salmonella
- Shigella
- Clostridium
- Staphylococcus aureus
- Escherichia coli
Urinary tract infections
- Escherichia coli
- Other Enterobacteriaceae
- Staphylococcus saprophyticus
- Pseudomonas aeruginosa


Overview of
Viral infections
Encephalitis/ meningitis
- JC virus
- Measles
- LCM virus
- Arbovirus
- Rabies
Common cold
- Rhinoviruses
- Parainfluenza virus
- Respiratory syncytial virus
Pharyngitis
- Adenovirus
- Epstein-Barr virus
- Cytomegalovirus
Gingivostomatitis
- Herpes simplex type 1
Cardiovascular
- Coxsackie B virus
Hepatitis
- Hepatitis virus types A, B, C, D, E
Skin infections
- Varicella zoster virus
- Human herpesvirus 6
- Smallpox
- Molluscum contagiosum
- Human papillomavirus
- Parvovirus B19
- Rubella
- Measles
- Coxsackie A virus
Sexually transmitted diseases
- Herpes simplex type 2
- Human papillomavirus
- HIV
Eye infections
- Herpes simplex virus
- Adenovirus
- Cytomegalovirus
Parotitis
- Mumps virus
Pneumonia
- Influenza virus, Types A and B
- Parainfluenza virus
- Respiratory syncytial virus
- Adenovirus
- SARS coronavirus
Myelitis
- Poliovirus
- HTLV-I
Gastroenteritis
- Adenovirus
- Rotavirus
- Norovirus
- Astrovirus
- Coronavirus
Pancreatitis
- Coxsackie B virus

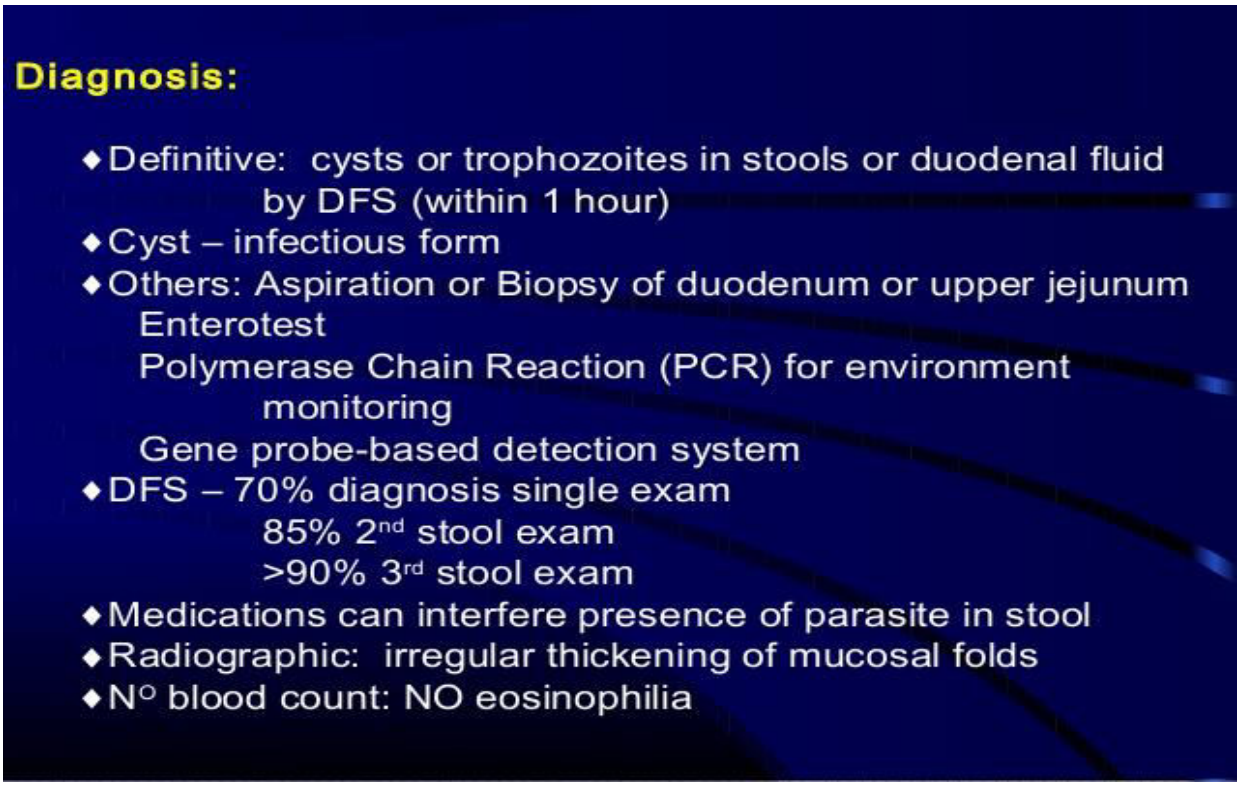

Coronavirus (COVID-19)
COVID-19 is a new coronavirus that has been identified as the cause of an outbreak of respiratory illness globally.
SEVERE SYMPTOMS
High fever (100.4 or higher)
Pneumonia
PRIMARY SYMPTOMS
Fever
Runny nose
Dry Cough
Shortness of breath
Fatigue
Body Aches
TRANSMISSION
Spread from person to person through sneezing or coughing.
Respiratory droplets containing the virus can remain on surfaces even after the ill person is no longer near.
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM


10 Parasite differentiates and enters saliva.
1 Mosquito takes a blood meal and injects Plasmodium.
2 Liver cell is infected.
human liver stages
3 Plasmodium multiplies.
merozoites
ruptured schizont
4 Plasmodium enters the blood.
immature ring stage
mature ring stage
mitosis
5 Gametes (1n) produced by meiosis
gametocytes
6 Mosquito takes a blood meal and ingests gametes.
7 Microgamete fertilizes macrogamete.
macrogamete
microgamete
8 Zygote (2n) forms.
9 Mitosis
ruptured oocysts
sporozoites
mosquito stages
human blood stages

Viral Diseases of the Circulatory and Lymphatic Systems					
Disease	**Pathogen**	**Signs and Symptoms**	**Transmission**	**Diagnostic Tests**	**Antimicrobial Drugs**
AIDS/HIV infection	Human immunodeficiency virus (HIV)	Flu-like symptoms during acute stage, followed by long period of clinical latency; final stage (AIDS) includes fever, weight loss, wasting syndrome, dementia, and opportunistic secondary infections leading to death	Contact with body fluids (e.g., sexual contact, use of contaminated needles)	Serological tests for antibodies and/or HIV antigens; nucleic acid test (NAT) for presence of virus	Antiretroviral therapy (ART) using various combinations of drugs
Burkitt lymphoma	Epstein-Barr virus (human herpesvirus-4 [HHV-4])	Rapid formation of malignant B-cell tumors, oral hairy leukoplakia; fatal if not promptly treated	Contact with body fluids (e.g., saliva, blood, semen); primarily affects patients immunocompromised by HIV or malaria	CT scans, tumor biopsy	Intensive alternating chemotherapy regimen
Chikungunya fever	Chikungunya virus	Fever, rash, joint pain	Transmitted between humans by *Aedes aegypti* and *A. albopictus* vectors	Viral culture, IFA, EIA, ELISA, PCR, RT-PCR	None
Cytomegalovirus infection	Cytomegalovirus (HHV-5)	Usually asymptomatic but may cause non-Epstein-Barr mononucleosis in adults; may cause developmental issues in developing fetus; in transplant recipients, may cause fever, transplant rejection, death	Contact with body fluids, blood transfusions, organ transplants; infected mothers can transmit virus to fetus transplacentally or to newborn in breastmilk, saliva	Histology, culture, EIA, IFA, PCR	Ganciclovir, valganciclovir, foscarnet, cidofovir
Dengue fever (breakbone fever)	Dengue fever viruses 1–4	Fever, headache, extreme bone and joint pain, abdominal pain, vomiting, hemorrhaging; can be fatal	Transmitted between humans by *A. aegypti* and *A. albopictus* vectors	Serologic testing, ELISA, and PCR	None
Ebola virus disease (EVD)	Ebola virus	Fever, headache, joint pain, diarrhea, vomiting, hemorrhaging in gastrointestinal tract, organ failure; often fatal	Contact with body fluids (e.g., blood, saliva, sweat, urine, feces, vomit); highly contagious	ELISA, IgM ELISA, PCR, virus isolation	None
Hantavirus pulmonary syndrome (HPS)	Hantavirus	Initial flu-like symptoms followed by pulmonary edema and hypotension leading to pneumonia and shock; can be fatal	Inhalation of dried feces, urine from infected mouse or rat	ELISA, Western blot, RIBA, RT-PCR	None
Hemorrhagic fever with renal syndrome	Hantavirus	Fever, headache, nausea, rash, or eye inflammation, followed by hemorrhaging and kidney failure; can be fatal	Inhalation of dried feces, urine from infected mouse or rat	ELISA, Western blot, RIBA, RT-PCR	None
Infectious mononucleosis	Epstein-Barr virus (HHV-4), cytomegalovirus (HHV-5)	Pharyngitis, fever, extreme fatigue; swelling of lymph nodes, spleen, and liver	Contact with body fluids (e.g., saliva, blood, semen)	Tests for antibodies to various EBV-associated antigens	None
Yellow fever	Yellow fever virus	Dizziness, fever, chills, headache, myalgia, nausea, vomiting, constipation, fatigue; moderate to severe cases may include jaundice, rash, mucosal hemorrhaging, seizures, shock, and death	From monkeys to humans or between humans via *Aedes* or *Haemagogus* mosquito vectors	Culture, serology, PCR	None for treatment; preventive vaccine available

Bacterial Infections of the Circulatory and Lymphatic Systems (continued)					
Disease	**Pathogen**	**Signs and Symptoms**	**Transmission**	**Diagnostic Tests**	**Antimicrobial Drugs**
Plague	*Yersinia pestis*	Bubonic: buboes, fever, internal hemorrhaging; septicemic: fever, abdominal pain, shock, DIC, necrosis in extremities; pneumonic: acute pneumonia, respiratory failure, shock. All forms have high mortality rates.	Transmitted from mammal reservoirs via flea vectors or consumption of infected animal; transmission of pneumonic plague between humans via respiratory aerosols	Culture of bacteria from lymph, blood, or sputum samples; DFA, ELISA	Gentamycin, fluoroquinolones, others
Puerperal sepsis	*Streptococcus pyogenes,* many others	Rapid-onset fever, shock, and death	Pathogens introduced during or immediately following childbirth	Wound, urine,or blood culture	As determined by susceptibility testing
Rat-bite fever	*Streptobacillus moniliformis, Spirillum minor*	Fever, muscle and joint pain, rash, ulcer	Bite from infected rat or exposure to rat feces or body fluids in contaminated food or water	Observation of the organism from samples and antibody tests	Penicillin
Relapsing fever	*Borrelia recurrentis, B. hermsii,* other *Borrelia* spp.	Recurring fever, headache, muscle aches	From rodent or human reservoir via body louse or tick vector	Darkfield microscopy	Doxycycline, tetracycline, erythromycin
Rheumatic fever	*Streptococcus pyogenes*	Joint pain and swelling, inflammation and scarring of heart valves, heart murmur	Sequela of streptococcal pharyngitis	Serology, electrocardiogram, echocardiogram	Benzathine benzylpenicillin
Rocky Mountain spotted fever	*Rickettsia rickettsii*	High fever, headache, body aches, nausea and vomiting, petechial rash; potentially fatal hypotension and ischemia due to blood coagulation	From rodent reservoir via tick vectors	Biopsy, serology, PCR	Doxycycline, chloramphenicol
Toxic shock syndrome (TSS)	*Staphylococcus aureus*	Sudden high fever, vomiting, diarrhea, hypotension, death	Pathogens from localized infection spread to bloodstream; pathogens introduced on tampons or other intravaginal products	Serology, toxin identification from isolates	Clindamycin, vancomycin
Toxic shock-like syndrome (STSS)	*Streptococcus pyogenes*	Sudden high fever, vomiting, diarrhea, acute respiratory distress syndrome (ARDS), hypoxemia, necrotizing fasciitis, death	Sequela of streptococcal skin or soft-tissue infection	Serology, blood culture, urinalysis	Penicillin, cephalosporin
Trench fever	*Bartonella quintana*	High fever, conjunctivitis, ocular pain, headaches, severe pain in bones of shins, neck, and back	Between humans via body louse vector	Blood culture, ELISA, PCR	Doxycycline, macrolide antibiotics, ceftriaxone
Tularemia (rabbit fever)	*Francisella tularensis*	Skin lesions, fever, chills, headache, buboes	Eating or handling infected rabbit; transmission from infected animal via tick or fly vector; aerosol transmission (in laboratory or as bioweapon)	DFA	Streptomycin, gentamycin, others

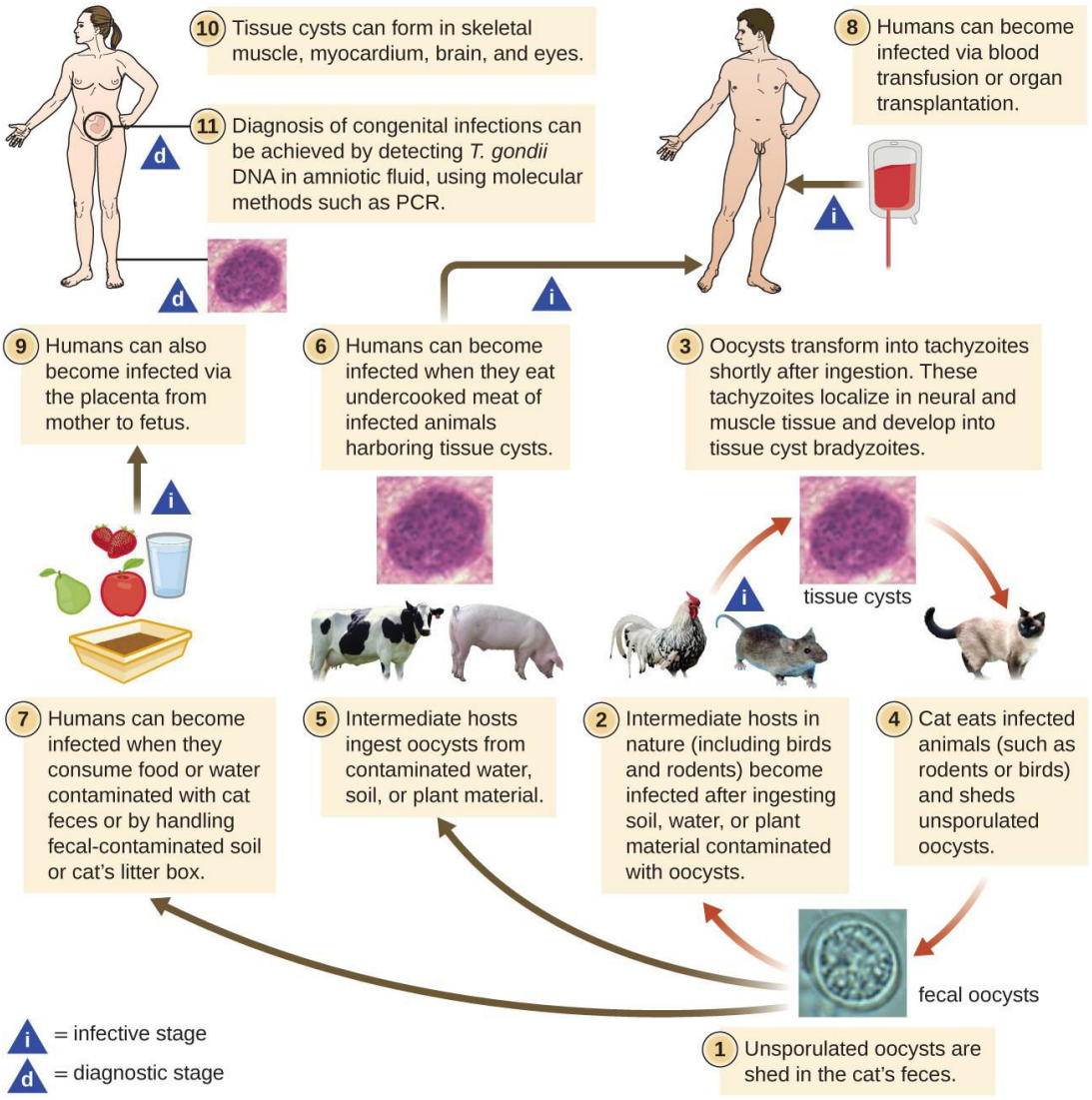
Fungal and Protozoan Infections of the Reproductive Tract					
Disease	**Pathogen**	**Signs and Symptoms**	**Transmission**	**Diagnostic Tests**	**Antimicrobial Drugs**
Trichomoniasis	*Trichomonas vaginalis*	Urethritis, vaginal or penile discharge; redness or soreness of female genitalia	Sexual contact	Wet mounts, NAAT of urine or vaginal samples; OSOM Trichomonas Rapid Test, Affirm VPII Microbial Identification Test	Metronidazole, tinidazole
Vaginal candidiasis (yeast infection)	*Candida* spp., especially *C. albicans*	Dysuria; vaginal burning, itching, discharge	Transmissible by sexual contact, but typically only causes opportunistic infections after immunosuppre-sion or disruption of vaginal microbiota	Culture, Affirm VPII Microbial Identification Test	Fluconazole, miconazole, clotrimazole, tioconazole, nystatin



Differential diagnosis of Haemoprotozoan diseases

Symptoms	Babesiosis	Theileriosis	Trypnosomiosis	Anaplasmosis
Fever	Present	Present	Present	Present
Lymph node	Normal	Swollen	Normal	Normal
Nervous sign	Absent	Absent	Present	Absent
Jaundice	Occasionally present	Present	Absent	Occasionally present
Abortion	Occasionally occurs	Absent	Absent	Occasionally occurs
Nasal discharge	Absent	Present	Absent	Absent
Lacrimation	Absent	Present	Absent	Absent
Pin point hemorrhages in conjunctiva	Absent	Present	Absent	Absent
Hemoglobinuria	Present	Absent	Absent	Absent

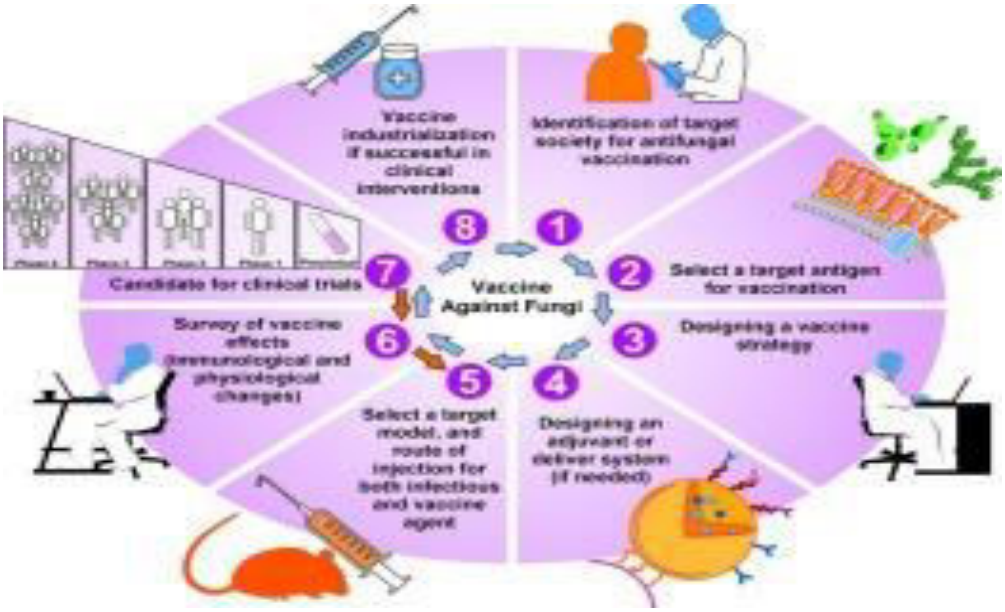
Protozoan Infections of the GI Tract					
Disease	**Pathogen**	**Signs and Symptoms**	**Transmission**	**Diagnostic Tests**	**Antimicrobial Drugs**
Amoebiasis (amoebic dysentery)	*Entamoeba histolytica*	From mild diarrhea to severe dysentery and colitis; may cause abscess on the liver	Fecal-oral route; ingestion of cysts from fecally contaminated water, food, or hands	Stool O&P exam, enzyme immunoassay	Metronidazole, tinidazole, diloxanide furoate, iodoquinol, paromomycin
Cryptosporidiosis	*Cryptosporidium parvum, Cryptosporidium hominis*	Watery diarrhea, nausea, vomiting, cramps, fever, dehydration, and weight loss	Contact with feces of infected mice, birds, farm animals; ingestion of contaminated food or water; exposure to contaminated water while swimming or bathing	Stool O&P exam, enzyme immunoassay, PCR	Nitazoxanide, azithromycin, and paromomycin
Cyclosporiasis	*Cyclospora cayetanensis*	Explosive diarrhea, fever, nausea, vomiting, cramps, loss of appetite, fatigue, bloating	Ingestion of contaminated food or water	Stool O&P exam using ultraviolet fluorescence microscopy	Trimethoprim-sulfmethoxazole
Giardiasis	*Giardia lamblia*	Diarrhea, nausea, stomach cramps, gas, greasy stool, dehydration if severe; sometimes malabsorption syndrome	Contact with infected individual or contaminated fomites; ingestion of contaminated food or water	Stool O&P exam; ELISA, direct fluorescence antibody assays	Metronidazole, tinidazole



Parasitic infections

Tinea Pedis (Athelete's Foot)	Tinea Corporis (Ringworms)	Yeast Infection (Candida)	Onychomycosis (Fungal Infection of Toe Nails)	Tinea Versicolor (Fungal Infection of Skin)	Tinea Cruris (Jock Itch)
- Peeling, cracking and scaling of feet - Redness, blisters/ softening, breaking down of skin - Itching, burning or both	- Itchy red ring shaped patch that can be scaly	-Itching and swelling around vagina - Burning sensation/pai n during urination or sexual intercourse - Redness and soreness in and around vagina - Unusual vaginal discharge	-Nail discoloration - Nail flaking - Nail thickening	-Affected area is lighter or darker than surrounding skin - Dry, itchy, scaly skin	- Redness in groin or buttocks - Chafing, irritation, itching, or burning in infected area - Red rash with circular shape and rough edges

Neuromycoses					
Disease	**Pathogen**	**Signs and Symptoms**	**Transmission**	**Diagnostic Tests**	**Antimicrobial Drugs**
Aspergillosis	*Aspergillus fumigatus*	Meningitis, brain abscesses	Dissemination from respiratory infection	CSF, routine culture	Amphotericin B, voriconazole
Candidiasis	*Candida albicans*	Meningitis	Oropharynx or urogenital	CSF, routine culture	Amphotericin B, flucytosine
Coccidioido-mycosis (Valley fever)	*Coccidioides immitis*	Meningitis (in about 1% of infections)	Dissemination from respiratory infection	CSF, routine culture	Amphotericin B, azoles
Cryptococcosis	*Cryptococcus neoformans*	Meningitis, granuloma formation in brain	Inhalation	Negative stain of CSF, routine culture	Amphotericin B, flucytosine
Histoplasmosis	*Histoplasma capsulatum*	Meningitis, granulomas in the brain	Dissemination from respiratory infection	CSF, routine culture	Amphotericin B, itraconazole
Mucormycosis	*Rhizopus arrhizus*	Brain abscess	Nasopharynx	CSF, routine culture	Amphotericin B, azoles



2.Diseases associated clinical samples for diagnosis:

Many different types of laboratory tests can identify microorganisms. Laboratory tests use a sample of blood, urine, sputum, or other fluid or tissue from the body. This sample may be

Stained and examined under a microscope

Cultured (placed in conditions that encourage the growth of microorganisms

Tested for antibodies (molecules produced by the person's immune system in response to the microorganism)

Tested for a microorganism's antigens (molecules from the microorganism that can trigger an immune response in the body)

Tested for genetic material (such as DNA or RNA) from the microorganism

No single test can identify every microorganism, and tests that work well for one microorganism often do not work well for another. Doctors must choose the test based on which microorganisms they think are most likely to cause a disorder.

Sometimes several different tests are done, typically in a specific order, based on the results of the previous test. Each test further narrows the possibilities. If the right test is not done, doctors may not identify the cause of infection.

When a microorganism is identified, doctors can then do tests to determine which drugs are most effective against it (susceptibility tests), and effective treatment can be started sooner.

A sample is taken from an area of the person's body likely to contain the microorganism suspected of causing the infection. Samples may include

Blood Sputum,Stool,Tissue,Cerebrospinal fluid Mucus from the nose, throat, or genital area

Some samples sent for testing, such as sputum, stool, and mucus from the nose or throat, normally contain many types of bacteria that do not cause disease. Doctors need to distinguish between these bacteria and those that could cause the person's illness.

Other samples come from areas that normally do not contain any microorganisms (that are sterile), such as urine, blood, or cerebrospinal fluid (the fluid that surrounds the brain and spinal cord). Finding any bacteria in such samples is abnormal as long as the area from which the sample was taken was first cleaned with an antiseptic to prevent contamination.

Staining and Examination Using a Microscope

Doctors sometimes can identify a microorganism simply by looking at it under a microscope.

Most samples are treated with stains. Stains are special dyes that color the microorganisms, causing them to stand out from the background. Some microorganisms have a distinctive size, shape, and stained color that enable doctors to recognize them.

However, many microorganisms look alike and cannot be distinguished using a microscope. Also, there must be enough of them, and they must be large enough to be seen with a microscope. For example, viruses cannot be identified using a microscope because they are too small.

For bacteria, doctors often first use Gram stain (a violet-colored stain). Bacteria are classified as follows.

Gram-positive (they look blue because they retain the violet Gram stain)

Gram-negative (they look red because they do not retain the stain)

Doctors can make some treatment decisions based on whether bacteria are gram-positive or gram-negative.In addition to Gram stain, other stains can be used depending on the microorganisms thought to be present.

Culture of Microorganisms

Many samples contain too few microorganisms to be seen using a microscope or to be identified using other tests. Thus, doctors usually try to grow the microorganism in a laboratory (called culture) until there are enough to identify.

Blood culture:

The sample is placed on a sterile dish (plate) or in a test tube that contains specific nutrients to encourage growth of microorganisms. Different nutrients are used depending on which microorganism doctors suspect is causing the infection. Often, doctors add substances to the dish or test tube to stop the growth of microorganisms that do not cause the disease doctors suspect.

Many microorganisms, such as the bacteria that cause urinary tract infections or strep throat, can easily be grown in a culture. Some bacteria, such as the bacteria that cause syphilis, cannot

be cultured at all. Other bacteria, such as those that cause tuberculosis, can be cultured but take weeks to grow. Some viruses can be cultured, but many cannot.

After the microorganisms are cultured, tests to identify them and to determine susceptibility and sensitivity to antimicrobial drugs are done.

Testing of a Microorganism's Susceptibility and Sensitivity to Antimicrobial Drugs

Although doctors know in general which antimicrobial drugs are effective against different microorganisms, microorganisms are constantly developing resistance to drugs that were previously effective. Thus, susceptibility testing is done to determine how effective various antimicrobial drugs are against the specific microorganism infecting the person. This testing helps doctors determine which drug to use for a particular person's infection (see Overview of Antibiotics : Selecting an Antibiotic).

Cultures are often used for susceptibility testing. Once a microorganism has been grown in a culture, doctors add different antimicrobial drugs to see which ones kill the microorganism. They also test how sensitive the microorganism is to a drug—that is, whether a small or a large amount of a drug is needed to kill the microorganism (sensitivity testing). If a large amount is needed to kill the microorganism in the laboratory, doctors usually do not use that drug.

Sometimes genetic testing can be used to detect genes in the microorganism that cause resistance to certain antimicrobial drugs. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria can be identified by testing for the *mecA* gene.

Because susceptibility testing occurs in the laboratory, the result does not always match what happens in the person's body when a drug is given. Factors related to the person receiving the drug can influence how effective a drug is (see also Overview of Response to Drugs). They include the following:

How well the person's immune system is working

How old the person is Whether the person has other disorders How the person's body absorbs and processes the drug

Tests That Detect Antibodies to or Antigens of Microorganisms

Some microorganisms, such as the bacteria that cause syphilis, cannot be cultured. To diagnose such infections, doctors may use a variety of tests called immunologic tests. These tests detect one of the following:

Antibodies, produced by the person's immune system in response to the microorganism

A microorganism's antigens (the molecules from the organism that trigger an immune response in the body)

Antibody tests

Antibody tests are usually done on a sample of the infected person's blood. They also can be done on samples of cerebrospinal fluid or other body fluids.

Antibodies are substances produced by a person's immune system to help defend against infection. They are produced by certain types of white blood cell when these white blood cells encounter a foreign substance or cell. It typically takes several days to produce the antibody.

An antibody recognizes and targets the specific foreign substance (antigen) that triggered its production, so each antibody is unique, made for a specific type (species) of microorganism. If a person has antibodies to a particular microorganism, it means that the person has been exposed to that microorganism and has produced an immune response. However, because many antibodies remain in the bloodstream long after an infection has resolved, finding antibodies to a microorganism does not necessarily mean the person is still infected. The antibodies may remain from a previous infection.

Doctors may test for several antibodies, depending on which infections they think are likely. Sometimes doctors just test whether an antibody is present or not. But usually they try to

determine how much antibody is present. They determine the amount of antibody by repeatedly diluting the sample in half until it no longer tests positive for the antibody. The more dilutions it takes until the test is negative, the more antibody there was in the infected person's sample.

Because it takes several days to weeks for the immune system to produce enough antibody to be detected, diagnosis of an infection may be delayed. Antibody tests done right after people become ill are often negative. Thus, doctors may take one sample immediately and then take another one several weeks later to see whether antibody levels have increased. If levels of an antibody are low on the first test after people become ill, finding an increase in the antibody levels several weeks later suggests an active, current (rather than a previous) infection.

Antigen tests

Antigens are substances that can trigger an immune response in the body. Microorganisms have antigens on their surface and inside them. Antigen tests detect the presence of a microorganism directly, so that doctors can diagnose an infection quickly, without waiting for a person to produce antibodies in response to the microorganism. Also, these tests can be used in people whose immune system cannot produce many antibodies, such as people who have recently had bone marrow transplantation or who have AIDS.

To do antigen tests, doctors take a sample from a person and mix it with a test antibody to the suspected microorganism. If there are antigens from that microorganism in the person's sample, they attach to the test antibody. Different methods can be used to detect the antigen-antibody combination. But whatever method is used, the presence of the antigen means that the microorganism is present and probably is the cause of the infection.

Nucleic acid–based tests

If a microorganism is difficult to culture or identify by other methods, doctors can do tests to identify pieces of the microorganism's genetic material. This genetic material consists of nucleic acids: deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

The polymerase chain reaction (PCR) is an example of this type of test. The PCR technique is used to produce many copies of a gene from a microorganism, making the microorganism much easier to identify.

Each genetic test is specific to only one specific microorganism. That is, a genetic test for hepatitis C virus detects only that virus and not any other. Thus, these tests are done only when a doctor already suspects a particular disease.

Most nucleic acid–based tests are designed to identify the presence of a microorganism (called qualitative testing). However, for certain infections, such as HIV and hepatitis C, tests can measure how much of the microorganism's genetic material is present (called quantitative testing) and thus determine how severe the infection is. Quantitative tests can also be used to monitor how well treatment is working.

Nucleic acid–based tests can sometimes be used to check the microorganisms for genes or gene mutations that make the microorganism resistant to a drug. However, these tests are not completely accurate because not all resistance mutations are known. Thus, tests cannot check for all the genes for resistance that may be present. Also, these tests are expensive, not widely available, and available for only a few microorganisms.

Other Tests Used to Identify Microorganisms

Tests that identify certain other unique characteristics of microorganisms are sometimes called

Non-nucleic acid–based identification tests

These tests are so named because they are not based on identifying the microorganism's genetic material, which consists of nucleic acids (DNA and RNA).For example, tests can be done to identify the following:The substances that the microorganism can grow in or grows best in when it is cultured, Enzymes produced by the microorganism (which help the microorganism infect cells or spread through tissues faster),Other substances in the microorganism (such as proteins and fatty acids) that help identify it.

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SCHOOL OF BIO AND CHEMICAL ENGINEEING

DEPARTMENT OF BIOTECHNOLOGY

UNIT II – MEDICAL LABORATORY TECHNOLOGY – SMB1607

UNIT II

Collection, Transport and Storage of specimens for viral diagnosis

Guideline objectives and target groups

1. Laboratory confirmation of infectious diseases.
2. Improve quality of specimens
3. Uniformity of sampling
4. Infection control issues

Target groups:

- Health care workers
- Medical administrators

General considerations and infection control issues

Important points to consider in collecting specimens for microbiological diagnosis include

1. What to collect? 2. When to collect? 3. How to collect?

Type of specimens to be collected depends on the infective disease. Specimens from localized infections.

Why collected from the site of infection. When systemic spread is known to occur blood cultures are carried out in addition. If the organism is known to be excreted from another body site, specimens from these sites may also be sampled in an attempt to isolate the pathogen. i.e. *Salmonella typi* from urine. Stage of the illness and whether the patient is on antibiotics also influence on suitability of a specimen for microbiological diagnosis.

Specimens should be collected before starting on treatment with antibiotics. Further isolations from the primary site of infection are more successful if the specimen is collected very early in the disease. In some diseases, the organism may appear at different body sites at different times of the disease process. In certain infective diseases the pathogen appear intermittently and therefore, more than one specimen collected at intervals may be needed. In some diseases where serological diagnosis is used, paired serum samples are collected 10- 14

days apart and both samples could be tested in order to compare the titres.

When it is necessary to collect a specimen, first inform the patient about the need of a sample for a particular test. It is also important to give instructions to the patient on the correct procedure of collecting the sample if it is done by the patient. (delete the part within the brackets. discuss on how to collect a proper specimen) Further tests should be coordinated in order to minimize the number of vene-punctures.General guidelines for collection, transport and storage of specimens for viral diagnosis

General specimens collected for virus detection

- Swabings from lesion sites (i.e. skin, throat) with swab heads transferred to viral transport medium that include antibiotics. Dry swabs are not acceptable.
- Scrapings of lesions to obtain infected cells (e.g. bases of vesicles, corneal ulcers).
- Aspirates of secretions or exudates (e.g. from posterior nasopharynx, conjunctiva, cervix).
- Excreta such as urine or faeces.
- Biopsy specimens obtained by needle aspirations, open exploration or endoscopy from liver, kidney, heart, lung, brain or intestine.

Blood

A - For peripheral blood leucocytes, blood is collected into a preservative free heparin tube.

B - For serological tests, 10ml blood sample is collected into a dry sterile container.

Transport and storage of specimens for Virology

a) Specimens for isolation of infectious virus should be transported to the laboratory without delay in order to maximize the recovery of the infectious agent.
b) When short delays are anticipated due to unavoidable circumstances or during transport over long distances optimum preservation of infectivity is obtained by placing the container in melting ice or refrigerating the sample at $4^{o}C$.
c) In general freezing the specimen should be avoided but if long delays are inevitable (>48 hours) specimen should be frozen at $-70^{o}C$, not $-20^{o}C$.
d) In the hospital environment, taking specimens at night should be avoided. But when this practice is unavoidable, it is important to place the container in the fridge rather than the freezer compartment.
e) Transport from a single point of origin of a large number of specimens inside a single plastic bag should be discouraged. Because, a single leaking container can lead to wastage of all specimens and potential source of infection to exposing staff in such situations.

Most satisfactory transport system is to provide racks to keep the blood /specimen - bottles upright and store the racks inside a sturdy insulated container provided with a carrying handle.

Transport of pathological specimens by air is governed by strict guidelines laid down by the International Air Transport Association (IATA). It is the responsibility of the shipper to adhere to current IATA regulations as the penalties for contravening IATA regulations are severe.

Collection, transport & storage

Collection, transport & storage of Urine <u>Urine specimens:</u>

Indications:

- To establish diagnosis, aetiology and antimicrobial sensitivity of an urinary tract infection.
- Diagnosis of enteric fevers, leptospirosis and legionellosis.

- Isolation/detection of viruses excreted in urine i.e. CMV, rubella virus, mumps virus and polyoma viruses.

General principles:

- Whenever possible, collect the urine sample for culture in the morning. Patient should be instructed the night before to refrain from passing urine until the specimen is collected in the morning.
- Patient should be given a sterile, dry, wide-necked leak proof screw- capped container.
- Explain to the patient the importance of collecting a specimen with minimum contamination as possible.
- Wash hands with soap and water before collecting the urine.
- Catching urine in 'midstream' is the goal. In the clean catch method, skin around the urethra is cleaned, and the patient urinates, stop urinating and then urinate into the collection container.

This method is difficult for young children.

A specimen of urine may sometimes have to be obtained by supra-pubic aspiration or by catheterisation from infants and very young children who cannot provide a good midstream specimen of urine

Collection of urine:

A. **Mid -stream urine (MSU) samples from females**:
   a) Clean the external genitalia with soap and water.
   b) Avoid contaminating the bottle mouth and the inside of the lid with hands while collecting the specimen
   c) Then holding the labia apart start passing urine. Discard the 1st part of the stream into the commode.
   d) Place the sterile container provided in the line of the flow of urine (still holding the labia apart) and collect about 20ml of urine from the midstream.

e) When sufficient sample has been collected into the container void the rest of the urine into the commode.

f) Replace the cap, wash hands and hand over the sample to the nursing or the laboratory staff immediately.

B. **Mid stream urine (MSU) samples from males**:

a) Retract the fore skin in uncircumcised males and clean the glans with water or normal saline.

b) Discard the first part of the stream into the commode.

c) Place the sterile container provided in the line of the flow of urine (still holding back the foreskin) and collect about 20ml of urine from the midstream.

d) Void the rest of the urine into the commode.

e) Replace the cap, wash hands and hand it over to the nursing or the laboratory staff immediately

C. **Bed-ridden patients:**

Nursing personnel must assist the cleansing procedure and in males foreskin should be retracted and glans should be cleaned before passing urine.Infants and young children:

D. **Mother is instructed to clean the external genitalia of the child, give plenty of fluid to drink.**

- Mother can collect as much urine as possible avoiding contamination when the child urinates.
- In very small children urine bags may be used and sample should be collected as soon as the child urinates and send to the lab within 2 hours.
- Supra-pubic aspiration is occasionally necessary in infants.

E. **Catheterised patients:**

- Do not collect urine from the urinary bag.

- Clamp the catheter tube for about 2 hours.
- Open the drainage tube and allow few mls of urine to pass into the urinary bag.
- With the help of a sterile syringe and needle collect urine and directly from the rubber tubing of the catheter after the cleaning the surface of the catheter with antiseptic (70% alcohol).
- When introducing the needle point it downwards towards the bag, this will prevent seeping of urine from the rubber tubing.
- Collect 10-15ml of urine into sterile dry screw capped bottle.
- Transport of urine specimens:
  - Properly collected urine sample should be labeled and sent to laboratory with a request form without delay preferably within 2 hours.
  - If a delay of more than 2 hours is anticipated refrigerate the specimen until dispatch. If no refrigerator available, keep the specimen on ice in an insulated flask.
  - If the delay is longer add 0.5gram of borax for 20ml of urine (1.8% w/v.)

F. **Collection of urine in suspected renal tuberculosis:**

Urine may be collected as three early morning samples or 24 hour sample.

i. **Three early morning urine samples:**

Collect the first urine passed (entire specimen) on three successive days. This is better than 24 hour sample. Store in $4^{o}C$ until all three samples are collected.

ii. **24 hour urine sample:**

This method of collection should be avoided as far as possible

Collect all the voided urine over a period of 24 hours into a clean, sterile dry and leak proof and sufficiently large container. Store in $4^{0}C$ until all 24 hour urine is collected.

G. **Collection of urine in suspected chronic prostatitis:**

- Collect a mid stream specimen as described for males and another 20ml of urine into a sterile dry, screw capped container after a prostatic massage. Prostatic massage should be avoided in patients with acute prosatitis to prevent the risk of potential bacteriaemia. .
- Label both specimens accurately and send to the laboratory as soon as possible.
- Refrigerate the specimens until dispatch.

H. Urine for viral studies:

Collect 5-10ml of MSU sample and refrigerate until dispatch.

3.4.2 Collection, transport & storage of stool samples

A. Faecal specimens

Indications:

- Investigation of diarrhoeal disease
- To identify Salmonella carriers
- Investigation of viral meningitis, encephalitis, acute paralytic disease or hand foot and mouth disease.

Procedure:

i. Request the patient to pass feaces into a clean, dry disinfectant free bedpan. Container need not be sterile. Advice the patient not to contaminate faeces with urine.

ii. Transfer a portion of the voided faeces preferably containing mucous, blood, pus blood shreds of epithelium into a clean dry disinfectant free leak proof container.

- Wide mouthed screw capped plastic disposable pot containing small plastic or wooden spoon which fits into the pot when closed is the most appropriate container.
- In the case of a glass container - it should be boiled or sterilized before use.

- In the case of liquid stools fill one third of the container
- If faeces is solid spoonful of faeces is collected using the spoon.
- If the specimen contains worms or tape worm segments, transfer these into a separate container and send them to the Parasitological investigations.

B. Rectal swabs

Indication:

- Only when it is not possible to obtain faeces
- A specimen of faeces is always better than a rectal swab.

Procedure:

- Use a sterile cotton wool swab moisten with sterile saline or transport medium. Do not use lubricating jelly.
- Insert the swab into the rectum through the anal sphincter, rotate and leave for about 10seconds and withdraw. Swab should be stained with faecal material
- If the swab could be processed in 2 hours replace the swab in a sterile empty test tube. If it is to be kept longer it should be inoculated into a transport medium.

C. When cholera is suspected:

A rectal catheter could be used to collect watery stool. Should be sent in alkaline transport medium i.e. Venketraman Ramakrishnan medium or alkaline peptone water

If transport media is not available and if the specimen has to be send to a distant laboratory impregnate a pledget sterile cotton wool or sterile filter paper with stool specimen , dry it and place in a sterile screw capped container and sent to the laboratory

D. Labeling and filling request forms for faecal specimens:

Label specimen and send to the lab with request form within 2 hours. Refrigerate until the dispatch. If amoebic dysentery suspected transport the specimen without delay.Transport media for faecal specimens:

If further delay is anticipated use a suitable transport medium to increase the chances of recovery of organism.

i. **Cary Blair medium:**

Samonella and Shigella may survive up to 48 hours, Campylobacter for about 6 hours. Insert a faecal swab into this semisolid transport medium, break off the swab stick jutting out of the bottle and replace the cap tightly.

ii. **Venketraman Ramakrishnan medium or alkaline peptone water:**

This is used for transport of faeces from suspected case of cholera. Transfer about 1ml of specimen into 10ml of medium.

E. **Collection of faeces for laboratory analysis to exclude the possibility of polio.**

- Two specimens taken 24-48 hours apart for virus studies.
- Should be collected within 2 weeks of onset of paralysis.
- Carefully seal the container and refrigerate or pack between frozen ice packs at 4-8^{o}C in a cold box and transport to the virus lab. Specimen kept in 4-8^{o}C should reach the lab in 72 hours of collection. If it is not possible pack in dry ice.

3.4.3 Collection, transport and storage of respiratory specimens

A. **Respiratory specimens for virology:**

- Throat swab
- Nasopharyngeal aspirates (NPA)
- Bronchoscopy specimens

i. **Throat swab for virus isolation**

Indications:

a) To establish microbial cause of pharyngitis
b) Isolation of respiratory viruses, herpes group of viruses, mumps, rubella, and enteroviruses from throat.

Procedure for collecting a throat swab:

a) Patient must not be treated with antiseptic mouth washes for 8 hours before swabbing
b) Throat swab should be collected by medical or other trained personnel.
c) Explain the procedure to the patient.
d) The patient should sit in front of a light source.
e) While the tongue is kept down with a tongue depressor, a sterile cotton wool or alginate swab moisten with VTM or sterile physiological saline is rubbed vigorously over each tonsil and posterior pharyngeal wall.
f) Care should be taken not to touch the tounge or buccal surfaces.
g) Break off the swab head into a sterile glass or plastic container containing VTM and send to the laboratory as soon as possible.

ii. **Nasopharyngeal aspirates (NPA) for virus isolation**

Indications:

- Bronchiolitis
- Investigation of other respiratory virus infections in a small child.

Procedure:

a) The specimens should be collected by a physician or other trained personnel
b) Explain the procedure to the patient.
c) The specimen is collected through the nose
d) Gently pass a sterile fine-bore catheter into patient's nostril and hence into upper pharynx (usually easier through left nostril but use right side if unsuccessful)

In adult insert the catheter 7-8 cm; in children length inserted depend on age. Hold babies in a sitting- up position because they are less likely to vomit.

e) After catheter is inserted into nostril, apply intermittent suction by placing thumb or finger over end of free arm of Y suction catheter.
f) Continue to apply intermittent suction while slowly withdrawing catheter and collecting mucous.

Whole process should take approximately 5-10 seconds depending on amount of mucous.

g) Dispense the specimen into a sterile container and deliver to laboratory as soon as possible with a completed request form.

h) Wash the specimen into the trap by sucking 4-5ml virus transport medium (VTM) through the catheter.

iii. **Bronchoscopy specimens for Virology:**

Indications:

- For the diagnosis of cytomegalovirus pneumonitis
- Biopsy, brushings and lavage specimens may be contaminated with normal upper respiratory tract flora that is carried into the lower respiratory tract with the passage of the bronchoscope.
- The specimens may also be contaminated with the local anesthetic solutions instilled into the upper airway during the bronchoscopy procedure.
- A special sheathed bronchial brush (microbiology specimen brush) enclosed within a telescopic inner and outer catheters, when available largely overcomes the problem of contamination.

3.4.4 Collection, transport & storage of Respiratory specimens for bacteriology

A. Lower

i. **Bronchoalveolar Lavage, Brush or Wash Endotracheal Aspirate**

Collection

a. Collect into a sputum trap
b. Place brush in sterile container with 1 ml saline

Transport device -Sterile container > 1 ml

Transport time and temperature-=< 2 hours at room temperature

Storage time-=< 2 hours at room temperature

Replica time- 1 per day

Comments

For quantitative analysis 40 – 80 ml fluid is needed. Collect into 1 ml saline.

ii. **Throat swabs**

Indications:

1. To establish microbial cause of pharyngitis
2. Investigation for carriers of *Corynebacterium diptheriae, Streptococcus pyogenes, Streptococcus pneumoniae, Haemophillus influenzae, Neisseria meningitides*.

Collection

(a) Depress tongue with tongue depressor

(b) Sample posterior pharynx, tonsils, inflamed areas with sterile swab

Transport and storage

Transport preferably in swab transport medium =<2 hours at room temperature and store =< 24 hours at room temperature

Comments

Throat swab cultures are contraindicated in epigllotitis. Swabs for Neisseria gonorrhoea are sent in charcoal containing transport medium and plated =< 12 hours after collection. Transportation in Bio-bags at room temperature is a better option.

3.4.5 Collection, transport & storage of Ear & Eye specimens

A. Ear specimens

a) Collection of specimens should be done by trained personnel

b) Collect the aspirate or swab in to a sterile leak proof container

c) Transfer to laboratory as soon as possible with a duly filled request form

d) If delay is anticipated send the specimen in anaerobic transport containers (if available) or Stuart's transport medium

B. Eye specimens

a) Collection of specimens should be done by trained personnel

b) Use a dry sterile cotton swab for collection and transfer to laboratory immediately with a duly filled request form

Transport media:

For bacterial pathogens – Stuart's transport medium For viruses – viral transport medium

If *Neisseria gonorrhoeae* is suspected **do not** refrigerate. Ideally streak on a culture plate obtained from the laboratory

If fungal infection is suspected send a kerato/corneal scraping collected by the ophthalmologist.

3.4.6 Collection and transport of specimens from the urogenital tract:

A. Urethral specimens

Indications:

- Urethritis in a male,
- Urethral discharge

Procedure:

- Patient should not have passed urine 2 hours before the specimen is collected.
- Cleans round the urethral opening using a sterile swab moisten with sterile normal saline.
- Gently massage the urethra from above downwards and collect a sample of pus with a sterile bacteriological loop or sterile swab or at least directly onto a clean slide.
- Ideally specimen should be inoculated onto a GC selective medium at the bedside.
- If this is not possible insert the swab into a container of Amies transport medium
- Break off the swab stick to allow the cap to be replaced tightly.
- Send it to the laboratory immediately with a request form
- Specimen for Chlamydial culture should be sent in chalmydia transport medium.
- Do not refrigerate specimens sent for GC culture

- Make two smear of the discharge on a two separate slides one for Gram stain and another for IF for Chalamydia

B. **Cervical specimens:**

- Moisten a vaginal speculum with sterile warm water and insert it into the vagina.
- Do not use antiseptics or gynaecological exploration cream.
- Samples for gonococcal and Chalmydial culture should be collected from the endocervix.
- Cervical mucus should be wiped off with a swab moisten with sterile physiological saline.
- Pass a sterile swab into the endocervical canal gently rotate the swab to obtain a specimen.
- Inoculate the specimen at the bedside onto a GC medium or insert the swab into a bottle containing Amies transport medium, close the bottle tightly.
- Make a smear on a slide for Gram staining and another for Chlamydia if facilities available. Vaginal specimens

For examination of yeasts, Trichomonas vaginalis and bacterial vaginosis – A high vaginal swab (HVS) is collected.

- Samples may be collected from the posterior fornix of the vagina using a sterile swab.
- Make a smear on a slide for gram staining

C. **Specimens from genital ulcers**

Genital ulcers can be caused by herpes virus, *Treponema pallidum, Haemophilus ducreyi*, *Calymmatobacterium granuulomatis* and *Chalmydia trachomatis*.

<u>Collection of specimens from a chancre:</u>

- Protective gloves should be worn
- Squeeze the ulcer between two fingers and clean the ulcer surface with saline.
- Remove any crusts if present.
- Wipe away the first few drops of blood

- Collect a sample of serous exudates by touching a clean glass slide to the lesion. Place a clean cover slip
- The specimen may be aspirated from the lesion or the enlarged lymph node using a sterile syringe.
- Examine immediately under a dark-field microscope.
- In case of secondary syphilis specimens may be collected from condylomata lata or mucous patches.

Collection of specimens from chancroid

(Haemophilus ducreyi)

Specimen should be obtained from the base of the ulcer.

When Infection with Chalmydia suspected:

Make a smear of exudates from bubo, urethra or cervix.

3.4.7 Collection, transport & storage of skin & subcutaneous specimens

General Considerations

- Disinfection of the site is critical. Contamination with normal skin bacteria must be avoided.
- If swabs must be used to collect the specimen, collect at least two swabs for every culture test ordered.
- These sites will be tested for both aerobic and anaerobic bacteria.

Collection container:

Preferred: Collect Aspirate in a sterile screw capped tube or in a capped syringe (with needle removed). Swabs in a swab transport system can be accepted if necessary.

- Be sure to send at least 2 swabs for every culture test ordered.
- If anaerobes are suspected, a separate sample should be collected and immediately placed into an anaerobic swab transport system.

Storage Requirements:-Room temperature.

Transport Conditions:

If delay in transport of more than one (1) hour, transfer a small amount of the sample to an anaerobic transport system and refrigerate.

Lesion-Superficial (Fungal)

Collection container:

- Sterile screw-cap container if delivering to lab or packed in a clean piece of paper

Storage Requirements:

- Room temperature.

Transport Conditions:

- Send promptly to the lab

Using a scalpel blade, scrape the periphery of the lesion border. Samples from scalp lesions should include hair that is selectively collected for examination. If there is nail involvement, obtain scrapings of debris or material beneath the nail plate.

Bacterial Wound-Superficial

Collection container:

- Sterile screw-capped tube, swab transport system

Storage Requirements:Room temperature.

Transport Conditions:

- Send promptly to the lab Syringe aspiration is preferable to swab collection.
- Disinfect the surface of the wound with 70% alcohol and then with 10% povidone-iodine. Or thoroughly clean the wound three times using new sterile saline moistened gauze 4x4. Flush well with sterile normal saline.
- Allow the disinfectant to dry prior to collecting the specimen.
- Using a 3-5 ml syringe with a 22-23 gauge needle, a physician will aspirate the deepest portion of the lesion.
- If the initial aspiration fails to obtain material, inject sterile 0.85% saline under the skin and repeat aspiration.Transfer material from syringe into sterile screw-capped tube and deliver promptly to the lab.
- If the specimen will be compromised by transferring it from the syringe, remove the needle and recap the syringe with a sterile cap.
- If swab is used, swab deep areas rather than lesion surface using 2 swabs or swab transport system using a 10 point back and forth motion, rotating the swab throughout the procedure.

Ulcers and Nodules

Collection container:

- Sterile screw-capped tube, swab transport system, or other appropriate transport system

Transport Conditions:

- Send promptly to the lab
- Clean the area with 70% alcohol and then with 10% povidone-iodine. Allow area to dry. Remove overlying debris.
- Curette the base of the ulcer of nodule.
- If exudate is present from ulcer or nodule, collect it with a syringe or sterile swab.
- Transfer material to appropriate transport system (sterile tube for aspirate, swab transport system for bacterial swab culture, viral transport media for viral swab culture.

Punch Skin Biopsies

Collection container:Sterile screw-cap tube Storage Requirements: Room temperature.

Transport Conditions:Send promptly to the lab

Criteria for Rejection:

- Specimen submitted in formalin
- Disinfect the surface with 70% alcohol and then with a 10% solution of povidone-iodine. Allow area to dry.

  Collect a 3-4 mm sample with a dermal punch.

Soft Tissue Aspirate

Collection container:Sterile screw-cap tube , Storage Requirements:Room temperature.

Transport Conditions:

- Send promptly to the lab. Disinfect the surface with 70% alcohol and then with a 10% solution of povidone-iodine. Allow area to dry.Aspirate the deepest portion of the lesion or sinus tract. Avoid contamination by the wound surface. Transfer material to sterile, screw cap container and deliver promptly to the lab.

Mycological specimens

Superficial sites-Hair-

Collection Procedure

- Select infected area. Remove at least 10 hairs. Entire hair shaft is necessary.

Transport Procedure

- Room Temperature

  Place hairs between two clean glass slides or in a clean envelope labeled with the patient's data.

b) Nails -

Collection Procedure

- Clean nail with 70% alcohol, scrape away the outer portion and obtain scrapings from the deeper infected areas.

Transport Procedure

- Room Temperature

c) Skin and interspaces -

Collection Procedure

- Clean skin with 70% alcohol. Scrape the entire lesion(s) and both sides of interspaces Transport Procedure
- Room Temperature Sub-cutaneous sites

a) Tissue Biopsy –

Collection Procedure

- Collect tissue aseptically from the center and edge of the lesion. Place specimens between moist gauze squares; add a small amount of sterile water or saline to keep tissue from drying out.

Transport Procedure

- Room Temperature

b) Mucus membranes

Collection Procedure

- Two swabs from the lesion to be collected

Transport Procedure

- Room temperature

3.4.8 Collection, Storage & Transport of Cerebrospinal Fluid (CSF)

A. Introduction:

Cerebrospinal Fluid (CSF) is collected from patients with clinically suspected infection of the Central Nervous System (CNS). CSF is used for general laboratory investigation (sugar, protein & cells) & for aetiological investigation to identify the etiological agent.

General precautions:

1. CSF samples are obtained by lumbar puncture and less commonly by a ventricular tap. CSF also can be collected from External Ventricular Drain (EVD) & from a CSF shunt (AV or VP).

2. Strict aseptic precautions should be adhered to during the collection of CSF to prevent organisms being introduced into CNS & to prevent contamination of the sample (Reference 1).
3. Adequate disinfection of the skin before the lumbar puncture or ventricular tap with an Iodine containing preparation followed by 70% alcohol (reference 1)
4. CSF for bacteriological investigations should be collected before administration of antibiotics. If antibiotics have been started, information should be entered in the request form (type, dose, duration). CSF for bacterial culture should not be kept or sent in ice.
5. Sample should be sent to laboratory with accompanying request form giving patient identification (name, age, sex, ward, BHT number, hospital) & clinical details (brief history, duration of symptoms)
6. It is difficult to isolate virus from CSF in viral meningitis / viral encephalitis. Therefore, other specimens (faeces, throat swab, and blood) should be collected & sent to the laboratory.

B. Sample collection

i. CSF obtained by lumbar puncture / by ventricular tap

a) Clean the skin as mentioned above (6. 2.3)
b) CSF is collected into 3 to 4 containers for
    - Sugar (fluoride containing bottle)
    - Proteins, cells & electrolytes
    - Bacterial culture & ABST*
    - Virology *

*should be collected into sterile, screw capped containers as the last two collections

ii. CSF obtained from EVD or shunt

- Clean the site of puncture with ethanol
- Using sterile needle & a syringe, aspirate CSF
- Collect into bottles as described above (B-General Precautions)

iii. **Additional specimens for bacteriological investigations**

- Blood culture
- Skin scrapings from petichiae in the case of Meningoccocal meningitis

iv. **Additional specimens for Biochemical investigations**

- Blood (3 – 5 ml) in a fluoride containing bottle for blood sugar

v. **Additional specimens for virological investigations**

- Throat swab into a dry sterile screw capped container with Virus Transport Medium (VTM)
- Faeces into a clean dry container (VTM not necessary)
- Blood into a clean dry container (VTM not necessary)

C. Sample storage & transport

1. CSF specimen should be sent to laboratory immediately (within 2 hours) and processed immediately.
Delay in examining CSF leads to disintegration of cells & reduces the chance of isolation of the pathogen.
2. Laboratory staff should be informed before the lumbar puncture (especially if it is done after hours), so that they would be ready to examine the specimen immediately.
3. If there is a delay, specimen for bacterial culture should be kept at room temperature. Do not refrigerate CSF specimens for bacterial culture.

4. **CSF specimen & additional specimens for virological investigations** should be sent to the laboratory **as soon as possible**. It is important to **transport these specimens at 4^{o} C** by using ice (refer to specimen transport for virological investigations).
5. **If there is delay in transport, specimen for viral studies, mycobacterial studies and for bacterial antigen detection should be refrigerated.**
6. **Blood culture Collection**

Disinfect rubber stopper of culture bottle – apply 70% Isopropyl alcohol to rubber stopper, wait for one minute before injecting the sample

Container and minimum volume

Adult : => 20 ml per set (5-7 ml in 50 ml container) Infant / Child : 1- 20 ml per set depending on the weight of the patient

Transport time and temperature-=< 2 hours of collection at room temperature

Storage time-=< 2 hours of collection at room temperature or per instructions

Replica limits-3 sets in 24 hours

Comments

Acute febrile episode:

Antibiotics to be started or changed immediately after 2 sets are taken from separate sites within 10 minutes.

Non-acute disease:

Antibiotics will not be started or changed immediately. 2 or 3 sets to be taken from separate sites within 24 hours at intervals no closer than 3 hours (before antibiotics)

Endocarditic:

3 sets from 3 separate sites within 1-2 hours preferably before antibiotics *P.U.O,* 2 or 3 sets from separate sites. => apart during a 24 hours period. If negative at 24 – 48 hours obtain 2 or 3 sets.

Collection procedure

Palpate vein before disinfection of venepuncture site.

1. Clean site with Iodine followed by 70% alcohol
    - Swab concentrically, starting at the centre with an Iodine preparation.
    - Allow Iodine to dry. Swab with alcohol
    - Do not palpate vein now without sterile gloves
    - Collect blood

3.4.9 Virus isolation & antigen detection

A. Sample collection for virological investigations

Introduction:

Different methods are available for the diagnosis of viral investigations. The quality & the reliability of the results depend on the proper sample collected at the correct time.

Methods of diagnosis available are

1. Detection of virus by electron microscopy (EM) / immune electron microscopy (IEM)
2. Detection of viral antigen
3. Detection of viral genome
4. Isolation of the virus
5. Detection of viral specific antibodies (IgM / IgG)

i) General precautions

1. Strict aseptic precautions should be adhered during the collection of specimens to prevent organisms being introduced into the body & to prevent contamination of the sample (Reference 1).
2. Person who collect the specimens should take universal / respiratory precautions to avoid exposure
3. Specimens should be collected into dry, sterile, screw capped containers .(Faeces can be collected into wide mouth dry, clean containers)
4. Each specimen should be labeled with patient name, BHT, ward, date of collection
5. Specimens should be sent to laboratory with accompanying request form giving patient identification (name, age, sex, ward, BHT number, hospital) & clinical details (brief history, duration of symptoms).
6. Specimens for virus isolation, antigen detection & for genome detection should be sent to the laboratory at $4^{o}C$ with out delay.
7. If there is a delay in transport, specimens for virus isolation, antigen detection & for genome detection should be kept in the refrigerator (at $4^{o}C$)

ii) **Type of specimen**

1. Blood
2. CSF
3. Swabs ((throat swab / nasal swab / swabs from genital lesions / swabs from skin lesions / conjunctival swabs
4. Respiratory secretions (nasopharyngeal aspirates / bronchial secretions / broncho- alveolar lavage
5. Faeces
6. Urine
7. Ante-mortem biopsy * / post mortem biopsy*
8. Corneal impressions / nuchal skin biopsy / CSF / Blood / Post mortem brain biopsy from suspected rabies patients**

*consult the virologist before sending the specimens

** consult the virologist at Rabies division / MRI before taking the specimens

iii) **Timing of specimen collection**

1. Specimens for antigen detection, genome detection & for virus isolation should be collected within 3 – 5 days of the onset of illness
2. Specimens (blood / CSF) for IgM detection should be collected 5 – 7 days after the onset of illness. If the first sample is negative, second sample should be collected about 7 days later.
3. Two specimens (blood) should be collected for IgG / Total antibody detection. Acute sample is collected within 7 days of onset & convalescent sample is collected 2 – 3 weeks later (Same samples can be used for IgM detection depending on the day of collection)

iv) **Collection of specimens**

1. Respiratory secretions, swabs & biopsy material should be collected into containers with VTM. Blood, CSF & faeces do not need VTM.

2. Before collecting specimens for genome detection, contact the laboratory to get specific instructions. Specimens may have to be collected into special buffers / into special containers.

v) Collection, storage & transport of blood for virological investigations

1. Blood for virological investigations is collected as per 4.1, 4.2 & 4.3
2. Blood should be collected into dry, sterile, screw capped container with out VTM.

Note – Special instructions for collection of blood for genome detection. Contact the laboratory before collection

3. Blood should be collected aseptically using universal precautions

4. Allow the blood to clot at room temperature
5. Blood collected for antigen detection, genome detection, virus isolation and for IgM detection should be transported to the laboratory without delay at 4 ^{o} C.
6. Blood collected for IgG / total antibody detection can be transported at room temperature.
7. If there is a delay in transport (for 1 -2 days), keep the blood samples at 4^{o}C and then transport at 4^{o}C.
8. If there is a delay more than 3 days, serum should be separated & it should be stored at – 20^{o}C.
9. Blood specimens intended for virological investigations should never be kept at 0^{o}C or below (frozen) with out separation of serum / plasma.
10. Blood for HIV serology should be sent to STD / AIDS laboratory

vi) Collection, storage & transport of CSF for virological investigations

1. CSF for genome detection & for virus isolation should be collected within 3 – 5 days of onset of illness. CSF for antibody detection (mainly for IgM) is collected after 5 – 7 days. If the IgM assay is negative collection of second sample 7 days later should be considered.

2. CSF should be collected into dry, sterile, screw capped container with out VTM.
3. CSF should be collected aseptically using universal precautions
4. CSF collected for genome detection, virus isolation and for IgM detection should be transported to the laboratory without delay at $4^{o}C$.
5. If there is a delay in transport (for 1 -2 days), keep the CSF samples at $4^{o}C$ & then transport at $4^{o}C$.
6. If there is a delay of more than 3 days, it should be stored at – $20^{o}C$.

vii) Collection, storage & transport of faeces for virological investigations

1. Faeces for antigen detection, genome detection & for virus isolation should be collected within 3 - 5 days of onset of illness.
2. Faeces should be collected into dry, preferably sterile, screw capped container with out VTM.
3. Patient should pass faeces into a washed bed pan (never use detergent to wash the bed pan). Using a plastic disposable spoon, about 8 g of faeces should be collected into the container.
4. Faeces collected for genome detection and virus isolation should be transport to the laboratory without delay at 4^oC.
5. If there is a delay in transport (for 1 -2 days), keep the samples at 4^oC and then transport at 4^oC.
6. If there is a delay more than 3 days, it should be stored at – 20^oC.

viii) Collection, storage & transport of respiratory specimens for virological investigations

1. Respiratory specimens for antigen detection, genome detection & for virus isolation should be collected within 3 - 5 days of onset of illness.
2. Should be collected aseptically using respiratory precautions
3. Should be collected into dry, sterile, screw capped container with VTM.
4. For methods of collection refer to Laboratory Manual MicrobiologySpecimens collected should be transported to the laboratory without delay at 4^oC.
5. If there is a delay in transport (for 1 -2 days), keep the samples at 4^oC and then transport at 4^oC.
6. If there is a delay of more than 3 days, it should be stored at – 20^oC.

ix) Collection, storage & transport of swabs & corneal smears for virological investigations

1. Swabs for antigen detection, genome detection & for virus isolation should be collected within 3 - 5 days of onset of illness.
2. Should be collected aseptically using universal precautions
3. Should be collected into dry, sterile, screw capped container with VTM.For methods of collection refer to Laboratory Manual Microbiology
4. Specimens collected should be transported to the laboratory without delay at 4^oC.

Note – Use a reverse cold chain box / regiform container with ice packs / sufficient amount of ice cubes to maintain the temperature

5. If there is a delay in transport (for 1 -2 days), keep the samples at 4^oC and then transport at 4^oC.
6. If there is a delay more than 3 days, it should be stored at – 20^oC.

x) **Collection, storage & transport of biopsy material for virological investigations**

1. Post mortem biopsy material for antigen detection, genome detection & for virus isolation should be collected as early as possible.
2. Should be collected aseptically using universal precautions
3. Should be collected into dry, sterile, screw capped container with VTM. Do not use fixatives / preservatives
4. For methods of collection refer to the virologist
5. Specimens collected should be transported to the laboratory without delay at 4^oC.
6. If there is a delay in transport (for 1 -2 days), keep the samples at 4^0C and then transport at 4^0C.
7. If there is a delay of more than 3 days, it should be stored at – 20^0C.

3.4.10 Immunological investigations Serum Immunoglobulins-

1. **Serum complement-** Collect 2 ml of venous blood in to a plain bottle Store at room temperature until the specimen is sent to the laboratory. If delay is anticipated in dispatching store at 4^0C.**C- reactive protein-** Collect 2 ml of venous blood in to a plain bottle. Store at 4^0C until the specimen is sent to the laboratory.
2. **Lymphocyte subsets** (by appointment only) **-** Collect 2 ml of venous blood in to a plain bottle. Store at room temperature until the specimen is sent to the laboratory. If delay is anticipated in dispatching store at 4^0C.

NBT test	patient has to be sent to the Immunology laboratory (by appointment only)
Lymphocyte function test	

3.4.11 Tissue & Biopsy specimens for microbiological investigations:

1. These specimens should be collected under strict aseptic conditions.
2. Send to the laboratory in a sterile screw capped container with ‘
3. sterile saline.

Indications:

1. 8ouLymph node biopsy - suspected mycobacterial disease
2. Biopsy of serous membranes for - suspected TB or any other chronic infection.
3. Gastric biopsy – for the detection of *Helicobacter pylori*
4. Brain biopsy – suspected Herpes encephalitis
5. Skin biopsy – leprosy, and other chronic skin ulcers

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SCHOOL OF BIO AND CHEMICAL ENGINEEING

DEPARTMENT OF BIOTECHNOLOGY

UNIT –III – MEDICAL LABORATORY TECHNOLOGY – SMB 1607

STAINNING

INTRODUCTION

The correct identification of micro organisms is of fundamental importance to microbial systematists as well as to scientists involved in many other areas of applied research and industry (e.g. agriculture, clinical microbiology and food production). Most bacteria are quite colourless and transparent and have a refractive index similar to that of the aqueous fluids in which they are suspended. Unless the diaphragm is carefully adjusted usually there is considerable difficulty in bringing the organisms into focus. Owing to the small size of bacteria little structural details can be seen with the ordinary light microscope unless the organisms are stained. The majority stain readily with aniline dyes. Some staining techniques, such as the Gram and Ziel Neelsen stains, although of great diagnostic value because of their differential staining properties for specific bacteria, reveal little internal structure. Other such as Feulgen stain for nuclear bodies, demonstrates specific structure. Because of its importance, different important stains are described in some detail.

HANGING DROP TECHNIQUE

The techniques employed are meant for microscopic observation of living bacteria. The motility

The study of bacterial morphology is performed in two ways:

1. Observing unstained cells live by hanging drop preparation.

2. Observing dead cells by making use of chemical nature of their unicellular, body. This is achieved by staining.

Hanging drop technique enables viewing of size shape, arrangement and motility of live microorganisms in fluid media. It requires the use of special ground slides. In this technique a loopful of bacterial suspension is placed in the centre of a cover slip. In the four corners tiny droplets of mineral oil are placed. The hollow ground slide is placed over the cover slip with the

depression side down and the slide is inverted quickly so that the water cannot run off to one side. However, the lack of contrast yields limited though valuable information. For pathogens one tube one plate method can be used. Each method has its advantage and limitations. The method you use will depend on which one is most suitable for the situation at hand.

Hanging Drop Preparation or Motility Test

1. Apply vaseline around the depression of the hanging-drop slide.
2. Using the inoculation loop, aseptically transfer one drop of the culture to the centre of a clean cover slip.
3. Invert the hanging-drop slide and centre its well over the drop of the culture, Press down on the edge of the cover slip so that the vaseline makes a firm seal.
4. Quickly and carefully turn the slide right side up so as to suspend the hanging drop in the well. Don't let the drop fall or touch the bottom of the well.
5. To examine, first locate its edge in centre of the microscopic field with low power objective and markedlv lower the light. The edge will be visible, as a bright wavy like against a dark background. Now the slide can be focused under oil immersion (Figure 6.1).

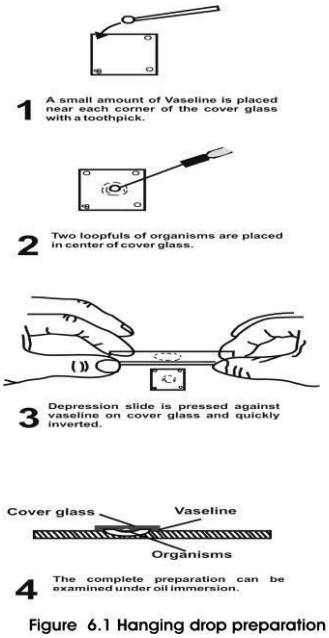


Figure 6.1 Hanging drop preparation

When working with pathogenic microorganisms such as the typhoid bacillus, it is too dangerous to attempt to determine motility with slide techniques. A much safer method is to culture the organisms in a special medium that can demonstrate the presence of motility. The procedure is to inoculate a tube of semisolid or SIM medium that can demonstrate the presence of motility. Both media have a very soft consistency that allows motile bacteria to migrate readily through them causing cloudiness. Following Figure 6.2 illustrates the inoculation procedure.

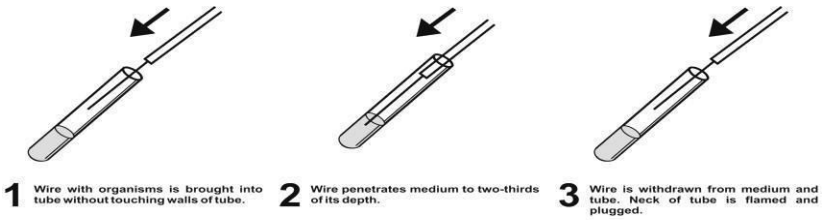


Figure 6.2 Stab technique for motility test

MICROBIAL STAINING

It is a chemical or a physical union between the dye and like component of a cell. If it is a chemical reaction a new compound is formed and a simple washing with water does not liberate the bound dye but if purely physical it is easy to decolorize such stained organism. Usually it is a combination of chemical and physical reactions.

The main advantages of staining are that it

(i) Provides contrast between microorganisms and their backgrounds, permitting differentiation among various morphological types;

(ii) Permits study of internal structures of the bacterial cell, such as the cell wall, vacuoles or nuclear bodies and other cellular structures;

(iii) and enables the bacteriologist to use higher magnifications.

Fixing

Before staining it is essential to fix the bacterial sample on to the slide. Smear is prepared in the following way:

(i) With a wire loop place a small drop of the broth culture or a loop full of bacteria on a clean slide.

(ii) Place a drop of water over it.

(iii) Spread the culture so as to form a thin film.

(iv) Allow slide to dry in the air or by holding it above a bunsen flame.

(v) Avoid excess heating.

The purpose of fixation is to kill the microorganisms, coagulate the protoplasm of the cell and cause it to adhere to the slide.

Type of Stains

SIMPLE STAINING

The use of a single stain to colour a bacterial organism is commonly referred as simple staining. All these dyes work well on bacteria as they have colour bearing ions (chromatophores) and are positively charged. The fact that bacteria are slightly negatively charged when the pH of the surrounding is near neutrality and produces a pronounced attraction between these cationic chromatophores and the organism so that the cell is stained. Such dyes are classified as basic dyes (Figure 6.3). Crystal violet and carbol fuschin are some other examples.

- Those dyes that have anionic chromatophores are called acidic dyes. Eosine (sodium^{+}eosine^{-}) is such a dye. The anionic chromatophores, eosine^{-} , will not stain bacteria because of the electrostatic repelling forces that are involved.

The staining times for most simple stains are relatively short, usually from 30 seconds to 2 minutes, depending on the affinity of the dye. After a smear has been stained for the required time, it is washed off gently, blotted dry, and examined directly under oil immersion. Such a

slide is useful in determining basic morphology and the presence or absence of certain kinds of granules.

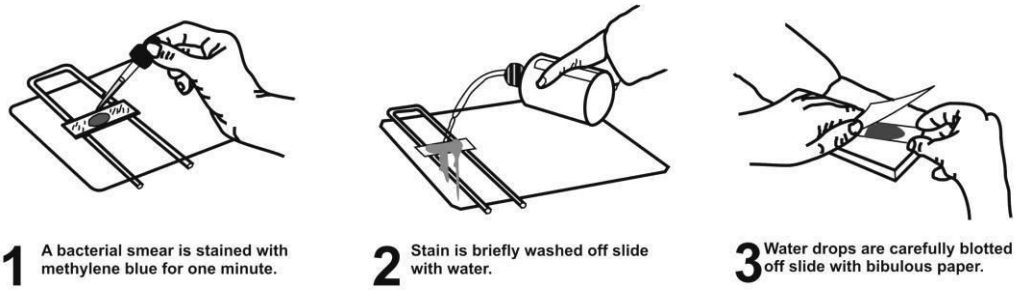


Figure 6.3 Demonstration of staining procedure

6.3.2.2 NEGATIVE STAINING

A better way to observe bacteria for the first time is to prepare a slide by a process called negative or background staining. This method consists of mixing the microorganisms in a small amount of nigrosine or india ink and spreading the mixture over the surface of the slide (nigrosine is far superior to india ink). Since these two pigments are not really bacterial stains, they do not penetrate the microorganisms; instead they obliterate the background, leaving the organisms transparent and visible in a darkened field. Although this technique has a limitation, it can be useful for determining cell morphology and size. Since no heat is applied to the slide, there is no shrinkage of the cell and consequently more accurate cell size determination result than with some other methods. This method is also useful for studying spirochaetes that does not stain readily with ordinary dyes.

Negative staining can be performed by one of the following methods. Figure 6.4 illustrates the more commonly used method in which the organisms are mixed in a drop of nigrosine and spread over the slide with another slide in order to prepare a smear that is thick at one end and feather thin at the other end. Somewhere between the too thick and too thin areas will be an ideal spot to study the organisms.

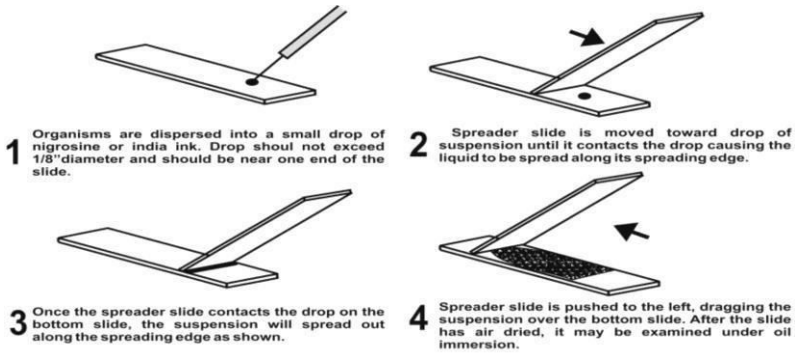


Figure 6.4 Demonstration of method of negative staining

In second method, the organisms are mixed in only a loopful of nigrosine instead of a full drop. The organisms are spread over a smaller area in the centre of the slide with an inoculating needle. No spreader slide is used in this method. It gives more accurate view of the bacterial cell (Figure 6.5).

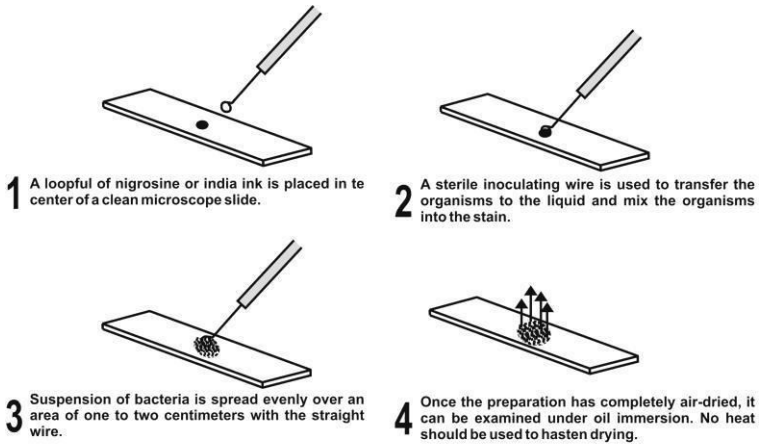
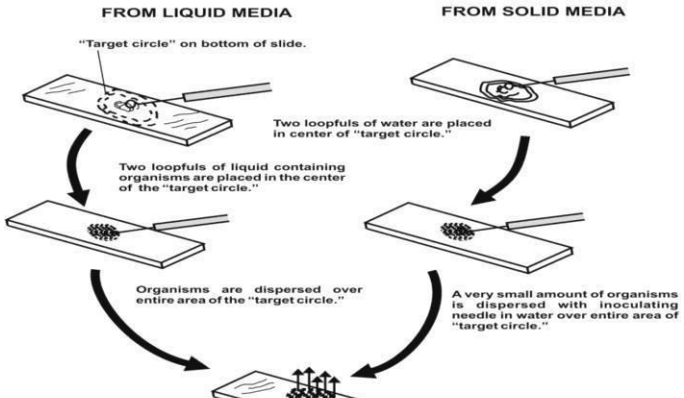


Figure 6.5 Method of negative staining by nigrosine

While negative staining is a simple enough process to make bacteria more visible with a brightfield microscope, it is of little help when one attempts to observe anatomical microstructures such as flagella, granule, and enodospore. Only by applying specific bacteriological stains to organisms one can see such organelles. However, success at bacterial staining depends on the preparation of a suitable smear of the organisms. A properly prepared bacterial smear is one that withstands one or more washings during staining without loss of organisms; should not be too thick; and does not result in excessive distortion due to cell shrinkage. The procedure is illustrated in Figure 6.6.



Differential Staining

Gram staining

Developed by Christian Gram in 1884; by using this procedure, bacteria are subdivided by their reaction to this stain into those which retain it, termed Gram-positive, and those which are decolourized, termed Gram-negative. Gram staining requires 4 different solutions.

(i) **A Basic Dye**: Discussed previously.

(ii) **A Mordant**: It increases the affinity or attraction between the cell and the dye, e.g. Iodine.

(iii) **A Decolorising agent**: It removes the dye from a stained cell, e.g., alcohol, acetone or ether.

(iv) **Counter stain**: It is a basic dye of a different colour than the initial one, e.g., Safranin.

Stain Preparation

(i) **Crystal Violet:** Dissolve 2 g of crystal violet in 20 mL of 95% ethanol. Dissolve 0.8 g of ammonium oxalate in 80 mL of distilled water. Mix these 2 solutions; stand for 24 h and f1lter.

(ii) **Gram's Iodine:** Dissolve 2 g of potassium iodide and 1 g of iodine in 300 mL of distilled water.

(iii) **Safranin:** Grind 0.25 g of Safranin in a mortar with 10 mL of 95% ethanol.

Principle

The differential response towards Gram reaction is attributed to the difference in the cell wall of these bacteria. Gram negative bacteria have cell wall generally thinner than those of gram positive bacteria. Gram negative ones have higher lipid content than the gram positive bacteria.

During staining of gram positive bacteria, the alcohol treatment extracts the lipid, which results in increased porosity or permeability of the cell wall. The CV-I (crystal violet iodine) complex can be extracted and the gram negative organism is decolorized. These cells subsequently take the colour of the safranin a *Counter stain.* The cell walls of gram positive bacteria, due to their different composition (lower lipid content) become dehydrated during treatment with alcohol presumably causes diminution in the pore diameter of the walls of peptioglycan and CV-I complex is trapped in the wall following ethanol treatment. The pore size decreases, permeability is reduced, and CV-I complex cannot be extracted. Hence these cells remain purple violet. While in gram negative bacteria the amount of peptidoglycan is very low hence the cross linking is reduced thus making space for crystal violet iodine complex to escape (Figure 6.7).

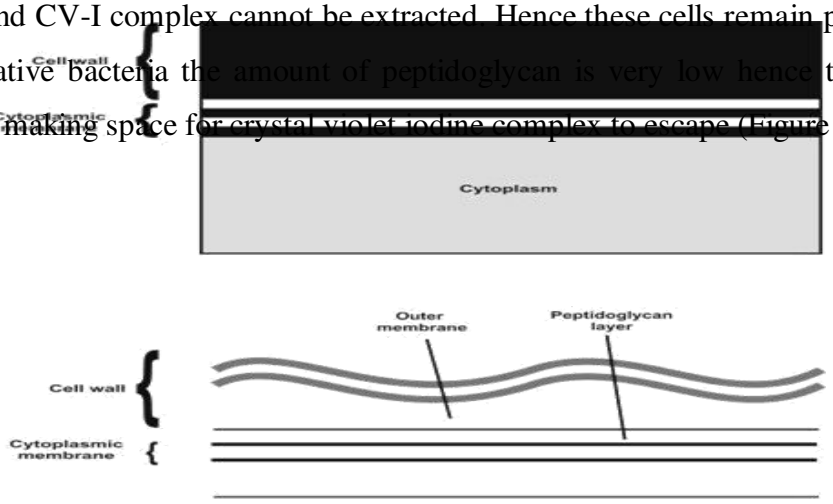


Figure 6.7 Cell Wall of Gram positive and Gram negative bacteria

Procedure

1. Prepare smear on a clean slide.

2. Stain with crystal violet for 30 seconds.

3. Rinse with water.

4. Flood the f1lm with Grams iodine and allow it to act for 30 sec. 5. Rinse with water.

6. Decolorise with 95% alcohol.

7. Rinse with water.

8. Counter stain with safranin for 20-30 sec. 9. Rinse with water and blot dry.

10. Examine under oil immersion objective.

Table 6.1 Steps in Gram Stain

Step	Process	Results	
		Gram+	Gram-
Initial stain Mordant Decolorisation Counterstain	CV for 30 sec. 12 for 30 sec. 95% alcohol for 10-20 sec. Safranin for 20-30 sec.	Stains purple Remains purple Remains purple Remains purple	Stains purple Remains purple Becomes colourless Stains pink

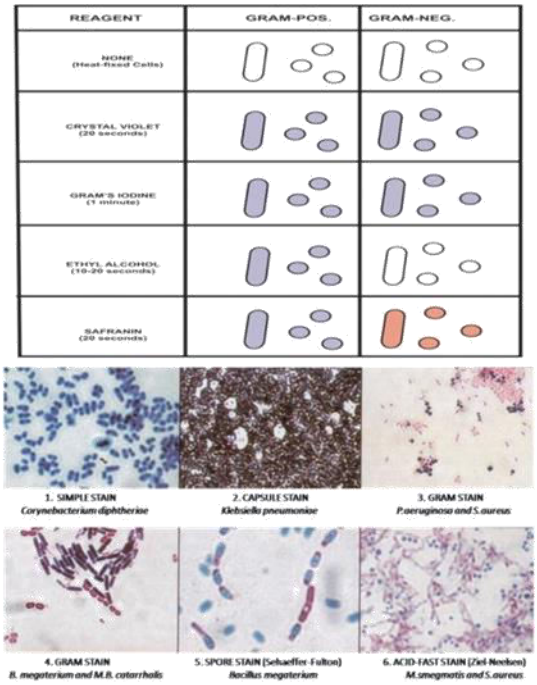


Figure 6.8 Demostration of the process of Gram staining and acid fast stain

Acid fast stain

The Ziel-Nehlson method employs carbol fuchsin, acid alcohol and a blue or green counter stain.

Acid fastness is a phenomenon that is found in certain types of bacteria where they resist the process of de-colorization that occurs when acid is used to wash a sample that contains these bacteria. These bacteria also resist staining and it may require heat and concentrated staining to colorize them. Once this colorization has taken place, it becomes difficult to decolorize using acid, or stain them with another color unless the heat and concentration technique is used. This is

why the term given to them is 'acid fast' just like one would use the term 'color fast' for cloth that does not leak color when washed (Figure 6.8).

The most common type of acid fast bacteria is mycobacteria. A strain of mycobacteria is responsible for the disease tuberculosis. In diagnostic medicine, the acid fast test may be used on a person in order to detect the existence of the bacteria that can cause tuberculosis. It is therefore used as a diagnosis of the disease because other symptoms of tuberculosis are not useful as they overlap with other conditions. Acid fast stain results can confirm the presence of the bacteria known as mycobacteria tuberculosis which is the bacteria responsible for causing tuberculosis. In laboratories where large number of sputum, gastric washings, urine, and other body fluid samples are tested for pathogenic mycobacteria, fluorochrome acid fast staining is used in conjunction with the Ziel-Nehlson method. The advantage of using a fluorescence method is that fluorochrome stained slides can be scanned under lower magnification, while a Ziel-Nehlson prepared slide must be examined under oil immersion (1000X magnification), fluorochrome stained slides can be examined with 60X or 100X magnification.

Stain Preparation

(i) Carbol fuchsin

1. Basic fuchsin 58 g
2. Crystalline phenol 25 g
3. 95% alcohol 50 mL
4. Distilled water to 500 mL

Dissolve the fuchsin and phenol in alcohol over a warm water bath, and then add water.

Filter, before use.

(ii)95% ethyl alcohol 970 mL

Concentrated HCl 30 mL

(iii) 0.5% methylene blue or malachite green in distilled water.

Procedure

Pour carbol fuchsin on the slide and heat carefully until steam rises. Stain for 3-5 min but do not allow to dry. Wash well with water. Decolorize using acid alcohol for 10-20 s. changing twice and counter stain for 2 min with methylene blue or malachite green. Acid fast organisms, e.g. *Mycobacterium tuberculosis and Mycobacterium leprae* stain well (Figure 6.9).

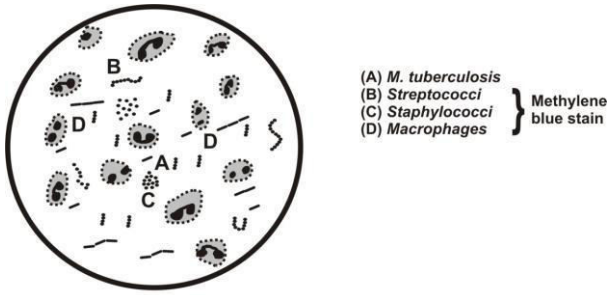


Figure 6.9 Carbol Fuchsin staining of acid fast bacteria

Structural Staining

Structural staining allows you to observe certain structures on bacteria. This is important because certain structures on bacteria can be antigenic or act as an endotoxin. Structural stains are more complex than simple ones and use more than one stain to differentiate cellular components. They are used to examine structural differences between bacterial groups or to provide contrast to different structures within the same organism. Some of these stains are explained below:

Endospore stain

Species of bacteria, belonging principally to the genera *Bacillus and Clostridium* produce extremely heat resistant structures called endospores. In addition to being heat-resistant, they are also resistant to many chemicals that destroy non-spore forming bacteria. This resistance to heat

and chemicals is due primarily to a thick, tough spore coat. They resist staining and, once stained they resist decolorisation and counter staining. Several methods are available that employ heat to provide stain penetration. However, the malachite green-Schaeffer and Fulton and Dorner

methods are commonly used by most bacteriologists. Thus endospore stains green but rest of the cell or a cell without endospore stains light red (Figure 6.10 and Figure 6.11).

Figure 6.10 Steps in the endospore stain

(i) The malachite green -Schaeffer and Fulton Method

Procedure

1. Prepare a thin smear on a clean slide.

2. Place the slide on staining rack above boiling water.

3. Cover the smear with small pieces of paper towel, keep saturated with malachite green (5% aqueous solution) and continue heating for 5 min.

4. Wash gently with water.

5. Counter stain with safranin for 30 seconds.

6. Wash with water and blot dry.

7. Examine under oil immersion objective.

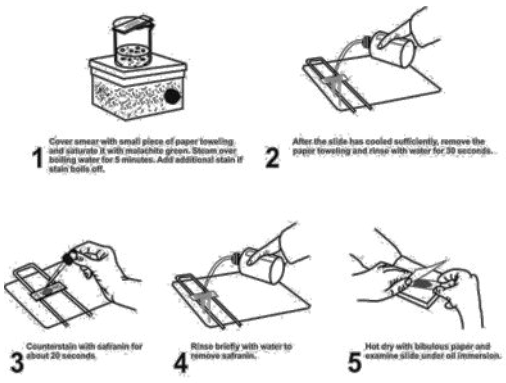


Figure 6.11 Malachite green -Schaeffer and Fulton Stain Method

Dorner method

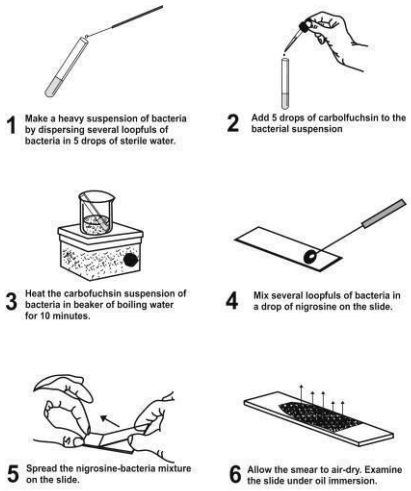


Figure 6.12 Dorner method of staining

The Dorner method for staining endospores produces a red spore within a colourless sporangium. Nigrosine is used to provide a dark background for contrast.The sporangium and endospore are stained during boiling in step 3, however, the sporangium is decolourized by the diffusion of safranin molecules into the nigrosine. The six steps involved in this technique are illustrated above (Figure 6.12).

Capsule stain

Place a small drop of Indian ink on a clean slide. Mix into it a small loopful of bacterial culture.

Drop a cover glass and blot off excess ink. Examine.

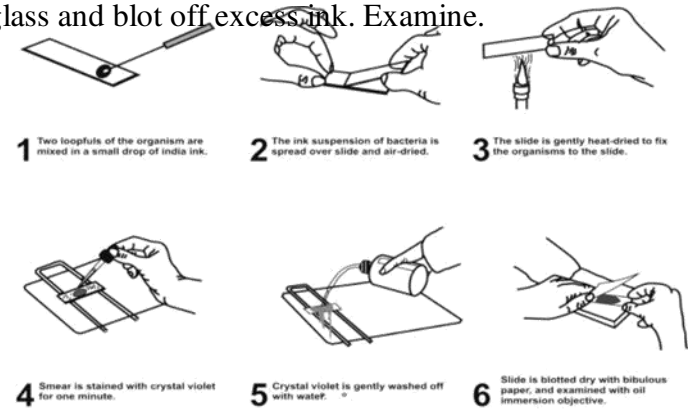


Figure 6.13 Process of Capsule staining

For dry preparations mix one loopful of Indian ink with one loopful of suspension of organism in 5% dextrose solutions at one end of a slide. Allow to dry and pour a. few drops of methyl alcohol on it keep the slide over the flame to fix. Stain for a few sec. with 0.5.% aq. solution of methyl violet. The capsule will appear as haloes in blue cell of bacterium under microscope (Figure 6.13).

Flagella stain

(i) **Mordant**

20% tannic acid solution	3 parts
5% tartar emetic solution	2 parts
Distilled water	5 parts

Mix and redissolve the heavy precipitate by boiling.

(ii) **Silver solution**: Dilute a saturated solution of silver sulphate with an equal volume of distilled water. Add 33% monoethylamine till the precipitate formed is redissolved. Solution can be stored for long term, heat gently before using (Figure 6.14).

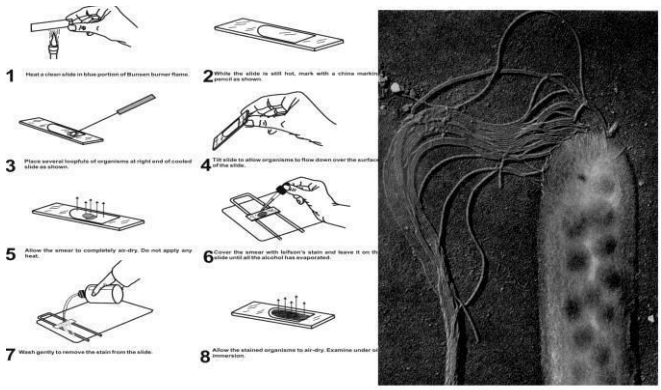


Figure 6.14 Demonstartion of flagella staining

Procedure

1. Take a clean slide and transfer a loopful of bacterial suspension holding the slide vertically. Allow the drop to run to the other end. Dry the film in air or in incubator.

2. Heat the mordant in a test tube to a boiling point and flood the slide with it wait for 2 minutes. Wash well in hot- water and finally in cold water. Allow to dry.

3. Cover the film with silver solution till thick portion of the film becomes dark to brown and metallic sheen appears on the edge.

4. Wash with water and blot dry.

5. For preparing a permanent slide dip the latter in a weak solution of gold chloride for 1 or 2 hr. Wash and mount in canada balasam.

FIGURE A-1. Blood collection for thin or thick blood films

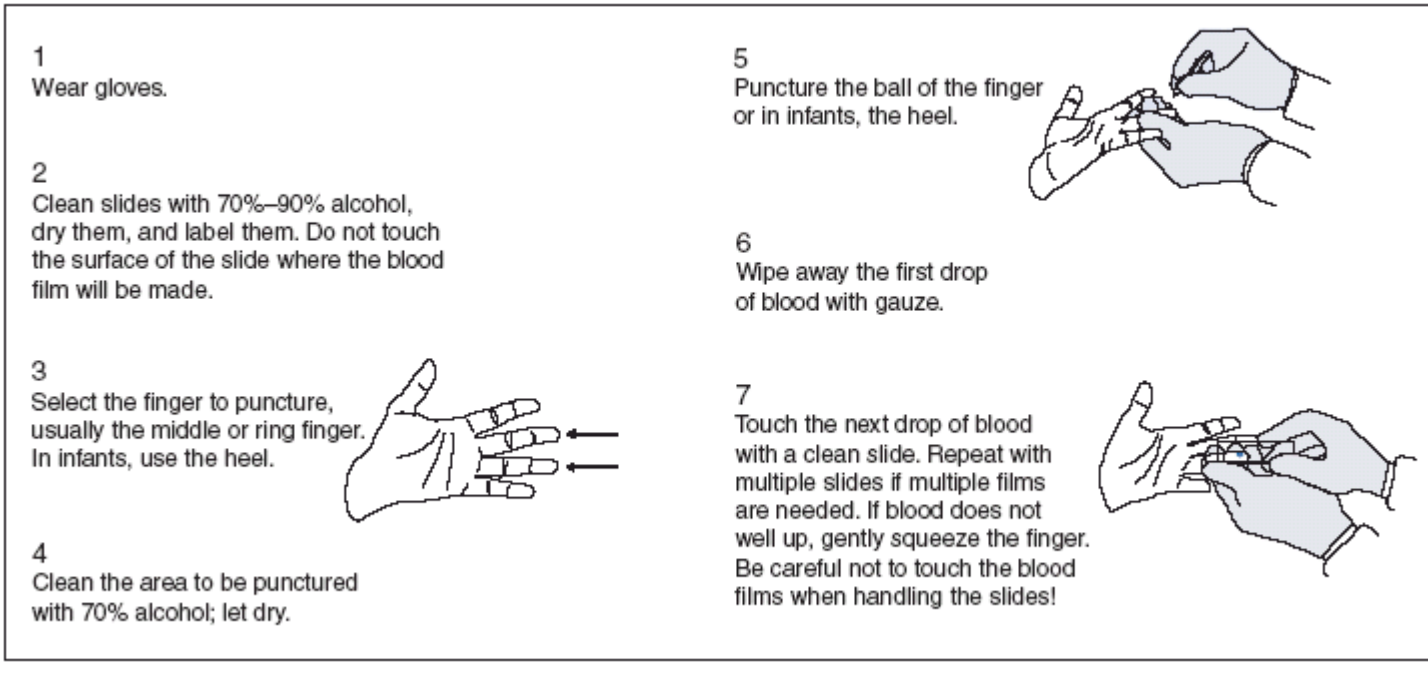
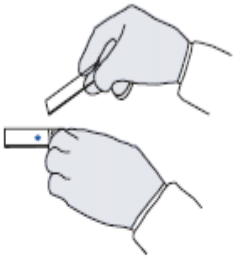


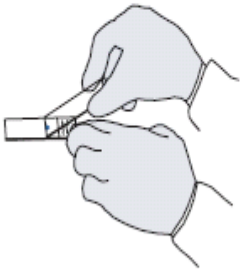
FIGURE A-2. Preparation of thin and thick blood films

1
Whenever possible, use separate slides for thick and thin films.

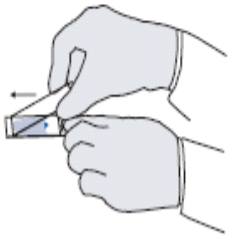
2
Thin film (a): Bring a clean spreader slide, held at a 45-deg angle, toward the drop of blood on the specimen slide.



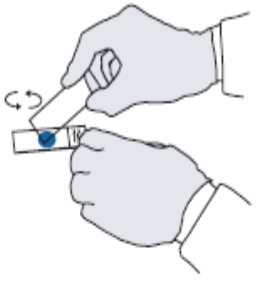
3
Thin film (b): Wait until the blood spreads along the entire width of the spreader slide.



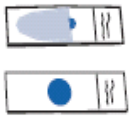
4
Thin film (c): While holding the spreader slide at the same angle, push it forward rapidly and smoothly.



5
Thick film: Using the corner of a clean spreader slide, spread the drop of blood in a circle the size of a dime (diameter 1–2 cm). Do not make the smear too thick or it will fall off the slide (you should be able to read newsprint through it).



6
Wait until the thin and thick films are completely dry. Fix the thin film with 100% (absolute) methanol. Do not fix the thick film.



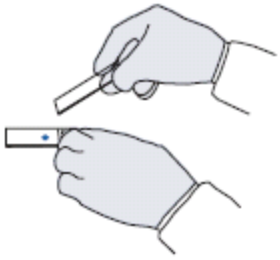
7
If both the thin and thick films must be made on the same slide, fix only the thin film with 100% (absolute) methanol. Do not fix the thick film.

8
When the thin and thick films are completely dry, stain them. Thick smears might take ≥1–2 hours to dry. Protect unstained blood smears from excessive heat, moisture, and insects by storing in a covered box.

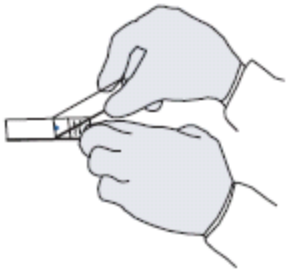
FIGURE A-2. Preparation of thin and thick blood films

1
Whenever possible, use separate slides for thick and thin films.

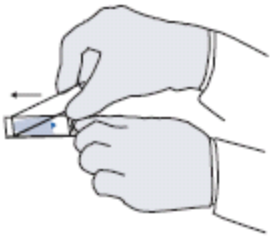
2
Thin film (a): Bring a clean spreader slide, held at a 45-deg angle, toward the drop of blood on the specimen slide.



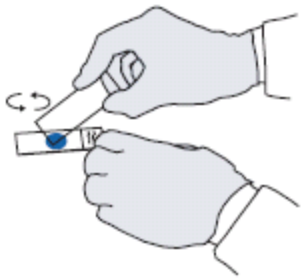
3
Thin film (b): Wait until the blood spreads along the entire width of the spreader slide.



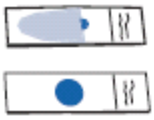
4
Thin film (c): While holding the spreader slide at the same angle, push it forward rapidly and smoothly.



5
Thick film: Using the corner of a clean spreader slide, spread the drop of blood in a circle the size of a dime (diameter 1–2 cm). Do not make the smear too thick or it will fall off the slide (you should be able to read newsprint through it).



6
Wait until the thin and thick films are completely dry. Fix the thin film with 100% (absolute) methanol. Do not fix the thick film.



7
If both the thin and thick films must be made on the same slide, fix only the thin film with 100% (absolute) methanol. Do not fix the thick film.

8
When the thin and thick films are completely dry, stain them. Thick smears might take ≥1–2 hours to dry. Protect unstained blood smears from excessive heat, moisture, and insects by storing in a covered box.

Preparation of Media and Reagents

Quality control (QC) of media

Each batch of media prepared in the laboratory and each new manufacturer's lot number of media should be tested using appropriate QC reference strains for sterility, the ability to support growth of the target organism(s), and/or the ability to produce proper biochemical reactions. A QC record should be maintained for all media prepared in the laboratory and purchased commercially; including preparation or purchase date and QC test results. Any unusual characteristic of the medium, such as color or texture, or slow growth of reference strains should be noted.

I. Routine agar and broth media

All agar media should be aseptically prepared and dispensed into 15x100 mm Petri dishes (15-20 ml per dish). After pouring, the plates should be kept at room temperature (25°C) for several hours to prevent excess condensation from forming on the covers of the dishes. For optimal growth, the plates should be placed in a sterile plastic bag and stored in an inverted position at 4ºC until use. All broth media should be stored in an appropriate container at 4ºC until use.

A. Blood agar plate (BAP): trypticase soy agar (TSA) + 5% sheep blood

A BAP is used as a general blood agar medium. It is used for growth and testing of *N.*

meningitidis and *S. pneumoniae.* The plate should appear a bright red color. If the plates appear dark red, they are either old or the blood was likely added when the agar was too hot. If so, the media should be discarded and a new batch should be prepared.

Media preparation

1. Prepare the volume of TSA needed in a flask according to the instructions given on the label of the dehydrated powder.

• It is convenient to prepare 500 ml of molten agar in a l-2 liter flask. If TSA broth powder is used, add 20 g agar into 500 ml of distilled water.

• The media should be heated and fully dissolved with no powder on the walls of the vessel before autoclaving.

2. Autoclave at 121°C for 20 minutes.

3. Cool to 60ºC in a water bath.

4. Add 5% sterile, defibrinated sheep blood (5 ml sheep blood can be added to 100 ml of agar).

1• If a different volume of basal medium is prepared, the amount of blood added must be adjusted accordingly to 5% (e.g., 50 ml of blood per liter of medium). Do NOT use human blood.

5. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

6. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow a *S. pneumoniae* or an *N. meningitidis* QC strain for 18-24 hours on a BAP at 35-37°C

with ~5% CO2 (or in a candle-jar).

2. Observe the BAP for specific colony morphology and hemolysis.

3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis).

• *N. meningitidis* should appear as large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.

• After 48 hours, the sterility test plate should remain clear.

B. Blood culture broth

Blood culture medium is used to grow *N. meningitidis, S. pneumoniae,* and *H. influenzae*.

Media preparation

1. Follow the manufacturer's instructions on the label of each bottle of dehydrated trypticase soy broth (TSB).

2. Add 0.25 g sodium polyanetholsulfonate (SPS) per liter of medium.

• SPS is especially important for recovery of *H. influenzae*.

3. Dispense in 20 ml (for a pediatric blood culture bottle) and 50 ml (for an adult blood culture bottle) amounts into suitable containers (tubes or bottles) with screw-caps with rubber diaphragms.

• The amount of liquid in the containers should make up at least two-thirds of the total

volume of the container.

24. Autoclave at 121ºC for 15 minutes.

5. Allow to cool and store medium at room temperature (25°C).

Quality control

1. Grow *N. meningitidis, S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a BAP or CAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Add 1-3 ml of sterile rabbit, horse, or human blood to 3 bottles of freshly prepared blood culture media.

3. Collect a loopful of overnight growth from each of the plates of bacteria and suspend it in 1-2 ml of blood culture broth (a different organism for each bottle).

4. Inoculate the bacterial suspensions into the 3 blood culture bottles.

5. Incubate the blood culture bottles at 35-37°C with ~5% CO2 (or in a candle-jar) for up to 7 days and observe for growth.

6. Subculture bacteria onto appropriate media at 14 hours and 48 hours.

7. As a sterility test, incubate an uninoculated blood culture bottle for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• All three bacteria should be recovered on appropriate media after 24 and 48 hours.

• After 48 hours, the sterility test plate should remain clear.

C. Chocolate agar plate (CAP)

CAP is a medium that supports the special growth requirements (hemin and NAD) needed for

the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO2 atmosphere. CAP has a reduced concentration of agar, which increases the moisture content of the medium. It can be prepared with heat-lysed horse blood, which is a good source of both hemin and NAD, although sheep blood can also be used. Growth occurs on a CAP because NAD is released from the blood during the heating process of chocolate agar preparation (the heating process also inactivates growth inhibitors) and hemin is available from non-hemolyzed as well as hemolyzed blood cells.

3**Media preparation**

1. Heat-lyse a volume of horse or sheep blood that is 5% of the total volume of media being prepared very slowly to 56°C in a water bath.

2. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

3. Place the plates in sterile plastic bags and store at 4ºC until use.

4. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Quality control

1. Grow *N. meningitidis, S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Observe the CAP for specific colony morphology and hemolysis.

Passing result:

• *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to

grey, opaque colonies on the CAP with no discoloration of the medium.

• *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.

• After 48 hours, the sterility test plate should remain clear.

D. CAP with bacitracin

CAP with bacitracin is a selective medium used to improve the primary isolation of *H. influenzae* from specimens containing a mixed flora of bacteria and/or fungi.

Media preparation

1. Prepare double strength TSA (20 g into 250 ml distilled water) as the basal medium.

2. Autoclave at 121ºC for 20 minutes.

3. Cool to 50°C in a water bath.

• Use a thermometer to verify the temperature in the water bath.

4. Prepare a solution of 2% hemoglobin (5 g in 250 ml distilled water). Mix the hemoglobin in 5-6 ml of distilled water to form a smooth paste. Continue mixing as the rest of the water is added.

45. Autoclave at 121ºC for 20 minutes.

6. Cool to 50ºC in a water bath.

7. Add the hemoglobin solution to the double strength TSA and continue to hold at 50ºC.

If a hemoglobin solution is unavailable, use the alternative method with defibrinated sheep blood described below:

1. Add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (5 ml blood to

100 ml agar) to full-strength TSA agar base (20 g in 500 ml distilled water).

• Do NOT use human blood.

2. Heat to 56ºC in a water bath then cool to 50ºC.

8. After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50ºC, add the growth supplement containing hemin and NAD to a final concentration of 1%. Mix the ingredients by gently swirling the flask in a figure 8 motion on the counter.

• Avoid forming bubbles.

9. Prepare a stock solution of bacitracin by suspending 3 g bacitracin in 20 ml distilled water. Filter-sterilize, dispense into 1 ml amounts, and store at -20°C or -70°C.

10. While the medium is still at 50ºC, add 1 ml stock solution of bacitracin (prepared in step 9) per 500 ml chocolate agar.

11. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

12. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow an *H. influenzae* QC strain for 18-24 hours on a CAP with bacitracin at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Observe the CAP with bacitracin for specific colony morphology and hemolysis.

3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with bacitracin with no discoloration of the medium.

5• After 48 hours, the sterility test plate should remain clear.

E. Chocolate agar with TSA and growth supplement

Chocolate agar with TSA and growth supplements is a medium that supports the special growth requirements (hemin and NAD) needed for the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO2 atmosphere.

Media preparation

1. Prepare double strength TSA (20 g into 250 ml distilled water) as the basal medium.

2. Autoclave at 121ºC for 20 minutes.

3. Cool to 50°C in a water bath.

• Use a thermometer to verify the temperature in the water bath.

4. Prepare a solution of 2% hemoglobin (5 g in 250 ml distilled water). Mix the hemoglobin in 5-6 ml of distilled water to form a smooth paste. Continue mixing as the rest of the water is added.

5. Autoclave at 121ºC for 20 minutes.

6. Cool to 50ºC in a water bath.

7. Add the hemoglobin solution to the double strength TSA and continue to hold at 50ºC.

If a hemoglobin solution is unavailable, use the alternative method with defibrinated sheep blood described below:

1. Add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (5 ml blood to 100 ml agar) to full-strength TSA agar base (20 g in 500 ml distilled water).

• Do NOT use human blood.

2. Heat to 56ºC in a water bath then cool to 50ºC.

8. After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50ºC, add the growth supplement containing hemin and NAD to a final concentration of 1%. Mix the ingredients by gently swirling the flask in a figure 8 motion on the counter.

• Avoid forming bubbles.

69. Dispense 20 ml in each 15x100 mm Petri dish. Allow the media to solidify and condensation to dry.

10. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow *N. meningitidis, S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Observe the chocolate agar with TSA and growth supplements for specific colony morphology and hemolysis.

3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to

grey, opaque colonies on the CAP with no discoloration of the medium.

• *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.

• After 48 hours, the sterility test plate should remain clear.

F. Chocolate agar with gonococcus medium (GC) base and growth supplement

Chocolate agar with GC base and growth supplement is a medium that supports the special growth requirements (hemin and NAD) needed for the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO2 atmosphere.

Media preparation

1. Suspend 7.2 g of GC agar base in 100 ml distilled water in a flask. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.

2. Autoclave the flask at 121ºC for 15 minutes.

3. Cool to 50ºC in a water bath.

4. Add 100 ml of warm distilled water to 2 g of soluble hemoglobin powder. Mix the powder with 5-10 ml of distilled water until a smooth paste is achieved. Gradually add the balance of water until the solution is homogenous. Continually stir the solution during the addition of water.

• Alternatively, 100 ml ready-made 2% sterile hemoglobin solution, warmed to 50ºC can be used.

75. Autoclave the solution at 121ºC for 15 minutes. Cool to 50ºC in a water bath.

6. Reconstitute lyophilized growth supplement containing NAD and hemin by aseptically

transferring 10 ml of the accompanying diluent with a sterile needle and syringe. Shake to assure complete solution. After reconstitution, use immediately, or store at 4ºC and use within 2 weeks.

7. Aseptically add 100 ml sterile hemoglobin solution and growth supplement to 100 ml of the GC agar base solution. Mix gently, but thoroughly, to avoid air bubbles in the agar.

8. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

9. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow *N. meningitidis, S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Observe the chocolate agar with TSA and growth supplements for specific colony morphology and hemolysis.

3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to grey, opaque colonies on the CAP with no discoloration of the medium.

• *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.

• After 48 hours, the sterility test plate should remain clear.

G. Chocolate agar slants

Chocolate agar slants for transport and short-term storage can be prepared in the same manner as described for agar plates with one difference: 4 ml of the medium should be dispensed into 16x125 mm screw-cap tubes and slanted before solidifying. Chocolate agar slants should look brown to brownish-red in color. Chocolate agar slants should tested for QC using the same methods used for QC testing of CAP.

8**H. Cystine trypticase agar (CTA) with 1% carbohydrate (a semi-solid medium)**

Media preparation

1. Follow the manufacturer's instructions for the amount of CTA medium to suspend in 900 ml of distilled water. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.

2. Autoclave the flask at 121°C for 15 minutes.

3. Cool to 50°C in a water bath.

4. Prepare a 10% glucose (also called dextrose) solution by adding 10 g glucose to 100 ml distilled water. Filter-sterilize using a 0.22 micron filter.

5. Aseptically add 100 ml of the 10% glucose solution from step 4 to 900 ml of CTA medium to obtain a final concentration of 1% glucose.

6. Aseptically dispense 7 ml of the medium into 16x125 mm screw-cap glass tubes.

7. Repeat this procedure for the remaining 3 carbohydrates: maltose, lactose, and sucrose.

8. Store at 4°C and warm to room temperature (25°C) before use.

Quality control

1. Grow *N. meningitidis, N. lactamica*, and *N. sicca* QC strains to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Allow the 4 CTA sugars, glucose, maltose, lactose, and sucrose, to warm to room temperature (25°C) and label the tubes with the name of the QC strain.

3. Remove 3-5 colonies from overnight growth on the BAP using a 1 µl disposable loop.

4. Stab the CTA sugar several times into the upper 10 mm of medium. Approximately 8 stabs with the same loopful are sufficient.

• Use a separate disposable loop for inoculating each carbohydrate to be tested.

5. Fasten the screw-cap of each tube loosely and place the tubes in a 35-37°C incubator without CO2. Incubate the CTA sugars for at least 72 hours (and up to 5 days) before discarding as negative.

6. Observe the CTA sugars for development of visible turbidity and color change to yellow.

9Passing result:

• Development of visible turbidity and a yellow color in the upper portion of the medium indicates growth of bacteria and production of acid and is interpreted as a positive test.

• *N. meningitidis* should utilize glucose and maltose, but not lactose or sucrose.

• *N. lactamica* should utilize glucose, maltose, and lactose, but not sucrose.

• *N. sicca* should utilize glucose, maltose, and sucrose, but not lactose.

I. *Haemophilus* test medium (HTM) plates

HTM is used for antimicrobial susceptibility testing for *H. influenzae*.

Media preparation

The Mueller-Hinton agar used to make HTM should be thymidine free to obtain consistent results if susceptibility to cotrimoxazole is to be tested.

1. Prepare a fresh hemin stock solution by dissolving 50 mg of hemin powder in 100 ml of 0.01 mol/L NaOH with heat and stirring until the powder is thoroughly dissolved.

2. Prepare a nicotinamide adenine dinucleotide (NAD) stock solution by dissolving 50 mg of NAD in 10 ml of distilled water; filter-sterilize.

3. Prepare Mueller-Hinton agar (MHA) from a commercially available dehydrated base according to the manufacturer's directions (or see protocol in section I.M.), adding 5 g of yeast extract and 30 ml of hemin stock solution to 1 L of MHA.

4. After autoclaving, cool the medium to 45°C to 50°C in a water bath.

5. Aseptically add 3 ml of the NAD stock solution.

6. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.

• Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.

• Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

• Using more or less agar will affect the susceptibility results.

7. Allow the media to solidify and condensation to dry.

8. The pH should be 7.2-7.4.

• Do not attempt to adjust the pH of the MHA test medium if it is outside the range.

109. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow a *H. influenzae* QC strain for 18-24 hours on a CAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Prepare a moderately heavy suspension of cells (comparable to a 1.0 McFarland standard) from overnight growth on the CAP in a suitable broth (trypticase soy, heart infusion, or peptone water) and mix well using a vortex.

3. Inoculate a HTM plate with 10 µl of the cell suspension using a sterile loop and streak for isolation.

4. Observe HTM plate for specific colony morphology.

5. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the HTM plate with no discoloration of the medium.

• After 48 hours, the sterility test plate should remain clear.

J. Heart infusion agar (HIA) and trypticase soy agar (TSA)

HIA and TSA are general purpose media used with or without blood for isolating and cultivating a number of microorganisms. The media should appear straw colored (yellowish to gold coloring). HIA and TSA are also used for determining the hemin (X factor) and NAD (V factor) growth requirements of *H. influenzae*.

Media preparation

1. Prepare the volume of HIA or TSA needed in a flask, according to the instructions on the label of the dehydrated medium.

• These media should be fully dissolved with no powder on the walls of the vessel, before autoclaving.

2. Autoclave at 121ºC for 20 minutes.

3. Cool to 50ºC in a water bath and dispense 20 ml into each 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

4. Place the plates in plastic bags and store at 4ºC until use.

11**Quality control**

1. Grow a *H. influenzae* QC strain for 18-24 hours on a CAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Prepare a moderately heavy suspension of cells (comparable to a 1.0 McFarland standard) from overnight growth on the CAP in a suitable broth (trypticase soy, heart infusion, or peptone water) and mix well using a vortex.

• Do not transfer any of the chocolate agar media from the plate to the cell suspension as even the smallest amount of agar will affect the test and may lead to misidentification of the bacteria.

3. Inoculate one half of the HIA or TSA plate with 10 µl of the cell suspension using a sterile loop or swab and allow the suspension to dry.

4. Place paper disks or strips containing hemin, NAD, and hemin/NAD on the inoculated plate after the inoculum has dried.

5. Carefully invert the plates and incubate for 18-24 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

6. Observe growth on the HIA or TSA plate around the paper disks or strips.

7. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing Result:

• *H. influenzae* should grow only around the hemin/NAD disk.

• After 48 hours, the sterility test plate should remain clear.

K. Heart infusion rabbit blood agar plate (HIA - rabbit blood)

HIA-rabbit blood is used for determining the hemolytic reaction of *Haemophilus* species. This medium should appear bright red and look very similar to BAP. If the medium is dark red, it should be discarded and a new batch should be prepared. Horse blood may be substituted for rabbit blood in this medium.

Media preparation

1. Prepare the volume of HIA needed in a flask according to the instructions on the label of the dehydrated medium.

2. Autoclave at 121ºC for 20 minutes.

3. Cool to 50ºC in a water bath.

124. Add 5% sterile, defibrinated rabbit blood (alternatively, 5 ml sheep blood can be added to 100 ml of agar).

5. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation

to dry.

6. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow a hemolytic *H. haemolyticus* QC strain for 18-24 hours on a HIA-rabbit blood at 35-37°C with ~5% CO2 (or in a candle-jar).

• After streaking the plate with the QC stain, stab the media with the inoculating loop.

2. Observe the HIA-rabbit blood for specific colony morphology and beta-hemolysis (clear).

3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *H. haemolyticus* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies and be surrounded by a distinct zone of complete hemolysis which appears as a clear halo surrounding the colonies.

• After 48 hours, the sterility test plate should remain clear.

L. Horse blood agar (blood agar base)

This highly nutritive medium may be used for the primary isolation of *H. influenzae* and for the determination of the hemolysis with *H. haemolyticus* or other bacteria.

Media preparation

1. Prepare the volume of blood agar base needed in a flask according to the manufacturer's instructions on the label of the dehydrated medium.

2. Autoclave at 121ºC for 15 minutes.

3. Cool to 50ºC in a water bath.

4. Add 5 ml horse blood per 100 ml of the medium.

5. Mix well and dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

136. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow a hemolytic *H. haemolyticus* QC strain for 18-24 hours on a horse blood agar plate at 35-37°C with ~5% CO2 (or in a candle-jar).

• After streaking the plate with the QC stain, stab the media with the inoculating loop.

2. Observe the horse blood agar for specific colony morphology and beta-hemolysis (clear).

3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *H. haemolyticus* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies and be surrounded by a distinct zone of complete hemolysis which appears as a clear halo surrounding the colonies.

• After 48 hours, the sterility test plate should remain clear.

M. MacConkey (MAC) agar

MacConkey Agar is used for the isolation and differentiation of lactose-nonfermenting, gram negative enteric bacteria from lactose-fermenting organisms. It is recommended that MAC medium be purchased commercially because preparing it with individual ingredients produces

variability among lots.

Media preparation

1. Prepare MAC according to manufacturer's instructions.

2. Sterilize the medium by autoclaving at 121°C for 15 minutes.

3. Cool to 50°C in a water bath.

4. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

5. Place the plates in sterile plastic bags and store at 4ºC until use.

14**Quality control**

1. Grow an *E. coli* QC strain for 18-24 hours on a MAC at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Observe the MAC for specific colony morphology.

3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *E. coli* should appear as pink to red colonies.

• After 48 hours, the sterility test plate should remain clear.

N. Modified Thayer-Martin (MTM) agar medium

MTM is a selective medium used to improve the primary isolation of *N. meningitidis* from specimens containing a mixed flora of bacteria and/or fungi. MTM is a chocolate agar base containing vancomycin, colistin, nystatin, and trimethoprim lactate.

Media preparation

1. Suspend 7.2 g of GC agar base in 100 ml distilled water in a flask. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.

2. Autoclave the flask at 121ºC for 15 minutes.

3. Cool to 50ºC in a water bath.

4. Add 100 ml of warm distilled water to 2 g of soluble hemoglobin powder. Mix the powder with 5-10 ml of distilled water until a smooth paste is achieved. Gradually add the balance of water until the solution is homogenous. Continually stir the solution during the addition of water.

• Alternatively, 100 ml ready-made 2% sterile hemoglobin solution, warmed to 50ºC can be used.

5. Autoclave the solution at 121ºC for 15 minutes. Cool to 50ºC in a water bath.

6. Reconstitute lyophilized growth supplement containing NAD and hemin by aseptically transferring 10 ml of the accompanying diluent with a sterile needle and syringe. Shake to assure complete solution. After reconstitution, use immediately or store at 4ºC and use within 2 weeks.

7. Aseptically add 100 ml sterile hemoglobin solution and growth supplement to 100 ml of the GC agar base solution. Mix gently, but thoroughly, to avoid air bubbles in the agar.

158. To the agar base solution, add the following ingredients:

3.0 µg/ml vancomycin

7.5 µg/ml colistin

12.5 units/ml nystatin

5.0 µg/ml trimethoprim lactate

9. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

10. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow a *N. meningitidis* QC strain for 18-24 hours on a MTM at 35-37°C with ~5% CO2 (or in a candle-jar).

2. Observe the MTM for specific colony morphology.

3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *N. meningitidis* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the MTM with no discoloration of the medium.

• After 48 hours, the sterility test plate should remain clear.

O. Mueller-Hinton agar

Mueller-Hinton agar (MHA) is used for making the media required for susceptibility testing for *N. meningitidis*, *S. pneumoniae,* and *H. influenzae*. It is recommended that dehydrated Mueller Hinton agar medium be purchased commercially because preparing it with individual ingredients can diminish the quality.

Media Preparation

1. Follow manufacturer's instructions to prepare MHA from a commercially available dehydrated base.

2. After autoclaving, cool the agar in a 45°C to 50°C water bath.

3. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.

• Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.

• Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

16• Using more or less agar will affect the susceptibility results.

4. Allow the media to solidify and condensation to dry.

5. The pH of MHA should be 7.2-7.4.

• Do not attempt to adjust the pH of the MHA test medium if it is outside the range.

6. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow an *E. coli* QC strain for 18-24 hours on a MHA plate at 35-37°C with ~5% CO2 (or in a candle-jar).

2. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• Observe for *E. coli* growth.

• After 48 hours, the sterility test plate should remain clear.

P. Mueller-Hinton agar with 5% sheep or horse blood

Mueller-Hinton agar with 5% sheep or horse blood is used for susceptibility testing for *N. meningitidis* and *S. pneumoniae*. It is recommended that dehydrated MHA medium be purchased commercially because preparing it with individual ingredients can diminish the quality.

Media preparation

1. Prepare MHA as described above in Section I.M. through step 2.

2. Add 5% sterile defibrinated sheep or horse blood to the medium at 5% (i.e., 50 ml blood per liter of medium or 25 ml blood to 500 ml medium.

3. The pH of MHA after the addition of blood should be 7.2-7.4.

• Do not attempt to adjust the pH if it is outside the range.

4. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.

• Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.

17• Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

• Using more or less agar will affect the susceptibility results.

5. Allow the media to solidify and condensation to dry.

6. Place the plates in sterile plastic bags and store at 4ºC until use.

Quality control

1. Grow a *S. pneumoniae* or a *N. meningitidis* QC strain for 18-24 hours on Mueller-Hinton

agar with 5% sheep or horse blood at 35-37°C with ~5% CO2 (or in a candle-jar).

2. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

• *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis).

• *N. meningitidis* should appear large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.

• After 48 hours, the sterility test plate should remain clear.

Q. Mueller-Hinton Broth

Mueller-Hinton broth is used to prepare the Mueller-Hinton broth (cation-adjusted).

Media Preparation

1. To 750 ml deionized H2O add:

3.0 g beef extract

17.5 g acid hydrolysate of casein (casamino acids)

1.5 g soluble starch

2. Adjust final volume to 1 liter.

3. Adjust pH to 7.3.

4. Autoclave the broth at 121°C for 20 minutes to sterilize.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO2 (or in

a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

18**R. Mueller-Hinton broth (cation-adjusted)**

Mueller-Hinton broth (cation-adjusted) is used to prepare dilutions equivalent McFarland standards with minimal loss of viability.

Media Preparation

1. Prepare a magnesium stock solution by dissolving 8.36 g of $MgCl_2 \cdot 6H_2O$ in 100 ml deionized H_2O to a final concentration of 10 ng/ml Mg^{2+}

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2. Prepare a calcium stock solution by dissolving 3.68 g of $CaCl_2 \cdot 2H_2O$ in 100 ml deionized H_2O to a final concentration of 10 mg/ml Ca^{2+}

.

3. Filter-sterilize both stock solutions.

4. To Mueller-Hinton broth, add the magnesium stock solution to a final concentration of 10-12.5 µg/ml Mg^{2+}

.

5. Add the calcium stock solution to a final concentration of 20-25 µg/ml Ca^{2+}

.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO_2 (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

S. Trypticase soy broth (TSB)

TSB is used for making suspensions of *H. influenzae* prior to testing for hemin and NAD requirements. Heart infusion broth, sterile saline, or PBS may be substituted for TSB.

Media preparation

1. Prepare the volume of TSB needed in a flask according to the instructions on the label of the dehydrated medium.

2. Dispense 5 ml into 15x125 mm tubes.

3. Autoclave at 121ºC for 20 minutes.

4. Cool and store at 4ºC.

19**Quality control**

1. Grow a *S. pneumoniae* QC strain for 18-24 hours on a BAP at 35-37°C with ~5% CO2 (or in a candle-jar).

• *H. influenzae* does not grow in TSB, but the medium should not be toxic to other bacteria. Therefore, *S. pneumoniae* should be used to QC for toxicity.

2. Inoculate a tube of TSB with a loop of overnight growth from the BAP and incubate overnight at 35ºC.

3. The broth should be turbid the next day. Subculture the broth onto a BAP to test for proper growth characteristics of *S. pneumoniae*.

4. Observe the BAP for specific colony morphology and hemolysis.

Passing result: *S. pneumoniae* colonies are small and appear grey to grey-green surrounded by a

distinct green halo (alpha-hemolysis).

II. Storage and transport media

A. Defibrinated sheep blood

Defibrinated sheep blood is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. Mechanically shake 30 ml sheep blood with sterile glass beads or a wooden stick device in a 125-250 ml Erlenmeyer flask at approximately 90 rpm for 7-9 minutes.

• Clotting factors will be visible in the flask as a translucent, fibrous web.

2. Remove the clotting factors using sterile forceps.

3. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

B. Dorset Transport medium

Dorset Transport medium is used for short term storage of *N. meningitidis, S. pneumoniae*, and *H. influenzae* isolates.

20**Media preparation**

1. Combine sterile 0.85 % saline solution with beaten whole hen's eggs at a 1:3 ratio.

2. Inspissate (i.e., thicken) the mixture in an electric inspissator at 80°C for 60 minutes.

3. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

C. Greaves solution

Greaves solution is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. To 200.0 ml distilled water, add:

10.0 g bovine albumin, fraction V

10.0 g L-glutamic acid, sodium salt

20.0 ml glycerol

2. Mix all ingredients listed below until they are completely dissolved.

3. Filter-sterilize the solution and transfer to a sterile flask.

4. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

21**D. Modified Trans-Isolate (MT-I) medium**

Modified Trans-Isolate medium (MT-I) was designed and developed to be a simple and inexpensive medium for transport of CSF and growth of meningococci in large scale epidemics

of meningococcal disease. It is less expensive and easier to produce than the standard T-I, and can be produced in most microbiology laboratories at approximately USD $0.50/bottle, providing a relatively inexpensive, rapidly available (reducing the production and shipping time and cost) transport medium and diagnostic tool for use during a meningitis epidemic. The ingredients of the MT-I formulation are similar to those in T-I, with modifications to eliminate costly ingredients and save time.

Laboratory evaluation has demonstrated that growth/survival of isolates of meningococci was equal in T-I and MT-I under several environmental conditions. It has not been evaluated under field conditions with clinical specimens yet. It does not support the growth of *H. influenzae*; therefore, it is not used for routine surveillance of agents of bacterial meningitis.

0.5 oz, sterile round, clear glass screw-cap (with rubber liner) bottles should be used.

MT-I bottles should be stored upright at 4

o

C when not in immediate use and warmed to room

temperature (25-30°C) before use. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature. MT-I media has a shelf life of 1 year with proper storage.

Media preparation

1. Weigh all ingredients listed below and place them into a 500mL Erlenmeyer flask.

8 g 2.0% gelatin

2 g 0.5% agar

2.5 g Tris-HCl

0.5 g Tris base

12 g 3.0% Tryptic Soy Broth

2. Add 350 ml distilled, deionized water to the flask (pH should be 7.5 at 25°C).

3. Place a magnetic stirring bar in the flask and place the flask on hot plate stirrer.

4. Bring the solution to boiling (90-100

o

C) to melt and dissolve the gelatin until the medium is

completely clear (about 30-45 minutes).

5. Remove the flask from the hot plate stirrer.

6. Mix 2.6 g 0.5% soluble starch with a small amount of cold water and dissolve completely.

7. Once the starch is evenly distributed throughout medium, add 1.6 g 0.4% activated charcoal and adjust water to 400ml.

228. Return the flask to the hot plate stirrer until all ingredients are thoroughly mixed (about 10 minutes).

• The solution should be liquid, appear black in color, and should not have any clumps.

9. Turn down the heat to low so the flask can be handled comfortably while dispensing the medium.

• Optional: At this step, the procedure can be stopped overnight if there is not enough time to dispense. Split the media into two flasks and place both flasks at 4ºC overnight. The next day, apply heat until fully melted and aliquot (see step 10).

10. While the media is being stirred, use a sterile serological pipette to remove 7 ml of medium

and dispense into each bottle.

11. Cap each bottle loosely with a screw cap.

12. Autoclave the bottles for 15 minutes at 121 C.

13. Tighten the caps as soon as possible after autoclaving.

14. Swirl the bottles to avoid the charcoal settling.

15. Slant the bottles overnight (or at least 4 hours) on wooden slanting stick (35 mm, 35 mm, 500 mm). • When slanted, the liquid should reach shoulder of the bottle.

16. Once the medium is firm, stand the bottles upright.

17. In approximately 1 hour, the weak slant should release some of the broth and the medium should appear biphasic.

18. Store at 4°C when not in use.

Quality control

1. Grow *N. meningitidis* and *S. pneumoniae* QC strains for 18-24 hours on a BAP or a CAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. For each organism, make a cell suspension equivalent to a 0.5 McFarland standard (~equivalent to 1.5x108

CFU/ml) and serially dilute it to achieve an inoculum size of 10^3

CFU/ml in brain heart infusion (BHI) broth.

3. Remove the screw cap from 3 MT-I bottles.

234. Inoculate the MT-I bottles with 100 µl of the 10^3

CFU/ml suspensions within 15 minutes of

preparation (inoculum size is 100 CFU) and replace the screw caps tightly.

• After inoculation and replacement of the screw-caps, invert each MT-I bottle several times to mix.

5. Slightly loosen the screw-caps of the MT-I bottles to allow some air exchange.

6. Incubate the MT-I bottles for 18-24 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

7. Close the screw-caps tightly and invert each MT-I bottle several times to mix.

8. Remove 10 µl of broth from each MT-I bottle and inoculate a BAP for the MT-Is containing *N. meningitidis* and *S. pneumoniae* and a CAP for the MT-I containing *H. influenzae*.

9. Streak for isolation with a sterile loop and incubate plates for 18-24 hours at 35-37°C with ~5% CO2 (or in a candle-jar) to detect growth of the QC strains.

Passing results:

• *N. meningitidis* should appear large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.

• *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis) on the BAP.

E. 10% skim milk and 15% glycerol solution

10% skim milk and 15% glycerol solution is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. Place 10 g dehydrated skim milk and 85 ml distilled water into flask A. Swirl to mix.

2. Place 15 ml of glycerol into flask B.

3. Autoclave both flasks at 115°C for 10 min, and exhaust the pressure carefully.

4. While still hot, pour the contents of flask A into flask B in a safety cabinet.

5. Store at 4°C when not in use.

24**Quality control**

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

F. Skim milk tryptone glucose glycerol (STGG) medium

STGG medium is used for transport and short term storage of nasopharyngeal swabs.

Media preparation

1. Add the following ingredients to 100 ml distilled water:

2 g skim milk powder

3 g TSB

0.5 g glucose

10 ml glycerol

2. Mix to completely dissolve all ingredients.

3. Dispense 1.0 ml amounts into 1.5 ml screw-cap vials.

4. Loosen the screw-caps and autoclave at 121°C for 10 minutes.

5. Tighten the caps after autoclaving and store at -20°C until use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO2 (or in

a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

G. Trans-Isolate (T-I) medium

T-I is used to transport CSF and grow *N. meningitidis, H. influenzae,* and *S. pneumoniae* from CSF. 10 cc tubing vials with rubber septum plugs and aluminum crimp seal caps should be used. T-I bottles should be stored upright at 4ºC when not in immediate use. T-I media should be warmed to room temperature (25ºC) before use. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature. T-I media has a shelf life of 1 year with proper storage.

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Media preparation

Diluent for solid and liquid phases: 3- (N-morpholino) propanesulfonic acid (MOPS) buffer; 0.1 M, pH 7.2

1. Dissolve 20.93g MOPS in 900ml distilled water.

2. Adjust to pH 7.2 with 1N NaOH.

3. Adjust volume to 1000 ml with distilled water.

Solid phase

Activated charcoal 2.0 g

Soluble starch 2.5 g

Agar 10.0 g

1. Suspend activated charcoal, soluble starch, and agar into 500 ml of MOPS buffer in a flask

and add a magnetic bar to the flask.

2. Heat on a magnetic stirrer hot plate to dissolve the charcoal and starch and melt the agar.

3. While stirring to keep the charcoal in suspension, use a sterile serological pipette to remove 5.0 ml and dispense into each serum bottle.

4. Cap each bottle with a piece of aluminum foil and autoclave at 121°C for 20 minutes.

5. Remove from the autoclave and slant the bottles until they reach room temperature (25°C), so that the apex of the agar reaches the shoulder of each bottle.

Liquid phase

Tryptic soy broth (TSB) 30.0 g

Gelatin 10.0 g

MOPS buffer 500.0 ml

1. Heat the TSB, gelatin, and MOPS buffer to completely dissolve the gelatin and avoid coagulation.

2. Autoclave the media at 121°C for 15 minutes.**Optional additive for growth of *H. influenzae***

1. Once the bulk liquid phase medium has cooled to room temperature (25°C) after autoclaving, add 10 ml of a sterile liquid growth supplement containing NAD and hemin aseptically to help support growth of *H. influenzae*.

• Alternatively, aseptically add 0.1 ml of the supplement to an individual T-I bottle (1% of the volume of both phases) or to a limited number of bottles, as needed.

• If a commercial growth supplement is used (preferred method), it should be a sterile product and can be added directly to the T-I medium.

• If the growth supplement is prepared in the laboratory, it should be filter-sterilized prior to being added to the T-I medium. To prepare the supplement in the laboratory:

1. Dissolve the lyophilized supplement into an appropriate diluent (usually water).

2. Filter-sterilize using a 0.45 micron pore size membrane.

3. Use immediately.

T-I medium

1. Dispense 5 ml of the liquid phase aseptically into each of the bottles containing the solid phase slants.

2. Seal the bottles with sterile rubber septum plugs and aluminum caps. Use a hand crimping tool to fasten the aluminum caps if an automated system is not available.

3. Store at 4°C when not in use.

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a BAP or CAP at 35-37°C with ~5% CO2 (or in a candle-jar).

2. For each organism, make a cell suspension equivalent to a 0.5 McFarland standard (~equivalent to $1.5X10^8$

CFU/ml) and serially dilute to achieve an inoculum size of 10^3

CFU/ml in brain heart infusion (BHI) broth.

3. Using sterile forceps pull the aluminum cover of 3 T-I bottles away from the rubber stopper and wipe the stopper with 70% isopropanol or ethanol.

• Do not use povidone-iodine.

274. Use a sterile syringe and needle to inoculate the T-I bottles with 100 µl of the 103 CFU/ml

suspensions within 15 minutes of preparation (inoculum size is 100 CFU).

• Use a new needle and syringe for each T-I. After inoculation and removal of the syringe and needle from the rubber stopper, invert each T-I bottle several times to mix.

5. Insert a sterilized T-I venting needle through the rubber stopper of each of the inoculated T-I bottles.

• Be sure that the venting needles do not touch the broth.

6. Incubate vented T-I bottles for 18-24 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

7. Remove and discard venting needles. Invert each T-I bottle several times to mix.

8. Use a sterile syringe and needle to remove 100-200 µl of broth from each T-I bottle and place the broth into a sterile, labeled 1.5 ml microcentrifuge tube. Inoculate a BAP for the T-Is containing *N. meningitidis* and *S. pneumoniae* and a CAP for the T-I containing *H. influenzae* with 10 µl of this broth.

9. Streak for isolated colonies with a sterile loop and incubate plates for 18-24 hours at 35-37°C with ~5% CO2 (or in a candle-jar) to detect growth of the QC strains.

Passing results:

• *N. meningitidis* should appear as large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.

• *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis) on the BAP.

• *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.

III.Miscellaneous reagents

A. Gram stain reagents

Ammonium oxalate-crystal violet

1. Dissolve 2.0 g certified crystal violet into 20.0 ml of 95% ethyl alcohol.

2. Dissolve 0.8 g ammonium oxalate into 80.0 ml distilled water.

3. Mix the two solutions together and allow them to stand overnight at room temperature (25°C).

4. Filter through coarse filter paper before use.

285. Store at room temperature (25°C).

Gram's iodine (protect solution from light)

1. Grind 1.0 g iodine (crystalline) and 2.0 g potassium iodide in a mortar. Small additions of distilled water may be helpful in preparing the solution.

2. Add to 300.0 ml distilled water.

3. Store at room temperature (25°C) in a foil-covered bottle.

Decolorizer is 95% ethyl alcohol

Counterstain (there are 2 options: safranin or carbol-fuchin)

Safranin

1. Add 2.5 g certified safranin-O to 100.0 ml 95% ethyl alcohol.

2. Add 10.0 ml safranin and ethyl alcohol solution made in step 1 to 90.0 ml distilled water.

3. Store at room temperature (25°C).

Ziehl-Nielsen carbol-fuchsin (may be a more effective counterstain than safranin)

1. Dissolve 0.3 g basic fuchsin in 10.0 ml 95% ethyl alcohol.

2. Add 5.0 ml melted phenol crystals to 95.0 ml distilled water.

3. Add the 5% phenol solution to the fuchsin solution and let stand overnight.

4. Filter through coarse filter paper.

5. Store at room temperature (25°C) in a foil-covered bottle for up to 1 year.

B. Iodine tincture

Iodine tincture is used as a skin antiseptic and should not be used to disinfect the rubber stoppers of T-I and blood culture bottles.

1. Add 1 g of iodine to 100 ml of 70% isopropyl alcohol.

2. Store at room temperature (25°C) in a foil-covered bottle for up to 1 year.

C. McFarland turbidity standards

1. Prepare a 1% solution of anhydrous $BaCl_2$ (barium chloride).

2. Prepare a 1% solution of H_2SO_4 (sulfuric acid).

3. Combine and completely mix the barium chloride and sulfuric acid solutions to form a turbid suspension of $BaSO_4$ in a specific proportion for each McFarland turbidity standard as shown in Table 1.

4. Place the resulting mixture in a foil-covered screw-cap tube.

5. Store the McFarland standard at room temperature (25°C) when not in use. Prepare a fresh

standard solution every 6 months. McFarland standard density solution will precipitate and clump over time, and it needs vigorous vortexing before each use. Mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred.

Table 1. McFarland turbidity standards

McFarland turbidity standard no. **0.5 1 2 3 4**

1% barium chloride (ml) 0.05 0.1 0.2 0.3 0.4

1% sulfuric acid (ml) 9.95 9.9 9.8 9.7 9.6

Approx. cell density (1x10^8 CFU/ml) 1.5 3.0 6.0 9.0 12.0

D. Phosphate buffered saline (PBS)

1. To 1000 ml distilled water, add the following ingredients for 0.1 M PBS:

7 g sodium dihydrogen phosphate

7 g disodium hydrogen phosphate

2. Mix to completely dissolve ingredients.

3. Adjust pH to 7.2 with 1 N acid or base.

4. Dispense buffer into a flask and autoclave at 121°C for 15 minutes.

5. Store at room temperature (25°C) for up to one year.

E. Physiological saline

1. Dissolve 8.5 g NaCl into 1 L distilled water.

2. Autoclave at 121°C for 15 minutes or sterilize using membrane filtration.

3. Store at room temperature (25°C) for up to 6 months.

Microscopic bacteria grow together in visible colonies. Learn the characteristics and vocabulary used to describe the appearance of those colonies and test your knowledge with practice examples in this lesson.

What Is a Bacterial Colony?

What do you know about bacteria? You probably know that they cause illnesses, that they are too small to see, and that, in the case of 'germs', they can be transmitted through the air via coughs and sneezes or found on surfaces. Maybe you even have some antibacterial soap or spray to get rid of these invisible, unwelcome guests. If you have ever had strep throat, you may have gone to your doctor and had your throat swabbed for a culture. In this lesson, you will learn what a bacterial culture looks like and how to observe and interpret a culture.

As we discussed, a **bacterium** (plural bacteria) is a single-celled organism too small to be seen without a microscope. In order to grow bacteria, known as culturing, the bacteria are spread onto the surface of agar contained within a petri dish. This agar is gel-like and contains all the food and nutrients that the bacteria need to grow.

As the bacteria consume the nutrients, they begin to grow and multiply. This generates thousands to millions to billions of cells that begin to pile up, becoming visible to the naked eye. This pile of cells originates from one cell and is called a **bacterial colony**. Each species of bacteria produces a colony that looks different from the colonies produced by other species of bacteria. Examination of the form and structure of bacterial colonies is termed **colony morphology** and is one of the first steps in characterizing and identifying a bacterial culture.

Colony Morphology

These are the characteristics used to accurately and consistently describe the morphology of a bacterial colony:

- Size
- Shape
- Color (also known as pigmentation)

- Texture
- Height (a.k.a. elevation)
- Edge (a.k.a. margin)

Each of these categories has its own vocabulary, allowing other scientists reading your description to paint an accurate picture of the colony.

The size of the colony can be described in two ways. The more accurate technique would be to measure the diameter of the colony with a ruler and report the size in millimeters. The second technique would simply be to describe the colonies as punctiform (tiny pinpoints), small, medium, or large.

Shape refers to the overall appearance of the colonies. The descriptors here are punctiform, circular, irregular, filamentous (has individual thin projections), or rhizoid (has thin, branching projections).

Some bacteria produce pigments, giving the colony a distinct color. Pigments span the entire color spectrum. Recording the color is the first step. In addition to describing the color, this is also the time to identify the colony as opaque (you can't see through it), translucent (you can see through it), dull, or shiny.

Texture refers to the characteristics of the colony surface. Colonies can be dry, mucoid (thick, stringy, and wet), moist, smooth, rough, rugose (wrinkled), or contain concentric rings.

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UNIT – IV – MEDICAL LABORATORY TECHNOLOGY – SMB 1607

Agglutination

Agglutination reactions involve particulate antigens capable of binding antibody molecules. Since antibody molecules are multivalent, suspended particulate antigens form large clumps or aggregates, easily visible without magnification, when exposed to specific antibodies. Antibodies that cause this reaction are referred to as agglutinins. Agglutination assays can be used to determine concentrations of specific antibodies in a patient's immune sera. A constant amount of a suspended particulate antigen is added to a series of tubes containing a twofold dilution of patient's immune serum, and the titer of antibody in the serum is the reciprocal of the highest serum dilution showing agglutination of the particulate antigen. Agglutination reactions are routinely used for identification and serotyping of a wide range of bacterial foodborne pathogens.

Blood Cell Antigens and Antibodies

Agglutination of red cells by antibody: a basic method

Agglutination tests are usually carried out in tubes, microtitre plates or using column agglutination (gel) technology, centrifugation or sedimentation. Rarely slide tests are used for emergency ABO and D grouping. For microplate tests, Tube tests.Add 1 volume of a 2% red cell suspension to 2–3 volumes of plasma in a disposable plastic tube. Mix well and leave undisturbed for the appropriate time (see below).Tubes.For agglutination tests, use medium-sized (75 × 10 or 12 mm) disposable plastic tubes. Similar tubes should be used for lysis tests when it is essential to have a relatively deep layer of serum to look through, if small amounts of lysis are to be detected. The level of the fluid must rise much higher than the concave bottom of the tubes.Glass tubes should always be used if the contents are to be heated to 50 °C or higher or if organic solvents are being used. Glass tubes, however, are difficult to clean satisfactorily, particularly small-bore tubes and cleaning methods such as those given on p. 564 should be followed carefully.Temperature and time of exposure of red cells to antibody

In blood group serology, tube tests are generally done at 37 °C, room temperature or both. There is some advantage in using a 20 °C waterbath rather than relying on ‘room temperature’, which in different countries and seasons may vary from 15 °C (or less) to 30 °C (or more).Sedimentation tube tests are usually read after 1–2 h have elapsed. Strong agglutination will, however, be obvious much sooner than this. In spin-tube tests, agglutination can be read after only 5–10 min incubation if the cell–plasma mixture is centrifuged.

Slide tests

These are used rarely in a few parts of the world. Because of evaporation, slide tests must be read within about 5 min. Reagents that produce strong agglutination within 1–2 min are normally used for rapid ABO and RhD grouping. Because the results are read macroscopically, strong cell suspensions should be used (35–45% cells in their own serum or plasma).

Reading results of tube tests

Only the strongest complete (C) grade of agglutination seems to be able to withstand a shake procedure without some degree of disruption, which may downgrade the strength of reaction.

The British Committee for Standards in Haematology (BCSH) Blood Transfusion Task Force has therefore recommended the following reading procedure.30

Microscopic reading.

It is essential that a careful and standardised technique be followed. Lift the tube carefully from its rack without disturbing the button of sedimented cells. Holding the tube vertically, introduce a straight-tipped Pasteur pipette. Carefully draw up a column of supernatant about 1 cm in length and then, without introducing an air bubble, draw up a 1–2 mm column of red cells by placing the tip of the pipette in the button of red cells. Gently expel the supernatant and cells onto a slide over an area of about 2 × 1 cm. It is important not to overload the suspension with cells and the method described earlier achieves this.

A scheme of scoring the results is given in Table 21-11.

Table 21-11. Scoring of results in red cell agglutination tests

Symbol	Agglutination Score*	Description
4 + or C (complete)	12	Cell button remains in one clump, macroscopically visible
3 +	10	Cell button dislodges into several large clumps, macroscopically visible
2 +	8	Cell button dislodges into many small clumps, macroscopically visible
1 +	5	Cell button dislodges into finely granular clumps, macroscopically just visible
(+) or w (weak)	3	Cell button dislodges into fine granules, only visible microscopically†
-	0	Negative result – all cells free and evenly distributed

* Titration scores are the summation of the agglutination scores at each dilution.

† May be further classified depending on the number of cells in the clumps (e.g. clumps of 12–20 cells [score 3]; 8–10 cells [score 2]; 4–6 cells [score 1]). This is the minimum agglutination that should be considered positive.

Macroscopic reading

A gentle agitation tip-and-roll ‘macroscopic’ method is recommended. It is possible to read agglutination tests macroscopically with the aid of a hand reading glass or concave mirror, but it is then difficult to distinguish reactions weaker than + (microscopic reading) from the

normal slight granular appearance of unagglutinated red cells in suspension. Macroscopic reading thus gives lower titration values than does microscopic reading, but the former is recommended. Follow the system of scoring in Table 21-11.

A good idea of the presence or absence of agglutination can often be obtained by inspection of the deposit of sedimented cells: a perfectly smooth round button suggests no agglutination, whereas agglutination is shown by varying degrees of irregularity, 'graininess', or dispersion of the deposit (Fig. 21-2).

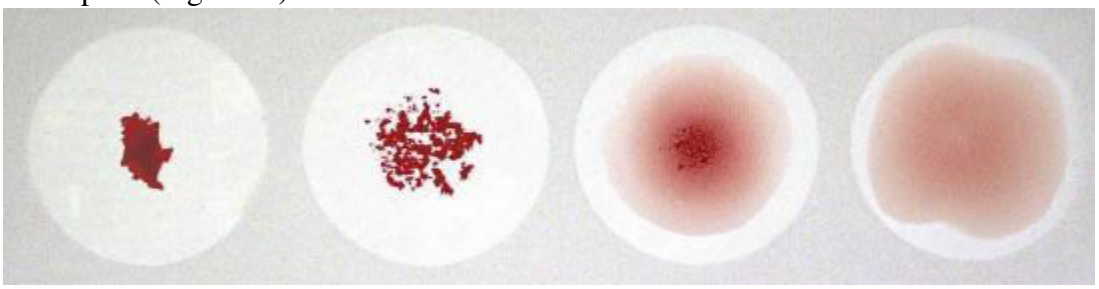

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Figure 21-2. Macroscopic appearances of agglutination in round-bottom tubes or hollow tiles. Agglutination is shown by various degrees of 'graininess'; in the absence of agglutination, the sedimented cells appear as a smooth round button, as on the extreme right.

Toxoplasma gondii

Agglutination and AC/HS Tests

The agglutination test using formalin-preserved whole tachyzoites is available commercially (bioMérieux, Marcy-l'Etoile, France) and detects IgG antibody. The test is very sensitive to IgM antibody, and "natural" IgM antibody causes nonspecific agglutination in sera that yield negative results when tested in the DT and the IFA test. This problem is avoided by including 2-mercaptoethanol in the test. The method is accurate, simple to perform, inexpensive, and excellent for screening purposes.307 This method, that is, with mercaptoethanol, should not be used for the measurement of IgM antibodies.

When two different compounds (i.e., acetone and formalin) are used to fix parasites for use in the agglutination test, a "differential" agglutination test (AC/HS test) results because the different antigenic preparations vary in their ability to recognize sera obtained during the acute and chronic stages of the infection.308 This test has proved useful in helping differentiate acute from chronic infections and is best used in combination with a battery of other tests. When the AC/HS yields a "nonacute" pattern, the infection has been present for at least 12 months from the time of serum sampling.309

Listeriosis

Serology

The agglutination reaction (Widal's test) demonstrates antibodies against O and H antigens of the various Listeria serovars. Unfortunately, because of the antigenic complexity of *L.*

monocytogenes, no agreement has been reached on the interpretation of agglutination reactions for diagnostic purposes.

Attempts to demonstrate complement-fixing *Listeria* antibodies date back to the 1930s.232 In one study, serum samples collected from 32 mothers with perinatal *Listeria* infection were compared with 128 samples from matched controls.233 The sensitivity and specificity of the complement fixation test were found to be 78% and 91%, respectively; however, the positive predictive value was only 75%. A titer of 1:8 or more is accepted as significant.233,234

Detection of antibodies to LLO has also been used to diagnose human listeriosis.235 Purified LLO incorporated into nitrocellulose filters is tested with serial dilutions of sera. Absorbed anti-LLO is identified using enzyme-labeled anti-human IgG. Sensitivity and specificity of the test is over 90%, and during a febrile gastroenteritis outbreak, it correlated well with clinical illness.215 Although these results are impressive, the technique is not available commercially. A precipitin test,236 indirect hemagglutination reaction,237 and antigen fixation test238 have also been described, showing apparent success but remaining unavailable on a widespread basis.

Leptospira Species (Leptospirosis)

Serology

The macroscopic slide agglutination test is the most useful test for rapid screening.18,19 Twelve serotypes of killed Leptospira (including strains responsible for most infections in the US) are included in this test. The microscopic agglutination test uses live organisms and is the gold standard for detecting antibodies to *Leptospira*.

Generally, agglutination test results are not positive until after the first week of infection; antibody levels peak 3 to 4 weeks after the onset of symptoms and can persist for years, although concentrations may decline over time. Demonstration of at least a fourfold rise in antibodies between acute and convalescent serum samples tested together is most definitive. Newer serologic tests may become useful and include indirect hemagglutination tests, passive microcapsule agglutination test, and enzyme immunoassays.20–24

Blood cell antigens and antibodies: erythrocytes, platelets, and granulocytes6

Tubes

For agglutination tests, use medium-sized (75 × 10 or 12 mm) disposable plastic or glass tubes. Similar tubes should be used for lysis tests when it is essential to have a relatively deep layer of serum to look through, if small amounts of lysis are to be detected. The level of the fluid must rise much higher than the concave bottom of the tubes.

Glass tubes should always be used if the contents are to be heated to 50°C or higher or if organic solvents are being used. Glass tubes, however, are difficult to clean satisfactorily, particularly small bore tubes, and cleaning methods such as those given on page 695 should be followed carefully.

Toxoplasmosis

Agglutination Test

The agglutination test [618,619] is available commercially in Europe and has been evaluated by a number of investigators [620–625]. The test employs whole parasites that have been preserved in formalin. The method is very sensitive to IgM antibodies. Nonspecific agglutination (apparently related to "naturally" occurring IgM *T. gondii* agglutinins) has been observed in persons devoid of antibody in the dye test and conventional IFA test [626]. These natural IgM antibodies do not cross the placenta but are detected at low titers as early as the second month of life. They do not develop in infants with maternal IgG antibody to *T. gondii*, however, so long as IgG antibody is present. False-positive results due to these natural antibodies may be avoided [627]. When they are, this test is excellent for wide-scale screening of pregnant women because it is accurate, simple to perform, and inexpensive [627,628]. A method that employs latex-tagged particles also may become available commercially [629–631].

Introduction to Immunoassay Product Technology in Clinical Diagnostic Testing

Slide agglutination tests are qualitative tests used to detect the presence of antibodies in serology laboratories and blood banks. Treated red blood cells or colored latex beads, coated with antigen, clump in the presence of antibody to the antigen. The degree of clumping may be measured using absorbance at 600 nm, and latex agglutination has been applied to quantitative assays.

Precipitin Assays

Precipitin assays, such as radial immunodiffusion and immunoelectrophoresis, are still used for certain applications, but these tend to be low volume assays, in specialist centers. In these types of assays, the presence of antibody (or antigen) in the sample causes the formation of a precipitate in agar containing antigen (or antibody). Radial immunodiffusion involves adding samples to circular holes cut in agar plates and the formation of a circular ring. In immunoelectrophoresis, proteins are first separated by electrophoresis before incubation with antibodies in a parallel trough, with the formation of precipitin arcs.

Nephelometry and Turbidimetry

Light-scattering immunoassays are based on the reaction between antigen and antibody to produce an aggregate or agglutinate large enough to scatter light over and above that scattered by the constituents of the reaction. Early agglutination assays depended on visual inspection to provide a semiquantitative result. Turbidimetry and nephelometry instrumentation were introduced to measure the extent of antigen–antibody combination. Turbidimetry is the measurement of light-scattering species in solution by means of a decrease in intensity of the incident beam after it has passed through the solution. It is measured by a detector 180° from the incident beam. Nephelometry detects light energy scattered or reflected toward a detector that is not in the direct path of the light beam. One of the early technological challenges was the reduction of stray light, and nephelometers were designed to measure at angles between 90° and 180° to take advantage of the increased forward scatter intensity caused by light scattering from larger particles. With advanced filters to suppress the undeflected light from the source, the

scattered light can be detected less than 30° from the direct light path, maximizing the sensitivity.

The use of turbidimetry and nephelometry predates radioimmunoassay (RIA) by many years (*see* The Foundations of Immunochemistry). Instruments to measure turbidimetry were introduced in 1938 and the introduction of nephelometry was first reported in 1951. Immunoprecipitation was adapted to run on the Technicon continuous flow autoanalyzer in 1972 to quantify immunoglobulins. Technicon also manufactured bench-top nephelometric immunoassay analyzers. The first nephelometers used tungsten light filaments but lasers were introduced from 1974. Leading products using this technology were the Behringwerke BNA and Hyland Laser Nephelometer. Beckman introduced the Immunochemistry System (ICS), initially using a quartz halogen or xenon lamp as the light source, but the introduction of lasers with high intensity and coherence greatly improved sensitivity and decreased the time required for nephelometric measurements.

The limitations of turbidimetry and nephelometry center around the measurements at antigen excess and matrix effects. The kinetics of immune complex formation are sufficiently different during antibody excess, equivalence, and antigen excess that systems employed computer algorithms that would automatically flag antigen excess where the signal has increased to a maximum value and then decreased. Matrix effects, as in highly lipemic samples, caused high background interference. An effective solution for minimizing this background was the implementation of rate nephelometry, where the initial sample blank was eliminated. In rate assays, measurements were made within the first few minutes of the reaction since the largest change in intensity of scattered light *versus* time is achieved during this interval, instead of the 30–60 min required for the measurement of pseudo-equilibrium methods. An example was the Beckman Array 360 ICS, which had two sets of optics and two flow cells for maximum throughput, and utilized a polarized laser beam that was split such that the first component was directed to the reaction container for the nephelometric analysis and the remaining beam component passed to a laser control light detector. Finally, the introduction of rate nephelometry inhibition allowed the measurement of low-molecular-weight haptens such as drugs. The drug of interest was attached to a carrier molecule such as albumin and added to the reaction to compete with the endogenous free hapten for the antihapten antibody. In the presence of free hapten, the immunoprecipitin is decreased because more antibody sites are saturated and therefore light scattering is decreased, resulting in an indirect relationship between the hapten concentration and the amount of light scatter. The Beckman IMMAGE ICS was a fully automated random-access system that used rate inhibition technology and near-infrared particles to increase the analytical sensitivity by 1000-fold to address low-molecular-weight analytes.

Light scattering immunoassays—particularly for the measurement of specific proteins—are still in common use in many general clinical chemistry analyzers.

African Trypanosomiasis

Serologic Assays

A card agglutination test (CATT) has been used for many years to screen for antibodies against *T. b. gambiense* using a mixture of abundant parasite antigens. This is a rapid, field-adapted test that detects antibodies by their ability to agglutinate antigen, but does not confirm the infection. Rather, a positive result should stimulate the search for parasites.

ELISA_ENZYME LINKED IMMUNOSORBENT ASSAY:

What is ELISA (enzyme-linked immunosorbent assay)?

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. Other names, such as enzyme immunoassay (EIA), are also used to describe the same technology. In an ELISA, an antigen must be immobilized on a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

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Introduction

ELISAs are typically performed in 96-well (or 384-well) polystyrene plates, which passively bind antibodies and proteins. It is this binding and immobilization of reagents that makes ELISAs so easy to design and perform. Having the reactants of the ELISA immobilized to the microplate surface makes it easy to separate bound from non-bound material during the assay. This ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes within a crude preparation.

A detection enzyme or other tag can be linked directly to the primary antibody or introduced through a secondary antibody that recognizes the primary antibody. It can also be linked to a protein such as streptavidin if the primary antibody is biotin labeled. The most commonly used enzyme labels are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Other enzymes have been used as well, but they have not gained widespread acceptance because of limited substrate options. These include β-galactosidase, acetylcholinesterase and catalase. A large selection of substrates is available for performing ELISA with an HRP or AP conjugate. The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection (spectrophotometer, fluorometer or luminometer).

ELISA formats

ELISAs can be performed with a number of modifications to the basic procedure. The key step, immobilization of the antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (labeled primary antibody) or indirectly (labeled secondary antibody). The most powerful ELISA assay format is the sandwich assay. This type of capture assay is called a “sandwich” assay because the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody. The sandwich format is used because it is sensitive and robust.

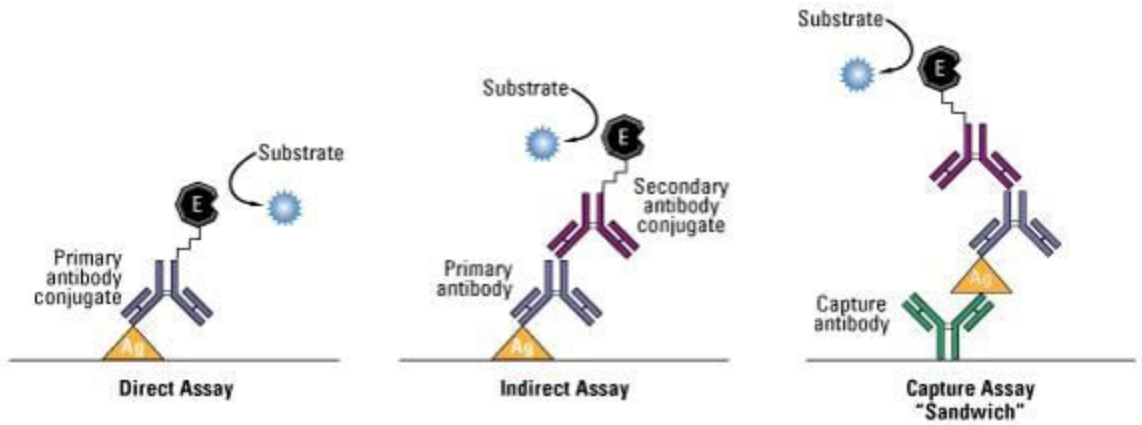


Diagram of common ELISA formats (direct vs. sandwich assays). In the assay, the antigen of interest is immobilized by direct adsorption to the assay plate or by first attaching a capture antibody to the plate surface. Detection of the antigen can then be performed using an enzyme-conjugated primary antibody (direct detection) or a matched set of unlabeled primary and conjugated secondary antibodies (indirect detection).

Direct vs. indirect detection ELISA strategies

Among the standard assay formats discussed and illustrated above, where differences in both *capture* and *detection* were the concern, it is important to differentiate between the particular strategies that exist specifically for the *detection* step. Irrespective of the method by which an antigen is captured on the plate (by direct adsorption to the surface or through a pre-coated "capture" antibody, as in a sandwich ELISA), it is the detection step (as either direct or indirect detection) that largely determines the sensitivity of an ELISA.

The direct detection method uses a labeled primary antibody that reacts directly with the antigen. Direct detection can be performed with an antigen that is directly immobilized on the assay plate or with the capture assay format. Direct detection while not widely used in ELISA is quite common for immunohistochemical staining of tissues and cells.

The indirect detection method uses a labeled secondary antibody for detection and is the most popular format for ELISA. The secondary antibody has specificity for the primary antibody. In a sandwich ELISA, it is critical that the secondary antibody be specific for the detection primary antibody only (and not the capture antibody) or the assay will not be specific for the antigen. Generally, this is achieved by using capture and primary antibodies from different host species (e.g., mouse IgG and rabbit IgG, respectively). For sandwich assays, it is beneficial to use secondary antibodies that have been cross-adsorbed to remove any secondary antibodies that might have affinity for the capture antibody.

Comparison of direct and indirect ELISA detection methods

Direct ELISA detection

Advantages	• Quick because only one antibody and fewer steps are used. • Cross-reactivity of secondary antibody is eliminated.
Disadvantages	• Immunoreactivity of the primary antibody might be adversely affected by labeling with enzymes or tags. • Labeling primary antibodies for each specific ELISA system is time-consuming and expensive. • No flexibility in choice of primary antibody label from one experiment to another. • Minimal signal amplification.
Indirect ELISA detection	
Advantages	• A wide variety of labeled secondary antibodies are available commercially. • Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection. • Maximum immunoreactivity of the primary antibody is retained because it is not labeled. • Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification. • Different visualization markers can be used with the same primary antibody.
Disadvantages	• Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal. • An extra incubation step is required in the procedure.

Fluorescent tags and other alternatives to enzyme-based detection can be used for plate-based assays. Despite not involving reporter-enzymes, these methods are also generally referred to as a type of ELISA. Likewise, wherever detectable probes and specific protein binding interactions can be used in a plate-based method, these assays are often called ELISAs despite not involving antibodies.

Other ELISA formats

Besides the standard direct and sandwich formats described above, several other styles of ELISA's exist:

Competitive ELISA is a strategy that is commonly used when the antigen is small and has only one epitope, or antibody binding site. One variation of this method consists of labeling purified antigen instead of the antibody. Unlabeled antigen from samples and the labeled antigen compete for binding to the capture antibody. A decrease in signal from the purified antigen indicates the presence of the antigen in samples when compared to assay wells with labeled antigen alone.

Watch this video about competitive ELISA methods

ELISPOT (enzyme-linked immunospot assay) refers to ELISA-like capture and measurement of proteins secreted by cells that are plated in PVDF-membrane-backed microplate wells. It is a "sandwich" assay in which the proteins are captured locally as they are secreted by the plated cells, and detection is with a precipitating substrate. ELISPOT is like a western blot in that the result is spots on a membrane surface.

In-cell ELISA is performed with cells that are plated and cultured overnight in standard microplates. After the cultured cells are fixed, permeabilized and blocked, target proteins are detected with antibodies. This is an indirect assay, not a sandwich assay. The secondary antibodies are either fluorescent (for direct measurement by a fluorescent plate reader or microscope) or enzyme-conjugated (for detection with a soluble substrate using a plate reader).

ELISA is nearly always performed using 96-well or 384-well polystyrene plates and samples in solution (i.e., biological fluids, culture media or cell lysates). This is the platform discussed in the remainder of this article.

Learn more

- ELISA Development and Optimization
- Spike-and-Recovery and Linearity-of-Dilution Assessment for ELISA
- Factors Affecting Signal Generation in ELISA
- ELISA Protocols
- ELISA Troubleshooting Guide
- Antibodies and Immunoassays Support Center
- ELISA Kits and Antibody Pairs Support Center
- Invitrogen Antibody Validation
- Antibodies Learning Center

Select products

- Find ELISA Kits by Target
- Ready-to-Use ELISA Kits
- Antibody Pair Kits
- Immunoassays
- Antibodies Search

Complete, ready-to-use ELISA kits

In addition to the individual components and general principles of ELISA discussed in the remainder of this article, ready-to-use sandwich ELISA kits are commercially available for detection of hundreds of specific cytokines, neurobiology analytes and phosphorylated proteins that are common targets of research interest.

For many targets, two kit types are available:

- **ELISA kits** contain pre-coated antibody-plates, detection antibodies, buffers, diluents, standards, and substrates. In addition to traditional ELISA kits with pre-coated plates include only the capture antibody when the sample is added, Thermo Fisher Scientific offers Instant ELISA kit plates that contain all of the necessary components including capture antibody and lyophilized detection antibody, streptavidin-HRP, and sample diluent. In addition, strip wells containing the standard for the standard curve are provided separately.

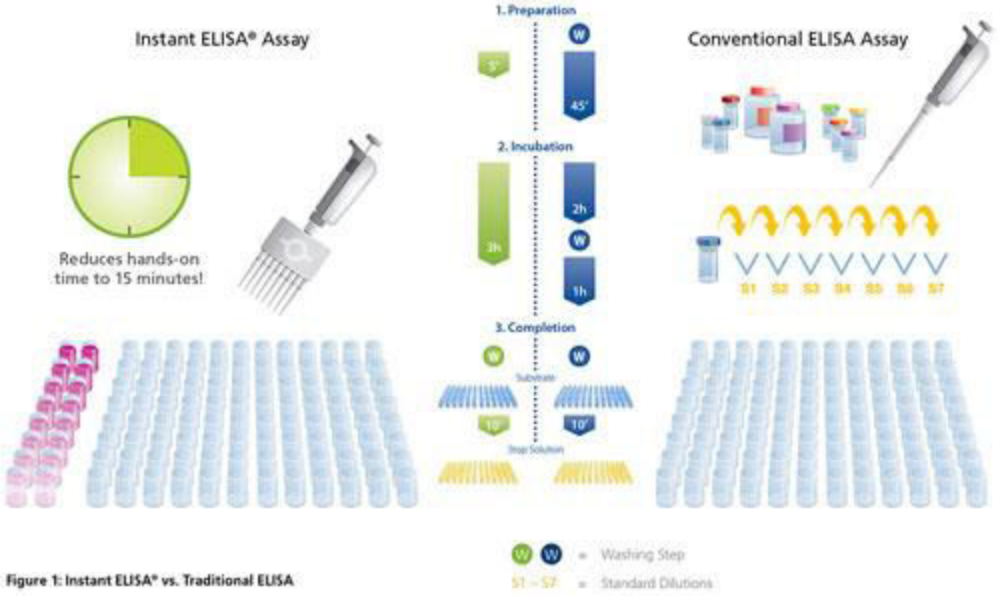


Figure 1: Instant ELISA® vs. Traditional ELISA

Comparison of instant ELISA technology vs. conventional ELISA procedures. In contrast to conventional ELISA kits, Thermo Scientific Invitrogen Instant ELISA kits were produced to include both the capture antibody and lyophilized detection antibody and other reagents required to develop an ELISA.

- **Antibody pair kits** contain only matched antibodies and standard (no plates or detection reagents).

This ELISA format selection guide compares characteristics of Thermo Fisher Invitrogen antibody pair kits and ELISA kits.

	Build-it-yourself	**Uncoated ELISA kits**	**Coated ELISA kits***	**Instant ELISA kits**
Ready-to-use reagents	No, an overnight coating process is required	Yes, an overnight coating process is required	Yes	Yes

Analytical sensitivity**	<10 pg/ml	<10 pg/ml	<10 pg/ml	<5 pg/ml
Dynamic range**	<5-250 pg/ml	<5-250 pg/ml	<5-250 pg/ml	<7.8-500 pg/ml
Incubation time**	4 h	3.5-4 h	2,5-4 h	2-3 h
Multiplexibility**	No	No	No	No
Number of targets measured per well	1	1	1	1
Readout	HRP-TMB (colorimetric)	HRP-TMB (colorimetric)	HRP-TMB (colorimetric)	HRP-TMB (colorimetric)
Instrumentation needed	Microplate reader, absorbance	Microplate reader, absorbance	Microplate reader, absorbance	Microplate reader, absorbance
Instrumentation read time	2 min	2 min	2 min	2 min

*Values in this table refer to our Standard Colorimetric kits. Ultrasensitive kits are also available.
**Every assay has its oven specifications. Please consult the protocol for your specific immunoassays/kits.

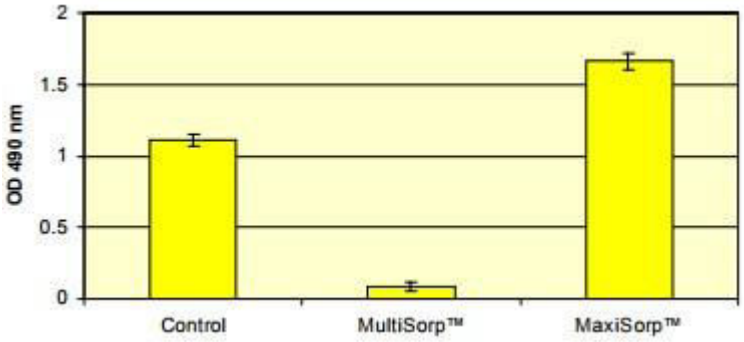
Selecting and coating ELISA plates

When developing a new ELISA for a specific antigen, the first step is to optimize the plate-coating conditions for the antigen or capture antibody. Begin by choosing an assay microplate (not tissue culture treated plates) with a minimum protein-binding capacity of 400 ng/cm². It is also important that the CV value (coefficient of variation) of the protein binding be low (<5% is preferred) so that there is limited deviation in values that should be identical in the assay results between wells and plates. The choice of plate color depends upon the signal being detected. Clear polystyrene flat bottom plates are used for colorimetric signals while black or white opaque plates are used for fluorescent and chemiluminescent signals. Visually inspect plates before use as imperfections or scratches in the plastic will cause aberrations when acquiring data from the developed assay.

Uncoated ELISA plates. Thermo Scientific Uncoated ELISA Plates are available with a variety of surfaces to optimize coating with the macromolecule of your choice. These plates are designed to deliver optimal results, lot-to-lot reliability and well-to-well reproducibility.

Plate coating is achieved through passive adsorption of the protein to the plastic of the assay microplate. This process occurs though hydrophobic interactions between the plastic and non-polar protein residues. Although individual proteins may require specific conditions or pretreatment for optimal binding, the most common method for coating plates involves adding a 2-10 µg/ml solution of protein dissolved in an alkaline buffer such as phosphate-buffered saline (pH 7.4) or carbonate-bicarbonate buffer (pH 9.4). The plate is left to incubate for several hours to overnight at 4-37° C. Typically, after removing the coating solution, blocking buffer is added to ensure that all remaining available binding surfaces of the plastic well are covered (see subsequent discussion). Coated plates can be used immediately or dried and stored at 4° C for later use, depending on the stability of the coated protein.

It is important to note that optimal coating conditions and plate binding capacity can vary with each protein and must be determined experimentally. With the exception of competition ELISAs, the plates are coated with more capture protein than can actually be bound during the assay in order to facilitate the largest working range of detection possible. Some proteins, especially antibodies, are best coated on plates at a concentration lower than the maximum binding capacity in order to prevent nonspecific binding in later steps by a phenomenon called "hooking". Hooking results from proteins getting trapped between the coating proteins which prevents effective washing and removal of unbound proteins. When hooking nonspecifically traps detection primary and secondary antibodies, high background signal results, lowering the signal to noise ratio and thus sensitivity of an assay. The following example illustrates how variations in polymer coatings may impact protein binding capacities.



IgG Binding on modified surfaces. The introduction of functional groups will affect the binding characteristics of the plastic polymer. This experiment demonstrates that surface modifications will affect binding of proteins. Comparison of adsorption of various proteins on non-treated control, Thermo Scientific Nunc MultiSorp Thermo Scientific Nunc MultiSorp and MaxiSorp flat-bottom plates indicates the importance of surface selection on assay optimization. Various molecules behave in distinctly different manners depending on the characteristics of the surface. For example, under basic conditions, IgG will adsorb to MaxiSorp modified polystyrene with significantly more capacity when compared with a non-treated control plate. In the case of MultiSorp, the functional groups on the surface restrict the protein absorption of IgG; evident by a binding capacity compared to the non-treated plate.

Pre-coated ELISA plates

For antibodies and proteins, coating plates by passive adsorption usually works well. However, problems can arise from passive adsorption, including improper orientation, denaturation, poor immobilization efficiency and binding of contaminants along with the target molecule. Antibodies can be attached to a microplate through the Fc region using Protein A, G, or A/G coated plates, which orients them properly and preserves their antigen binding capability. Fusion proteins can be attached to a microplate in the proper orientation using glutathione, metal-chelate, or capture-antibody coated plates. Peptides and other small molecules, which typically do not bind effectively by passive adsorption, can be biotinylated and attached with high efficiency to a streptavidin or NeutrAvidin protein coated plate. Biotinylated antibodies also can be immobilized on plates precoated with biotin-binding proteins. Using pre-coated plates in this manner physically separates the antigen or capture antibody from the surface of the plate as a protection from its denaturing effects.

NeutrAvidin Coated Plates—Standard Capacity

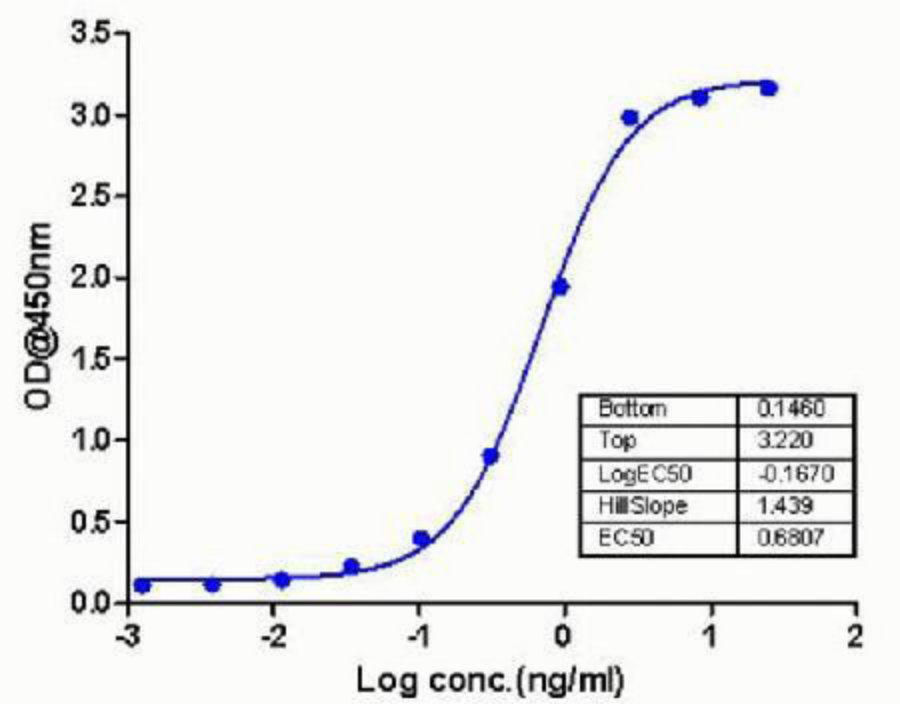
Pierce NeutrAvidin Coated—High Capacity

Pierce NeutrAvidin Coated—High Sensitivity

There is a wide selection of high-performance surface coated plates (pre-coated and pre-blocked) in 96-well and 384-well format (black, clear or white). These coated microplates can be used for ELISA development and other plate-based assays with standard or fluorescence plate readers.

Antibodies and probes for ELISA

Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. Monoclonals have an inherent monospecificity toward a single epitope that allows fine detection and quantitation of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible. Then a monoclonal is used as the detecting antibody in the sandwich assay to provide improved specificity. In addition to the use of traditional monoclonal antibodies, recombinant monoclonal antibodies may also be utilized for ELISA. For example, Invitrogen rabbit recombinant antibodies are derived from antibody-producing cell lines engineered to express specific antibody heavy and light chain DNA sequences. Compared to traditional monoclonal antibodies derived from hybridomas, recombinant antibodies are not susceptible to cell-line drift or lot-to-lot variation, thus allowing for peak antigen specificity. The example that follows presents data produced using a recombinant rabbit monoclonal antibody.



Sandwich ELISA. This experiment was performed using ABfinity EGF Recombinant Rabbit Monoclonal Antibody at 2 µg/mL. A standard curve was plotted with full length recombinant EGF protein with concentrations ranging from 0.3 pg/mL to 12.5 ng/mL. An anti-EGF antibody conjugated to biotin was used as a detector at a concentration of 2 µg/mL.

An important consideration in designing a sandwich ELISA is that the capture and detection antibodies must recognize two different non-overlapping epitopes. When the antigen binds to the capture antibody, the epitope recognized by the detection antibody must not be obscured or altered. Capture and detection antibodies that do not interfere with one another and can bind simultaneously are called "matched pairs" and are suitable for developing a sandwich ELISA. Many primary antibody suppliers provide information about epitopes and indicate pairs of antibodies that have been validated in ELISA as matched pairs.

Another design consideration in choosing antibodies is cost. A polyclonal antibody is generally less expensive (~5 fold) to produce than a monoclonal. The specificity gained by using monoclonals for both the capture and detecting antibody must be weighed against the cost and time required for producing two monoclonal antibodies. Preparing a “self-sandwich” ELISA assay, where the same antibody is used for the capture and detection, can limit the dynamic range and sensitivity of the final ELISA.

Blocking buffers and wash buffers

The binding capacity of microplate wells is typically higher than the amount of protein coated in each well. The remaining surface area must be blocked to prevent antibodies or other proteins from adsorbing to the plate during subsequent steps. A blocking buffer is a solution of irrelevant protein, mixture of proteins, or other compound that passively adsorbs to all remaining binding

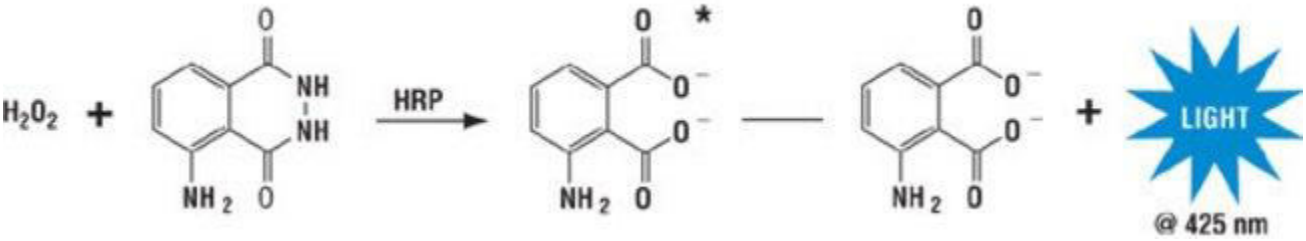
surfaces of the plate. The blocking buffer is effective if it improves the sensitivity of an assay by reducing background signal and improving the signal-to-noise ratio. The ideal blocking buffer will bind to all potential sites of nonspecific interaction, eliminating background altogether, without altering or obscuring the epitope for antibody binding.

When developing any new ELISA, it is important to test several different blockers for the highest signal: noise ratio in the assay. Many factors can influence nonspecific binding, including various protein: protein interactions unique to the samples and antibodies involved. The most important parameter when selecting a blocker is the signal: noise ratio, which is measured as the signal obtained with a sample containing the target analyte as compared to that obtained with a sample without the target analyte. Using inadequate amounts of blocker will result in excessive background and a reduced signal: noise ratio. Using excessive concentrations of blocker may mask antibody-antigen interactions or inhibit the enzyme, again causing a reduction of the signal: noise ratio. No single blocking agent is ideal for every occasion and empirical testing is essential for true optimization of the blocking step.

In addition to blocking, it is essential to perform thorough washes between each step of the ELISA. Washing steps are necessary to remove nonbound reagents and decrease background, thereby increasing the signal: noise ratio. Insufficient washing will allow high background, while excessive washing might result in decreased sensitivity caused by elution of the antibody and/or antigen from the well. Washing is performed in a physiologic buffer such as Tris-buffered saline (TBS) or phosphate-buffered saline (PBS) without any additives. Usually, a detergent such as 0.05% Tween-20 is added to the buffer to help remove nonspecifically bound material. Another common technique is to use a dilute solution of the blocking buffer along with some added detergent. Including the blocking agent and adding a detergent in wash buffers helps to minimize background in the assay. For best results, use high-purity detergents to prevent introduction of impurities that will interfere with the assay such enzyme inhibitors or peroxides.

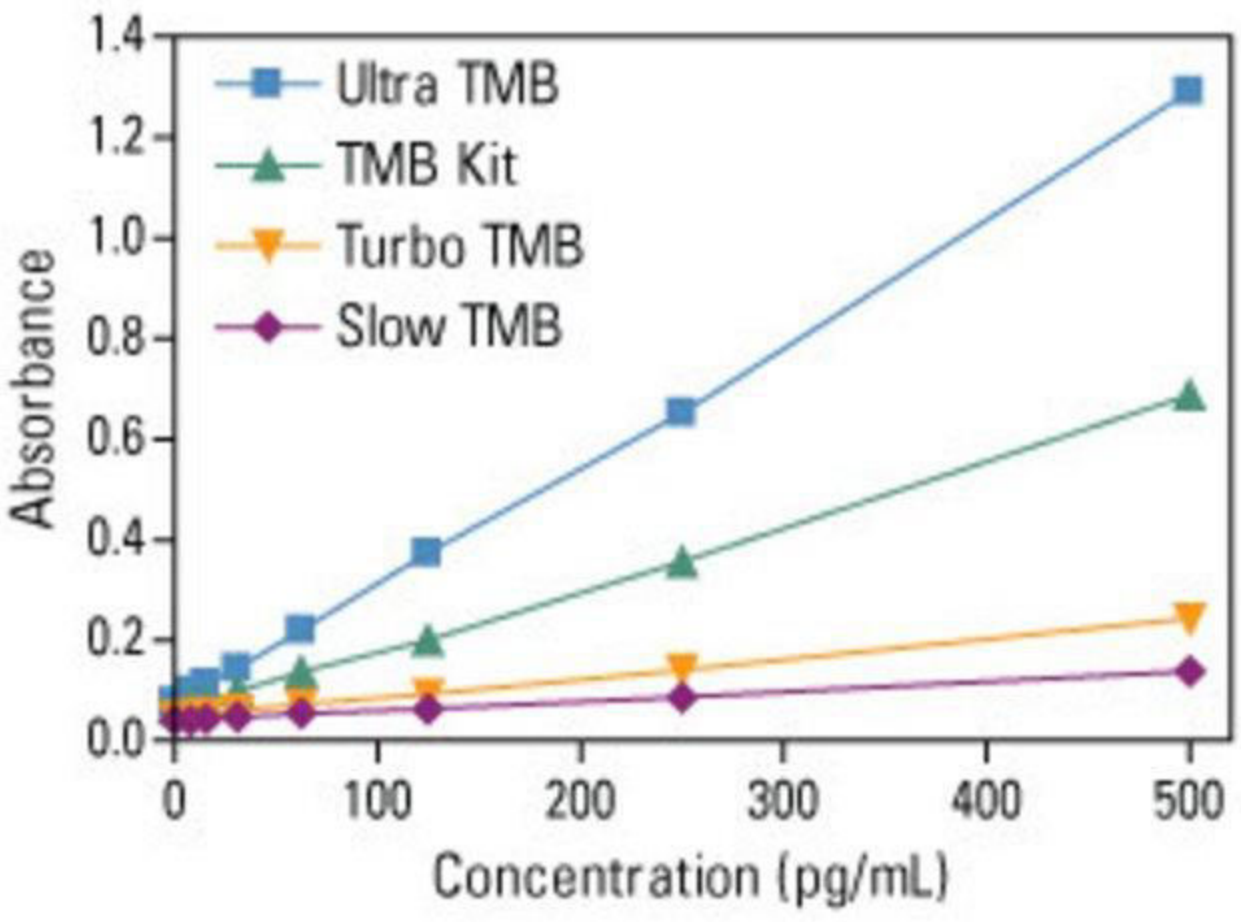
Detection strategies for ELISA

The final stage in all ELISA systems is a detection step. Unless a radioactive or fluorescent tag was used, this involves the introduction of an enzyme substrate. The enzyme converts the substrate to a detectable product. If an ELISA has been constructed and developed properly, then the intensity of signal produced when the substrate is added will be directly proportional to the amount of antigen captured in the plate and bound by the detection reagents. Enzyme-conjugated antibodies (especially those involving horseradish peroxidase, HRP) offer the most flexibility in detection and documentation methods for ELISA because of the variety of substrates available for chromogenic, chemifluorescent and chemiluminescent imaging. The following illustration describes the chemical reaction associated with luminol, a reagent that exhibits chemiluminescence.



Luminol reaction. This illustrates the chemiluminescent reaction used by the Thermo Scientific SuperSignal ELISA Pico Substrate for HRP.

Though not as sensitive as fluorescent or chemiluminescent substrates, chromogenic ELISA substrates allow direct visualization and enable kinetic studies to be performed. Furthermore, chromogenic ELISA substrates are detected with standard absorbance plate readers common to many laboratories. Fluorescent ELISA substrates are not as common and require a fluorometer that produces the correct excitation beam to cause signal emission to be generated from the fluorescent tag. Though best used with a luminometer plate reader, chemiluminescent substrates can be detected by various means including digital camera systems. Once drawback of using chemiluminescent substrates for ELISA is the signal intensity can vary more than with other substrates. For assays requiring many plates to be read, this can present a problem if the signal begins to decay before plates are read. For this reason, it is important to make sure the assay has been optimized with the substrate in order to avoid misinterpreting signal-fade in a sample as low antigen abundance. In the representative experiment that follows, the performance of multiple TMB (3, 3', 5, 5'-tetramethylbenzidine)-based kits were compared. TMB, a common chromogenic substrate for HRP, yields a blue color when oxidized. In the example that follows, the performance of multiple TMB substrates are compared using ELSIA.



Principle of Immunofluorescence

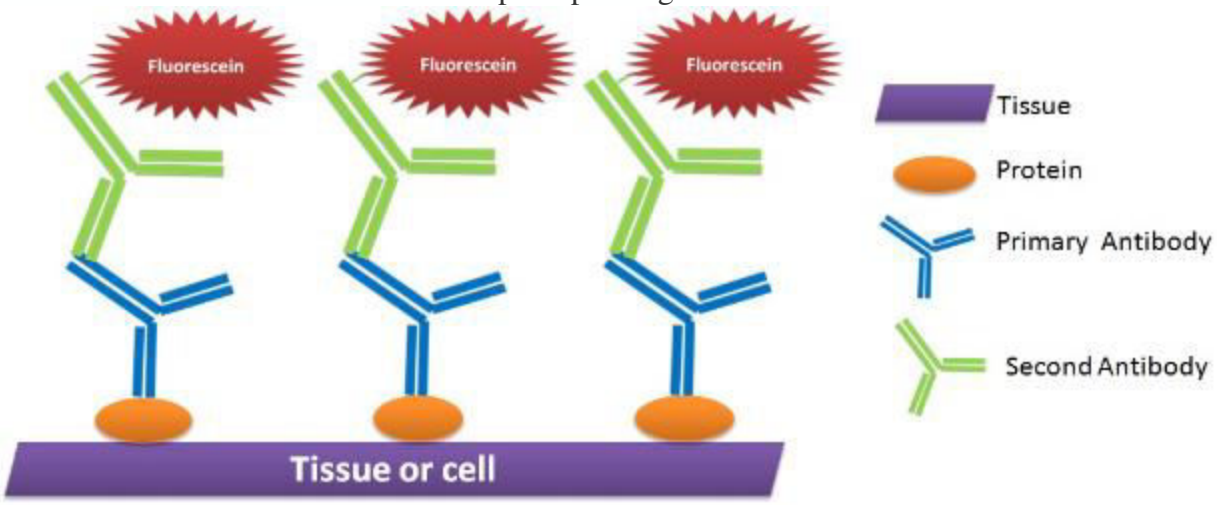
Immunofluorescence is an assay which is used primarily on biological samples and is classically defined as a procedure to detect antigens in cellular contexts using antibodies. The specificity of antibodies to their antigen is the base for immunofluorescence. The biological samples include tissue and cells. Immunofluorescence allows researchers to evaluate whether or not cells in a particular sample express the antigen in question. In cases where an immunopositive signal is found, immunofluorescence also allows researchers to determine which subcellular compartments are expressing the antigen. Immunofluorescence can be used on cultured cell lines, tissue sections, or individual cells.

Immunofluorescence may be used to analyze the distribution of proteins, glycans, and small biological and non-biological molecules. Immunofluorescence has been widely used in biological research and medical research yield and becomes one most important and effective method

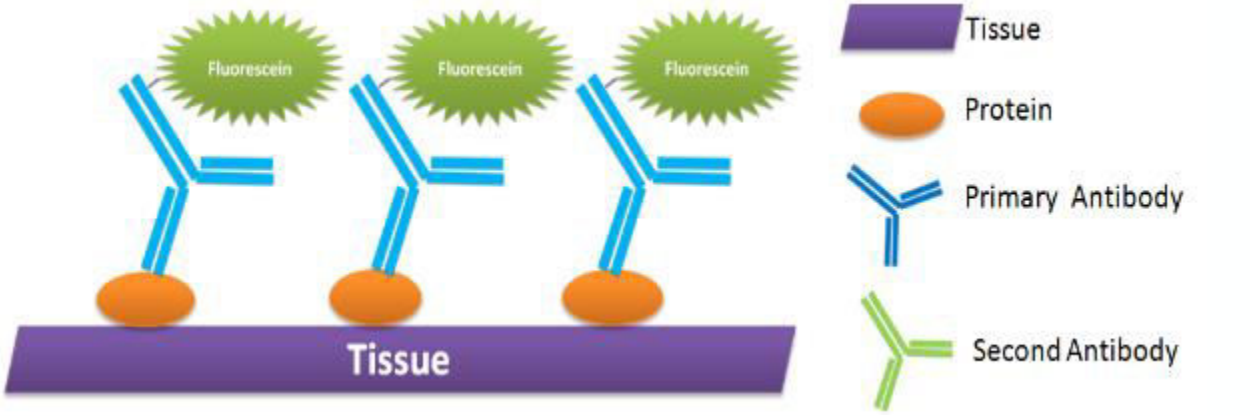
There are two different immunofluorescence assay which include indirect immunofluorescence assay and direct immunofluorescence assay.For indirect immunofluorescence assay, the protocol

mainly include tissue or tell treparation, tissue or cell fixation, serum blocking, primary antibody incubation, marked second antibody incubation, staining, result judgment and imaging. For direct immunofluorescence assay, there are only marked primary antibody been incubated without second antibody and other steps are same.

Indirect Immunofluorescence / IF/ ICCprinciple diagram



Direct Immunofluorescence / IF/ ICCprinciple diagram



POLYMERASE CHAIN REACTION

Introduction

In this chapter, the use of nucleic acids in molecular diagnostic testing will be described. Detailed disease area examples will be discussed to illustrate technical capabilities as well as the medical relevance of such testing.

Polymerase chain reaction (PCR) was invented by Kary B. Mullis in the 1980s. Fundamentally, PCR is a cyclic process designed to specifically replicate (amplify) nucleic acid sequences from as little as one to a few strands of DNA. The target DNA is heated to separate double-stranded DNA sequences; short oligonucleotide "primers" that define the portion of the genome to be replicated bind to the target DNA. The primers are extended by a DNA polymerase making a copy of the target DNA. After multiple cycles in which the concentration of the replicated target DNA increases exponentially, the amplified product (amplicon) can be visualized by gel electrophoresis or measured by detection of labeled PCR product (amplicon) by incubation with additional reagents to produce color or by fluorescent probe detection. This technique improved the ability to diagnose a number of diseases by enabling identification of many human pathogens that had previously been difficult to detect due to their low concentration in the sample. With the addition of a reverse transcription step to the original PCR process, RNA could be converted to cDNA and then replicated with the PCR process. Thus, the utility of the technique was broadened to detect RNA viruses and eukaryotic mRNA.

A key technical improvement was introduced by Higuchi et al.[4, 5] who developed real-time polymerase chain reaction (RT-PCR), which follows the kinetics of the PCR and detects PCR products during the process of amplification (Figure 1). With RT- PCR, accurate and reproducible quantitation of pathogen concentration could be incorporated into the amplification process. RT-PCR is used to monitor a pathogen's kinetic replication processes over time, and measurement of viral load is now widely employed to monitor the success of treatment of viral and other diseases.

Figure 1.

Principles of Real-Time PCR. (A) During the polymerization step, the template is amplified by primers supplied in the reaction mix. The amplicon allows for annealing of sequence-specific, labeled probes. As a new strand is synthesized, the probes will be displaced, the label cleaved off, and a fluorescent signal proportional to the amount of the cleaved probe is generated. (B) Fluorescence is measured and recorded at each cycle of PCR. Cycle threshold (Ct) is defined as the fractional PCR cycle number in which the sample fluorescence signal reaches a level above an assigned fluorescence threshold. The Ct value indicates the beginning of the exponential amplification of the template DNA or RNA and is proportional to the concentration of the sample.[6]

Clinical microbiology was one of the first fields to adopt PCR and, later, RT-PCR, due to the sensitivity and specificity of the technique for detecting nucleic acids of pathogenic microorganisms.

Perkin-Elmer developed the first thermal cycler instrument in December 1985. The first commercial *in vitro* PCR diagnostic products were created when the California company, Cetus, entered into a partnership with Kodak in February 1986.[7] The first reagent kit, the "Gene-Amp PCR reagent kit" and the thermal cycler were commercially available in November 1987.[8]

In January 1989, it was announced that Roche had entered into an agreement with Cetus to develop diagnostic applications for PCR. A new area of molecular diagnostics began using PCR to detect genes and pathogen genomes to diagnose diseases since that time.[7]

At Cetus, it was decided in the late 1980s that the forensic applications of PCR represented a stand-alone business that could be operated in-house, and, therefore, the applications were not sold to a partner.[7] In 1990, the first forensic PCR kit, developed by Cetus, was sold by Perkin-Elmer and became useful for identification of individual humans. The nucleic acid, DNA, became a mainstay of the justice system in 1997 when the FBI announced the selection of 13 short tandem repeat (STR) DNA loci to constitute the core of a national database—Combined DNA Index System (CODIS). By the time a review was published in 2006,[9] 5 million profiles of individuals existed in CODIS. By 2003, almost 1 million samples were being processed annually using core STR loci as part of parentage testing.[10]

Over the past decades, RT-PCR technology has continued to develop, optimize, and expand in the clinical laboratory for the identification, detection, and quantitation of a variety of pathogen uses. Automated instrument platforms were created to facilitate the workflow and allow for accurate and precise processing of patient samples in a highly automated manner.

2. Nucleic acid detection in molecular diagnostics

For molecular diagnostic purposes, since each microbe has a unique complement of DNA (or RNA, for many viral pathogens), nucleic acids are the ideal molecular fingerprint aiding identification. Particularly useful are RT-PCR and PCR, with their enzyme-driven processes for amplifying RNA/DNA *in vitro*, to analyze levels of microbial DNA in clinical samples for which other detection methods require higher concentrations, or are too time-consuming or cumbersome to detect.

2.1. The importance of nucleic acid measurement for HIV-1

With the emergence of the global HIV epidemic in the 1980s, it became evident that following the viral kinetics in infected subjects can aid significantly in understanding the progression of HIV-1 infection to stage 3, i.e., the disease of AIDS. Before viral load tests, many researchers believed that HIV-1 infection underwent dormant periods. Viral load tests showed that HIV-1 replication in the human body is a continual and gradually progressive process and that the viral replication is always active.[11] A typical pattern of HIV-1 infection is shown in Figure 2.

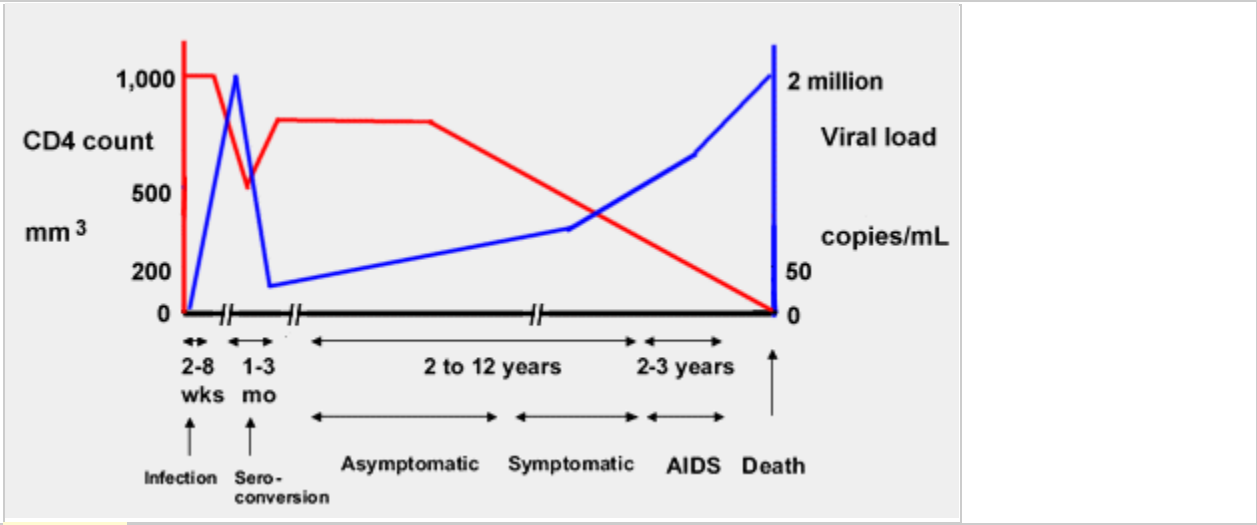


Figure 2.

Viral Kinetics of HIV-1 Infection. After HIV-1 infection, during the acute disease phase, viral load is high, followed by a strong CD4 cell decline. After seroconversion and establishment of chronic infection, the viral load reaches a viral set point phase (at approximately 14 weeks following infection) from which it continues to rise as the CD4 cell count declines over several years. A CD4 cell count of fewer than 200 cells/mm3 is one of the qualifications for a diagnosis of stage 3 infection (AIDS). Source: http://i-base.info/ttfa/section-2/214-how-cd4-and-viral-load-are-related/[12]

With the development of the first antiretroviral (ARV) agents (such as AZT, zidovudine) and later, the establishment of highly active antiretroviral therapy (HAART), understanding the kinetics of suppression of viral replication and detection of antiviral resistance became a major focal point in guiding and caring for patients. Viral load monitoring was the first direct approach to personalized healthcare, determining the activity of ARV medicines in an individual patient at specific time points.

Suppressing the HIV-1 viral load to undetectable levels (<50 copies per mL) is the primary goal of HAART.[13] This level of suppression should be achieved by 24 weeks after starting combination therapy. HIV-1 viral load is the most important predictor of response to treatment with HAART.[15] Failure of HAART to adequately suppress viral load is termed virologic failure. Levels of HIV-1 RNA higher than 200 copies per mL are considered virologic failure, and should prompt further testing for potential viral resistance.

In 1992, to aid in this therapeutic approach the first commercially available PCR-based diagnostics were marketed. The AMPLICOR CT (*Chlamydia trachomatis*) Test and the AMPLICOR HIV-1 MONITOR Test were the first PCR-based molecular diagnostic tests. During the first half of the 1990s, the sensitivity of these early commercial products was moderate. By 1996 to 1997, through technical improvements, the next generation of tests could detect and measure viral loads as low as 400 or 500 copies/mL. Since 1998, most tests used routinely in clinical practice accurately detect and measure HIV-1 RNA as low as 40 or 50 copies/mL.[18] For academic research purposes, several groups have described an ultrasensitive or single copy assay that can detect 5 copies/mL or even 1 copy/mL.[19]

In addition to RT-PCR, a range of other nucleic acid-based techniques are also employed to measure HIV-1 RNA viral load, such as branched DNA (bDNA) assay[20] and nucleic acid sequence based amplification (NASBA).[21] NASBA was developed in 1991 by J. Compton, who defined it as "a primer-dependent technology that can be used for the continuous amplification of nucleic acids in a single mixture at one temperature."[22]
These techniques were also employed to study the relative effectiveness of ARV drugs in clinical trials during the very active HIV drug discovery decades from 1990 to 2010.[23]

Principle of bDNA and NASBA. (A) Target RNA is captured with a bifunctional Capture Extender oligonucleotide probe that hybridizes to the target molecule and a Capture Probe that is covalently attached to a substrate (e.g., a microtitre plate well or a bead). A Signal Amplification complex (Preamplifier and Amplifier with labeled probes) containing a number of alkaline phosphatase enzymes is then hybridized to the target molecule via a Label Extender probe. Source: http://www.diacarta.com/article.php?id=38[24] (B) NASBA works as follows: An RNA template is added to the reaction mixture and reverse transcriptase synthesizes the opposite, complementary DNA strand. RNAse H destroys the RNA template from the DNA–RNA complex (RNAse H only destroys RNA in RNA–DNA hybrids, but not single-stranded RNA). A second primer attaches to the 5' end of the DNA strand. Reverse transcriptase again synthesizes another DNA strand from the attached primer, resulting in double-stranded DNA. T7 RNA polymerase continuously produces complementary RNA strands off this template which results in amplification. Finally, a molecular beacon is employed to detect the amplified product and allow for quantitation. Source: http://www.biomerieux.com.co/servlet/srt/bio/colombia/dynPage?open=CLM_CLN_PRD&doc=CLM_CLN_PRD_G_PRD_CLN_87&pubparams.sform=3&lang=es_co[25]
Various nucleic acid detection techniques, discussed above, are employed for the detection and quantitation of HIV-1 infection. Molecular diagnostics may carry, in general, a larger cost burden than other laboratory techniques that detect pathogens via shed surface proteins or antibodies in human serum. However, the speed, specificity, and sensitivity of molecular testing offers a number of advantages over the more "traditional" methods that it has replaced, such as culture, which is slow and labor-intensive, or hybridization or similar techniques that are often imprecise, insensitive, or for which the interpretation of results is often subjective. Molecular methods also offer advantages over measuring antigens, such as p24, or disease markers, such as CD4 cells, in people infected with HIV-1. Early monitoring of the status of patients with HIV-1 infection used the CD4 cell count to determine progression of disease, and many resource-limited settings still employ this technique today. CD4 cells are the white blood T-cells that are specifically targeted by HIV due to their surface receptor repertoire and depleted as infection progresses. The CD4 cell count provides a measure of the immune function of the human host and is a late marker of disease progression. The measurement is used in establishing thresholds for the initiation and discontinuation of opportunistic infection (OI) prophylaxis and in assessing the urgency to initiate HAART. It is recommended that ARV therapy be initiated when the CD4 cell count falls below 200–350 cells/mm^3, depending on the availability of ARV medicines in a given country.
Measurement of CD4 cells using the current technology is imprecise. Since certain standard-of-care recommendations, such as initiation of prophylaxis against *Pneumocystis carinii* pneumonia

(an OI common in HIV-1 patients), have been made, treatment may be based on a single CD4 cell count, and CD4 measurement error may have important clinical consequences. Often the use of confirmatory tests is recommended and both tests need to be below a certain threshold limit.[27] Therefore, additional cost is incurred by the confirmatory testing, and the advantage of using the more inexpensive CD4 cell count test is lost.

After initiation of ARV therapy, due to suppression of the HIV-1 viral load, the immune system is allowed to recover and the CD4 cell count increases. For most patients on therapy, an adequate response is defined as an increase in CD4 count in the range of 50–150 cells/mm^3 during the first year of HAART, generally with an accelerated response in the first 3 months of treatment. The CD4 count response to HAART varies widely, but a poor CD4 response in a patient with viral suppression is rarely an indication for modifying an ARV regimen. In patients with consistently suppressed viral loads who have already experienced HAART-related immune reconstitution, the CD4 count provides only limited information.[28]

A second biomarker used in HIV-1 laboratory testing is the viral core protein p24. This biomarker can be measured in the patient's blood in early acute infection, often before antibodies to the viral onslaught are detectable. A negative result for the antigen does not rule out infection, because the test lacks exquisite sensitivity; i.e., the test should not be used to verify noninfection. Antigen detection signals infection, however, and positive results in seronegative individuals can be an effective, although not cost-effective, means to identify early infection. The p24 antigen test can be of value in blood screening, for identification of acute infection, for monitoring infection, and to assist in the diagnosis of infection in the newborn. It has been used for detecting early infection in rape cases, for identifying infection after occupational exposure, and for assisting in the resolution of indeterminate Western blot results.[29]

As both CD4 cell count and p24 have caveats briefly discussed above, HIV-1 RNA viral load analysis by nucleic acid testing has, in many clinical situations, replaced less predictive methods of measurement of these biomarkers.

2.2. Other therapeutic areas

2.2.1. Microbiology and infectious diseases

Real-time PCR revolutionized the means by which clinical laboratories identify human pathogens. It is estimated that <1% of bacteria present on earth have been described using cultivation technology.[30] Additionally, various pathogens, particularly mycobacteria and fungi, require prolonged periods of cultivation, necessitating administration of empiric antimicrobial therapy while a laboratory result is awaited. Due to the limitations of cultivation technology, PCR amplification and sequencing-based methods are able to also reveal novel microbes associated with human diseases. Hence, cultivation-independent methods offer a potential for rapid diagnosis, thus preventing antibiotic selection pressure and emergence of resistant pathogen infections. Additionally, molecular testing is able to identify hazardous microbes without risk to laboratory staff as well as speed isolation of a given patient harboring highly infectious pathogens into a quarantine setting.

As opposed to monitoring during care of chronic viral infections, such as HIV-1, HBV, and HCV, most tests in the microbiology diagnostic assay repertoire are qualitative, aimed at the detection of the pathogens, which then warrant follow-up evaluation.

Several reviews of real-time PCR in clinical microbiology have been published; among others, the review by Espy et al. in 2006 is a comprehensive guide. Important pathogens diagnosed with

molecular testing at the time of publication[32] of this chapter were agents for disease areas such as:

- Respiratory infections, such as adenovirus, Mycoplasma pneumoniae, Mycobacterium tuberculosis, Legionella spp, and Streptococcus pneumonia
- Genitourinary/sexually transmitted infections with PCR assays for C. trichomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, and human papillomavirus
- Central nervous system infections dominated by herpes simplex, varicella zoster, and West Nile Virus (WNV)
- Gastrointestinal infections with, most notably, Clostridium difficile

In recent years infectious disease surveillance and monitoring of antibiotic resistance has also been added to PCR-based molecular diagnostic tests, such as detection of gram-negative bacilli and vancomycin-resistant enterococcus species.

Finally, the host of viral pathogens causing human disease are generally identified, quantitated, and managed via PCR-based laboratory tests. Important examples are diagnosis and management of hepatitis B and C, herpes virus family infections, and influenza epidemic outbreaks.

2.2.2. Blood screening

Annually, millions of people worldwide receive blood transfusions or blood-derived products. Around the world, more than 92 million blood donations are collected every year.[33] From these, a single whole-blood donation can be transfused in up to three people, and blood-derived products from a single donation may be given to hundreds of patients.[34, 35] Although testing and policy decisions have combined to make blood supplies in many countries among the safest in the world, there still exists some risk of transfusion-transmitted infection (TTI) with blood-borne diseases (e.g., HIV, hepatitis, WNV). Laboratory screening of donated blood and blood products for infectious diseases is a key safety measure in protecting patients and preventing the spread of serious diseases.

Nucleic acid testing (NAT) by PCR- or transcription-mediated amplification (TMA) technology detects the presence of viral infection by directly testing for viral nucleic acids and can be used to screen whole blood and plasma samples. Commonly used NAT assays detect HIV-1 RNA, HCV RNA, HBV DNA, and WNV RNA.[38]

NAT technology has revolutionized the ability of blood centers to efficiently test for and reduce infusions of potentially infectious blood units while continuing to ensure on-time availability of blood and blood products for patients. The global trend toward adopting this technology clearly demonstrates its effectiveness for increasing the safety of blood supplies.

2.2.3. Human genetics—Testing via nucleic acid markers

Besides the exploration of human pathogen diagnostics, molecular testing has been employed to identify a myriad of human host markers predominately via DNA found in any human cell.

2.2.3.1. Prenatal diagnosis

Prenatal diagnosis employs a variety of techniques to determine the health and condition of an unborn fetus. There are three purposes of prenatal diagnosis: (1) to enable timely medical or surgical treatment of a condition before or after birth, (2) to give the parents the chance to abort a fetus with the diagnosed condition, and (3) to give parents the chance to prepare psychologically, socially, financially, and medically for a baby with a health problem or disability or for the likelihood of a stillbirth.

Congenital anomalies account for 276,000 perinatal deaths by pregnancy Week 4 annually on a global basis. The aim of prenatal screening is to detect birth defects, such as neural tube defects; chromosome abnormalities (e.g., Down Syndrome, fragile X syndrome); and genetic disorders and other conditions (e.g., spina bifida, cleft palate, Tay Sachs disease, sickle cell anemia, thalassemia, cystic fibrosis, and muscular dystrophy). Screening can also be used for prenatal sex discernment.

There is a variety of noninvasive and invasive techniques available for prenatal diagnosis. Each should be applied only during specific time periods of a pregnancy for greatest utility.

Traditionally, amniocentesis, performed at pregnancy Weeks 14–20, was employed to sample the amniotic fluid, which contains fetal cells, for analysis of chromosomal defects. Risks with amniocentesis are uncommon, but include fetal loss. The increased risk for fetal mortality following amniocentesis is about 0.5% above what would normally be expected. Collected embryonic cells from the amniotic sac need to be cultured for the chromosomal analysis. This process is cumbersome, carried out in specialized laboratories only, and requires a time period of 1–2 weeks, including transport of the sample.

Similarly, chorionic villi sampling can provide information about the fetus' health and development status as early as at 10 weeks of pregnancy. Miscarriage rates are higher in this procedure compared to amniocentesis, up to 1.9%. Test results are obtained within 2 weeks and require specialized laboratories and culturing techniques.

In recent years, analysis of cell-free DNA shed from fetal cells in the maternal blood has become a molecular technique to investigate congenital defects as early as pregnancy Weeks 9–10. High-throughput shotgun sequencing of the plasma of pregnant women results in obtaining about 5 million sequence tags per patient sample. Using this technology, in 2008, Fan et al. were able to identify aneuploid pregnancies, with trisomy detected at gestational ages as early as ~10 weeks. Shotgun sequencing is carried out on a next-generation sequencing platform such as Illumina. In 2010, Chiu et al. studied 753 pregnant females using a 2-plex massively parallel maternal plasma DNA sequencing, and trisomy was diagnosed with z-score greater than 3.[43] The test demonstrated 100% sensitivity, 97.9% specificity, positive predictive value of 96.6%, and negative predictive value of 100%.

The main advantages of these protocols are that they can be used earlier than the current prenatal testing protocols and, unlike current protocols, that there is no risk of spontaneous abortion. Noninvasive prenatal diagnosis (NIPD) has been implemented in the United Kingdom (UK) and parts of the United States (US).

2.2.3.2. Inherited diseases

Carrier screening, testing of parents in preparation for pregnancy, is used to identify genetic mutations that could cause serious inherited disorders. Some of the more common disorders for which screening is done are cystic fibrosis, sickle cell disease, thalassemia, and Tay-Sachs disease. These disorders are recessive, which means that a person must inherit a defective gene from each parent to have the disease. If both parents are carriers of a disorder, the child will have a one-in-four chance of inheriting one defective gene from each of the parents and having the disorder. This type of testing is offered to individuals who have a family history of a genetic disorder and to individuals in certain ethnic groups with an increased risk of specific genetic conditions. For the testing procedure, venous blood is collected and sent to specialized laboratories. There, the DNA contained in the human blood cells is amplified via PCR and, for

example, a next-generation sequencing platform is utilized to investigate the genotype of a set of genes in a cost-efficient manner.

Newborn screening is used just after birth to identify genetic disorders that can be treated early in life. Early detection, diagnosis, and intervention can prevent death or disability and enable children to reach their full potential. Each year, millions of babies in the US are routinely screened, using molecular tests performed on a few drops of blood obtained from their heels, for certain genetic, endocrine, and metabolic disorders, and are also tested for hearing loss prior to discharge from a hospital or birthing center. All states currently test infants for phenylketonuria (a genetic disorder that, if left untreated, causes intellectual disability) and congenital hypothyroidism (a disorder of the thyroid gland).

The expansion of the screening panel to approximately 30 heritable metabolic conditions occurred from 1997 to 2007 with the introduction of tandem mass spectrometry (MS/MS), a technology that detects multiple disease biomarkers simultaneously in a single specimen. This technique employs the screening of blood spots for inborn errors of metabolism by electrospray MS/MS with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. More recently, other markers, based on nucleic acid analysis of the newborn genetic makeup,[48] such as sickle cell disease, alpha-1-antitrypsin deficiency, and Factor V Leiden, have been added.

2.2.3.3. Cancer markers

Cervical cancer is the 7th most common cause of cancer death in Europe for females, and the 15th most common cause of cancer death overall. According to currently available US Centers for Disease Control (CDC) Fast Stats [49] cervical cancer mortality in the US in 2010 was ~4,000 or ~2.5 deaths per 100,000 females.

The global statistics provided by Cancer Research UK are far more saddening. Worldwide, there were more than ~275,000 deaths from cervical cancer in 2010 that accounted for ~10% of female cancer deaths.

The Papanicolaou test—aka Pap test, Pap smear, cervical smear, or smear test—was historically the method of cervical screening used to detect potentially precancerous and cancerous cells in the endocervical canal of the female reproductive system. Atypical findings were followed with more sensitive diagnostic procedures, and, if warranted, interventions that aimed to prevent progression to cervical cancer.

In March 2014, the FDA's Medical Devices Advisory Committee Microbiology Panel voted unanimously to approve the cobas® 4800 HPV Test (Roche Molecular Systems) and recommended that this real-time PCR HPV test replace the Pap smear as the first-line standard of care for cancer screening, another use of nucleic acid testing in molecular diagnostics.

Another wide-ranging use of molecular tests using PCR can be found in the disease area of colorectal cancers where tumor nucleic acids are analyzed for the presence of mutations or other markers. Historically, all colorectal cancers (CRCs) have been considered a single disease entity sharing the same cause, clinical characteristics, and treatment outcomes. However, through analysis of precursor lesions and hereditary forms of the disease, it has now become clear that CRC is a complex and heterogeneous disorder. Although microsatellite instability (MSI) testing has been used for more than a decade for identifying patients with Lynch syndrome, with the recent growth in personalized cancer care, other molecular tests to identify the genetic makeup of individual cancers have become increasingly more important in making therapeutic decisions.

Novel medicines in oncology and relevant biomarker tests are now often developed side-by-side. Current indications for standard-of-care molecular testing in colorectal carcinomas include identifying hereditary cancer syndromes, such as Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer [HNPCC]), and testing for *KRAS* mutational status as a predictor of response to antiepidermal growth factor receptor (EGFR) agents such as cetuximab. In the case of Lynch syndrome, multiple mononucleotide markers are detected via a fluorescent multiplex PCR-based method. A tumor tissue specimen (with tumor cellularity of >20%) and normal tissue specimen are amplified using PCR for 5–7 microsatellite markers. Patterns of normal and tumor genotypes are compared for each marker and scored as MSI-High, MSI-Low, or MS-Stable. Analysis for somatic mutations in the V600E hot spot in the BRAF gene may be indicated for tumors that are scored as MSI-High or show loss of MLH1 expression, because this mutation has been found in sporadic MSI-High tumors but not in HNPCC-associated cancer. *KRAS* mutations have been convincingly associated in randomized clinical trials with poor response to cetuximab and panitumumab. Activating mutations in *KRAS* serve to isolate this signaling pathway from the effects of EGFR and render EGFR inhibition ineffective. Recent advances have shown that only tumors with wild-type *KRAS* show significant response to these agents. Accumulated data from both randomized and nonrandomized studies, reviewed by Jimeno et al., suggest that patients with CRCs whose tumors show *KRAS* mutations should not receive EGFR-targeting monocloncal antibody therapy. This led to the so-called codiagnostic assays with guidance language in both the test intended use information and drug package insert detailing use of the molecular test results for physicians and patients. This approach of diagnostic testing prior to prescription of costly and not always easy-to-tolerate medicines will dominate personalized healthcare in the future.

Currently, most assays can be performed on small quantities of formalin-fixed paraffin-embedded–derived tumor DNA. The pathologist must carefully select the tumor block to minimize dilution of tumor DNA by contaminating normal cells, such as fibroblasts, endothelial cells, and inflammatory cells; a target of at least 10% tumor cells is recommended for most assays.

Cancer research continues to focus on new molecular markers.[55] The integration of molecular markers into existing histomorphologic classifications in surgical pathology has already provided additional stratification for a more accurate prognosis. Furthermore, a molecular definition of cancer may often guide therapy and allow the monitoring of residual disease.

3. Conclusion and a future outlook

The introduction of nucleic acid testing into clinical laboratories has vastly improved detection of infections. Chronic viral infection can be treated with tests at hand that are adequate to inform the physician if the patient is responding, developing resistance, or being cured. The safety of the blood supply was dramatically improved on a global basis with the introduction of nucleic acid testing for blood-borne pathogens. Expectant parents can be informed of the genetic risks of a pregnancy and the inherited diseases for which a developing fetus or a newborn may be treated. Finally, today, patients diagnosed with cancer can experience a much more tailored approach to therapy, maximizing success and efficiency and minimizing costs to both themselves and the healthcare system.

The past decade has seen the number of commercial molecular tests used in practice increase fivefold. In 2013, 60% of the molecular diagnostics tests were sold by five companies: Roche, Becton Dickenson, Abbott, Hologic, and Qiagen.[56] However, in recent years the number of companies developing molecular tests has grown remarkably. Roughly, 350 companies are now active in development of molecular diagnostics,[56] highlighting the utility and importance of nucleic acid testing in healthcare today.

As molecular testing becomes more widely available and applicable to healthcare globally, it is not surprising that the next-wave nucleic acid testing will penetrate the markets in emerging and developing countries. For example, ARV regimens are becoming more widely available, including in sub-Saharan Africa to manage the large numbers of HIV-infected individuals, and state-of-the-art viral load testing will need to accompany the expansion of these regimens. It is a challenge to the manufacturers of nucleic acid tests to adapt technologies and platforms to resource-limited settings. The future of molecular testing may involve reduction in time to test result as well as reduction in assay and instrument complexity and number and training expertise of staff required to perform such assays.

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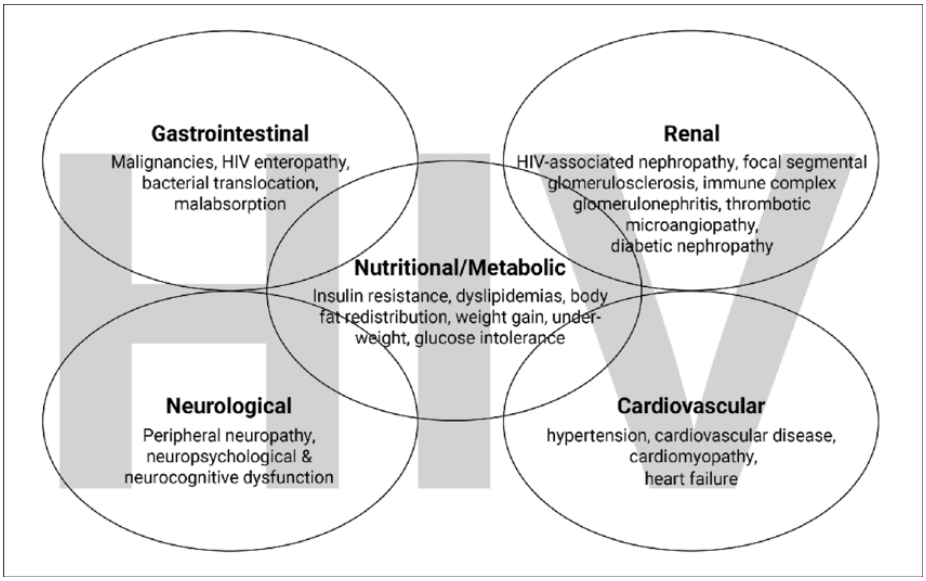
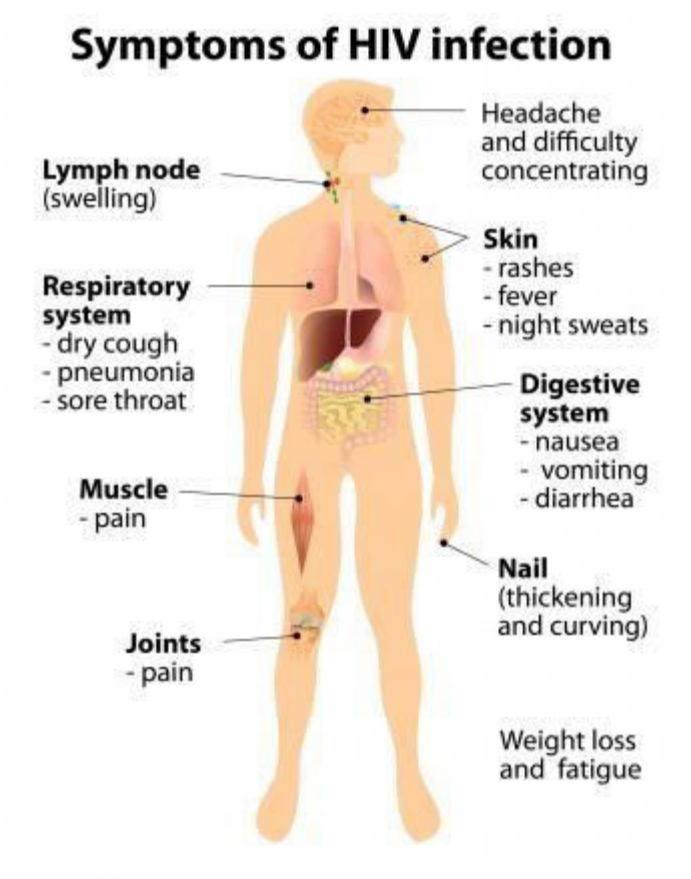
SCHOOL OF BIO AND CHEMICAL ENGINEEING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – V – MEDICAL LABORATORY TECHNOLOGY – SMB 1607



Complications of HIV



AIDS is a disease of human immune system caused by infection with human immunodeficiency virus (HIV). The initial symptoms of this disease may be a brief period of influenza like illness. AIDS is a disease which may be continued a prolonged period without any symptoms. As the infection progresses, it interfere more and more with immune system and makes the infected person so weak to get susceptible to common infections like Tuberculosis, tumors and other general infections.

The HIV virus attacks on the T-cells in the immune system and makes your body so weak that it can be easily affected by bacteria, virus and fungi. In starting weeks, it can show symptoms like headache, fever, sore joints and muscles etc. AIDS is the extreme condition of this infection. When the infection spread, it

attacks on the human immune system making it harder to fight with diseases even normal infections.

Stages of HIV

The HIV infection is divided into three stages;

- Acute HIV Infection
- Chronic HIV Infection
- AIDS/ Advanced Infection

Acute HIV Infection: This is the first stage of HIV infection. Generally, the symptoms of HIV infection do not appear instantly after the infection. So, people don?t know right away when they are infected with HIV. It takes around two to four weeks for the initial symptoms of HIV to appear. This stage starts when the symptoms of HIV start appearing. This stage is also called primary HIV infection or acute retroviral syndrome. The flu-like sickness which is a main symptom in this stage is known as acute HIV infection.

Chronic HIV Infection: This is the second stage of HIV infection. In this stage, the immune system loses battle with HIV and the flu like symptoms disappear. The other symptoms may take months or years to appear. Doctors call this stage asymptomatic or clinical latent period. In this stage, the virus starts replicating in the body that gradually weakens the immune system. You may not look or feel sick, so there are chances that you can pass HIV to others. So, the early testing for HIV is very important, even if you are feeling fine.

AIDS/Advanced Infection: This is the third and advanced stage of HIV infection. In this stage, your CD4 T-cell number goes below 200 and your immunity decreases drastically that make you more susceptible to opportunistic infections.

Reasons of HIV infection

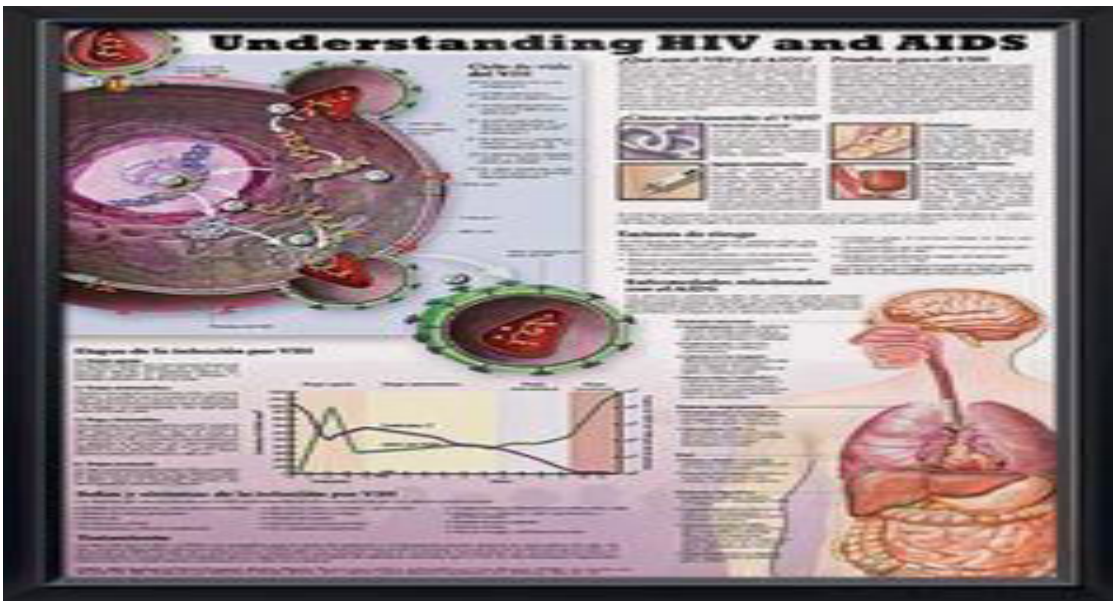
Aids can be transmitted from person to person in many ways:

- By unprotected intercourse with infected person
- By Blood transfusion
- From mother to a child (By birth), it can also be spread by breastfeeding
- Through oral sex, in some cases it can possible by deep kissing
- By the use of hypodermic needles

Symptoms of AIDS

Aids can be transmitted from person to person in many ways:

- Rapid weight loss
- Recurring fever or extreme night sweats
- Sore throat
- Extreme tiredness, Muscles and joint pain


Understanding HIV and AIDS

HIV - Human Immunodeficiency Virus
AIDS - Acquired Immune Deficiency Syndrome.

Introduction: AIDS was first clinically recognized in 1981. Initially it was reported among HOMOSEXUAL MEN and INTRAVENOUS DRUG USERS. Later, it was seen in individuals receiving *blood transfusions, children of *mothers injecting drugs, engaging in *unprotected sex.

Prior to the discovery of HIV, the diagnosis of AIDS was based on the clinical finding of opportunistic infections. *Opportunistic infections are caused by micro organisms to which normal individuals are generally resistant. However, when the immune system is affected by HIV infection, then the previously harmless microorganism cause disease and this can prove fatal.

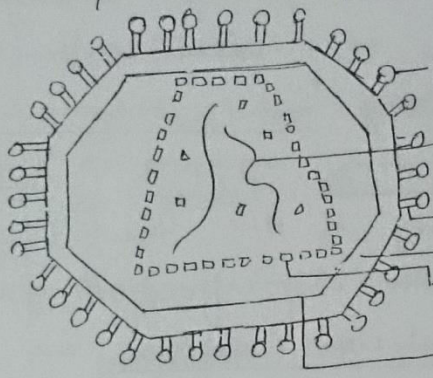
Some principal Opportunistic Infections associated with AIDS

(1) Toxoplasmosis - infect tissue of central nervous system.
* (2) Pneumocystis carinii - (protozoan) found in lungs, causes serious pneumonia.
* (3) Mycobacterium tuberculosis - cause tuberculosis (virulent)
(4) Mycobacterium avium - recurring fever, serious weightloss, digestive abnormalities.
(5) Herpes simplex (virus) cause minor infections of skin.
* (6) Kaposi's sarcoma - most common type of cancer associated with AIDS. It is a slow-growing tumour of the skin.

* very important, sure mentionable

(2) HIV -2 causal agent in West Africa.
There are genetically closely related retrovirus of the lentivirus family.

Diagrammatic representation of HIV virus.



Stages of AIDS

(I) Acute illness, flu-like symptoms, recurring fever for days to several months.
(II) Chronic asymptomatic infection that can last for about 10 yrs.
(III) Swollen lymph nodes; flu-like symptoms, last for several months.
(IV) AIDS - leads to death, usually occurs in 1-2 years.

THE VIRUS AND PATHOGENESIS

→ HIV RNA contains 9 genes.
→ Major genes associated with the virus are
(1) gag (2) pol (3) env
→ (1) gag - this gene encodes for core proteins, matrix protein and nucleocapsid.
(2) pol - encodes for 3 enzymes
- reverse transcriptase
- integrase

* gp 120, gp 41 - refer to the diagram

MECHANISM

→ It is the gp 120 that interacts with the CD4 molecule present on target cells. (T_H cells)

→ Recent research unfold theories of other receptor interactions in addition to that of CD4 - gp120. These are the CK-5 (chemokine) found on the surface of T-cells and macrophages.

→ This CK-5 interacts with the gp 41 envelope protein.

→ Thus, both CD4 - gp120 as well as CK-5 - gp41 interaction ensure a successful entry of the virus (HIV) into the target cells.

→ After the entry of HIV into target cell, the two identical strands of viral RNA enter the cytoplasm along with viral enzymes reverse transcriptase, protease, RNase H and integrase.

→ The enzyme reverse transcriptase immediately begins the process of transcribing the viral RNA into DNA. The DNA then becomes double stranded, the original viral RNA ‡ DNA complex is degraded by RNase H.

→ The viral DNA then penetrates the nuclear envelop and enters the nucleus. Enzyme integrase is responsible for integrating the viral DNA into cell genome.

→ If the cell is not activated to respond to a particular antigen, the integrated virus remains

→ This initial infection responsible for the 'flu-like' symptoms, which last for years.

→ As soon as the cell becomes activated by ~~to~~ an antigen, it triggers a series of reactions that codes for the synthesis of desired antibody but this protein synthetic mechanism also trigger the expression and production of the integrated viral products by the normal cells.

→ The transcription of the viral genome is mediated by the production of three accessory viral genes :-

(1) tat - speed up transcription of integrated DNA.

(2) nef - modifies the host to suit for HIV viral particles production.

(3) rev - promotes expression of the HIV late structural genes.

Additional genes Vif and Vpr (vpu in HIV-2) encode protein that increases the level of HIV virions.

→ The enzyme protease is involved in ~~cleansing~~ cleaving the translational products into their individual proteins, because they are synthesized in single long transcriptional piece from each of the 3 major genes.

→ Transcription and subsequent translation yields the viral RNA or genetic product along with the structural components. The genes assemble spontaneously

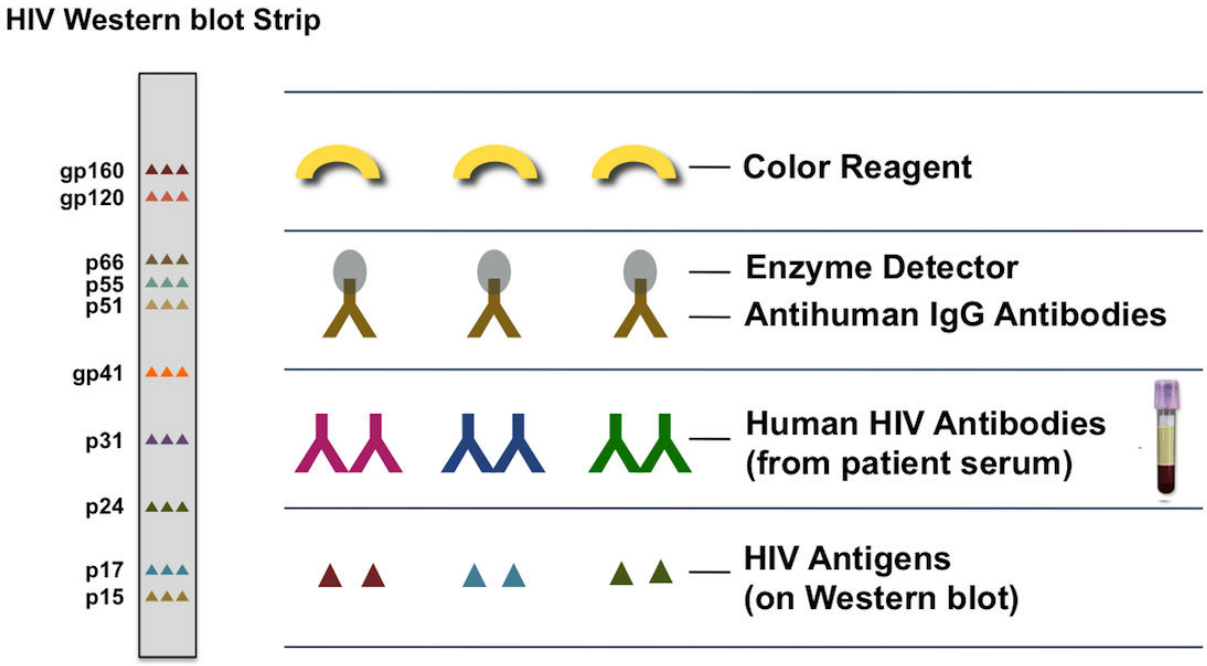
virus is then available to infect and other CD4 cell.

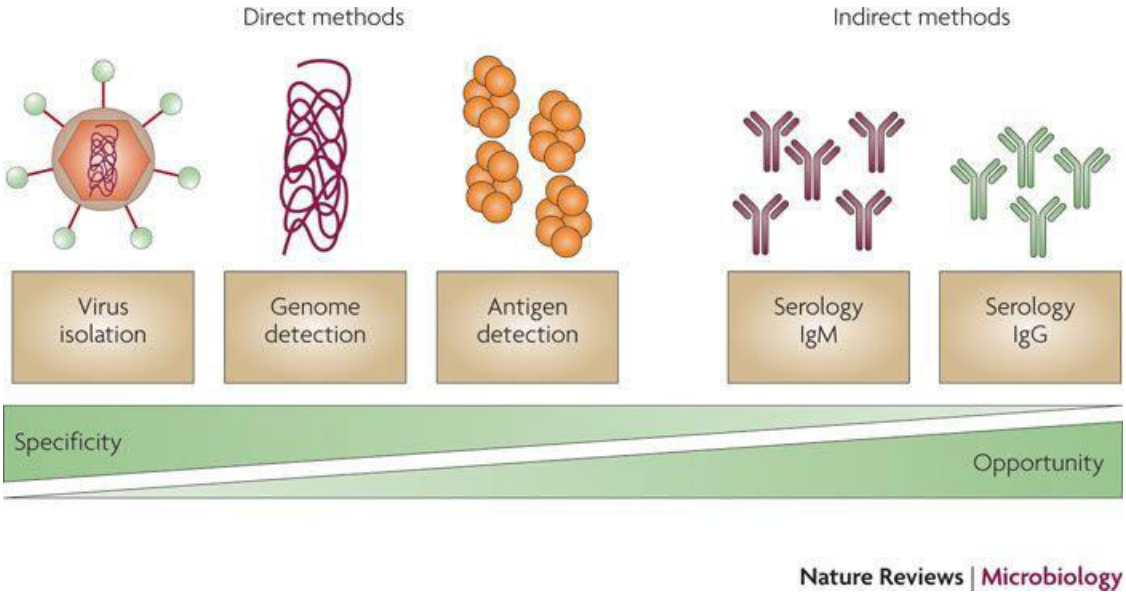
Thus, we can conclude that HIV binds to the CD4 molecule of helper T cells and then gains entry to these cells. Proliferation of the virus may end with destruction of the CD4 + cells when the CD4 + cells are sufficiently reduced in number, individuals become susceptible to the opportunistic infections and finally AIDS develops.

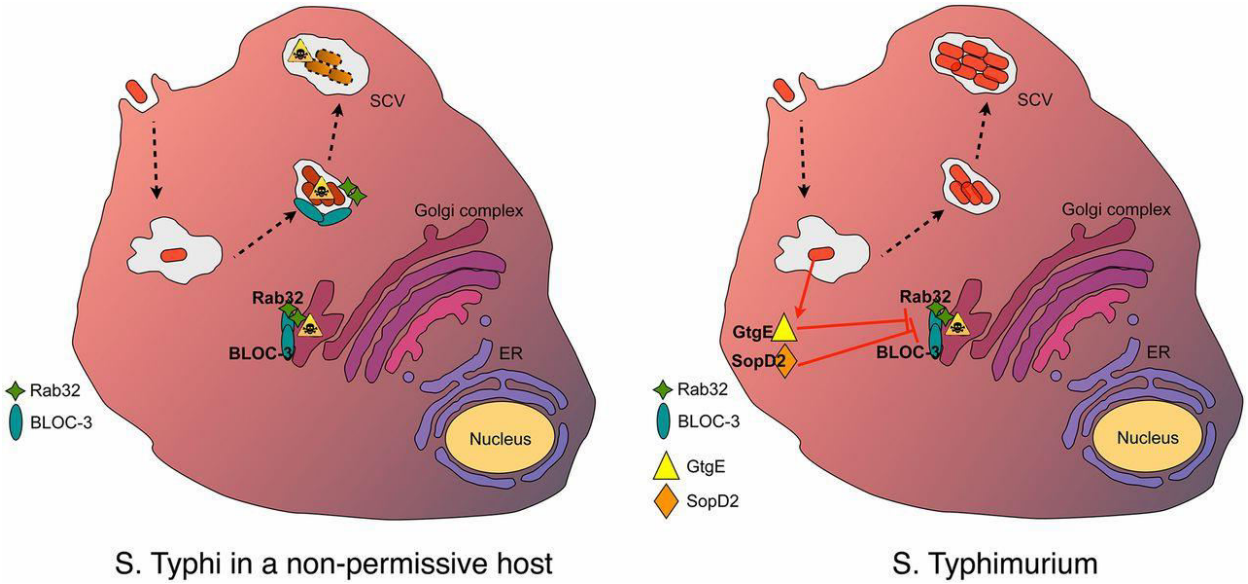
Depletion of CD4 t-lymphocytes results in a decrease of cytokines, such as interleukin 2 and gamma interferon.

Thus, the AIDS virus arrests the cytokine production from the Helper T cells and affect the immune response even towards minor infections. Therefore the individual has to be kept in an antiseptic surrounding with lots of care.

This virus spread only and only through unsafe blood transfusion, from mother to child or during unprotective sex and does not spread during shaking hands, sharing bed, utensils or clothes with the patients.

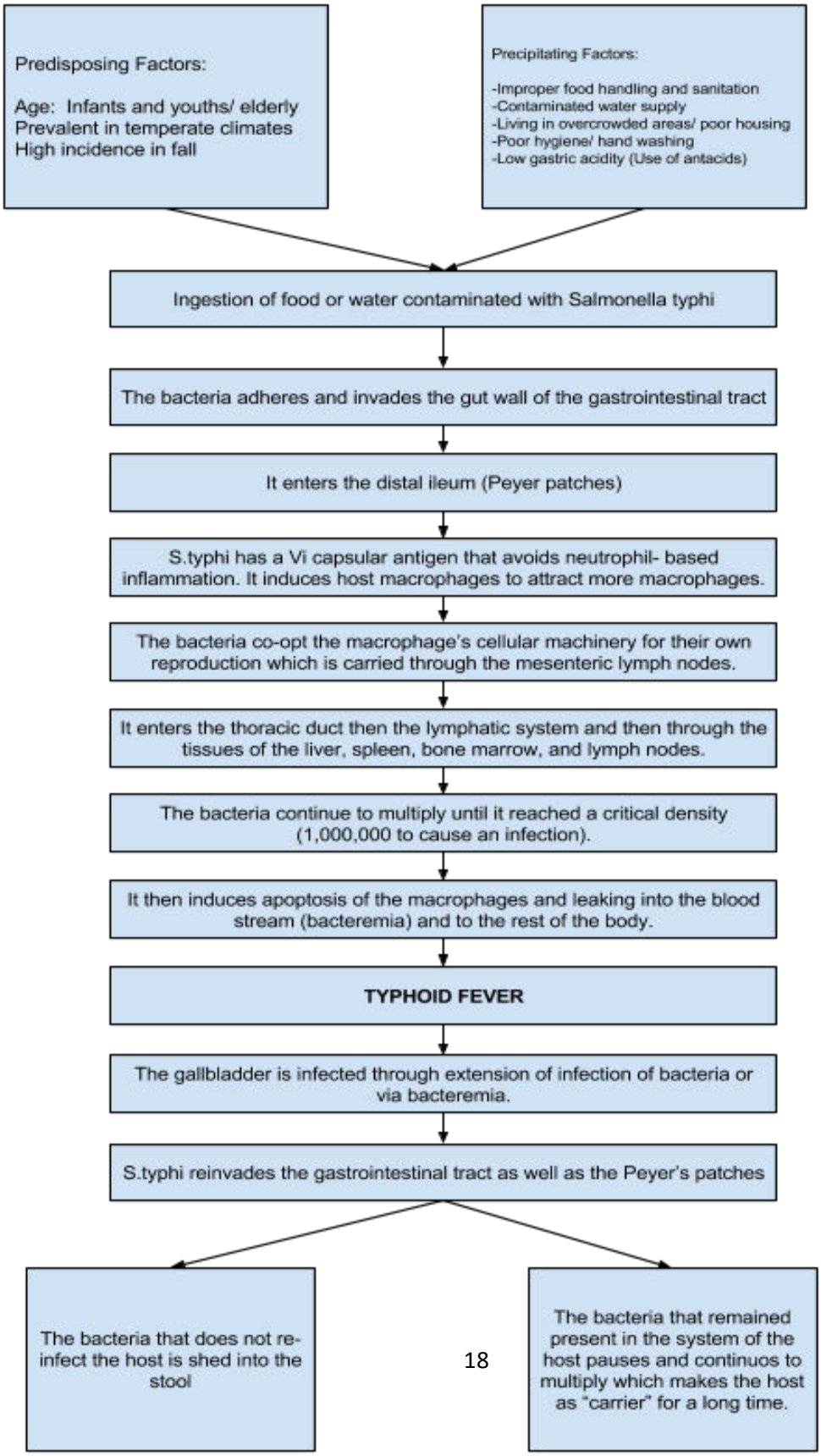

HIV Western blot Strip
gp160
gp120
p66
p55
p51
gp41
p31
p24
p17
p15
Color Reagent
Enzyme Detector
Antihuman IgG Antibodies
Human HIV Antibodies
(from patient serum)
HIV Antigens
(on Western blot)


Direct methods
Indirect methods
Virus isolation
Genome detection
Antigen detection
Serology IgM
Serology IgG
Specificity
Opportunity
Nature Reviews | Microbiology



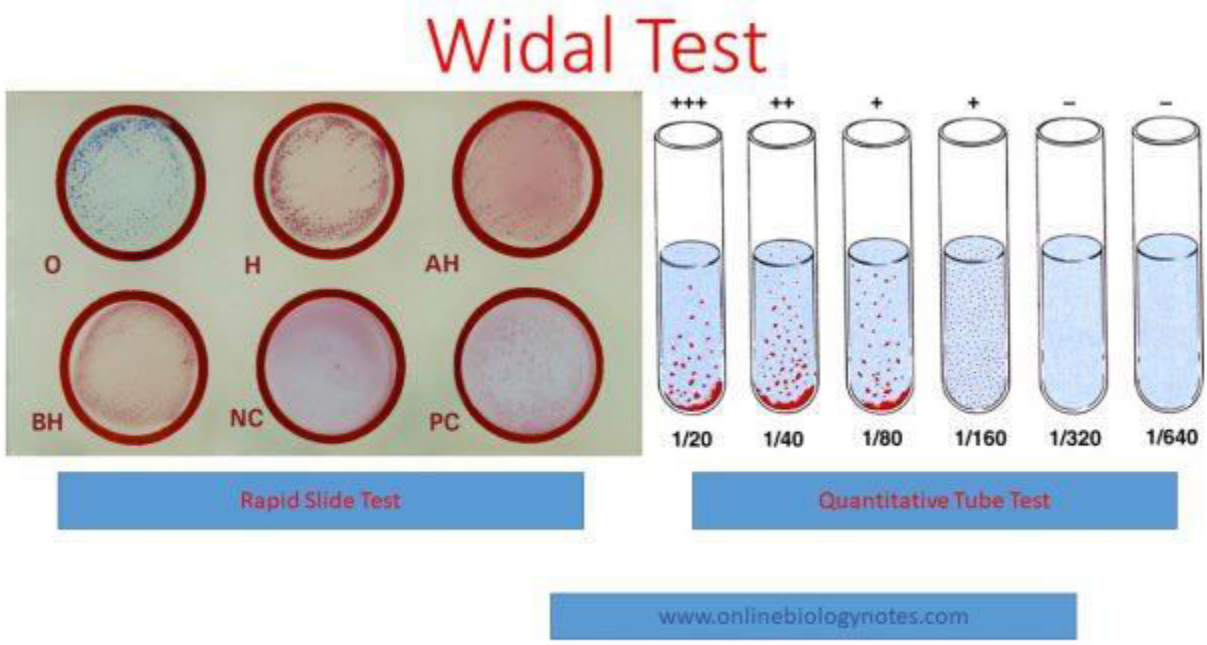
S. Typhi in a non-permissive host

S. Typhimurium


Predisposing Factors:
Age: Infants and youths/ elderly
Prevalent in temperate climates
High incidence in fall
Precipitating Factors:
-Improper food handling and sanitation
-Contaminated water supply
-Living in overcrowded areas/ poor housing
-Poor hygiene/ hand washing
-Low gastric acidity (Use of antacids)
Ingestion of food or water contaminated with Salmonella typhi
The bacteria adheres and invades the gut wall of the gastrointestinal tract
It enters the distal ileum (Peyer patches)
S.typhi has a Vi capsular antigen that avoids neutrophil- based inflammation. It induces host macrophages to attract more macrophages.
The bacteria co-opt the macrophage's cellular machinery for their own reproduction which is carried through the mesenteric lymph nodes.
It enters the thoracic duct then the lymphatic system and then through the tissues of the liver, spleen, bone marrow, and lymph nodes.
The bacteria continue to multiply until it reached a critical density (1,000,000 to cause an infection).
It then induces apoptosis of the macrophages and leaking into the blood stream (bacteremia) and to the rest of the body.
TYPHOID FEVER
The gallbladder is infected through extension of infection of bacteria or via bacteremia.
S.typhi reinvades the gastrointestinal tract as well as the Peyer's patches
The bacteria that does not re-infect the host is shed into the stool
The bacteria that remained present in the system of the host pauses and continuos to multiply which makes the host as "carrier" for a long time.

Widal test: Introduction, Principle, Procedure, Result interpretation, Applications and limitations

March 7, 2018 Gaurab Karki Tests 0



Widal test: Introduction, Principle, Procedure, Result interpretation, Applications and limitations

Introduction

- Widal test is a serological test which is used for the diagnosis of enteric fever or typhoid fever. The test was developed by Greembaum and Widal in 1896. Typhoid or enteric fever is caused by a gram negative bacteria Salmonella enterica (Salmonella Typhi or Salmonella Paratyphi), found in the intestine of man. Salmonella paratyphi also causes Typhoid but of a milder form.

- Salmonella possess O antigen on their cell wall and h antigen on their flagella. On infection, these antigen stimulates the body to produce specific antibodies which are released in the blood. The Widal test is used to detect these specific antibodies in the serum sample of patients suffering from typhoid using antigen-antibody interactions. These specific antibodies can be detected in the patient's serum after 6 days of infection (fever).
- Salmonella Typhi possesses O antigen on the cell wall and H antigen on flagella. Salmonella Paratyphi A and S. Paratyphi B also possess O antigen on their cell wall and but have AH and BH antigen on their flagella respectively.

Principle of Widal test:

- Widal test is an agglutination test in which specific typhoid fever antibodies are detected by mixing the patient's serum with killed bacterial suspension of Salmonella carrying specific O, H, AH and BH antigens and observed for clumping ie. Antigen-antibody reaction. The main principle of Widal test is that if homologous antibody is present in patient's serum, it will react with respective antigen in the suspension and gives visible clumping on the test slide or card.

Requirements for widal test:

i) Fresh serum, stored at 2-8° Serum should not be heated or inactivated.
ii) The complete kit containing five vials containing stained Salmonella antigen

- S. Typhi———-O antigen
- S. Tyhhi———- H antigen
- S. Paratyphi ——AH antigen
- S. Paratyphi ——BH antigen

iii) Widal positive control
iv) Widal test card or slide
v) Applicator stick

Procedure of Widal test:

- Widal test can be done in two ways-one is rapid test on slide and another is tube test in which result may be obtained after one night of incubation.

I. Rapid slide test:

1. Clean the glass slide or test card supplied in the kit well and make it dry.
2. Label the circles (1, 2, 3, 4, 5 and 6) in the test card as O, H, AH, BH, Negative control and Positive control
3. Place a drop of undiluted test serum in each of the four labelled circle (1, 2, 3 and 4) ie O, H, AH and BH and place a drop of Negative control serum in circle 5 and Positive control in circle 6.
4. Place a drop of antigen O, H, AH and BH in circle 1, 2, 3, and 4 respectively and no antigen in circle 5 and O/H antigen in circle 6.
5. Mix the content of each circle with a separate wooden applicator stick and spread to fill the whole area of the individual circle.
6. Rock the test card for a minute and observe for agglutination.

*if agglutination is visible within 1 minute, proceed for quantitative slide test or tube test for the quantitative estimation of the titre of the antibody.

II. Quantitative slide test:

1. Clean the test card and make it dry
2. Put 0.005 ml, 0.001 ml, 0.02ml, 0.04ml and 0.08ml of undiluted serum in 1st, 2nd, 3rd, 4th and 5th circles respectively in the test card.
3. Add a drop of appropriate antigen suspension which showed agglutination in rapid slide test, to each of the above circles.
4. Mix the contents of each circle with a separate wooden applicator stick.
5. Rock the slide slowly for 1 minute and observe for agglutination.
6. The titre of the antibody is the highest dilution of serum up to which there is clear agglutination.
7. Repeats steps 1 to 6 with all the antigens, which showed agglutination in rapid slide test.

The serum volumes in the quantitative slide test corresponds approximately to the tube test is given below:

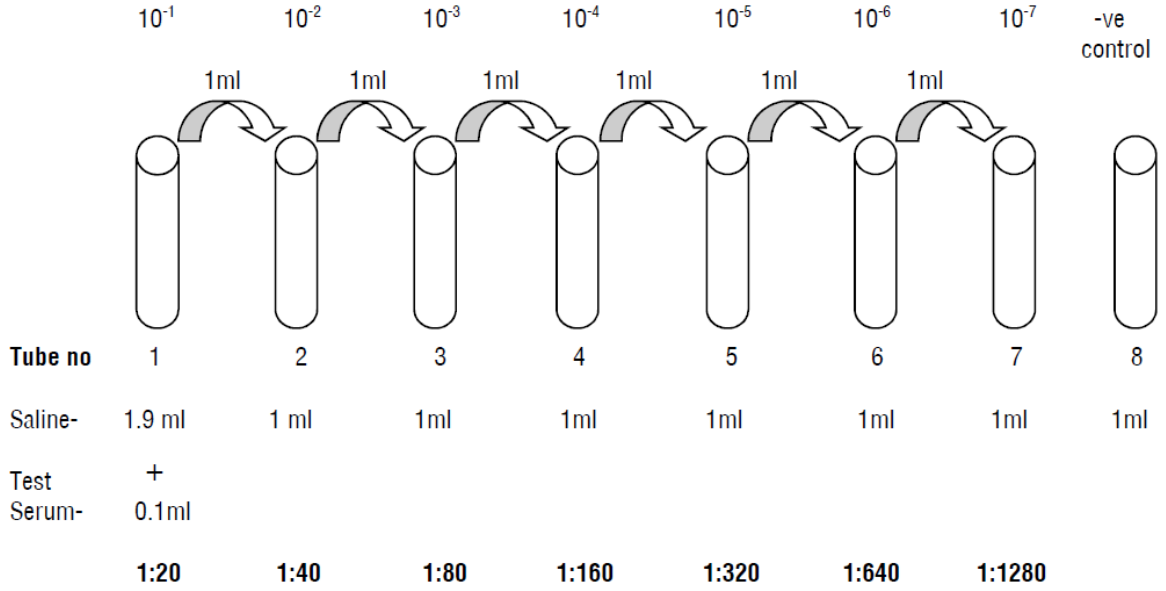
Circle	Serum volume	Antigen drop	Approx. test tube titre
1st	0.08 ml	1 drop	1 : 20
2nd	0.04 ml	1 drop	1 : 40
3rd	0.02 ml	1 drop	1 : 80
4th	0.01 ml	1 drop	1 : 160
5th	0.005 ml	1drop	1 : 320

III. Quantitative tube test:

1. Take a set of 8 clean dry test tubes (Kahn tubes) and label as 1, 2,3, 4, 5, 6, 7 and 8 for O antibody detection.
2. Similarly, take 3 sets of 8 test tubes and label then as 1, 2…8.
3. Dilute the serum samples as follows:

- Pipette into the tube No.1 of all sets 1.9 ml of isotonic saline.
- To each of the remaining tubes (2 to 8) add 1.0 ml of isotonic saline.
- To the tube No.1 tube in each row add 0.1 ml of the serum sample to be tested and mix well.
- Transfer 1.0 ml of the diluted serum from tube no.1 to tube no.2 and mix well.
- Transfer 1.0 ml of the diluted sample from tube no.2 to tube no.3 and mix well. Continue this serial dilution till tube no.7 in each set.
- Discard 1.0 ml of the diluted serum from tube No.7 of each set.
- Tube No.8 in all the sets, serves as a saline control. Now the dilution of the serum sample achieved in each set is as follows: Tube No. : 1 2 3 4 5 6 7 8 (control) Dilutions 1:20 1:40 1:80 1:160 1:320 1:640 1:1280.

4. Add a drop of appropriate widal test antigen to all the test tubes
5. Mix well and incubate at 37°C for 16-20 hours and examine for agglutination.



6. Antibody titre is the highest dilution of serum showing clear agglutination.

Test tube	1	2	3	4	5	6	7	8
Dilution	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280	Control (saline)

Result interpretation of Widal test:

- Antibody titre greater than 1 : 80 is considered significant and usually suggests positive test for Salmonella infection.
- Low titres are often in normal individuals
- A single positive is less significant than the rising antibody titre, since rising titre is considered to be a definite evidence of infection.

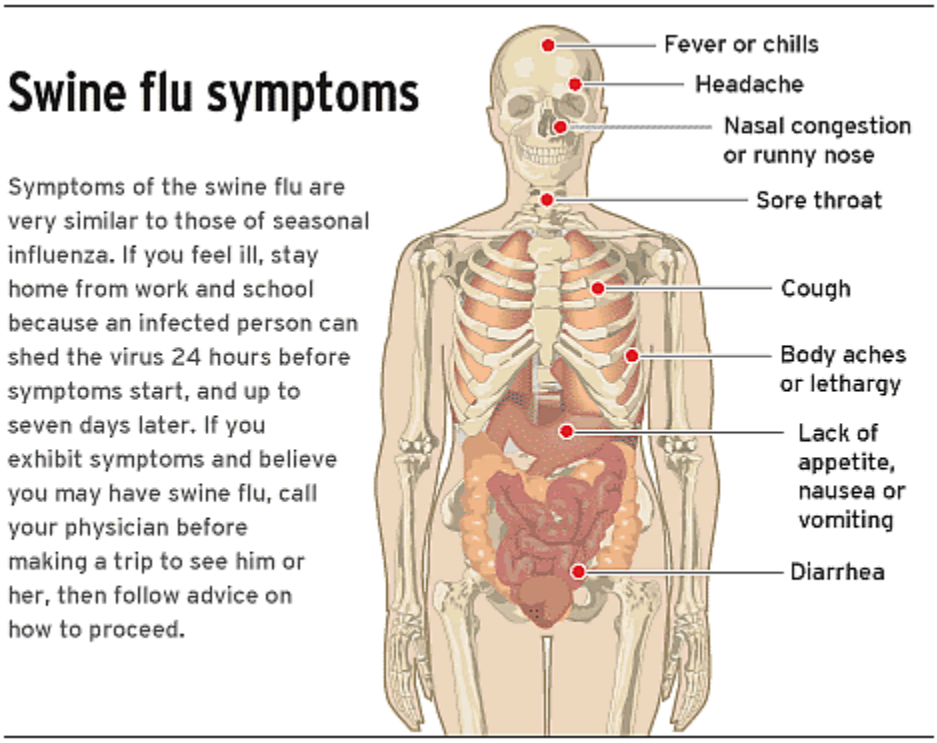
Applications of Widal test:

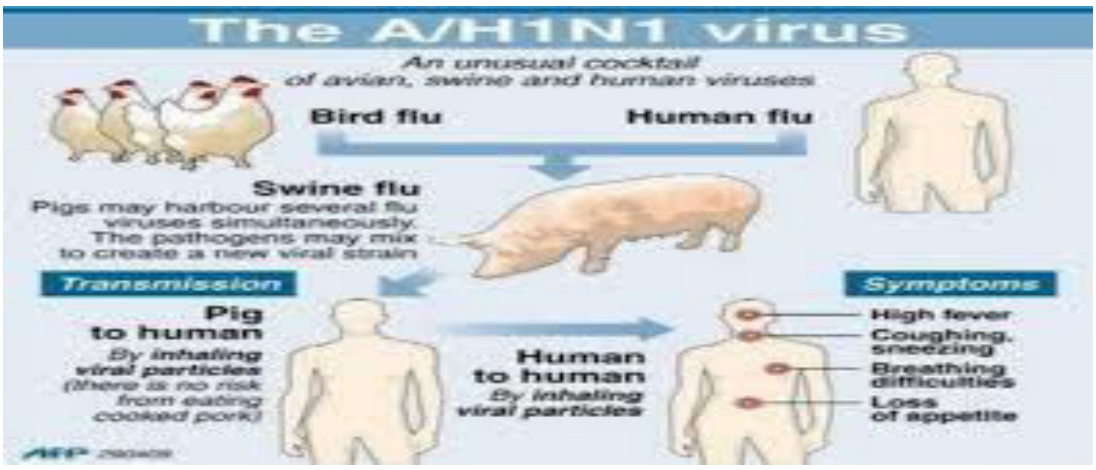
- Rapid test for screening typhoid fever in endemic areas.
- When culture facilities is not available, Widal test is very reliable
- Use for both Salmonella Typhi and Salmonella Paratyphi

Limitations of Widal test:

- Widal test is time consuming to find antibody titre and often times when diagnosis is reached it is too late to start an antibiotic regimen.
- Widal test may be falsely positive in patients who have had previous vaccination or infection with S.Typhi.
- Widal test cannot distinguish between a current infection and a previous infection or vaccination against typhoid.

- Widal test shows cross-reactivity with other Salmonella species.
- False positive Widal test results are also known to occur in typhus, acute falciparum malaria (particularly in children), chronic liver disease associated with raised globulin levels and disorders such as rheumatoid arthritis, myelomatosis and nephrotic syndrome.
- Widal test should be interpreted in the light of baseline titers in a healthy local population. The antibody levels found in a healthy population however, may vary from time to time and in different areas, making it difficult to establish a cut off level of baseline antibody in a defined area and community.
- Severe hypoproteinaemia may also prevent a rise in 0 and H antibody titres. False negative Widal tests may be due to antibody responses being blocked by early antimicrobial treatment or following a typhoid relapse.
- In low typhoid endemic areas, weak and delayed O and H antibody responses limit the usefulness of the Widal test. Variations also exist between laboratories in the performance and reading of Widal tests which compromise further the reliability of the test.
- The World Health Organization (WHO) has said that due to the various factors that can influence the results of a Widal test, it is best not to rely too much on this test.




The A/H1N1 virus
An unusual cocktail of avian, swine and human viruses
Bird flu
Human flu
Swine flu
Pigs may harbour several flu viruses simultaneously. The pathogens may mix to create a new viral strain
Transmission
Pig to human
By inhaling viral particles
(there is no risk from eating cooked pork)
Human to human
By inhaling viral particles
Symptoms
High fever
Coughing, sneezing
Breathing difficulties
Loss of appetite

Swine FLU

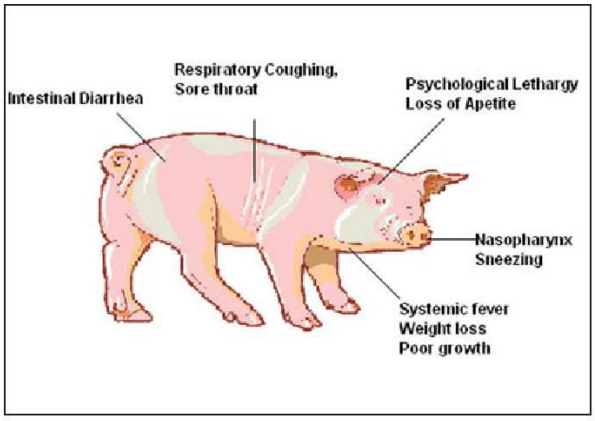


Figure 4: Main symptoms of Swine flu in Pigs[5]

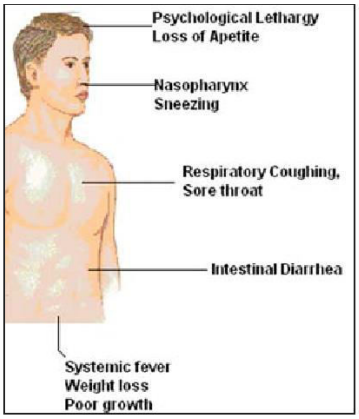
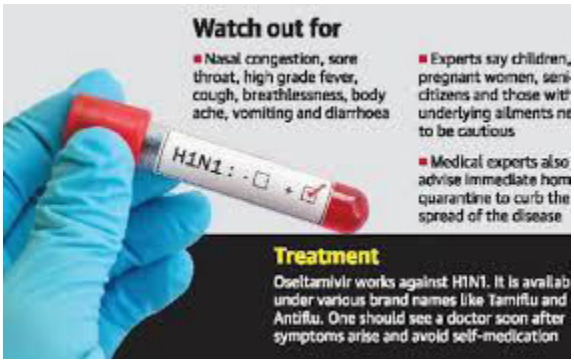
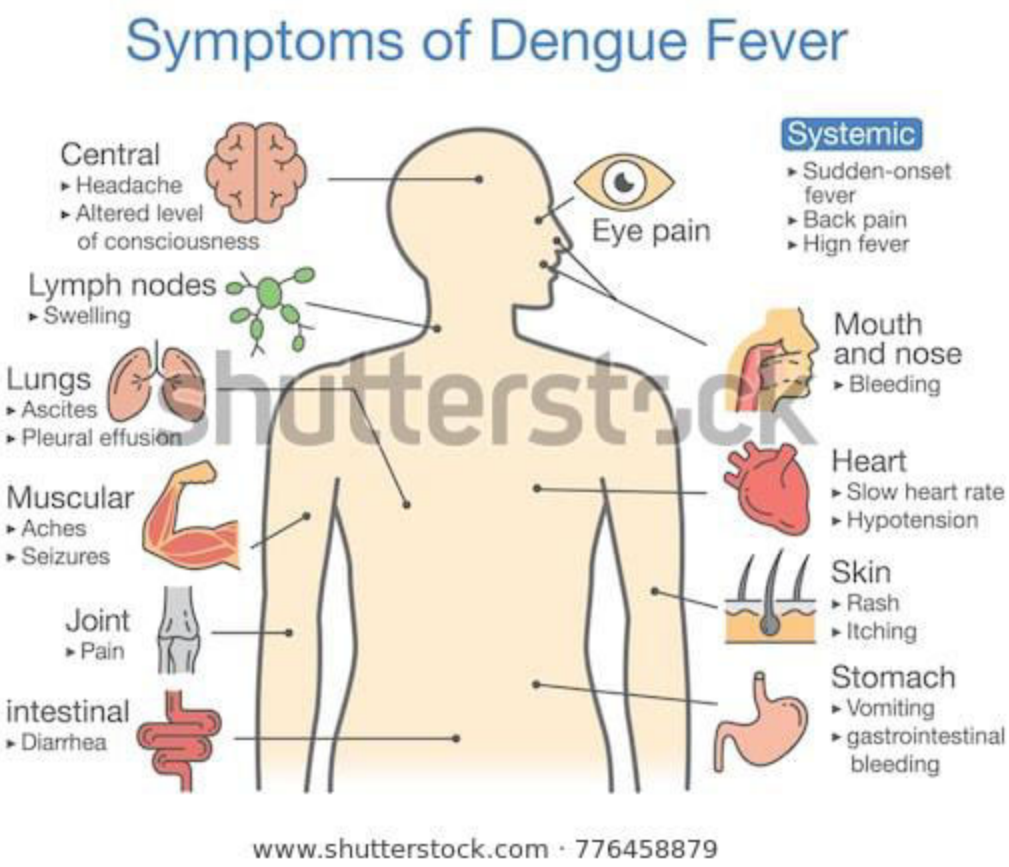


Figure 5: Main symptoms of swine flu in humans[118]




Symptoms of Dengue Fever
Central
▸ Headache
▸ Altered level of consciousness
Lymph nodes
▸ Swelling
Lungs
▸ Ascites
▸ Pleural effusion
Muscular
▸ Aches
▸ Seizures
Joint
▸ Pain
intestinal
▸ Diarrhea
Eye pain
Systemic
▸ Sudden-onset fever
▸ Back pain
▸ Hign fever
Mouth and nose
▸ Bleeding
Heart
▸ Slow heart rate
▸ Hypotension
Skin
▸ Rash
▸ Itching
Stomach
▸ Vomiting
▸ gastrointestinal bleeding
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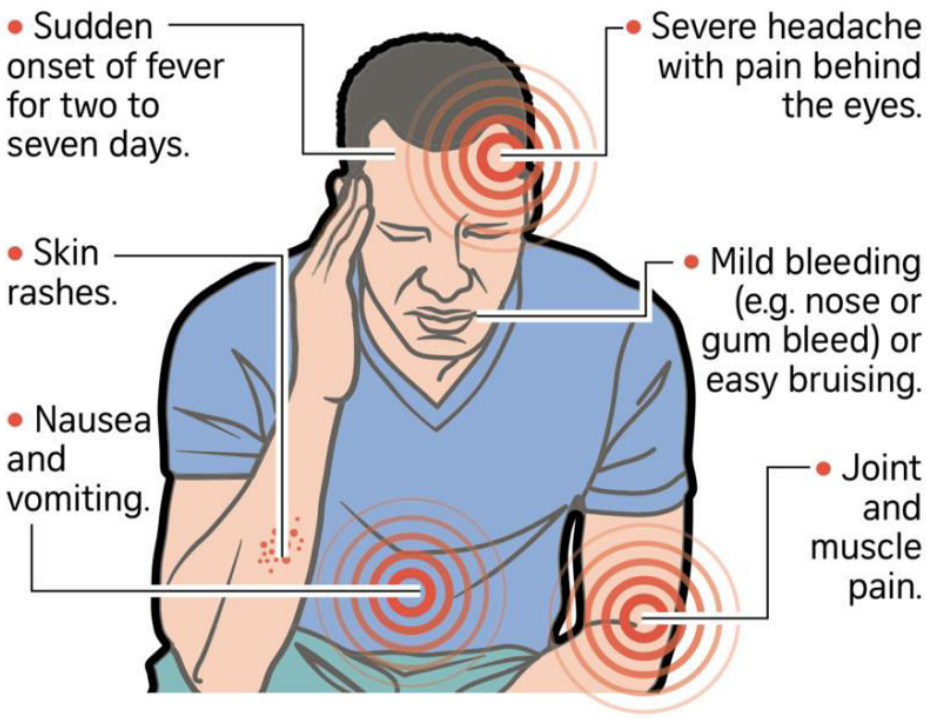
Dengue fever

SYMPTOMS



Risks of severe dengue fever

In rare cases, dengue fever may progress to dengue haemorrhagic fever or dengue shock syndrome. These are severe forms of the infection that can result in death. Symptoms include:

- More severe bleeding problems (gum bleeding, nose bleeding, bleeding into the skin and internal organs, black/tarry stool).
- Plasma leakage (abdominal pain, persistent vomiting).

These usually develop after the start of recovery from the initial infection.

Source: MINISTRY OF HEALTH SUNDAY TIMES GRAPHICS

Dengue (DENV)

Currently, there are about 2.5 billion people (40% of the world's population) living in hazardous areas at risk of becoming infected by dengue. This infection is endemic in at least 100 countries in Asia, the Pacific, America, Africa, and the Caribbean. The World Health Organization estimates that between 50 and 100 new cases are reported each year. In Mexico, there were 17,795 confirmed cases reported in 2016 (including dengue with and without severe or significant alert symptoms),and there have been 541 confirmed cases in the first ten weeks of 2017; out of these 541, 76 are from Nuevo Leon.

Pathophysiology

Dengue (DENV)

The DENV virus contains a single positive-stranded RNA. For the incorporation of the virions to the cells to occur, the interaction of the primary glycoprotein (E) with the receptors on the cellular surface is critical; these include heparan sulfate or lectins (DC SIGN and GLEC5A). Moreover, they can also bind to immunoglobin-type surface receptors in the presence of antibodies against glycoprotein-E or membrane precursor protein (pre-M)8 (Fig. 1). After the fusion of the viral and cell membranes through acidified endocytic vesicles, the viral RNA enters the cytoplasm along the other viral proteins. Non-structural NS5 proteins are the polymerase of RNA dependent of RNA, which fits viral proteins as well as cell proteins to form the replication complex, transcribing viral RNA to produce the RNA template, beginning the replication of viral genetic material. The DENV virus enters the organism through a mosquito bite, infecting the Langerhans cells and fibroblasts.17 The viremia starts three days after the vector bite, is detectable 6–18 hours before the onset of the signs and symptoms, and concludes at the moment of solving the fever.18 The immunological response of the organism to the virus is complex, with elevated levels of interferon (IFN) α and β, which are part of the type-I IFN group contributing to

the elimination of viral particles. However, viral proteins are capable of inhibiting the production of interferons as well as the infected cells' antiviral function.[19] The antibody answer is mainly directed at specific determinants of each serotype, from these, the primary targets are protein E, membrane precursor protein (pre-M) and NS1 protein. When there is a primary infection with one of the four serotypes of the DENV virus, it will generally provide long-term immunity to infections with viruses of the same serotype. Nevertheless, it does not work for a virus of a different serotype, which may result in a secondary infection.

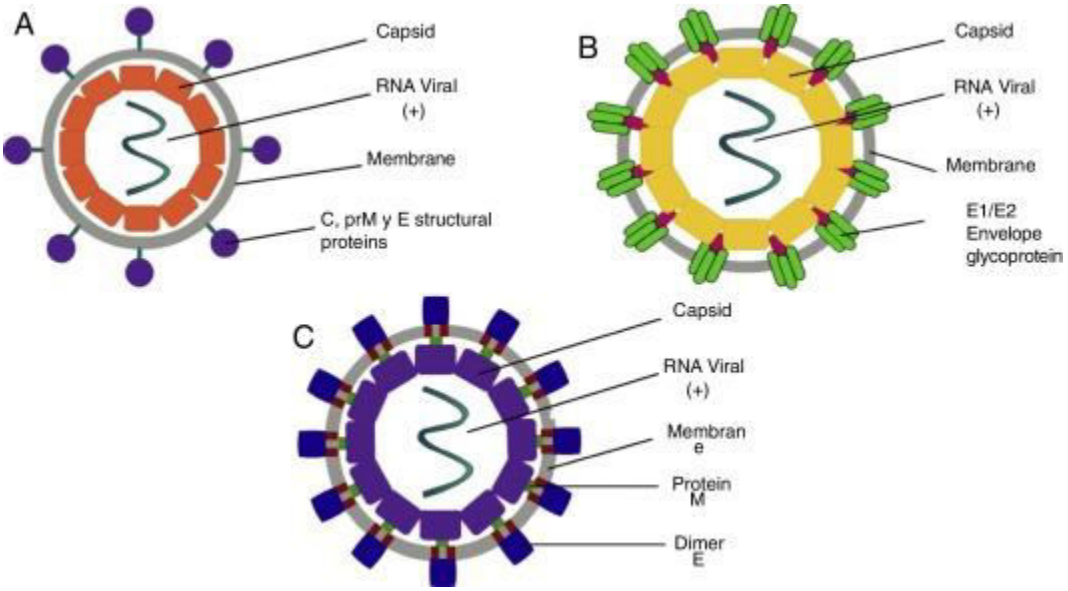


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Figure 1. Structure of a viral particle of DENV (A), CHIKV (B) and ZIKV (C). DENV: Dengue virus; CHIKV: Chikungunya virus; ZIKV: Zika Virus. Source: Modis,[8] Coudera,[22] and Weaver.[15]

Once an immunological answer is assembled, this will cause most of the symptoms in the patient, hence the presence of the capillary leak syndrome, which is caused by the actions of FNT-α IL-8, IFN-γ, IL-2, and some fractions of the serum complement like C3a and C5a. Thus, the intensity of the immunological response will be determined by the magnitude of the viral infection.[20, 21]

Transmission methods

Infections by DENV can occur through multiple paths of transmission, the most important being the mosquito bite. Once the virus has entered the organism, it causes tissue tropism (Fig. 2).

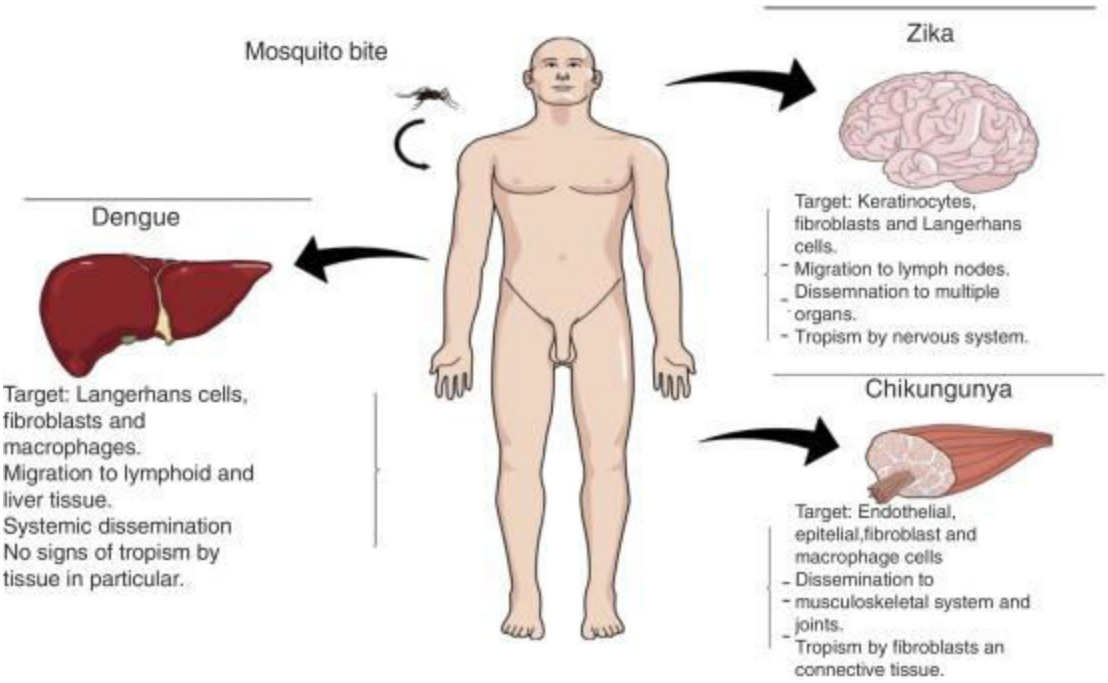

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Figure 2. Tropisms presented by the Dengue, Chikungunya and Zika viruses. Sources: Wu,17 Couderc,22 and Lanciotti.27

The infection transmission through these viruses is mainly caused by:

•
Mosquito bite: The primary vector is *Aedes aegypti*, which feeds itself during the day, transmitting the virus. This vector is found in tropical and subtropical areas (latitude: 45° north to 35° south), presenting a wide distribution.33 Another vector is *Aedes albopictus*, which is more tolerant to cold conditions and presents a global distribution much broader than *Aedes aegypti*. However, its bite is less frequent in humans.

•
Nosocomial transmission: this section includes transmission by blood components, by accidents with sharp objects and splatters in mucocutaneous areas. However, these last two mentioned do not present evidence of an efficient transmission through these pathways and has only been documented as an anecdote.

•
Vertical transmission: Depending on the virus, this is the preferred method of transmission (Table 1).

Table 1. Modes of DENV transmission.

Dengue
- Mosquito bite from *Ae. Aegypti*, and to the lesser extent, *Ae. Albopictus*. - Nosocomial transmission by blood components, puncturing and splashing. The latter do not constitute an efficient transmission mechanism and have only been documented anecdotally. - Vertical transmission in maternal infection 10 days before delivery.

Clinical manifestations

Clinical characteristics caused by these viruses tend to be similar. Nevertheless, we ought to keep in mind the signs and symptoms which predominate in each one, directing us to the proper diagnosis (Table 2).

Table 2. Clinical manifestations of DENV.

Dengue
- Presents an incubation period with a range of 3 - 14 days. - Dengue WITHOUT warning signs: fever (>38 ° C) and two of the following: Nausea/vomiting, rash, headache, retroocular pain, myalgias or arthralgias, leukopenia, positive tourniquet test. (This phase is known as the febrile phase). - Dengue WITH warning signs: Abdominal pain, persistent vomiting, clinical accumulation of fluid (ascites, pleural effusion), bleeding from mucous membranes, lethargy, hepatomegaly (>2 cm), increased hematocrit with rapid decrease in platelets. - Severe dengue: Includes the characteristics of dengue infection plus a manifestation secondary to plasma leakage: Shock, accumulation of fluids with respiratory distress, severe bleeding, severe organ involvement (AST or ALT >1000 IU/L, alteration of the conscience and organ failure). Critical phase in which thrombocytopenia, a rise in activated partial thromboplastin time and a decrease in fibrinogen levels is observed. At the end of the infectious disease by DENGUE is the convalescent phase, characterized by the resolution of most symptoms.

Immunophysiopathological mechanisms of viral infections by DENV

Dengue

- Innate response: After the insect bite, the virus infects the target cells (Langerhans cells, interstitial dendritic cells, and mononuclear phagocytic cells)19, 35 thanks to the non-integrin adhesion molecule-3 (DC-SIGN)36 and the mannose receptor (CD206),37 allowing Rab5-mediated endocytosis. Pattern recognition receptors (PRRs) in these cells are responsible for detecting viral particles or nucleic acids. Most immunocomplexes formed by antibodies and antigens clear from circulation through the reticuloendothelial system.38, 39 Moreover, viral antigens expressed on the surface of infected cells may be recognized by NK cells and eliminated through cellular cytotoxicity. Subsequently, a viral replication occurs in the spleen, lymph nodes, bone marrow and muscle and from there, disseminate into systemic circulation.

- Cellular immune response (T lymphocytes): Infection by DENV of the dendritic cells stimulates T lymphocytes CD4+ and T CD8+, producing pro-inflammatory cytokines [TNF-α (Tumor Necrosis Factor Alpha) and IFN-γ (Interferon-gamma)], that is, in the case of the Th1 cells, the production of IFN-γ, TNF-α, IL-2 (Interleucina-2) and CCL4 (C-C Motif Chemokine Ligand 4) and for Th2 cells, the liberation of IL-4 and IL-13.40, 41, 42 A more significant activity of the T CD8+ cells during secondary infection has been proven,43 as well as crossed recognition in the case of severe dengue with a decrease of cytolytic and cytotoxic activity without altering the production of cytokines.41 During secondary infection, a response mediated by specific T CD8+ cells occurs, which is characterized by the activation of markers, active proliferation, high apoptosis levels and low avidity by the virus causative of the primoinfection. A response of the T cells which correlates with the severity of the disease has been observed.

- Humoral immune response (Lymphocytes B/antibodies): An early response has been observed to be mediated by IgM, subsequently, a response by IgG (predominated by the IgG1 and IgG3 subclasses) occurs, recognizing antigens E, NS3 and NS5 in the primoinfection by DENV, while there is a broader recognition in reinfection (E, C, prM, NS1, NS3, and NS5), presenting a more significant response against E protein.44 Lymphocytes B activity is stimulated during secondary infection as a result of the memory generated in primoinfection. In the case of heterotopic infection by DENV, high amounts of antibodies are produced, enhancing the infection rather than having a neutralizing effect on the virus. This response is called antibody-dependent enhancement (ADE), which acts by connecting viruses with their target cells, mainly monocytes, and macrophages, through receptors to the Fc portion of antibodies (FcyR). FcyRIa and FcyRIIa are the mediators of this ineffectiveness by DENV, which bind IgG.45 Non-neutralizing heterological antibodies recognize viral epitopes and stimulate the dependent ineffectiveness of Fc.

- Chemokines: Chemokine receptors are expressed on the leukocyte surface, are linked to G protein and contain seven transmembrane domains.48 CC-type chemokines play an important

role in the recruitment of leukocytes, with a greater importance placed on CCL4/MIP-1b and CCL3/MIP-1a, both ligands to the CCR1 receptor, and CCL2/MCP-1 for the ligand CCR2. The CCR1 receptor has been described to not play a significant role in the pathogenesis of DENV. Moreover, the relevance of the CCR4 receptor has been observed in hepatic damage and inflammation.

- Other immunoregulator factors: Immunoregulation of the organism facing infection by DENV occurs through the response of mediated signaling by STAT1 and STAT2. In the case of platelets, we observed the increased IL-1b expression after DENV exposure, freeing microparticles through mechanisms dependent on NLRP3 and IL-1B, a secretion dependent on caspase-1 by the platelets.

- Interleukin profiles as a response to viral infection: Induction of Th17 cells is through TCR activation in the presence of cytokines, which activate STAT3, including IL-6, IL-21, and IL-23. Its polarization is characterized by the expression of the CC6 chemokines receptor and its Cc120 ligand, producing IL-17a, IL-17F, IL-21, and IL-22. The receptor of the IL-22 is expressed in non-hematopoietic cells, which helps regulation mediation by IL-22 of the response of local tissue during the infection and/or inflammation, having a protective and regenerative effect.50, 51 On the other hand, IL-17 has an inflammation-inductor effect.52 Elevated levels of TNF-α, IL-6, IL-8, CCL2, CCL3, CXCL10 and IFN-γ have been proven in primary and secondary infections by DENV in humans, in addition to reporting protective activity by IFN-γ, which stimulates cellular resistance to primary infection by DENV, controlling nitric oxide synthase two production. In the case of TNF-α, it has been linked to a rise in its production during episodes of severe dengue.19 Inflammatory mediators act on the endothelium, altering its permeability, resulting in the extravasation of fluid to extracellular space.

- TLRs: Toll-like receptors and the cytoplasmic receptor of the RNA helicase box DExD/H family (Gen 5 associated with the differentiation of melanoma (MDA5) are the two most important sensor families present in the cells of mammals to detect nucleic acids, which, once they recognize the virus, activate two critical families of transcriptional factors: Interferon regulating factors and NF-kB, both producing IFN-α/β and inflammatory cytokines. TLR3 has been observed to recognize and restrict DENV replication in multiple cellular lineages, just like TLR7 has been linked to induced production of IFN-1 by DENV in dendric cells.

- Original antigenic sin: The immune system's inability to gather a response against the same microorganism, but with antigenic variations. In the case of reinfection by DENV, it always involves different viral serotypes, triggering a different immunological response to the primoinfection, altering B and T cell reactivation, not having an optimal avidity for the viral epitopes corresponding to the new infection.

Immunological diagnosis

For diagnosis, early detection of viral components in serological samples ought to be documented, either directly or indirectly. However, the type of test used for detection will depend on the stage of the disease (Table 3 and Fig. 3).

Table 3. Diagnosis of infections by DENV.

Dengue (serological)
- 1st week: Detection of viral DNA by means of RT-PCR or detection of non-structural antigenic protein type 1 (NS1). - IgM: A titration elevated 4 times over its base state is obtained. - IgG: In the case of a primary response, titration levels will be low. In secondary infections, there will be a rapid increase in titration from the 4th day.

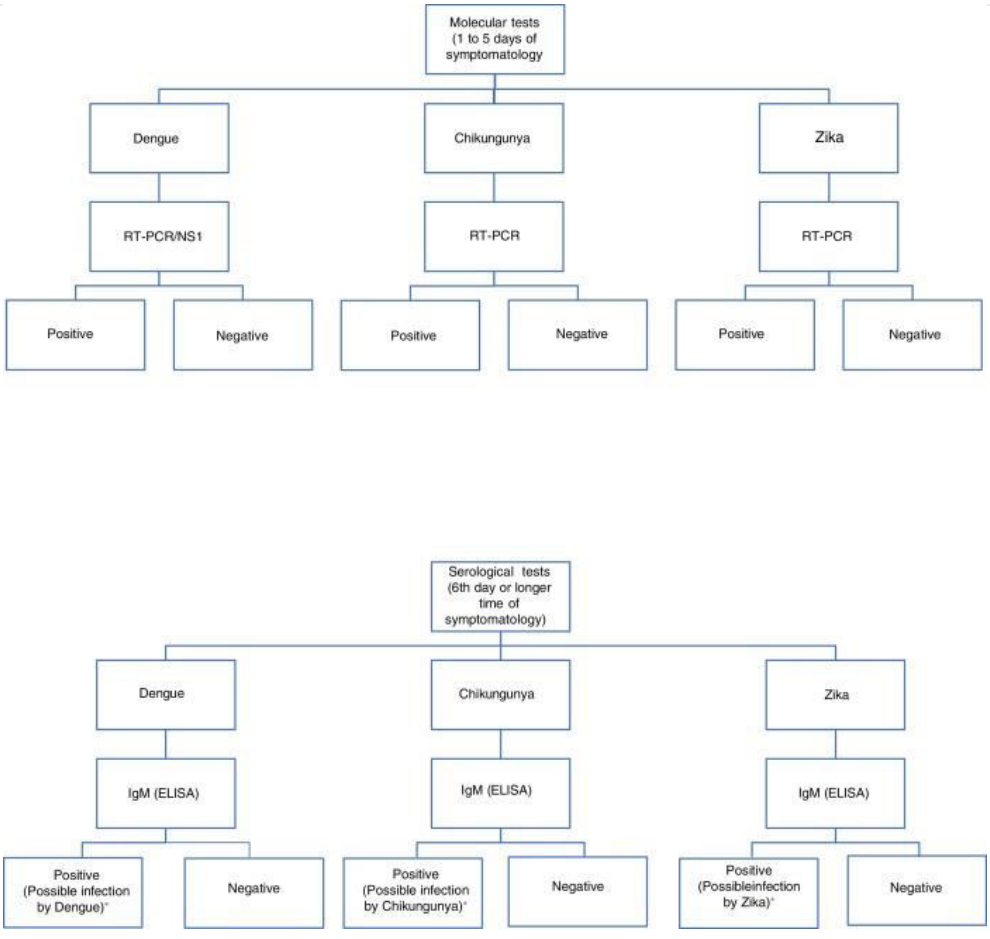

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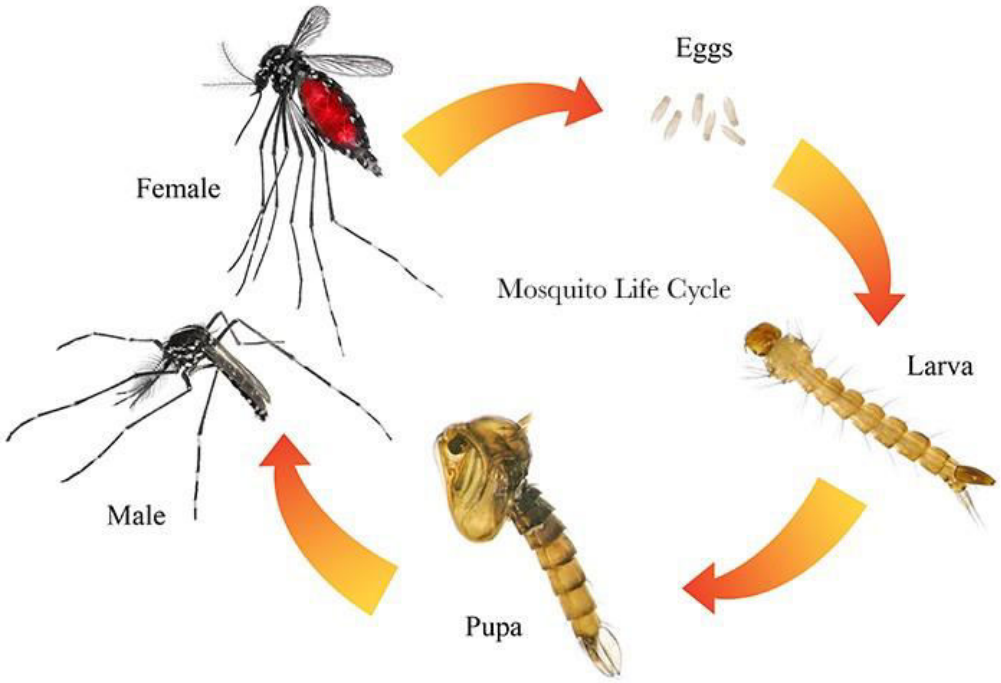
Figure 3. Algorithm for the detection of arboviruses in cases where there is a suspicion of infection by Dengue, Chikungunya or Zika. ELISA: Enzyme-Linked ImmunoSorbent Assay; IgM: Immunoglobulin M; RT-PCR: real-time polymerase chain reaction; NS1: Non-structural antigenic protein type 1. Adaptation of the algorithm proposed by the *Pan-American Health Organization.[71]

Treatment

There is no specific treatment for each one of these infections.[23, 28, 34] Management is symptomatic, depending on the patients' needs and on the current stage of their disease. (Table 4) Today, multiple vaccines for infections caused by arboviruses are being developed; in the case of DENV, there is one vaccine (CYD-TDV) which consists of attenuated virus (recombinants), which is tetravalent and consists of single or multiple doses (5 doses). These vaccines induce the production of neutralizing antibodies against all 4 DENV serotypes, as well as a response from the T cells against structural antigens. Currently, its efficiency in the first two years after the application of the first dose has been reported.[72] The vaccine has been approved by the WHO in over 10 countries (Mexico, the Philippines, Brazil, El Salvador, Costa Rica, Paraguay, Guatemala, Peru, Indonesia, Thailand and Singapore).

Table 4. Management of infections by DENV.

Dengue	Chikungunya	Zika
- Outpatient treatment: oral hydration with solutions containing electrolytes and glucose to replace the losses caused by fever and vomiting. Paracetamol (not aspirin, ibuprofen, or other NSAIDs) should be used in case of high fever. - Hospital management: Patients with signs of alarm, comorbidities (pregnancy, children, the elderly, obesity, diabetes mellitus, renal failure, chronic hemolytic diseases). - Urgent management: Critical phase of the disease (severe plasma leakage with shock and/or fluid accumulation). They should be referred to intensive care, in addition to receiving transfusions of blood components.		



What is Dengue?

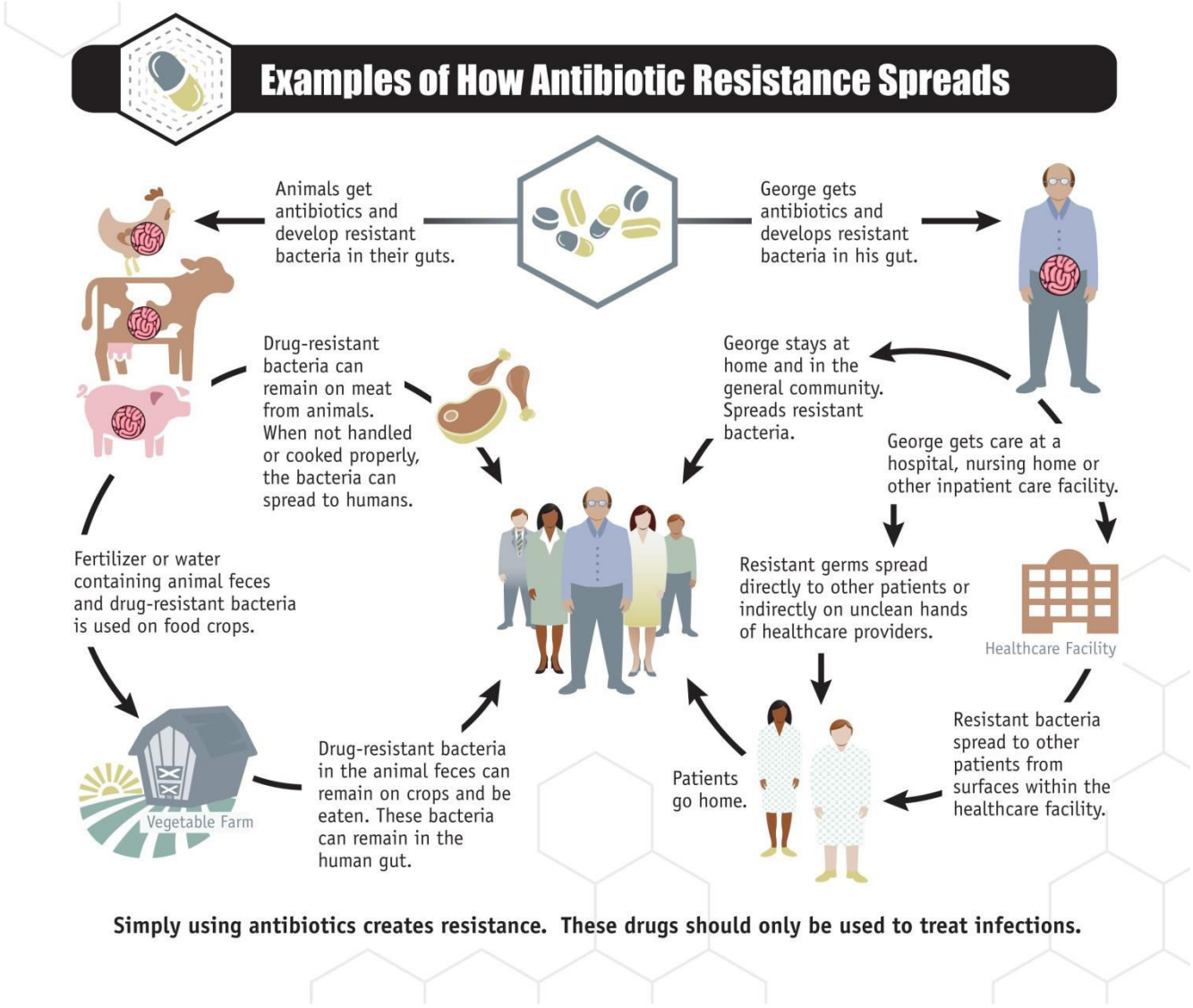
Dengue, commonly called dengue fever, is a mosquito-borne disease that occurs in tropical and sub-tropical parts of the world. In mild cases, symptoms are similar to the flu: fever, rash, and muscle and joint pain. In severe cases, Dengue can cause severe bleeding, low blood pressure, and even death.

Because it is carried by mosquitoes, the transmission dynamics of Dengue are related to climate variables such as temperature and precipitation. Although the relationship to climate is complex, a growing number of scientists argue that climate change is likely to produce distributional shifts that will have significant public health implications worldwide.

The ability to accurately forecast the number of people in a given area that will be infected with Dengue is of great value to public health officials, physicians, and ultimately anyone at risk of infection.

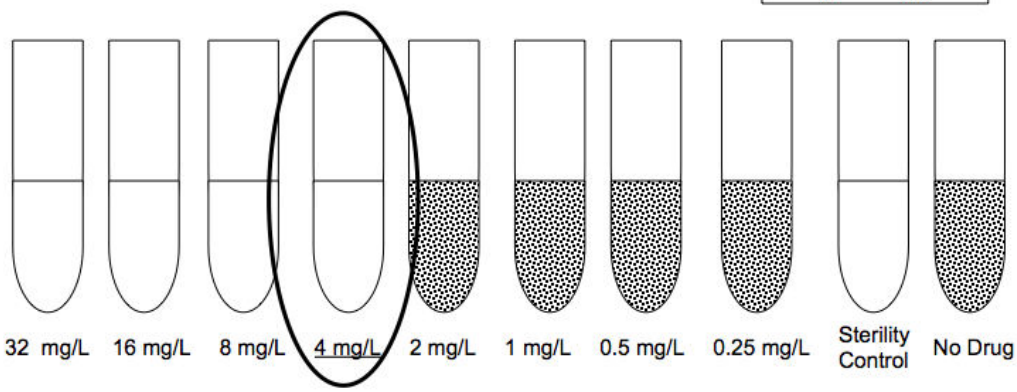
Aedes aegypti and "Domain Knowledge"

After a female Aedes aegypti feeds on blood, she lays an average of 100 to 200 eggs and can do this up to five times in her lifespan. Eggs are laid on damp surfaces in areas likely to temporarily flood, such as tree holes and man-made containers like barrels, drums, jars, pots, buckets, flower vases, plant saucers, tanks, discarded bottles, tins, tires, water cooler, etc. and a lot more places where rain-water collects or is stored. She lays her eggs separately, unlike most species. Not all eggs are laid at once, but they can be spread out over hours or days, depending on the availability of suitable substrates. Eggs will most often be placed at varying distances above the waterline. Additionally, she will not lay the entire clutch at a single site, but rather spread out the eggs over several sites. The eggs can survive in a dry environment for over a year but when they are in a wet environment they can hatch in as little as two days. The remaining growth stages to adulthood can occur in as little as six days (denguevirusnet.com).

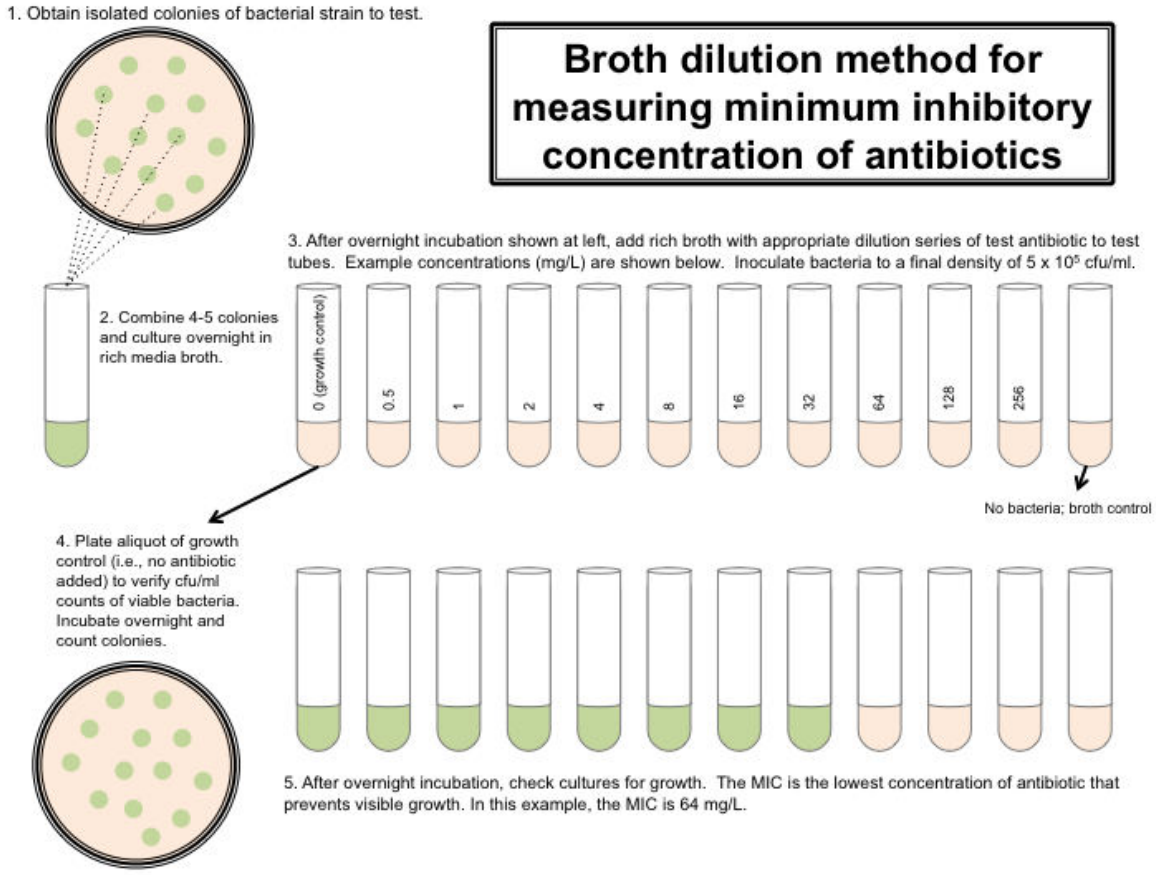


Simply using antibiotics creates resistance. These drugs should only be used to treat infections.

Minimal Inhibitory Concentration (MIC) of Ampicillin for *E. coli*



- Serial 2-fold dilutions of ampicillin in Mueller-Hinton broth
- Inoculum 5X10^5 CFU/ml (clear, non-turbid); Incubate 18 hrs. @35° C.
- Assess turbidity, visually
- Concentration of drug in first tube without visible turbidity is the MIC (4 mg/L)



Dilution methods can be carried out in 2 ways

A. Broth dilution

Broth dilution testing allows the option of providing both quantitative (MIC) and qualitative (category interpretation) results. MIC can be helpful in establishing the level of resistance of a particular bacterial strain and can substantially affect the decision to use certain antimicrobial agents.

Broth dilution can again be performed by 2 ways

1. Macro dilution: Uses broth volume of 1 ml in standard test tubes .
2. Microdilution: Uses about 0.05 to 0.1 ml total broth volume and can be performed in a microtiter plate or tray .

The procedure for both macro and microdilution are same except the volume of the broth.

B. Agar dilution

MIC of an antibiotic using broth dilution method is determined by using the following procedure

1. Preparation of antibiotic stock solution
2. Preparation of antibiotic dilution range
3. Preparation of agar dilution plates
4. Preparation of inoculum
5. Inoculation
6. Incubation
7. Reading and interpreting results

Preparation of antibiotic Stock solution.

Antibiotic stock solution can be prepared by commercially available antimicrobial powders (with given potency). The amount needed and the diluents in which it can be dissolved can be calculated by using either of the following formulas to determine the amount of antimicrobial powder (1) or diluent (2) needed for a standard solution:

$$(1)\text{Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration } (\mu g/mL)}{\text{Potency } (\mu g/mg)}$$

or

$$(2)\text{Volume (mL)} = \frac{\text{Weight (mg)} \cdot \text{Potency } (\mu g/mg)}{\text{Concentration } (\mu g/mL)}$$

Prepare antimicrobial agent stock solutions at concentrations of at least 1000 µg/mL (example: 1280 µg/mL) or 10 times the highest concentration to be tested, whichever is greater.

Because microbial contamination is extremely rare, solutions that have been prepared aseptically but not filter sterilized are generally acceptable. If desired, however, solutions may be sterilized by **membrane filtration**. Dispense small volumes of the sterile stock solutions into sterile glass, polypropylene, polystyrene, or polyethylene vials; carefully seal; and store (preferably at −60 °C or below, but never at a temperature warmer than −20 °C and never in a self-defrosting freezer). Vials may be thawed as needed and used the same day.

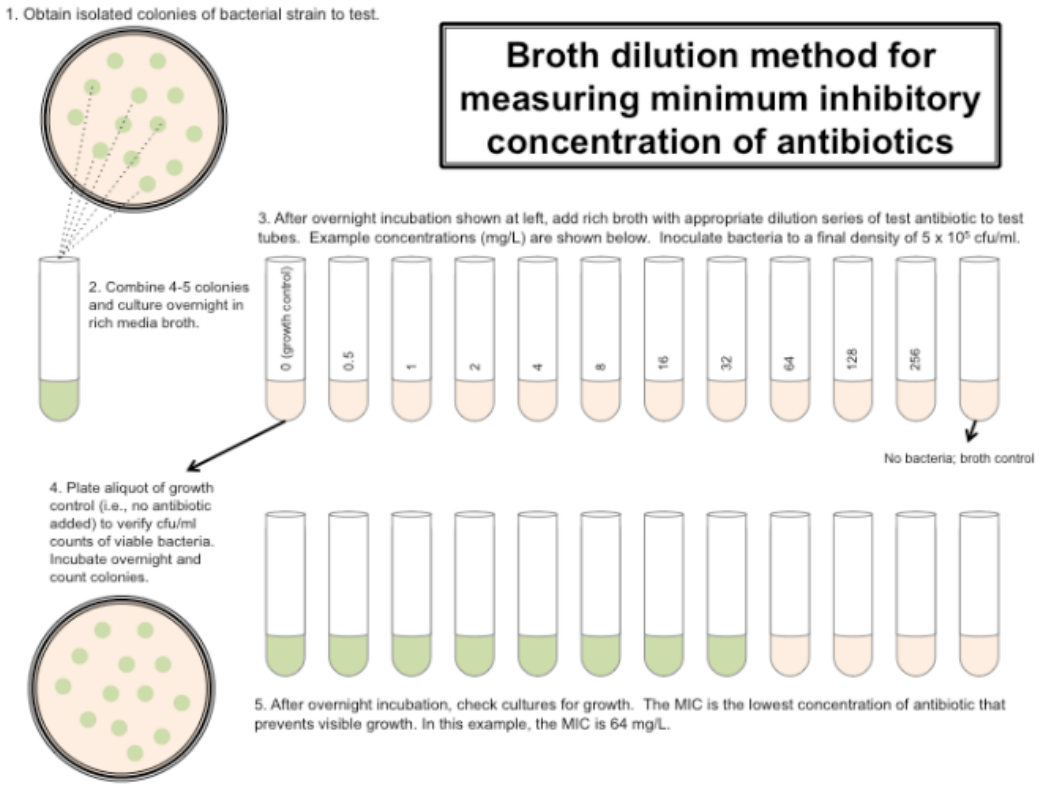
Preparation of antibiotic dilution range

- Use sterile 13- x 100-mm test tubes to conduct the test. If the tubes are to be saved for later use, be sure they can be frozen.
- Close the tubes with loose screw-caps, plastic or metal closure caps, or cotton plugs.
- Prepare the final two fold (or other) dilutions of antimicrobial agent volumetrically in the broth. A minimum final volume of 1 mL of each dilution is needed for the test.

Note: For, microdilution,only 0.1 ml is dispensed into each of the 96 wells of a standard tray.

Preparation of inoculum

1. Prepare the inoculum by making a direct broth suspension of isolated colonies selected from an 18- to 24-hour agar plate (use a non-selective medium, such as ***blood agar***).
2. Adjust the suspension to achieve a turbidity equivalent to a **0.5 McFarland turbidity standard**. This results in a suspension containing approximately 1 to 2 x 10^8 colony forming units (CFU)/mL for *Escherichia coli* ATCC®a 25922.
3. Compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.
4. Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in broth so, after inoculation, each tube contains approximately 5 x 10^5 CFU/mL.Note: This can be accomplished by diluting the 0.5 McFarland suspension 1:150, resulting in a tube containing approximately 1 x 10^6 CFU/mL. The subsequent 1:2 dilution in step 3 brings the *final inoculum to 5 x 10^5 CFU/mL.*



Broth dilution method for measuring minimum inhibitory concentration of antibiotics. (image source:labome.com)

Inoculation

Within 15 minutes after the inoculum has been standardized as described above, add 1 mL of the adjusted inoculum to each tube containing 1 mL of antimicrobial agent in the dilution series (and a positive control tube containing only broth), and mix.

This results in a 1:2 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculums.

Incubation:

Incubate the inoculated tubes at 35 ± 2 ºC for 16 to 20 hours in an ambient air incubator. To maintain the same incubation temperature for all cultures, do not stack microdilution trays more than four high.

Interpretation

Compare the amount of growth in the wells or tubes containing the antimicrobial agent with the amount of growth in the growth-control wells or tubes (no antimicrobial agent) used in each set of

tests when determining the growth end points. For a test to be considered valid, acceptable growth (≥ 2 mm button or definite turbidity) must occur in the growth-control well.

The lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) is recorded as the minimal inhibitory concentration or MI