MINISTRY OF HEALTH CARE OF UKRAINE
Kharkiv National Medical University
D.P. Grynyov department of microbiology, virology and immunology

STANDARD PROTOCOLS

TO LABORATORY CLASSES IN SPECIAL MICROBIOLOGY

for the II and III year English media students

PART 3

student_____year_____group

Surname_____

_____

Teacher_____

_____

Kharkiv 2018

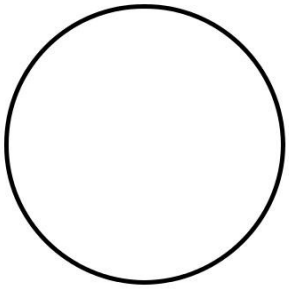
Standard protocols to laboratory classes in special microbiology for the II and III year English media students of medical and dentistry faculties / M.M. Mishyna, Yu.A. Mozgova, N.I. Kovalenko, T.M. Zamaziy, O.O. Vovk. – Kharkiv: KNMU, 2018. – 152 p.

Special microbiology

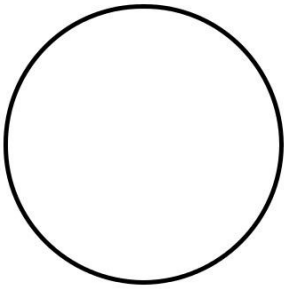
Protocol № 33

Theme: Grampositive cocci (staphylococci, streptococci).

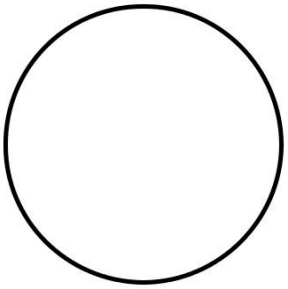
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Staphylococcus aureus (Gram stain)



Streptococcus pyogenes (Gram stain)

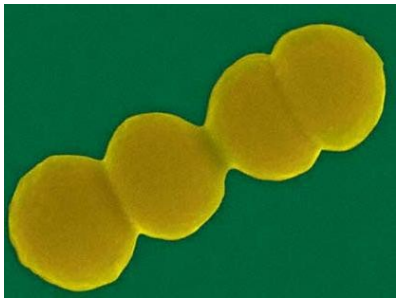


Streptococcus pneumoniae (Methylene blue stain)

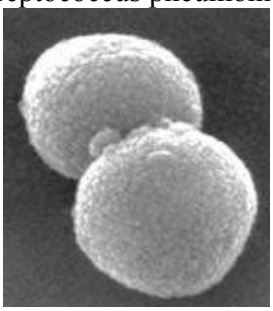
II. Study scanning electron micrographs:

Staphylococcus

Streptococcus pyogenes



Streptococcus pneumoniae



III. Study nutrient media for cultivation of staphylococci and streptococci:

а) MPB and MPA, blood agar, egg yolk salt agar for staphylococci; б) serum and sugar broth for streptococci;

в) ascetic broth for pneumococci;

г) Hiss media.

IV. Study culture and biochemical properties of staphylococci:

a) growth on MPB: _____;

b) growth on MPA: _____;

c) growth on blood agar: _____;

d) growth on egg yolk salt agar: _____;

g) media with mannitol (only *S. aureus* can ferment mannitol) and optochin (S. pneumoniae is sensitive while S. mitis (normal flora) is resistant).

e) sugar fermentation and protein hydrolysis by staphylococci on Hiss media and MPB:

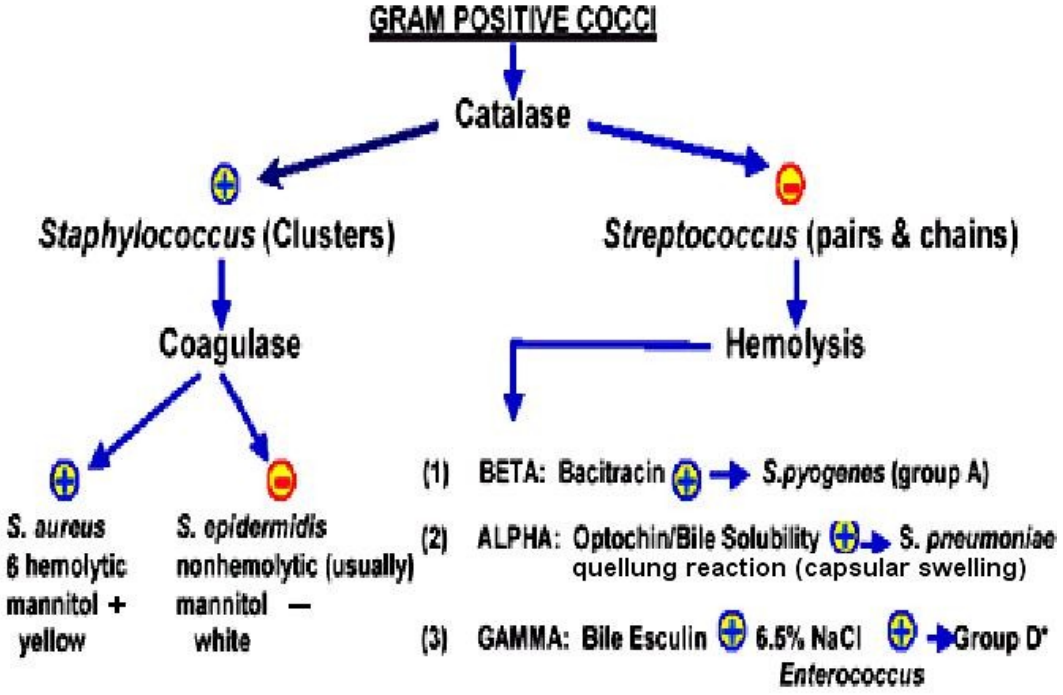
Species	Lactose	Glucose	Mannitol	Maltose	Sucrose	MPB	
						H_2S	Indol
S.aureus	acid	acid	acid	acid	acid	+	-
S.epidermidis	acid	acid	-	acid	acid	+	-
S.saprophyticus	acid	acid	acid	acid	acid	+	-

V. Spesific therapy of staphylococcal infections - immunoglobulin.
Spesific prophilaxis of Streptococcus pneumoniae infections - groups A, C, AC, and ACYW135 capsular polysaccharide vaccines are available.

VI. Therapy of staphylococcal infections: a first- and second-generations cephalosporin: cefasolin, cefuroxim, cefaclor, cefoxitin; a third-generation cephalosporin: cefotaxime or ceftriaxone**;** penicillins: oxacillin, ampicillin, amoxicilin+clavulanic acid (Amoxiclav), ampicillin+oxacillin (Ampiox); fluroquinolones: lomefloxacin, moxifloxacin, levofloxacin; vancomycin, clindamycin, erythromycin, tetracycline, etc.

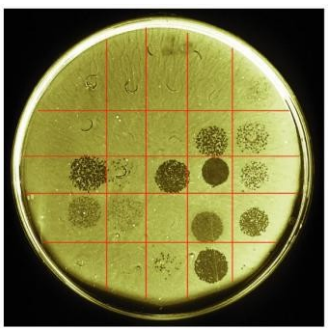
VII. Study antibiotics sensitivity testing of Staphylococci – disk diffusion test.

VIII. Study the schemes of laboratory diagnosis of staphylococcal and streptococcal infections.



Phage typing after Fisher (to detect origin of the source of infection):

- It is the identification of bacterial species and strains by determining their susceptibility to various phages.
  - The testing strain is infected by bacteriophages.
  - After incubation, a lytic phage infection will result in plaques = no bacterial growth.



ADDING THEORETICAL MATERIAL

Bacteria in the genus *Staphylococcus* are pathogens of man and other mammals. Traditionally they were divided into two groups on the basis of their ability to clot blood plasma (the coagulase reaction). The coagulase-positive staphylococci constitute the most pathogenic species *S. aureus*. The coagulase-negative staphylococci (CNS) are now known to comprise over 30 other species. The CNS are common commensals of skin, although some species can cause infections. It is now obvious that the division of staphylococci into coagulase positive and negative is artificial and indeed, misleading in some cases. Coagulase is a marker for *S. aureus* but there is no direct evidence that it is a virulence factor. Also, some natural isolates of *S.aureus* are defective in coagulase. Nevertheless, the term is still in widespread use among clinical microbiologists.

Staphylococci can cause many forms of infection. (1) *S. aureus* causes superficial skin lesions (boils, styes) and localized abscesses in other sites. (2) *S. aureus* causes deep-seated infections, such as osteomyelitis and endocarditis and more serious skin infections (furunculosis). (3) *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and, with *S. epidermidis*, causes infections associated with indwelling medical devices. (4) *S. aureus* causes food poisoning by releasing enterotoxins into food. (5) *S. aureus* causes toxic shock syndrome by release of superantigens into the blood stream. (6) *S.saprophiticus* causes urinary tract infections, especially in girls. (7) Other species of staphylococci (*S. lugdunensis, S. haemolyticus, S. warneri, S. schleiferi, S. intermedius*) are infrequent pathogens.

Structure. Staphylococci are Gram-positive cocci 1μm in diameter. They form clumps.

Classification. *S. aureus* and *S. intermedius* are coagulase positive. All other staphylococci are coagulase negative. They are salt tolerant and often hemolytic. Identification requires biotype analysis.

S aureus colonizes the nasal passage and axillae. *S. epidermidis* is a common human skin commensal. Other species of staphylococci are infrequent human commensals. Some are commensals of other animals.

Pathogenesis. *S. aureus* expresses many potential virulence factors. (1) Surface proteins that promote colonization of host tissues. (2) Factors that probably inhibit phagocytosis (capsule, immunoglobulin binding protein A). (3) Toxins that damage host tissues and cause disease symptoms. Coagulase-negative staphylococci are normally less virulent and express fewer virulence factors. *S.epidermidis* readily colonizes implanted devices.

Antibiotic Resistance. Multiple antibiotic resistance is increasingly common in *S. aureus* and *S. epidermidis*. Methicillin resistance is indicative of multiple resistance. Methicillin-resistant *S.aureus* (MRSA) causes outbreaks in hospitals and can be epidemic.

Diagnosis is based on performing tests with colonies. Tests for clumping factor, coagulase, hemolysins and thermostable deoxyribonuclease are routinely

used to identify *S.aureus*. Commercial latex agglutination tests are available. Identification of *S epidermidis* is confirmed by commercial biotyping kits.

Identification of Staphylococci in the Clinical laboratory

Staphylococci are Gram-positive cocci about 0.5 – 1.0 μm in diameter. They grow in clusters, pairs and occasionally in short chains. The clusters arise because staphylococci divide in two planes. The configuration of the cocci helps to distinguish micrococci and staphylococci from streptococci, which usually grow in chains. Observations must be made on cultures grown in broth, because streptococci grown on solid medium may appear as clumps. Several fields should be examined before deciding whether clumps or chains are present.

Catalase Test

The catalase test is important in distinguishing streptococci (catalase-negative) staphylococci which are catalase positive. The test is performed by flooding an agar slant or broth culture with several drops of 3% hydrogen peroxide. Catalase-positive cultures bubble at once. The test should not be done on blood agar because blood itself will produce bubbles.

Isolation and Identification

The presence of staphylococci in a lesion might first be suspected after examination of a direct Gram stain. However, small numbers of bacteria in blood preclude microscopic examination and require culturing first.

The organism is isolated by streaking material from the clinical specimen (or from a blood culture) onto solid media such as blood agar, tryptic soy agar or heart infusion agar. Specimens likely to be contaminated with other microorganisms can be plated on yolk salt agar, milk salt agar or mannitol salt agar containing 7.5% sodium chloride, which allows the halo-tolerant staphylococci to grow. Ideally a Gram stain of the colony should be performed and tests made for catalase and coagulase production, allowing the coagulase-positive *S. aureus* to be identified quickly. Another very useful test for *S. aureus* is the production of thermostable deoxyribonuclease. *S. aureus* can be confirmed by testing colonies for agglutination with latex particles coated with immunoglobulin G and fibrinogen which bind protein A and the clumping factor, respectively, on the bacterial cell surface. These are available from commercial suppliers (e.g., Staphaurex). The most recent latex test (Pastaurex) incorporates monoclonal antibodies to serotype 5 and 8 capsular polysaccharides in order to reduce the number of false negatives. (Some recent clinical isolates of *S. aureus* lack production of coagulase and/or clumping factor, which can make identification difficult.)

Because *S. aureus* is a major cause of nosocomial and community-acquired infections, it is necessary to determine the relatedness of isolates collected during the investigation of an outbreak. Typing systems must be reproducible, discriminatory, and easy to interpret and to use. The traditional method for typing *S. aureus* is phage-typing. This method is based on a phenotypic marker with poor reproducibility.

Pathogenesis of *S. aureus* Infections.

S. aureus expresses many cell surface-associated and extracellular proteins that are potential virulence factors. For the majority of diseases caused by this

organism, pathogenesis is multifactorial. Thus it is difficult to determine precisely the role of any given factor. This also reflects the inadequacies of many animal models for staphylococcal diseases.

However, there are correlations between strains isolated from particular diseases and expression of particular factors, which suggests their importance in pathogenesis. With some toxins, symptoms of a human disease can be reproduced in animals with pure proteins. The application of molecular biology has led to recent advances in the understanding of pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced and proteins purified. This has facilitated studies at the molecular level on their modes of action, both in in vitro and in model systems. In addition, genes encoding putative virulence factors have been inactivated, and the virulence of the mutants compared to the wild-type strain in animal models. Any diminution in virulence implicates the missing factor. If virulence is restored when the gene is returned to the mutant then "Molecular Koch's Postulates" have been fulfilled. Several virulence factors of S. aureus have been confirmed by this approach.

S. aureus cells express on their surface proteins that promote attachment to host proteins such as laminin and fibronectin that form part of the extracellular matrix. Fibronectin is present on epithelial and endothelial surfaces as well as being a component of blood clots. In addition, most strains express a fibrinogen/fibrin binding protein (the clumping factor) which promotes attachment to blood clots and traumatized tissue. Most strains of *S. aureus* express fibronectin and fibrinogen-binding proteins.

The receptor which promotes attachment to collagen is particularly associated with strains that cause osteomyelitis and septic arthritis. Interaction with collagen may also be important in promoting bacterial attachment to damaged tissue where the underlying layers have been exposed.

Evidence that these staphylococcal matrix-binding proteins are virulence factors has come from studying defective mutants in vitro adherence assays and in experimental infections. Mutants defective in binding to fibronectin and to fibrinogen have reduced virulence in a rat model for endocarditis, suggesting that bacterial attachment to the sterile vegetations caused by damaging the endothelial surface of the heart valve is promoted by fibronectin and fibrinogen. Similarly, mutants lacking the collagen-binding protein have reduced virulence in a mouse model for septic arthritis. Furthermore, the soluble ligand-binding domain of the fibrinogen, fibronectin and collagen-binding proteins expressed by recombinant methods strongly blocks interactions of bacterial cells with the corresponding host protein.

Capsular Polysaccharide

The majority of clinical isolates of *S. aureus* express a surface polysaccharide of either serotype 5 or 8. This has been called a microcapsule because it can be visualized only by electron microscopy after antibody labeling, unlike the copious capsules of other bacteria which are visualized by light microscopy. *S. aureus* isolated from infections expresses high levels of polysaccharide but rapidly loses it upon laboratory subculture. The function of the

capsule is not clear. It may impede phagocytosis, but in in vitro tests this was only demonstrated in the absence of complement. Conversely, comparing wild-type and a capsule defective mutant strain in an endocarditis model suggested that polysaccharide expression actually impeded colonization of damaged heart valves, perhaps by masking adhesins.

Protein A

Protein A is a surface protein of *S. aureus* which binds immunoglobulin G molecules by the Fc region. In serum, bacteria will bind IgG molecules the wrong way round by this non-immune mechanism. In principle this will disrupt opsonization and phagocytosis. Indeed mutants of *S. aureus* lacking protein A are more efficiently phagocytozed in vitro, and studies with mutants in infection models suggest that protein A enhances virulence.

Leukocidin

S. aureus can express a toxin that specifically acts on polymorphonuclear leukocytes. Phagocytosis is an important defense against staphylococcal infection so leukocidin should be a virulence factor. This toxin is discussed in more detail in the next section.

S. aureus can express several different types of protein toxins which are probably responsible for symptoms during infections. Some damage the membranes of erythrocytes, causing hemolysis; but it is unlikely that hemolysis is relevant in vivo. The leukocidin causes membrane damage to leukocytes and is not hemolytic. Systemic release of α-toxin causes septic shock, while enterotoxins and TSST-1 cause toxic shock.

Membrane Damaging Toxins

(A) The best characterized and most potent membrane-damaging toxin of *S. aureus* is α-toxin. It is expressed as a monomer that binds to the membrane of susceptible cells. Subunits then oligomerize to form hexameric rings with a central pore through which cellular contents leak.

Susceptible cells have a specific receptor for α-toxin which allows low concentrations of toxin to bind, causing small pores through which monovalent cations can pass. At higher concentrations, the toxin reacts non-specifically with membrane lipids, causing larger pores through which divalent cations and small molecules can pass. However, it is doubtful if this is relevant under normal physiological conditions.

In humans, platelets and monocytes are particularly sensitive to α-toxin. They carry high affinity sites which allow toxin to bind at concentrations that are physiologically relevant. A complex series of secondary reactions ensue, causing release of eicosanoids and cytokines which trigger production of inflammatory mediators. These events cause the symptoms of septic shock that occur during severe infections caused by *S. aureus*.

The notion that α-toxin is a major virulence factor of *S. aureus* is supported by studies with the purified toxin in animals and in organ culture. Also, mutants lacking α-toxin are less virulent in a variety of animal infection models.

(B) β-toxin is a sphingomyelinase which damages membranes rich in this lipid. The classical test for β-toxin is lysis of sheep erythrocytes. The majority of

human isolates of *S.aureus* do not express β-toxin. A lysogenic bacteriophage is inserted into the gene that encodes the toxin. This phenomenon is called negative phage conversion. Some of the phages that inactivate the β-toxin gene carry the determinant for an enterotoxin and staphylokinase.

In contrast the majority of isolates from bovine mastitis express β-toxin, suggesting that the toxin is important in the pathogenesis of mastitis. This is supported by the fact that β-toxin-deficient mutants have reduced virulence in a mouse model for mastitis.

(C) The δ-toxin is a very small peptide toxin produced by most strains of *S. aureus*. It is also produced by *S. epidermidis* and *S. lugdunensis*. The role of δ-toxin in disease is unknown.

(D) The γ-toxin and the leukocidins are two-component protein toxins that damage membranes of susceptible cells. The proteins are expressed separately but act together to damage membranes. There is no evidence that they form multimers prior to insertion into membranes. The γ-toxin locus expresses three proteins. The B and C components form a leukotoxin with poor hemolytic activity, whereas the A and B components are hemolytic and weakly leukotoxic.

The classical Panton and Valentine (PV) leukocidin is distinct from the leukotoxin expressed by the γ-toxin locus. It has potent leukotoxicity and, in contrast to γ-toxin, is non-hemolytic. Only a small fraction of *S. aureus* isolates (2% in one survey) express the PV leukocidin, whereas 90% of those isolated from severe dermonecrotic lesions express this toxin. This suggests that PV leukocidin is an important factor in necrotizing skin infections.

PV-leukocidin causes dermonecrosis when injected subcutaneously in rabbits. Furthermore, at a concentration below that causing membrane damage, the toxin releases inflammatory mediators from human neutrophils, leading to degranulation. This could account for the histology of dermonecrotic infections (vasodilation, infiltration and central necrosis).

S U P E R A N TI G EN S : E NT ER O T OX I NS A ND T O XI C S H OC K S Y N DR OM E T OX I N

S. aureus can express two different types of toxin with superantigen activity, enterotoxins, of which there are six serotypes (A, B, C, D, E and G) and toxic shock syndrome toxin (TSST-1). Enterotoxins cause diarrhea and vomiting when ingested and are responsible for staphylococcal food poisoning. When expressed systemically, enterotoxins can cause toxic shock syndrome (TSS) - indeed enterotoxins B and C cause 50% of non-menstrual TSS. TSST-1 is very weakly related to enterotoxins and does not have emetic activity. TSST-1 is responsible for 75% of TSS, including all menstrual cases. TSS can occur as a sequel to any staphylococcal infection if an enterotoxin or TSST-1 is released systemically and the host lacks appropriate neutralizing antibodies. Tampon-associated TSS is not a true infection, being caused by growth of *S aureus* in a tampon and absorption of the toxin into the blood stream. TSS came to prominence with the introduction of super-absorbent tampons; and although the number of such cases has decreased dramatically, they still occur despite withdrawal of certain types of tampons from the market.

Superantigens stimulate T cells non-specifically without normal antigenic recognition. Up to one in five T cells may be activated, whereas only 1 in 10,000 are stimulated during antigen presentation. Cytokines are released in large amounts, causing the symptoms of TSS. Superantigens bind directly to class II major histocompatibility complexes of antigen-presenting cells outside the conventional antigen-binding grove. This complex recognizes only the Vβ element of the T cell receptor. Thus any T cell with the appropriate Vβ element can be stimulated, whereas normally antigen specificity is also required in binding.

E P I D ER M O L Y TI C (E XF O L I A TI VE) TO XI N (E T)

This toxin causes the scalded skin syndrome in neonates, with widespread blistering and loss of the epidermis. There are two antigenically distinct forms of the toxin, ETA and ETB. There is evidence that these toxins have protease activity. Both toxins have a sequence similarity with the *S. aureus* serine protease, and the three most important amino acids in the active site of the protease are conserved. Furthermore, changing the active site of serine to a glycine completely eliminated toxin activity. However, ETs do not have discernible proteolytic activity but they do have esterase activity. It is not clear how the latter causes epidermal splitting. It is possible that the toxins target a very specific protein which is involved in maintaining the integrity of the epidermis.

OTHER EXTRACELLULAR PROTEINS

Coagulase is not an enzyme. It is an extracellular protein which binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. This is the basis of the tube coagulase test, in which a clot is formed in plasma after incubation with the *S. aureus* broth-culture supernatant. Coagulase is a traditional marker for identifying *S. aureus* in the clinical microbiology laboratory. However, there is no evidence that it is a virulence factor, although it is reasonable to speculate that the bacteria could protect themselves from host defenses by causing localized clotting. Notably, coagulase deficient mutants have been tested in several infection models but no differences from the parent strain were observed.

There is some confusion in the literature concerning coagulase and clumping factor, the fibrinogen-binding determinant on the *S. aureus* cell surface. This is partly due to loose terminology, with the clumping factor sometimes being referred to as bound coagulase. Also, although coagulase is regarded as an extracellular protein, a small fraction is tightly bound on the bacterial cell surface where it can react with prothrombin. Finally, it has recently been shown that the coagulase can bind fibrinogen as well as thrombin, at least when it is extracellular. Genetic studies have shown unequivocally that coagulase and clumping factor are distinct entities. Specific mutants lacking coagulase retain clumping factor activity, while clumping factor mutants express coagulase normally.

Staphylokinase

Many strains of *S. aureus* express a plasminogen activator called staphylokinase. The genetic determinant is associated with lysogenic bacteriophages. A complex formed between staphylokinase and plasminogen

activates plasmin-like proteolytic activity which causes dissolution of fibrin clots. The mechanism is identical to streptokinase, which is used in medicine to treat patients suffering from coronary thrombosis. As with coagulase there is no evidence that staphylokinase is a virulence factor, although it seems reasonable to imagine that localized fibrinolysis might aid in bacterial spreading.

S. aureus can express proteases, a lipase, a deoxyribonuclease (DNase) and a fatty acid modifying enzyme (FAME). The first three probably provide nutrients for the bacteria, and it is unlikely that they have anything but a minor role in pathogenesis. However, the FAME enzyme may be important in abscesses, where it could modify anti-bacterial lipids and prolong bacterial survival. The thermostable DNase is an important diagnostic test for identification of *S. aureus*.

Since the beginning of the antibiotic era *S. aureus* has responded to the introduction of new drugs by rapidly acquiring resistance by a variety of genetic mechanisms including (1) acquisition of extrachromosomal plasmids or additional genetic information in the chromosome via transposons or other types of DNA insertion and (2) by mutations in chromosomal genes.

Many plasmid-encoded determinants have recently become inserted into the chromosome at a site associated with the methicillin resistance determinant. There may be an advantage to the organism having resistance determinants in the chromosome because they will be more stable. There are essentially four mechanisms of resistance to antibiotics in bacteria: (1) enzymatic inactivation of the drug, (2) alterations to the drug target to prevent binding, (3) accelerated drug efflux to prevent toxic concentrations accumulating in the cell, and (4) a by-pass mechanism whereby an alternative drug-resistant version of the target is expressed.

Antimicrobial Drugs

Ever since the first use of penicillin, *S. aureus* has shown a remarkable ability to adapt. Resistance has developed to new drugs within a short time of their introduction. Some strains are now resistant to most conventional antibiotics. It is worrisome that there do not seem to be any new antibiotics on the horizon. Any recent developments have been modifications to existing drugs.

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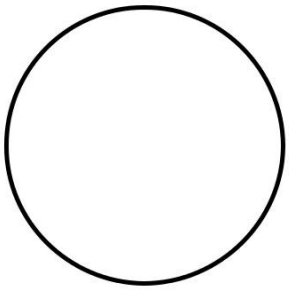
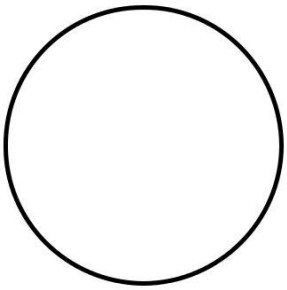
Protocol № 34

Theme: Gramnegative cocci (gonococci, meningococci).

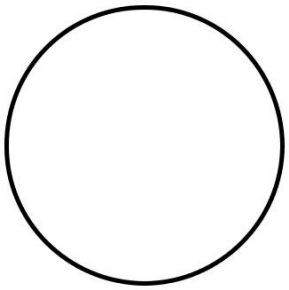
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



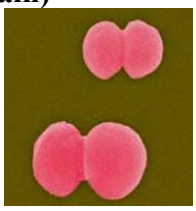
Neisseria meningitidis (Gram stain)



Neisseria gonorrhoeae (Gram stain)



Neisseria gonorrhoeae (Methylene blue stain)

II. Study scanning electron micrograph of Neisseria 

III. Study nutrient media for cultivation of meningococci and gonococci:

a) serum agar;
b) ascetic agar;
c) chocolate agar,
d) MTM (Martin-Thayer medium) contains antibiotics to inhibit normal body flora:
- vancomycin to inhibit gram-positive bacteria;
- colistin to inhibit gram-negative bacteria;
- trimethoprim to suppress *Proteus*;
- nystatin to inhibit yeast.

IV. Identification of *Neisseria:*

1. Oxidase test.

The oxidase test is based on the bacterial production of an oxidase enzyme. Cytochrome oxidase, in the presence of oxygen, oxidizes the para-amino dimetheylanaline oxidase test reagent in a Taxo-N® disc. In the **immediate test**, oxidase-positive reactions will turn a **rose color within 30 seconds**. Oxidase-negative will not turn a rose color. This reaction only lasts a couple of minutes.

All *Neisseria* are oxidase positive. The oxidase test can be performed using a Taxo N® disc. A moistened Taxo N® disc can be placed on a growing culture and a blackening of the colonies surrounding the disc indicates a positive oxidase test. All oxidase-positive cultures would be gram stained to confirm gram-negative diplococci.

The ability to grow on MTM chocolate agar and the positive oxidase test indicate the organism is most likely a pathogenic *Neisseria*. Identification of genus and species can be confirmed by carbohydrate fermentation.

2. Carbohydrate fermentation.

The various species of *Neisseria* can be differentiated according to fermentation patterns using glucose and maltose media. If fermentation occurs, acid end products cause the phenol red pH indicator to turn yellow. *N. gonorrhoeae* ferments only glucose whereas *N. meningitidis* ferments glucose and maltose. Nonpathogenic neisseriae usually ferment only sucrose.

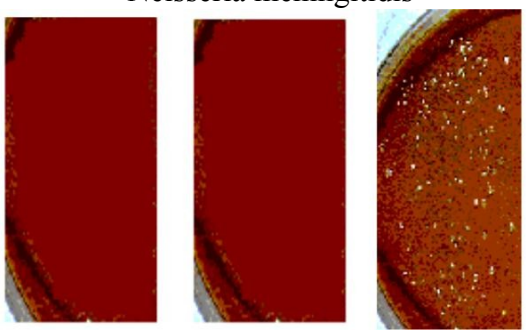
S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

Carbohydrate fermentation by gonococci and meningococci:

Species	Glucose	Maltose
Neisseria gonorrhoeae	acid	-
Neisseria meningitidis	acid	acid

V. Differentiation of Neisseria meningitides and Neisseria catarrhalis on serum and MPA at different temperatures:

Neisseria meningitidis



37^0 C MPA | 22^0 C serum agar | 37^0 C serum agar

Result:

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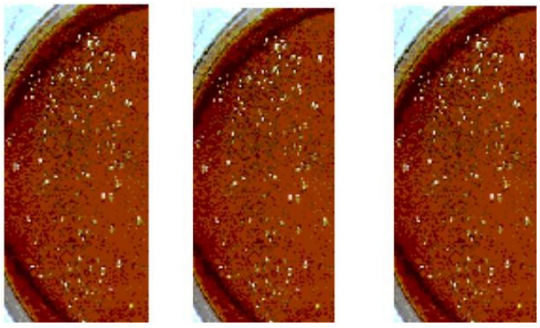
Neisseria catarrhalis



37^0 C MPA | 22^0 C serum agar | 37^0 C serum agar

Result:

_____

_____

VI. Therapy of gonococcal and meningococcal infections: a third-generation cephalosporin: cefixime or ceftriaxone**;** penicillins: oxacillin, ampicillin, ampicillin+oxacillin (Ampiox); fluroquinolones: ciprofloxacine, lomefloxacin, moxifloxacin, levofloxacin.

VII. Study the scheme of laboratory diagnosis of gonococcal and meningococcal infections.

1. Microscopy - intracellular gram negative diplococci.

2. Immunefluorescence.

3. Culture of specimen under increased CO_2 tension.

To isolate *N. meningitidis*, cultures are taken from the **nasopharynx, blood, CSF, and skin lesions**.

To diagnose genital gonorrhea in males, the sample is taken from the **urethra**, in females - from the **cervix and the rectum.**

Identification of colonies by: oxidase test, gram-staining,

carbohydrate fermentation reactions: *N. gonorrhoeae* ferments **only glucose** whereas *N. meningitidis* ferments **glucose and maltose**.

4. Precipitation test, ELISA - detection of meningococcal antigen by antimeningococcal serum on cerebrospinal fluid or from skin lesions for rapid identification.

5. Serology: CFT (diagnosis of chronic gonorrhea).

ADDING THEORETICAL MATERIAL

The ***Neisseriaceae*** are a family of Beta Proteobacteria consisting of Gram-negative aerobic bacteria from fourteen genera (Bergey's 2005), including *Neisseria, Chromobacterium, Kingella,* and *Aquaspirillum.* The genus *Neisseria* contains two important human pathogens, *N. gonorrhoeae* and *N. meningitidis. N. gonorrhoeae* causes gonorrhea, and *N. meningitides* is the cause of meningococcal

meningitis. *N. gonorrhoeae* infections have a high prevalence and low mortality, whereas *N. meningitides* infections have a low prevalence and high mortality.

Neisseria gonorrhoeae infections are acquired by sexual contact and usually affect the mucous membranes of the urethra in males and the endocervix and urethra in females, although the infection may disseminate to a variety of tissues. The pathogenic mechanism involves the attachment of the bacterium to nonciliated epithelial cells via pili and the production of lipopolysaccharide endotoxin. Similarly, the lipopolysaccharide of *Neisseria meningitides* is highly toxic, and it has an additional virulence factor in the form of its antiphagocytic capsule. Both pathogens produce IgA proteases which promote virulence. Many normal individuals may harbor *Neisseria meningitides* in the upper respiratory tract, but *Neisseria gonorrhoeae* is never part of the normal flora and is only found after sexual contact with an infected person (or direct contact, in the case of infections in the newborn).

Neisseria gonorrhoeae

Neisseria gonorrhoeae is a Gram-negative coccus, 0.6 to 1.0 μm in diameter, usually seen in pairs with adjacent flattened sides. The organism is frequently found intracellularly in polymorphonuclear leukocytes (neutrophils) of the gonorrhea pustular exudate. Fimbriae or pili, which play a major role in adherence, extend several micrometers from the cell surface *Neisseria gonorrhoeae* possesses a typical Gram-negative outer membrane composed of proteins, phospholipids, and lipopolysaccharide (LPS). However, neisserial LPS is distinguished from enteric LPS by its highly-branched basal oligosaccharide structure and the absence of repeating O-antigen subunits. For these reasons, neisserial LPS is referred to as lipooligosaccharide (LOS). The bacterium characteristically releases outer membrane fragments called "blebs" during growth. These blebs contain LOS and probably have a role in pathogenesis if they are disseminated during the course of an infection.

N. gonorrhoeae is a relatively fragile organism, susceptible to temperature changes, drying, uv light, and other environmental stresses. Strains of *N. gonorrhoeae* are fastidious and variable in their cultural requirements, so that media containing hemoglobin, NAD, yeast extract and other supplements are needed for isolation and growth of the organism. Cultures are grown at 35-36 degrees in an atmosphere of 3-10% added CO_2.

The disease **gonorrhea** is a specific type of urethritis that practically always involves mucous membranes of the urethra, resulting in a copious discharge of pus, more apparent in the male than in the female. The first usage of the term "gonorrhea", by Galen in the second century, implied a "flow of seed". For centuries thereafter, gonorrhea and syphilis were confused, resulting from the fact that the two diseases were often present together in infected individuals. Paracelsus (1530) thought that gonorrhea was an early symptom of syphilis. The confusion was further heightened by the classic blunder of English physician John Hunter, in 1767. Hunter intentionally inoculated himself with pus from a patient with symptoms of gonorrhea and wound up giving himself syphilis! The causative agent of gonorrhea, *Neisseria gonorrhoeae,* was first described by A. Neisser in 1879, in

the pustular exudate of a case of gonorrhea. The organism was grown in pure culture in 1885, and its etiological relationship to human disease was later established using human volunteers in order to fulfill the experimental requirements of Koch's postulates.

Gonorrheal infection is generally limited to superficial mucosal surfaces lined with columnar epithelium. The areas most frequently involved are the urethra, cervix, rectum, pharynx, and conjunctiva. Squamous epithelium, which lines the adult vagina, is not susceptible to infection by the *N. gonorrhoeae.* However, the prepubescent vaginal epithelium, which has not been keratinized under the influence of estrogen, may be infected. Hence, gonorrhea in young girls may present as vulvovaginitis. Mucosal infections are usually characterized by a purulent discharge.

Uncomplicated gonorrhea in the adult male is an inflammatory and pyogenic infection of the mucous membranes of the anterior urethra. The most common symptom is a discharge that may range from a scanty, clear or cloudy fluid to one that is copious and purulent. Dysuria (difficulty in urination) is often present. Inflammation of the urethral tissues results in the characteristic redness, swelling, heat, and pain in the region. There is intense burning and pain upon urination.

Endocervical infection is the most common form of uncomplicated gonorrhea in women. Such infections are usually characterized by vaginal discharge and sometimes by dysuria. About 50% of women with cervical infections are asymptomatic. Asymptomatic infections occur in males, as well. Males with asymptomatic urethritis are an important reservoir for transmission and are at increased risk for developing complications. Asymptomatic males and females are a major problem as unrecognized carriers of the disease.

In the male, the organism may invade the prostate resulting in prostatitis, or extend to the testicles resulting in orchitis. In the female, cervical involvement may extend through the uterus to the fallopian tubes resulting in salpingitis, or to the ovaries resulting in ovaritis. As many as 15% of women with uncomplicated cervical infections may develop pelvic inflammatory disease (PID). The involvement of testicles, fallopian tubes or ovaries may result in sterility. Occasionally, disseminated infections occur. The most common forms of disseminated infections are a dermatitis-arthritis syndrome, endocarditis and meningitis.

Ocular infections by *N. gonorrhoeae* can have serious consequences of corneal scarring or perforation. Ocular infections (ophthalmia neonatorum) occur most commonly in newborns who are exposed to infected secretions in the birth canal. Part of the intent in adding silver nitrate or an antibiotic to the eyes of the newborn is to prevent ocular infection by *N. gonorrhoeae.*

Pathogenesis. Gonorrhea in adults is almost invariably transmitted by sexual intercourse. The bacteria adhere to columnar epithelial cells, penetrate them, and multiply on the basement membrane. Adherence is mediated through pili and opa (P.II) proteins. although nonspecific factors such as surface charge and hydrophobicity may play a role. Pili undergo both phase and antigenic variation.

The bacteria attach only to microvilli of nonciliated columnar epithelial cells. Attachment to ciliated cells does not occur.

Most of the information on bacterial invasion comes from studies with tissue culture cells and human fallopian tube organ culture. After the bacteria attach to the nonciliated epithelial cells of the fallopian tube, they are surrounded by the microvilli, which draw them to the surface of the mucosal cell. The bacteria enter the epithelial cells by a process called parasite-directed endocytosis. During endocytosis the membrane of the mucosal cell retracts and pinches off a membrane-bound vacuole (phagosome) that contains the bacteria. The vacuole is transported to the base of the cell, where the bacteria are released by exocytosis into the subepithelial tissue. The neisseriae are not destroyed within the endocytic vacuole, but it is not clear whether they actually replicate in the vacuoles as intracellular parasites.

A major porin protein, P.I (Por)**,** in the outer membrane of the bacterium is thought to be the invasin that mediates penetration of a host cell. Each *N. gonorrhoeae* strain expresses only one type of Por; however, there are several variations of Por that partly account for different antigenic types of the bacterium.

Neisseria gonorrhoeae can produce one or several outer membrane proteins called Opa (P.II) proteins. These proteins are subject to phase variation and are usually found on cells from colonies possessing a unique opaque phenotype called O+. At any particular time, the bacterium may express zero, one, or several different Opa proteins, and each strain has 10 or more genes for different Opas.

Rmp (P.III) is an outer membrane protein found in all strains of *N. gonorrhoeae.* It does not undergo antigenic variation and is found in a complex with Por and LOS. It shares partial homology with the OmpA protein of *Escherichia coli.* Antibodies to Rmp, induced either by a neisserial infection or by colonization with *E. coli,* tend to block bactericidal antibodies directed against Por and LOS. In fact, anti-Rmp antibodies may increase susceptibility to infection by *N.gonorrhoeae.*

During infection, bacterial lipooligosaccharide (LOS) and peptidoglycan are released by autolysis of cells. Both LOS and peptidoglycan activate the host alternative complement pathway, while LOS also stimulates the production of tumor necrosis factor (TNF) that causes cell damage. Neutrophils are immediately attracted to the site and feed on the bacteria. For unknown reasons, many gonococci are able to survive inside of the phagocytes, at least until the neutrophils themselves die and release the ingested bacteria.

Neisserial LOS has a profound effect on the virulence and pathogenesis of *N.gonorrhoeae.* The bacteria can express several antigenic types of LOS and can alter the type of LOS they express by some unknown mechanism. Gonococcal LOS produces mucosal damage in fallopian tube organ cultures and brings about the release of enzymes, such as proteases and phospholipases, that may be important in pathogenesis. Thus, gonococcal LOS appears to have an indirect role in mediating tissue damage. Gonococcal LOS is also involved in the resistance of *N. gonorrhoeae* to the bactericidal activity of normal human serum. Specific LOS

oligosaccharide types are known to be associated with serum-resistant phenotypes of *N. gonorrhoeae.*

N. gonorrhoeae can utilize host-derived N-acetylneuraminic acid (sialic acid) to sialylate the oligosaccharide component of its LOS, converting a serum-sensitive organism to a serum-resistant one. Organisms with nonsialylated LOS are more invasive than those with sialylated LOS but organisms with sialylated LOS are more resistant to bactericidal effects of serum. There is also antigenic similarity between neisserial LOS and antigens present on human erthyrocytes. This similarity to "self" may preclude an effective immune response to these LOS antigens by maintaining the immunotolerance of the host.

N. gonorrhoeae is highly efficient at utilizing transferrin-bound iron for *in vitro* growth; many strains can also utilize lactoferrin-bound iron. The bacteria bind only human transferrin and lactoferrin. This specificity is thought to be, in part, the reason these bacteria are exclusively human pathogens.

Strains of *N. gonorrhoeae* produce two distinct extracellular IgA1 proteases which cleave the heavy chain of the human immunoglobulin at different points within the hinge region. Split products of IgA1 have been found in the genital secretions of women with gonorrhea, suggesting that the bacterial IgA1 protease is present and active during genital infection. It is thought that the Fab fragments of IgA1 may bind to the bacterial cell surface and block the Fc-mediated functions other immunoglobulins.

Occasionally, as described above, invading *Neisseria gonorrhoeae* enter the bloodstream causing a Gram-negative bacteremia which may lead to a disseminated bacterial infection. Asymptomatic infections of the urethra or cervix usually serve as focal sources for bacteremia. Strains of *N. gonorrhoeae* that cause disseminated infections are usually resistant to complement and the serum bactericidal reaction. This accounts for their ability to persist during bacteremia. In Gram-negative bacteremias of this sort, the effect of bacterial endotoxin can be exacerbated by the lyis of bacterial cells which may simply liberate soluble LOS.

Treatment. The current CDC Guidelines recommend treatment of all gonococcal infections with antibiotic regimens effective against resistant strains. Currently recommended antimicrobial agents are ceftriaxone, cefixime, ciprofloxacin, or oflaxacin.

Neisseria meningitidis

The bacterium *Neisseria meningitidis,* the **meningococcus,** is identical in its staining and morphological characteristics to *Neisseria gonorrhoeae.* However, at the ultrastructural level, *N. meningitidis* has a prominent antiphagocytic polysaccharide capsule. *N. meningitidis* strains are grouped on the basis of their capsular polysaccharides, into 12 serogroups, some of which are subdivided according to the presence of outer membrane protein and lipopolysaccharide antigens.

Neisseria meningitidis is usually cultivated in a peptone-blood base medium in a moist chamber containing 5-10% CO_2. All media must be warmed to 37 degrees prior to inoculation as the organism is extremely susceptible to

temperatures above or below 37 degrees. This trait is rather unique among bacteria. Also, the organism tends to undergo rapid autolysis after death, both in vitro and in vivo. This accounts for the dissemination of lipopolysaccharide (endotoxin) during septicemia and meningitis.

The organism tends to colonize the posterior nasopharynx of humans, and humans are the only known host. Individuals who are colonized are carriers of the pathogen who can transmit disease to nonimmune individuals. The bacterium also colonizes the posterior nasopharynx in the early stages of infection prior to invasion of the meninges. Most individuals in close contact with a case of meningococcal meningitis become carriers of the organism. This carrier rate can reach 20 percent of the contact group before the first case is recognized, and may reach as high as 80 percent at the height of an epidemic.

Structure and Classification. The only distinguishing structural feature between *N. meningitidis* and *N. gonorrhoeae* is the presence of a polysaccharide capsule in the former. The capsule is antiphagocytic and is an important virulence factor.

Meningococcal capsular polysaccharides provide the basis for grouping the organism. Twelve serogroups have been identified (A, B, C, H, I, K, L, X, Y, Z, 29E, and W135). The most important serogroups associated with disease in humans are A, B, C, Y, and W135. The chemical composition of these capsular polysaccharides is known. The prominent outer membrane proteins of *N. meningitidis* have been designated class 1 through class 5. The class 2 and 3 proteins function as porins and are analogous to gonococcal Por. The class 4 and 5 proteins are analogous to gonococcal Rmp and Opa, respectively. Serogroup B and C meningococci have been further subdivided on the basis of serotype determinants located on the class 2 and 3 proteins. A handful of serotypes are associated with most cases of meningococcal disease, whereas other serotypes within the same serogroup rarely cause disease. All known group A strains have the same protein serotype antigens in the outer membrane. Another serotyping system exists based on the antigenic diversity of meningococcal LOS.

Pathogenesis. Infection with *N. meningitidis* has two presentations, meningococcemia, characterized by skin lesions, and acute bacterial meningitis. The fulminant form of disease (with or without meningitis) is characterized by multisystem involvement and high mortality.

Infection is by aspiration of infective bacteria, which attach to epithelial cells of the nasopharyngeal and oropharyngeal mucosa, cross the mucosal barrier, and enter the bloodstream. If not clear whether blood-borne bacteria may enter the central nervous system and cause meningitis.

The mildest form of disease is a transient bacteremic illness characterized by a fever and malaise; symptoms resolve spontaneously in 1 to 2 days. The most serious form is the fulminant form of disease complicated by meningitis. The manifestations of meningococcal meningitis are similar to acute bacterial meningitis caused by other bacteria such as *Streptococcus pneumoniae, Haemophilus influenzae,* and *E. coli.* Chills, fever, malaise, and headache are the

usual manifestations of infection. Signs of meningeal inflammation are also present.

Clinical manifestations of *N. meningitidis* infection. The onset of meningococcal meningitis may be abrupt or insidious. Infants with meningococcal meningitis rarely display signs of meningeal irritation. Irritability and refusal to take food are typical; vomiting occurs early in the disease and may lead to dehydration. Fever is typically absent in children younger than 2 months of age. Hypothermia is more common in neonates. As the disease progresses, apnea, seizures, disturbances in motor tone, and coma may develop.

In older children and adults, specific symptoms and signs are usually present, with fever and altered mental status the most consistent findings. Headache is an early, prominent complaint and is usually very severe. Nausea, vomiting, and photophobia are also common symptoms.

Neurologic signs are common; approximately one-third of patients have convulsions or coma when first seen by a physician. Signs of meningeal irritation such as spinal rigidity, hamstring spasms and exaggerated reflexes are common.

Petechiae (minute hemorrhagic spots in the skin) or purpura (hemorrhages into the skin) occurs from the first to the third day of illness in 30 to 60% of patients with meningococcal disease, with or without meningitis. The lesions may be more prominent in areas of the skin subjected to pressure, such as the axillary folds, the belt line, or the back.

Fulminant meningococcemia occurs in 5 to 15% of patients with meningococcal disease and has a high mortality rate. It begins abruptly with sudden high fever, chills, myalgias, weakness, nausea, vomiting, and headache. Apprehension, restlessness, and delirium occur within the next few hours. Widespread purpuric and ecchymotic skin lesions appear suddenly. Typically, no signs of meningitis are present. Pulmonary insufficiency develops within a few hours, and many patients die within 24 hours of being hospitalized despite appropriate antibiotic therapy and intensive care.

Virulence Factors. For a time, the virulence of *Neisseria meningitidis* was attributed to the production of an "exotoxin" that could be recovered from culture filtrates of the organism. But when studies revealed that antitoxin reacted equally well with washed cells as culture filtrate, it was realized that the bacteria underwent autolysis during growth and released parts of their cell walls in a soluble form. Hence, the major toxin of *N. meningitidis* is its lipooligosaccharide, LOS, and its mechanism is endotoxic. The other important determinant of virulence of *N. meningitidis* is its antiphagocytic polysaccharide capsule.

The human nasopharynx is the only known reservoir of *N. meningitidis.* Meningococci are spread via respiratory droplets, and transmission requires aspiration of infective particles. Meningococci attach to the nonciliated columnar epithelial cells of the nasopharynx. Attachment is mediated by fimbriae and possibly by other outer membrane components. Invasion of the mucosal cells occurs by a mechanism similar to that observed with gonococci. Events involved after bloodstream invasion are unclear and how the meningococcus enters the central nervous system is not known.

Epidemiology. The meningococcus usually inhabits the human nasopharynx without causing detectable disease. This carrier state may last for a few days to months and is important because it not only provides a reservoir for meningococcal infection but also stimulates host immunity. Between 5 and 30% of normal individuals are carriers at any given time, yet few develop meningococcal disease. Carriage rates are highest in older children and young adults. Attack rates highest in infants 3 months to 1 year old. Meningococcal meningitis occurs both sporadically (mainly groups B and C meningococci) and in epidemics (mainly group A meningococci), with the highest incidence during late winter and early spring. Whenever group A strains become prevalent in the population, the incidence of meningitis increases markedly.

Treatment. Penicillin is the drug of choice to treat meningococcemia and meningococcal meningitis. Although penicillin does not penetrate the normal blood-brain barrier, it readily penetrates the blood-brain barrier when the meninges are acutely inflamed. Either chloramphenicol or a third-generation cephalosporin such as cefotaxime or ceftriaxone is used in persons allergic to penicillins.

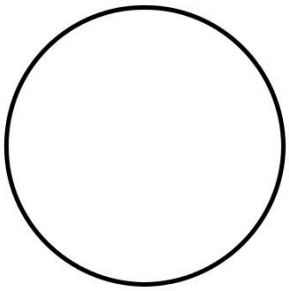
Control. Groups A, C, AC, and ACYW135 capsular polysaccharide vaccines are available.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

Protocol № 35

Theme: Microbiological diagnosis of diseases caused by E. coli

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Escherichia coli
(Gram stain)

II. Study culture and biochemical properties of E. coli:

Growth on MPB	Growth on MPA		Growth on Endo agar	Carbohydrates fermentatiom						
	Slantculture	Plateculture		Glucose	Lactose	Maltose	Mannitol	Sucrose	**Indo**	**H_2S**
Turbidity	Colorles bright	Colorless smooth bright colonies	Red colonies	Acid, gas	Acid, gas	Acid, gas	Acid, gas	-	+	-

IV. Study antigenic properties E. coli.

Strains E. coli that cause infections

Infection	**Serological group**		
	O-Ag	H-Ag	K-Ag
Intestinal			
Enterotoxigenc	O6, O8, O11, O15, O20, 0114, O115, O153,O166 и др.	H4, H7, H9, H11, H12, H19, H20, H28, H40	-
Enteropathogenic	O18, O26, O44, O55, O111, O112 и др.	H2, H6, H7, H11, H12, H14, H18	-
Enteroinvasive	O28, O29, O115, O136, O143 и др.	-	-
Enterohemorrhagic	O26, O157	H6, H7, H8, H11	-
Urinary tract infections	O1, O2, O4, O6, O7, O8, O9, O11, O18 и др.	-	K1, K2, K5, K12, K13
Bacterimia	O1, O2, O4, O6, O7, O8, O9, O11, O18 и др.	-	K1, K2, K5, K12, K15, K23
Meningitidis	O1, O6, O7, O16, O18, O83	-	K1

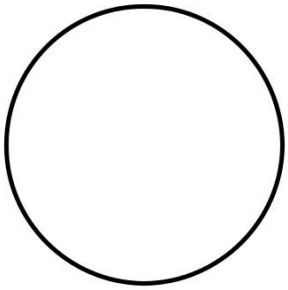
While *Escherichia coli* is **one of the dominant normal flora in the intestinal tract** of humans and animals, **some strains can cause infections of the intestines**.

- **Enterotoxigenc *E. coli* (ETEC)** produce enterotoxins that cause the loss of sodium ions and water from the small intestines resulting in a watery diarrhea. Over half of all travelers' diarrhea is due to ETEC; almost 80,000 cases a year in the U.S.
- **Enteropathogenic *E. coli* (EPEC)** causes an endemic diarrhea in areas of the developing world, especially in infants younger than 6 months. The bacterium disrupts the normal microvilli on the epithelial cells of the small intestines resulting in maladsorbtion and diarrhea.
- **Enteroinvasive *E. coli* (EIEC)** invade and kill epithelial cells of the large intestines causing a dysentery-type syndrome similar to *Shigella* common in underdeveloped countries.
- **Enterohemorrhagic *E. coli* (EHEC)**, such as *E. coli* 0157:H7, produce a shiga-like toxin that kills epithelial cells of the large intestines causing hemorrhagic colitis, a bloody diarrhea. In rare cases, the shiga-toxin enters the blood and is carried to the kidneys where, usually in children, it damages vascular cells and causes hemolytic uremic syndrome. *E. coli* 0157:H7 is thought to cause more than 20,000 infections and up to 250 deaths per year in the U.S.
- **Diffuse aggregative *E. coli* (DAEC)** causes watery diarrhea in infants 1-5 years of age. They stimulate elongation of the microvilli on the epithelial cells lining the small intestines. **Enteroaggregative *E. coli* (EAEC)** is a cause of persistant diarrhea in developing countries. It probably causes diarrhea by adhering to mucosal epithelial cells of the small intestines and interfering with their function.

V. Self-work of students:

A. Isolation of pure culture from "feces of patient with enteric fever":

- study growth on Endo agar: red glistening smooth colonies;
- prepare a smear, stain after Gram, microscopy



Gram stain

B. Seeding "blood of patient with enteric fever" on bile broth (5 ml blood in seering, bile broth (50ml)).

VI. Study the scheme of laboratory diagnosis of E.coli infections.

Specimen: feces.

1 step **Seeding on Endo or MacConkey media**

2 step **culture properties** **slide AT with O-sera**

Gram stain

Seeding on Ressel medium

3 step **Identification: Hiss media,** **Tube AT with O- and K sera**

MPB (protein hydrolysis)

ADDING THEORETICAL MATERIAL

Escherichia coli

Theodor Escherich first described *E. coli* in 1885, which he isolated from the feces of newborns.

The GI tract of most warm-blooded animals is colonized by *E. coli* within hours or a few days after birth. The bacterium is ingested in foods or water or obtained directly from other individuals handling the infant. The human bowel is usually colonized within 40 hours of birth. *E. coli* can adhere to the mucus overlying the large intestine. Once established, an *E. coli* strain may persist for months or years. Resident strains shift over a long period (weeks to months), and more rapidly after enteric infection or antimicrobial chemotherapy that perturbs the normal flora. The basis for these shifts and the ecology of *Escherichia coli* in the intestine of humans are poorly understood despite the vast amount of information on almost every other aspect of the organism's existence. The entire DNA base sequence of the *E. coli* genome has been known since 1997.

E. coli is the head of the large bacterial family, *Enterobacteriaceae*, the enteric bacteria, which are facultatively anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease. The *Enterobacteriaceae* are among the most important bacteria medically. A number of genera within the family are human intestinal pathogens (e.g. *Salmonella*, *Shigella*, *Yersinia*). Several others are normal colonists of the human gastrointestinal tract (e.g. *Escherichia*, *Enterobacter*, *Klebsiella*), but these bacteria, as well, may occasionally be associated with diseases of humans.

Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent. Wild-type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O_2. Under anaerobic conditions it will grow by means of fermentation, producing characteristic "mixed acids and gas" as end products. However, it can also grow by means of anaerobic respiration, since it is able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport processes. In part, this adapts *E. coli* to its intestinal (anaerobic) and its extraintestinal (aerobic or anaerobic) habitats.

E. coli can respond to environmental signals such as chemicals, pH, temperature, osmolarity, etc., in a number of very remarkable ways considering it is a unicellular organism. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Or it can stop swimming and grow fimbriae that will specifically attach it to a cell or surface receptor. In response to change in temperature and osmolarity, it can vary the pore diameter of its outer membrane porins to accommodate larger molecules (nutrients) or to exclude inhibitory substances. With its complex mechanisms for regulation of metabolism the bacterium can survey the chemical contents in its environment in advance of synthesizing any enzymes that metabolize these compounds. It does not wastefully produce enzymes for degradation of carbon

sources unless they are available, and it does not produce enzymes for synthesis of metabolites if they are available as nutrients in the environment.

E. coli is a consistent inhabitant of the human intestinal tract, and it is the predominant facultative organism in the human GI tract; however, it makes up a very small proportion of the total bacterial content. The anaerobic *Bacteroides* species in the bowel outnumber *E. coli* by at least 20:1. however, the regular presence of *E. coli* in the human intestine and feces has led to tracking the bacterium in nature as an indicator of fecal pollution and water contamination. As such, it is taken to mean that, wherever *E. coli* is found, there may be fecal contamination by intestinal parasites of humans.

Escherichia coli in the Gastrointestinal Tract. The commensal *E. coli* strains that inhabit the large intestine of all humans and warm-blooded animals comprise no more than 1% of the total bacterial biomass.

Pathogenesis of *E. coli.* Over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H, and K antigens. At one time serotyping was important in distinguishing the small number of strains that actually cause disease. Thus, the serotype O157:H7 (O refers to somatic antigen; H refers to flagellar antigen) is uniquely responsible for causing HUS (hemolytic uremic syndrome). Nowadays, particularly for diarrheagenic strains (those that cause diarrhea) pathogenic *E. coli* are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic *E. coli* usually requires that the isolates first be identified as *E. coli* before testing for virulence markers.

Pathogenic strains of *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis). The diseases caused (or not caused) by a particular strain of *E. coli* depend on distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses.

Summary of the virulence determinants of pathogenic *E. coli*:

Adhesins CFAI/CFAII Type 1 fimbriae P fimbriae S fimbriae Intimin (non-fimbrial adhesin) EPEC adherence factor	**Invasins** Hemolysin Shigella-like "invasins" for intracellular invasion and spread	**Motility/ chemotaxis** Flagella	**Toxins** LT toxin ST toxin Shiga toxin Cytotoxins Endotoxin (LPS)
Genetic attributes Genetic exchange by transduction and conjugation Transmissible plasmids R factors and drug resistance plasmids Toxin and other virulence plasmids Siderophores and siderophore uptake systems Pathogenicity islands	**Antiphagocytic surface properties** Capsules K antigens LPS	**Defense against serum bactericidal reactions** LPS K antigens	**Defense against immune responses** Capsules K antigens LPS antigenic variation

Urinary Tract Infections. Uropathogenic *E. coli* (UPEC) cause 90% of the urinary tract infections (UTI) in anatomically-normal, unobstructed urinary tracts. The bacteria colonize from the feces or perineal region and ascend the urinary tract to the bladder. Bladder infections are 14-times more common in females than males by virtue of the shortened urethra. The typical patient with uncomplicated cystitis is a sexually-active female who was first colonized in the intestine with a uropathogenic *E. coli* strain. The organisms are propelled into the bladder from the periurethral region during sexual intercourse. With the aid of specific adhesins they are able to colonize the bladder.

The adhesin that has been most closely associated with uropathogenic *E. coli* is the P fimbria (or pyelonephritis-associated pili [PAP]). The letter designation is derived from the ability of P fimbriae to bind specifically to the P blood group antigen which contains a D-galactose-D-galactose residue. The fimbriae bind not only to red cells but to a specific galactose dissaccharide that is found on the surfaces uroepithelial cells in approximately 99% of the population.

The frequency of the distribution of this host cell receptor plays a role in susceptibility and explains why certain individuals have repeated UTI caused by *E. coli*. Uncomplicated *E. coli* UTI virtually never occurs in individuals lacking the receptors.

Uropathogenic strains of *E. coli* possess other determinants of virulence in addition to P fimbriae. *E. coli* with P fimbriae also possess the gene for Type 1 fimbriae, and there is evidence that P fimbriae are derived from Type 1 fimbriae by insertion of a new fimbrial tip protein to replace the mannose-binding domain of Type 1 fimbriae. In any case, Type 1 fimbriae could provide a supplementary mechanism of adherence or play a role in aggregating the bacteria to a specific manosyl-glycoprotein that occurs in urine.

Uropathogenic strains of *E. coli* usually produce siderophores that probably play an essential role in iron acquisition for the bacteria during or after colonization. They also produce hemolysins which are cytotoxic due to formation of transmembranous pores in host cell membranes. One strategy for obtaining iron and other nutrients for bacterial growth may involve the lysis of host cells to release these substances. The activity of hemolysins is not limited to red cells since the alpha-hemolysins of *E. coli* also lyse lymphocytes, and the beta-hemolysins inhibit phagocytosis and chemotaxis of neutrophils.

Another factor thought to be involved in the pathogenicity of the uropathogenic strains of *E. coli* is their resistance to the complement-dependent bactericidal effect of serum. The presence of K antigens is associated with upper urinary tract infections, and antibody to the K antigen has been shown to afford some degree of protection in experimental infections. The K antigens of *E.coli* are "capsular" antigens that may be composed of proteinaceous organelles associated with colonization (e.g., CFA antigens), or made of polysaccharides. Regardless of their chemistry, these capsules may be able to promote bacterial virulence by decreasing the ability of antibodies and/or complement to bind to the bacterial surface, and the ability of phagocytes to recognize and engulf the bacterial cells. The best studied K antigen, K-1, is composed of a polymer of N-acetyl neuraminic

acid (sialic acid), which besides being antiphagocytic, has the additional property of being an antigenic disguise.

Neonatal Meningitis. It affects 1/2,000-4,000 infants. Eighty percent of *E. coli* strains involved synthesize K-1 capsular antigens (K-1 is only present 20-40% of the time in intestinal isolates).

E. coli strains invade the blood stream of infants from the nasopharynx or GI tract and are carried to the meninges.

The K-1 antigen is considered the major determinant of virulence among strains of *E. coli* that cause neonatal meningitis. K-1 is a homopolymer of sialic acid. It inhibits phagocytosis, complement, and responses from the host's immunological mechanisms. K-1 may not be the only determinant of virulence, however, as siderophore production and endotoxin are also likely to be involved.

Neonatal meningitis requires antibiotic therapy that usually includes ampicillin and a third-generation cephalosporin.

Intestinal Diseases Caused by *E. coli.* As a pathogen, *E. coli* is best known for its ability to cause intestinal diseases. Five classes (virotypes) of *E. coli* that cause diarrheal diseases are now recognized: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E.coli* (EAEC). Each class falls within a serological subgroup and manifests distinct features in pathogenesis.

Enterotoxigenic *E. coli* (ETEC). ETEC is an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. In the U.S., it has been implicated in sporadic waterborne outbreaks, as well as due to the consumption of soft cheeses, Mexican-style foods and raw vegetables. The diseases vary from minor discomfort to a severe cholera-like syndrome. ETEC are acquired by ingestion of contaminated food and water, and adults in endemic areas evidently develop immunity. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded.

ETEC may produce a heat-labile enterotoxin (LT) that is similar in molecular size, sequence, antigenicity, and function to the cholera toxin (Ctx). It is an 86kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors that are recognized by the cholera toxin (i.e., GM1), and its enzymatic activity is identical to that of the cholera toxin.

ETEC may also produce a heat-stable toxin (ST) that is of low molecular size and resistant to boiling for 30 minutes. There are several variants of ST, of which ST1a or STp is found in *E. coli* isolated from both humans and animals, while ST1b or STh is predominant in human isolates only. The ST enterotoxins are peptides of molecular weight about 4,000 daltons. Their small size explains why they are not inactivated by heat. ST causes an increase in cyclic GMP in host cell cytoplasm leading to the same effects as an increase in cAMP. ST1a is known to act by binding to a guanylate cyclase that is located on the apical membranes of host cells, thereby activating the enzyme. This leads to secretion of fluid and electrolytes resulting in diarrhea.

The infective dose of ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly and the infirm may be susceptible to lower numbers.

ETEC adhesins are fimbriae which are species-specific. For example, the K-88 fimbrial Ag is found on strains from piglets; K-99 Ag is found on strains from calves and lambs; CFA I, and CFA II, are found on strains from humans. These fimbrial adhesins adhere to specific receptors on enterocytes of the proximal small intestine.

Symptoms ETEC infections include diarrhea without fever. The bacteria colonize the GI tract by means of a fimbrial adhesin, e.g. CFA I and CFA II, and are noninvasive, but produce either the LT or ST toxin.

Enteroinvasive *E. coli* (EIEC). EIEC closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes a dysentery-like diarrhea with fever. EIEC apparently lack fimbrial adhesins but do possess a specific adhesin that, as in *Shigella*, is thought to be an outer membrane protein. Also, like *Shigella*, EIEC are invasive organisms. They do not produce LT or ST toxin.

The primary source for EIEC appears to be infected humans. Although the infective dose of *Shigella* is low (in the range of 10 to few hundred cells), volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults. Unlike typical *E.coli*, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose. Pathogenicity of EIEC is primarily due to its ability to invade and destroy colonic tissue. The invasion phenotype, encoded by a high molecular weight plasmid, can be detected by PCR and probes for specific for invasion genes.

Enteropathogenic *E. coli* (EPEC). EPEC induce a profuse watery, sometimes bloody, diarrhea. They are a leading cause of infantile diarrhea in developing countries. Outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Pathogenesis of EPEC involves a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells and a non fimbrial adhesin designated intimin, which is an outer membrane protein that mediates the final stages of adherence. They do not produce ST or LT toxins.

Adherence of EPEC strains to the intestinal mucosa is a very complicated process and produces dramatic effects in the ultrastructure of the cells resulting in rearrangements of actin in the vicinity of adherent bacteria. The phenomenon is sometimes called "attachment and effacing" of cells. EPEC strains are said to be "moderately-invasive", meaning they are not as invasive as *Shigella,* and unlike ETEC or EAEC, they cause an inflammatory response. The diarrhea and other symptoms of EPEC infections probably are caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins. Through volunteer feeding studies the infectious dose of EPEC in healthy adults has been estimated to be 10^6 organisms.

Some types of EPEC are referred to as diffusely adherent *E. coli* (DAEC), based on specific patterns of adherence. They are an important cause of traveler's diarrhea in Mexico and in North Africa.

Enteroaggregative *E. coli* (EAEC). The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. This suggests that the organisms produce an enterotoxin of some sort. Recently, a distinctive heat-labile plasmid-encoded toxin has been isolated from these strains, called the EAST (EnteroAggregative ST) toxin. They also produce a hemolysin related to the hemolysin produced by *E. coli* strains involved in urinary tract infections. The role of the toxin and the hemolysin in virulence has not been proven. The significance of EAEC strains in human disease is controversial.

Enterohemorrhagic *E. coli* (EHEC). EHEC are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS). EHEC are characterized by the production of verotoxin or Shiga toxins(Stx). Although Stx1 and Stx2 are most often implicated in human illness, several variants of Stx2 exist.

There are many serotypes of Stx-producing *E. coli*, but only those that have been clinically associated with HC are designated as EHEC. Of these, O157:H7 is the prototypic EHEC and most often implicated in illness worldwide. The infectious dose for O157:H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes. EHEC infections are mostly food or water borne and have implicated undercooked ground beef, raw milk, cold sandwiches, water, unpasteurized apple juice and vegetables.

EHEC are considered to be "moderately invasive". Nothing is known about the colonization antigens of EHEC but fimbriae are presumed to be involved. The bacteria do not invade mucosal cells as readily as *Shigella*, but EHEC strains produce a toxin that is virtually identical to the Shiga toxin. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause HUS. The toxin is phage encoded and its production is enhanced by iron deficiency.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

Diarrheagenic *E. coli* - virulence determinants and characteristics of disease:

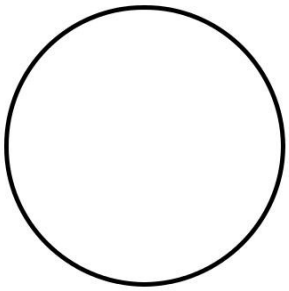
ETEC	**EIEC**	**EPEC**	**EAEC**	**EHEC**
- fimbrial adhesins e.g. CFA I, CFAII, K88. K99 - non invasive - produce LT and/or ST toxin - watery diarrhea in infants and travelers; no inflammation, no fever	- nonfimbrial adhesins, possibly outer membrane protein - invasive (penetrate and multiply within epithelial cells) - does not produce shiga toxin - dysentery-like diarrhea (mucous, blood), severe inflammation, fever	- non fimbrial adhesin (intimin) EPEC adherence factor (EAF) enables localized adherence of bacteria to intestinal cells - moderately invasive (not as invasive as *Shigella* or EIEC) - does not produce LT or ST; some reports of shiga-like toxin - usually infantile diarrhea; watery diarrhea with blood, some inflammation, no fever; symptoms probably result mainly from invasion rather than toxigenesis	- adhesins not characterized - non invasive produce ST-like toxin (EAST) and a hemolysin - persistent diarrhea in young children without inflammation or fever	- adhesins not characterized, probably fimbriae - moderately invasive - does not produce LT or ST but does produce shiga toxin - pediatric diarrhea, copious bloody discharge (hemorrhagic colitis), intense inflammatory response, may be complicated by hemolytic uremia

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

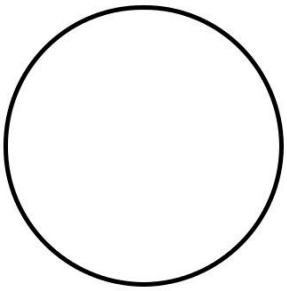
Protocol № 36

The theme: Microbiological diagnosis of diseases caused by Salmonella.

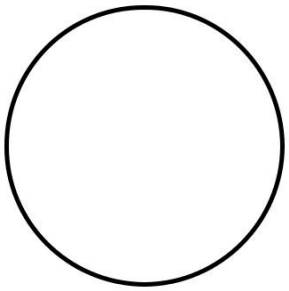
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



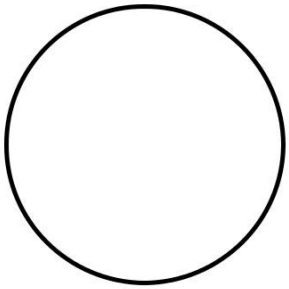
Salmonella typhi (Gram stain)



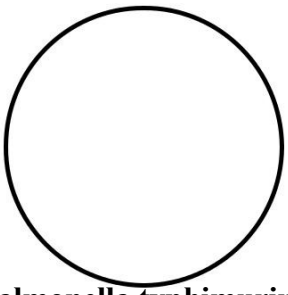
Salmonella paratyphi A (Gram stain)



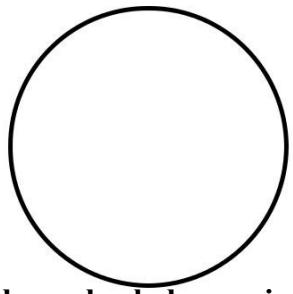
Salmonella paratyphi B (Gram stain)



Salmonella enteritidis (Gram stain)



Salmonella typhimurium (Gram stain)



Salmonela choleraesuis (Gram stain)

II. Study culture and biochemical properties of Salmonella:

a) growth on MPA: _____;
b) growth on Endo agar: _____;
c) growth on Ressel agar: _____;
d) indol production: _____;
g) H_2S production: _____.

III. Study antigenic structure of salmonella (Caufmann and Wait scheme):

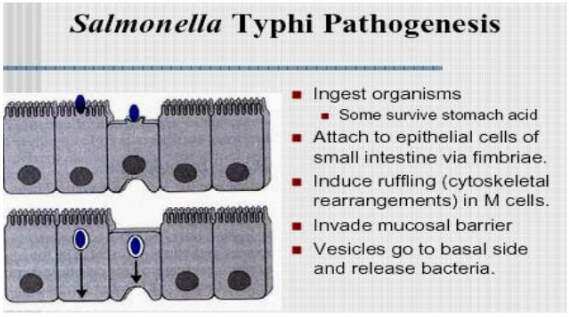
Species	S antigen	H antigen	
		1st phase	2nd phase
Group A		a, b	-
S. paratyphi A	1, 2, 12		
Group B			
S. paratyphi B	1, 4, 5, 12	-	e, n, x
S. abortus ovis	4, 12	c	1, 6
S. typhimurium	1, 4, 5, 12	i	1, 2
Group C			
S. paratyphic	6, 7, Vi	c	1, 5
S. cholerae suis	6, 7	c	1, 5
S. newport	6, 8	eh	1, 2
S. dusseldorf	6, 8	z4, z24	-
Group D			
S. typhi	9, 12Vi	d	-
S. enteritidis	1, 9, 12	g, m	-
S. moscow	9, 12	gq	-

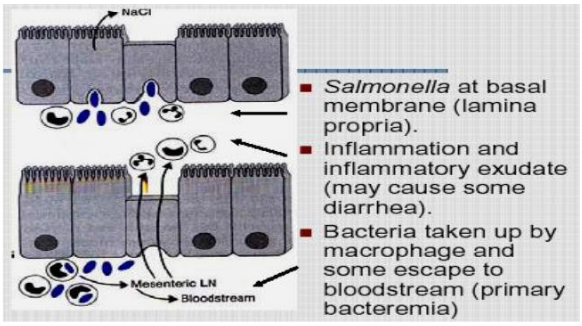
Group E S. anatum S. Cambridge	 3, 10 3, 15	 eh eh	 1, 6 i, w

IV. Study biochemical properties of Salmonella typhi and paratyphi A and B:

	Glucose	**Lactose**	**Maltose**	**Mannitol**	**Sucrose**	**Indol**	**H_2S**
S.typhi	Acid	_	Acid	Acid	_	_	+
S.paratyphi A	Acid, gas	_	Acid, gas	Acid, gas	_	_	_
S.paratyphi B	Acid, gas	_	Acid, gas	Acid, gas	_	_	+

V. Study Salmonella typhi pathogenesis:





VI. Study the scheme of laboratory diagnosis of enteric fever.

Specimen: blood (1st week), feces (from 5th day).

1 step **Seeding on Endo or MacConkey media**

2 step **culture properties** **slide AT with O-sera and H-sera**

Gram stain

Seeding on Ressel medium

3 step **Identification: Hiss media (sugars fermentation), MPB (protein hydrolysis)**

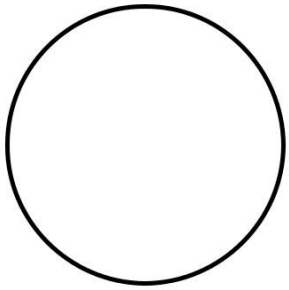
Serology (from 2nd week):

1. Tube agglutination test (Widal test) with O- and H-antigens to diagnose acute infection.
2. Passive hemagglutination test (PHAT) with Vi-antigen to diagnose convalescence and carrier state.

V. Self-work of students:

A. Continue of isolation of pure culture from "feces of patient with enteric fever":

- study growth on Ressel agar: there is no change of color of medium;
- prepare a smear of lactose negative culture, stain after Gram, microscopy:

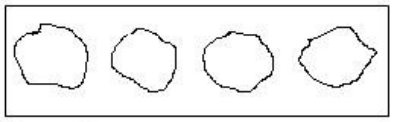


Gram stain

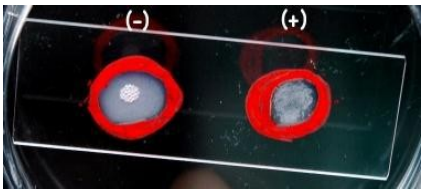
S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

- perform slide agglutination test for differentiation of Salmonella:

1. On a clean glass slide, draw two circles (1 cm in diameter) heavily with a red wax marker.
2. Place the slide in an empty Petri dish.
3. With a Pasteur pipette place a drop of the cell suspension within each circle. Make sure the drops appear as even, cloudy suspensions.
4. Without touching the dropper to the cells, place a drop of the anti-O4 antiserum on one suspension and a drop of anti-O9 on the other.
5. After several minutes, hold up the Petri dish and observe the slide from the bottom. Gently tap the dish to effect some mixing of the cell suspension. Where there is a reaction between antibodies in the antiserum and their homologous antigens on the cell wall of the bacteria, the cells will agglutinate, and the drop will appear to contain many small particles. Where there is no agglutination, the cell suspension will maintain its original, evenly-cloudy (grainy) appearance.
6. Discard the slide appropriately (or clean it off for reuse) and discard the Petri plate into the proper container. (Do not place glass petri plates into the pails with the disposable plastics!)



For S.typhi test is positive with Salmonella group, O-9 and Hd sera.



Serological reactions for *Salmonella.* The negative reaction (-). The positive reaction (+).

- seeding on Hiss media and MPA for detection indol and H_2S

production. **B.** Result of growth on bile broth: turbidity.

VI. Study ingredients and scheme of Widal test

Ingrediantds:

Patient's serum, dilution 1:50;
Diagnosticums (antigens) O and H (S.typhi, S.paratyphi A, S.paratyphi B);
Physiological solution.

Scheme of Widal test

Row	Ingredient	Serum dilution				
		1:100	1:200	1:400	1:800	Contol
1	Physiological solution	1,0	1,0	1,0	1,0	1,0
	Patient's serum (1:50)	1,0 →	1,0 →	1,0 →	1,0 →	
	S.typhi (OH)	3 drops	3 drops	3 drops	3 drops	3 drops
2	Physiological solution	1,0	1,0	1,0	1,0	1,0
	Patient's serum (1:50)	1,0 →	1,0 →	1,0 →	1,0 →	
	S.typhi (O)	3 drops	3 drops	3 drops	3 drops	3 drops
3	Physiological solution	1,0	1,0	1,0	1,0	1,0
	Patient's serum (1:50)	1,0 →	1,0 →	1,0 →	1,0 →	
	S.paratyphi A	3 drops	3 drops	3 drops	3 drops	3 drops
4	Physiological solution	1,0	1,0	1,0	1,0	1,0
	Patient's serum (1:50)	1,0 →	1,0 →	1,0 →	1,0 →	
	S.paratyphi B	3 drops	3 drops	3 drops	3 drops	3 drops

Surname _____ Date _____

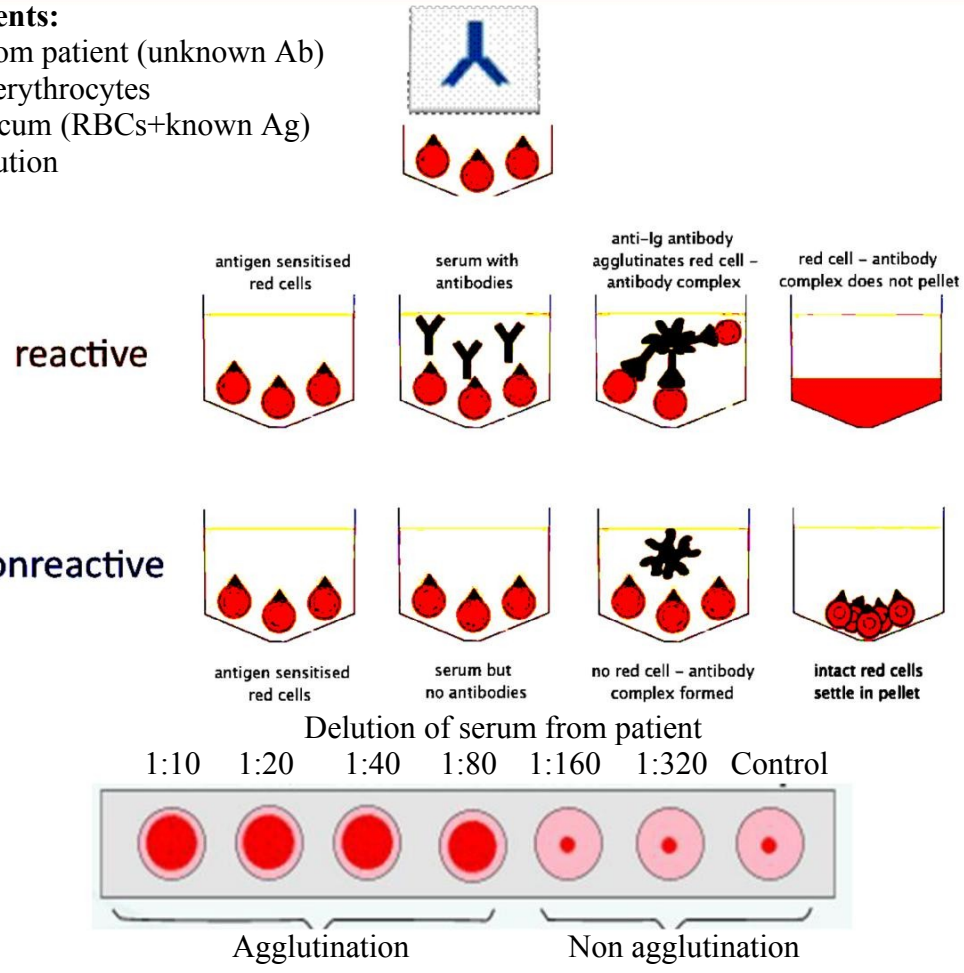
VII. Study results of Widal test

Antigenes	Serum dilution															
	1:100	1:200	1:400	1:800	1:100	1:200	1:400	1:800	1:100	1:200	1:400	1:800	1:100	1:200	1:400	1:800
S.typhi OH	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	-
S.typhi O	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-
S.paratyphi A OH	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
S.paratyphi A H	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
S.paratyphi B OH	-	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-
S.paratyphi B O	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Type of reaction	Infection				Postvaccination				Immunity (enteric fever)				Need to repeat			

VIII. Study scheme and principle of passive Vi-hemagglutination test with erythrocytic diagnosticum for enteric fever.

I. Components:

1. Serum from patient (unknown Ab)
2. Specific erythrocytes diagnosticum (RBCs+known Ag)
3. NaCl solution



IX. Therapy of enteric fever: a third-generation cephalosporin: cefixime or ceftriaxone**;** aminoglycosides; fluroquinolones: ciprofloxacine, lomefloxacin, moxifloxacin, levofloxacin.

ADDING THEORETICAL MATERIAL

Salmonella is a Gram-negative facultative rod-shaped bacterium in the same proteobacterial family as *Escherichia coli,* the family *Enterobacteriaceae,* trivially known as "enteric" bacteria. *Salmonella* is nearly as well-studied as *E. coli* from a structural, biochemical and molecular point of view, and as poorly understood as *E. coli* from an ecological point of view. Salmonellae live in the intestinal tracts of warm and cold blooded animals. Some species are ubiquitous. Other species are specifically adapted to a particular host. In humans, *Salmonella* are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a foodborne infection/intoxication.

Salmonella nomenclature. The genus *Salmonella* is a member of the family *Enterobacteriaceae.* It is composed of bacteria related to each other both phenotypically and genotypically. *Salmonella* DNA base composition is 50-52 mol % G+C, similar to that of *Escherichia, Shigella,* and *Citrobacter.* The bacteria of the genus *Salmonella* are also related to each other by DNA sequence.

Salmonella nomenclature has been controversial since the original taxonomy of the genus was not based on DNA relatedness, rather names were given according to clinical considerations, e.g., *Salmonella typhi, Salmonella choleraesuis, Salmonella abortus-ovis,* and so on. When serological analysis was adopted into the Kauffmann-White scheme in 1946, a *Salmonella* species was defined as "a group of related fermentation phage-type" with the result that each *Salmonella* serovar was considered as a species. Since the host-specificity suggested by some of these earlier names does not exist (e.g., *S. typhi-murium, S. cholerae-suis* are in fact ubiquitous), names derived from the geographical origin of the first isolated strain of the newly discovered serovars were next chosen, e.g., *S. london, S. panama, S. stanleyville.*

Antigenic Structure. As with all *Enterobacteriaceae,* the genus *Salmonella* has three kinds of major antigens with diagnostic or identifying applications: somatic, surface, and flagellar.

Somatic (O) or Cell Wall Antigens. Somatic antigens are heat stable and alcohol resistant. Cross-absorption studies individualize a large number of antigenic factors, 67 of which are used for serological identification. O factors labeled with the same number are closely related, although not always antigenically identical.

Surface (Envelope) Antigens. Surface antigens, commonly observed in other genera of enteric bacteria (e.g., *Escherichia coli* and *Klebsiella),* may be found in some *Salmonella* serovars. Surface antigens in *Salmonella* may mask O antigens, and the bacteria will not be agglutinated with O antisera. One specific surface antigen is well known: the Vi antigen. The Vi antigen occurs in only three *Salmonella* serovars (out of about 2,200): Typhi, Paratyphi C, and Dublin. Strains of these three serovars may or may not have the Vi antigen.

Flagellar (H) Antigens. Flagellar antigens are heat-labile proteins. Mixing salmonella cells with flagella-specific antisera gives a characteristic pattern of

agglutination (bacteria are loosely attached to each other by their flagella and can be dissociated by shaking). Also, antiflagellar antibodies can immobilize bacteria with corresponding H antigens.

A few *Salmonella enterica*serovars (e.g., Enteritidis, Typhi) produce flagella which always have the same antigenic specificity. Such an H antigen is then called monophasic. Most *Salmonella* serovars, however, can alternatively produce flagella with two different H antigenic specificities. The H antigen is then called diphasic. For example, Typhimurium cells can produce flagella with either antigen i or antigen 1,2. If a clone is derived from a bacterial cell with H antigen i, it will consist of bacteria with i flagellar antigen. However, at a frequency of 10^{-3}- 10^{-5}, bacterial cells with 1,2 flagellar antigen pattern will appear in this clone.

Habitats. The principal habitat of the salmonellae is the intestinal tract of humans and animals. *Salmonella* serovars can be found predominantly in one particular host, can be ubiquitous, or can have an unknown habitat. Typhi and Paratyphi A are strictly human serovars that may cause grave diseases often associated with invasion of the bloodstream. Salmonellosis in these cases is transmitted through fecal contamination of water or food. Gallinarum, Abortusovis, and Typhisuis are, respectively, avian, ovine, and porcine *Salmonella* serovars. Such host-adapted serovars cannot grow on minimal medium without growth factors (contrary to the ubiquitous *Salmonella* serovars).

Ubiquitous (non-host-adapted) *Salmonella* serovars (e.g., Typhimurium) cause very diverse clinical symptoms, from asymptomatic infection to serious typhoid-like syndromes in infants or certain highly susceptible animals (mice). In human adults, ubiquitous *Salmonella* organisms are mostly responsible for foodborne toxic infections.

The pathogenic role of a number of *Salmonella* serovars is unknown. This is especially the case with serovars from subspecies II to VI. A number of these serovars have been isolated rarely (some only once) during a systematic search in cold-blooded animals.

Salmonella in the Natural Environment. Salmonellae are disseminated in the natural environment (water, soil, sometimes plants used as food) through human or animal excretion. Humans and animals (either wild or domesticated) can excrete *Salmonella* either when clinically diseased or after having had salmonellosis, if they remain carriers. *Salmonella* organisms do not seem to multiply significantly in the natural environment (out of digestive tracts), but they can survive several weeks in water and several years in soil if conditions of temperature, humidity, and pH are favorable.

Isolation and Identification of *Salmonella.* A number of plating media have been devised for the isolation of *Salmonella.* Some media are differential and nonselective, i.e., they contain lactose with a pH indicator, but do not contain any inhibitor for non salmonellae (e.g., bromocresol purple lactose agar). Other media are differential and slightly selective, i.e., in addition to lactose and a pH indicator, they contain an inhibitor for nonenterics (e.g., MacConkey agar and eosin-methylene blue agar).

The most commonly used media selective for *Salmonella* are SS agar, bismuth sulfite agar, Hektoen enteric (HE) medium, brilliant green agar and xylose-lisine-deoxycholate (XLD) agar. All these media contain both selective and differential ingredients and they are commercially available.

Media used for *Salmonella* identification are those used for identification of all *Enterobacteriaceae.* Most *Salmonella* strains are motile with peritrichous flagella, however, nonmotile variants may occur occasionally. Most strains grow on nutrient agar as smooth colonies, 2-4 mm in diameter. Most strains are prototrophs, not requiring any growth factors. However, auxotrophic strains do occur, especially in host-adapted serovars such as Typhi and Paratyphi A.

Characteristics shared by most *Salmonella* strains belonging to subspecies I:

- Motile, Gram-negative bacteria
- Lactose negative; acid and gas from glucose, mannitol, maltose, and sorbitol; no Acid from adonitol, sucrose, salicin, lactose
- ONPG test negative (lactose negative)
- Indole test negative
- Methyl red test positive
- Voges-Proskauer test negative
- Citrate positive (growth on Simmon's citrate agar)
- Lysine decarboxylase positive
- Urease negative
- Ornithine decarboxylase positive
- H_2S produced from thiosulfate
- Do not grow with KCN
- Phenylalanine and tryptophan deaminase negative
- Gelatin hydrolysis negative.

Pathogenesis of *Salmomella* Infections in Humans. *Salmonella* infections in humans vary with the serovar, the strain, the infectious dose, the nature of the contaminated food, and the host status. Certain serovars are highly pathogenic for humans; the virulence of rather rare serovars is unknown. Strains of the same serovar are also known to differ in their pathogenicity. An oral dose of at least 10^5 *Salmonella typhi* cells are needed to cause typhoid in 50% of human volunteers, whereas at least 10^9 *S. typhimurium* cells (oral dose) are needed to cause symptoms of a toxic infection. Infants, immunosuppressed patients, and those affected with blood disease are more susceptible to *Salmonella* infection than healthy adults.

In the pathogenesis of typhoid the bacteria enter the human digestive tract, penetrate the intestinal mucosa (causing no lesion), and are stopped in the mesenteric lymph nodes. There, bacterial multiplication occurs, and part of the bacterial population lyses. From the mesenteric lymph nodes, viable bacteria and LPS (endotoxin) may be released into the bloodstream resulting in septicemia.

Salmonella excretion by human patients may continue long after clinical cure. Asymptomatic carriers are potentially dangerous when unnoticed. About 5% of patients clinically cured from typhoid remain carriers for months or even years. Antibiotics are usually ineffective on *Salmonella* carriage (even if salmonellae are

susceptible to them) because the site of carriage may not allow penetration by the antibiotic.

Salmonellae survive sewage treatments if suitable germicides are not used in sewage processing. In a typical cycle of typhoid, sewage from a community is directed to a sewage plant. Effluent from the sewage plant passes into a coastal river where edible shellfish (mussels, oysters) live. Shellfish concentrate bacteria as they filter several liters of water per hour. Ingestion by humans of these seafoods (uncooked or superficially cooked) may cause typhoid or other salmonellosis. Salmonellae do not colonize or multiply in contaminated shellfish.

Typhoid is strictly a human disease. The incidence of human disease decreases when the level of development of a country increases (i.e., controlled water sewage systems, pasteurization of milk and dairy products). Where these hygienic conditions are missing, the probability of fecal contamination of water and food remains high and so is the incidence of typhoid.

Foodborne *Salmonella* toxic infections are caused by ubiquitous *Salmonella* serovars (e.g., Typhimurium). About 12-24 hours following ingestion of contaminated food (containing a sufficient number of *Salmonella*), symptoms appear (diarrhea, vomiting, fever) and last 2-5 days. Spontaneous cure usually occurs.

Salmonella may be associated with all kinds of food. Contamination of meat (cattle, pigs, goats, chicken, etc.) may originate from animal salmonellosis, but most often it results from contamination of muscles with the intestinal contents during evisceration of animals, washing, and transportation of carcasses. Surface contamination of meat is usually of little consequence, as proper cooking will sterilize it (although handling of contaminated meat may result in contamination of hands, tables, kitchenware, towels, other foods, etc.). However, when contaminated meat is ground, multiplication of *Salmonella* may occur within the ground meat and if cooking is superficial, ingestion of this highly contaminated food may produce a *Salmonella* infection. Infection may follow ingestion of any food that supports multiplication of *Salmonella* such as eggs, cream, mayonnaise, creamed foods, etc.), as a large number of ingested salmonellae are needed to give symptoms. Prevention of *Salmonella* toxic infection relies on avoiding contamination (improvement of hygiene), preventing multiplication of *Salmonella* in food (constant storage of food at 4°C), and use of pasteurized and sterilized milk and milk products. Vegetables and fruits may carry *Salmonella* when contaminated with fertilizers of fecal origin, or when washed with polluted water.

The incidence of foodborne *Salmonella* infection/toxication remains reletavely high in developed countries because of commercially prepared food or ingredients for food. Any contamination of commercially prepared food will result in a large-scale infection. In underdeveloped countries, foodborne *Salmonella* intoxications are less spectacular because of the smaller number of individuals simultaneously infected, but also because the bacteriological diagnosis of *Salmonella* toxic infection may not be available. However, the incidence of *Salmonella* carriage in underdeveloped countries is known to be high.

Salmonella epidemics may occur among infants in pediatric wards. The frequency and gravity of these epidemics are affected by hygienic conditions, malnutrition, and the excessive use of antibiotics that select for multiresistant strains.

Salmonella enteritidis Infection. Egg-associated salmonellosis is an important public health problem in the United States and several European countries. *Salmonella enteritidis*, can be inside perfectly normal-appearing eggs, and if the eggs are eaten raw or undercooked, the bacterium can cause illness.

Most types of *Salmonella* live in the intestinal tracts of animals and birds and are transmitted to humans by contaminated foods of animal origin. Stringent procedures for cleaning and inspecting eggs were implemented in the 1970s and have made salmonellosis caused by external fecal contamination of egg shells extremely rare. However, unlike eggborne salmonellosis of past decades, the current epidemic is due to intact and disinfected grade A eggs. The reason for this is that *Salmonella enteritidis* silently infects the ovaries of hens and contaminates the eggs before the shells are formed.

A person infected with the *Salmonella enteritidis* usually has fever, abdominal cramps, and diarrhea beginning 12 to 72 hours after consuming a contaminated food or beverage. The illness usually lasts 4 to 7 days, and most persons recover without antibiotic treatment. However, the diarrhea can be severe, and the person may be ill enough to require hospitalization. The elderly, infants, and those with impaired immune systems (including HIV) may have a more severe illness. In these patients, the infection may spread from the intestines to the bloodstream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics.

Exotoxins. *Salmonella* strains may produce a thermolabile enterotoxin that bears a limited relatedness to cholera toxin both structurally and antigenically. This enterotoxin causes water secretion in rat ileal loop and is recognized by antibodies against both cholera toxin and the thermolabile enterotoxin (LT) of enterotoxinogenic *E. coli,* but it does not bind in vitro to ganglioside GM1 (the receptor for E. coli LT and cholera ctx). Additionally, a cytotoxin that inhibits protein synthesis and is immunologically distinct from Shiga toxin has been demonstrated. Both of these toxins are presumed to play a role in the diarrheal symptoms of salmonellosis.

Antibiotic Susceptibility. During the last decade, antibiotic resistance and multiresistance of *Salmonella* spp. have increased a great deal. The cause appears to be the increased and indiscriminate use of antibiotics in the treatment of humans and animals and the addition of growth-promoting antibiotics to the food of breeding animals. Plasmid-borne antibiotic resistance is very frequent among *Salmonella* strains involved in pediatric epidemics (e.g., Typhimurium, Panama, Wien, Infantis). Resistance to ampicillin, streptomycin, kanamycin, chloramphenicol, tetracycline, and sulfonamides is commonly observed. Colistin resistance has not yet been observed.

Vaccination Against Typhoid Fever. Three types of typhoid vaccines are currently available for use: (1) an oral live-attenuated vaccine; (2) a parenteral

heat-phenol-inactivated vaccine; (3) a newly licensed capsular polysaccharide vaccine for parenteral use. A fourth vaccine, an acetone-inactivated parenteral vaccine, is currently available only to the armed forces.

NOTE: No typhoid vaccine is 100% effective and is not a substitute for being careful about what you eat or drink.

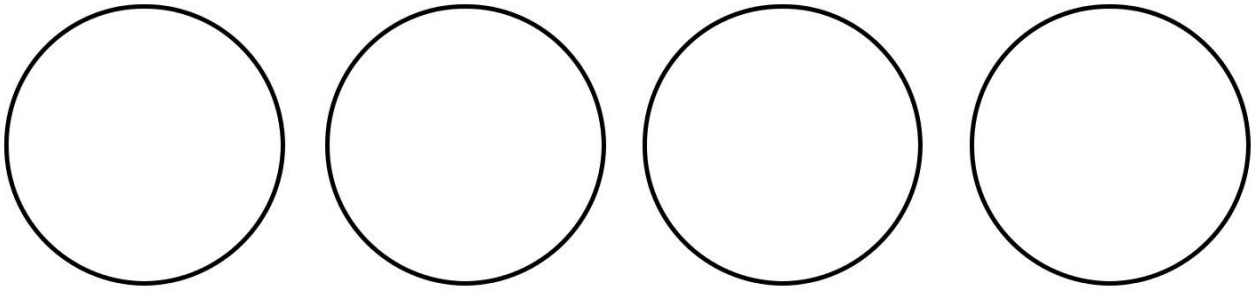
Routine typhoid vaccination is not recommended, but typhoid vaccine is recommended for travellers to parts of the world where typhoid is common, people in close contact with a typhoid carrier, and laboratory workers who work with *Salmonella typhi* bacteria.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

Protocol № 37

The theme: Microbiological diagnosis of shigellosis.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Shigella dysenteriae (Gram stain) | **Shigella flexneri (Gram stain)** | **Shigella boydii (Gram stain)** | **Shigella sonnei (Gram stain)**

II. Study culture and biochemical properties of Shigella:

a) growth on MPA: _____*;* and MPB: _____;

b) growth on Endo agar: _____*;*

c) growth on Ressel agar: _____;

d) indol production: Shigella do not produce indol exept _____;

g) H_2S production: _____.

Biochemical properties of Shigella

Species	Carbohydrates fermantation					Indol production	Toxinproduction
	Glucose	Mannitol	Dulcit	Lactose	Sucrose		
S.dysenteriae	+	-	-	-	-	-	+
S.flexneri	+	+	+	-	-	±	-
S.boydii	+	+	+	-	-	-	-
S.sonnei	+	+	-	+ (slowly)	+ (slowly)	±	-

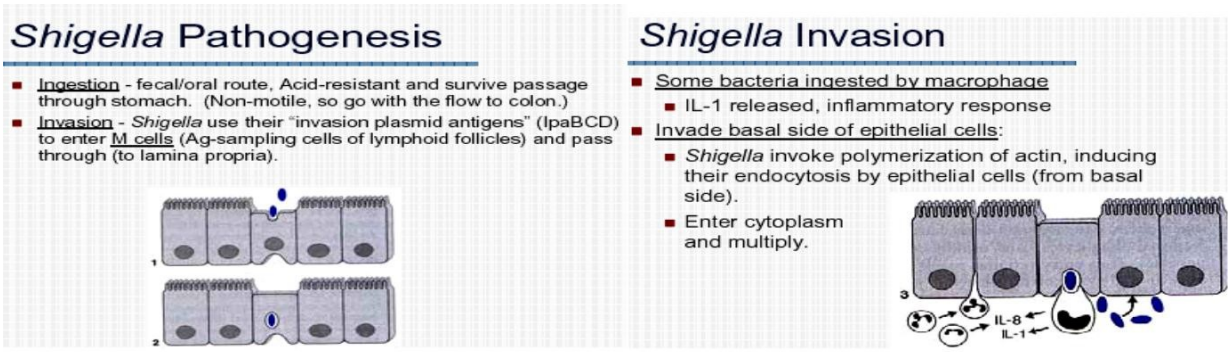
III. Study antigenic properties of Shigella.

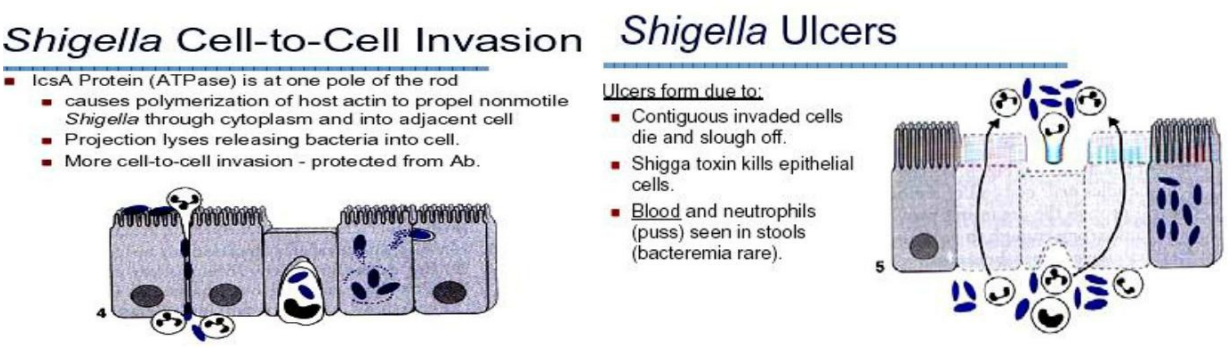
Classification of genus Shigella

Subgroup	Species	Serovar	Subserovar
A	S. dysenteriae	1-10	-
B	S. flexneri	1 2 3 4 5 6	1a, 1b 2a, 2b 3a, 3b 4a, 4b 5a, 5b -
C	S. boydii	1-15	-
D	S. sonnei	-	-

Surname _ Date _ _ _ _ _ _ _ _ _ _ _

IV. Study Shigella pathogenesis:





V. Self-work of students: isolation of pure culture from "feces of patient with cholera" on 1% basdic peptone water and agar.

VI. Study the scheme of laboratory diagnosis of dysentery.
Specimen: feces.

1 step **Seeding on Endo or MacConkey media**

2 step **culture properties** **slide AT with O-sera**
Gram stain
Seeding on Ressel medium

3 step **Identification: Hiss media (sugars fermentation), MPB (protein hydrolysis)**

ADDING THEORETICAL MATERIAL

Symptoms of shigellosis include abdominal pain, tenesmus, watery diarrhea, and/or dysentery (multiple scanty, bloody, mucoid stools). Other signs may include abdominal tenderness, fever, vomiting, dehydration, and convulsions.

Structure, Classification, and Antigenic Types. Shigellae are Gram-negative, nonmotile, facultatively anaerobic, non-spore-forming rods. *Shigella* are differentiated from the closely related *Escherichia coli* on the basis of pathogenicity (dysenteric stool is scant and contains blood, mucus, and inflammatory cells), physiology (failure to ferment lactose or decarboxylate lysine) and serology. The genus is divided into four serogroups with multiple serotypes: A

(*S.dysenteriae*, 12 serotypes); B (*S. flexneri*, 6 serotypes); C (*S. boydii*, 18 serotypes); and D (*S.sonnei*, 1 serotype).

Pathogenesis. Infection is initiated by ingestion of shigellae (usually via fecal-oral contamination). An early symptom, diarrhea (possibly elicited by enterotoxins and/or cytotoxin), may occur as the organisms pass through the small intestine. The hallmarks of shigellosis are bacterial invasion of the colonic epithelium and inflammatory colitis. These are interdependent processes amplified by local release of cytokines and by the infiltration of inflammatory elements. Colitis in the rectosigmoid mucosa, with concomitant malabsorption, results in the characteristic sign of bacillary dysentery: scanty, unformed stools tinged with blood and mucus, and/or febrile diarrhea.

Clinical Presentation. Shigellosis has two basic clinical presentations: (1) watery diarrhea associated with vomiting and mild to moderate dehydration, and (2) dysentery characterized by a small volume of bloody, mucoid stools, and abdominal pain (cramps and tenesmus). Possible complications of shigellosis include bacteremia, convulsions and other neurological complications, reactive arthritis, and hemolytic-uremic syndrome.

Host Defenses. Inflammation, copious mucus secretion, and regeneration of the damaged colonic epithelium limit the spread of colitis and promote spontaneous recovery. Serotype-specific immunity is induced by a primary infection, suggesting a protective role of antibody recognizing the lipopolysaccharide (LPS) somatic antigen. Other *Shigella* antigens include enterotoxins, cytotoxin, and plasmid-encoded proteins that induce bacterial invasion of the epithelium.

Toxins. Enterotoxins designated ShET1 and ShET2 have been identified, and the genetic loci encoding these toxins have been localized to the chromosome and plasmid, respectively. ShET1 is neutralized by convalescent sera from volunteers challenged with *S. flexneri* 2a, suggesting that this toxic moiety is expressed by shigellae growing in the human intestine. The ShET1 locus is present on the chromosome of *S. flexneri* 2a, but it is only occasionally found in other serotypes. In contrast, ShET2 is more widespread and detectable in 80% of shigellae representing all four species. These enterotoxins may elicit the diarrheal prodrome that often precedes bacillary dysentery; however, their role in the disease process remains to be defined by controlled challenge studies using toxin-negative mutants.

S dysenteriae serotype 1 expresses Shiga toxin, an extremely potent, ricin-like, cytotoxin that inhibits protein synthesis in susceptible mammalian cells. This toxin also has enterotoxic activity in rabbit ileal loops, but its role in human diarrhea is unclear, since shigellae apparently express a number of enterotoxins. More importantly, Shiga toxin is associated with the hemolytic-uremic syndrome, a complication of infections with *S. dysenteriae* 1.

Epidemiology. Humans are the primary reservoir of *Shigella* species, with captive subhuman primates as accidental hosts. In developing countries with prevailing conditions of inadequate sanitation and overcrowded housing, the infection is transmitted most often by the excreta of infected individuals via direct fecal-oral contamination. Flies may contribute to spread from feces to food. The most common species, *S. dysenteriae* and *S. flexneri*, are also the most virulent. Shigellosis is endemic in developing countries were sanitation is poor. Typically 10 to 20 percent of enteric disease, and 50% of the bloody diarrhea or dysentery of young children, can be characterized as shigellosis, and the

prevalence of these infections decreases significantly after five years of life. In developed countries, single-source, food or water-borne outbreaks occur sporadically, and pockets of endemic shigellosis can be found in institutions and in remote areas with substandard sanitary facilities.

Diagnosis. Shigellosis can be correctly diagnosed in most patients on the basis of fresh blood in the stool. Neutrophils in fecal smears is also a strongly suggestive sign. Nonetheless, watery, mucoid diarrhea may be the only symptom of many *S. sonnei* infections, and any clinical diagnosis should be confirmed by cultivation of the etiologic agent from stools. Positive cultures are most often obtained from blood-tinged plugs of mucus in freshly passed stool specimens obtained during the acute phase of disease. Rectal swabs may also be used to culture shigellae if the specimen is processed rapidly or is deposited in a buffered glycerol saline holding solution. Isolation of shigellae in the clinical laboratory typically involves an initial streaking for isolation on differential/selective media with aerobic incubation to inhibit the growth of the anaerobic normal flora. Commonly used primary isolation media include MacConkey, Hektoen Enteric Agar, and *Salmonella-Shigella* (SS) Agar. These media contain bile salts to inhibit the growth of other Gram-negative bacteria and pH indicators to differentiate lactose fermenters (Coliforms) from non-lactose fermenters such as shigellae. A liquid enrichment medium (Hajna Gram-negative broth) may also be inoculated with the stool specimen and subcultured onto the selective/differential agarose media after a short growth period. Following overnight incubation of primary isolation media at 37° C, colorless, non-lactose-fermenting colonies are streaked and stabbed into tubed slants of Kligler's Iron Agar or Triple Sugar Iron Agar. In these differential media, *Shigella* species produce an alkaline slant and an acid butt with no bubbles of gas in the agar. This reaction gives a presumptive identification, and slide agglutination tests with antisera for serogroup and serotype confirm the identification.

Sensitive and rapid methodology for identification of both EIEC and *Shigella* species utilizes DNA probes that hybridize with common virulence plasmid genes or DNA primers that amplify plasmid genes by polymerase chain reaction (PCR). Enzyme-linked immunosorbent assay (ELISA) using antiserum or monoclonal antibody recognizing Ipa proteins can also be used to screen stools for enteroinvasive pathogens.

Treatment. The oral rehydration treatment developed by the World Health Organization has proven effective and safe in the treatment of acute diarrhea, provided that the patient is not vomiting or in shock from severe dehydration. In the latter case, intravenous fluid replacement is required until initial fluid and electrolyte losses are corrected. With proper hydration, shigellosis is generally a self-limiting disease, and the decision to prescribe antibiotics is predicated on the severity of disease, the age of the patient, and the likelihood of further transmission of the infection. Effective antibiotic treatment reduces the average duration of illness from approximately 5–7 days to approximately 3 days and also reduces the period of *Shigella* excretion after symptoms subside. Absorbable drugs such as ampicillin (2 g/day for 5 days) are likely to be effective when the isolate is sensitive. Trimethoprim (8 mg/kg/day) and sulfamethoxazole (40 mg/kg/day) will eradicate sensitive organisms quickly from the intestine, but resistance to this agent is increasing. Ciprofloxacin (1 g/day for 3 days) is effective against multiple drug resistant strains.

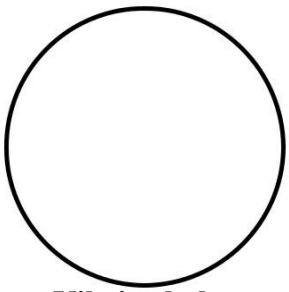
Control. Prevention of fecal-oral transmission is the most effective control strategy. Severe dysentery is treated with ampicillin, trimethoprim-sulfamethoxazole, or, in patients over 17 years old, a 4-fluorquinolone such as ciprofloxacin. Vaccines are not currently available, but some promising candidates are being developed.

Surname _____ Date _____

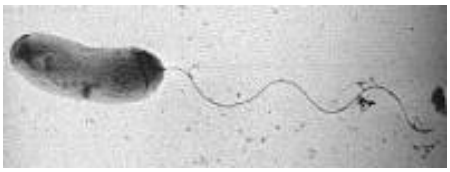
Protocol № 38

Theme: Microbiological diagnosis of cholera.

I. Observe the smears below: V. cholera (stained after Gram) and motility (in phase contrast microscope). Using appropriately colored pencils draw the following cells.



Vibrio cholera
(Gram stain)



Vibrio cholera
(ESM)

II. Study culture and biochemical properties of Vibrio cholera:
a) growth on 1% peptone basic water: _____;
b) growth on basic agar: _____.

Biochemical properties of (Chanberg triad) V. cholera, El-Tor and cholera-like vibrios.
Fermantation of:
Mannose_____;
sucrose:_____;
arabinose: _____.

III. Study differential features of biovars of V. cholera:

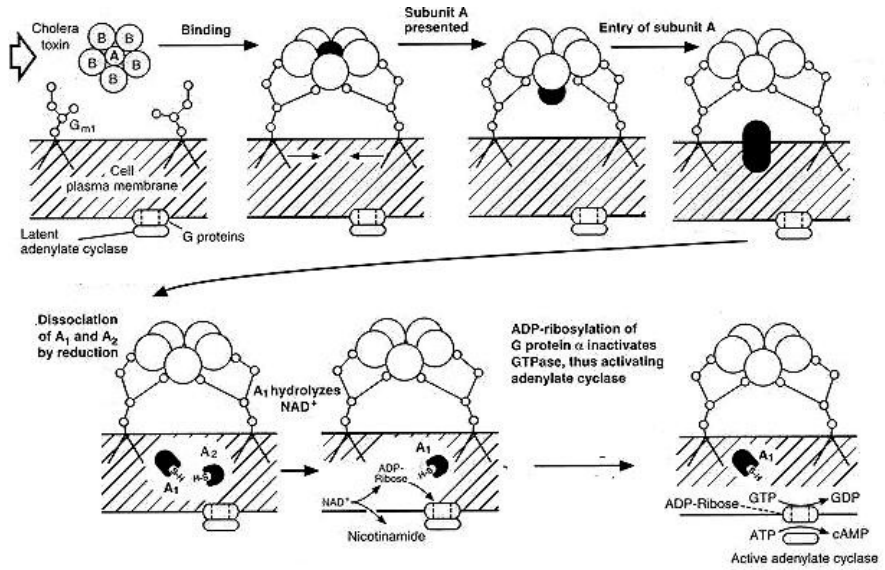
Properties	V.cholerae biovar asiaticae	V.cholerae biovar eltor	Serovar O139 (Bengal)
Foges-Proskower reaction	± (often -)	± (often +)	± (often +)
Polimyxin B sensitivity	+	-	-
Hemolysis of sheep erythrocytes	-	+	-
Agglutination of chicken erythrocytes	-	± (often +)	± (often +)
Sensitivity to classical bacteriophage	+	-	-
Sensitivity to El-Tor bacteriophage	-	+	-
Hexamin test	-	+	-

IV. Study specific therapy of cholera (bacteriophage) and specific profilaxis (vaccine, choleragen-anatoxin).

V. Study antibiotics for therapy of cholera (tetracyclines, chloramphenicol, Co-trimoxasol).

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

IV. Study mechanism of action of cholera enterotoxin:



V. Self-work of students:

Continue of isolation of pure culture from "feces of patient with cholera":

- study growth on 1% basic peptone water and agar: bluish thin pellicle and colorless transparent glistening colonies.
- prepare a smear from the isolated colorless colony, stain after Gram, microscopy
- perform slide agglutination test with O-1 cholera serum: test is positive.

Positive test Negative test

VI. Study the scheme of laboratory diagnosis of cholera.

Specimen: feces, food, raising water.

1 step **Rapid methods: Microscopy. IF**

Seeding on 1% basic water and basic agar.

2 step **Culture properties.** **Slide AT with O1-serum.**

(6-8 h) **Gram staim. IF.**

Seeding on basic agar in Petri dish.

3 step **Culture properties (colonies)**

(12-14 h) **Seeding on lactose-sucrose mesium**

4 step **Biochemical tests** **Slide AT with O1-serum and Ogava and Inaba sera**

(18-24 h) **Differentiation of El-Tor and classical vibrios**

Phagetyping

ADDING THEORETICAL MATERIAL

Cholera and *Vibrio cholerae*

C LI N I C AL M A NI F ES TA T I O NS

Cholera is a potentially epidemic and life-threatening secretory diarrhea characterized by numerous, voluminous watery stools, often accompanied by vomiting, and resulting in hypovolemic shock and acidosis. It is caused by certain members of the species *Vibrio cholera* which can also cause mild or inapparent infections. Other members of the species may occasionally cause isolated outbreaks of milder diarrhea whereas others—the vast majority—are free-living and not associated with disease.

S TR U C T UR E , C L AS S I F I C AT I O N, A N D A N T I G E NI C T Y P ES

Vibrios are Gram-negative, highly motile curved rods with a single polar flagellum. They tolerate alkaline media that kill most intestinal commensals, but they are sensitive to acid. Numerous free-living vibrios are known, some potentially pathogenic. Until 1992, cholera was caused by only two serotypes, Inaba (AC) and Ogawa (AB), and two biotypes, classical and El Tor, of toxigenic O group 1 *V. cholerae*. These organisms may be identified by agglutination in O group 1-specific antiserum directed against the lipopolysaccharide component of the cell wall and by demonstration of their enterotoxigenicity. In 1992, cholera caused by serogroup O139 (synonym "Bengal" the 139th and latest serogroup of *V. cholera* to be identified) emerged in epidemic proportions in India and Bangladesh. This serovar is identified by 1) absence of agglutination in O group 1 specific antiserum; 2) by agglutination in O group 139 specific antiserum; and 3) by the presence of a capsule.

P A T HO G E N ES I S

Cholera is transmitted by the fecal-oral route. Vibrios are sensitive to acid, and most die in the stomach. Surviving virulent organisms may adhere to and colonize the small bowel, where they secrete the potent cholera enterotoxin (CT, also called "choleragen"). This toxin binds to the plasma membrane of intestinal epithelial cells and releases an enzymatically active subunit that causes a rise in cyclic adenosine 5^1-monophosphate (cAMP) production. The resulting high intracellular cAMP level causes massive secretion of electrolytes and water into the intestinal lumen.

H O S T D EF E NS ES

Gastric acid, mucus secretion, and intestinal motility are the prime nonspecific defenses against *V. cholerae*. Breastfeeding in endemic areas is important in protecting infants from disease. Disease results in effective specific immunity, involving primarily secretory immunoglobulin (IgA), as well as IgG antibodies, against vibrios, somatic antigen, outer membrane protein, and/or the enterotoxin and other products.

E P I D EM I O L O GY

Cholera is endemic or epidemic in areas with poor sanitation; it occurs sporadically or as limited outbreaks in developed countries. In coastal regions it may persist in shellfish and plankton. Long-term convalescent carriers are rare.

Enteritis caused by the halophile *V. parahaemolyticus* is associated with raw or improperly cooked seafood.

D I A GN O S I S

The diagnosis is suggested by strikingly severe, watery diarrhea. For rapid diagnosis, a wet mount of liquid stool is examined microscopically. The characteristic motility of vibrios is stopped by specific antisomatic antibody. Other methods are culture of stool or rectal swab samples on TCBS agar and other selective and nonselective media; the slide agglutination test of colonies with specific antiserum; fermentation tests (oxidase positive); and enrichment in peptone broth followed by fluorescent antibody tests, culture, or retrospective serologic diagnosis. More recently the polymerase chain reaction (PCR) and additional genetically-based rapid techniques have been recommended for use in specialized laboratories.

C O N TR OL

Control by sanitation is effective but not feasible in endemic areas. A good vaccine has not yet been developed. A parenteral vaccine of whole killed bacteria has been used widely, but is relatively ineffective and is not generally recommended. An experimental oral vaccine of killed whole cells and toxin B-subunit protein is less than ideal. Living attenuated genetically engineered mutants are promising, but such strains can cause limited diarrhea as a side effect. Antibiotic prophylaxis is feasible for small groups over short periods.

Other Vibrio Infections

Other serogroups of *V. cholera* may cause diarrheal disease and other infections but are not associated with epidemic cholera. *Vibrio parahaemolyticus* is an important cause of enteritis associated with the ingestion of raw or improperly prepared seafood. Other *Vibrio species*, including *V. vulnificus*, can cause infections of humans and other animals including fish. *Campylobacter* species (formerly included with vibrios) can cause enteritis. *C. pylori*, now known as *Helicobacter pylori*, is associated with gastric and duodenal ulcers.

Vibrios are highly motile, gram-negative, curved or comma-shaped rods with a single polar flagellum. Of the vibrios that are clinically significant to humans, *Vibrio cholera* O group 1, the agent of cholera, is the most important. *Vibrio cholera* was first isolated in pure culture by Robert Koch in 1883, although it had been seen by other investigators, including Pacini, who is credited with describing it first in Florence, Italy, in 1854.

Cholera is a life-threatening secretory diarrhea induced by an enterotoxin secreted by *V.cholerae*.

Other vibrios may also be clinically significant in humans, and some are known to cause diseases in domestic animals. Nonpathogenic vibrios are widely distributed in the environment, particularly in estuarine waters and seafoods. For this reason, isolation of a vibrio from a patient with diarrheal disease does not necessarily indicate an etiologic relationship.

Clinical Manifestations

Following an incubation period of 6 to 48 hours, cholera begins with the abrupt onset of watery diarrhea. The initial stool may exceed 1 L, and several liters of fluid may be secreted within hours, leading to hypovolemic shock. Vomiting usually accompanies the diarrheal episodes. Muscle cramps may occur as water and electrolytes are lost from body tissues. Loss of skin turgor, scaphoid abdomen, and weak pulse are characteristic of cholera. Various degrees of fluid and electrolyte loss are observed, including mild and subclinical cases. The disease runs its course in 2 to 7 days; the outcome depends upon the extent of water and electrolyte loss and the adequacy of water and electrolyte repletion therapy. Death can occur from hypovolemic shock, metabolic acidosis, and uremia resulting from acute tubular necrosis.

The cholera vibrios are Gram-negative, slightly curved rods whose motility depends on a single polar flagellum. Their nutritional requirements are simple. Fresh isolates are prototrophic (i.e., they grow in media containing an inorganic nitrogen source, a utilizable carbohydrate, and appropriate minerals). In adequate media, they grow rapidly with a generation time of less than 30 minutes. Although they reach higher population densities when grown with vigorous aeration, they can also grow anaerobically. Vibrios are sensitive to low pH and die rapidly in solutions below pH 6; however, they are quite tolerant of alkaline conditions. This tolerance has been exploited in the choice of media used for their isolation and diagnosis.

Until 1992, the vibrios that caused epidemic cholera were subdivided into two biotypes: classical and El Tor. Classical *V. cholera* was first isolated by Koch in 1883. Subsequently, in the early 1900s, some vibrios resembling *V. cholerae* were isolated from Mecca-bound pilgrims at the quarantine station at El Tor, in the Sinai Peninsula, that had been established to try to control cholera associated with pilgrimages to Mecca. These vibrios resembled classical *V.cholerae* in many ways but caused lysis of goat or sheep erythrocytes in a test known as the Greig test. Because the pilgrims from whom they were isolated did not have cholera, these hemolytic El Tor vibrios were regarded as relatively insignificant except for the possibility of confusion with true cholera vibrios. In the 1930s, similar hemolytic vibrios were associated with relatively restricted outbreaks of diarrheal disease, called paracholera, in the Celebes. In 1961, cholera caused by El Tor vibrios erupted in Hong Kong and spread virtually worldwide. Although in the course of this pandemic most *V. cholerae* biotype El Tor strains lost their hemolytic activity, a number of ancillary tests differentiate them from vibrios of the classical biotype.

The operational serology of the cholera vibrios which belong in O antigen group 1 is relatively simple. Both biotypes (El Tor and classical) contain two major serotypes, Inaba and Ogawa. These serotypes are differentiated in agglutination and vibriocidal antibody tests on the basis of their dominant heat-stable lipopolysaccharide somatic antigens. The cholera group has a common antigen, A, and the serotypes are differentiated by the type-specific antigens, B (Ogawa) and C (Inaba). An additional serotype, Hikojima, which has both specific antigens, is rare. *V. cholerae* O139 appears to have been derived from the pandemic El Tor

biotype but has lost the characteristic O1 somatic antigen; it has gained the ability to produce a polysaccharide capsule; it produces the same cholera enterotoxin; and it seems to have retained the epidemic potential of O1 strains.

The cholera vibrios cause many distinctive reactions. They are oxidase positive. The O group 1 cholera vibrios almost always fall into the Heiberg I fermentation pattern; that is, they ferment sucrose and mannose but not arabinose, and they produce acid but not gas. *Vibrio cholerae* also possesses lysine and ornithine decarboxylase, but not arginine dihydrolase. Freshly isolated agar-grown vibrios of the El Tor biotype, in contrast to classical *V. cholerae*, produce a cell-associated mannose-sensitive hemagglutinin active on chicken erythrocytes. This activity is readily detected in a rapid slide test. In addition to hemagglutination, numerous tests have been proposed to differentiate the classical and El Tor biotypes, including production of a hemolysin, sensitivity to selected bacteriophages, sensitivity to polymyxin, and the Voges-Proskauer test for acetoin. El Tor vibrios originally were defined as hemolytic. They differed in this characteristic from classical cholera vibrios; however, during the most recent pandemic, most El Tor vibrios (except for the recent isolates from Texas and Louisiana) had lost the capacity to express the hemolysin. Most El Tor vibrios are Voges-Proskauer positive and resistant to polymyxin and to bacteriophage IV, whereas classical vibrios are sensitive to them. As both biotypes cause the same disease, these characteristics have only epidemiologic significance. Strains of the El Tor biotype, however, produce less cholera enterotoxin, but appear to colonize intestinal epithelium better than vibrios of the classical variety. Also, they seem some what more resistant to environmental factors. Thus, El Tor strains have a higher tendency to become endemic and exhibit a higher infection-to-case ratio than the classical biotype.

Pathogenesis

Cholera is exclusively a disease of the small bowel. To establish residence and multiply in the human small bowel (normally relatively free of bacteria because of the effective clearance mechanisms of peristalsis and mucus secretion), the cholera vibrios have one or more adherence factors that enable them to adhere to the microvilli. Several hemagglutinins and the toxin-coregulated pili have been suggested to be involved in adherence but the actual mechanism has not been defined. In fact, there may be multiple mechanisms. The motility of the vibrios may affect virulence by enabling them to penetrate the mucus layer. They also produce mucinolytic enzymes, neuraminidase, and proteases. The growing cholera vibrios elaborate the cholera enterotoxin. The subsequent cAMP-mediated cascade of events has not yet been delineated, but the final effect is hypersecretion of chloride and bicarbonate followed by water, resulting in the characteristic isotonic voluminous cholera stool. The stool of an actively purging, severely ill cholera patient can resemble rice water—the supernatant of boiled rice.

Recovery from cholera probably depends on two factors: elimination of the vibrios by antibiotics or the patient's own immune response, and regeneration of the poisoned intestinal epithelial cells. Treatment with a single 200-mg dose of doxycycline has been recommended. As studies in volunteers demonstrated

conclusively, the disease is an immunizing process. Patients who have recovered from cholera are solidly immune for at least 3 years.

Cholera vaccines consisting of killed cholera bacteria administered parenterally have been used since the turn of the century. Combined preparations of bacterial somatic antigen and toxin antigen have been reported to act synergistically in stimulating immunity in laboratory animals; that is, the combined protective effect is closer to the product than to the sum of the individual protective effects.

Epidemiology

Humans apparently are the only natural host for the cholera vibrios. Cholera is acquired by the ingestion of water or food contaminated with the feces of an infected individual. Previously, the disease swept the world in six great pandemics and later receded into its ancestral home in the Indo-Pakistani subcontinent. In 1961, the El Tor biotype (a subset distinguished by physiologic characteristics) of *V. cholerae*, not previously implicated in widespread epidemics, emerged from the Celebes (now Sulawesi), causing the seventh great cholera pandemic. In the course of their migration, the El Tor biotype cholera vibrios virtually replaced *V. cholerae* of the classic biotype that formerly was responsible for the annual cholera epidemics in India and East Pakistan (now Bangladesh). The pandemic that began in 1961 is now heavily seeded in Southeast Asia and in Africa. It has also invaded Europe, North America, and Japan, where the outbreaks have been relatively restricted and self-limited because of more highly developed sanitation. Several new cases were reported in Texas in 1981 and sporadic cases have since been reported in Louisiana and other Gulf Coast areas. This now endemic focus appears to be due to a clone which is unique from the pandemic strain. In 1991, the pandemic strain hit Peru with massive force and has since spread through most of the Western Hemisphere, causing more than a million cases. Fortunately, mortality has been less than 1 percent because of the effectiveness of oral rehydration therapy. The vibrios surprised us again, in 1992, with the emergence of O139 in India and Bangladesh. For a while it appeared that O139 would replace O1 (both classical and El Tor) but it has exhibited quiescent periods when O1 reemerges.

Cholera appears to exhibit three major epidemiologic patterns: heavily endemic, neoepidemic (newly invaded, cholera-receptive areas), and, in developed countries with good sanitation, occasional limited outbreaks. These patterns probably depend largely on environmental factors (including sanitary and cultural aspects), the prior immune status or antigenic experience of the population at risk, and the inherent properties of the vibrios themselves, such as their resistance to gastric acidity, ability to colonize, and toxigenicity. In the heavily endemic region of the Indian subcontinent, cholera exhibits some periodicity; this may vary from year to year and seasonally, depending partly on the amount of rain and degree of flooding. Because humans are the only reservoirs, survival of the cholera vibrios during interepidemic periods probably depends on a relatively constant availability of low-level undiagnosed cases and transiently infected, asymptomatic individuals. Long-term carriers have been reported but are extremely rare. The classic case occurred in the Philippines, where “cholera Dolores” harbored cholera vibrios in

her gallbladder for 12 years after her initial attack in 1962. Her carrier state resolved spontaneously in 1973; no secondary cases had been associated with her well-marked strain. Recent studies, however, have suggested that cholera vibrios can persist for some time in shellfish, algae or plankton in coastal regions of infected areas and it has been claimed that they can exist in "a viable but nonculturable state."

During epidemic periods, the incidence of infection in communities with poor sanitation is high enough to frustrate the most vigorous epidemiologic control efforts. Although transmission occurs primarily through water contaminated with human feces, infection also may be spread within households and by contaminated foods. Thus, in heavily endemic regions, adequate supplies of pure water may reduce but not eliminate the threat of cholera.

In neoepidemic cholera-receptive areas, vigorous epidemiologic measures, including rapid identification and treatment of symptomatic cases and asymptomatically infected individuals, education in sanitary practices, and interruption of vehicles of transmission (e.g., by water chlorination), may be most effective in containing the disease. In such situations, spread of cholera usually depends on traffic of infected human beings, although spread between adjacent communities can occur through bodies of water contaminated by human feces. John Snow was credited with stopping an epidemic in London, England, by the simple expedient of removing the handle of the "Broad Street pump" (a contaminated water supply) in 1854, before acceptance of the "germ theory" and before the first isolation of the "Kommabacillus" by Robert Koch.

In such developed areas as Japan, Northern Europe, and North America, cholera has been introduced repeatedly in recent years, but has not caused devastating outbreaks; however, Japan has reported secondary cases and, in 1978, the United State experienced an outbreak of about 12 cases in Louisiana. In that outbreak, sewage was infected, and infected shellfish apparently were involved. Interestingly, the hemolytic vibrio strain implicated was identical to one that caused an unexplained isolated case in Texas in 1973.

Diagnosis

Rapid bacteriologic diagnosis offers relatively little clinical advantage to the patient with secretory diarrhea, because essentially the same treatment (fluid and electrolyte replacement) is employed regardless of etiology. Nevertheless, rapid identification of the agent can profoundly affect the subsequent course of a potential epidemic outbreak. Because of their rapid growth and characteristic colonial morphology, *V. cholerae* can be easily isolated and identified in the bacteriology laboratory, provided, first, that the presence of cholera is suspected and, second, that suitable specific diagnostic antisera are available. The vibrios are completely inhibited or grow somewhat poorly on usual enteric diagnostic media (MacConkey agar or eosin-methylene blue agar). An effective selective medium is thiosulfate-citrate-bile salts-sucrose (TCBS) agar, on which the sucrose-fermenting cholera vibrios produce a distinctive yellow colony. However, the usefulness of this medium is limited because serologic testing of colonies grown on it occasionally proves difficult, and different lots vary in their productivity. This

medium is also useful in isolating *V. parahaemolyticus*. They can also be isolated from stool samples or rectal swabs from cholera cases on simple meat extract (nutrient) agar or bile salts agar at slightly alkaline pH values. Following observation of characteristic colonial morphology with a stereoscopic microscope using transmitted oblique illumination, microorganisms can be confirmed as cholera vibrios by a rapid slide agglutination test with specific antiserum. Classic and El Tor biotypes can be differentiated at the same time by performing a direct slide hemagglutination test with chicken erythrocytes: all freshly isolated agar-grown El Tor vibrios exhibit hemagglutination; all freshly isolated classic vibrios do not. In practice, this can be accomplished with material from patients as early as 6 hours after streaking the specimen in which the cholera vibrios usually predominate. However, to detect carriers (asymptomatically infected individuals) and to isolate cholera vibrios from food and water, enrichment procedures and selective media are recommended. Enrichment can be accomplished by inoculating alkaline (pH 8.5) peptone broth with the specimen and then streaking for isolation after an approximate 6-hour incubation period; this process both enables the rapidly growing vibrios to multiply and suppresses much of the commensal microflora.

The classic case of cholera, which includes profound secretory diarrhea and should evoke clinical suspicion, can be diagnosed within a few minutes in the prepared laboratory by finding rapidly motile bacteria on direct, bright-field, or dark-field microscopic examination of the liquid stool. The technician can then make a second preparation to which a droplet of specific anti-*V.cholerae* O group 1 antiserum is added. This quickly stops vibrio motility. Another rapid technique is the use of fluorescein isothiocyanate-labeled specific antiserum (fluorescent antibody technique) directly on the stool or rectal swab smear or on the culture after enrichment in alkaline peptone broth. For cultural diagnosis, both nonselective and selective (TCBS) media may be used. Although demonstration of typical agglutination essentially confirms the diagnosis, additional conventional tests such as oxidase reaction, indole reaction, sugar fermentation reactions, gelatinase, lysine, arginine, and ornithine decarboxylase reactions may be helpful. Tests for chicken cell hemagglutination, hemolysis, polymyxin sensitivity, and susceptibility to phage IV are useful in differentiating the El Tor biotype from classic *V. cholerae*. Tests for toxigenesis may be indicated.

Diagnosis can be made retrospectively by confirming significant rises in specific serum antibody titers in convalescents. For this purpose, conventional agglutination tests, tests for rises in complement-dependent vibriocidal antibody, or tests for rises in antitoxic antibody can be employed. Convenient microversions of these tests have been developed. Passive hemagglutination tests and enzyme-linked immunosorption assays (ELISAs) have also been proposed.

Cultures that resemble *V. cholerae* but fail to agglutinate in diagnostic antisera (nonagglutinable or non-O group 1 vibrios) present more of a problem and require additional tests such as oxidase, decarboxylases, inhibition by the vibriostatic pteridine compound 0/129, and the “string test.” The string test demonstrates the property, shared by most vibrios and relatively few other genera,

of forming a mucus-like string when colony material is emulsified in 0.5 percent aqueous sodium deoxycholate solution. Additional tests for enteropathogenicity and toxigenesis may be useful. Genetically based tests such as PCR are increasingly being used in specialized laboratories.

Control

Treatment of cholera consists essentially of replacing fluid and electrolytes. Formerly, this was accomplished intravenously, using costly sterile pyrogen-free intravenous solutions. The patient's fluid losses were conveniently measured by the use of buckets, graduated in half-liter volumes, kept underneath an appropriate hole in an army-type cot on which the patient was resting. Antibiotics such as tetracycline, to which the vibrios are generally sensitive, are useful adjuncts in treatment. They shorten the period of infection with the cholera vibrios, thus reducing the continuous source of cholera enterotoxin; this results in a substantial saving of replacement fluids and a markedly briefer hospitalization. Note, however, that fluid and electrolyte replacement is all-important; patients who are adequately rehydrated and maintained will virtually always survive, and antibiotic treatment alone is not sufficient.

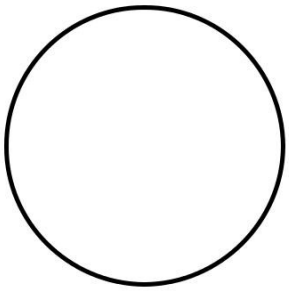
Cholera is essentially a disease associated with poor sanitation. The simple application of sanitary principles—protecting drinking water and food from contamination with human feces—would go a long way toward controlling the disease. However, at present, this is not feasible in the underdeveloped areas that are afflicted with epidemic cholera or are considered to be cholera receptive. Meanwhile, development of a vaccine that would effectively prevent colonization and manifestations of cholera would be extremely helpful. As indicated above, such vaccines are presently being tested. Antibiotic or chemotherapeutic prophylaxis is feasible and may be indicated under certain circumstances. It also should be mentioned that the incidence of cholera is significantly higher in formula-fed than in breast-fed babies.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

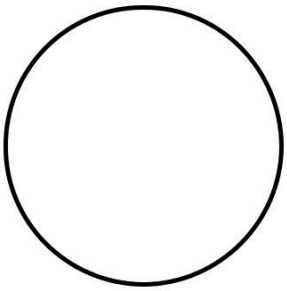
Protocol № 39

Theme: Microbiological diagnosis of opportunistic infections (Pseudomonas aeruginosa, Proteus spp., Klebsiella spp.).

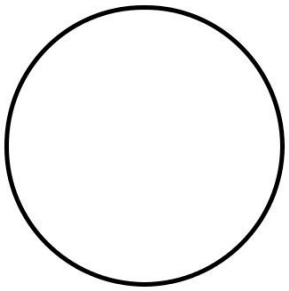
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Pseudomonas aeruginosa (Gram stain)



Proteus vulgaris (Gram stain)



Klebsiella pneumoniae (Burry-Gins stain)

II. Study nutrient media for cultivation of:

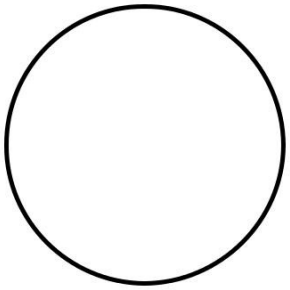
a) P. aeruginosa: agar with citylpyridinium chloride, MPA with chicken embryo albumen, blood agar, eosin-methylthionine blue agar.

b) Proteus spp.: MacConkey agar, Endo agar, eosin-methylene blue agar, bismuth sulfite agar, brilliant green agar, triple sugar iron agar, blood agar, tryptic soy agar (TSA).

c) Klebsiella spp.: MacConkey agar, Endo agar.

III. Examine colonies of P. aeruginosa, K.pneumoniae and P. vulgaris on nutrient media.

IV. Self-work of students: preparation a smear of capsule-producing bacteria, stain after Burry-Gins, microscopy.



Klebsiella pneumoniae, Burry-Gins stain

V. Study the scheme of laboratory diagnosis of **P. aeruginosa** infections.

Specimen: sputum, pus, wound exudates, conjunctival exudates, urine, feces, blood, liquor.

1. Culture: the ability to grow at 42° C distinguishes it from many other Pseudomonas species Identification:

- Biochemical tests:
  - lactose-negative,
  - oxidase-positive,
  - catalase-positive,
  - liquefaction of gelatin.
- Green pigment production on MPA with chicken embryo albumen.
- Serotyping, pyocyonintyping, phagetyping.

2. Serology (detection of antibodies to exotoxin A): CFT, PHAT.

VI. Study the scheme of laboratory diagnosis of **Proteus spp.** infections.

Specimen: pus, wound exudates, conjunctival exudates, nasopharyngeal aspirate, urine, feces.

1. Culture on blood agar (swarming effect), on MacConkey agar (fishy odor, non-lactose fermenting colonies), on triple sugar iron agar (red with H_2S production).

Identification:

- Biochemical tests:
  - lactose-negative,
  - catalase- and nitrate-positive,
  - methyl red-positive,
  - urease-positive,
  - H_2S-positive,
  - oxidase-negative,
  - Voges-Proskauer - variable.

Differentiation of P. mirabilis and P. vulgaris:

- Indole test: P. mirabilis (negative) and P. vulgaris (positive).
- Citrate test: P. mirabilis (positive) and P. vulgaris (negative).
- Slide agglutination test with polyvalent O-sera and type-specific H-sera.

- Phagetyping.

VII. Study the scheme of laboratory diagnosis of **Klebsiella** infections.

Specimen: sputum, wound exudates, urine.

1. Microscopy: Gram stain. Klebsiella appear as short, plump, gram-negative bacilli. Burry-Gins stain. They are usually surrounded by a capsule that appears as a clear space.

Specimen: nasal swab, sputum, wound exudates, blood, urine, pleural fluid, peripheral or central intravenous access sites, urinary catheters, respiratory support equipment.

2. Culture: Klebsiella grow well on most ordinary media.

Identification:

- Biochemical tests:
  - lactose-positive,
  - urease-positive,
  - methyl red-positive,
  - indole-negative (K. oxytoca and some strains of K. pneumoniae are exceptions).
  - H_2S-negative.

- Slide AT and IF with type-specific capsular sera.
- Phagetyping.

3. Serology (scleroma): CFT, PHAT (detection of antibodies).

ADDING THEORETICAL MATERIAL

Pseudomonas aeruginosa is a common Gram-negative, rod-shaped bacterium that can cause disease in plants and animals, including humans. A species of considerable medical importance, *P. aeruginosa* is a multidrug resistant pathogen recognised for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms, and its association with serious illnesses – hospital-acquired infections such as ventilator-associated pneumonia and various sepsis syndromes.

The organism is considered opportunistic insofar as serious infection often occurs during existing diseases or conditions – most notably cystic fibrosis and traumatic burns. It is also found generally in the immunocompromised but can infect the immunocompetent as in hot tub folliculitis. Treatment of *P. aeruginosa*

infections can be difficult due to its natural resistance to antibiotics. When more advanced antibiotic drug regimens are needed adverse effects may result.

It is citrate, catalase, and oxidase positive. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in low-oxygen atmospheres, thus has colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, its versatility enables the organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonizations occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal. Because it thrives on moist surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is also able to decompose hydrocarbons and has been used to break down tarballs and oil from oil spills. *P. aeruginosa* is not extremely virulent in comparison with other major pathogenic bacterial species – for example *Staphylococcus aureus* and *Streptococcus pyogenes* – though *P. aeruginosa* is capable of extensive colonization, and can aggregate into enduring biofilms.

P. aeruginosa is a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion. This organism can achieve anaerobic growth with nitrate or nitrite as a terminal electron acceptor. When oxygen, nitrate, and nitrite are absent, it is able to ferment arginine and pyruvate by substrate-level phosphorylation. Adaptation to microaerobic or anaerobic environments is essential for certain lifestyles of *P. aeruginosa*, for example, during lung infection in cystic fibrosis and primary ciliary dyskinesia, where thick layers of lung mucus and alginate surrounding mucoid bacterial cells can limit the diffusion of oxygen. *P. aeruginosa* growth within the human body can be asymptomatic until the bacteria form a biofilm, which overwhelms the immune system. These biofilms are found in the lungs of people with cystic fibrosis and primary ciliary dyskinesia, and can prove fatal.

It is the most common cause of infections of burn injuries and of the outer ear (otitis externa), and is the most frequent colonizer of medical devices (e.g., catheters). *Pseudomonas* can be spread by equipment that gets contaminated and is not properly cleaned or on the hands of healthcare workers. *Pseudomonas* can, in rare circumstances, cause community-acquired pneumonias, as well as ventilator-associated pneumonias, being one of the most common agents isolated in several studies. Pyocyanin is a virulence factor of the bacteria and has been known to cause death in *C. elegans* by oxidative stress. However, salicylic acid can inhibit pyocyanin production. One in ten hospital-acquired infections is from *Pseudomonas*. Cystic fibrosis patients are also predisposed to P. aeruginosa infection of the lungs. *P. aeruginosa* may also be a common cause of "hot-tub rash" (dermatitis), caused by lack of proper, periodic attention to water quality. Since these bacteria like moist environments, such as hot tubs and swimming pools, they can cause skin rash or swimmer's ear. *Pseudomonas* is also a common cause of postoperative infection in radial keratotomy surgery patients. The organism is also associated with the skin lesion ecthyma gangrenosum. *P.*

aeruginosa is frequently associated with osteomyelitis involving puncture wounds of the foot, believed to result from direct inoculation with *P. aeruginosa* via the foam padding found in tennis shoes, with diabetic patients at a higher risk.

P. aeruginosa uses the virulence factor exotoxin A to inactivate eukaryotic elongation factor, eukaryotic cells cannot synthesize proteins and necrotise. The release of intracellular contents induces an immunologic response in immunocompetent patients. In addition *P.aeruginosa* uses an exoenzyme, ExoU, which degrades the plasma membrane of eukaryotic cells, leading to lysis.

Depending on the nature of infection, an appropriate specimen is collected and sent to a bacteriology laboratory for identification. As with most bacteriological specimens, a Gram stain is performed, which may show Gram-negative rods and/or white blood cells. *P. aeruginosa* produces colonies with a characteristic "grape-like" or "fresh-tortilla" odor on bacteriological media. In mixed cultures, it can be isolated as clear colonies on MacConkey agar (as it does not ferment lactose) which will test positive for oxidase. Confirmatory tests include production of the blue-green pigment pyocyanin on cetrimide agar and growth at 42 ^{0}C.

When *P. aeruginosa* is isolated from a normally sterile site (blood, bone, deep collections), it is generally considered dangerous, and almost always requires treatment. However, *P. aeruginosa* is frequently isolated from nonsterile sites (mouth swabs, sputum, etc.), and, under these circumstances, it may represent colonization and not infection. The isolation of *P. aeruginosa* from nonsterile specimens should, therefore, be interpreted cautiously, and the advice of a microbiologist or infectious diseases physician/pharmacist should be sought prior to starting treatment. Often, no treatment is needed.

Identification

Test	**Results**
Gram Stain	-
Oxidase	+
Indole Production	-
Methyl Red	-
Voges-Proskaeur	-
Citrate	+
Hydrogen Sulfide Production	-
Urea Hydrolysis	+
Phenylalanine Deaminase	-
Lysine Decarboxylase	-
Motility	+
Gelatin Hydrolysis	+
Acid from lactose	-

acid from glucose	+
acid from maltose	-
acid from mannitol	+
acid from sucrose	-
nitrate reduction	+
DNAse	-
Lipase	+
Pigment	+ (bluish green pigmentation)
Catalase	+

Proteus vulgaris is a rod-shaped, nitrate-reducing, indole+ and catalase-positive, hydrogen sulfide-producing, Gram-negative bacterium that inhabits the intestinal tracts of humans and animals. It can be found in soil, water, and fecal matter. It is grouped with the Enterobacteriaceae and is an opportunistic pathogen of humans. It is known to cause wound infections and other species of its genera are known to cause urinary tract infections.

The genus *Proteus*, and in particular *P. vulgaris*, has undergone a number of major taxonomic revisions. In 1982, *P. vulgaris* was separated into three biogroups on the basis of indole production. Biogroup one was indole negative and represented a new species, *P. penneri*, while biogroups two and three remained together as *P. vulgaris*.

Cause and epidemiology

- Nosocomial infections
- *P. mirabilis* causes 90 % of *Proteus* infections.
- *P. vulgaris* and *P. penneri* are easily isolated from individuals in long-term care facilities and hospitals and from patients with underlying diseases or compromised immune systems.
- Patients with recurrent infections, those with structural abnormalities of the urinary tract, those who have had urethral instrumentation, and those whose infections were acquired in the hospital have an increased frequency of infection caused by *Proteus* and other organisms (e.g., *Klebsiella*, *Enterobacter*, *Pseudomonas*, enterococci, and staphylococci).

Lab identification. According to laboratory fermentation tests, *P. vulgaris* ferments glucose and amygdalin, but does not ferment mannitol or lactose. *P. vulgaris* also tests positive for the methyl red (mixed acid fermentation) test and is also an extremely motile organism.

The optimal growing conditions of this organism is in a facultative anaerobic environment with an average temperature of about 40 ^{0}C.

P. vulgaris may yield the following results:

- Positive for glucose fermentation (with gas production)
- Negative for lysine and ornithine

- Positive for hydrogen sulfide production and indole production
- Negative for adonitol and lactose
- Negative for arabinose, sorbitol and dulcitol
- Positive for the phenylalanine test and the Harnstoff urea test

P. vulgaris can test positive or negative for citrate, negative urease test, is capable of hydrolysis of gelatin.

Antibiotics to which *P. vulgaris* is known to be sensitive:

- Ciprofloxacin
- Ceftazidime
- Netilmicin
- Sulbactam or cefoperazone
- Meropenem
- Piperacillin/tazobactam
- Ampicillin/sulbactam.

Klebsiella pneumoniae is a Gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar.

Although found in the normal flora of the mouth, skin, and intestine, it can cause destructive changes to human and animal lungs if aspirated (inhaled), specifically to the alveoli (in the lungs) resulting in bloody sputum. In the clinical setting, it is the most significant member of the *Klebsiella* genus of the Enterobacteriaceae. *K. oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, *Klebsiella* species have become important pathogens in nosocomial infections.

It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions. As a free-living diazotroph, its nitrogen-fixation system has been much-studied, and is of agricultural interest, as *K. pneumoniae* has been demonstrated to increase crop yields in agricultural conditions.

Members of the *Klebsiella* genus typically express two types of antigens on their cell surfaces. The first, O antigen, is a component of the lipopolysaccharide (LPS), of which 9 varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties. Both contribute to pathogenicity and form the basis for serogrouping.

It is closely related to *K. oxytoca* from which it is distinguished by being indole-negative and by its ability to grow on melezitose but not 3-hydroxybutyrate.

As a general rule, *Klebsiella* infections are seen mostly in people with a weakened immune system. Most often, illness affects middle-aged and older men with debilitating diseases. This patient population is believed to have impaired respiratory host defenses, including persons with diabetes, alcoholism, malignancy, liver disease, chronic obstructive pulmonary diseases, glucocorticoid therapy, renal failure, and certain occupational exposures (such as papermill workers). Many of these infections are obtained when a person is in the hospital for some other reason

(a nosocomial infection). Feces are the most significant source of patient infection, followed by contact with contaminated instruments.

The most common condition caused by *Klebsiella* bacteria outside the hospital is pneumonia, typically in the form of bronchopneumonia and also bronchitis. These patients have an increased tendency to develop lung abscess, cavitation, empyema, and pleural adhesions. It has a death rate around 50%, even with antimicrobial therapy. The mortality rate can be nearly 100% for people with alcoholism and bacteremia.

In addition to pneumonia, *Klebsiella* can also cause infections in the urinary tract, lower biliary tract, and surgical wound sites. The range of clinical diseases includes pneumonia, thrombophlebitis, urinary tract infection, cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, meningitis, and bacteremia and septicemia. For patients with an invasive device in their bodies, contamination of the device becomes a risk; for example, neonatal ward devices, respiratory support equipment, and urinary catheters put patients at increased risk. Also, the use of antibiotics can be a factor that increases the risk of nosocomial infection with *Klebsiella* bacteria. Sepsis and septic shock can follow entry of the bacteria into the blood.

Klebsiella ranks second to *E. coli* for urinary tract infections in older people. It is also an opportunistic pathogen for patients with chronic pulmonary disease, enteric pathogenicity, nasal mucosa atrophy, and rhinoscleroma. New antibiotic-resistant strains of *K. pneumoniae* are appearing.

To get a *K. pneumoniae* infection, a person must be exposed to the bacteria. In other words, *K. pneumoniae* must enter the respiratory tract to cause pneumoniae, or the blood to cause a bloodstream infection. In healthcare settings, *K. pneumoniae* bacteria can be spread through person-to-person contact (for example, contaminated hands of healthcare personnel, or other people via patient to patient) or, less commonly, by contamination of the environment; the role of transmission directly from the environment to patients is controversial and requires further investigation. However, the bacteria are not spread through the air. Patients in healthcare settings also may be exposed to *K. pneumoniae* when they are on ventilators, or have intravenous catheters or wounds. Unfortunately, these medical tools and conditions may allow *K.pneumoniae* to enter the body and cause infection. *Klebsiella* organisms are often resistant to multiple antibiotics. Current evidence implicates plasmids as the primary source of the resistance genes. *Klebsiella* species with the ability to produce extended-spectrum beta-lactamases (ESBL) are resistant to virtually all beta-lactam antibiotics, except carbapenems. Other frequent resistance targets include aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and trimethoprim/sulfamethoxazole.

Infection with carbapenem-resistant Enterobacteriaceae (CRE) or carbapenemase-producing Enterobacteriaceae is emerging as an important challenge in health-care settings. One of many CREs is carbapenem-resistant *Klebsiella pneumoniae* (CRKP).

CRKP is resistant to almost all available antimicrobial agents, and infections with CRKP have caused high rates of morbidity and mortality, in particular among

persons with prolonged hospitalization and those critically ill and exposed to invasive devices (e.g., ventilators or central venous catheters). The concern is that carbapenem is often used as a drug of last resort when battling resistant bacterial strains. New slight mutations could result in infections for which healthcare professionals can do very little, if anything, to treat patients with resistant organisms.

A number of mechanisms cause carbapenem resistance in the Enterobacteriaceae. These include hyperproduction of ampC beta-lactamase with an outer membrane porin mutation, CTX-M extended-spectrum beta-lactamase with a porin mutation or drug efflux, and carbapenemase production.

To prevent spreading *Klebsiella* infections between patients, healthcare personnel must follow specific infection-control precautions, which may include strict adherence to hand hygiene (preferably using an alcohol based hand rub (60-90%) or soap and water if hands are visibly soiled. Alcohol based hand rubs are effective against these Gram-negative bacilli) and wearing gowns and gloves when they enter rooms where patients with *Klebsiella*–related illnesses are housed. Healthcare facilities also must follow strict cleaning procedures to prevent the spread of *Klebsiella*.

To prevent the spread of infections, patients also should clean their hands very often, including:

- Before preparing or eating food
- Before touching their eyes, nose, or mouth
- Before and after changing wound dressings or bandages
- After using the restroom
- After blowing their nose, coughing, or sneezing
- After touching hospital surfaces such as bed rails, bedside tables, doorknobs, remote controls, or the phone.

K. pneumonia can be treated with antibiotics if the infections are not drug-resistant. Infections by *K. pneumoniae* can be difficult to treat because fewer antibiotics are effective against them. In such cases, a microbiology laboratory must run tests to determine which antibiotics will treat the infection. As with many bacteria, the recommended treatment has changed as the organism has developed resistances. The choice of a specific antimicrobial agent or agents depends on local susceptibility patterns and on the part of the body infected. For patients with severe infections, a prudent approach is the use of an initial short course (48–72 h) of combination therapy, followed by a switch to a specific monotherapy once the susceptibility pattern is known for the specific patient.

If the specific *Klebsiella* in a particular patient does not show antibiotic resistance, then the antibiotics used to treat such susceptible isolates include ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanate, ceftazidime, cefepime, levofloxacin, norfloxacin, gatifloxacin, moxifloxacin, meropenem and ciprofloxacin. Some experts recommend the use of meropenem for patients with ESBL-producing *Klebsiella*. The claim is that meropenem produces the best bacterial clearing.

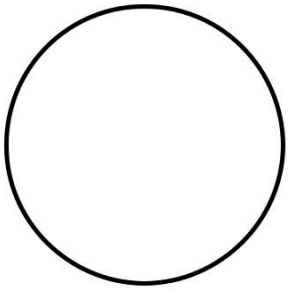
The use of antibiotics is usually not enough. Surgical clearing (frequently done as interventional radiology drainage) is often needed after the patient is started on antimicrobial agents.

Surname_____ Date_____

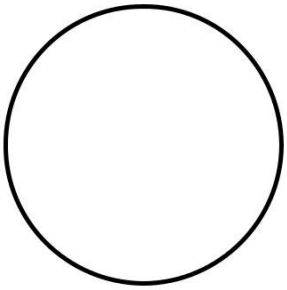
Protocol № 40

Theme: Microbiological diagnosis of diphtheria.

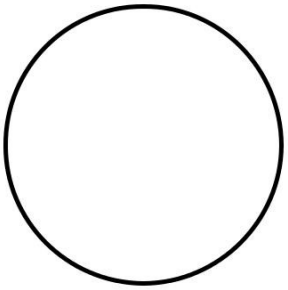
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Corynebacterium diphtheriae (Gram stain)



Corynebacterium diphtheriae (Neisser stain)



Corynebacterium diphtheriae (Loeffler stain with methylene blue)

II. Study nutrient media for cultivation of Corynebacterium diphtheriae:

Enrichment media:

a) Roux medium (clotted serum);

b) Loeffler medium (clotted serum + meat peptone agar + glucose).

Selective media:

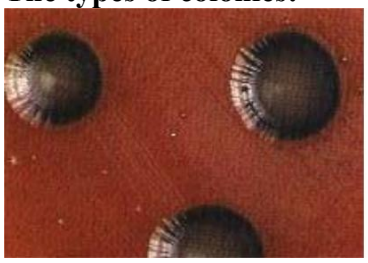
a) Tinsdal-Sadikova medium (cystin tellurite serum medium);

b) blood tellurite agar.

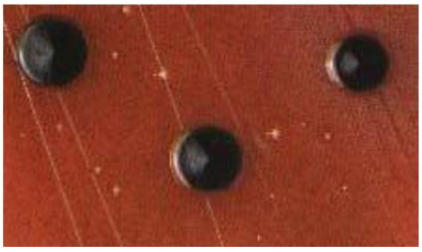
III. Study culture characteristic features of C. diphtheriae variants:

Properties	Biovar		
	gravis	intermedius	mitis
Growth on tellurite agar	Large dry greysh-black colonies with undulate edge (daisy head)	Small dry greysh-black colonies with translucent periphery, convex centre and undulate edge (frog egg colony)	Small smooth shiny black flat colonies with central elevation and entire edge
Growth in broth	Pelicle, turbidity, granular deposit	Turbidity following by clearing and granular deposit	Diffuse turbidity with pellicle later on
Hemolysis on blood agar	_+	+	+

The types of colonies:



Gravis: daisy head colony with grayish black centre and semi translucent periphery



Mitis: shiny black, flat colonies with central elevation (poached-egg colony)

Intermedius: frog egg colonies with dull granular centre and smooth glistening periphery

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

IV. Study biochemical properties of C. diphtheriae compare to diphtheroids and other corynebacteria:

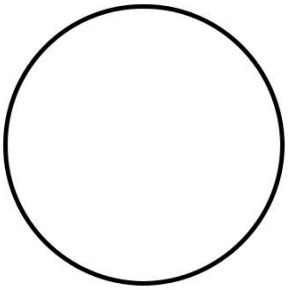
Tests	**Corynebacterium diphtheriae**		**Corynebacterium pseudodiphthericum** (Hofmann rod)	**Corynebacterium xerosis** (diphtheroids)
	gravis	mitis		
Glucose	acid	acid	-	acid
Sucrose	-	-	-	acid
Starch	acid	-	-	-
Cysteinase test with cystein	Blackness of medium around dril		-	-
Urease test with urea	-	-	+redness	

V. Study specific therapy of diphtheria (diphtheria anti-toxin) and specific profilaxis (formaldehyde-inactivated diphtheria toxin – **toxoid, the DPT (diphtheria, pertussis, tetanus) vaccine).**

VI. Study antibiotics for therapy of diphtheria: Erythromycin (orally or by injection), procaine penicillin G given intramuscularly. Patients with allergies to penicillin G or erythromycin can use rifampin or clindamycin.

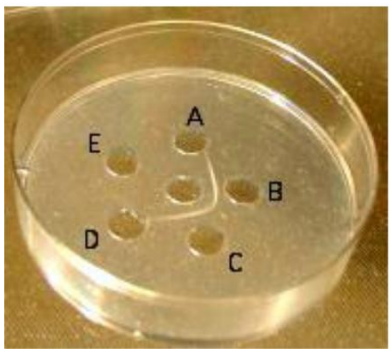
IV. Self-work of students:

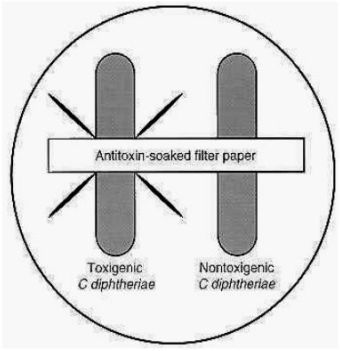
- stain a smear after Gram, microscopy

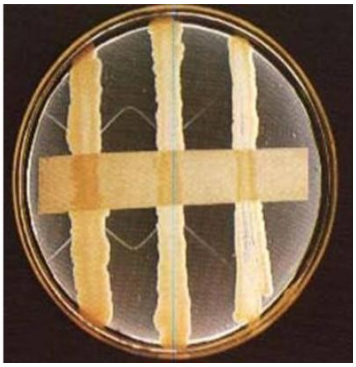


Gram stain

- study assay for toxigenicity of C. diphtheriae (the Elek immunodiffusion test):







Result: _____.

V. Study the scheme of laboratory diagnosis of diphtheria.

Specimen: secretions of the nose, throat (tonsil), pharynx, larynx, wounds, blood, skin.

1. Microscopy:

- Methylene blue stain shows metachromatic granules.
- Gram stain shows Gram-positive pleomorphic rods arranged in “Chinese letter” formations

2. Bacteriological methods.

3. **Detection of exotoxin production** by gel precipitation tests.

ADDING THEORETICAL MATERIAL

Corynebacterium diphtheriae infects the nasopharynx or skin. Toxigenic strains secrete a potent exotoxin which may cause diphtheria. The symptoms of diphtheria include pharyngitis, fever, swelling of the neck or area surrounding the skin lesion. Diphtheritic lesions are covered by a pseudomembrane. The toxin is distributed to distant organs by the circulatory system and may cause paralysis and congestive heart failure.

Corynebacterium diphtheriae is a nonmotile, noncapsulated, club-shaped, Gram-positive bacillus. Toxigenic strains are lysogenic for one of a family of corynebacteriophages that carry the structural gene for diphtheria toxin, *tox*. *Corynebacterium diphtheriae* is classified into biotypes (mitis, intermedius, and gravis) according to colony morphology, as well as into lysotypes based upon corynebacteriophage sensitivity. Most strains require nicotinic and pantothenic acids for growth; some also require thiamine, biotin, or pimelic acid. For optimal production of diphtheria toxin, the medium should be supplemented with amino acids and must be deferrated.

Asymptomatic nasopharyngeal carriage is common in regions where diphtheria is endemic. In susceptible individuals, toxigenic strains cause disease by multiplying and secreting diphtheria toxin in either nasopharyngeal or skin lesions. The diphtheritic lesion is often covered by a pseudomembrane composed of fibrin, bacteria, and inflammatory cells. Diphtheria toxin can be proteolytically cleaved into two fragments: a N-terminal fragment A (catalytic domain), and fragment B (transmembrane and receptor binding domains). Fragment A catalyzes the NAD^+-dependent ADP-ribosylation of elongation factor 2, thereby inhibiting protein synthesis in eukaryotic cells. Fragment B binds to the cell surface receptor and facilitates the delivery of fragment A to the cytosol.

Protective immunity involves an antibody response to diphtheria toxin following clinical disease or to diphtheria toxoid (formaldehyde-inactivated toxin) following immunization.

Corynebacterium diphtheriae is spread by droplets, secretions, or direct contact. *In situ* lysogenic conversion of nontoxigenic strains to a toxigenic phenotype has been documented. Infection is spread solely among humans, although toxigenic strains have been isolated from horses. In regions where immunization programs are maintained, isolated outbreaks of disease are often associated with a carrier who has recently visited a subtropical region where diphtheria is endemic. Large-scale outbreaks of disease may occur in populations where active immunization programs are not maintained.

Clinical diagnosis depends upon culture-proven toxigenic *C. diphtheriae* infection of the skin, nose, or throat combined with clinical signs of nasopharyngeal diphtheria (e.g., sore throat, dysphagia, bloody nasal discharge, pseudomembrane). Toxigenicity is identified by a variety of *in vitro* (e.g., gel immunodiffusion, tissue culture) or *in vivo* (e.g., rabbit skin test, guinea pig challenge) methods.

Immunization with diphtheria toxoid is extraordinarily effective. Diphtheria patients must be promptly treated with antitoxin to neutralize circulating diphtheria toxin.

Diphtheria is a paradigm of the toxigenic infectious diseases. In 1883, Klebs demonstrated that *Corynebacterium diphtheriae* was the agent of diphtheria. One year later, Loeffler found that the organism could only be cultured from the nasopharyngeal cavity, and postulated that the damage to internal organs resulted from a soluble toxin. By 1888, Roux and Yersin showed that animals injected with sterile filtrates of *C. diphtheriae* developed organ pathology indistinguishable from that of human diphtheria; this demonstrated that a potent exotoxin was the major virulence factor.

Diphtheria is most commonly an infection of the upper respiratory tract and causes fever, sore throat, and malaise. A thick, gray-green fibrin membrane, the pseudomembrane, often forms over the site(s) of infection as a result of the combined effects of bacterial growth, toxin production, necrosis of underlying tissue, and the host immune response. Recognition that the systemic organ damage was due to the action of diphtheria toxin led to the development of both an effective antitoxin-based therapy for acute infection and a highly successful toxoid vaccine.

Although toxoid immunization has made diphtheria a rare disease in those regions where public health standards mandate vaccination, outbreaks of diphtheria still occur in nonimmunized and immunocompromised groups. In marked contrast, widespread outbreaks of diphtheria reaching epidemic proportions have been observed in those regions where active immunization programs have been halted.

There are two types of clinical diphtheria: nasopharyngeal and cutaneous. Symptoms of pharyngeal diphtheria vary from mild pharyngitis to hypoxia due to airway obstruction by the pseudomembrane.

The skin lesions in cutaneous diphtheria are usually covered by a gray-brown pseudomembrane. Life-threatening systemic complications, principally loss of motor function (e.g., difficulty in swallowing) and congestive heart failure, may develop as a result of the action of diphtheria toxin on peripheral motor neurons and the myocardium.

The clinical diagnosis of diphtheria requires bacteriologic laboratory confirmation of toxigenic *C. diphtheriae* in throat or lesion cultures. For primary isolation, a variety of media may be used: Loeffler agar, Mueller-Miller tellurite agar, or Tinsdale tellurite agar. Sterile cotton-tipped applicators are used to swab the pharyngeal tonsils or their beds. Calcium alginate swabs may be inserted through both nares to collect nasopharyngeal samples for culture. Since diphtheritic lesions are often covered with a pseudomembrane, the surface of the lesion may have to be carefully exposed before swabbing with the applicator.

Following initial isolation, *C. diphtheriae* may be identified as mitis, intermedius, or *gravis* biotype on the basis of carbohydrate fermentation patterns and hemolysis on sheep blood agar plates. The toxigenicity of *C. diphtheriae* strains is determined by a variety of *in vitro* and *in vivo* tests. The most common *in vitro* assay for toxigenicity is the Elek immunodiffusion test. This test is based on

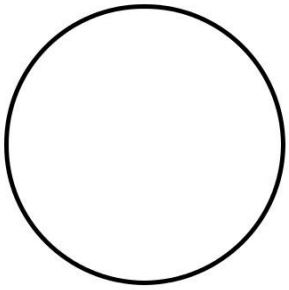
the double diffusion of diphtheria toxin and antitoxin in an agar medium. A sterile, antitoxin-saturated filter paper strip is embedded in the culture medium, and *C.diphtheriae* isolates are streak-inoculated at a 90° angle to the filter paper. The production of diphtheria toxin can be detected within 18 to 48 hours by the formation of a toxin-antitoxin precipitin band in the agar. Alternatively, many eukaryotic cell lines (e.g., African green monkey kidney, Chinese hamster ovary) are sensitive to diphtheria toxin, enabling *in vitro* tissue culture tests to be used for detection of toxin. Several sensitive *in vivo* tests for diphtheria toxin have also been described (e.g., guinea pig challenge test, rabbit skin test).

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _ _

Protocol № 41

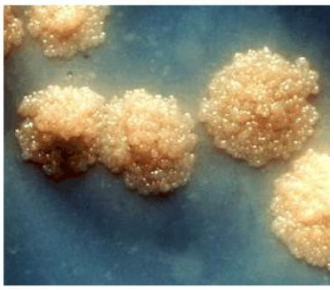
Theme: Microbiological diagnosis of tuberculosis.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



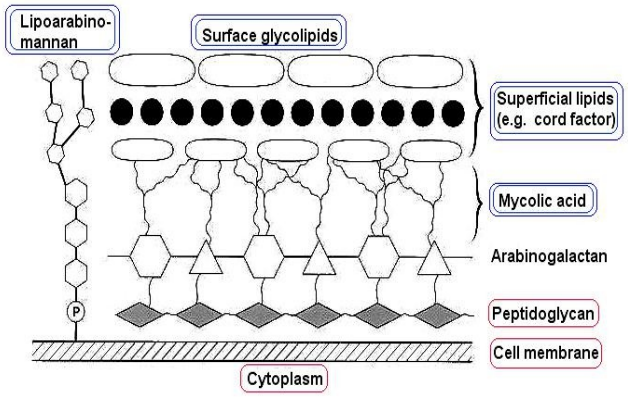
Mycobacterium tuberculosis (Ziehl-Neelsen stain)

Serpentine cords stain



Colonies of Mycobacterium tuberculosis

II. Study complex cell wall structure of mycobacteria:



II. Study nutrient media for cultivation of M.tuberculosis: glycerol broth, Lowenstain-Jensen medium, glycerol-potato agar, Petraniani agar.

III. Stude biological preparations for allergic test (**tuberulin** or **PPD (purified protein derivative) for Mantoux test)** and specific profilaxis of tuberculosis (**BCG** vaccine (Bacillus of Calmette and Guerin).

IV. Study antimicrobial drugs for therapy of tuberculosis:

- First line: isoniazid, rifampin, pyrazinamide, ethambutol, streptomycin (injectables only)
- Second line: cycloserine, kanamycin, capreomycin, rifabutin, fluoroquinolones (levofloxacin), viomycin, clarithromycin, azithromycin.

V. Study the scheme of laboratory diagnosis of tuberculosis.
Specimens: sputum, bronchial or gastric washings, pleural fluid, urine, or cerebrospinal fluid.
1. Microscopy: detection of acid-fast bacilli via the Ziehl-Neelsen method.
2. Bacteriological method.
M. tuberculosis can be differentiated from most other mycobacteria by the production of niacin.
3. Polymerase chain reaction (PCR).
4. Tuberculin skin test (Mantoux test or PPD skin test) detects **delayed hypersensitivity (cell cytotoxity).** A positive test indicates that the person has developed a **cellular immunity (T cells)** as a result of either a **previous or a current infection**.

ADDING THEORETICAL MATERIAL

Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. The disease affects 1.8 billion people/year which is equal to one-third of the entire world population.

Mycobacterium tuberculosis is the etiologic agent of tuberculosis in humans. Humans are the only reservoir for the bacterium.

Mycobacterium bovis is the etiologic agent of TB in cows and rarely in humans. Both cows and humans can serve as reservoirs. Humans can also be infected by the consumption of unpasteurized milk. This route of transmission can lead to the development of extrapulmonary TB, exemplified in history by bone infections that led to hunched backs.

Other human pathogens belonging to the *Mycobacterium* genus include *Mycobacterium avium* which causes a TB-like disease especially prevalent in AIDS patients, and *Mycobacterium leprae,* the causative agent of leprosy.

Mycobacterium tuberculosis (MTB) was the cause of the "White Plague" of the 17th and 18th centuries in Europe. During this period nearly 100 percent of the European population was infected with MTB, and 25 percent of all adult deaths were caused by MTB (Note: The White Plague is not to be confused with the "Black Plague", which was caused by *Yersinia pestis* and occurred about 3 centuries earlier).

General Characteristics. *Mycobacterium tuberculosis* is a fairly large nonmotile rod-shaped bacterium distantly related to the Actinomycetes. Many non pathogenic mycobacteria are components of the normal flora of humans, found most often in dry and oily locales. The rods are 2-4 micrometers in length and 0.2-0.5 um in width.

Mycobacterium tuberculosis is an obligate aerobe. For this reason, in the classic case of tuberculosis, MTB complexes are always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological characteristic that may contribute to its virulence.

Two media are used to grow MTB: Middlebrook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium. MTB colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing MT. It takes 4-6 weeks to get visual colonies on either type of media.

Chains of cells in smears made from in vitro-grown colonies often form distinctive serpentine cords. This observation was first made by Robert Koch who associated cord factor with virulent strains of the bacterium.

MTB is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. If a Gram stain is performed on MTB, it stains very weakly Gram-positive or not at all (cells referred to as "ghosts").

Mycobacterium species are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds. One acid-fast staining method for *Mycobacterium tuberculosis* is the Ziehl-Neelsen stain. When this method is used, the MTB. smear is fixed, stained with carbol-fuchsin (a pink dye), and decolorized with acid-alcohol. The smear is counterstained with methylene-blue or certain other dyes. Acid-fast bacilli appear pink in a contrasting background.

The cell wall structure of *Mycobacterium tuberculosis* deserves special attention because it is unique among procaryotes, and it is a major determinant of virulence for the bacterium. The cell wall complex contains peptidoglycan, but otherwise it is composed of complex lipids. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of MTB's cell wall consists of three major components, mycolic acids, cord factor, and wax-D.

Mycolic acids are unique alpha-branched lipids found in cell walls of *Mycobacterium* and *Corynebacterium.* They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. Mycolic Acids are thought to be a significant determinant of virulence in MTB. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum.

Cord Factor is responsible for the serpentine cording mentioned above. Cord factor is toxic to mammalian cells and is also an inhibitor of PMN migration. Cord factor is most abundantly produced in virulent strains of MTB.

Wax-D in the cell envelope is the major component of Freund's complete adjuvant (CFA).

The high concentration of lipids in the cell wall of *Mycobacterium tuberculosis* have been associated with these properties of the bacterium:

- Impermeability to stains and dyes
- Resistance to many antibiotics
- Resistance to killing by acidic and alkaline compounds
- Resistance to osmotic lysis via complement deposition
- Resistance to lethal oxidations and survival inside of macrophages.

TB infection means that MTB is in the body, but the immune system is keeping the bacteria under control. The immune system does this by producing macrophages that surround the tubercle bacilli. The cells form a hard shell that keeps the bacilli contained and under control. Most people with TB infection have a positive reaction to the tuberculin skin test. People who have TB infection but not TB disease are NOT infectious, i.e., they cannot spread the infection to other people. These people usually have a normal chest x-ray. TB infection is not considered a case of TB disease. Major similarities and differences between TB infection and TB disease are given in the table below.

Tuberculosis: Infection vs Disease

TB Infection	TB disease in lungs
MTB present	MTB present
Tuberculin skin test positive	Tuberculin skin test positive
Chest X-ray normal	Chest X-ray usually reveals lesion
Sputum smears and cultures negative	Sputum smears and cultures positive
No symptoms	Symptoms such as cough, fever, weight loss
Not infectious	Often infectious before treatment
Not defined as a case of TB	Defined as a case of TB

Predisposing factors for TB infection include:

- Close contact with large populations of people, i.e., schools, nursing homes, dormitories, prisons, etc.
- Poor nutrition
- iv drug use
- Alcoholism
- HIV infection is the № 1 predisposing factor for MTB infection. 10 percent of all HIV-positive individuals harbor MTB.

Stages of the Disease. The following stages that will be explained are for a MTB - sensitive host. It should be realized that, as stated previously, only a small percent of MTB infections progress to disease and even a smaller percent progress all the way to stage 5. Usually the host will control the infection at some point.

Disease progression depends on:

- Strain of MTB
- Prior exposure
- Vaccination
- Infectious dose
- Immune status of the host.

Stage 1

Droplet nuclei are inhaled. One droplet contains no more than 3 bacilli. Droplet nuclei are so small that they can remain air-borne for extended periods of time. The most effective (infective) droplet nuclei tend to have a diameter of 5 micrometers. Droplet nuclei are generated by during talking coughing and sneezing. Coughing generates about 3000 droplet nuclei. Talking for 5 minutes generates 3000 droplet nuclei but singing generates 3000 droplet nuclei in one minute. Sneezing generates the most droplet nuclei by far, which can spread to individuals up to 10 feet away.

Tuberculosis begins when droplet nuclei reach the alveoli. When a person inhales air that contains droplets most of the larger droplets become lodged in the upper respiratory tract (the nose and throat), where infection is unlikely to develop. However, the smaller droplet nuclei may reach the small air sacs of the lung (the alveoli), where infection begins.

Stage 2

Begins 7-21 days after initial infection. MTB multiplies virtually unrestricted within unactivated macrophages until the macrophages burst. Other macrophages begin to extravasate from peripheral blood. These macrophages also phagocytose MTB, but they are also unactivated and hence can not destroy the bacteria.

Stage 3

At this stage lymphocytes begin to infiltrate. The lymphocytes, specifically T-cells, recognize processed and presented MTB antigen in context of MHC molecules. This results in T-cell activation and the liberation of cytokines including gamma interferon (IFN). The liberation of IFN causes in the activation of macrophages. These activated macrophages are now capable of destroying MTB.

It is at this stage that the individual becomes tuberculin-positive. This positive tuberculin reaction is the result of the host developing a vigorous cell mediated immune (CMI) response. A CMI response must be mounted to control an MTB infection. An antibody mediated immune (AMI) will not aid in the control of a MTB infection because MTB is intracellular and if extracellular, it is resistant to complement killing due to the high lipid concentration in its cell wall.

Although a CMI response is necessary to control an MTB infection, it is also responsible for much of the pathology associated with tuberculosis. Activated macrophages may release lytic enzymes and reactive intermediates that facilitate the development of immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including Interleukin 1 (IL-l), tumor necrosis factor (TNF), and gamma IFN.

It is also at this stage that tubercle formation begins. The center of the tubercle is characterized by "caseation necrosis", meaning it takes on a semi-solid or "cheesy" consistency. MTB cannot multiply within these tubercles because of the low pH and anoxic environment. MTB can, however, persist within these tubercles for extended periods.

Stage 4

Although many activated macrophages can be found surrounding the tubercles, many other macrophages present remain unactivated or poorly activated. MTB uses these macrophages to replicate, and hence, the tubercle grows.

The growing tubercle may invade a bronchus. If this happens, MTB infection can spread to other parts of the lung. Similarly, the tubercle may invade an artery or other blood supply line. The hematogenous spread of MTB may result in extrapulmonary tuberculosis otherwise known as milliary tuberculosis. The name "milliary" is derived from the fact that metastasizing tubercles are about the same size as a millet seed, a grain commonly grown in Africa.

The secondary lesions caused by milliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes and peritoneum. These lesions are of two types:

1. Exudative lesions result from the accumulation of PMN's around MTB. Here the bacteria replicate with virtually no resistance. This situation gives rise to the formation of a "soft tubercle".

2. Productive or granulomatous lesions occur when the host becomes hypersensitive to tuberculoproteins. This situation gives rise to the formation of a "hard tubercle".

Stage 5

For unknown reasons, the caseous centers of the tubercles liquefy. This liquid is very conducive to MTB growth, and the organism begins to rapidly multiply extracellularly. After time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture. This results in cavity formation. This also allows MTB to spill into other airways and rapidly spread to other parts of the lung.

As stated previously, only a very small percent of MTB infections result in disease, and even a smaller percentage of MTB infections progress to an advanced stage. Usually the host will begin to control the infection at some point. When the primary lesion heals, it becomes fibrous and calcifies. When this happens the lesion is referred to as the Ghon complex. Depending on the size and severity, the Ghon complex may never subside. Typically, the Ghon complex is readily visible upon chest X-ray.

Small metastatic foci containing low numbers of MTB may also calcify. However, in many cases these foci will contain viable organisms. These foci are referred to Simon foci. The Simon foci are also visible upon chest X-ray and are often the site of disease reactivation.

Virulence Mechanisms and Virulence Factors

The virulence of *Mycobacterium tuberculosis* is extraordinarily complicated and multifaceted. Although the organism apparently does not produce any toxins, it possesses a huge repertoire of structural and physiological properties that have been recognized for their contribution to mycobacterial virulence and to pathology of tuberculosis. Some of the general properties of *Mycobacterium tuberculosis* that render it virulent are discussed below. This section is followed by a more specific discussion of the complex array of virulence determinants exhibited by this pathogen. This should not be surprising for one of the most successful human pathogens to have evolved.

Some general properties of *Mycobacterium tuberculosis* that contribute to its virulence. Special mechanisms for cell entry. The tubercle bacillus can bind directly to mannose receptors on macrophages via the cell wall-associated mannosylated glycolipid, LAM, or indirectly via certain complement receptors or Fc receptors.

Intracellular growth. MTB can grow intracellularly. This is an effective means of evading the immune system. In particular, antibodies and complement are ineffective. Once MTB is phagocytosed, it can inhibit phagosome-lysosome fusion by secretion of a protein that modifies the phagosome membrane. It may remain in the phagosome or escape from the phagosome, in either case, finding a protected environment for growth in the macrophage.

Detoxification of oxygen radicals. MTB interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by three mechanisms:

1. Compounds including glycolipids, sulfatides and LAM down regulate the oxidative cytotoxic mechanism.
2. Macrophage uptake via complement receptors may bypass the activation of a respiratory burst.
3. The oxidative burst may be counteracted by production of catalase and superoxide dismutase enzymes.

Antigen 85 complex. This complex is composed of a group of proteins secreted by MTB that are known to bind fibronectin. These proteins may aid in walling off the bacteria from the immune system and may facilitate tubercle formation.

Slow generation time. Because of MTB's slow generation time, the immune system may not readily recognize the bacteria or may not be triggered sufficiently to eliminate them.

High lipid concentration in cell wall. This accounts for impermeability and resistance to antimicrobial agents, resistance to killing by acidic and alkaline compounds in both the intracellular and extracellular environment, and resistance to osmotic lysis via complement deposition and attack by lysozyme.

Cord factor. Cord factor (trehalose 6, 6' dimycolate) is a glycolipid found in the cell walls of mycobacteria, which causes the cells to grow in serpentine cords. It is primarily associated with virulent strains of MTB. It is known to be toxic to mammalian cells and to be an inhibitor of PMN migration. Its exact role in MTB virulence is unclear, although it has been shown to induce granulomatous reactions identical to those seen in TB.

M. tuberculosis virulence is studied both in tissue culture, using macrophages, dendritic cells or pneumocytes, and in animal models, primarily mice. Tissue culture models are easier and more humane to work with and give faster results, but they are limited to studying early stages of infection. Ultimately, only in animal models can all the stages of TB be studied.

Since sequencing the *Mycobacterium tuberculosis* genome in 1998, genetic methods are more commonly used to study the bacterium's virulence. The usual genetic approaches to study virulence are to disrupt, inactivate, modify, delete or complement a gene and assess the effects in the macrophage or mouse model.

Oxidative stress proteins. Most aerobic organisms have enzymes that degrade peroxides and superoxide, which are normal byproducts of aerobic respiration, but also are toxic oxygen radicals. These enzymes, generally superoxide dismutases, catalases and peroxidases, are also important for the response to various external oxidative stresses. Since phagocytic cells produce oxygen radicals during the respiratory burst to kill invading bacteria, it is not surprising that these enzymes may contribute to *M. tuberculosis* virulence. Enzymes found in *M. tuberculosis* that combat oxygen radicals include AhpC, an alkyl hydroperoxide reductase that detoxifies organic hydroxyperoxides, and SodA and SodC, two species of superoxide dismutase that degrade superoxides, which

are normal by-products of aerobic respiration and are also produced by the phagocytic respiratory burst.

Nitrate reductase. *M. tuberculosis* was originally thought to be an obligate aerobe, but there are numerous experimental indicators that the bacterium can grow in microareophilic environments, especially during the later stages of infection, e.g., in lung granulomas. Wild type *M. tuberculosis* has been shown to possess an inducible nitrate reductase (NarG encoded by *narG*) which allows respiration using NO_3 as a final electron acceptor. If anaerobic or microareophilic growth is an important feature of *M. tuberculosis* physiology during infection, the existence of nitrate reductase could be a significant factor in sustaining growth under these conditions.

Adherence. The specific bacterial adhesins involved in the complex interaction between *M. tuberculosis* and the human host are largely unknown. Nevertheless, a few potential adherence factors have been considered, including the heparin-binding hemagglutin (HbhA), a fibronectin-binding protein, and a polymorphic acidic, glycine-rich protein, called PE-PGRS. HbhA is a surface-exposed protein that is involved in binding *Mycobacterium tuberculosis* to epithelial cells but not to phagocytes. It could be involved in extrapulmonary spread after the initial long-term colonization of the host. Fibronectin-binding proteins (FbpA), first identified as the α-antigen (Antigen 85 complex), can bind to the extracellular matrix protein fibronectin in vitro. This property may represent a mechanism of tissue colonization. The surface-exposed PE-PGRS proteins found in *M. tuberculosis* and *Mycobacterium bovis* also show fibronectin-binding properties.

It has been shown recently that *Mycobacterium tuberculosis* produces pili during human infection, which could be involved in initial colonization of the host.

Clinical Identification and Diagnosis of Tuberculosis. The diagnosis of tuberculosis requires detection of acid-fast bacilli in sputum via the Ziehl-Neelsen method as previously described.

The organisms must then be cultured from sputum. First, the sputum sample is treated with NaOH. This kills other contaminating bacteria but does not kill the MTB present because cells are resistant to alkaline compounds by virtue of their lipid layer.

The media used for growth of MTB and the the resulting colony morphology have been described previously. However, methods of culturing can take 4-6 weeks to yield visible colonies. As a result, another method is commonly used call the BACTEC System. The media used in the BACTEC system contains radio-labeled palmitate as the sole carbon source. As MTB multiplies, it breaks down the palmitate and liberates radio-labeled CO_2. Using the BACTEC system, MTB growth can be detected in 9-16 days vs 4-6 weeks using conventional media.

Skin Testing is performed as the tuberculin or Mantoux test. PPD (purified protein derivative) is employed as the test antigen in the Mantoux test. PPD is generated by boiling a culture of MTB, specifically Old Tuberculin (OT). 5 TU (tuberculin units), which equals 0.000l mg of PPD, in a 0.1 ml volume is intracutaneously injected in the forearm. The test is read within 48-72 hours.

Administering the Mantoux test. The test is considered positive if the diameter of the resulting lesion is 10 mm or greater. The lesion is characterized by erythema (redness) and swelling and induration (raised and hard). 90% of people that have a lesion of 10 mm or greater are currently infected with MTB or have been previously exposed to MTB. 100% of people that have a lesion of 15 mm or greater are currently infected with MTB or have been previously exposed to MTB.

False positive tests usually manifest themselves as lesser reactions. These lesser reactions could indicate prior exposure or infection with other mycobacteria or vaccination with BCG. However, in places were the vaccine is not used, lesser reactions should be regarded as highly suspicious.

False negatives are rarer than false positives but are especially common in AIDS patients as they have an impaired CMI response. Other conditions such as malnutrition, steroids, etc., can rarely result in a false negative reaction.

Tuberculosis Treatment. Because administration of a single drug often leads to the development of a bacterial population resistant to that drug, effective regimens for the treatment of TB must contain multiple drugs to which the organisms are susceptible. When two or more drugs are used simultaneously, each helps prevent the emergence of tubercle bacilli resistant to the others. However, when the in vitro susceptibility of a patient's isolate is not known, which is generally the case at the beginning of therapy, selecting two agents to which the patient's isolate is likely to be susceptible can be difficult, and improper selection of drugs may subsequently result in the development of additional drug-resistant organisms.

Hence, tuberculosis is usually treated with four different antimicrobial agents The course of drug therapy usually lasts from 6-9 months. The most commonly used drugs are rifampin (RIF) isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) or streptomycin (SM). When adherence with the regimen is assured, this four-drug regimen is highly effective. Based on the prevalence and characteristics of drug-resistant organisms, at least 95% of patients will receive an adequate regimen (at least two drugs to which their organisms are susceptible) if this four-drug regimen is used at the beginning of therapy. Furthermore, a patient who is treated with the four-drug regimen, but who defaults therapy, is more likely to be cured and not relapse when compared with a patient treated for the same length of time with a three-drug regimen.

Drugs used to treat TB disease. From left to right isoniazid, rifampin, pyrazinamide, and ethambutol. Streptomycin (not shown) is given by injection.

Prevention. A vaccine against MTB is available. It is called BCG (Bacillus of Calmette and Guerin, named after the two Frenchmen that developed it). BCG consists of a live attenuated strain derived from *Mycobacterium bovis*. This strain of *Mycobacterium* has remained avirulent for over 60 years.

The vaccine is not 100 % effective. Studies suggest a 60-80% effective rate in children.

Multidrug-Resistant Tuberculosis (MDR TB) and Extensively Drug-Resistant Tuberculosis (XDR TB). Resistance to anti-TB drugs can occur when these drugs are misused or mismanaged. Examples include when patients do not

complete their full course of treatment; when health-care providers prescribe the wrong treatment, the wrong dose, or length of time for taking the drugs; when the supply of drugs is not always available; or when the drugs are of poor quality.

Multidrug-resistant tuberculosis (MDR TB) is TB that is resistant to at least two of the best anti-TB drugs, isoniazid and rifampicin. These drugs are considered first-line drugs and are used to treat all persons with TB disease.

Extensively drug resistant TB (XDR TB) is a relatively rare type of MDR TB. XDR TB is defined as TB which is resistant to isoniazid and rifampin, plus resistant to any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). Because XDR TB is resistant to first-line and second-line drugs, patients are left with less effective treatment options, and cases often have worse treatment outcomes.

Both MDR TB and XDR TB are more common in TB patients that do not take their medicines regularly or as prescribed, or who experience reactivation of TB disease after having taken TB medicine in the past.

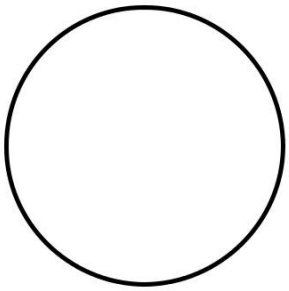
Persons with HIV infection or other conditions that can compromise the immune system are at highest risk for MDR TB and XDR TB. They are more likely to develop TB disease once infected and have a higher risk of death from disease.

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

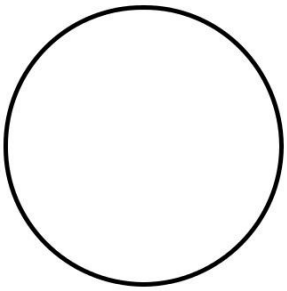
Protocol № 42

Theme: Microbiological diagnosis of *whooping cough and Haemophylus influenzae infections.*

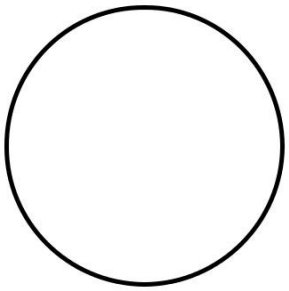
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Bordetella pertusis (Gram stain)



Bordetella parapetusis (Gram stain)



***Haemophylus influenzae* (Gram stain)**

II. Study nutrient media for cultivation of Bordetella:
   a) casein-charcoal agar;
   b) Bordeux-Gengou medium (glycerol-potato-blood agar).

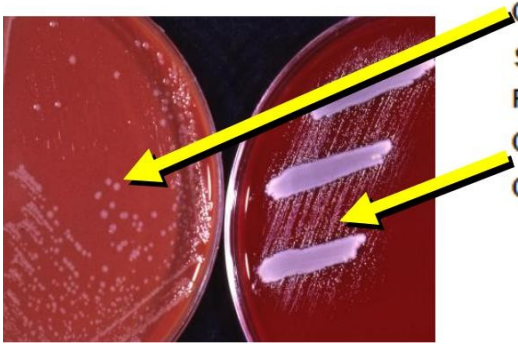
III. Study nutrient media for cultivation of Haemophylus influenzae:
   a) chocolate agar;
   b) Mueller-Hinton agar.

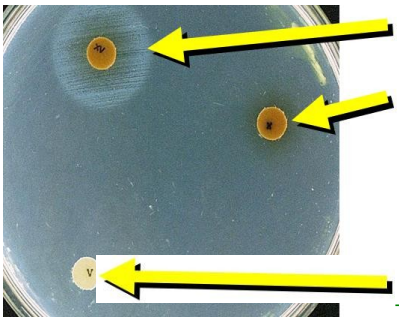
IV. Study biological preparations for laboratory diagnosis, specific prophylaxis and therapy of: whooping cough: agglutinating sera for agglutination test; inactivated monovaccine; DTP vaccine with inactivated B.pertusis bacteria; human immunoglobulin.
Haemophylus influenzae infection: conjugated H. influenzae b vaccine (capsular antigen+ diphtheria or tetanus toxoids).

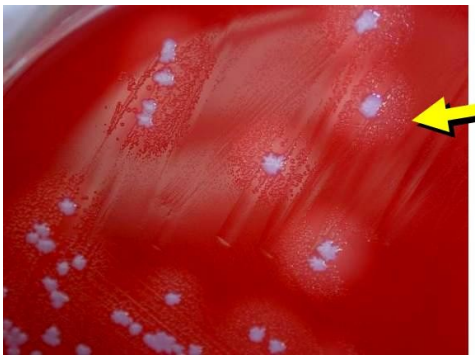
V. Study the scheme of laboratory diagnosis of **whooping cough.**

Specimen: nasopharyngeal aspirate (cough plate method), nasopharyngeal swab.
1. IF (detection of antigen).
2. Culture on Bordet-Gengou medium. The organism grows as small transparent colonies. Time of growth: B. pertusis – 48-72 h, B. parapertusis – 24-72 h.
Identification: slide AT to distinguish from *B. parapertussis* and *B. bronchosepticus*.
Biochemical tests: B.pertusis: urease-negative, citrate-negative,
B.parapertusis: urease-positive, citrate-negative.
3. Serology (detection of antibodies): AT, CFT.

VI. Study the scheme of laboratory diagnosis of **Haemophylus influenzae** infections.
Specimen: liquor.
1. **IF** (detection of capsular antigen).
Specimen: nasopharyngeal swab, pus, sputum, blood, liquor.
2. Culture: Chocolate agar.
Identification: Species of Haemophilus require either or both of the two factors for growth and can be used to differentiate the species. The X factor is haemin and the V factor is nicotinamide adenine dinucleotide (NAD). The factors are incorporated into filter paper disks which are placed on a blood free medium previously inoculated with the organism under test. After incubation, the presence or absence of growth around the disks indicates a requirement for that particular factor.







Biochemical tests:
- catalase-positive,
- urease-positive,
- indol-positive,
- nitrate reduction-positive,
- nonhemolytic,
- H_2S-negative,
- oxydase-negative.

Serology(detection of capsular antigen): AT, ELISA, immunoelectrophoresis.

ADDING THEORETICAL MATERIAL

Bordetella pertussis is a Gram-negative, aerobic, pathogenic, encapsulated coccobacillus of the genus *Bordetella*, and the causative agent of pertussis or whooping cough. Unlike *B. bronchiseptica*, *B. pertussis* is not motile. Its virulence factors include pertussis toxin, filamentous haemagglutinin, pertactin, fimbria, and tracheal cytotoxin. The bacterium is spread by airborne droplets; its incubation period is 9–10 days on average (range 6–20 days). Humans are the only known reservoir for *B. pertussis*.

Diagnosis. A nasopharyngeal or an oropharynx swab is sent to the bacteriology laboratory for Gram stain (Gram-negative, coccobacilli, diplococci arrangement), growth on Bordet-Gengou agar to select for the organism, which shows mercury drop-like colonies. *B. pertussis* can also be detected by PCR, which is more sensitive than culture.

Several diagnostic tests are available, especially ELISA kits. These are designed to detect FHA and/or PT antibodies of IgG, IgA, or IgM. Some kits use a combination of antigens which lead to a higher sensitivity, but might also make the interpretation of the results harder, since one cannot know which antibody has been detected.

The organism is oxidase positive, but urease, nitrate reductase, and citrate negative. It is also not motile.

Whooping cough (pertussis) is an infection of the respiratory systemcaused by the bacterium *Bordetella pertussis* (or *B. pertussis*). It mainly affects babies younger than 6 months old who aren't yet protected by immunizations, and kids 11 to 18 years old whose immunity has started to fade.

Whooping cough causes severe coughing spells, which can sometimes end in a "whooping" sound when the child breathes in.

The incubation period (the time between infection and the start of symptoms) for whooping cough is usually 7 to 10 days, but can be as long as 21 days.

The first symptoms of whooping cough are similar to those of a common cold:

- runny nose, sneezing, mild cough, low-grade fever.

After about 1 to 2 weeks, the dry, irritating cough evolves into coughing spells. During a coughing spell, which can last for more than a minute, a child may turn red or purple. At the end of a spell, the child may make the characteristic whooping sound when breathing in or may vomit. Between spells, the child usually feels well.

While many infants and younger kids with whooping cough develop the coughing fits and accompanying whoop, not all do. And sometimes babies don't cough or whoop as older kids do. Infants may look as if they're gasping for air with a reddened face and may actually stop breathing (this is called apnea) for a few seconds during very bad spells.

Adults and teens may have milder or different symptoms, such as a prolonged cough (rather than coughing spells) or coughing without the whoop.

The last stage consists of another few weeks of recovery with gradual clearing of symptoms. In some children, the recovery period can last for months.

Pertussis is highly contagious. The bacteria spread from person to person through tiny drops of fluid from an infected person's nose or mouth. These may become airborne when the person sneezes, coughs, or laughs. Others then can become infected by inhaling the drops or getting the drops on their hands and then touching their mouths or noses.

Infected people are most contagious during the earliest stages of the illness for up to about 2 weeks after the cough begins. Antibiotics shorten the period of contagiousness to 5 days following the start of antibiotic treatment.

Whooping cough can be prevented with the pertussis vaccine, which is part of the DTaP (diphtheria, tetanus, acellular pertussis) immunization.

DTaP immunizations are routinely given in five doses before a child's sixth birthday. For additional protection in case immunity fades, experts recommend that kids ages 11-18 get a booster shot of the new combination vaccine (called Tdap), ideally when they're 11 or 12 years old.

The Tdap vaccine is similar to DTaP but with lower concentrations of diphtheria and tetanus toxoid. It also should be given to adults who did not receive it as preteens or teens. The vaccine is also recommended for all pregnant women during the second half of each pregnancy, regardless of whether or not they had the vaccine before, or when it was last given.

Getting the vaccine is especially important for people who are in close contact with infants, because babies can develop severe and potentially life-threatening complications from whooping cough. An adult's immunity to whooping cough lessens over time, so getting vaccinated and protecting yourself against the infection also helps protect your infant or child from getting it.

As is the case with all immunization schedules, there are important exceptions and special circumstances. Your doctor will have the most current information.

People who live with or come into close contact with someone who has pertussis should receive antibiotics to prevent the spread of the disease, even if they've already been vaccinated against it. Young kids who have not received all five doses of the vaccine may need a booster dose if exposed to an infected family member.

Treatment. Whooping cough is treated with antibiotics. Many experts believe that antibiotics are most effective in shortening the length of the infection when they're given in the first stage of the illness, before coughing spells begin. But even if antibiotics are started later, they're still important because they can stop the spread of the pertussis infection to others. Ask your doctor whether preventive antibiotics or vaccine boosters for other family members are needed.

Some kids with whooping cough need to be treated in a hospital. Babies and younger children are more likely to be hospitalized because they're at greater risk for problems like pneumonia. Whooping cough can be life-threatening for infants younger than 6 months, so they almost always need hospital treatment.

Other potential complications include difficulty breathing, periods of apnea, needing oxygen (particularly during a coughing spell), and dehydration.

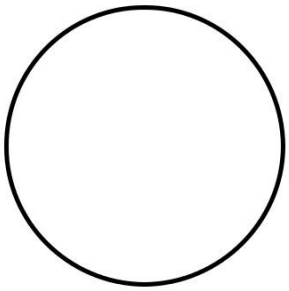
Kids with whooping cough may vomit or not eat or drink much because of the coughing. So offer smaller, more frequent meals and encourage your child to drink lots of fluids. Watch for signs of dehydration, including thirst, irritability, restlessness, lethargy, sunken eyes, a dry mouth and tongue, dry skin, crying without tears, and fewer trips to the bathroom to pee (or in infants, fewer wet diapers).

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

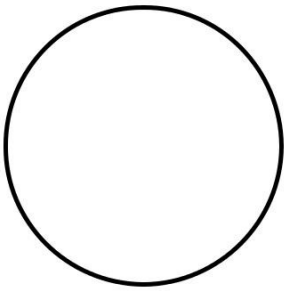
Protocol № 43

Theme: Microbiological diagnosis of anaerobic diseases.

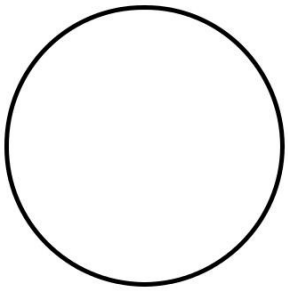
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



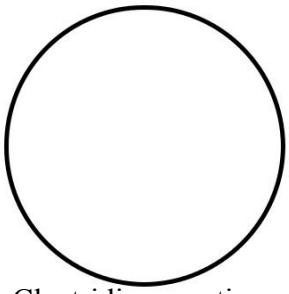
Clostridium tetani
(Gram stain)



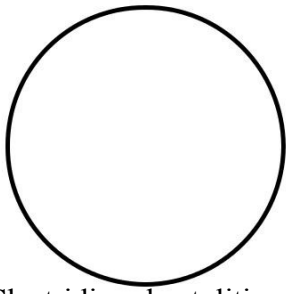
Clostridium botulinum
(Gram stain)



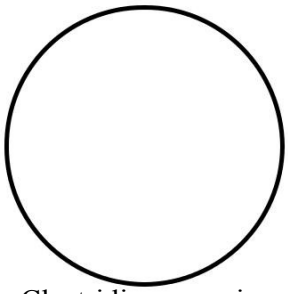
Clostridium perfringens
(Gram stain)



Clostridium septicum
(Gram stain)



Clostridium hystoliticum
(Gram stain)



Clostridium novyi
(Gram stain)

II. Study classification of obligative anaerobic bacteria:

Sporforming	**Nonsporforming anaerobs**	
	Gram negative	**Gram positive**
C.botulinum	**Rods**	
C.tetani	Bacteroides	Actinomyces
C.perfringens	Prevotella	Bifidobacterium
C.novyi	Fusobacterium	Eubacterium
C.septicum	Leptotrichia	Propionibacterium
C.histolyticum	**Cocci**	
C.dificille	Veilonella	Peptococcus
		Peptostreptococcus
	Spirochaetes	
		Treponema

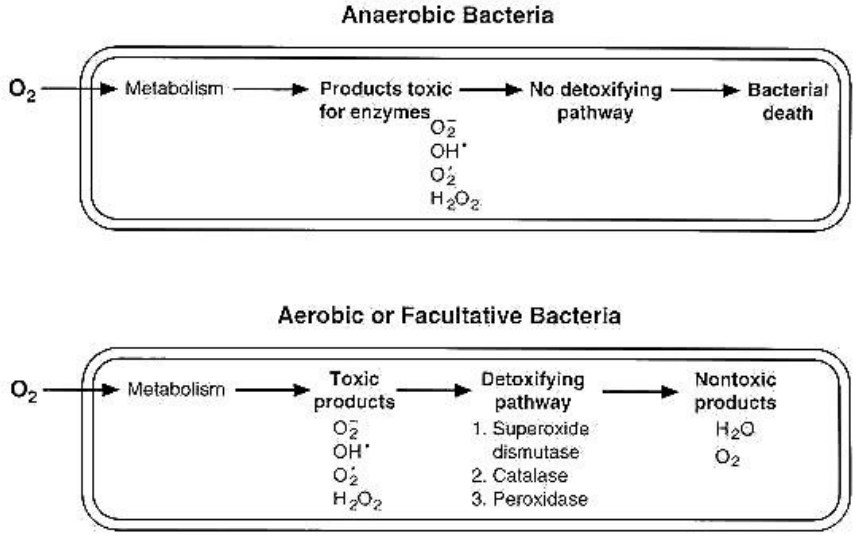
III. Study equipment and nutrient media for cultivation of anaerobic bacteria:
а) anaerostat, Petri dish presenting Fortner method;
б) Kitt-Tarozzi medium, Wilson-Bler medium, milk under oil;
в) Winial tube.

IV. Self-work of students: describe the growth on Kitt-Tarozzi medium; prepare a smear, stain after Gram, microscopy

Gram stain

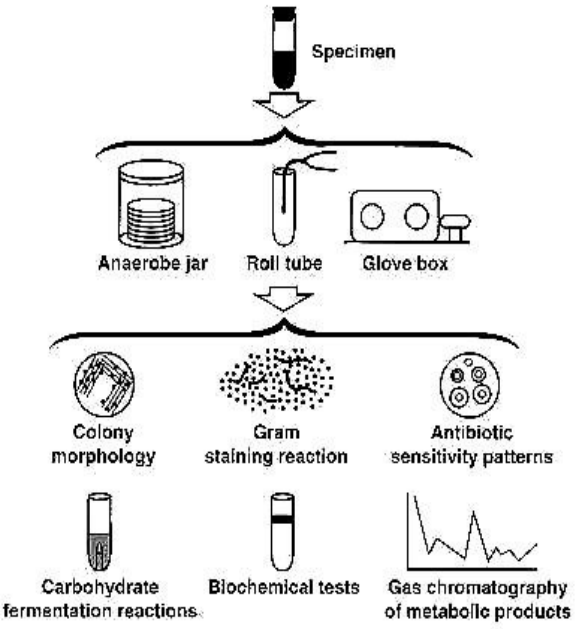
Result of growth on Kitt-Tarozzi medium: turbidity, sediment.

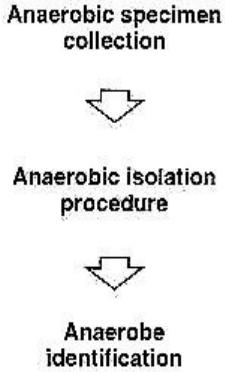
V. Study effects of oxygen on aerobic, anaerobic, and facultative anaerobic bacteria:



VI. Stude biological preparations for specific therapy and profilaxis of tetanus, gas gangrene and botulism:
a) tetanus antitoxin, botulinum antitoxic sera; gas gangrene antitoxic sera; b)
tetanus toxoid, as part of the DPT (DTP) vaccine or the DT (TD) vaccine.

VII. Study the scheme of laboratory diagnosis of tetanus, botulism and gas gandrene.





1. Microscopy: Gram stains of wound smears: don't often proove useful!
2. Culture on blood agar heated anerobically.
3. Biological methos (neutralization test): an anaerobically prepared culture may be injected into mice, with control mice protected by antitoxin.

ADDING THEORETICAL MATERIAL

Clostridium botulinum is a gram positive, obligate anaerobic, spore-forming, rod-shaped bacterium, are commonly found in soils and marine sediments throughout the world.

C. botulinum may be found in any region of the world. Since it is found in the soil, it may contaminate vegetables cultivated in or on the soil. It also colonizes the gastro-intestinal tract of fishes, birds and mammals.

Main Toxins. Although the seven neurotoxins (A, B, C, D, E, F and G) are genetically distinct, they possess similar molecular weights and have a common subunit structure. The toxins are synthesized as single chain polypeptides with a molecular mass of approximately 150 kDa. In this form, the toxin molecules have relatively little potency as neuromuscular agents. Neurotoxin activation requires a two-step modification in the tertiary structure of the protein.

Routes of exposure:

1) Oral - Foodborne botulism is caused by ingestion of food contaminated by a preformed neurotoxin of the bacterium Clostridium botulinum. Home-preserved foods containing fish, vegetables, or potatoes are often involved in outbreaks of botulism. High acid content foods are rarely involved. C. botulinum spores are heat-resistant but the toxin is heat-labile. Boiling food to ensure thorough heating of the interior should destroy the toxin

Infant botulism is a result of colonization of the intestinal tract after ingestion of spores of C. botulinum. The infant intestinal tract often lacks both the protective bacterial flora and the clostridium-inhibiting bile acids found in normal adult intestinal tract. Most cases occur before the age of 6 months.

2) Inhalation - recent concern about the use of C. botulinum neurotoxin aerosol in a terrorist attack has drawn attention to the potential risk to public health and the need for preventive measures to be developed.

3) Dermal - neither the spores nor the neurotoxins are able to penetrate intact skin. However damaged skin may be affected.

4) Eye - no data available.

5) Parenteral - wound botulism as the result of spore inoculation though open wound.

Botulism is characterised by symmetrical, descending, flaccid paralysis of motor and autonomic nerves usually beginning with cranial nerves. It occurs when neuromuscular transmission is interrupted by a protein neurotoxin produced by the spore-forming, obligate anaerobic bacterium Clostridium botulinum. Paralysis begins with the cranial nerves, then affects the upper extremities, the respiratory muscles, and, finally, the lower extremities in a proximal-to-distal pattern. In severe cases, extensive respiratory muscle paralysis leads to ventilatory failure and death unless supportive care is provided.

There are five clinical categories of botulism:

1) foodborne botulism;

2) wound botulism;

3) infant botulism;

4) adult infectious botulism;

5) inadvertent, following botulinum toxin injection.

Foodborne botulism. Onset generally occurs 18 to 36 hours after exposure (range, 6 hours to 8 days). Initial symptoms can include nausea, vomiting, abdominal cramps or diarrhoea. After the onset of neurologic symptoms, constipation is typical. Dry mouth, blurred vision, and diplopia are usually the earliest neurologic symptoms. They are followed by dysphonia, dysarthria, dysphagia, and peripheral muscle weakness. Symmetric descending paralysis is characteristic of botulism. Wound botulism This can be defined as clinical evidence of botulism following lesions, with a resultant infected wound and no history suggestive of foodborne illness. Except for the gastrointestinal symptoms, the clinical manifestations are similar to those seen in foodborne botulism. However, the incubation period is much longer as time is required for the incubation of spores, growth of clostridium and release of toxins (4 to 14 days).

Infant botulism. This is caused by the absorption of toxin produced by Clostridium botulinum that colonize the intestinal tracts of infants under one year of age. It is often associated with ingestion of honey and the first clinical sign is usually constipation. After a few weeks, progressive weakness and poor feeding are observed. The weakness is symmetrical and descending. It evolves over hours or several days. The infant is afebrile and has a weak cry, has either absent or diminished spontaneous movements, decreased sucking, floppy head and decreased motor response to stimuli. The autonomic nervous system manifestations include dry mucous membranes, urinary retention, diminished gastro-intestinal motility, fluctuation of heart rate, and changes in skin colour. Duration of hospitalisation may last from a few days to six months.

Adult infectious botulism. It occurs as a result of intestinal colonization with C. botulinum and in vivo toxin production in a manner similar to that of infant botulism. These patients often have a history of abdominal surgery, achlorhydria, Crohn’s disease or recent antibiotic treatment. The disease may simulate a GuillainBarré Syndrome. Inadvertent botulism This has been reported in patients who have been treated with intramuscular injections of botulinum toxin. Marked clinical weakness is observed as well as electrophysiologic abnormalities.

Diagnosis.

Foodborne botulism. This should be suspected in a patient with acute onset of gastro-intestinal symptoms associated with autonomic (dry mouth, difficulty focusing eyes) and cranial nerves dysfunction (ptosis, diplopia, dysarthria, dysphagia). A history of home-prepared or home-preserved food (often, inadequately pasteurized vegetables) and similar symptoms in people who have shared the same food increases likelihood of the diagnosis. The initial diagnosis should be made on the basis of history and physical findings. Confirmatory tests may take days to be performed. Serum, stools and suspected food should be tested for the presence of botulism. The mouse inoculation test is still the most reliable method. Stool specimens should be cultured for C. botulinum as a confirmatory test. Isolation of C. botulinum organism devoid of toxin from the suspected food has little significance.

Wound botulism. Specimens of wound exudate, a tissue sample, or a swab sample should be obtained for anaerobic culture in addition to a serum toxin assay. A stool specimen should be obtained in order to exclude food or intestinal colonization as sources of toxin. Infant botulism This should be suspected in an infant with constipation, poor feeding, diminished sucking and crying ability, neck and peripheral muscle weakness, or ventilatory distress. Stool cultures for C. botulinum and testing for the presence of toxin in the stool should be performed in such patients.

Adult infectious botulism. This is a rare disease and should be suspected in patients with some abnormality of the gastrointestinal tract who develop cranial nerve autonomic dysfunction, and muscular weakness. Stool cultures for C. botulinum and testing for the presence of toxin should be performed. Endogenous antibody production to botulinum toxin has been described. Inadvertent botulism This may be suspected in patients with recent history of botulin A toxin injection, especially into big muscles for systemic effect, or perhaps, in a suicide attempt.

Clostridium tetani is a gram-positive, spore-forming, motile, anaerobic bacillus. Typically measuring 0.3 to 0.5 μm in width and 2 to 2.5 μm in length, the vegetative form often develops long filament-like cells in culture. Motility is produced by peritrichous flagellae coating the cell surface. With sporulation, *C. tetani* loses its flagellae and takes on the more characteristic drumstick-like appearance reflecting spore formation in the terminal position. *C.tetani* is a strict anaerobe that grows optimally at 33° to 37° C; however, depending on the strain, growth can occur at 14° to 43° C. It can be cultured in a variety of anaerobic growth media such as thioglycolate, casein hydrolysate, and cooked meat. Growth is enhanced in media supplemented with reducing substances at a neutral to alkaline pH. On blood agar, the organism produces characteristic compact colonies extending in a meshwork of fine filaments. Growth is usually accompanied by the production of gas and is associated with a fetid odor.

The biochemical activity of *C. tetani* is limited. In general, it does not ferment sugars, although some strains will ferment glucose. Gelatin is slowly hydrolysed but other proteins used in laboratory tests are not digested. Indole is produced slowly, but not hydrogen sulphide. Neither lecithinase nor lipase is produced. Gas–liquid chromatography of broth culture extracts reveals the major bacterial products to be acetic, butyric and propionic acids.

Sporulation depends on a variety of factors that include pH, temperature, and medium composition. Spores can be promoted at 37° C and in the presence of oleic acid, phosphates, 1% to 2% sodium chloride, protein, and magnesium. In contrast, acidification, high (≥ 41° C) or low (≤ 25° C) temperatures, glucose, assorted saturated fatty acids, antibiotics, and potassium inhibit spore formation. The germination of spores requires anaerobic conditions and is enhanced by the presence of lactic acid and chemicals toxic to cells.

If not exposed to sunlight, *C. tetani* spores can persist in soil for months to years. Spores are resistant to boiling and a variety of disinfectants. Inactivation of spores requires 15 to 24 hours in a solution of phenol (5%), formalin (3%),

chloramine (1%), or hydrogen peroxide (6%). Use of aqueous iodine or 2% glutaraldehyde at pH 7.5 to 8.5 kills spores within 3 hours; autoclaving at 120° C and 15 psi destroys them within 15 to 20 minutes.

The most common source of environmental exposure to *C. tetani* and spores is soil, where the organism is widely but variably distributed. It is difficult to compare studies of the distribution of the organism in nature because of differences in methodology. Most studies suggest that viable spores are more common in soils with an alkaline pH and in nutrient-rich soils in warm, moist climates that could more easily support multiplication of the bacillus.

Animals are also *C. tetani* reservoirs. Both herbivores and omnivores can harbor tetanus bacilli and spores in their intestines, disseminating the organism in their feces. Fecal carriage has been reported in 10% to 20% of horses and 25% to 30% of dogs and guinea pigs; fecal specimens from several other species, including sheep, cattle, and small mammals, also were found to contain *C. tetani*. Attempts to quantify the frequency of human intestinal colonization have produced varied results ranging from 0% to 40%. Rural residents tend to have higher rates of intestinal carriage than city dwellers. *C. tetani* spores have also been detected in street dust and the dust and air of surgical operating theaters.

Tetanus toxin, also known as tetanospasmin, is one of two toxins elaborated by the organism. The other, tetanolysin, is a hemolysin.

Generalized tetanus is the most commonly described form of clinical tetanus, accounting for up to 80% of cases in which symptoms commonly develop in a descending pattern. Difficulty opening the mouth due to tetanic spasms of the masseter, known as trismus or lockjaw, is the most common initial symptom. Lockjaw is usually followed by neck stiffness and difficulty swallowing. This is followed by stiffness and spasms of the back, abdominal and chest muscles, as well as flexion and extension of the limbs. The muscular spasms can be extremely forceful, resulting in hyperthermia, rhabdomyolysis, and even vertebral fractures. Although the toxin is epileptogenic and the spasms may resemble seizure activity, true seizures appear to be rare, except in the circumstance of hypoxia. Tachycardia and hypertension due to autonomic instability are also commonly seen in generalized tetanus and are a major source of morbidity and mortality. Classic descriptions of generalized tetanus note distortion of the face by muscle spasms, resulting in risus sardonicus, as well opistotonic posturing of the back. Death due to generalized tetanus is usually due to hypoxia secondary to chest wall spastic immobility, hyperthermia, and autonomic instability, and the rate has dramatically declined in the modern era in developed nations. The onset of symptoms, although variable, is usually about 8 days after an identified injury (range 3–21 days), but in 10% to 20% of cases, a wound or other portal for the bacteria cannot be identified. The incubation period has a direct relation to prognosis, with more rapid onset of symptoms a harbinger of a more serious disease due to greater elaboration of toxin. The course of generalized spasms is usually 3 to 4 weeks, but milder and more localized spasms can occur for up to several months.

Localized Tetanus. Although many patients develop spasms around the site of elaboration or a wound during the course of their disease, in a minority of cases

(10% to 15%), these spasms remain restricted without the appearance of symptoms of generalized tetanus.5 These patients are referred to as having localized or local tetanus. Although the contractions can involve an entire limb and be sustained for many weeks, it is a milder form of the disease in which mortality is rare (1%). Localized tetanic spasm as an initial presentation of generalized tetanus is also relatively rare in humans. Cases of localized tetanus have been seen in previously but apparently inadequately vaccinated persons.

Diagnosis is based on clinical signs.

Treatment consists of cleaning and aggressively debriding the infected wound, administration of tetanus antitoxin, vaccination with tetanus toxoid, administration of antibiotics both parenterally and flushed into the cleaned wound, a sedative or tranquilizer and a muscle relaxant. Antibiotics to which the bacilli of *C. tetani* are susceptible include penicillin, erythromycin, clindamycin, tetracycline, chloramphenicol and metronidazole.

The disease can be controlled and prevented by following good sanitation measures, aseptic surgical procedures, and vaccination programs. Tetanus toxoid vaccine is available and very effective for stimulating long-term immunity. Animals should be vaccinated two to three times during the first year of life. Does and ewes should receive booster vaccinations within 2 months of parturition to ensure colostral antibodies. For sheep, goats, and cattle, the tetanus toxoid vaccine is available in combination with other clostridial diseases. Tetanus antitoxin can be administered as a preventative or in the face of disease as an adjunct to therapy.

Clostridial gas gangrene is a highly lethal necrotizing soft tissue infection of skeletal muscle caused by toxin- and gas-producing *Clostridium* species. The synonym clostridial myonecrosis better describes both the causative agent and the target tissue. Prior to the advent of antibiotics and mobile army surgical hospitals, as many as 5 % of battlefield injuries were complicated by this condition. Presently, 90 % of contaminated wounds demonstrate clostridial organisms, but fewer than 2 % develop clostridial myonecrosis. This underscores the importance of host and local wound factors in the development of this process, rather than the mere presence of the organisms in the wound.

Clostridial myonecrosis may also occur following surgery, most often of the gastrointestinal or biliary tract, and following septic abortions. *Clostridium perfringens, Clostridium septicum,* and *Clostridium histolyticum* are the principal causes of trauma-associated gas gangrene, and their incidence increases dramatically in times of war, hurricanes, earthquakes, and other mass-casualty conditions. There has also been an increased incidence of spontaneous gas gangrene caused by *C septicum* in association with gastrointestinal abnormalities and neutropenia.

Similarly, over the last 15 years, a toxic shock–like syndrome associated with *Clostridium sordellii* infection has been increasingly recognized in individuals skin-popping black tar heroin and in women undergoing childbirth or other gynecologic procedures, including medically induced abortion.

Clostridia are gram-positive, anaerobic, spore-forming bacilli commonly found throughout nature (with the exception of the North African desert). Cultivated rich soil has the highest density of organisms. In addition, clostridia have been isolated from normal human colonic flora, skin, and the vagina. More than 150 *Clostridium* species have been identified, but only 6 have been demonstrated to be capable of producing the fulminant condition known as clostridial gas gangrene. Usually, more than 1 species is isolated from clinical specimens.

Clostridium perfringens, previously known as *Clostridium welchii,* is the most common cause of clostridial gas gangrene (80-90 % of cases). Other clostridia species responsible for the condition include *Clostridium novyi* (40 %), *Clostridium septicum* (20 %), *Clostridium histolyticum* (10 %), *Clostridium bifermentans* (10 %), and *Clostridium fallax* (5 %).

Infections are characterized by a very low level of host inflammation in response to organism-associated exotoxins. In fact, it is more of a response to the exotoxins than a classic immune response to invading organisms. Purulence is often absent. The process of myonecrosis can spread as fast as 2 cm/h. This results in systemic toxicity and shock that can be fatal within 12 hours. Overwhelming shock with accompanying renal failure usually leads to death.

Infection requires 2 conditions to coexist. First, organisms must be inoculated into the tissues. Second, oxygen tension must be low enough for the organisms to proliferate. These organisms are not strict anaerobes; 30 % oxygen tension in the tissues allows for free growth of these bacteria, but 70 % oxygen tension restricts their growth. Inoculation of organisms into low oxygen tension tissues is followed by an incubation period that usually ranges from 12-24 hours. However, this period can be as brief as 1 hour or as long as several weeks. The organisms then multiply and produce exotoxins that result in myonecrosis.

Although exotoxins appear to be tissue-destructive soluble antigens produced by clostridia. They include lecithinase, collagenase, hyaluronidase, fibrinolysin, hemagglutinin, and hemolysin toxins. *C. perfringens* produces at least 17 identifiable exotoxins that are used for species typing (eg, type A, type B, type C).

Theta toxin causes direct vascular injury, cytolysis, hemolysis, leukocyte degeneration, and polymorphonuclear cell destruction. These effects on leukocytes may explain the relatively minor host inflammatory response that is observed in tissues of patients with clostridial myonecrosis.

Kappa toxin, also produced by *C. perfringens,* is a collagenase that facilitates the rapid spread of necrosis through tissue planes by destroying connective tissue.

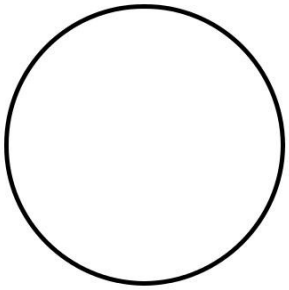
Alpha toxin is produced by most clostridia and has phospholipase C activity. This potent lecithinase causes lysis of red blood cells, myocytes, fibroblasts, platelets, and leukocytes. It also may decrease cardiac inotropy and trigger histamine release, platelet aggregation, and thrombus formation.

Surname_____ Date_____

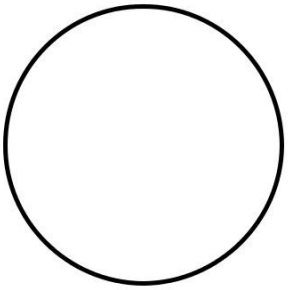
Protocol № 44

Theme: Microbiological diagnosis of plague and tularemia.

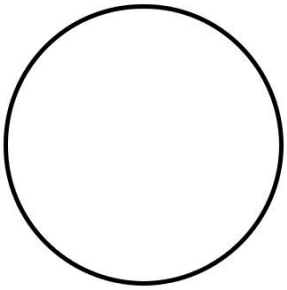
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



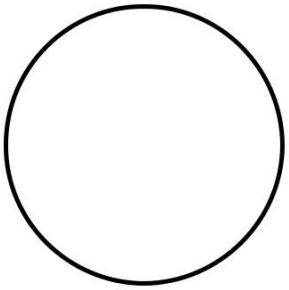
Yersinia pestis (Methylene-blue stain)



Yersinia pestis (Romanowski-Giemsa stain)



Francisella tularensis (Romanowski-Giemsa stain)



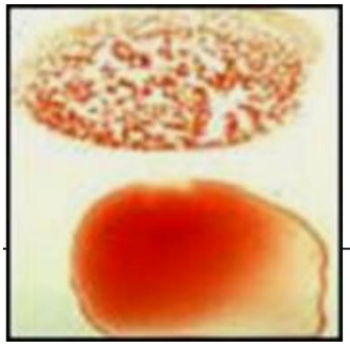
Francisella tularensis (Gram stain)

II. Study biological preparations for laboratory diagnosis, specific profilaxis and therapy of plague and tularemia:
- antiplague bacteriophage; antiplague equine serum; live attenuated plague vaccine;
- tularemic diagnosticum for agglutination test; tularemic fluorescent serum; tularin; live attenuated dry tulkaremic vaccine.

III. Study cheme of agglutination test for serological diagnosis of tularemia.

Ingredients, ml	Tubes						
	1	2	3	4	5	6 Antigen control	7 Serum control
Physiological solution NaCl	-	0,5	0,5	0,5	0,5	0,5	0,5
Patient's serum (1:25)	0,5 →	0,5 →	0,5 →	0,5 →	0,5 →	-	0,5
Diagnosticum	0,5	0,5	0,5	0,5	0,5	0,5	-
Dilution of serum	1:50	1:100	1:200	1:400	1:800	-	1:50
Incubation at 37^0 C for 2 hours, then at 18^0C for 18-20 hours							
Result							

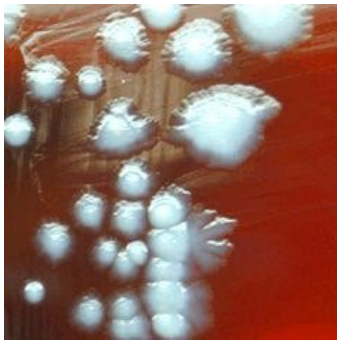
IV. Performing of slide agglutination test (blood-drop test) for serological diagnosis of tularemia.



A

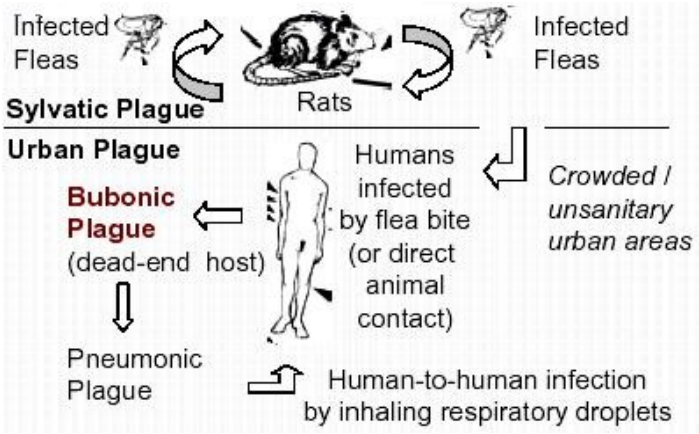
Result:
A – _____;
B – _____.

V. Study morphology of Y.pestis colonies:



Yersinia pestis on sheep blood agar.
After 48 to 72 hours, grey-white to slightly yellow opaque raised, irregular "fried egg" morphology; alternatively colonies may have a "hammered copper" shiny surface.

VI. Routes of transsmition of plague:



VI. Study the scheme of laboratory diagnosis of **plague**.

Specimen: lymph node aspirate, sputum, blood.

1. Microscopic examination: by Gram stain.

2. Culture in sheep blood agar (SBA) and brain heart infusion broth (BHI).

Identification: character of colonies. Fluorescent antibody assay (IF). Slide agglutination test. Sensitivity to phage.

3. Biological method: guinea pig inoculation.

Specimen: tissues and exudates of dead animals.

4. Ring precipitation test.

V. Study the scheme of laboratory diagnosis of **tularemia**.

Specimen: sputum, lymph node aspirates.

1. Gram stain: small, gram-negative coccobacilli.

2. IF

3. PCR.

Specimen: pharyngeal secretions, sputum, blood.

4. Mouse inoculation.

5. Culture: cysteine-glucose-blood agar.

6. Serology:

- **Microagglutination** assay can detect antibodies beginning 10 days. A 4-fold rise or a single titer of >1:128 is diagnostic.
- **Tube agglutination** (a 4-fold increase or a single titer of > in titer 1:160)

7. Skin allergic test.

ADDING THEORETICAL MATERIAL

The genus Yersinia, a member of the family Enterobacteriaceae, consists of 11 species, of which 3 are human pathogens (Y. pestis, Y. pseudotuberculosis, and Y. enterocolitica). The type species, **Y. pestis**, is a gram-negative, nonmotile, non-spore-forming coccobacillus (0.5 to 0.8 mm in diameter and 1 to 3 mm long) that exhibits bipolar staining with Giemsa staining. The organism grows at temperatures from 4 up to 40 ^{0}C (optimum at 28 to 30 ^{0}C); the optimum pH for growth ranges between 7.2 to 7.6; however, extremes of pH 5 to 9.6 are tolerated. Y. pestis has typical cell wall and whole-cell lipid compositions and an enterobacterial antigen, in common with other enteric bacteria. Its lipopolysaccharide is characterized as rough, possessing core components but lacking extended O-group side chains; while there is no true capsule, a carbohydrate-protein envelope, termed capsular antigen or fraction 1 (F1), forms during growth above 33 ^{0}C. This facultative anaerobe possesses a constitutive glyoxylate bypass and unregulated L-serine deaminase expression but lacks detectable adenine deaminase, aspartase, glucose 6-phosphate dehydrogenase, ornithine decarboxylase, and urease activities, as well as a possible lesion in a-ketoglutarate dehydrogenase. At all temperatures, Y. pestis has nutritional requirements for L-isoleucine, L-valine, L-methionine, L-phenylalanine, and glycine (or L-threonine); these auxotrophies, some of which are capable of reversion, are due to cryptic genes. At 37 ^{0}C, the organism has additional requirements for biotin, thiamine, pantothenate, and glutamic acid. These metabolic requirements preclude a saprophytic existence; Y. pestis is an obligate parasite.

Three biotypes (or biovars) of Y. pestis are recognized on the basis of conversion of nitrate to nitrite and fermentation of glycerol. Biotype antiqua is positive for both characteristics, orientalis forms nitrite but does not ferment glycerol, and mediaevalis ferments glycerol but does not form nitrite from nitrate. Strains of the three biotypes exhibit no difference in their virulence or pathology in animals or humans.

Plague is a zoonotic disease primarily affecting rodents; humans play no role in the long-term survival of Y. pestis. Transmission between rodents is accomplished by their associated fleas. While infection can occur by direct contact or ingestion, these routes do not normally play a role in the maintenance of Y. pestis in animal reservoirs. Fleas acquire Y. pestis from an infected blood meal. Infection in the flea is restricted to the alimentary canal with other organs and tissues including salivary glands, reproductive organs, and the hemocele being unaffected. The organism is not transmitted transovarially, and artificially infected larvae clear the organism within 24 h. Consequently, maintenance of plague in nature is absolutely dependent upon cyclic transmission between fleas and mammals. Two days after an infected blood meal, the stomach exhibits clusters of brown specks containing Y. pestis. These develop into cohesive dark brown masses, containing bacilli, a fibrinoid-like material, and probably hemin, which extend throughout the stomach and into the proventriculus and esophagus. The

proventriculus, which separates the stomach and esophagus, is a sphincter-like organ with needle-like teeth directed back toward the stomach; it aids in the rupture of blood cells and normally prevents regurgitation of a blood meal. Between days 3 and 9 after the infected blood meal, the bacterial masses may completely block the proventriculus, extend into the esophagus, and prevent ingested blood from reaching the stomach. As the hungry flea repeatedly attempts to feed, the blood sucked from the mammalian host distends the esophagus, mixes with bacilli, and is regurgitated into the mammalian host when the feeding attempt is terminated. While not all blocked fleas transmit plague, blockage is an important process in ensuring transmission; transmission from unblocked fleas is exceedingly rare.

Through the start of the third pandemic, transmission from urban rodents (especially rats) was the norm. Currently, most human plague cases in the world are classified as sylvatic plague (contracted from rural wild animals such as squirrels, chipmunks, marmots, voles, gerbils, mice, and rabbits). Two important new trends in plague epidemiology include increased peridomestic transmission as residential areas encroach upon formerly rural enzootic foci and significant transmission by domestic cats. Consequently, plague has become a potential but rare occupational risk factor for veterinary workers in areas of endemic foci. However, bubonic plague remains the most common form of the disease, with a flea bite being the primary form of transmission to humans. Progression to secondary pneumonic plague is rare. Although transmission by cats is often emphasized, ground and rock squirrels remain the most common animal source of human infections. Rabbit involvement appears to be declining—nearly all cases occurred prior to 1987.

Bubonic Plague. Bubonic plague is the classic form of the disease. Patients usually develop symptoms of fever, headache, chills, and swollen, extremely tender lymph nodes (buboes) within 2 to 6 days of contact with the organism either by flea bite or by exposure of open wounds to infected materials. In addition, gastrointestinal complaints such as nausea, vomiting, and diarrhea are common. Skin lesions infrequently develop at the initial site of an infection. Soreness in the affected lymph nodes will sometimes precede swelling, and any of the lymph node areas can be involved, depending upon the site of the initial infection. Buboes are typically found in the inguinal and femoral regions but also occur in other nodes.

Septicemic Plague. Primary septicemic plague is generally defined as occurring in a patient with positive blood cultures but no palpable lymphadenopathy. Clinically, plague septicemia resembles septicemias caused by other gram-negative bacteria. Patients are febrile, and most have chills, headache, malaise, and gastrointestinal disturbances. There is some evidence that patients with septicemic plague have a higher incidence of abdominal pain than do bubonic plague patients. The mortality rate for people with septicemic plague is fairly high, ranging from 30 to 50%, probably because the antibiotics generally used to treat undifferentiated sepsis are not effective against Y. pestis.

Pneumonic Plague. Primary pneumonic plague is a rare but deadly form of the disease that is spread via respiratory droplets through close contact with an

infected individual. It progresses rapidly from a febrile flu-like illness to an overwhelming pneumonia with coughing and the production of bloody sputum. The incubation period for primary pneumonic plague is between 1 and 3 days. Plague patients may developed pneumonia secondary to either the bubonic or septicemic form of the disease. In general, patients who develop secondary plague pneumonia have a high fatality rate.

Diagnosis. A clinical diagnosis of plague is generally based on the patient symptoms and exposure history. Thus, bubonic plague is suspected in a patient with a painful, swollen lymph node, fever, and prostration who has been exposed to fleas, rodents, or other animals. Septicemic plague is harder to diagnose on clinical grounds since the symptoms resemble those of other gram-negative septicemias. Likewise, pneumonic plague has been mistaken for other pulmonary syndromes. Recent data indicate that pneumonic plague should be suspected in persons exposed to infected pets, especially cats. In any case, if possible, samples should be obtained for epidemiological and laboratory diagnostic purposes before treatment is begun. However, treatment should not be delayed by waiting for the laboratory results. All suspected cases of plague are reported to the local and state health departments, and the diagnosis is confirmed by the Centers for Disease Control and Prevention (CDC). Only presumptive or confirmed cases are officially reported by the CDC to the WHO.

Laboratory Diagnosis. A laboratory diagnosis of plague is based on bacteriological and/or serological evidence. Samples for analysis can include blood (if possible, four samples taken at 30-min intervals), bubo aspirates, sputum, cerebrospinal fluid in patients with plague meningitis, and scrapings from skin lesions, if present. Staining techniques such as the Gram, Giemsa stain can provide supportive but not presumptive or confirmatory evidence of a plague infection. A positive fluorescent-antibody test can be used as presumptive evidence of a Y. pestis infection. The antibody is directed against purified F1, a capsular antigen expressed predominantly at 37^0C. Samples that have been refrigerated for more than 30 h, from cultures that were incubated at temperatures less than 35 ^{0}C, or from fleas will be negative. To confirm a diagnosis of plague by bacteriological means, it is necessary to isolate the organism. Y. pestis grows readily on most routine laboratory culture media but takes 2 days to achieve visible colonies. The colonies are opaque and smooth with irregular edges that have a “hammered-metal” appearance when magnified. A presumptive identification of Y. pestis can be made on the basis of biochemical tests. Lysis by a specific bacteriophage is used by the CDC to conclusively identify Y. pestis. Although not a rapid diagnostic technique, a serological response is often used retrospectively to confirm cases of plague. Paired serum samples, either acute and convalescent phases or convalescent and post-convalescent phases, are best, but a single serum sample can be used to provide presumptive evidence of plague. The samples are analyzed at the CDC for the presence of anti-F1 antibodies by a passive hemagglutination test. A fourfold rise or fall in the titer of paired serum samples is considered confirmatory for plague.

Treatment. All patients suspected of having bubonic plague should be placed in isolation until 2 days after starting antibiotic treatment to prevent the potential spread of the disease should the patient develop secondary plague pneumonia. Streptomycin has been used to treat plague for over 45 years and still remains the drug of choice. Because streptomycin is bacteriolytic, it should be administered with care to prevent the development of endotoxic shock. Due to its toxicity, patients are not usually maintained on streptomycin for the full 10-day treatment regimen but are gradually switched to one of the other antibiotics, usually tetracycline. The tetracyclines are also commonly used for prophylactic therapy, while chloramphenicol is recommended for the treatment of plague meningitis. While Y. pestis is susceptible to penicillin in vitro, this antibiotic is considered ineffective against human disease. Other, newer antibiotics have been used to successfully treat experimental plague infections in mice but are not generally used to treat human cases. Antibiotic-resistant strains are rare and are not increasing in frequency.

Prevention of Disease. Both antibiotics and vaccines have been used to prevent Y. pestis infections from occurring in the first place. The tetracyclines are popular antibiotics for plague prophylaxis. Usually, antibiotics are given as prophylactic measures only to close contacts of pneumonic plague patients. There are two types of plague vaccine currently used in various parts of the world. The live vaccine is derived from a Pgm2 attenuated strain, usually related to EV76, while the killed vaccine uses a formalin-fixed virulent strain of Y. pestis.

Only people at high risk for Y. pestis infection take the vaccine. These include individuals who work with or are potentially exposed to fully virulent strains and military personnel serving in areas where plague is endemic.

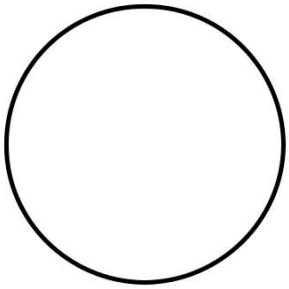
Control of Plague. Given the complexity of its life cycle, including the number and variety of potential animal and vector hosts involved, it is unlikely that plague will ever be completely eradicated by human endeavors.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

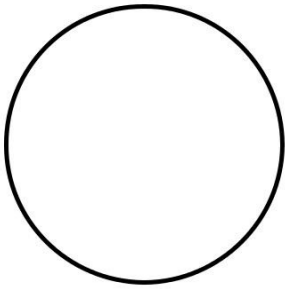
Protocol № 45

Theme: Microbiological diagnosis of brucellosis.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Brucella abortus (Gram stain)



Brucella abortus (Kozlovsky stain)

II. Study biological preparations for laboratory diagnosis, specific profilaxis and therapy of brucellosis: brucella diagnosticum for Rait and Haddlson agglutination test;
brucellin for skin allergic test;
inactivated vaccine.

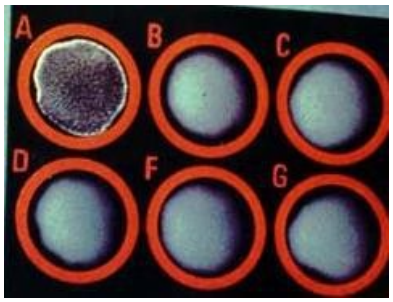
III. Self-work of students:

a) performing of slide agglutination test for detection of antibodies in patient's serum.

Sheme of Haddlson agglutination test

Ingredients, ml	Number of squer					
	1	2	3	4	5 – Serum control	6 – Antigen control
Physiological solution NaCl	-	-	-	-	0,03	0,03
Patient's serum	0,08	0,04	0,02	0,01	0,02	-
Diagnosticum	0,03	0,03	0,03	0,03	-	0,03

Haddlson slide agglutination test



Result: Test is positive in a sample _____.

IV. Study the scheme of laboratory diagnosis of brucellosis.

Specimens: blood, bone marrow, urine, bile, milk.

1. Culture on blood agar plates. Incubation during 1 month. *B. abortus* grow better in 5% CO_2. Identification: slide agglutination test, sensitivity to phage, H_2S production.

2. Biological method: mouse or guinea pig inoculation.

3. Skin allergic test with brucellin 15-20 days after exposure.

4. Serology: CFT, slide agglutination test (Hadlson test), tube agglutination test (Write test), Coomb's test, passive hemagglutination test, IF, ELISA.

ADDING THEORETICAL MATERIAL

Bacteria of the genus *Brucella* cause disease primarily in domestic, feral and some wild animals and most are also pathogenic for humans. In animals, brucellae typically affect the reproductive organs, and abortion is often the only sign of the disorder.

Three species (*B melitensis, B abortus, B suis*) are important human pathogens; *B canis* is of lesser importance. Species are differentiated by production of urease and H_2S, dye sensitivity, cell wall antigens and phage sensitivity. The major species are divided into multiple biovars.

Brucellosis is a severe acute febrile disease caused by bacteria of the genus *Brucella*. Relapses are not uncommon; focal lesions may occur in bones, joints, genitourinary tract, and other sites. Hypersensitivity reactions can follow occupational exposure. Infection may be subclinical. Chronic infections may occur.

Human brucellosis is either an acute febrile disease or a persistent disease with a wide variety of symptoms. It is a true zoonosis in that virtually all human infections are acquired from animals. The disease is controlled by the routine practice of pasteurizing milk and milk products, as well as by comprehensive campaigns to eradicate the disease by destroying domestic animals which exhibit positive serologic reactions to brucellae. Vaccines providing some protection to cattle, sheep and goats are available.

The presentation of brucellosis is characteristically variable.

Brucella enter the human body through the mouth, conjunctivae, respiratory tract and abraded skin. Organisms spread, possibly in mononuclear phagocytes, to reticuloendothelial sites. Small granulomas reveal a mononuclear response; hypersensitivity is a major factor.

The incubation period is often difficult to determine but is usually from 2 to 4 weeks. The onset may be insidious or abrupt. Subclinical infection is common.

In the simplest case, the onset is influenzalike with fever reaching 38 to 40°C. Limb and back pains are unusually severe, however, and sweating and fatigue are marked. The leukocyte count tends to be normal or reduced, with a relative lymphocytosis. On physical examination, splenomegaly may be the only finding. If the disease is not treated, the symptoms may continue for 2 to 4 weeks. Many patients will then recover spontaneously but others may suffer a series of exacerbations. These may produce an undulant fever in which the intensity of fever and symptoms recur and recede at about 10 day intervals. Anemia is often a feature. True relapses may occur months after the initial episode, even after apparently successful treatment.

Most affected persons recover entirely within 3 to 12 months but some will develop complications marked by involvement of various organs, and a few may enter an ill-defined chronic syndrome. Complications include arthritis, often sacroiliitis, and spondylitis (in about 10 percent of cases), central nervous system effects including meningitis (in about 5%), uveitis and, occasionally, epididymoorchitis. In contrast to animals, abortion is not a feature of brucellosis in

pregnant women. Hypersensitivity reactions, which may mimic the symptoms of an infection, may occur in individuals who are exposed to infective material after previous, even subclinical, infection.

Brucellae are Gram-negative coccobacilli (short rods) measuring about 0.6 to 1.5 μm by 0.5-0.7 μm. They are non-sporing and lack capsules or flagella and, therefore, are non-motile. The outer cell membrane closely resembles that of other Gram-negative bacilli with a dominant lipopolysaccharide (LPS) component and three main groups of proteins. The guanine-plus-cytosine content of the DNA is 55-58 moles/cm. No *Brucella* species has been found to harbor plasmids naturally although they readily accept broad-host-range plasmids.

The metabolism of the brucellae is mainly oxidative and they show little action on carbohydrates in conventional media. They are aerobes but some species require an atmosphere with added CO_2 (5-10 percent). Multiplication is slow at the optimum temperature of 37°C and enriched medium is needed to support adequate growth.

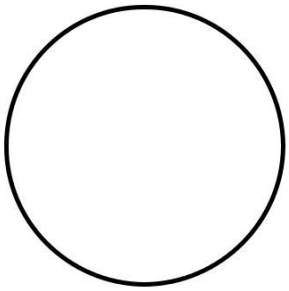
Brucella colonies become visible on suitable solid media in 2-3 days. The colonies of smooth strains are small, round and convex but dissociation, with loss of the O chains of the LPS, occurs readily to form rough or mucoid variants. These latter forms are natural in *B. canis* and *B. ovis* as the LPS of these lack O chains.

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

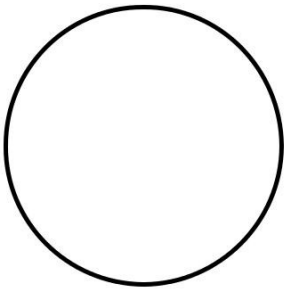
Protocol № 46

Theme: Microbiological diagnosis of anthrax.

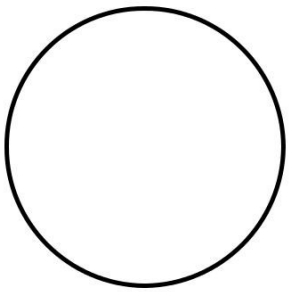
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Smear-imprint
(Methylene blue stain)



Central spore
(Gram stain)

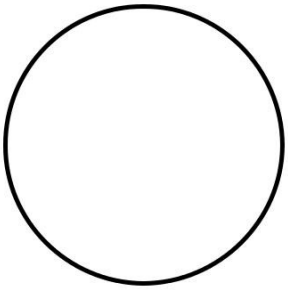


Streptobacilli
(Gram stain)

II. Study biological preparations for laboratory diagnosis, specific profilaxis and therapy of anthrax:
precipitating serum for Ascoly test;
anthraxin for skin allergic test; bactreriophage;
immunoglubulin; live attenuated vaccine.

III. Self-work of students:

a) prepare a smear of B. anthracoides, stain after Gram, microscopy



Gram stain

IV. Study the scheme of laboratory diagnosis of anthrax.
Specimens: vesicular fluid, fluid from under the eschar, blood, sputum, or spleen or lymph node aspirates.
1. Microscopy: stain with methylene blue or Gram stain; immunofluorescence.
2. Culture on blood agar plates.
Identification: absence of hemolysis, lack of motility, gelatin hydrolysis, susceptible to lysis by gamma phage, test with penicillin.
3. Biological method: mouse or guinea pig inoculation.
4. Ascoly test: ring precipitation test.
Specimen: tissues and exudates of dead animals.

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

ADDING THEORETICAL MATERIAL

Anthrax is a serious infectious disease caused by gram-positive, rod-shaped bacteria known as Bacillus anthracis. Anthrax can be found naturally in soil and commonly affects domestic and wild animals around the world. People can get sick with anthrax if they come in contact with infected animals or contaminated animal products. Contact with anthrax can cause severe illness in both humans and animals.

Anthrax is not contagious, which means you can't catch it like the cold or flu.

Domestic and wild animals such as cattle, sheep, goats, antelope, and deer can become infected when they breathe in or ingest spores in contaminated soil, plants, or water. In areas where domestic animals have had anthrax in the past, routine vaccination can help prevent outbreaks.

People get infected with anthrax when spores get into the body. When anthrax spores get inside the body, they can be "activated." When they become active, the bacteria can multiply, spread out in the body, produce toxins (poisons), and cause severe illness.

Anthrax is most common in agricultural regions of Central and South America, sub-Saharan Africa, central and southwestern Asia, southern and eastern Europe, and the Caribbean. Anthrax is more common in developing countries and countries that do not have veterinary public health programs that routinely vaccinate animals against anthrax.

The anthrax bacillus, *Bacillus anthracis,* was the first bacterium shown to be the cause of a disease. In 1877, Robert Koch grew the organism in pure culture, demonstrated its ability to form endospores, and produced experimental anthrax by injecting it into animals.

Bacillus anthracis is very large, Gram-positive, sporeforming rod, 1 - 1.2µm in width x 3 - 5µm in length. The bacterium can be cultivated in ordinary nutrient medium under aerobic or anaerobic conditions. Genotypically and phenotypically it is very similar to *Bacillus cereus,* which is found in soil habitats around the world, and to *Bacillus thuringiensis,* the pathogen for larvae of *Lepidoptera.* The three species have the same cellular size and morphology and form oval spores located centrally in a nonswollen sporangium.

Cultivation. Several nonselective and selective media for the detection and isolation of *Bacillus anthracis* have been described, as well as a rapid screening test for the bacterium based on the morphology of microcolonies.

Differential Characteristics of *B. anthracis B. cereus and B. thuringiensis*

Characteristic	*B. anthracis*	*B. cereus* and *B. thuringiensis*
growth requirement for thiamin	+	-
hemolysis on sheep blood agar	-	+
glutamyl-polypeptide capsule	+	-

lysis by gamma phage	+	-
motility	-	+
growth on chloral hydrate agar	-	+
string-of-pearls test	+	-

The most common form of the disease in humans is cutaneous anthrax, which is usually acquired via injured skin or mucous membranes. A minor scratch or abrasion, usually on an exposed area of the face or neck or arms, is inoculated by spores from the soil or a contaminated animal or carcass. The spores germinate, vegetative cells multiply, and a characteristic gelatinous edema develops at the site. This develops into papule within 12-36 hours after infection. The papule changes rapidly to a vesicle, then a pustule (malignant pustule), and finally into a necrotic ulcer from which infection may disseminate, giving rise to septicemia. Lymphatic swelling also occurs within seven days. In severe cases, where the blood stream is eventually invaded, the disease is frequently fatal.

Another form of the disease, inhalation anthrax ("woolsorters" disease), results most commonly from inhalation of spore-containing dust where animal hair or hides are being handled. The disease begins abruptly with high fever and chest pain. It progresses rapidly to a systemic hemorrhagic pathology and is often fatal if treatment cannot stop the invasive aspect of the infection.

Gastrointestinal anthrax is analogous to cutaneous anthrax but occurs on the intestinal mucosa. As in cutaneous anthrax, the organisms probably invade the mucosa through a preexisting lesion. The bacteria spread from the mucosal lesion to the lymphatic system. Intestinal anthrax results from the ingestion of poorly cooked meat from infected animals. Gastrointestinal anthrax is rare but may occur as explosive outbreaks associated with ingestion of infected animals. Intestinal anthrax has an extremely high mortality rate.

Meningitis due to *B. anthracis* is a very rare complication that may result from a primary infection elsewhere.

Bacillus anthracis clearly owes its pathogenicity to two major determinants of virulence: the formation of a poly-D-glutamyl capsule, which mediates the invasive stage of the infection, and the production of the multicomponent anthrax toxin which mediates the toxigenic stage.

Antibiotics should be given to unvaccinated individuals exposed to inhalation anthrax. Penicillin, tetracyclines and fluoroquinolones are effective if administered before the onset of lymphatic spread or septicemia, estimated to be about 24 hours.

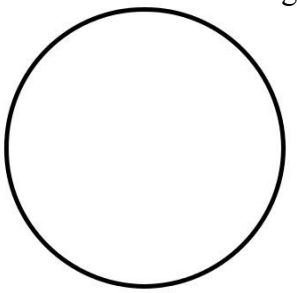
The anthrax vaccine consists of a series of six doses with yearly boosters. The first vaccine of the series must be given at least four weeks before exposure to the disease. This vaccine protects against anthrax that is acquired through the skin and it is believed that it would also be effective against inhaled spores.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

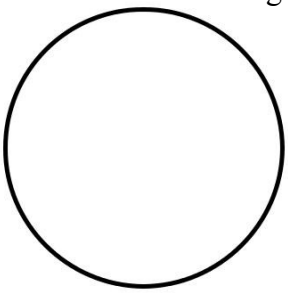
Protocol № 47

Theme: Microbiological diagnosis of syphilis.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Treponema pallidum
(Morosov stain)



Treponema pallidum
(Burry stain)

II. Study the scheme of laboratory diagnosis of syphilis.

Diagnostic Test	Method or Examination
Microscopy	Dark field microscopy
	Direct Immunofluorescence antibody staining (FA)
Culture	**Not used**
Serology	Nontreponemal VDRL, RPR, CFT
Treponemal tests	FTA-ABS, MHA-TP, CFT, ELISA, immobilization test

Serology: The diagnosis of syphilis in most patients is made by serological testing. The two general types of tests are biologically nonspecific (nontreponemal) tests and the specific treponemal tests.

Nontreponemal tests measure IgG and IgM antibodies (reagin antibodies) developed against lipids from damaged cells during the early stage of the disease. The antigen used for the nontreponemal tests is cardiolipin, derived from beef heart. The two tests used commonly are the Venereal Disease Research Laboratory (VDRL) test and the rapid plasma reagin (RPR) test. Both tests measure coagulation of cardiolipin antigen by the patient's serum. Both tests are rapid, although complement in serum must be inactivated for 30 minutes before the VDRL test can be performed. Only the VDRL test can be used to test cerebrospinal fluid from patients with suspected neurosyphilis.

Treponemal tests are specific antibody tests used to confirm positive reactions with the VDRL or RPR tests. The treponemal tests can be positive before the nontreponemal tests become positive in early syphilis. Treponemal test may also remain positive when the nonspecific tests revert to negative in some patients who have late syphilis. The tests most commonly used are Fluorescent Treponemal Antibody Absorption (FTA-ABS) test and the Microhemagglultination Test for T. pallidum (MHA-TP). The MHA-TP is technically easier to perform and interpret than the FTA-ABS tests.

ADDING THEORETICAL MATERIAL

The genus *Treponema* contains both pathogenic and nonpathogenic species. Human pathogens cause four treponematoses: syphilis (*T. pallidum* subsp *pallidum*), yaws (*T. pallidum* subsp *pertenue*), endemic syphilis (*T. pallidum* subsp *endemicum*), and pinta (*T. carateum*). Nonpathogenic treponemes may be part of the normal flora of the intestinal tract, the oral cavity, or the genital tract. Some of the oral treponemes have been associated with gingivitis and periodontal disease.

Treponemes are helically coiled, corkscrew-shaped cells, 6 to 15 μm long and 0.1 to 0.2 μm wide. The organisms stain poorly with aniline dyes. Treponemes in tissues can be visualized by silver impregnation methods. Live treponemes, which are too slender to be seen by conventional light microscopy, can be visualized by using dark-field microscopy. *Treponema pallidum* subsp *pallidum* exhibits characteristic motility that consists of rapid rotation about its longitudinal axis and bending, flexing, and snapping about its full length. They have an outer membrane which surrounds the periplasmic flagella, a peptidoglycan-cytoplasmic membrane complex, and a protoplasmic cylinder. Multiplication is by binary transverse fission. Treponemes have not yet been cultured in vitro.

Classification of the pathogenic treponemes is based primarily upon the clinical manifestations of the respective diseases they cause. *Treponema pallidum* subsp *pallidum* causes venereal syphilis; *T. pallidum* subsp *pertenue* causes yaws; *T. pallidum* subsp *endemicum* causes endemic syphilis; and *T. carateum* causes pinta. Venereal syphilis is transmitted by sexual contact; the other diseases are transmitted by close nonvenereal contact.

Treponemes are highly invasive pathogens which often disseminate relatively soon after inoculation. Evasion of host immune responses appears to be, at least in part, due to the unique structure of the treponemal outer membrane (i.e., its extremely low content of surface-exposed proteins). Although treponemes lack classical lipopolysaccharide (endotoxin), they possess abundant lipoproteins which induce inflammatory processes.

Various studies suggest that both cellular and humoral processes contribute to host defenses against treponemal infection. Clearance of treponemes from local sites appears to be due to phagocytosis by macrophages.

Humans are the only source of treponemal infection; there are no known nonhuman reservoirs. Venereal syphilis is distributed worldwide, and over the past several decades has become a significant public health problem in many underdeveloped countries. Infectivity rates correspond to the most sexually active age groups. Following the adoption of penicillin as the mainstay of syphilotherapy, the number of new syphilis cases progressively decreased until 1958, after which the trend reversed and a steady increase has occurred. The late 1980's experienced a major increase in the incidence of early syphilis cases which was largely related to crack cocaine usage among inner city minorities. Improved surveillance methods have helped to control this syphilis epidemic. Despite extensive eradication campaigns, yaws remains widespread in the tropics. Pinta remains endemic in Central and South America, and endemic syphilis is present in certain

regions of the Middle East. The pathogenic treponemes have many cross-reacting antigens, and untreated infection is believed to confer partial protection against the other treponemal diseases.

Diagnosis relies heavily on clinical manifestations. In addition, the finding of treponemes within exudative lesions and positive serology aids the diagnosis.

Definitive diagnosis of syphilis is complicated by the inability to cultivate *T.pallidum* subsp *pallidum* in vitro. Clinical manifestations, demonstration of treponemes in lesion material, and serologic reactions are used for diagnosis. In many cases, clinical manifestations are highly characteristic. If manifestations include one or more cutaneous exudative lesions, motile treponemes can often be visualized within lesion exudate by dark-field microscopy.

Serologic tests are a mainstay of syphilis diagnosis. They are the only means of identifying asymptomatically infected individuals. More than 200 serologic tests have been developed over the years and fall into two general categories:

(1) "nontreponemal" tests, which measure antibodies directed against lipid antigens, principally cardiolipin, thought to be derived from host tissues. They are the Venereal Disease Research Laboratory (VDRL) and Rapid Plasma Reagin (RPR) tests;

(2) "treponemal," which detect antibodies directed against protein constituents of *T. pallidum* subsp *pallidum*, examples are the Fluorescent *T.pallidum* Antibody-Absorption (FTA-ABS) and Microhemagglutination for *T.pallidum* (MHA-Tp) tests.

Both treponemal and nontreponemal serological tests have been highly standardized by the Centers for Disease Control and Prevention (CDC). The sensitivity of the nontreponemal and treponemal tests varies with the stage of the disease. The results of nontreponemal tests usually parallel the extent of infection; titers tend to be highest during secondary syphilis and subside during subclinical infection (latency) or following antibiotic therapy. The treponemal tests often remain reactive for life.

Two terms relevant to syphilis serodiagnostic testing are sensitivity and specificity. The perfect test, not yet developed, would detect 100 percent of the treponemal infections and would be nonreactive in all other diseases. Sensitivity refers to the ability to detect the tested variable, in this case syphilis. A false-negative occurs when serum from a syphilitic patient fails to react. Specificity refers to the ability to recognize when the variable is not present (i.e., to exclude syphilis in nonsyphilitic patients). A false-positive occurs when serum from a nonsyphilitic patient reacts positively. In general, treponemal tests are more sensitive and more specific than the nontreponemal tests. However, mathematical models have shown that maximal sensitivity and specificity are achieved if patients are screened with a nontreponemal test and positive sera confirmed by a treponemal test. A number of clinical conditions may cause false-positive nontreponemal tests. These include leprosy, tuberculosis, malaria, infectious mononucleosis, collagen disorders, systemic lupus erythematosus, rheumatoid arthritis, pregnancy, and drug abuse. Individuals with false-positive nontreponemal tests are identified by virtue of the fact that their treponemal tests are nonreactive.

Congenital syphilis is difficult to diagnose in asymptomatically infected neonates because maternal antibodies (IgG) which pass through the placenta and enter the fetal circulation cause reactivity in both nontreponemal and treponemal tests. In uninfected infants, such maternal antibodies disappear by 3 months. Because of the presence of maternal antibodies in the newborn, quantitative VDRL or RPR tests should be performed monthly over the first 6 months. If the titer increases or stabilizes and does not decrease, congenital syphilis is indicated and the baby should be treated accordingly.

Penicillin remains the drug of choice for treating syphilis. Penicillin resistance has not yet emerged, unlike the situation for gonorrhea. In non-penicillin-allergic patients without central nervous system involvement, infection is usually treated with benzathine penicillin G, a long-acting penicillin preparation which produces treponemicidal levels in serum for up to ten days. Patients with central nervous system involvement (neurosyphilis) should receive high dose intravenous penicillin for 10 to 14 days. Penicillin-allergic, nonpregnant patients with early syphilis can be treated with tetracycline. Penicillin-allergic, pregnant patients and patients with neurosyphilis must be desensitized to penicillin because of the lack of effective alternative therapies.

Control of venereal and nonvenereal treponematoses is based upon active surveillance and treatment of contacts.

Yaws, caused by *T. pallidum* subsp *pertenue*, predominates in the tropical areas of Africa, South America, India, Indonesia, and the Pacific Islands. Its highly contagious nature is indicated by an estimated 50 million cases worldwide. Transmission occurs through nonsexual human-to-human contact. Most cases are in children and adolescents. In endemic areas, 75 percent of the population contract yaws before reaching 20 years of age.

The primary lesion, or mother yaw, develops within 2 to 4 weeks at the site of skin entry as a painless erythematous papule or group of papules. Lesions enlarge and ulcerate, exuding a serous fluid with a bloody tinge that is swarming with organisms. These lesions heal within one to several months, leaving an atrophic, depressed scar. The treponemes disseminate, and, within 1 to 12 months, secondary lesions evolve that are quite similar to the mother yaw. Crops of these lesions develop initially on the face and moist areas of the body and then spread to the trunk and arms. Infection of the soles and palms is characteristic, as it is in syphilis. Elevated granulomatous papules may enlarge to a diameter of 5 cm and then heal, leaving areas of depigmentation. Successive crops of these lesions occur for many months. Histopathology is similar to that observed in syphilis, with minimal vascular changes and no endothelial cell proliferation. The late destructive stage, sometimes called tertiary yaws, involves treponemal infection of the bones and periosteum, especially the long bones of legs and forearms, and the bones of the feet and hands. Pathologic findings are similar to those seen in the tertiary stage of syphilis. Highly destructive gummas also may occur within the bones and soft tissues. Diagnosis depends on geographic location, clinical manifestations, demonstration of treponemes within exudates, and positive serology. In areas in

which syphilis and yaws coexist, definitive diagnosis is unnecessary since both can be readily eradicated by penicillin.

Pinta, caused by *T. carateum*, is endemic in the tropical areas of Central and South America. Recently, the total number of cases has been estimated at 500,000. Transmission occurs through human-to-human nonsexual contact. Most cases initially occur in children and adolescents.

The primary lesion develops within 2 to 6 months at the site of skin entry as a flat, erythematous papule or group of papules. These lesions and occasional satellite lesions enlarge over several months and produce plaques with scaly surfaces. Secondary lesions occur after 2 to 18 months or longer and involve ulceration and hyperchromic patches. Typically the hands, feet, and scalp are infected. Late stages of pinta involve patches of hyperchromia and achromia, irregular acanthosis, and epidermal atrophy. Lesions heal initially with hyperpigmentation but, over time, become depigmented and hyperkeratotic due to scarring. The treponemes disturb normal melanin pigmentation and produce the characteristic skin manifestations within 2 to 5 years.

The different stages of this disease are not clearly separated, and overlap of manifestations is common. Diagnosis relies on geographic location, clinical manifestations, demonstration of organisms in exudates, and positive serology. Penicillin is the antibiotic of choice. Contrary to syphilis and yaws, in which the lesions heal rapidly following antibiotic treatment, pinta lesions may require 1 year to fully resolve. After primary or early secondary manifestations, skin pigmentation returns to normal. In later manifestations, however, pigmentation remains altered permanently.

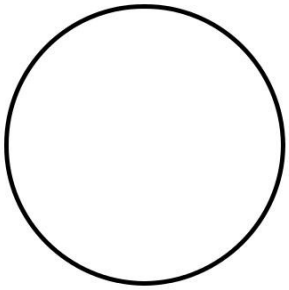
Endemic syphilis, caused by *T. pallidum* subsp *endemicum*, is found in the desert areas of the Middle East and Central and South Africa. Transmission is through human-to-human nonsexual contact. Most cases are contracted by children past the age of two years. Transmission of endemic syphilis, like that of yaws and pinta, is associated with poor hygiene. Clinical manifestations can be quite similar to those of syphilis and yaws. The site of entry is usually the mucous membranes of the eyes and mouth. The primary lesion, a small papule, is detectable in only one percent of cases. After two to three months, secondary lesions or plaques develop in mucous membranes, skin, muscles, and bone. These oozing papules erode, harden, become condylomatous, and eventually heal. Clinical manifestations are then not apparent for 5 to 15 years (latency). Late endemic syphilis develops in the skin and skeletal system. Skin lesions may be superficial, nodular, or tuberous, or they may be highly destructive, deep gummas. Destructive bone lesions frequently localize in the tibia. Diagnosis depends on geographic location, clinical manifestations, treponemes in the exudate, and positive serology. Penicillin eradicates endemic syphilis.

Surname _____ Date _____

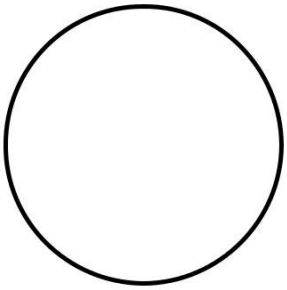
Protocol № 48

Theme: Microbiological diagnosis of leptospirosis and relapsing fevers.

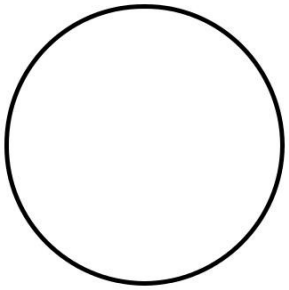
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Leptospira (Burry stain)



Leptospira (Silver stain)



Borrelia recurrentis (Giemsa stain)

II. Study the scheme of laboratory diagnosis of relapsing fever

Disease	Method	Specimen	Time
Epidemic relapsing fever	**Microscopic examination** 1. Dark-field microscopy 2. Burry stain 3. Giemsa stain	Blood	acute stage
	Serology Rickenberg-Brusine test	Serum	from the 2nd –3rd weeks
Endemic relapsing fever	**Microscopic examination** 1. Dark-field microscopy 2. Burry stain 3. Giemsa stain	Blood	during illness
	Biological method Inoculation of guinea pig	Blood	during illness

III. Study the schemes of laboratory diagnosis of leptospirosis

Method	Specimen	Time
Microscopic examination Giemsa stain	Blood	the 1st –3rd days of disease
Bacteriological method Isoletion on Ulengout medium	Blood, urine	from the 7ts day to 3rd month
Serology 1. Microagglutination-lysis test with living culture of Leptospira 2. CFT	Serum	from the 6th day
Biological method (ickteric leptospirosis) Inoculation of guinea pig	Blood	the first days of illness

Surname_____ Date_____

ADDING THEORETICAL MATERIAL

Leptospirosis, also called Weil's disease, is an infection you can catch from animals.

Leptospira interrogans causes leptospirosis, a usually mild febrile illness that may result in liver or kidney failure.

Leptospira is a flexible, spiral-shaped, Gram-negative spirochete with internal flagella. *Leptospira* has the general structural characteristics that distinguish spirochetes from other bacteria The cell is encased in a three- to five-layer outer membrane or envelope. Beneath this outer membrane are the flexible, helical peptidoglycan layer and the cytoplasmic membrane; these encompass the cytoplasmic contents of the cell. The structures surrounded by the outer membrane are collectively called the protoplasmic cylinder. An unusual feature of the spirochetes is the location of the flagella, which lie between the outer membrane and the peptidoglycan layer. They are referred to as periplasmic flagella. The periplasmic flagella are attached to the protoplasmic cylinder subterminally at each end and extend toward the center of the cell. The number of periplasmic flagella per cell varies among the spirochetes. The motility of bacteria with external flagella is impeded in viscous environments, but that of spirochetes is enhanced. The slender (0. 1 μm by 8 to 20 μm) leptospires are tightly coiled, flexible cells. In liquid media, one or both ends are usually hooked. Leptospires are too slender to be visualized with the bright-field microscope but are clearly seen by dark-field or phase microscopy. They do not stain well with aniline dyes. *Leptospira interrogans* has many serovars based on cell surface antigens.

The leptospires are the most readily cultivated of the pathogenic spirochetes. They have relatively simple nutritional requirements; long-chain fatty acids and vitamins B_1 and B_{12} are the only organic compounds known to be necessary for growth. When cultivated in media of pH 7.4 at 30°C, their average generation time is about 12 hours. Aeration is required for maximal growth. They can be cultivated in plates containing soft (1 percent) agar medium, in which they form primarily subsurface colonies.

Leptospira enters the host through mucosa and broken skin, resulting in bacteremia. The spirochetes multiply in organs, most commonly the central nervous system, kidneys, and liver. They are cleared by the immune response from the blood and most tissues but persist and multiply for some time in the kidney tubules. Infective bacteria are shed in the urine. The mechanism of tissue damage is not known. Serum antibodies are responsible for host resistance.

Leptospirosis is a worldwide zoonosis affecting many wild and domestic animals. Humans acquire the infection by contact with the urine of infected animals. Human-to-human transmission is extremely rare. The primary reservoir hosts are wild animals such as rodents, which can shed leptospires throughout their lifetimes. Domestic animals are also an important source of human infections. Leptospires have been isolated from approximately 160 mammalian species in the temperate zone. The disease is more widespread in tropical countries, where the infectious agent may be one of many serotypes carried by a large variety of hosts.

Direct or indirect contact with urine containing virulent leptospires is the major means by which leptospirosis is transmitted. As mentioned above, leptospires from urine-contaminated environments, such as water and soil, enter the host through the mucous membranes and through small breaks in the skin. Moist environments with a neutral pH provide suitable conditions for survival of leptospires outside the host. Urine-contaminated soil can remain infective for as long as 14 days. In humans, leptospirosis has occurred in an infant being breast-fed by a mother with the disease. The cellular structure of leptospires causes them to be susceptible to killing by adverse conditions such as dehydration, exposure to detergents, and temperatures above 50 °C. Most cases of leptospirosis occur during summer and fall.

Diagnosis. Clinical manifestations of leptospirosis are too variable and nonspecific to be diagnostically useful, microscopic demonstration of the organisms, serologic tests, or both are used in diagnosis. The microscopic agglutination test is most frequently used for serodiagnosis. The organisms can be isolated from blood or urine on commercially available media, but the test must be requested specifically because special media are needed. Isolation of the organisms confirms the diagnosis.

Treatment with tetracycline and penicillin G is effective.

Control. Human leptospirosis can be controlled by reducing its prevalence in wild and domestic animals. Although little can be done about controlling the disease in wild animals, leptospirosis in domestic animals can be controlled through vaccination with inactivated whole cells or an outer membrane preparation.

Animal vaccination and eradication of rodents are important. No human vaccine is available.

Borrelia recurrentis (louse borne) and *B. hermsii* and *B. turicatae* (tick borne) cause relapsing fevers: influenza-like febrile diseases that follow a relapsing and remitting course. Myocarditis is a rare sequela. *Borrelia burgdorferi* causes Lyme disease, a multisystem, relapsing febrile disease with a rash and manifestations such as arthritis, carditis, and neuritis.

Borrelia is a flexible, spiral-shaped, Gram-negative spirochete with internal flagella. *Borrelia* species are differentiated primarily on the basis of vectors and DNA homology. *Borrelia* cells average 0.2 to 0.5 μm by 4 to 18 μm and have fewer coils. Seven to twenty periplasmic flagella originate at each end and overlap at the center of the cell. Basal bodies of periplasmic flagella of borreliae resemble those in Gram-positive bacteria. Because of their larger diameter, borreliae are more readily stained with aniline dyes than are other spirochetes. Their lipid components are unusual in that they include cholesterol. The nutritional requirements of the borreliae are more complex than those of leptospires. Glucose, amino acids, long-chain fatty acids, N-acetylglucosamine, and several vitamins are some of their required organic nutrients. The borreliae are microaerophilic organisms. *Borrelia hermsii* has a generation time of 12 hours when cultivated in artificial media at 35 °C compared with only 6 to 10 hours in the mouse.

Borrelia is transmitted to humans by the body louse or ticks. *Borrelia recurrentis,* the cause of louse-borne (epidemic) relapsing fever, is carried by the human louse *Pediculus humanus*. The relapsing-fever borreliae cause recurrent febrile bacteremias separated by remissions during which the borreliae are sequestered in tissues; each resurgence involves a change in cell surface antigens. Lyme disease may have different manifestations at different times; recurrences and late sequelae may appear for many years.

Borrelia appears to be resistant to nonspecific host defense mechanisms and elicits an inflammatory response consisting of mononuclear cells. These spirochetes are rapidly killed in vitro by the antibody-complement system. Immunity to the borrelioses is primarily humoral, and immune serum passively protects experimental animals from infection. Several genospecies of *B.burgdorferi* have been reported. Serum antibodies are responsible for host resistance.

The tick-borne relapsing fever is called endemic relapsing fever because it occurs whenever humans are exposed to infected ticks. The pathogenesis of borrelial diseases is not understood. The tick-borne relapsing fevers and Lyme disease are zoonoses with rodents as the major reservoir; incidence and distribution depend mainly on the biology of the tick vectors. Louseborne relapsing fever has no animal reservoir and causes epidemics in crowded, unsanitary populations.

The clinical diagnosis is confirmed by serology and also by microscopic visualization of the organism in blood of relapsing fever patients. Clinical features of the relapsing fevers other than their recurring pattern are not diagnostic. Diagnosis is based primarily on demonstration of the spirochetes in blood during febrile episodes by dark-field examination, use of stained blood smears, or mouse inoculation. Antibody detection by indirect immunofluorescence assay is available.

The characteristic expanding red skin lesion, erythema migrans, is diagnostic for Lyme disease. However, 30 percent of patients do not develop this rash. The usual symptoms of early disease (fever, fatigue, headache, and muscle and joint pain) are too nonspecific to be diagnostic. Although *B. burgdorferi* has been isolated from blood, skin, and cerebrospinal fluid, this is a low yield procedure and is not recommended. Serologic tests are used most commonly for diagnosis of Lyme disease.

Early Lyme disease can be effectively treated with oral tetracyclines and semisynthetic penicillins. Arthritic and neurologic disorders are treated with high-dose intravenous penicillin G or ceftriaxone. Patients who have failed to respond to penicillin or tetracycline therapy have been effectively treated with ceftriaxone.

Relapsing fevers and Lyme disease are prevented by avoiding the vectors. It is important to be aware of endemic areas and to take proper precautions. When in potential tick habitats, one should wear clothing that covers as much of the skin as possible and use tick repellents. Periodic skin inspection and tick removal prevent Lyme disease. A Lyme disease vaccine may be available in the near future. The relapsing-fever and Lyme disease borreliae have similar antibiotic susceptibilities. Areas known to harbor infected ticks and lice should be avoided. Tetracycline is an effective treatment. No vaccines are available.

Protocol № 49

Theme: ***Pathogenic protozoa. Malaria.***

I. Observe the smears below. Using appropriately colored pencils draw the following cells.

Plasmodium falceparum ring stage (Giemsa stain)

Plasmodium ovale mature schizont (Giemsa stain)

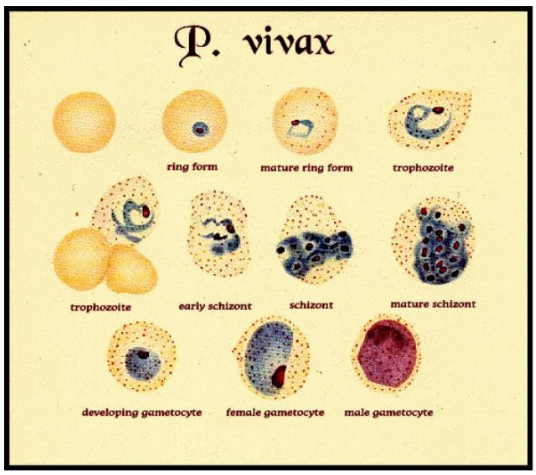
Plasmodium vivax mature schizont (Giemsa stain)

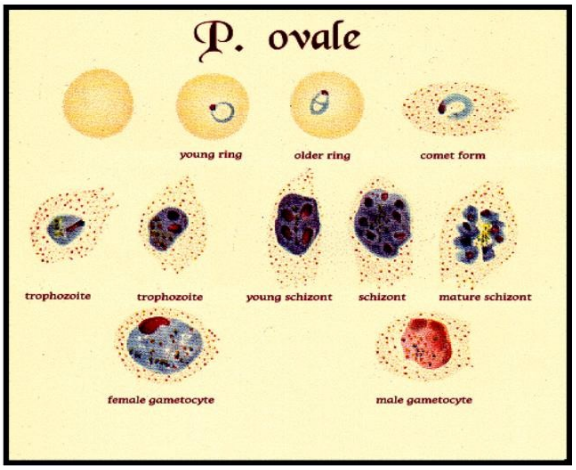
Plasmodium vivax bursting out of red blood cells (Giemsa stain)

Plasmodium vivax hametocyte (Giemsa stain)

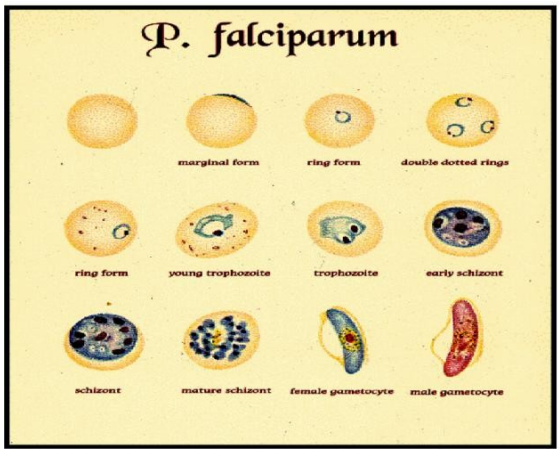
Plasmodium falciparum hametocytes (Giemsa stain)

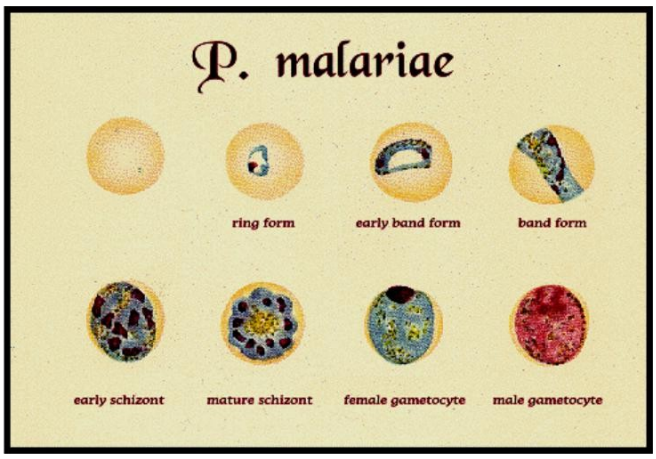
II. Study intracellular forms of Plasmodias within erythrocytes:





Surname _____ Date _____





III. Study antimalarial agents:

Mode of action		Potent action against gametocytic form	Combined
Tissue stage	Erythrocytic stage		
Histoschizotropic 1. Pyrimethamine 2. Proquanil (Bigumal) 3. Quinocide 4. Primaquine	**Hematoschizotropic** 1. Pyrimethamine 2. Proquanil 3. Chloroquine 4. Quinine 5. Mefloquine	1. Pyrimethamine 2. Proquanil 3. Quinocide 4. Primaquine	1. Pyrimethamine + Sulfamethapirasine 2. Pyrimethamine + sulfadoxine

IV. Study differenciation of Plasmidias in blood smear:

Feature	P.vivax	P.ovale	P.malariae	P.falciparum
Erythrocytes:				
Enlarged, pale pink	+	+	-	-
Ovoid, irregular edge	-	+	-	-
Granular appearance of the cytoplasm of an infected erythrocyre				
- small, crawed, red (Schuffner's dots)	+	-	-	-
- large, less crawed (Jaims' dots)	-	+	-	-
- single large pink spotes (Mauer's dots' clefts)	-	-	-	+
Parasite:				
Presence of all stages	+	+	+	-
Ring stage (quantity of parasites in an erythrocyte)	**1**	**2-3**	**1**	**2-3**
Elongated trophozoites	-	-	+	-
Round gametocytes	+	+	+	-
Crescent or banana shaped gametocytes	-	-	-	+

ADDING THEORETICAL MATERIAL

Malaria is caused by the Plasmodium parasite. The parasite can be spread to humans through the bites of infected mosquitoes.

These are:

- Plasmodium falciparum – mainly found in Africa, it's the most common type of malaria parasite and is responsible for most malaria deaths worldwide
- Plasmodium vivax – mainly found in Asia and South America, this parasite causes milder symptoms than Plasmodium falciparum, but it can stay in the liver for up to three years, which can result in relapses
- Plasmodium ovale – fairly uncommon and usually found in West Africa, it can remain in your liver for several years without producing symptoms
- Plasmodium malariae – this is quite rare and usually only found in Africa

The plasmodium parasite is spread by female Anopheles mosquitoes, which are known as "night-biting" mosquitoes because they most commonly bite between dusk and dawn.

If a mosquito bites a person already infected with malaria, it can also become infected and spread the parasite on to other people. However, malaria can't be spread directly from person to person.

Once you're bitten, the parasite enters the bloodstream and travels to the liver. The infection develops in the liver before re-entering the bloodstream and invading the red blood cells.

The parasites grow and multiply in the red blood cells. At regular intervals, the infected blood cells burst, releasing more parasites into the blood. Infected blood cells usually burst every 48-72 hours. Each time they burst, you'll have a bout of fever, chills and sweating.

Malaria can also be spread through blood transfusions and the sharing of needles, but this is very rare.

Symptoms of malaria can develop as quickly as seven days after you're bitten by an infected mosquito.

Typically, the time between being infected and when symptoms start (incubation period) is 7 to 18 days, depending on the specific parasite you're infected with. However, in some cases it can take up to a year for symptoms to develop.

The initial symptoms of malaria are flu-like and include:

- a high temperature (fever)
- headache
- sweats
- chills
- vomiting.

With some types of malaria, the fever occurs in 48-hour cycles. During these cycles, you feel cold at first with shivering. You then develop a fever,

accompanied by severe sweating and fatigue. These symptoms usually last between 6 and 12 hours.

Other symptoms of malaria can include:

- muscle pains
- diarrhoea
- generally feeling unwell.

Malaria is a serious illness that can be fatal if not diagnosed and treated quickly. Pregnant women, babies, young children and the elderly are particularly at risk.

The Plasmodium falciparum parasite causes the most severe malaria symptoms and most deaths.

As complications of severe malaria can occur within hours or days of the first symptoms, it's important to seek urgent medical help as soon as possible.

Antimalarial medication is used to prevent and treat malaria.

You should always consider taking antimalarial medicine when travelling to areas where there's a risk of malaria. Visit your GP or local travel clinic for malaria advice as soon as you know when and where you're going to be travelling.

It's very important to take the correct dose and finish the course of antimalarial treatment. If you're unsure, ask your GP or pharmacist how long you should take your medication for.

It's usually recommended you take antimalarial tablets if you're visiting an area where there's a malaria risk as they can reduce your risk of malaria by about 90%.

The type of antimalarial tablets you will be prescribed is based on the following information:

- where you're going
- any relevant family medical history
- your medical history, including any allergies to medication
- any medication you're currently taking
- any problems you've had with antimalarial medicines in the past
- your age
- whether you're pregnant.

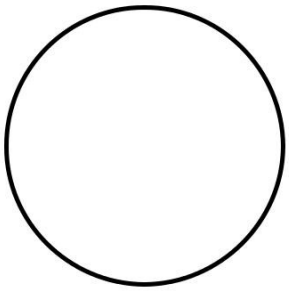
You may need to take a short trial course of antimalarial tablets before travelling. This is to check that you don't have an adverse reaction or side effects. If you do, alternative antimalarials can be prescribed before you leave.

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

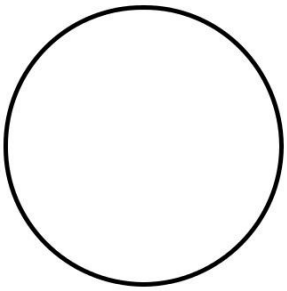
Protocol № 50

Them: ***Pathogenic protozoa. Causative agents of leishmaniosis, toxoplasmosis, lambliosis, trichomonosis and amebiasis.***

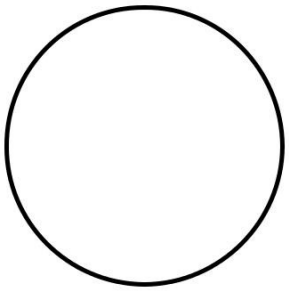
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



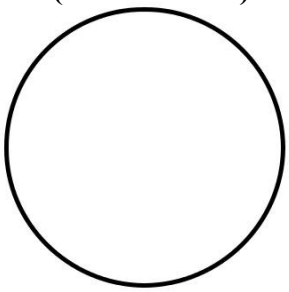
Leishmania in cell culture (Giemsa stain)



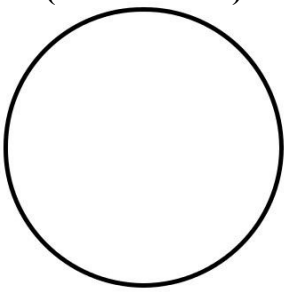
Leishmania in tissue (Giemsa stain)



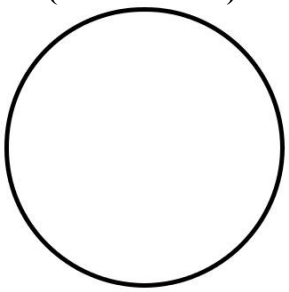
Toxoplasma (Giemsa stain)



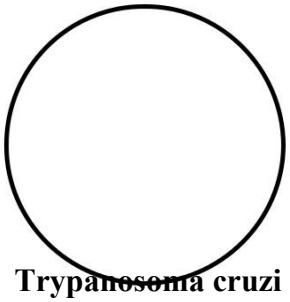
Trichomonas vaginalis (Methylene blue stain)



Giardia lamblia (Giemsa stain)



Entamoeba hystolytica (Ferriferous hematoxilyne stain)



Trypanosoma cruzi (Giemsa stain)

Balantidium coli (Ferriferous hematoxilyne stain)

II. Review of Intestinal, Urogenital, and Tissue Protozoa of Man:

Disease	Parasite	Means of human infection	Usual location in man	Clinical features	Laboratory diagnosis
Amebiasis	Entamoeba histolytica	Ingestion of cysts	Large intestine, liver, lungs	Diarrhea, localized abdominal pain, abscesses	Trophozoites, cysts in stool, serological test
Balantidiasis	Balantidium coli	Ingestion of cysts	Large intestine	Diarrhea, colitis	Trophozoites or cysts in stool
Disease	**Parasite**	**Means of**	**Usual**	**Clinical**	**Laboratory**

		human infection	location in man	features	diagnosis
Trichomoniasis	Trichomonas vaginalis	Sexual intercourse	Vagina and male urethra and prostate	Vaginitis, urethritis, burning, itching, leukorrhea	Trophozoites in urine and vaginal discharge
Toxoplasmosis	Toxoplasma gondii	Infected meat, hemotransfusion, transplacentally	Cells of RES, brain, muscles, lung, etc.	Lymphocyto-sis, lymphadenitis congenital toxoplasmosis	Serologic tests (CFR, ELISA)
Visceral leishmaniasis (Kala-azar)	Leishmania donovani	Bite of infected female Phlebotomus sand flies	Cells of RES	Hepatospleno megaly, anemia	Smears and culture of bone marrow
Cutaneous leishmaniosis	Leishmania tropica	Bite of infected female Phlebotomus sandflies	Epithelial cells of skin and mucous membranes	Ulcers of skin and mucous membranes	Smears and culture of ulcer specimen
African trypanosomosis (sleeping sickness)	Trypanosima gambiense, T.rhodesiense	Bite of infected tsetseflies	Blood, lymph nodes, CNS	Winterbottom ’s sign, fever, “sleeping sickness”	Films of blood, lymph node aspirate, spinal fluid
American trypanosomosis (Chagas’ disease)	Trypanosoma cruzi	Feces of infected reduviid bugs	Cells of RES, heart, brain	Romana’s sign, myocarditis	Films and culture of blood

III. Study the most effective drugs against protozoa:

Infecting organism	Drug of choice
Trichomonas vaginalis	Metronidazole
Giardia lamblia	Metronidazole
Entamoeba histolytica	Metronidazole
Balantidium coli	Tetracycline
Toxoplasma gondii	Pyrimethamine plus sulfadiazine, Co-trimoxazole

ADDING THEORETICAL MATERIAL

Entamoeba histolytica is an enteric protozoan parasite with worldwide distribution. It is responsible for amoebic dysentery (bloody diarrhea) and invasive extraintestinal amebiasis (such as liver abscess, peritonitis, pleuropulmonary abscess). Other species of Entamoeba-*E. hartmanii, E. coli* and *E. dispar* does not cause diseases but their trophozoite is difficult to distinguish from those of *E.histolytica* by light microscopy.

Mode of transmission: Feco-oral route, via the ingestion of contaminated food or water containing mature **quadrinucleate cyst** of *Entamoeba histolytica*. Trophozoites if ingested **would not survive** exposure to the gastric environment.

Infective form: Mature quadrinucleate cyst; it is **spherical in shape** with refractile wall (Note: ***Giardia lamblia*** cyst also has four nuclei, but the cyst is oval in shape).

Geographical distribution: Worldwide, more common in the tropics and sub tropics, especially in areas with poor sanitation (developing and under-developed countries).

Habitat: Trophozoites of *E. histolytica* live in the mucosal and submucosal layers of the **large intestine of man**. Life cycle has two stage: motile trophozoite and non-motile cyst. Trophozoites are found in intestinal lesions, extra-intestinal lesions and diarrheal stools where as cyst predominate in non-diarrheal stools.

Infection by *Entamoeba histolytica* occurs by ingestion of **mature quadrinucleate cysts** in fecally contaminated food, water, or hands. The quadrinucleate cyst is **resistant to the gastric environment** and passes unaltered through the stomach.

When they cyst of *E. histolytica* reaches caecum or lower part of ileum excystation occurs and an amoeba with four nuclei emerges and that divides by binary fission to form eight trophozoites.

Trophozoites migrate to the large intestine and lodge in to the submucosal tissue.

Trophozoites grow and multiply by binary fission in large intestine (Trophozoite phase of life cycle is responsible for producing characteristics lesion of amoebiasis).

The trophozoites invade the colonic epithelium and secrete enzymes that cause localized necrosis. Little inflammation occurs at the site.

As the lesion reaches the muscularis layer, a typical "**flask shaped**" ulcer forms, that can undermine and destroy large areas of intestinal epithelium.

Progression into submucosa leads to invasion of the portal circulation by the trophozoites.

Certain number of trophozoites are discharged in to the lumen of the bowel and are transformed into cystic forms.

The cysts thus formed are unable to develop in the same host and therefore necessitate a transference to another susceptible host. The cysts are passed in the feces.

Diagnosis of intestinal amebiasis rests on **finding either trophozoites in diarrheal stools or cysts in formed stools.** Diarrheal stools should be examined **within one hour of collection** to see the ameboid motility of the trophozoite. The trophozoite **characteristically contain ingested red blood cells.**

Characteristics of Stool:

1. **Macroscopic appearance of stool**: Offensive dark brown semisolid stool, acid in reaction, admixed with blood, mucus and much fecal matter.

2. **General microscopic examination**:

- Presence of charcot-Leyden crystals. Scanty cellular exudates, and consists of only the nuclear masses ("pyknotic bodies).
- *E. histolytica* infection is distinguished from bacillary dysentery by the lack of high fever and absence PMN leukocytosis

Laboratory diagnosis methods:

A. Microscopy:

E. histolytica can be distinguished from other amoebas by two major criteria

1. **Nature of the nucleus of the trophozoite.** The *E. histolytica* nucleus has a small central nucleolus and fine chromatin granules along the border of the nuclear membrane. The nuclei of other amebas are quite different. **Note:** The trophozoites of *Entamobea dispar*, a nonpathogenic species of Entamoeba, are morphologically indistinguishable from those of *E. histolytica.*

2. **Cyst size and number of its nuclei.** Mature cysts of *E. histolytica* are smaller than those of *Entamoeba coli* and contain four nuclei, where as *E. coli* cysts have eight nuclei.

B. Antigen detection: detection of *E.histolytica* antigen in the stool. C.

Serologic testing is useful for the diagnosis of invasive amebiasis.

D. Detection of nucleic acid of this protozoan parasite by PCR based assay.

Giardiasis is an infection in your small intestine. It's caused by a microscopic parasite called *Giardia lamblia*. Giardiasis spreads through contact with infected people. And you can get giardiasis by eating contaminated food or drinking contaminated water. Pet dogs and cats also frequently contract giardia.

G. lamblia are found in animal and human feces. These parasites also thrive in contaminated food, water, and soil, and can survive outside a host for long periods of time. Accidentally consuming these parasites can lead to an infection.

The most common way to get giardiasis is to drink water that contain *G.lamblia*. Contaminated water can be in swimming pools, spas, and bodies of water, such as lakes. Sources of contamination include animal feces, diapers, and agricultural runoff.

Contracting giardiasis from food is less common because heat kills the parasites. Poor hygiene when handling food or eating produce rinsed in contaminated water can allow the parasite to spread.

Giardiasis also spreads through personal contact. For example, unprotected anal sex can pass the infection from one person to another.

Changing a child's diaper or picking up the parasite while working in a day care center are also common ways to become infected. Children are at high risk for giardiasis because they're likely to encounter feces when wearing diapers or potty training.

Some people can carry giardia parasites without experiencing any symptoms. Symptoms of giardiasis generally show up one or two weeks after exposure. Common symptoms include:

- fatigue
- nausea
- diarrhea or greasy stools
- loss of appetite
- vomiting
- bloating and abdominal cramps
- weight loss

- excessive gas
- headaches
- abdominal pain.

Diagnosis. It is necessary to submit one or more stool samples for testing. A technician will check your stool sample for giardia parasites. You could have to submit more samples during treatment. Your doctor may also perform an enteroscopy. This procedure involves running a flexible tube down your throat and into your small intestine. This will allow your doctor to examine your digestive tract and take a tissue sample.

Treatment. In most cases, giardiasis eventually clears up on its own. Your doctor might prescribe medication if your infection is severe or prolonged. Most doctors will recommend treatment with antiparasitic drugs, rather than leaving it to clear up on its own. Certain antibiotics are commonly used to treat giardiasis:

- Metronidazole is an antibiotic that needs to be taken for five to seven days. It can cause nausea and leave a metallic taste in your mouth.
- Tinidazole is as effective as metronidazole, and often treats giardiasis in a single dose.
- Nitazoxanide is a popular option for children because it's available in liquid form and only needs to be taken for three days.
- Paromomycin has a lower chance of causing birth defects than other antibiotics, although pregnant women should wait until after delivery before taking any medication for giardiasis. This medication is given in three doses over the course of 5 to 10 days.

Giardiasis can lead to complications such as weight loss and dehydration from diarrhea. The infection can also cause lactose intolerance in some people. Children under 5 years old who have giardiasis are at risk for malnutrition, which can interfere with their physical and mental development.

To prevent giardiasis thoroughly wash your hands, especially if work in places where germs spread easily, such as day care centers.

Ponds, streams, rivers, and other bodies of water can all be sources of giardia. Don't swallow water if you go swimming in one of these. Avoid drinking surface water unless it's been boiled, treated with iodine, or filtered. Bring bottled water with you when you go hiking or camping.

When traveling in a region where giardiasis occurs, don't drink tap water. Avoid brushing your teeth with tap water. Keep in mind that tap water can also be present in ice and other beverages. Avoid eating uncooked local produce.

Balantidium coli is the largest protozoan parasite in humans and causes a disease called balantidiasis. It belongs to the ciliophora phylum and is the only protozoan ciliate to infect humans. It goes through two development phases; a cyst and a trophozoite. **Trophozoites** are 0.03–0.15 mm long and 0.025–0.12 mm wide. Their shape is either spherical or oblong. Their surface is covered with cilia and are able to move around. Trophozoites have both a micronucleus and a macronucleus, which both are normally visible. The macronucleus is bigger and sausage-shaped whereas the micronucleus is less notable. **Cysts** are spherical and 0.04–0.06 mm in

diameter. They have a tough multilayered shell which protects them against stomach acid of the host, when ingested. They are usually destroyed at a pH lower than five (normal pH of a healthy stomach is about three). Some people are weakened by other diseases and thus the cysts are not killed. Unlike trophozoites, cysts cannot reproduce and do not have any cilia for moving.

The **life cycle** of *Balantidium coli* begins, when a human eats food or water that has been contaminated with infective cysts. If the cysts survive through the stomach, trophozoites are formed in the small intestine. Trophozoites live in the cecum and the colon of the large intestine. They live and feed in the lumen but sometimes penetrate the mucosa. They multiply by transverse binary fission in the intestinal wall. Some trophozoites return to the lumen and encyst (transform into cysts) once the feces dry up. The cysts are formed either in the large intestine or outside of the body. If the feces get in contact with vegetables or drinking water, humans might ingest the cysts.

About 1 % of the world's population is infected with balantidiasis. Most infections occur in developing countries where feces are more likely to get in contact with food and drinking water. In addition to humans, pigs and other animals carry the disease. People who raise pigs have bigger risk of getting infected with balantidiasis.

Balantidiasis is often **asymptomatic**. But in some cases the patient might have**diarrhea**, **weight loss** and **dysentery**. Dysentery is an inflammatory disorder of the intestine, particularly of the colon, that causes severe diarrhea containing blood and/or mucus in the feces with **stomach pain** and **fever**. Untreated dysentery cases **can be fatal**.

Diagnosis can be made by finding trophozoites from a stool or tissue sample (collected during endoscopy). Cysts are rarely found. Trophozoites are passed irregularly and quickly destroyed outside the colon. For this reason, many stool samples are usually required to confirm the disease.

Balantidiasis is **treated** with tetracycline according to the instructions of your health care provider. Tetracycline is not recommended for pregnant women or children under 8 years old. If the drug is not available, then iodoquinol and metronidazole can be used.

Balantidiasis infections can be **prevented** by following proper hygiene practices. Do not use human feces as fertilizer in agriculture. Wash your hands after going to the toilet and before meal. Only drink pure water. Wash vegetables and cook meat properly. Infective *Balantidium coli* cysts are killed by heat.

Trichomonas vaginalis is likely the most common non-viral sexually transmitted infection (STI) in the world. While not a reportable disease, the World Health Organization estimated that nearly 90 % of these infections occurred among people living in resource-limited settings. TV is more prevalent that *Chlamydia trachomatis, Neisseria gonorrhoeae*, and syphilis combined. The global prevalence of TV has been estimated at 8.1 % for women and 1.0 % for men. These rates may be underestimates as they are derived from studies that used microscopy rather

than the more sensitive nucleic acid amplification tests (NAAT) and no formal surveillance systems exist.

TV is a flagellated parasitic protozoan, typically pyriform but occasionally amoeboid in shape, extracellular to genitourinary track epithelium with a primarily anerobic lifestyle. The individual organism is 10–20 μ m long and 2–14 μ m wide. Four flagella project from the anterior portion of the cell and one flagellum extends backwards to the middle of the organism, forming an undulating membrane. An axostyle extends from the posterior aspect of the organism. TV has a large genome (strain G3, 176,441,227 bp) with ~ 60,000 protein coding genes organized into six chromosomes. TV is a highly predatory obligate parasite that phagocytoses bacteria, vaginal epithelial cells and erythrocytes and is itself ingested by macrophages. TV uses carbohydrates as its main energy source via fermentative metabolism under aerobic and anaerobic conditions. Incubation time is generally between 4 and 28 days.

TV primarily infects the squamous epithelium of the genital tract. TV resides in the female lower genital tract and the male urethra and prostate, where it replicates by binary fission. TV is transmitted among humans, its only known host, primarily by sexual intercourse. Infection may persist for long periods, possibly months or even years, in women but generally persists less than 10 days in males. The parasite does not appear to have a cyst form and does not survive well in the external environment, but can survive outside the human body in a wet environment for more than three hours.

While thought to be rare, evidence of non-sexual transmission via fomites and possibly water has been described. TV can be infected with double-stranded RNA (dsRNA) viruses that may have important implication for trichomonal virulence and disease pathogensis.

The majority of women (85 %) and men (77 %) with TV are asymptomatic. One third of asymptomatic women become symptomatic within 6 months. Among those who do have symptoms, they include urethral discharge and dysuria. Among women, common sites of infection include the vagina, urethra and endocervix. Symptoms include vaginal discharge (which is often diffuse, malodorous, yellow-green), dysuria, itching, vulvar irritation and abdominal pain. The normal vaginal pH is 4.5, but with TV infection this increases markedly, often to >5. *Coplitis macularis* or strawberry cervix is seen in about 5 % of women, though with colposcopy this rises to nearly 50 %. Other complications include infection of the adnexa, endometrium, and Skene and Bartholin glands. In men, it can cause epididymitis, prostatitis, and decreased sperm cell motility.

The diagnosis of TV is becoming more precise and more tests have become available in the last decade. Wet mount microscopy has been used for many decades to diagnose TV. The test is inexpensive, low technology and is point of care, however, it is insensitive, particularly in men. Sensitivities range from 50–70 % depending on the expertise of the reader and should be read within 10 min of collection. While culture has better sensitivity that wet mount, in women it is more expensive, time consuming, and also demonstrates poor sensitivity in men. The lack of sensitivity of culture has been found in longitudinal studies of TV

treatment. One study of HIV- and one study of HIV+ women found that that after single dose MTZ treatment, TV infection was non-detectable for months via culture and then reappeared in the absence of reported sexual exposure underscoring the need for more sensitive testing than culture.

Nucleic acid probe techniques are the most sensitive tests, are moderately priced and fast, but require instrumentation. These tests are not considered point-of-care.

It has been generally thought that only vaginal specimens should be collected for TV testing among women. There is, however, some evidence that endocervical specimens are suitable.

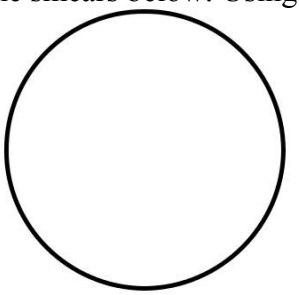
For nearly four decades, metronidazole (MTZ) has been the treatment of choice for TV. MTZ belongs to the 5-nitroimidazole drug family and is reported to have about a 95 % success rate in curing TV along with its related compounds such as tinidazole (TNZ) and seconidazole.

Surname _____ Date _____

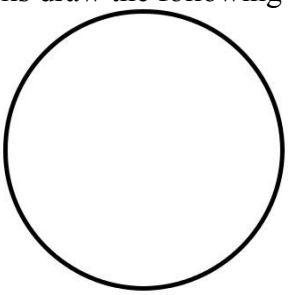
Protocol № 51

Theme: Microbiological diagnosis of mycosis.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Candida albicans (budding cells) (Gram stain)



Candida albicans (pseudohypha and hypha) (Gram stain)

II. Study human diseases caused by fungi.

1. **Superficial**: Outer skin layer - no immune response caused mostly by yeasts (Dandruff)
2. **Cutaneous**: Epidermal layers local, skin and other keratinized tissues - evoke immune response Tinea (Ringworm, Athlete's foot, jock itch) caused by Dermatophytosis: : dermatophytes – *Trichophyton, Epidermophyton Microsporum*
3. **Subcutaneous**: Chronic inf.of subdermal tissues - surgical intervention as it may result in local granuloma formation and spread: via lymphatics
   Egs:Sporothrichosis(*Sporothrixschenckii)*,Chromomycosis(*Phialophoras*pp.), Chromoblastomycosis (*Fonsecaea* spp.) , Maduromycosis/mycetoma (mixed)
4. **Systemic**: Mostly originating in the lung caused by virulent dimorphic exogen source fungi inhalation Eg: Histoplasmosis , Coccidioidomycosis, Blastomycosis.
5. **Opportunistic**: In immunocompromised host (AIDS; altered mucosal flora due to antibiotics): Eg: mostly Candidiasis and Aspergillus spp., Penicillium and other molds- exogen or endogen source

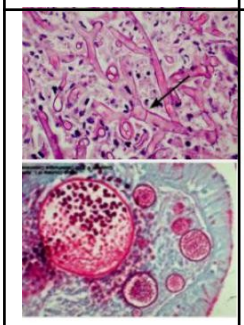
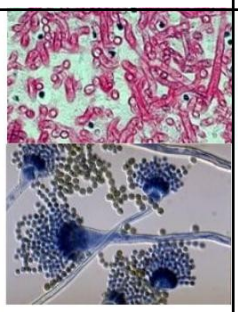
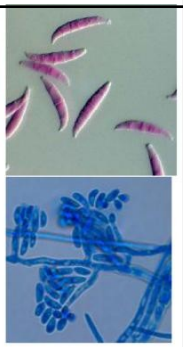
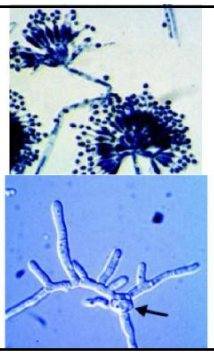
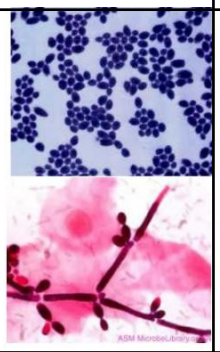
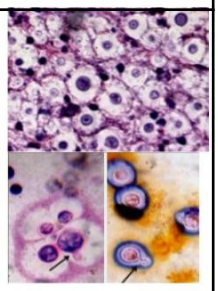
III. Study antifungal agents.

Antifungal drugs	Site of action
I. Antibiotics a. **Polyenes** - Amphotericin B Nystatin Natamycin, Hamycin	bind to ergosterol in the cell membrane causing leakage of material
b. **Heterocyclic benzofurans**- Griseofulvin,	inhibits mitosis in dermatophytes, inhibits chitin synthesis in cell wall
II. Azoles **a. Imidazoles** - Ketoconazole, Miconazole, Clotrimazole,Econazole , Tioconazole, Enilconazole **b.Triazoles**-Fluconazole, Itraconazole,Voriconazole,Posaconazole, Ravuconazole	intefere with ergosterol synthesis, damage permeability of the cell membrane
III. Antimetabolites-5-Flucytosine	interferes with DNA and RNA synthesis
IV. Echinocandins- Caspofungin, ,Cilofungin, Micafungin, Anidulafungin	inhibit cell wall synthesis by targeting glucans
V. Allylamines -TerbinafineButenafine, Naftifine, Amorolfine ,)	inhibit squalene epoxidase and ergosterol synthesis
VI. Others(Topical)- Ciclopirox olamine, haloprogin,tolnaftate,, undecylenicacid, ,quinidochlor, Thiabendazole, salicylic acid, benzoicacid, Sodiumthiosulphate, Copper sulphate, Dichlorophen, Monosulfiram, Sodium iodide, Lufenuron	

Surname _ Date _ _ _ _ _ _ _ _ _ _ _

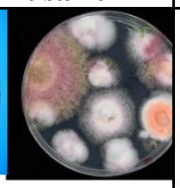
IV. Study the scheme of laboratory diagnosis of opportunistic mycosis.
Specimen: skin scrapings, pus, sputum, blood, liquor, biopsy, vaginal discharge or bronchoscopic washings.

1. Microscopy:

Zygomycetes	Aspergillum	Fusarium	Penicillium	Candida	Cryptococcus
					
Nonseptate, ribbon-like hyphae with branch at right angles, sporangium with different stages of sporangiospore	Septate hyphae, dichotomously branched; Conidiophores and conidiospores	Macro- and microconidia	Conidiophores and conidiospores. Septate hyphae	Yeast cells, pseudohyphae, true hyphae	Budding yeast(arrow) Cells are round with a thick mucoid capsule and have a single bud

2. Culture.

Special media: Sabouraud solid and liquid media, Potato agar, Brain heart medium. Incubation at 25-30 °C for 24-48 hours.

Zygomycetes	Aspergillum	Fusarium	Penicillium	Candida	Cryptococcus
					

3. Serology: Titer of antibodies (AT, CFT, indirect IF, ELISA). Detection of mannan antigen of C. albicans (ELISA, IF, AT).

4. PCR.

5. Skin allergic test: candidiasis (Candida albicans, Candida krusei, Candida tropicalis), **aspergillosis.**

ADDING THEORETICAL MATERIAL

Candidiasis is a fungal infection caused by yeasts that belong to the genus *Candida*. There are over 20 species of Candida yeasts that can cause infection in humans, the most common of which is *Candida albicans*. Candida yeasts normally reside in the intestinal tract and can be found on mucous membranes and skin without causing infection; however, overgrowth of these organisms can cause symptoms to develop. Symptoms of candidiasis vary depending on the area of the body that is infected.

Candidiasis that develops in the mouth or throat is called "thrush" or oropharyngeal candidiasis. Candidiasis in the vagina is commonly referred to as a "yeast infection". Invasive candidiasis occurs when *Candida species* enter the bloodstream and spread throughout the body.

Candidiasis in the mouth and throat can have many different symptoms, including:

- White patches on the inner cheeks, tongue, roof of the mouth, and throat (photo showing candidiasis in the mouth)
- Redness or soreness
- Cottony feeling in the mouth
- Loss of taste
- Pain while eating or swallowing
- Cracking and redness at the corners of the mouth.

Symptoms of candidiasis in the esophagus usually include pain when swallowing and difficulty swallowing.

Diagnosis & Testing. To diagnose candidiasis in the mouth or throat is simply by looking inside. To take a small sample from the mouth or throat and send to a laboratory for testing, usually to be examined under a microscope.

Treatment of candidiasis in the mouth, throat, or esophagus is usually treated with antifungal medicine for 7 to 14 days. These medications include clotrimazole, miconazole, or nystatin. For severe infections, the treatment is usually fluconazole or another type of antifungal medicine.

Risk & Prevention. Candidiasis in the mouth, throat, or esophagus is uncommon in healthy adults. People who are at higher risk for getting candidiasis in the mouth and throat include babies, especially those younger than one month old, and people who: wear dentures, have diabetes, have cancer, have HIV/AIDS, take antibiotics or corticosteroids, including inhaled corticosteroids for conditions like asthma, take medications that cause dry mouth or have medical conditions that cause dry mouth, smoke.

To prevent candidiasis in the mouth and throat includes maintain good oral health, rinse mouth or brush teeth after using inhaled corticosteroids.

Candidiasis in the vagina is commonly called a "vaginal yeast infection". Other names for this infection are "vaginal candidiasis," "vulvovaginal candidiasis," or "candidal vaginitis."

The symptoms of vaginal candidiasis include:

- Vaginal itching or soreness
- Pain during sexual intercourse
- Pain or discomfort when urinating
- Abnormal vaginal discharge

Although most vaginal candidiasis is mild, some women can develop severe infections involving redness, swelling, and cracks in the wall of the vagina.

Diagnosis & Testing. By taking a small sample of vaginal discharge to be examined under a microscope or sent to a laboratory for a fungal culture.

Treatment. Vaginal candidiasis is usually treated with antifungal medicine applied inside the vagina or a single dose of fluconazole taken by mouth.

Risk & Prevention. Vaginal candidiasis is common, though more research is needed to understand how many women are affected. Women who are more likely to get vaginal candidiasis include those who: are pregnant, use hormonal contraceptives (for example, birth control pills), have diabetes, have a weakened immune system, are taking or have recently taken antibiotics.

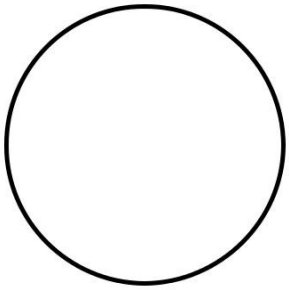
Invasive candidiasis. A Candida bloodstream infection, which is the most common form of invasive candidiasis, is called candidemia.Can be treated with antifungal medication, and antifungal medication is often given to prevent the infection from developing in certain patient groups.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

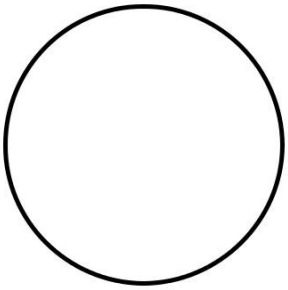
Protocol № 52

Theme: Microbiological diagnosis of Rickettsiosis.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Rickettsia typhi (Zdrodovsky stain)



Rickettsia typhi (Giemsa stain)

II. Study classification of rickettsia:

Medically Important Rickettsias

Bacteria	Disease	Vector	Reservoir
R.prowazekii	Epidemic typhus	Louse	Humans, squirred fleas, flying squirrels
R.typhi	Murine typhus	Flea	Wild rodents
R.rickettsii	Rocky Mountain spotted fever	Tick	Ticks, wild rodents
R.ackari	Rickettsialpox	Mite	Mites, wild rodents
R. conorii	Boutonneuse fever	Tick	Ticks, wild rodents
R. sibirica	Siberian tick typhus	Tick	Ticks, wild rodents
R. australis	Australian tick typhus	Tick	Ticks, wild rodents
R. japonica	Oriental spotted fever	Tick	Ticks, wild rodents
R. africae	African tick bite fever	Tick	Ticks, wild rodents
E.chaffensis	Erlichiosis	Tick	Ticks
O.tsutsugamushi	Scrub typhus	Mite	Mites, wild rodents

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

Epidemiology of erlichiosis

Organism	Disease	Vector	Reservoir	Distribution
***E. canis* subgroup**				
E chaffeensis	**Human monocytic ehrlichiosis**	**Lone Star tick**	**Tick**	**Southeastern, Mid-Atlantic and South Central United States**
***E. phagocytophilia* subgroup**				
***E.equi* (probably)**	**Human granulocytic ehrlichiosis**	**Deer and dog ticks**	**Deer, dogs**	**Wisconsin, Minnesota, Connecticut**
***E. sennetsu* subgroup**				
E. sennetsu	**Sennetsu fever**	**Unknown**	**Unknown**	**Japan**

III. Study the scheme of laboratory diagnosis of epidemic typhus.

Time of disease	Methods	
	Serological	**Biological**
$3^{th} – 5^{th}$ day 11^{th} day	1. Agglutination test. 2. Passive hemagglutination test 3. CFT	Intraperitoneal inoculation of quinea pig to developscrotal phenomena

IV. Study the scheme of laboratory diagnosis of rickettsiosis.

1. Immunohistochemical visualization of rickettsiae in tissues.

2. Isolation of rickettsiae from blood.

3. PCR.

4. Serology: indirect IF, ELISA, latex agglutination test.
Patients with **epidemic typhus** initially have IgM followed by IgG; Patients with **Brill-Zinsser disease** initially have IgG.

ADDING THEORETICAL MATERIAL

Rickettsiae

The rickettsiae are a diverse collection of obligately intracellular Gram-negative bacteria found in ticks, lice, fleas, mites, chiggers, and mammals. They include the genera *Rickettsiae, Ehrlichia, Orientia*, and *Coxiella*. These zoonotic pathogens cause infections that disseminate in the blood to many organs.

Rickettsia species are small, Gram-negative bacilli that are obligate intracellular parasites of eukaryotic cells. This genus consists of two antigenically defined groups: spotted fever group and typhus group, which are related; scrub typhus rickettsiae differ in lacking lipopolysaccharide, peptidoglycan, and a slime layer, and belong in the separate, although related, genus *Orientia*.

Rickettsia and *Orientia* species are transmitted by the bite of infected ticks or mites or by the feces of infected lice or fleas. From the portal of entry in the skin, rickettsiae spread via the bloodstream to infect the endothelium and sometimes the vascular smooth muscle cells. *Rickettsia* species enter their target

cells, multiply by binary fission in the cytosol, and damage heavily parasitized cells directly.

The geographic distribution of these zoonoses is determined by that of the infected arthropod, which for most rickettsial species is the reservoir host.

Rickettsia species cause Rocky Mountain spotted fever, rickettsialpox, other spotted fevers, epidemic typhus, and murine typhus. *Orientia* (formerly *Rickettsia*) *tsutsugamushi* causes scrub typhus. Patients present with febrile exanthems and visceral involvement; symptoms may include nausea, vomiting, abdominal pain, encephalitis, hypotension, acute renal failure, and respiratory distress.

Rickettsioses are difficult to diagnose both clinically and in the laboratory. Cultivation requires viable eukaryotic host cells, such as antibiotic-free cell cultures, embryonated eggs, and susceptible animals. Confirmation of the diagnosis requires comparison of acute- and convalescent-phase serum antibody titers.

Rickettsia species are susceptible to the broad-spectrum antibiotics, doxycycline, tetracycline, and chloramphenicol. Prevention of exposure to infected arthropods offers some protection. A vaccine exists for epidemic typhus but is not readily available.

Ehrlichia sennetsu, E. chaffeensis, and the human granulocytic ehrlichia are genetically distinct and are easily distinguished antigenically.

Ehrlichia species cause ehrlichioses that vary in severity from a life-threatening febrile disease that resembles Rocky Mountain spotted fever, except for less frequent rash, to an infectious mononucleosis-like syndrome.

A reservoir of *E chaffeensis* is deer, and for both human monocytic and granulocytic ehrlichiosis are transmitted when ticks bite human skin and inoculate organisms, which then spread by the bloodstream. Macrophages or neutrophils have cytoplasmic vacuoles that contain ehrlichiae dividing by binary fission in each of these ehrlichioses.

Sennetsu ehrlichiosis has been documented in Japan and Malaysia. Human infections with *E. chaffeensis* and *E. phagocytophila* like organisms have been found recently. Human monocytic ehrlichiosis originates in most of the Atlantic, southeastern, and south central states from New Jersey to Texas. Human granulocytic ehrlichiosis has been identified in the upper midwest and New England thus far.

Clinical and laboratory clues must be confirmed serologically or by polymerase chain reaction detection of specific ehrlichial DNA.

Coxiella burnetii causes Q fever, which may present as an acute febrile illness with pneumonia or as a chronic infection with endocarditis.

Coxiella burnetii varies in size and has an endospore-like form. This species has lipopolysaccharide and phage type diversity.

Coxiella burnetii organisms are transmitted to the human lungs by aerosol from heavily infected placentas of sheep and other mammals and disseminate in the bloodstream to the liver and bone marrow, where they are phagocytosed by

macrophages. Growth within phagolysosomes is followed by formation of T-lymphocyte-mediated granulomas. In the few patients who develop serious chronic Q fever, heart valves contain organisms within macrophages.

Q fever is found worldwide. It is associated mainly with exposure to infected placentas and birth fluids of sheep and other mammals.

The disease is difficult to diagnose clinically, and cultivation poses a biohazard. Therefore, serology is the mainstay of laboratory diagnosis.

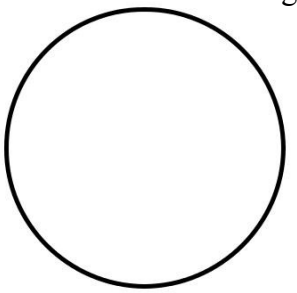
Antibiotics are effective against acute Q fever. A vaccine containing killed phase I organism shows promise in protecting against infection.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

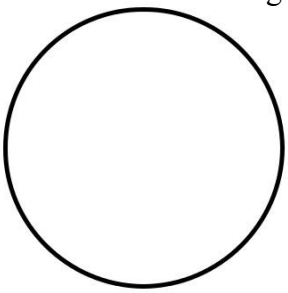
Protocol № 53

Theme: Microbiological diagnosis of Chlamydia and Mycoplasmas infections.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Mycoplasma pneumoniae
(Giemsa stain)



Chlamydia trachomatis
(Giemsa stain)

II. Study human diseases caused by Chlamydia and Mycoplasmas:

Medically Important Chlamydiae

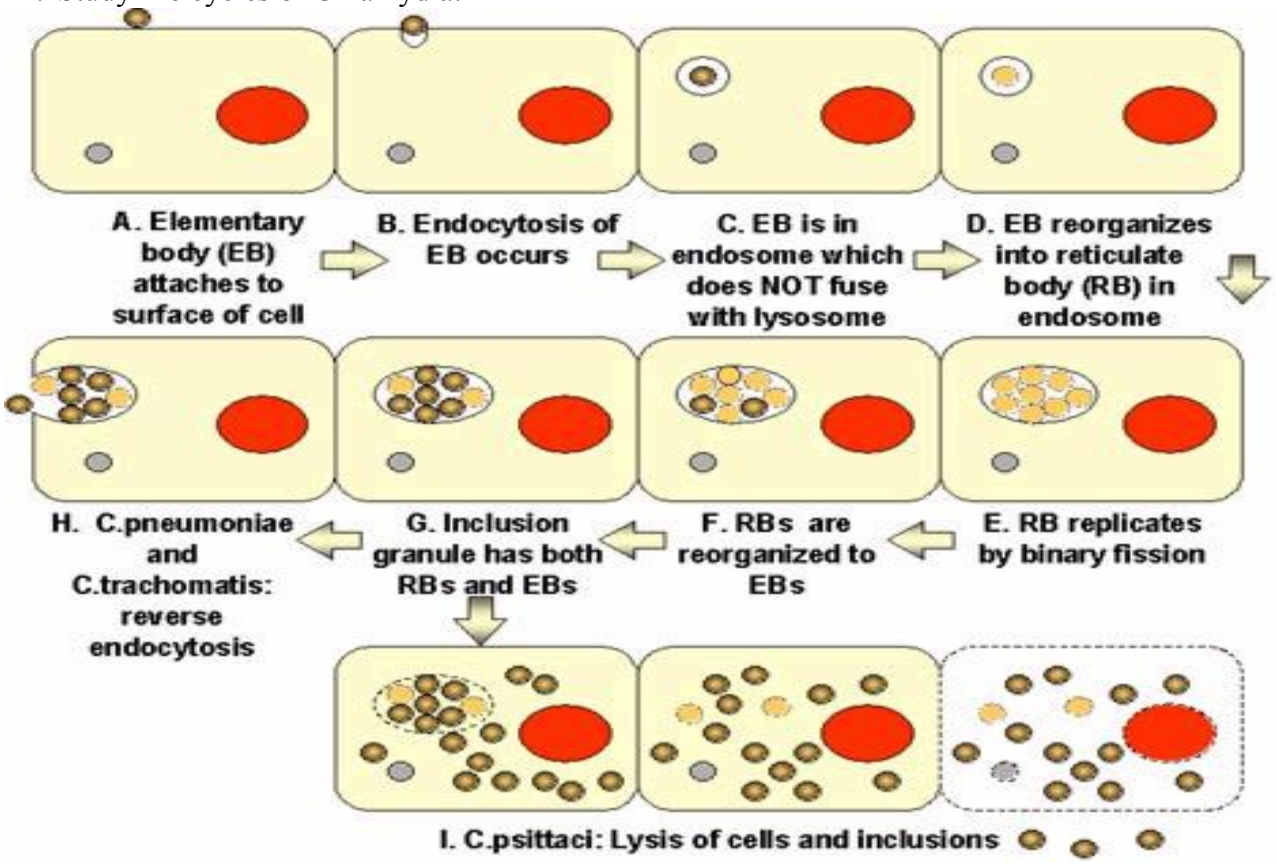
Species	Serotype	Disease
Chlamydia trachomatis	A, B, Ba, C	Trachoma, a serious eye infection.
	D,F,G,H,I,J,K	- Nongonococcal urethritis and genital tract diseases, the most common sexually transmitted bacterial disease. - Reiter's syndrome (urethritis, conjunctivitis, arthritis). - Conjunctivitis and pneumonia of newborn.
	L_{1-3}	Lymphogranuloma venereum
Chlamydophylla psittaci		Pneumonia ("psittacosis", "ornitosis" or "parrot fever"), bronchitis, upper respiratory infection
Chlamydophylla pneumoniae		Mild pneumonia

Medically Important Mycoplasmas

Organism	Habitat	Disease
M. pneumoniae	Human respiratory tract	Upper respiratory tractdisease, tracheobronchitis, atypical pneumonia, pericarditis, meningitis, polyartritis
M. hominis	Human respiratory and genital tract	Pyelonephritis, pelvic inflammatory disease, postpartum fever
M. genitalium	Human respiratory and genital tract	Nongonococcl urethritis
U. urealyticum	Human genitourinary tract	Nongonococcl urethritis
M. fermentans	Human genitourinary tract	Pyelonephritis, arthritis
M. orale	Human mouth	Periodontitis
M. salivarium	Human mouth	Periodontitis
M.arthritidis	Sinovial liquid	Arthritis

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

III. Study life cycles of Chlamydia:



IV. Study the scheme of laboratory diagnosis of **chlamydiosis**.
Specimen: scrapings from the eyes or the urogenital tract.
1. Microscopic examination of Giemsa stained cell for the presence of inclusion bodies.
2. Culture in cell culture or yolk sac of chicken embryo.
Identification: infected cells are examined for the presence of iodine-staining inclusion bodies. Iodine stains glycogen in the inclusion bodies of *C. trachomatis*.
3. Direct IF detects antigens.
4. Serology (detection of high titer IgM is indicative of a recent infection): **ELISA, CFT**.
5. PCR.

V. Study the scheme of laboratory diagnosis of **mycoplasmosis**.
1. Direct IF detects antigens.
2. Culture: A medium consists of heart infusion, peptone, yeast extract, salts, glucose or arginine, and horse serum (5 to 20 %).
Identification: biochemical tests, serologic tests (growth inhibition on agar by specific antiserum), urease activity.
3. Serology:
CFT - from 4 - 6 weeks after infection. A fourfold rise in titer is indicative of a recent infection.
Cold agglutination - Cold agglutinins are antibodies that agglutinate human erythrocytes at 40C but not at 370C.
ELISA - for IgM.
4. PCR.

ADDING THEORETICAL MATERIAL

All mycoplasma infections have one thing in common, though. They're caused by tiny living things called bacteria.

Unlike other bacteria, the ones that lead to mycoplasma infections don't have cell walls. That's important because many antibiotics kill bacteria by weakening those walls. Since mycoplasma bacteria don't have them, some antibiotics, like penicillin, won't work against them.

There are about 200 types of mycoplasma bacteria, but most of them are harmless. The ones you may have to worry about are:

- *Mycoplasma pneumoniae*
- *Mycoplasma genitalium*
- *Mycoplasma hominis*
- *Ureaplasma urealyticum*
- *Ureaplasma parvum*

Mycoplasma pneumoniae

This type causes lung infections. About a third of people who get infected come down with a mild form of pneumonia called "walking pneumonia." Most people, especially children, will get "tracheobronchitis," a fancy name for a chest cold.

You can catch one of these infections when someone who is sick coughs or sneezes and sends droplets with the bacteria into the air.

In *Mycoplasma pneumoniae* infection symptoms are like:

- Sore throat
- Cough
- Fever
- Fatigue
- Headache

To treat infection, these types of antibiotics may be suggested:

- Fluoroquinolones like levofloxacin or moxifloxacin
- Macrolides such as azithromycin or erythromycin
- Tetracyclines like doxycycline

Mycoplasma genitalium

You get this if you have sex with someone who's infected. Some people don't have any symptoms.

In woman symptoms may be: pain during sex, bleed from the vagina after sex, a discharge from the vagina.

In man, the infection can cause: Urethritis, stinging or burning when pee, discharge from the penis.

For treatment antibiotics may be:

- Fluoroquinolones like levofloxacin or moxifloxacin
- Macrolides such as azithromycin
- Tetracyclines like doxycycline

Partner may need to get treated, too.

Mycoplasma hominis

These bacteria live in the urinary tract and genitals of about half of all women and fewer men. But if you're in general good health, you don't have to worry. They rarely cause an infection. Women with a weakened immune system -- your body's defense against germs -- are most at risk.

The bacteria can also pass from a mother to her baby during childbirth.

In woman, these bacteria may be linked to pelvic inflammatory disease, an infection of reproductive organs. They can also lead to problems in pregnant, such as:

- Ectopic pregnancy (the embryo grows outside the uterus)
- Early delivery
- Miscarriage.

Mycoplasma hominis can also cause a fever and infection in newborn baby.

Ureaplasma urealyticum and Ureaplasma parvum

Most healthy women have these bacteria in their cervix or vagina, and a smaller number of men also have them in their urethra. Normally, they don't cause any problems.

Ureaplasma can spread during sex. Infected pregnant woman can pass the bacteria to baby in the womb or during childbirth.

Some symptoms women can get are:

- It hurts when you pee
- Belly pain
- Pain, odor, or discharge from the vagina
- Swelling at the opening of the urethra
- Discharge from the urethra

Men who are infected can get inflammation of the urethra, called urethritis.

During pregnancy, the bacteria can lead to infections in both the mother and the baby. Problems in newborn babies can include low birth weight, pneumonia, bacteria in the blood, called septicemia.

An antibiotic to treat the infection have to be percribed:

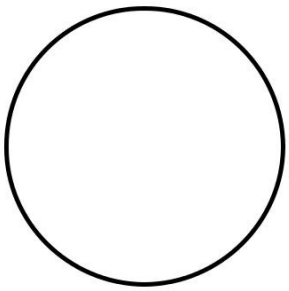
- Fluoroquinolones like moxifloxacin
- Macrolides such as azithromycin
- Tetracyclines like doxycycline

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _

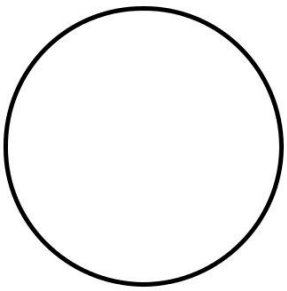
Protocol № 54, part 1

Theme: Sanitary microbiology. <u>Part 1.</u> Sanitary microbiology of water.

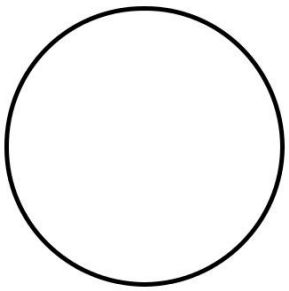
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



E. coli
(Gram stain)



C. perfringens
(Gram stain)



E. faecalis
(Gram stain)

II. Introduction to microbiological parameters to the sanitary guality of environmental samples:

1. Heterotrophic plate count.
2. Coliform bacteria group: total coliform group; total coliform E.coli; total Enterococcus; Klebsiella.

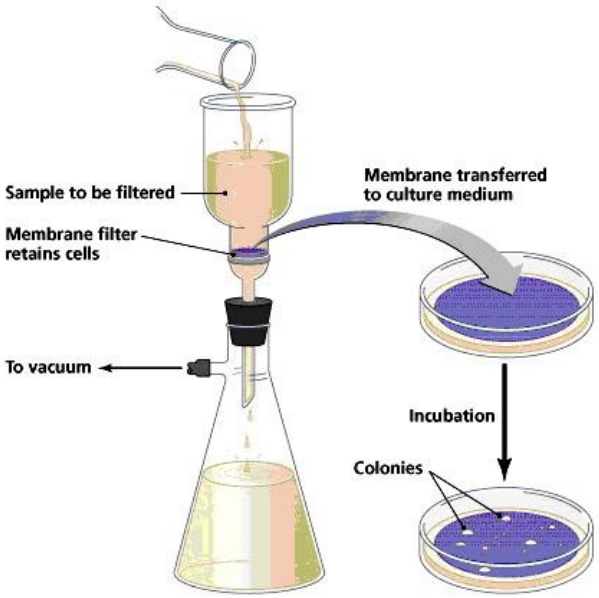
III. Regulation for drinking water quality: minimum coliform monitoring

Coli-titer: the least of water (ml), a coliform (Bacteria group of Escherichia coli – BGEC) is revealed in wich. For drinking water: coli-titer>333ml.

Coli-index: amount of BGEC in 1 litre of water. For drinking water: coli-index<3.

IV. Laboratory quality assurance and quality control:

1. Collecting and handling water samples for microbiological examinations
2. Direct measurement of bacteria growth: dilution water,
   plate count (pour, spred, streak),
   membrane filtration technique.



Membrane filtration technique

ADDING THEORETICAL MATERIAL

Sanitary microbiology is a science based on the detection of risks associated with the production, manufacture and consumption of foodstuffs, air and water. It has been established that environment facts determine the survival, growing and inactivation of the microorganisms. These risks are commonly associated with the presence of microbiological hazards and represent a serious problem from the Public Health viewpoint. The types of microorganisms in products depend on the way they have been elaborated, transportated, stored, taken or prepared before eating. The study of the sanitary and bacteriological objects of external environment is needed for warning development of infections, understanding of infection origin. For example, the absence of coliforms in a water sample does not give absolute assurance of the absence of pathogenic organisms, but it is a good indication. Certain other types of microbial pathogens such as viruses (hepatitis A, poliomyelitis, norovirus) and protozoa (giardia, cryptosporidium, entamoeba) can be present in the absence of bacterial indicators. Experience has shown that disinfection of water supplies to achieve zero coliform counts has generally been successful at preventing water-borne disease. Therefore every specialist must know the basic ways of pathogens transmission, to know the basic bacteriological indicators of contamination of external environment objects, able to estimate the results of sanitary and bacteriological examination. Primary objective: to be able to conduct and evaluate the sanitary and bacteriological investigation of water, soil, air, and foodstuffs.

The importance of the provision of a wholesome supply of drinking water has been recognised since at least the times of the Romans with major advances being made during the nineteenth century. Knowledge, understanding and good practice has continued to be gained and developed over time with consequential benefits for public health. The provision of safe drinking water is one of the most important steps that can be taken to improve the health of a community by preventing the spread of water-borne disease. The maintenance of a sufficient supply of wholesome drinking water is a complex undertaking in which individuals from many disciplines have a role.

The use of indicator organisms, in particular the coliform group, as a means of assessing the potential presence of water-borne pathogens has been paramount to protecting public health. These are based upon the principle of the detection of selected bacteria that are indicative of either contamination or deterioration of water quality through the use of simple bacteriological tests. This has been the foundation upon which protection of public health from water-borne disease has been developed. The relatively rare occasions where bacterial or viral illnesses have been caused through public drinking water supplies stand testament to the success of the indicator principle and improvements in water treatment.

Indicator organisms are used to assess the microbiological quality of water. For many pathogens, such as viruses and protozoan parasites, reliable indicators are not available. Even if there were, there is no absolute correlation between the number of indicator organisms and (a) the actual presence or numbers of enteric pathogens or (b) the risk of illness occurring. The use of indicator bacteria, in particular Escherichia coli (E.coli) and the coliform bacteria, as a means of assessing the potential presence of water-borne pathogens has been paramount to protecting public health. The analysis of large volumes of

sample for faecal indicator bacteria using membrane filtration procedures can be very useful in assessing water treatment efficiency

Many pathogens are present only under specific conditions and, when present, occur in low numbers compared with other micro-organisms. Whilst the presence of coliform bacteria does not always indicate a public health threat, their detection is a useful indication The bacteriological examination of water is particularly important as it remains the most sensitive method for detecting faecal and, therefore, potentially dangerous contamination. Chemical analysis is, nevertheless, an important aid to the hygienic assessment of a water supply. However, the major role of chemical analysis is to provide process control information for water treatment and for monitoring compliance with prescribed standards. Chemical tests that give additional information on whether faecal contamination may be present include turbidity, colour, total organic carbon, nitrate, nitrite and ammonia.

Tests for colony count bacteria growing at 37 °C and 22 °C enable a count to be determined of the heterotrophic bacterial population of the water. The bacteria grown in these tests are not indicators of faecal contamination

Coliform bacteria belong to the family Enterobacteriaceae and share similar cultural characteristics. Typical genera encountered in water supplies are Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Serratia and Yersinia. Coliform bacteria are defined as Gram-negative, non-spore-forming, rod shaped bacteria which are capable of aerobic and facultative anaerobic growth in the presence of bile-salts or other surface-active agents with similar growth-inhibiting properties. They usually ferment lactose at 37 °C within 48 hours, possess the enzyme β-galactosidase and are oxidase-negative. Faecal coliform bacteria possess the characteristics of coliform bacteria but are able to carry out lactose fermentation at 44 °C. The term “faecal coliform” is not precise and has been used to describe coliform bacteria thought to be of faecal origin. The term “thermotolerant coliform” has been used to describe presumptive faecal coliform bacteria.

When coliform bacteria are isolated from drinking water supplies it is often useful to determine which species of coliform bacteria are present, particularly if problems recur, in order to determine the source and significance of the coliform bacteria being recovered. The potential source of coliform bacteria in water supplies result from sub-optimal operation of water treatment processes or ingress of contamination from breaches in the integrity of the distribution system. These include for example, leaking hatches on service reservoirs, contamination via air-valves and stop valves, infiltration into mains and service reservoirs, cross connections and back-flow effects. Coliform bacteria can be present in domestic plumbing systems with kitchen taps and sinks being recognised sources of these organisms.

E. coli is a coliform bacterium and has historically been regarded as the primary indicator of faecal contamination of both treated and untreated water. As a coliform bacterium it is a member of the family Enterobacteriaceae, and is capable of fermenting lactose or mannitol at 44 °C, usually within 24 hours, and produces indole from tryptophan. Most of the E. coli strains possess the enzyme β-glucuronidase, which can be detected using specific fluorogenic or chromogenic substrates.

Colony counts are enumerations of the general population of heterotrophic bacteria present in water supplies. The enumerations may represent bacteria whose natural habitat is

the water environment or those that have originated from soil or vegetation. Historically, these bacteria have been enumerated on bacteriologically nutrient-rich media with incubation at 37 °C and 22 °C. It is well recognised, however, that only a small fraction of the viable bacterial population present in water is enumerated by the procedures normally employed. Despite this, monitoring of water supplies for colony count bacteria can be useful for monitoring trends in water quality or detecting sudden changes in quality.

An important benefit of determining colony counts at both 37 °C and 22 °C, particularly if carried out regularly from the same site and location, is that the data generated can provide an indication of seasonal and longer-term changes in the general bacteriological quality of the water. Many heterotrophic bacteria are able to multiply within the distribution system network by utilising nutrients derived either from fixtures and fittings or from assimilable or particulate organic carbon in the water. Changes in colony numbers may, therefore, be indicative of the use of inappropriate materials or changes in the quality of the source water. Drinking water supplies derived from surface waters tend to support higher numbers of heterotrophic bacteria than those derived from groundwater sources.

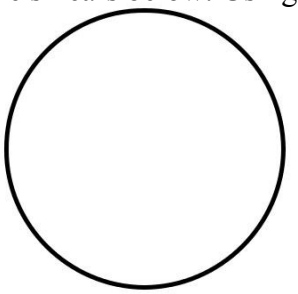
E. coli and related coliform bacteria, intestinal enterococci and Clostridium perfringens are currently recommended for use as indicator organisms of faecal contamination in water. Other micro-organisms have been suggested for this purpose and these include the Bacteroides fragilis group, Bifidobacterium species or Rhodococcus coprophilus. Also bacteriophages that infect the coliform bacteria (coliphages) and the Bacteroides fragilis group have been used. Although some of these alternative indicator organisms have been applied with varying degrees of success to environmental waters, they are not considered suitable for the assessment of water treatment efficacy or treated water quality. E. coli and related coliform bacteria, intestinal enterococci and Clostridium perfringens are currently recommended for use as indicator organisms of faecal contamination in water. Other micro-organisms have been suggested for this purpose and these include the Bacteroides fragilis group, Bifidobacterium species or Rhodococcus coprophilus. Also bacteriophages that infect the coliform bacteria (coliphages) and the Bacteroides fragilis group have been used. Although some of these alternative indicator organisms have been applied with varying degrees of success to environmental waters, they are not considered suitable for the assessment of water treatment efficacy or treated water quality.

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

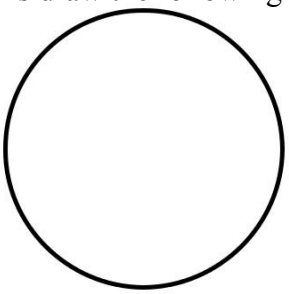
Protocol № 54, part 2

Theme: Sanitary microbiology. *Part 2. Sanitary microbiology of air.*

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



S. aureus
(Gram stain)



S. pyogenes
(Gram stain)

The detection of airborne microbial contamination

Air quality plays a key role in the pharmaceutical, biotechnological and food and beverage industries, in hospitals and in the field of occupational and environmental protection. The most frequently used method today for sampling airborne organisms is based on the Andersen principle that traps particles on culture media by impaction. In this method, air is suctioned through a sieve, accelerated and directed against a culture medium plate. Due to their inertia, airborne organisms are prevented from being swept away by the diverted stream of air and are impacted onto the culture medium plate. After sampling, the culture medium plate is incubated and the colonies grown are counted as colony-forming units/m3 of air (cfu/m3).

1. MD8 airscan® - Air Sampler for Microbiological Air Monitoring

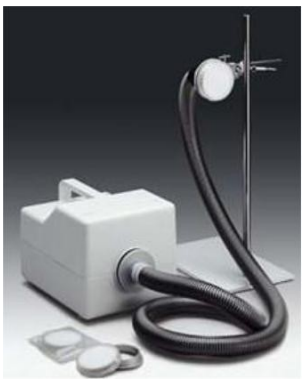
The system consists of the MD8 airscan® air sampler and disposable gelatine filter units. The system is routinely used for the quantitative detection of airborne organisms, mainly in sterile areas of class A and B (classification according to "EU-Guide for GMP"), isolators, or blow-fill-seal machines.

The very high air flow rate of 8 m^3/h enables isokinetic sample removal at flow speed usual in laminar flow as well as for the filtration of 1 m^3 air very quickly (less than 8 minutes). The filter unit can be placed remote from the air sampler.

Gelatine filter disposables are individually packed, presterillized and ready-to-connect units, each consisting of a gelatine membrane filter and a holder.

After a sample is taken the gelatin membrane filter is placed directly onto an agar plate. The gelatin dissolves on the moist surface so that the microorganisms come into direct contact with the nutrients. The plates are then incubated and the colonies counted to check the level of microorganisms in the atmosphere.

The air flow, sample rate and other operational parameters can be saved by the end user after shutdown by using the in-built electronics in the system. This should save calibration time upon re-use. The device weighs 2.5 kg and is 300 mm long, 135 mm wide and has a depth of 165 mm.



MD8 airscan® - Air Sampler

Gelatine Membrane Filters

2. The AirPort MD8 air sampler.

Sartorius Stedim Biotech has developed a new system for sampling airborne microorganisms that allows impaction onto culture media plates, where the plates function directly as collection heads. This means that the collection properties are integrated right into the culture media plates. Metal sieve plates or metal collection heads with slots, which have to be sterilized for routine samplings on a regular basis, are eliminated. Now, non-sterile sieves or slots have become a thing of the past.

The geometry of the culture medium plate and the 400 holes in the sieve plate yield exceptional sampling efficiency, which is generally higher than that of other impaction samplers.

This new method uses the AirPort MD8 air sampler to pump the air stream. BACTairs™ are ready-to-connect to the AirPort MD8.

The AirPort MD8 air sampler

BACTair ™

ADDING THEORETICAL MATERIAL

The main objective **of sanitary and microbiological research of air** — a hygienic and epidemiological assessment of the air environment, and also development of a complex of the actions directed to prevention of aerogenic transfer of causative agents of infectious diseases. At an assessment of a sanitary condition of the enclosed space depending on research problems define OMCh, existence of SPM (stafilokokk, and - and the beta and hemolitic streptococci which are kontamination indicators microflora of a nasopharynx of the person). Due to the development of the biotechnology industry using various microorganisms producers of BAV the risk of emission in the atmosphere of big concentration of microbes, including with the changed genotype significantly increased. At the same time, the production technology of some substances directly includes

periodic release of microorganisms. Specified gives to a problem of control of microflora of atmospheric air and disinfecting of emissions of the biotechnology enterprises special relevance.

Microflora of air

Microbic impurity of air has non-constant and local character, that is the microflora of air depends on the place and time of sampling. In the summer the obsemenyonnost of air is several times higher, than in the winter. Atmospheric air with microorganisms over the large cities is especially saturated. By consideration of qualitative structure of microflora of air it is necessary to distinguish microflora of atmospheric air and air of premises.

Microflora of atmospheric air. In the SPM atmospheric air (staphylococcus and streptococci) find only in 3,7% of the samples taken in places of big accumulation of people. Among microorganisms the types living in the soil dominate. In atmospheric air generally meet three groups of microorganisms.

• **Chromogenic cocci** in sunny days make up to 70-80% of all flora (the pigment protects bacteria from insolation).

• **Soil sporous and putrifactive microorganisms**. Their contents sharply increase in dry and windy weather.

• **Mold mushrooms and yeast**. Their contents increase at air humidity increase.

Unlike air of the enclosed space, in atmospheric air constantly there are self-cleaning processes. This process happens thanks to rainfall, insolation, temperature influences and other factors. In turn atmospheric air in itself — a factor of clarification of air of premises.

The microflora of air of the enclosed space is more uniform and rather stable. Among microorganisms inhabitants of a nasopharynx of the person, including the pathogenic types getting to air at cough, sneezing or conversation dominate. The main source of air pollution by pathogenic types — bacillicarriers. Level of microbic pollution depends mainly on density of settling, activity of the movement of people, a sanitary condition of the room, including dust impurity, ventilation, frequency of airing, a way of cleaning, degree of illumination and other conditions. So, regular airings and wet cleaning of rooms reduces an air obsemenyonnost by 30 times (in comparison with control rooms). Self-cleaning of air of the enclosed space does not happen.

Indicator microorganisms of a sanitary condition of air

Research of air in MPI is conducted once a quarter at the current sanitary inspection of the State sanitary and epidemiological surveillance by the center; once a month bacteriological laboratories of hospitals and according to epidemiological indications. In system of hygienic and anti-epidemic actions at the current sanitary inspection define quantity of SPM in 1 m3 of air. At the current supervision carry golden staphylococcus, streptococci, gram-negative bacteria and mushrooms to SPM (in drugstores). OMCh — normalized, but after all a relative indicator.

• **In air of hospital rooms** golden staphylococcus and streptococci dominate. The ratio of microorganisms averages 70 and 30% respectively. At the

same time in 1 m3 of air of operational halls, postoperative chambers, dressing rooms, intensive care units, delivery rooms they have to be absent.

• **Due to growth of frequency of the diseases** caused by gram-negative bacteria standards included definition of their quantity in 1 m3 of air of premises of MPI.

• The special case — **air of pharmaceutical rooms** where because of existence of germicides bacteria can quickly perish but remains mushrooms therefore they need to be revealed at research of air of drugstores.

• **Additional criteria**. As an indicator of dust content and lack of wet cleaning regard presence of spore-forming sticks, and an indicator of the increased humidity — mold mushrooms. An indicator of bad illumination — lack of chromogenic forms of bacteria (sometimes this indicator can be determined by a task of phthisiatricians).

For allocation **of microorganisms from air** use the following methods.

Sedimentation method of research of air (Koch's method). Usually use for establishment of structure of microflora in the enclosed space. Pyotri's cups with Wednesday display in various places y open for a certain time; then incubate and reveal specific accessory of microorganisms. Most often in air define OMCh (apply cups with MPA; time of exposure of 10-30 min.), the maintenance of stafilokokk [is applied by cups with a vitelline sole-vym an agar (ZhSA); time of exposure of 15 min.], determine the maintenance of streptococci by epidemic indications (apply cups with KA; time of exposure of 10-15 min.). At inspection of air of drugstores make determination of content of mushrooms, using cups with Saburo's circle. At a quiet condition of air on the area of 100 cm2 settles so many microorganisms how many they contain in 0,01 ^{cm3} of air.

Filtrational method of research of air. Pass a stream of the taken-away air through a sheet of water, then carry out allocation and identification of the microorganisms which got to water.

The methods based on shock action of a current of air. Special devices allow to determine precisely quantitative planting of air by specific microorganisms. The mechanism of catching of microflora is based on "shock pribivnom" action of a current of air which passes through a narrow crack and hits against a wet surface of a medium. During selection Pyotri's cup rotates together with a little table that provides hypodispersion of microorganisms on a surface of Wednesday. Big advantage of this method — a possibility of crops of a certain volume of air.

Conditions of circulation of microorganisms in air. Indicator microorganisms of a sanitary condition of air.

Microorganisms are airborne in a condition of an aerosol. Allocate three main phases of a bacterial aerosol.

The drop, or large particle phase consists of the bacterial cells surrounded with a water-salt cover. Diameter of particles is about 0,1 mm and more. Particles settle quickly enough: duration of stay in air makes several seconds, and movement speed — on average 30 cm / page.

The smallparticle phase is formed when drying particles of the first phase and consists of the bacterial cells which kept only chemically connected water on the surface and free water in cells. In this phase of a particle have the smallest sizes, easily move air flows, a long time are in it in a suspension. It is the steadiest phase as diameter of the majority of particles does not exceed 0,05 mm, and the speed of sedimentation of particles makes, on average, 0,013 cm / page. At the same time the speed of their movement exceeds 30 cm / with therefore they can dissipate on long distances. This phase constitutes the greatest epidemiological danger as in its structure the majority of activators of droplet infections, especially unstable to external influences extends (for example, the causative agent of whooping cough).

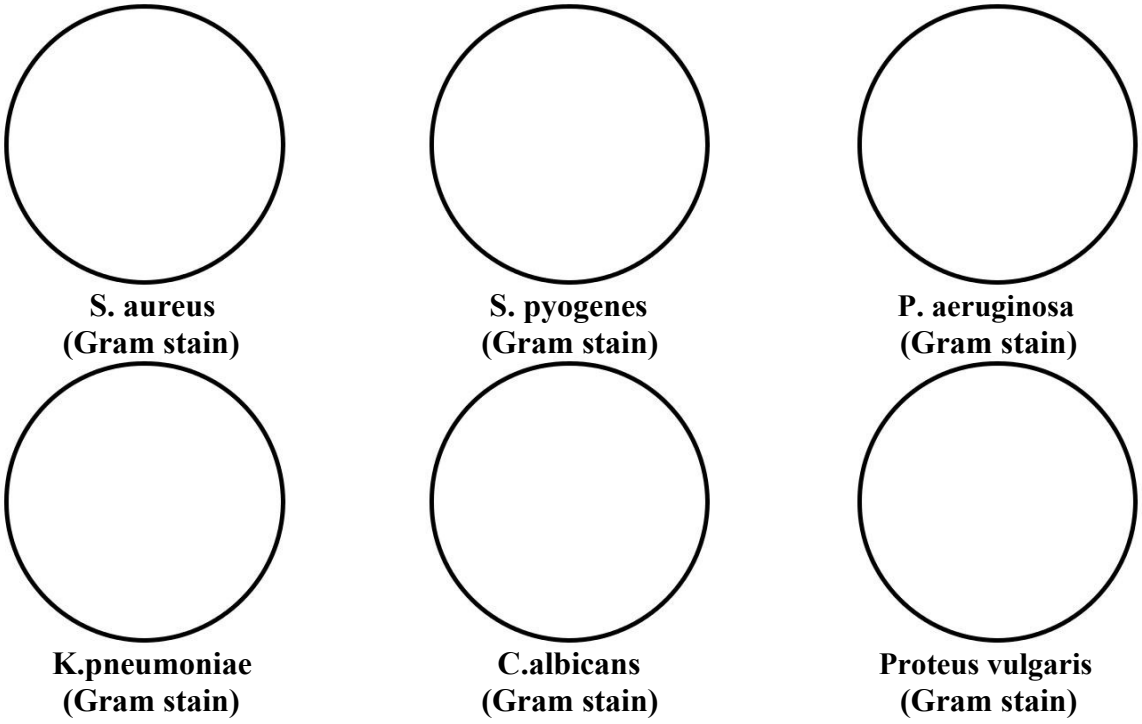
Phase of "bacterial dust". From the first two phases of a bacterium can pass into structure of more coarse particles settling in the form of dust on various objects forming so-called "bacterial dust". Its important property — ability is easy to be dispersed under influence even of small air flows. Particle size varies from 0,01 to 1 mm. Depending on particle size and the speed of air currents, the speed of their movement is in limits of 0,5-30 cm / page. Owing to long stay in a suspension and ability of particles to get into distal departments of lungs, finely divided "bacterial pyl also constitutes epidemiological danger. This phase of a bacterial aerosol prevails in air of premises and with it the pathogenic microorganisms steady against drying dissipate (a mycobacterium, a clostridium, staphylococcus, streptococci, mushrooms).

Surname_____ Date_____

Protocol № 55

Theme: Clinical microbiology. ***Hospital infections.***

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



S. aureus (Gram stain) | **S. pyogenes (Gram stain)** | **P. aeruginosa (Gram stain)**

K.pneumoniae (Gram stain) | **C.albicans (Gram stain)** | **Proteus vulgaris (Gram stain)**

II. Study risk factors, sources, etiology, pathogenesis and microbiological diagnosis of hospital infections caused by pathogenic and opportunistic bacteria.

Iatrogenic risk factors include:

1) Many of hospital strains demonstrate a **high degree of drug resistance** because they have been exposed to antibiotics and disinfectants over many generations in the hospital setting. Infections due to such organisms tend to be difficult to treat.

2) Major invasive procedures, diagnostic or therapeutic (eg, intubation, endoscopy, and catheterization), are carried out only in hospitals. The slightest lapse in asepsis during these procedures can lead to infection.

3) Advances in treatment of cancer, organ transplantation, implanted prostheses and other sophisticated medical technologies: **Radiation therapy, chemotherapy, general anesthesia, steroid drugs, and immunosuppressant drugs** (widely used to prevent rejection of transplanted organs) further weaken the immune systems of many hospital patients and enhance the risk of infection to patients.

Organizational risk factors:

1) The hospital environment is heavily laden with a wide variety of
pathogens: patients shed them from their bodies;
hospital personnel spread them through their hands and clothes.
pathogens are present in the hospital dust and air, and sometimes even in antiseptic lotions and ointments.
contamination of hospital food or water may cause outbreaks of infections.

2) Because of frequent exposure, many medical personnel house pathogenic organisms as part of their **normal bacterial flora**.

3) Medical personal moving rapidly from patient to patient create a **chain of transmission** which can readily transmit infectious diseases,

Patient risk factors include the severity of illness, immunocompromised state by underlying disease or treatment, malnutrition, age; and length of stay. Many hospital patients are, therefore, highly susceptible to infection.

Sources of nosocomial infections: Nosocomial infections can come from either exogenous or endogenous sources

Endogenous infections are caused by opportunistic pathogens that come from a patient's own normal flora. Endogenous infections may be acquired due to: patients being immunologically depressed; as a consequence of a loss of microbial antagonism due to antibiotic treatment; in the course of an invasive procedure; due to injury.

Exogenous infections come from sources outside of the patient's own body. Hospital pathogens can be found incorporated among the normal flora of staff or on fomites.

III. Study microbiocenosis of human body.

Normal flore is the mixture of organisms regularly found at any anatomical site is referred to as the normal flora.

Opportunistic microflora: bacteria which cause a disease in a compromised host which typically would not occur in a healthy (noncompromised) host.

The benefits of the normal flora to the human host:

1. synthesize and excrete vitamins (B and K), and break down food stuffs that are normally indigestible by the host into components that can be digested.

2. prevent colonization by pathogens by competing for attachment sites or for essential nutrients.

3. antagonize other bacteria through the production of antibacterial chemicals (**bacteriocins**) which inhibit or kill nonindigenous species.

4. stimulate the development of certain tissues, i.e., the caecum and certain lymphatic tissues (Peyer's patches) in the GI tract.

5. stimulate the production of cross-reactive antibodies. Low levels of antibodies produced against the normal flora cross react with pathogens, and thereby prevent infection.

ANATOMIC LOCATION OF NORMAL FLORA

- **Skin** (104-105 microbes/cm2)
  - S.epidermidis (in 85-100% of normal individuals)
  - S.aureus (in 10-40%)
  - Propionibacterium (in 45-100% of teenagers)
  - Corynebacterium
- **Oral cavity**
  - Lactobacillus (in neonates)
  - Gram-positive cocci (in breast-fed babies)
  - Gram-negative bacilli (in bottle-fed babies)
  - Streptococcus spp.

Anaerobes:

  - Peptostreptococcus spp.
  - Veillonella spp.
  - Fusobacterium spp.
  - Bacteroides spp.
  - Treponema spp.

- **Upper respiratory tract**
  - Nose: S.aureus, S.epidermidis
  - Nasopharynx: Corynebacterium spp., Neisseria spp., *S. pneumoniae, S. pyogenes, H. influenzae, N. meningitidis*

- **The lower respiratory tract (trachea, bronchi, and pulmonary tissues)** are virtually free of microorganisms, mainly because of the efficient cleansing action of the ciliated epithelium which lines the tract.
- **Gastrointestinal tract**
  - Stomach: H. pylori
  - Colon: (1011per gram - 90%-anaerobes)
    Bifidobacteria are prevalence
    Bacteroides
    Streptococci
    C.perfringens
    Lactobacilli
    E. coli is prevalence
    Enterococci
- **Genitourinary tract**
  - Uretra
    S. *epidermidis,*
    E. faecalis
    alpha-hemolytic streptococci
    E. coli, Proteus
  - Vagina
    Lactobacillus
    Bacteroides
    Corynebacterium
    E. coli

IV. Dyisbacteriosis: classification, methods of laboratory diagnosis and treatment.

Dysbiosis (also called **dysbacteriosis**) refers to a condition with microbial imbalances (change of structure and quantitative ratio of a microflora) on or within the body. Dysbiosis is most prominent in the degestive tract or on the skin, but can also occur on any exposed surface or mucous membrane such as the vagina, lungs, mouth, nose, sinuses, ears, nails, or eyes.

Symptoms of Dysbacteriosis:

- Diarrhea
- Weight Gain
- Vaginal Yeast Infections
- Diabetes and Autoimmune Disorders
- Dandruff and Fungal Toenails
- Fatigue and Tiredness
- Depression and Anxiety
- Frequent Sinus Infections
- Constipation

In treatment of the dysbacteriosis of intestines associated with antibiotics now key position occupy **probiotics** – the preparations containing microorganisms which make positive impact on an intestinal microbiocenosis (Bifidobacterium bifidum, Lactobacillus acidophilus, Lactobacillus GG, Lactobacillus fermentum, Enterococcus faecium SF68, S. termophilus, Saccharomyces boulardii). The listed microorganisms are a part of numerous preparations, both monobacterial, and combined.

ADDING THEORETICAL MATERIAL

A nosocomial infection is contracted because of an infection or toxin that exists in a certain location, such as a hospital. People now use nosocomial infections interchangeably with the terms health-care associated infections (HAIs) and hospital-acquired infections. For a HAI, the infection must not be present before someone has been under medical care.

One of the most common wards where HAIs occur is the intensive care unit (ICU), where doctors treat serious diseases. About 1 in 10 of the people admitted to a hospital will contract a HAI. They're also associated with significant morbidity, mortality, and hospital costs.

As medical care becomes more complex and antibiotic resistance increases, the cases of HAIs will grow. The good news is that HAIs can be prevented in a lot of healthcare situations. Read on to learn more about HAIs and what they may mean for you.

For a HAI, the infection must occur:

- up to 48 hours after hospital admission
- up to 3 days after discharge
- up to 30 days after an operation
- in a healthcare facility when someone was admitted for reasons other than the infection

Symptoms of HAIs will vary by type. The most common types of HAIs are:

- urinary tract infections (UTIs)
- surgical site infections
- gastroenteritis
- meningitis
- pneumonia

The symptoms for these infections may include:

- discharge from a wound
- fever
- cough, shortness of breathing
- burning with urination or difficulty urinating
- headache
- nausea, vomiting, diarrhea

People who develop new symptoms during their stay may also experience pain and irritation at the infection site. Many will experience visible symptoms.

Bacteria, fungus, and viruses can cause HAIs. Bacteria alone cause about 90 percent of these cases. Many people have compromised immune systems during their hospital stay, so they're more likely to contract an infection. Some of the common bacteria that are responsible for HAIs are:

Bacteria	Infection type
Staphylococcus aureus (*S. aureus*)	blood
Escherichia coli (*E. coli*)	UTI
Enterococci	blood, UTI, wound
Pseudomonas aeruginosa (*P. aeruginosa*)	kidney, UTI, respiratory

Of the HAIs, *P. aeruginosa* accounts for 11 percent and has a high mortality and morbidity rate.

Bacteria, fungi, and viruses spread mainly through person-to-person contact. This includes unclean hands, and medical instruments such as catheters, respiratory machines, and other hospital tools. HAI cases also increase when there's excessive and improper use of antibiotics. This can lead to bacteria that are resistant to multiple antibiotics.

Anyone admitted to a healthcare facility is at risk for contracting a HAI. For some bacteria, your risks may also depend on:

- your hospital roommate
- age, especially if you're more than 70 years old
- how long you've been using antibiotics
- whether or not you have a urinary catheter
- prolonged ICU stay
- if you've been in a coma
- if you've experienced shock
- any trauma you've experienced
- your compromised immune system

Your risk also increases if you're admitted to the ICU. The chance of contracting a HAI in pediatric ICUs is 6.1 to 29.6 percent. A study found that nearly 11 percent of roughly 300 people who underwent operations contracted a HAI. Contaminated areas can increase your risk for HAIs by almost 10 percent.

HAIs are also more common in developing countries. Studies show that five to 10 percent of hospitalizations in Europe and North America result in HAIs. In areas such as Latin America, Sub-Saharan Africa, and Asia, it's more than 40 percent.

Many doctors can diagnose a HAI by sight and symptoms alone. Inflammation and/or a rash at the site of infection can also be an indication. Infections prior to your stay that become complicated don't count as HAIs. But you should still tell your doctor if any new symptoms appear during your stay.

You also may be required to talk a blood and urine test as to identify the infection.

Treatments for these infections depend on the infection type. Your doctor will likely recommend antibiotics and bed rest. Also, they'll remove any foreign devices such as catheters as soon as medically appropriate.

To encourage a natural healing process and prevent dehydration, your doctor will encourage a healthy diet, fluid intake, and rest.

Early detection and treatment are vital for HAIs. Many people are able to make a full recovery with treatment. But people who get HAIs usually spend 2.5 times longer in the hospital.

In some cases, a HAI can seriously increase your risk for life-threatening situations. The Centers for Disease Control and Prevention (CDC) estimate that around 2 million people contract HAIs. About 100,000 of those cases result in death.

The responsibility of HAI prevention is with the healthcare facility. Hospitals and healthcare staff should follow the recommended guidelines for sterilization and disinfection. Taking steps to prevent HAIs can decrease your risk of contracting them by 70 percent or more. However, due to the nature of healthcare facilities, it's impossible to eliminate 100 percent of nosocomial infections.

Some general measures for infection control include:

- Screening the ICU to see if people with HIAs need to be isolated.
- Identifying the type of isolation needed, which can help to protect others or reduce chances of further infection.
- Observing hand hygiene, which involves washing hands before and after touching people in the hospital.
- Wearing appropriate gear, including gloves, gowns, and face protection.
- Cleaning surfaces properly, with recommended frequency.
- Making sure rooms are well ventilated.

To reduce the risk of UTIs, your healthcare provider can:

- Follow the aseptic insertion technique to minimize infection.
- Insert catheters only when needed and remove when no longer needed.
- Change catheters or bags only when medically indicated.
- Make sure the urinary catheter is secured above the thigh and hanging below the bladder for unobstructed urine flow.
- Keep a closed drainage system.

Talk to your doctor about any concerns you have during a procedure.

Nosocomial infections, or healthcare associated infections occur when a person develops an infection during their time at a healthcare facility. Infections that appear after your hospital stay must meet certain criteria in order for it to qualify as a HAI.

If new symptoms appear within 48 hours of admission, three days after discharge, or 30 days after an operation, talk to your doctor. New inflammation, discharge, or diarrhea could be a symptom of a HAI.

Surname_____ Date_____

QUESTIONS TO EXAMINATION IN MICROBIOLOGY, IMMUNOLOGY AND VIROLOGY

SPECIAL BACTERIOLOGY AND PROTOZOOLOGY

1. Staphylococci, their biological properties. Classification, practical value.

2. Methods of microbiological diagnosis of staphylococcal infections. Immunity in staphylococcal diseases: Preparations for the specific prophylaxis and terapy.

3. Streptococci, their biological properties, classification. Toxins, enzymes of pathogenicity.

4. Streptococci, their role in pathology of man. Pathogenesis of streptococcal diseases. Toxins and enzymes of pathogenicity of streptococci. Immunity. Methods of microbiological diagnosis of streptococcal diseases.

5. Streptococci of pneumonia, their biological properties. Pathogenicity for man and animals. Microbiological diagnosis of pneumococcal diseases.

6. Meningococci, their biological properties, classification. Pathogenesis and microbiological diagnosis of meningococcal diseases and carriage of bacteria. Differentiation of meningococci and Gram-negative diplococci of the nasopharynx.

7.Gonococci. Biological properties, classification, pathogenesis and microbiological diagnosis of the diseases. Prophylaxis and specific therapy of gonorrhea and blennorhea.

8. The causative agent of Typhus. Properties. Pathogenesis of the disease, immunity. Laboratory diagnosis, assessment of methods. Specific prophylaxis.

9. Escherichiae, their main properties. Pathogenic serological types of Escherichiae, their differentiation. Significance in the pathology of man. Microbiological characteristics of coli enterites.

10. Salmonella as causative agents of typhoid fever and paratyphoids A and B. Biological properties, antigenic structure. Pathogenesis of the disease. Immunity.

11.Shigellae. Pathogenesis of dysentery, role of toxins and enzymes of pathogenicity. Immunity. Methods of microbiological diagnosis of dysentery.

12. Cholera vibrio, its biological properties, biological variants. Pathogenesis and immunity in cholera. Methods of microbiological diagnosis of cholera. Specific prophylaxis and therapy of cholera.

13. Pathogenetic fundamentals for microbiological diagnosis of typhoid fevers and paratyphoids A and B. Methods of microbiological diagnosis.

14.Corynebacteria, their characteristics. Biological variants of diphtheria bacilli. Production of the toxin. Genetic determinants of toxigenicity.

15. Diphtherial bacillus, its biological properties. Characteristics of the exotoxin. Pathogenesis of diphtheria, immunity. Microbiological diagnosis of diphtheria. Differentiation of the causative agent of diphtheria from saprophytic Corynebacteria. Specific prophylaxis and therapy of diphteria, revealing of the antitoxic immunity.

16. Pathogenic mycobacteria, their role in pathology of man. The causative agent of tuberculosis, its properties. Types of tuberculous bacteria. Pathogenesis, microbiological diagnosis of tuberculosis.

17. Immunity in tuberculosis. Specific prophylaxis and treatment of tuberculosis.

18. The causative agent of tularemia, its biological properties. Pathogenesis, immunity. Methods of microbiological diagnosis and specific prophylaxis of tularemia.

19. The causative agent of plague, its biological characteristics. Pathogenesis, immunity. Methods of diagnosis and specific prophylaxis.

20. The causative agent of anthrax, its biological properties. Pathogenesis, immunity. Methods of microbiological diagnosis and specific prophylaxis of anthrax.

21. Brucellae, their types and differentiation. Pathogenesis and immunity in brucellosis. Methods of microbiological diagnosis of brucellosis and their assessment. Preparations for specific prophylaxis and therapy.

22. Clostridia of tetanus, their properties. Formation of the toxin. Pathogenesis of tetanus in man. Microbiological diagnosis, specific prophylaxis and therapy.

23. Clostridia of botulinum, their properties. Formation of the toxin. Pathogenesis of botulinum in man. Microbiological diagnosis, specific prophylaxis and therapy.

24. General comparative characteristic of anaerobic bacteria, their significance in pathology of man. Peculiarities in microbiological diagnosis of the diseases, caused by anaerobes. Bacteroides, their biological properties.

25. Causative agents of the anaerobic infections of wounds, their properties, pathogenesis and microbiological diagnosis. Methods of specific prophylaxis and therapy of the anaerobic wound infection.

26. Klebsiella, their role in pathology of man. Characteristics of Klebsiella pneumonia, ozena, rhinoscleroma. Microbiological diagnosis, specific prophylaxis.

27. Bordetellae, their properties. The causative agents of whooping cough; their morphological, cultural and antigenic properties. Microbiological diagnosis and specific prophylaxis of whooping cough.

28. Haemophilus influenzae, its morphological, cultural and antigenic properties. Microbiological diagnosis and specific prophylaxis of haemophilus infection.

29. Opportunistic microorganisms, their biological properties and role in pathology of man. Pseudomonas aeruginosa and Proteus. Etiological role in purulent processes. Significance in etiology of hospital infections. Microbiological diagnosis.

30. The family of Spirochaetaceae: Leptospira and Borrelia, their characteristics and classification. Pathogenesis of the disease, immunity, microbiological diagnosis of the diseases. Specific prophylaxis, therapy.

31. The causative agent of syphilis. Morphological and cultural characteristic. Pathogenesis and immunity. Microbiological diagnosis and specific therapy of syphilis.

32. Pathogenic protozoa, biological properties. Classification, role in pathology of man.

33. Toxoplasma gondii, their morphology, peculiarities of cultivation. Pathogenesis of the diseases. Microbiological diagnosis. Specific prophylaxis.

34. Plasmodia malariae, their characteristics. Pathogenesis of malaria. Microbiological diagnosis. Specific prophylaxis, therapy.

35. Entamoeba histolytica, its morphology. Pathogenesis of the diseases. Microbiological diagnosis. Therapy.

36. Trichomonas vagimalis, its morphology. Pathogenesis of the diseases. Microbiological diagnosis. Therapy.

37. Giardia lamblia, its morphology. Pathogenesis of the diseases. Microbiological diagnosis. Therapy.

38. Leishmania, species, morphology. Pathogenesis of the diseases. Microbiological diagnosis. Therapy.

39. Mycoplasmatales, classification. Biological properties, methods of cultivation. Role in pathology of man. Microbiological diagnosis of mycoplasmoses.

40. Chlamydia, classification. Biological properties, methods of cultivation. Role in pathology of man. Microbiological diagnosis of chlamydiases.

41. Rickettsiae, their biological properties. Classification. Rickettsiae as causative agents of diseases in man. Pathogenesis of the disease, laboratory diagnosis, specific prophylaxis.

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