MICROBIOLOGICAL LABORATORY DIAGNOSTIC METHODS

Microscopic-preparation of smears from investigating material, staining by Gram method and microscopy. Description of microorganisms by morphology and staining properties.

Bacteriological method-isolation of pure culture from investigating material and identification of them by morphology, staining properties (tinctorial), cultural properties, bio-chemical activities, antigenic structure, toxigenicity, etc.

Serological: revealing of specific antibodies or antigens in blood serum by immunological reactions (agglutination, precipitation, complement fixation, lysis, immune enzyme, RIA).

Skin-allergic tests-revealing of development of infectious allergy (DTH) in some infectious diseases (tuberculosis, brucellosis, anthrax).

Biological: infection of pathogenic material into susceptible animal organism and isolation of pure culture from these organisms.

Choosing of pathological material (feces, urine, blood, mucous, sputum, etc.) depends on clinical diagnosis, pathogenesis of infections, stages of infections, and choused method of examination.

ANTIGEN-ANTIBODY REACTIONS IN THE LABORATORY

Reactions of antigens and antibodies are highly specific. An antigen will react only with antibodies elicited by itself or by a closely related antigen. Because of the great specificity, reactions between antigens and antibodies are suitable for identifying one by using the other. *This is the basis of serologic reactions.*

Types of diagnostic tests: Many properties of diagnostic tests are performed in the immunological laboratory. Most of these tests can be designed to determine the presence of either antigen or antibody. To do this, one of the components, either antigen or antibody, is known and the other is unknown.

Immunoelectrophoresis- mmunoelectrophoresis combines electrophoresis immunodiffusion (immune precipitation in gel). This method can be used for analyzing complex antigens in biological fluids. A glass slide is covered with molten agar or agarose. A well for antigen and a trough for antiserum is cut on it. Antigen well is filled with antigen mixture (human serum). The slid is then placed in an electric field for about an hour to allow for the electrophoretic migration of various antigens. Different antigens will migrate at different rates or even in different directions, depending upon their size and charge and the conditions of electrophoresis. After the completion of electrophoresis, antiserum trough is filled with appropriate antiserum (antiserum to whole human serum). Antigens and antibodies diffuse towards each other, resulting in the formation of precipitin bands, for individual antigens and antibodies, whenever they are both in zones of optimal proportions, in 18-24hours. Because immunoelectrophoresis uses electric charge in addition to diffusion, it is more likely to separate antigen than is simple diffusion alone. By this method, over 30 different antigens can be identified in human serum. This technique is useful for detection of normal and abnormal serum proteins.

Radioimmine assay (RIA)-This method is used for the quantitation of antigens or haptens that can be radioactively labeled. It is based on the competition for specific antibody between the labeled (known) and unlabeled (unknown) concentration of material. The complexes that form between the antigen and antibody can be separated and amount of radioactivity measured. The more unlabeled antigen is present, the less radioactivity there is in the complex. The concentration of the unknown (unlabeled) antigen or hapten is determined by comparison with the effect of standards. RIA is a highly sensitive method and is

commonly used to assay hormones or drugs in serum. The radioallergosobent test (RAST) is a specialized RIA that is used to measure the amount of serum IgE antibody which reacts with a known allergen (antigen).

Enzyme-Linked Immunosorbent Assay (ELISA) – the method can be used for the quantitation of either antigens or antibodies in patient specimens. It is based on covalently linking an enzyme to a known antigen or antibody, reacting the enzyme -linked material with the patient's specimen, and the assaying for enzyme activity by adding the substrate of the enzyme. The method is nearly as sensitive as RIA yet requires no special equipment or radioactive labels.

For measurement of antibody, known antigens are fixed to a surface (eg, the bottom of small wells on a plastic plate), incubated with dilutions of the patient's serum, washed, and then reincubated with antibody to human IgG labeled with an enzyme, eg, horseradish peroxidase. Enzyme activity is measured by adding the substrate for the enzyme and estimating the color reaction in a spectrophotometer. The amount of antibody bounds in proportional to the enzyme activity. The titer of antibody in the patient's serum is the highest dilution of serum that gives a positive color reaction.

Immunofluorescence (Fluorescent antibody) - Fluorescent dyes eg, fluorescein and rhoamine, can be covalently attached to antibody molecules and made visible by ultraviolet (UV) light in the fluorescence microscope. Such "labeled" antibody can be used to identify antigens, eg, on the surface of bacteria(such as streptococci and treponemas), in cells in histologic section, or in other specimens. The immunofluorescence reaction is **direct** when known labeled antibody interacts directly with unknown antigen and **indirect** when a two-stage process is used. For example, known antigen is attached to a slide, the patient's serum (unlabeled) is added, and the preparation is washed. If the patient's serum contains antibody against the antigen, it will remain fixed to it on the slide and can be detected on addition of a fluorescent dye-labeled antibody to human IgG and examination by UV microscopy. The indirect test is often more sensitive than direct immunofluorescence, because more labeled antibody adheres per antigenic site. Furthermore, the labeled antiglobulin becomes a "universal reagent", ie, it is independent of the nature of the antigen used because the antibody to IgG is reactive with all human IgG.

Immunoblot (Western Blot) - is a technique that combines electrophoresis with ELISA to separate and identify protein antigens in a sample. It has many research applications, but its main clinical use is to confirm positive ELISA screening tests for antibodies against HIV. The ELISA tests are simpler and cheaper, but they give a small percentage of false positive results; therefore, positive results need to be confirmed by different separated by electrophoresis in a polyacrylamide gel. Smaller proteins migrate faster than larger ones. The resulting bands of separated antigens can react with specific antibodies, but antibodies do not diffuse well into these gels. Therefore, it is necessary to transfer the antigens present in the bands from the gel by blotting them onto a filter. To test for antibodies against HIV, commercial kits are available in which antigens of the HIV virus have been electrophoresed and blotted onto the filter, which is then cut into strips for each test. Each sample to be tested for antibodies to HIV is applied to a strip and incubated to allow specific antibodies to react with the viral antigens. To detect the antigen – antibody combinations formed, a label is used, usually an enzyme – labeled anti-HGG, as in ELISA, or sometimes a radioactive label. The Western blot is a more laborious and expensive technique than the ELISA; however, it offers greater specificity, since antigens are identified by two criteria: their size and their reactivity with antibodies.

GRAM – POSITIVE COCCI

S T A P H Y L O C O C C I

The Staphylococcus, S. aureus, was discovered by R. Koch (1878), and later isolated from furuncle pus by L. Pasteur (1880). It was described as the causative agent of numerous suppurative processes and studied in detail by F. Rosenbach (1884).

Staphylococci are included in the class **Bacteria,** family **Micrococcaceae,** genus **Staphylococcus.** According to the contemporary classification Staphylococci are classified into 20 species and 15 subspecies, which we classify into 2 groups: coagulase positive, coagulase negative. Three of them ecologically are connected with human organism:

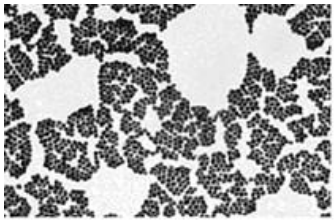
S. aureus produces golden pigment (S. aureus)

S. epidermidis produces white pigment (S. albus)

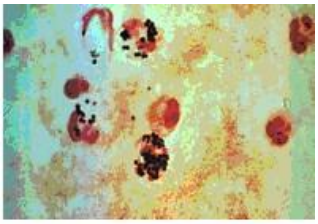
S. saprophyticus produces yellow pigment (S. citreus)

Other species are parasitic on animals. Some are members of the normal flora of the skin and mucous membranes of humans; others cause suppuration, abscess, a variety of pyogenic infections, and even a fatal septicaemia, they can cause 120 nosologic forms.

Morphology: Staphylococci are spherical in shape, 0.6-1.5μm in diameter. They are arranged characteristically in grape-like clusters (in smears from pure culture). Cluster formation is due to cell division occurring in more than one plane with daughter cells remaining close together. In smears from liquid media and pus, the cocci appear singly or in pairs, short chains, but not clusters. Staphylococci are Gram-positive organisms which are nonmotile, non-sporing and, with the exception of rare strains, non-capsulated.



Staphylococci in the smear from pure culture



Staphylococci in the smear from pathological material

Cultivation: Staphylococci are facultative anaerobes. They grow well on ordinary nutrient media with a pH of 7.2-7.4 at a temperature of 37º C. At room temperature with adequate aeration and subdued light the organisms produce golden, white, lemon-yellow, and other pigments known as lipochromes. These pigments do not dissolve in water but are soluble in ether, alcohol. They are most readily formed on milk agar and potatoes at tempera- ture of 20-25º C. The **selective medias** for Staphylococcus are salt agars: **yolk-salt agar** and **milk- salt agar**: Salt (6-15%) agars are good selective plating media and are useful for staphylococci isolation from food, dust, faeces and pus where mixed bacterial flora is expected. On **yolk-salt agar** they form smooth (S-form), convex, shiny, opaque colonies. Round the colonies they form pearl zone (lecithinase activity). On milk-salt agar pigment production is enhanced. Pigment is not formed in liquid media.

On **blood agar** colonies are similar to those on salt agar, but may be surrounded by a zone of ß-hemolysis (haemolytic activity).

Sugar broth (as an enrichment media) is used for examination of blood (sepsis).

Fermentative properties: Staphylococci produce enzymes which cause lysis of proteins and sugars. They hydrolyze proteins and form H2S, liquefy gelatin (as a funnel), don't form indole; fermentation of sugars (glucose, maltose, lactose, saccharose) with acid formation, without gas. Fermentation of **glucose and**

mannite in anaerobic condition has differential diagnostic meaning. There is no indole production in cultures. S. aureus is catalase-positive and oxidase –negative.

Staphylococcus aureus strains usually exhibit the following characteristics:

1. coagulase positive; 2.greater biochemical activity, (differential diagnostic is fermentation of mannite and glucose in anaerobic condition); 3.produce clear hemolysis on blood agar; 4. produce a golden yellow pigment; 5.liquefy gelatine; 6.produce phosphatase; 7.in a medium containing potassium tellurite, reduce tellurite to form black colonies; 8.produce pearl zone on yolk-salt agar; 9.produce thermostable nucleases which can be demonstrated by the ability of boiled cultures to degrade DNA in an agar diffusion test 10.Urease activity is positive for S. epidermidis and S. saprophyticus.

Staphylococci are variably sensitive to many antimicrobial drugs. They are resistant to many types of penicillin (β-lactamates) because they produce **beta-lactamase enzyme.**

Antigenic structure: Antigenic structure of S. aureus is very complex. Staphylococci contain antigenic polysaccharides and proteins as well as other substances important in cell wall structure. These include:

1. Capsular polysaccharide: A few strains of S. aureus are capsulated and these tend to be more virulent than non-capsulated strains. The capsule is composed of antigenic polysaccharide. It prevents ingestion of the organism by polymorphonuclear leucocytes. The capsule may promote adherence of the organism to host cells and to prosthetic devices.

2. Teichoic acid (which are polymers, are linked to the peptidoglycan and can be antigenic). Teichoic acid is a major antigenic determinant of all strains of S. aureus. Teichoic acids function in the specific adherence of gram-positive bacteria to mucosal surface. Teichoic acid participates in staining of bacteria also.

3. Peptidoglycan: (is a polysaccharide polymer containing linked subunits, provides rigid exoskeleton of the cell wall). It stimulates both humoral and cellular immune responses in the host. The antibodies against peptidoglycan possess opsonizing activity; however, increased levels of antibodies may predispose some patients to immune complex disorders. In addition to their role in providing rigidity and resilience to the staphylococcal cell wall, peptidoglycan and teichoic acid also have several biologic activities that are thought to contribute to virulence. These properties include the ability to activate complement, to inhibit chemotaxis of inflammatory cells, and to stimulate antibody production.

4. Protein A: Protein A is a group - specific antigen and is a cell wall component of many S. aureus strains that binds IgG molecules, non-specifically, through Fc region leaving specific Fab sites free to combine with specific antigen. When suspension of such sensitized cells is treated with homologous (test) antigen, the antigen combines with free Fab sites of IgG attached to staphylococci cells. This is known as ***co-agglutination.***

5. Cross reacting antigens.

Pathogenicity: Pathogenicity of Staphylococcus aureus depends on invasive properties, capsule formation, which has antiphagocytic ability; protein A, which inactivate complement. The pathogenic factors are combined with toxin production and enzymes too.

They produce several **toxins:**

Leukocidin: Leukocidin act to damage polymorphonuclear leucocytes and especially macrophages (antiphagocytic action), and can produce dermonecrosis.

Hemolysins (ά, β, γ and δ): These toxins have haemolytic activity. **Alpha** toxin is the most important in pathogenicity. This toxin lyses erythrocytes. It is also leucocidal (is toxic for human macrophages and platelets and causes degranulation of polymorphonuclear leucocytes through disruption of their lysosomes), dermonecrotic, cardiotoxic (alfa toxin has a powerful action on the vascular smooth muscle) and neurotoxic. This **is lethal** toxin.

Enterotoxins: An important cause of food poisoning. Enterotoxins (A to F) are

soluble, heat-stable (they resist boiling for 30 minutes) and resistant to the action of gut enzymes. Enterotoxins are produced when S. aureus grows in protein food (e.g., creams, creamery products, dairy products). By mechanism of action it is **cytotoxin.** The usual incubation period ranges from 2-6 hours after the ingestion of food. Patient develops nausea, vomiting and diarrhoea. The duration of acute symptoms is usually less than 24 hours. In addition to the ability to cause nausea and vomiting, enterotoxins are pyrogenic, mitogenic and are capable of producing thrombocytopenia and hypotension. Enterotoxin F is reported to be responsible for the **Toxic shock syndrome (TSS):** In humans, the toxin is associated with fever, shock, and multi-system involvement, including a desquamative skin rash.

Toxic shock syndrome toxin (TSST): TSST is the same as the enterotoxin F.

TSST and the enterotoxins are recognized as **superantigens,** i.e. they are potent activators of T- lymphocytes without relation to their epitope specificity resulting in the liberation of cytokines such as tumour necrosis factor, interleukins 1, 2; interferon gamma and they bind with high affinity to mononuclear cells. These characteristics partly explain the florid and multisystem nature of the clinical conditions associated with these toxins.

Exfoliative toxin: This is **epidermolytic** toxin. This toxin is responsible for scalded skin syndrome in young children, causes impetigo of the new-born (characterized by isolated pustules that become crusted and rupture).

Staphylococci produce **enzymes:**

Hyaluronidase, which breaks down hyaluronic acid, a component of connective tissue, which facilitates the spread of bacteria to adjacent areas(known as spreading factor).

Coagulase accelerates the formation of a fibrin clot from its precursor, fibrinogen (this clot may protect the bacteria from phagocytosis by walling off the infected area and by coating the organisms with a layer of fibrin).

Fibrinolysin (staphylokinase): This enzyme dissolves coagulated plasma and probably aids in the rapid spread of bacteria through tissues.

Lecithinase, which is hydrolyses lecithin in the cell membrane resulting in destruction of the membrane and widespread cell death.

Lipase ,which participates in adhesion and invasion.

DNA-ase –plays a role in pathogenesis of diseases.

Resistance: Staphylococci are among the more resistant of non-sporing bacteria. They are relatively resistant to drying, heat (they withstand 50º C for 30 minutes), a 3 per cent phenol solution kills the organisms in 15-20 minutes. 1% chloramine kills the staphylococci in 2-5 minutes. Staphylococci are very sensitive to certain aniline dyes, particularly to brilliant green which is used for treating pyogenic skin diseases caused by these organisms.

Staphylococci are variably sensitive to many antibacterial drugs, they can develop drug resistance. They produce **Beta-lactamase** which causes resistance to penicillin and similar drugs (beta-lactamates).

Pathogenesis and diseases in man: Staphylococci, particularly S. epidermidis, are members of the normal flora of the human skin and respiratory and gastrointestinal tracts. Nasal carriage of S. aureus occurs in 40-50% of humans. Staphylococci are also found regularly on clothing, bed linen, and other fomites in human environment.

Staphylococci enter the body through the skin and mucous membranes. When they overcome the lymphatic barrier and penetrate into the blood, staphylococcal septicaemia sets in. Both the exotoxins and the bacterial cells play an important role in pathogenesis of diseases caused by these organisms.

Staphylococci are responsible for a number of **local lesions** in humans: abscess, furuncle, carbuncle, osteomyelitis, dermatitis eczema, peritonitis, meningitis, etc, 120 nosologic forms.

In some cases Staphylococci may give rise to a **secondary infection** in individuals suffering from smallpox, influenza, and wounds, as well as postoperative suppurations.

Staphylococcal sepsis and staphylococcal pneumonia in children are particularly severe diseases.

Staphylococci play an essential part in **mixed infections**, and are found together with streptococci in cases of wound infections, diphtheria, and tuberculosis.

S. aureus infection can also result from direct **contamination of a wound,** e.g., postoperative staphylococcal wound infection or infection trauma (chronic osteomyelitis subsequent to an open fracture, meningitis following skull fracture).

If S. aureus **disseminates and bacteremia ensues**, endocarditis, acute hematogenous osteomyelitis, meningitis, or pulmonary infection can develop (table 1).

Food poisoning due to staphylococcal enterotoxin is characterized by a short incubation period (1-8 hours); violent nausea, vomiting, and diarrhoea; and rapid convalescence. There is no fever

Table 1 **Staphylococcal diseases**

Skin and soft tissue	Folliculitis, furuncle (boil), abscess(particularly breast abscess) wound infection, carbuncle, impetigo, paronychia, less often cellulitis
Musculoskeletal	Osteomyelitis, arthritis, bursitis, pyomyositis
Respiratory	Tonsillitis, pharyngitis, sinusitis, otitis, bronchopneumonia, lung abscess, empyema, rarely pneumonia
Central nervous system	Abscess, meningitis, intracranial thrombophlebitis
Endovascular	Bacteremia, septicaemia, pyemia, endocarditis
Urinary	Staphylococci are uncommon in routine urinary tract infections, though they do cause infection in association with local instrumentation, implants or diabetes

Immunity: Immunity acquired after staphylococcal diseases is due to phagocytosis and the presence of antibodies (antitoxins, precipitins, agglutinins). The phagocytic and humoral factors act together and supplement each other. Post-infectious immunity following staphylococcal diseases is of low grade and short duration.

Treatment: diseases are treated with antibiotics, sulphonamides, and anti-staphylococcal gamma-globulin.

The agents of choice for severe infections are penicillinaseresistant penicillins, since 70–80% of all strains produce penicillinase. These penicillins are, however, ineffective against methicillin-resistant strains, and this resistance applies to all betalactams.

During Staphylococcal chronic infections anatoxin, autovaccine are used.

Epidemiology and prevention. S. aureus is a frequent colonizer of skin and mucosa. High carrier rates (up to 80%) are the rules among hospital patients and staff. The principle localization of colonization in these persons is the anterior nasal mucosa area, from where the bacteria can spread to hands or with dust into the air and be transmitted to susceptible persons. S. aureus is frequently the causal pathogen in nosocomial infections. Certain strains are known to cause hospital epidemics. Identification of the epidemic strain requires differentiation of relevant infection isolates from other ubiquitous strains. Lysotyping (see p. 186) can be used for this purpose, although use of molecular methods to identify genomic DNA “fingerprints” is now becoming more common. The most important preventive measure in hospitals iswashing the hands thoroughly before medical and nursing procedures. Intranasal application of antibiotics (mupirocin) is a method of reducing bacterial counts in carriers.

The general precautionary measures include: hygiene in working and everyday-life conditions, treatment of vitamin deficiency, prevention of traumatism and excess perspiration, observance of rules of hygiene in maternity hospitals, surgical departments, and children’s institutions.

Routine disinfection of hospital premises (surgical departments, maternity wards) and bacteriological examination of the personnel for carriers is used. Examination of pathogenic staphylococci resistant to antibiotics is important.

For prophylaxis is recommended staphylococcal anatoxin and bacteriophage. For the formation of passive response antistaphylococcal immunoglobulin and donor's antistaphylococcal hyperimmune sera is recommended.

Laboratory diagnosis: Diagnostic methods are (picture 1):

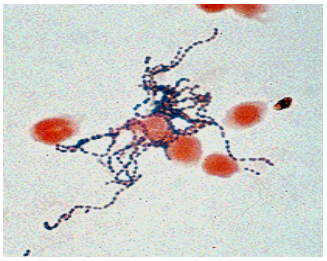
1.Microscopic
2.Bacteriological

S T R E P T O C O C C I

The streptococcus was discovered by T. Billroth (1874) in tissues of patients with erysipelas and wound infections and by Pasteur in patients with sepsis. Streptococci are placed in the family Streptococaceae. They are part of the normal flora of humans and animals. Some of them are human pathogens. The most important of them is **Streptococcus pyogenes** causing pyogenic infections.

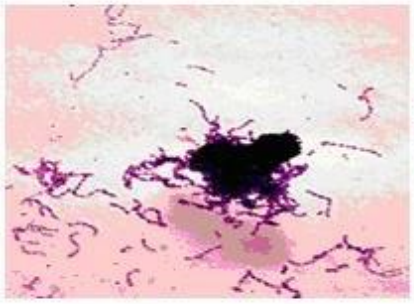
Streptococcus pyogenes

Morphology: The streptococci are gram-positive spherical cells measuring 0.5 to 1.0μm in diameter and form chains (they divide in only one plane and tendency of cells to remain united results in the development of the characteristic chains). They are non-motile, do not form spores. Some strains are capsulated. In smears from cultures grown on solid media the streptococci are usually present in pairs or in short chains, while in smears from broth cultures they form long chains or clusters.

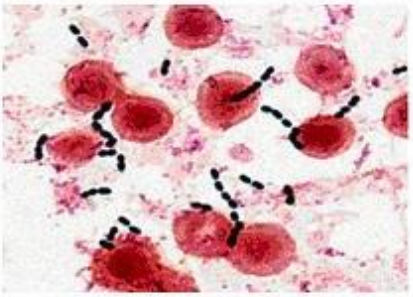


electron microscopy

Immersion microscopy



from pure culture



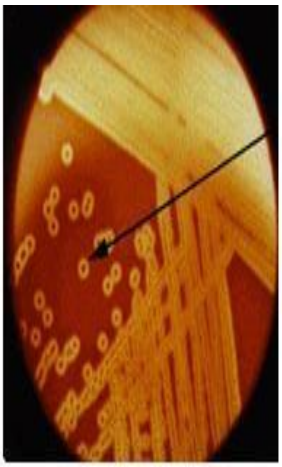
from pathological material

Cultivation: The majority of streptococci are aerobes and facultative anaerobes. The optimal temperature for growth is 37º C.

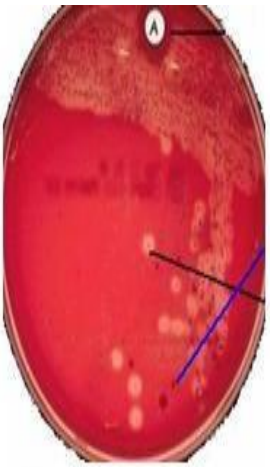
The organisms show poor growth on ordinary meat-peptone agar, and grow well on **selective medias** - sugar, blood, serum, ascitic agars. On **solid media** they produce small, translucent colonies. In **sugar broth** medium growth is in the form of fine-granular precipitates on the walls and at the bottom of the tube.

Some streptococcal strains cause haemolysis on blood agar. Based upon their haemolytic properties, streptococci can be classified into three groups:

1. **ß-haemolytic** streptococci: these organisms produce a wide (2-4mm in diameter) clear zone of complete haemolysis in which no red blood cell is visible on microscopic examination.
2. **α-haemolytic** streptococci: The colonies are surrounded by a narrow zone (1-2 mm) of haemolysis**;** they produce a green zone round the colony, as a result of conversion of haemoglobin into methaemoglobin. The streptococci producing α-haemolysis are also known as streptococci viridans.
3. **γ-haemolytic** streptococci: These organisms do not produce any haemolysis on blood agar. Enterococcus faecalis is an important organism of this group.



β – hemolysis



β and γ hemolysis

Fermentative properties: Streptococci ar

and do not reduce nitrates to nitrites. They coagulate milk; dissolve fibrin, ferment glucose, maltose, lactose, saccharose with acid formation. S. pyogenes is soluble in bile (40%) and are not soluble in 10per cent bile, unlike pneumococci. Streptococci are catalase negative (table 1).

Antigenic structure: By the group-specific polysaccharide (C substance) the streptococci are classified into 20 serological groups which are designated by capital letters: A, B, C, D….(Lancefield classification). S. pyogenes is in **A group.**

Depending on the type-specific **M, T and R** protein antigens the serological group A is subdivided into serotypes (about 100 serotypes). The **M protein** is the most important of these. It acts as a **virulence factor** by inhibiting phagocytosis (it binds IgG molecules, non-specifically, through Fc region leaving specific Fab sites free to combine with specific antigen). The T and R proteins have no relation to virulence.

Various structural components of S. pyogenes exhibit **antigenic cross reaction** with different tissues of the human body. Antigenic relationships have been demonstrated between capsular hyaluronic acid and human synovial fluid, cell wall protein and myocardium, group A carbohydrate and cardiac valves, cytoplasmic membrane antigens and vascular intima and peptidoglycan and skin.

Pathogenicity: The pathogenetic factors are: adherence, colonization, invasion, secretion of toxins and enzymes.

Toxin production: Streptococci produce exotoxins with various activities:

Haemolysins:

a) **Streptolysin-S** is nucleoprotein with haemolytic and cytotoxic activities, non-antigenic, oxygen stable and sensitive to heat and acid. It is responsible for hemolysis around the surface colonies. In addition to causing ß-hemolysis, it is able to inhibit chemotaxis and phagocytosis. It can cause destruction of cell's ***lysosomal membranes.*** Therefore, this haemolysin appears to be an important factor of group A streptococcal infections.
b) **b)Streptolysin-O** (oxygen labile) is protein and is strongly antigenic. It is heat labile. SLO induces brisk response, usually within 10-14 days. Anti streptolysin O(ASO) appears in sera following streptococcal infection. Estimation of this antibody (the titre of **ASO** antibodies can be important in the diagnosis of rheumatic fever) is a standard serological procedure for the retrospective diagnosis of infection with S. pyogenes.

It can cause ***lysis of erythrocytes***, destruction of cell's ***lysosomal membranes*** leading to ***necrosis of tissues.*** Streptolysin-O is toxic for **leucocytes,** produces **haemolytic**, **cytotoxic, leukotoxic, cardiotoxic** activities.

Leukocidin, which destructive to leucocytes (inhibition of phagocytosis); occurs in highly virulent strains.

Erythrogenic (Dick, scarlatinal) toxin: It is a protein, antigenic, relatively heat stable. This is superantigen. This toxin is responsible for characteristic skin rash with pharyngitis and tonsillitis in scarlet fever. It is produced only by certain strains of S. pyogenes lysogenized by a bacteriophage carrying the gene for the toxin. This toxin is neutralized by antibodies found in the convalescent sera. This property has been used for developing susceptibility and diagnostic tests for scarlet fever (Dick test gives positive result in persons lacking antitoxin, i.e., susceptible person). This test is known to be only of historical importance as scarlet fever is no longer a common or serious disease.

Cytotoxins: Peptides, which destroy kidney cells and cause glomerulonephritis.
Cardiohepatic toxin, which causes myocardia's affection and forms granules in liver.
Enzymes:

Fibrinolysin (streptokinase) activates plasminogen to form plasmin, which dissolves fibrin clots. It can be used to lyse thrombi in the coronary arteries of heart attack patients. Fibrinolysin plays a biological role in streptococcal infections by breaking down the fibrin

barrier around the lesions and facilitating the spread of infection.

Desoxyribonucleases – D Nase (streptodornase) depolymerizes DNA in exudates or necrotic tissue. Pyogenic exudates contain large amounts of DNA, derived from the nuclei of necrotic cells. Sterptodornase helps to liquefy the thick pus and may be responsible for the thin serous character of streptococcal exudates.

Hyaluronidase degrades hyaluronic acid, which is the ground substance of subcutaneous tissue. Hyaluronidase is known as a spreading factor because it facilitates the rapid spread of S. pyogenes in skin infections.

Pathogenesis: Streptococci cause a wide variety of infections. Streptococci are mainly spread by the air droplet route. S. pyogenes causes three types of diseases: 1. **Pyogenic** diseases such as local pyogenic inflammation, abscesses, phlegmona, lymphadenitis, pyelitis, otitis, sinusitis, meningitis, peritonitis, and pharyngitis, tonsillitis (inflammation of the pharyngeal and tonsillar mucosa). S. pyogenes is the most common bacterial cause of sore throat. Invading the blood, streptococci produce a serious septic condition. 2. **Toxigenic** diseases such as scarlet fever and toxic shock syndrome. 3. **Immunologic** diseases such as rheumatic fever and acute glomerulonephritis (certain strains of group A streptococci contain cell membrane antigens that cross-react with human heart tissue antigens- autoimmune processes develop).

Epidemiology and resistance: Many streptococci are members of the normal flora of the human body. The source of infection is carriers and sick persons. The main ways of spreading are droplet; and endogenous which occur during immunodeficiency.

S. pyogenes is a delicate organism. It can be killed by heating at 54º C for 30minutes. It is also killed by usual strengths of disinfectants, but is more resistant to crystal violet than many other bacteria. Streptococci live for a long time at low temperature, are resistant to desiccation, and survive for many months in pus and sputum. When exposed to a temperature of 70ºC, they are destroyed within one hour. A 3-5 per cent phenol solution kills the organisms within 15 minutes.

Immunity: Resistance against streptococcal diseases is type-specific. Thus, a host who has recovered from infection by one group A streptococcal is relatively insusceptible to reinfection by the same type but fully susceptible to infection by another type.

Immunity against erythrogenic toxin is due to antitoxin in blood. Immunity after scarlet fever is stable, life-long. Antibody to streptolysin O (antistreptolysin O) develops following infection; it blocks hemolysis by streptolysin O but does not indicate immunity.

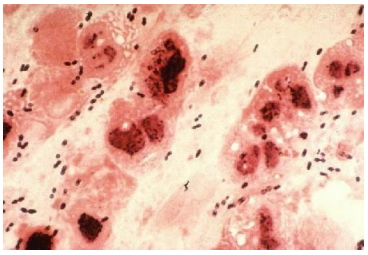
Immunity after streptococcal infections is of a low grade and short duration.

Therapy. The agents of choice are penicillin G or V. Resistance is unknown. Alternatives are oral cephalosporins or macrolide antibiotics, although resistance to the latter can be expected. In treatment of septic shock, a polyvalent immunoglobulin is used to inactivate the PSE.

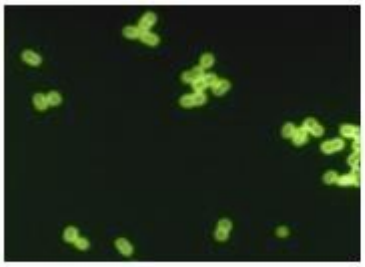
Epidemiology and prophylaxis. There is no specific prophylaxis (vaccines). Streptococcal infections are prevented by the practice of general hygienic measures in everyday life.

Infection frequency varies according to geographical area, season, and age. Humans are the only pathogen reservoir for S. pyogenes. Transmission is by direct contact (smear infection) or droplets. The incubation period is one to three days. The incidence of carriers among children is 10–20%, but can be much higher depending on the epidemiological situation. Carriers and infected persons are no longer contagious 24 hours after the start of antibiotic therapy. Microbiological follow-up checks of patients and first-degree contacts are not necessary (exception: rheumatic history). In persons with recurring infections or with acute rheumatic fever in their medical histories, continuous penicillin prophylaxis with a long-term penicillin is appropriate (e.g., 1.2 million IU benzathine penicillin per month).

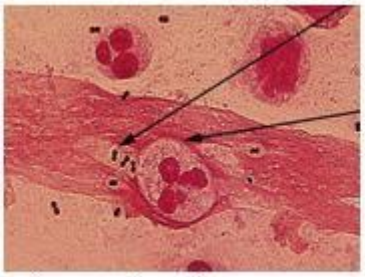
STREPTOCOCCUS PNEUMONIAE



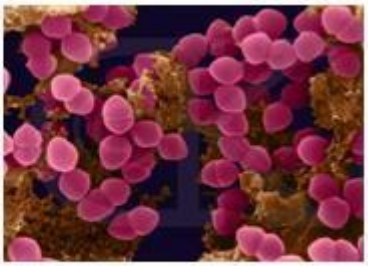
Morphology: Streptococcus pneumoniae is gram-positive, about 1μm in diameter, lancet (flame) shape, and arranged in pairs (diplococci), capsule forming, non-motile and non-sporing organisms. It is inhabitant of upper respiratory tract of human and some animals, causes infection primarily of the respiratory tract, conjunctivitis, otitis, meningitis. They differ from streptococci chiefly in their morphology, optochin sensitivity and possession of a specific polysaccharide capsule.



Luminescence microscopy



Immersion microscopy



Electron microscopy

Cultural characteristics: Pneumococcus is **a**erobe and facultative anaerobe**.** Optimum temperature is 37ºC, pH 7.8 and grows only in enriched media (blood, serum, ascitic agar). Pneumococci form a small round colony, primarily dome-shaped and later developing a central plateau with an elevated rim. In broth they produce diffuse turbidity. Pneumococci are ***ά*-hemolytic** on blood agar. For growth they need 5-10% CO_2 for primary isolation.

Biochemical reactions: Pneumococci ferment glucose, saccharose, lactose and inulin with the production of acid but no gas. **Fermentation of inulin by pneumococci** is a useful test for differentiating them from streptococci which do not ferment it. Pneumococci are bile soluble (40%). Bile solubility is a constant property of pneumococci and hence is of diagnostic importance. They are highly sensitive to **optochin** (ethyl hydrocuprein hydrochloride). Optochin sensitivity test is used for identification of pneumococci and distinguishing them from viridans streptococci, both of which produce α –haemolysis on blood agar. Pneumococci are catalase and oxidase negative.

Antigenic structure: The most important antigen of the pneumococci is the **capsular polysaccharide** which is **type specific.** By the capsular antigen pneumococci are subdivided into 85 serotypes. The antigenic structure depends on **M** protein. The somatic portion of the pneumococcus contains an M protein that is characteristic for each type and a group-specific carbohydrate that is common to all pneumococci. The carbohydrate can be precipitated by C-reactive protein, a substance found in the serum of certain patients.

Production of disease: Pneumococci produce disease through their ability to multiply in the tissues. The virulence of the organism is a function of its capsule, which prevents or delays ingestion of encapsulated cells by phagocytes. The virulence is connected with C-polysaccharide, M-protein, which is antiphagocytic, hemolysins (S-streptolysin), leukocidin, and enzymes: peptidase, which destroys SIgA, hyaluronidase, which is a spreading factor (degrades hyaluronic acid).

Immunity: Immunity to infection with pneumococci is type-specific and depends on both antibodies to capsular polysaccharide and intact phagocytic function.

Treatment: Streptococci are sensitive to many antimicrobial drugs. Some species are interesting for medical microbiology:

Group A streptococci are among the most important human pathogens. The great majority of haemolytic streptococci that produce human infections belong to group A.

Haemolytic streptococcus group A is known as S. pyogenes.

Group B (S. agalactica) streptococci colonize the genital tract of some women and cause neonatal meningitis and sepsis.

Group C streptococci colonize the respiratory and urinary tracts.

Group D streptococci (Enterococcus faecalis), which are pathogenic for human and animals occur as a part of the normal flora in the gut, are noted for their ability to cause urinary, biliary, and cardiovascular infections.

Group H and K is noted during endocarditis.

Oral streptococci (S. mutans, S. salivarius) occur in dental caries.

Penicillin is still the antibiotic of choice. There have been reports of high-frequency occurrence of strains resistant to penicillin (South Africa, Spain, Hungary, USA). These strains are still relatively rare in Germany, Switzerland, and Austria (5–10%). Macrolide antibiotics are an alternative to penicillins, but resistance to them is also possible.

Penicillin resistance is not due to penicillinase, but rather to modified penicillin-binding proteins (PBPs) to which penicillins have a lower level of affinity. PBPs are required for murein biosynthesis. Biochemically, penicillin resistance extends to cephalosporins as well. However, certain cephalosporins (e.g., ceftriaxone) can be used against penicillin-resistant pneumococci due to their higher levels of activity.

Epidemiology and prophylaxis. Pneumococcal infections are endemic and occur in all seasons, more frequently in the elderly. Humans are the natural pathogen reservoir.

The vaccine product Pneumovax! is available for immunization purposes. It contains 25mg of the purified capsule polysaccharides of each of 23 of the most frequent serovars. Eighty to ninety percent of all isolated pneumococci have antigens contained in this vaccine, which is primarily indicated in persons with predisposing primary diseases. There is also a seven-valent conjugate vaccine that is effective in children under two years of age. Exposure prophylaxis is not necessary.

Diagnostic laboratory tests for Streptococcal infections:

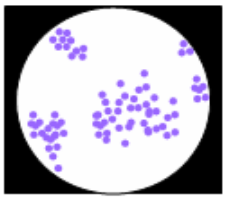
1. **Microscopic:** Test material is obtained from the pus of wounds, inflammatory exudate, tonsillar swabs, blood, urine, and foodstuffs. Tests include microscopy of pus smears.
2. **Bacteriological:** Isolation of the pure culture and its identification (Table1).
3. **Biological**: White mice are sensitive to pneumococcus.

S. aureus Coagulase-positive; colonies golden yellow. Local purulent infections: furuncles, carbuncles, bullous impetigo, wound infections, sinusitis, otitis media, mastitis puerperalis, ostitis, postinfluenza pneumonia, sepsis. Toxin-caused illnesses: food poisoning, dermatitis exfoliativa, toxic shock syndrome

Table1 Differences between Streptococcus pneumonia and pyogenes

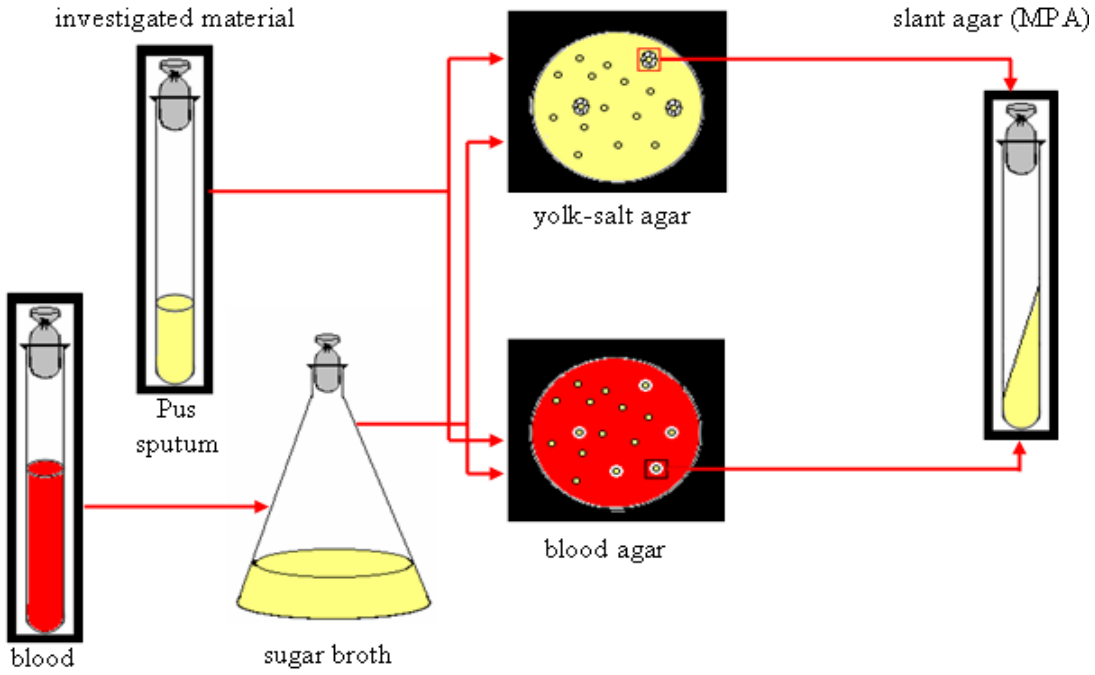
	S. pneumonia	S. pyogenes
1. morphology:		
▪ Shape	Flame shape	Round or oval cocci
▪ Arrangement	In pairs (diplococci)	In chain
▪ Capsule	Present	Absent
2. Cultural characteristic:		
• On blood agar medium	After 24 hours incubation, colonies are round, moist, mucoid, transparent and surrounded by 2-3mm zone of ά-haemolysis. On further incubation, the colonies develop a central depression with raised rim(draughtsman colonies)	After 24 hours incubation colonies are small, semitransparent, surrounded by 2-3mm of β-haemolysis
• In liquid medium	Uniform turbidity	Fine-granular precipitates on the walls and at the bottom of the tube.
3. Bile solubility (40%)	Positive	Positive
4. Inulin fermentation	Positive	Negative
5. Optochin sensitivity	Positive	Negative
6. Formation of:		
O-streptolysin	-	+
S-streptolysin	+	+
Streptokinase	-	+
Hyaloronidase	+	+
Proteinase	+	+
DNA-ase	+	+
Leukocidin	+	+
Peptidase	+	-
7. Fermentation off:		
Lactose	+	+
Mannitol	-	-
Glycerol	-	-
Salicyn	-	+

Staphylococcus aureus

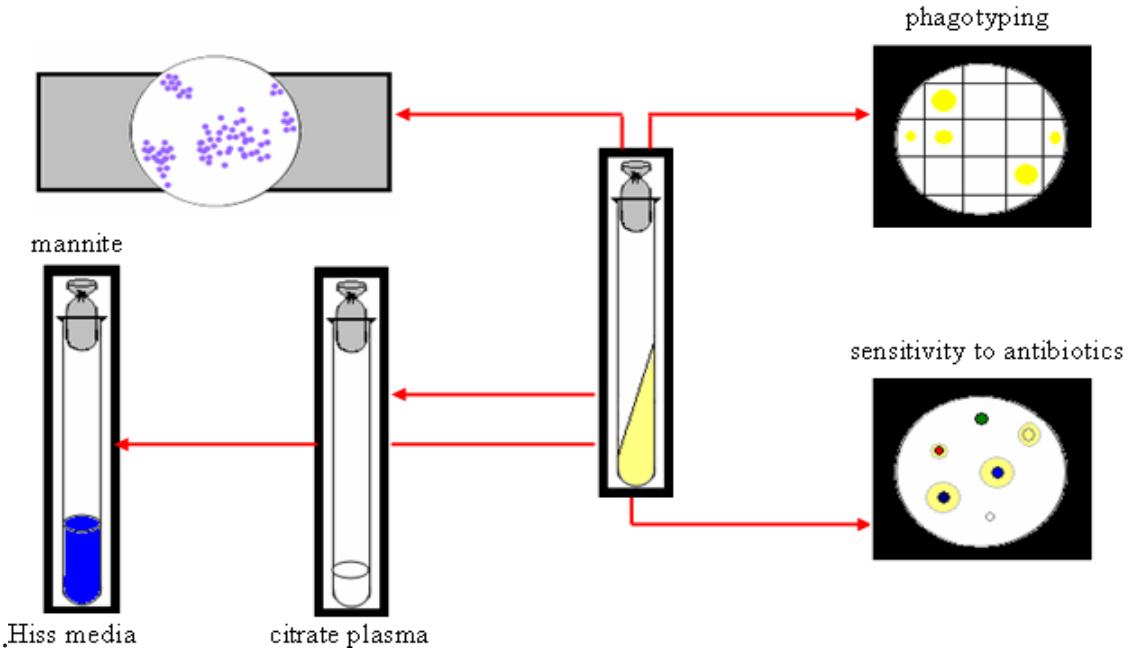


Diagnostic methods
1.Microscopic
2.Bacteriological

Bacteriological examination



Identification



Streptococcus pneumoniae

Streptococcus pyogenes

METHODS

MICROSCOPIC
BACTERIOLOGICAL
BIOLOGICAL

BIOLOGICAL

BACTERIOLOGICAL

Investigated material

Sputum, pus

Serum agar

Blood agar

Smear

IDENTIFICATION

Examination of serovars

Gram staining

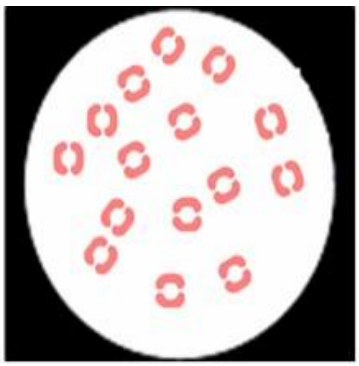
AR

Hiss's media

Bile

EXPRESS DIAGNOSES "Quellung reaction"

serums- I type II type III type

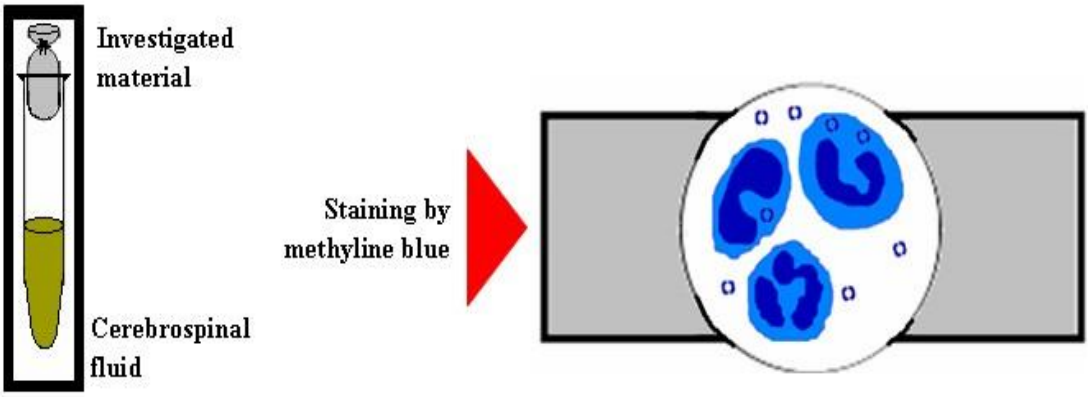


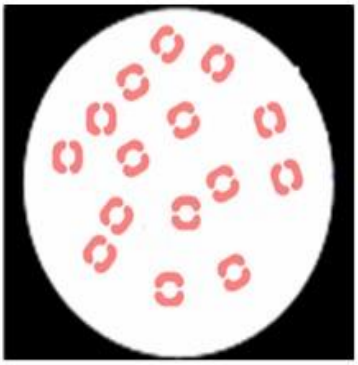
NEISSERIA MENINGITIDIS

METHODS

MICROSCOPIC
BACTERIOLOGICAL

BACTERIOSCOPIC METHOD



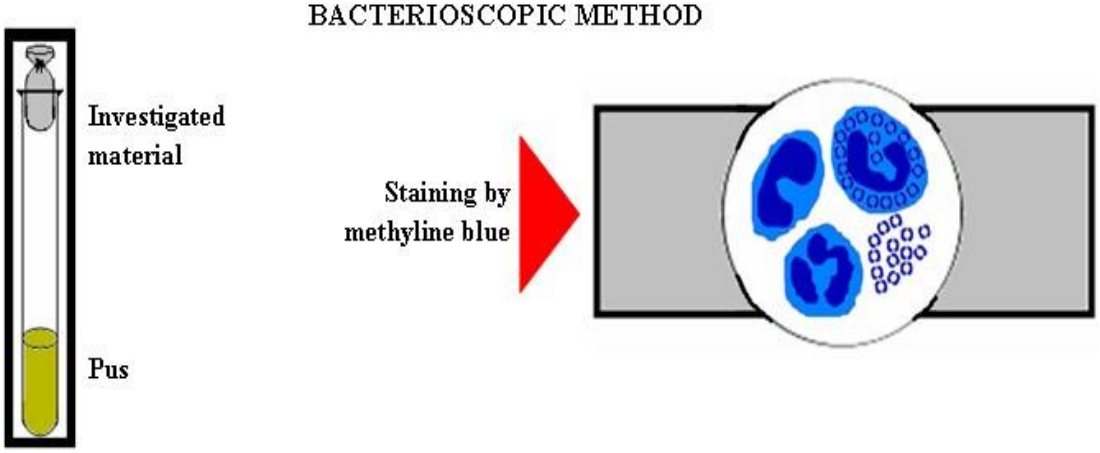


NEISSERIA GONORRHOEAE

METHODS

MICROSCOPIC
BACTERIOLOGICAL

BACTERIOSCOPIC METHOD



GRAM – NEGATIVE COCCI

The genus Neisseria consists of gram negative, aerobic, non-sporulating, nonmotile, oxidase-positive cocci, typically arranged in pairs (diplococcus). N. meningitidis and N. gonorrhoeae are the primary human pathogens of the genus. Besides the two important pathogens, N. meningitidis and N. gonorrhoeae, the family contains many other genuses: **Moraxella, Acinetobacter, Kingella, which** occur as commensals (conditionally pathogenic) and saprophytes.

N. meningitidis and N. gonorrhoeae are pathogenic for human and typically are found associated with or inside polymorphonuclear cells. Most importantly, the two species are differentiated by the usual clinical presentations of the diseases they cause: meningococci typically are found in the upper respiratory tract and cause meningitis, while gonococci cause genital infections.

GONORRHOEAE

Neisseria gonorrhoeae (Gonococcus)

Morphology: N. gonorrhoeae is Gram-negative, non-motile diplococcus, approximately 0.8 μm in diameter, non-capsulated. They are kidney (coffee-bean) shaped. Gonococci possess pili on their surface. Pili facilitate adhesion of the cocci to mucosal surfaces and promote virulence by inhibiting phagocytosis. Under the action of chemotherapeutic preparations they can transfer into L-forms.

Cultural characteristics: Gonococci grow best on media containing complex organic substances such as heated blood, hemin, and animal proteins and in an atmosphere con- taining 5-10% CO_2. They are aerobic. Optimal temperature for growth is 37ºC; pH-7.2-7.4. On ascites agar they form small, round, transparent, convex colonies (S-type).

Biochemical reactions: Gonococci ferment **glucose** with producing acid only. They are catalase and cytochrome oxidase positive. They lack protein lysing activity.

Antigenic structure: Gonococci are antigenically heterogenous. They are capable of changing their surface structures and antigenic structure. The antigenicity is connected with:

1. **Pili**: which are hairlike structures, act as virulence factors by promoting attachment to host cells. Pili undergo antigenic variations (16serotypes).
2. **Cell membrane proteins:** Outer membrane proteins show antigenic diversity, which helps in typing gonococcal strains. These proteins act as ligands attaching the coccus to the host cells. They also form transmembrane channels (proteins) which play a role in the exchange of molecules across the outer membrane.
3. Lipopolysaccharides of the cell wall.
4. **Superficial polysaccharide K antigen.**

Toxicity in gonococcal infections is largely due to the **endotoxic** effect of LPS.

Virulence factors are: fimbria, capsule, LPS of the cell wall which possesses endotoxic and antiphagocytic activities, SIgA- protease, β-lactamase enzyme, production of which depends on R plasmids.

Resistance: The gonococcus is a very delicate organism, readily killed by heat, drying and antiseptics.

Pathogenicity: Gonorrhoeae is a venereal disease which has been known since ancient times. The name gonorrhoeae (meaning flow of seed) was first employed by Galen in150AD. The disease is acquired by sexual contact. Gonococci require cylindric epithelium. They attack mucous membrane of the genitourinary tract, eye, rectum, and throat producing acute suppuration that may lead to tissue invasion; this is followed by chronic inflammation and fibrosis. The incubation period is 2-8days. In males the disease starts as an acute urethritis with yellow, creamery pus containing gonococci in a large number, and painful urination. The process may extend to the epididymis. In females, the primary infection **(acute gonorrhoeae)** is in the endocervix and extends to the urethra and vagina, giving rise to mucopurulent discharge. It may then progress to the uterine tubes, causing salpingitis, fibrosis and obliteration of the tubes. Infertility occurs in 20% of women

with gonococcal salpingitis. **Chronic** gonococcal cervicitis or proctitis are often asymptomatic.

Blennorrhea, gonococcal ophthalmia neonatorum, an infection of eye of the newborn, is acquired during passage through an infected birth canal. Initial conjunctivitis rapidly progresses and, if untreated, results in blindness. For prevention of gonococcal ophthalmia neonatorum, instillation of tetracycline, erythromycin or silver nitrate into the conjunctival sac of the newborn is used.

Immunity: Repeated gonococcal infections are common. Protective immunity to reinfection does not appear to develop as part of the disease process, because of the antigenic variety of gonococci.

Treatment: The agent of choice used to be penicillin G. In recent years, however, the percentage of penicillinase-producing strains has increased considerably all over the world. For this reason, third-generation cephalosporins are now used to treat uncomplicated cases of gonorrhea. They are applied in a single dose (e.g., ceftriaxone, 250–500mg i.m.). Good results have also been reported with single-dose oral application of fluorinated 4-quinolones (e.g., 0.5 g ciprofloxacin or 0.4 g ofloxacin).

Penicillin Resistance in Gonococci The determinants of high-level penicillin resistance in gonococci are small, nonconjugative plasmids, which are mobilized by a conjugative helper plasmid for transmission from one gonococcal cell to another. The penicillin resistance plasmids code for the TEM betalactamase that occurs frequently in Enterobacteriaceae. It is therefore assumed that the penicillinase gene in gonococci derived from the Enterobacteriaceae gene pool. Low-level, inherent resistance to penicillin is based on chromosomal genes (penA, penB) that code for penicillin-binding proteins with reduced affinity to penicillin. These genes are products of mutations.

Epidemiology and prevention. Gonorrhea is a worldwide sexually transmitted disease that occurs only in humans. Its level of annual incidence in developed countries is estimated at 12 cases per 1000 inhabitants. The actual figures are likely to be much higher due to large numbers of unreported cases. A reduction in incidence seen in recent years may be due to AIDS prophylaxis. Protective immunization for high-risk persons is not feasible due to the antigen variability of the organism as described above. Stopping the spread of gonorrhea involves mainly rapid recognition of infections and treatment accordingly. One hundred percent prevention of ophthalmia neonatorum is possible with a single parenteral dose of 125mg ceftriaxone. Local prophylaxis is also practiced using a 1% solution of silver nitrate or eye ointments containing 1% tetracycline or 0.5% erythromycin.

Diagnostic laboratory tests:

1. **Acute gonorrheae:** Microscopic method- direct examination of gram-stained smear of pus. This can be used to demonstrate the characteristic intracellular gram negative diplococci in symptomatic urethritis (phenomenon of incomplete phagocytosis).
2. **Chronic gonorrheae:**
   a) bacteriological method
   b) serological method: Bordet-Gengou CFR.

MENINGITIS
(Neisseria meningitidis)

Morphology: Meningococci (N. meningitidis) are gram-negative cocci that resemble paired coffee bean, non-motile diplococcus, and 0.6-1μm in diameter. They form prominent polysaccharide capsule. They are non-sporing and non motile.

Cultural characteristics: Meningococci do not grow on ordinary media**.** The organisms grow best on blood agar (chocolate agar), serum agar incubated at 37°C in an atmosphere of 5-8 % CO_2. They are strict aerobes. On solid media, after incubation for 24hours, the colonies are small (about 1mm in diameter), translucent, round, convex, bluish grey, with a smooth surface(S-type). Growth is poor in liquid media, producing a granular

turbidity with little or no surface growth.

The biochemical activity of meningococci is feebly marked: they ferment glucose and maltose with acid formation. Indole and hydrogen sulphide are not produced and nitrates are not reduced. They are catalase and oxidase positive.

Antigenic structure: N. meningitidis possesses a polysaccharide capsule and on the basis of this it has been subdivided into 13 serogroups. The most important serogroups associated with disease in humans are A, B, C, D, X, Y, Z, 29E, W-135, H, I, K, L. Serogroups H, I, K and L have been isolated from carriers and have not been associated with disease. Meningococcal antigens are found in blood and cerebrospinal fluid of patients with active disease. By the cell wall proteins they are subdivided into serotypes, which are designated by Arabic numerals (1,2,3, ….).

Ecology: Meningococci are very delicate organisms, being highly susceptible to heat, desiccation, alterations in pH and disinfectants.

Epidemiology: The human nasopharynx is the only reservoir of the meningococcus. Asymptomatic nasopharyngeal carriers rarely contract the illness but serve to infect their contacts. Transmission is essentially by airborne droplets or less often by fomites. During interepidemic periods, the carrier rate is about 5-10 per cent. An increase in carrier rate heralds the onset of an epidemic. During epidemics the carrier rates in closed communities may go up to 90 per cent. Meningitis is common in children between 3 months and 5 years of age. Epidemics usually occurs in semi-closed communities living in crowded conditions, as in jails and ships formerly, and in army camps in recent times.

Meningococci colonize the membranes of the nasopharynx and become part of the transient flora of the upper respiratory tract without producing symptoms. The organisms attach to epithelial cells with the aid of pili. From nasopharynx meningococci can enter the bloodstream producing bacteremia and spread to specific sites, such as the meninges or joints, or be disseminated throughout the body. The most important manifestations of disease are **nasopharyngitis, meningococcemia and meningitis.**

Fulminant meningococcemia (Waterhouse-Friderchsen syndrome) is more severe, with high fever, shock and hemorrhagic rash; there may be disseminated intravascular coagulation, and adrenal insufficiency.

Cerebrospinal meningitis is the most common complication of meningococcemia. It usually begins suddenly with fever, headache, vomiting, stiff neck and progresses to coma within a few hours.

The important virulence factors of meningococci are:

1. A polysaccharide capsule that enables the organism to resist phagocytosis by polymorphonuclear leukocytes.
2. Endotoxin-LPS of the cell wall, which causes fever, shock and other pathophysiologic changes (in purified form, endotoxin can reproduce many of the clinical manifestations of meningococcemia).
3. An immunoglobulin A protease, which by cleaving secretory Ig A helps the bacteria to attach to the membranes of the upper respiratory tract.
4. Pili: Meningococci possess pili on their surface which allow intimate contact with host cell and organisms release endotoxin.
5. Outer membrane proteins.
6. They produce enzymes **hyaluronidase**, **neuraminidase**, **fibrinolysin,** which promote their invasion in tissues, SIgA- protease and **plasmocoagulase.**

Immunity: Immunity to meningococcal infection is associated with the presence of specific, complement-dependent, bactericidal antibodies in the serum. Immunity is tense, lifelong and relapse occurs rarely.

Prophylaxis: Monovalent and polyvalent vaccins containing the capsular polysaccharides of groups A, C, W-135 and Y are available. Meningococcal chemical vaccine is used.

Laboratory diagnosis:

Methods (picture 3):

1. Microscopic (RIF)
2. Bacteriological -isolation of pure culture (in ristomycin containing media (ristomycin inhibits gra positive microbes in investigated material) and identification (table 1).
3. Serological (PHAR).

Table 1 Differences between pathogenic and non-pathogenic meningococci

	Non-pathogenic	Pathogenic
1.Growth on ordinary nutrient media	+	-
2.Growth temperature	the borders are high	22^{o}- $39\ ^{o}$ C
3.Biochemical activity	high	low (only fermentation of glucose and maltose)
4. Pigment formation	+	-

Therapy. The antibiotic of choice is penicillin G. Very good results have also been obtained with third-generation cephalosporins, e.g., cefotaxime or ceftriaxone. It is important to start treatment as quickly as possible to prevent delayed damage. The advantage of cephalosporins is that they are also effective against other meningitis pathogens due to their broad spectrum of action (with the exception of Listeria monocytogenes).

Epidemiology and prevention. Meningococcal infections are more frequent in the winter and spring months. Transmission of meningococci is by droplet infection. Humans are the only pathogen reservoir. Sources of infection include both carriers and infected persons with manifest disease. In developed countries, meningitis occurs sporadically or in the form of minor epidemics in more or less isolated collectives (work camps, recruiting camps, school camping facilities). The incidence level is approximately 12 cases per 100 000 inhabitants per year. In parts of the developing world (African meningitis belt) the level is higher. Lethality runs to 85% if the disease is left untreated, but is reduced to less than 1% if treatment is begun early enough. Prophylactic antibiosis is indicated for those in close contact with diseased persons (e.g., in the same family). Prophylactic measures also include treatment of carriers to eliminate this reservoir, whereby minocylin or rifampicin must be used instead of penicillin G. Prophylactic immunization can be achieved with a vaccine made from the purified capsule polysaccharides A, C, Y, and W-135. There is no serogroup B vaccine, since the capsule in serogroup B consists of polyneuraminic acid, which the immune system does not recognize as a foreign substance.